ASPERGILLUS-DERIVED MYCOTOXINS IN THE FEED AND FOOD CHAIN

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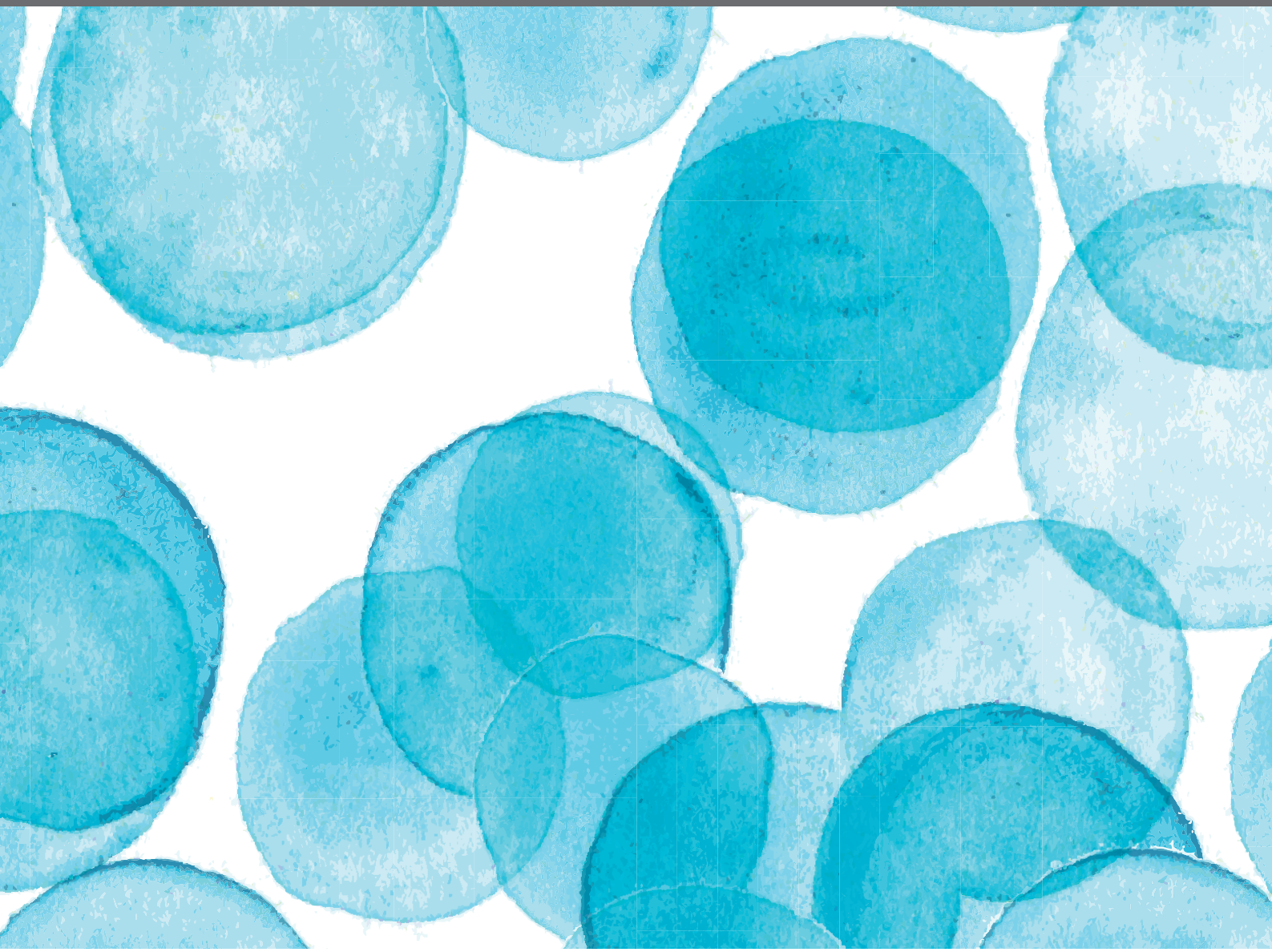

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ASPERGILLUS-DERIVED MYCOTOXINS IN THE FEED AND FOOD CHAIN

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Editorial: *Aspergillus*-Derived Mycotoxins in the Feed and Food Chain

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Editorial on the Research Topic

***Aspergillus*-Derived Mycotoxins in the Feed and Food Chain**

Aspergillus-produced mycotoxins can enter the feed and food chain at many points in both pre-harvest and post-harvest. Although current climate changes seem to speed up the world-wide spread of mycotoxigenic fungi including the *Aspergilli* and also facilitate the production of these harmful secondary metabolites the factors governing these disadvantageous global processes are only partly understood or even have remained completely hidden until now. This Research Topic summarizes our knowledge on *Aspergillus*-derived mycotoxins especially focusing on *three* major areas of on-going research: (i) toxicological, medical, veterinary aspects, prevalence, detection, risk assessment, control strategies, (ii) ecology and biological control of mycotoxigenic *Aspergilli* in the fields, and (iii) pre-harvest and post-harvest management of mycotoxigenic *Aspergilli* and their mycotoxin production. We hope that the wealth of information generously provided by the *Aspergillus* mycotoxin research community will help the hard work of all those experts, who are active in this important field, and the papers collected here will be instructive and illuminating readings for students and the public as well.

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TOXICOLOGICAL, MEDICAL, VETERINARY ASPECTS, PREVALENCE, DETECTION, RISK ASSESSMENT, CONTROL STRATEGIES

Toxicological, Medical and Veterinary Aspects

Aspergillus-derived mycotoxins (aflatoxins, ochratoxins, gliotoxin, fumonisins, sterigmatocystin, patulin, etc.) represent a remarkably versatile group of fungal secondary metabolites considering both their chemical structures and adverse physiological effects in humans (Ráduly et al.). Although current food safety measures are often adequate to prevent the accumulation of these mycotoxins in the food chain further interdisciplinary research is eagerly needed to elaborate more effective prevention strategies of mycotoxicoses, to reach a deeper understanding of the deleterious consequences of both sole and combined mycotoxin exposures at various stages of life, and to invent novel diagnostic tools and therapeutic procedures to mitigate both acute and chronic mycotoxin poisonings (Ráduly et al.). To prevent primary and secondary (*via* food of animal origin) aflatoxicoses in humans is of paramount importance (Peles et al.). Fortunately, the accurate physiological effects, the existent transformation and detoxification pathways and the mechanisms of channeling of harmful aflatoxins into food raw materials have been elucidated in important

livestock like poultry, pigs and ruminants, and a wide spectrum of biocontrol and detoxification products are available to prevent harmful aflatoxins from entering the feed and food chain in animal husbandry (Peles et al.).

Prevalence and Detection of Aflatoxins

Food and feed contamination by aflatoxins create food insecurity around the world. Two overviews detail the prevalence of *Aspergillus* section *Flavi* and the occurrence of aflatoxins in raw peanuts and peanut-based products (Norlia et al.) and in food and feed (Mahato et al.). Since even a low aflatoxin concentration is hazardous for human and livestock, the identification and quantification of AFs is a major challenge to guarantee food safety. The demand for determination of aflatoxins triggered extensive research and method development, and, in the last decades, increasingly faster and more sensitive analytical techniques proved to be promising, but, only a few of them have gained applicability in routine analysis. The study of Miklós et al. provides guidance on the current performance characteristics of various analytical methods for determination of aflatoxins in different food and feed matrices, and highlights their limitations for practical use, i.e., the absence of processes applied for reduction of large laboratory samples to the few grams for extraction (Miklós et al.) or the fact that the repeatability or reproducibility, if reported, was based on a few spiked samples (Miklós et al.). This guide helps in the decision to choose the most appropriate method that meets the practical requirements of fast and sensitive control of their contamination. Special references are devoted to new methods developed for masked AFs that are unable to be identified by routine analysis processes (Mahato et al.) and for concomitant detection of aflatoxins and their major fungi precursors *Aspergillus flavus* and *Aspergillus parasiticus* in stored maize by a simultaneous run of a Display Mediated Immuno-polymerase Chain Reaction (PD-IPCR) for aflatoxins and a conventional real-time PCR (RT-PCR) for aflatoxin producers (Ren, Yue et al.).

Risk Assessment and Control Strategies for Aflatoxins

Despite of prevention methods and strict regulations, *Aspergillus*-derived mycotoxins are still present in the feed and food chain, as well as the mycotoxicoses (Ráduly et al.). Quantitative exposure assessment is a methodology developed to evaluate the probable intake of chemical substances *via* food. The study of Serraino et al. calculated the Estimated Daily Intake, the Hazard Index, and the fraction of hepatocarcinoma cases (HCC) due to AFM_1 exposure in different population groups in Italy. A low risk of HCC was predicted but the variability of climatic conditions throughout years justifies a continuous monitoring of aflatoxins and an update of the risk assessment. To implement appropriate control measures, a special focus is devoted to the aflatoxin management and the impact of climate change on AFs production, and of control strategies of AFs in terms of innovative processing technologies applied for pre-and post-harvest aflatoxins management in combination with either biological, physical, chemical or genetic engineering methods (Mahato et al.; Ráduly et al.).

ECOLOGY AND BIOLOGICAL CONTROL OF MYCOTOXIGENIC *ASPERGILLI* IN THE FIELDS

Interactions of the *Aspergilli* and Their Mycotoxins With Plants and the Soil Micro-and Macrobiota

The remarkably complex and dynamic network of soil microbiota and macrobiota determining the ecological niches the mycotoxigenic *Aspergilli* can enter and fill in is still waiting to be described and deciphered in details (Pfliegler et al.). Ecological factors influencing the production and fate of fungal secondary metabolites including mycotoxins like sterigmatocystin/aflatoxins, gliotoxin, ochratoxins, patulin, and cyclopiazonic acid as well as the versatile interactions of these molds with plants (e.g., *A. flavus* with peanut, maize, and cotton), other microorganisms (including fungi, prokaryotes, and protists) and animals (first of all with arthropods) need to be clarified to reach a deeper understanding of these ecosystems and also to develop novel biocontrol and mycotoxin biodegradation technologies for plant and food protection (Pfliegler et al.). In the last decades, various and powerful omics techniques helped us to shed light on the fine details of the molecular mechanisms of host-pathogen interactions especially in *A. flavus*-maize and *A. flavus*-groundnut relations (Soni et al.). Not surprisingly, a plethora of proteins and genes have been identified with definite or hypothesized functions in the resistance of these agricultural crops to aflatoxin contaminations, which has opened the way to the development of novel molecular breeding technologies in this important field (Soni et al.).

Monitoring Atoxigenic Biocontrol *Aspergillus flavus* Genotypes in Fields

Application of non-aflatoxigenic *A. flavus* strains to prevent aflatoxin contamination under field conditions is one of the leading pre-harvest strategies to control this harmful, carcinogenic mycotoxin in the feed and food chain. Interactions of these biocontrol agents with the indigenous soil populations of aflatoxigenic fungi has been the subject of extensive research for a long time. Interestingly, the Afla-Guard strain originally isolated from naturally infected peanut in Georgia and belonging to lineage IB performed better in maize fields in the south-eastern United Stated (Alabama, Georgia, North Carolina) than the AF36 strain in lineage IC and isolated from cottonseed in Arizona as indicated by shifts in genetic diversities (Lewis et al.). In Ghana, 12 atoxigenic African *A. flavus* VCGs (AAVs) were identified and the biocontrol potential of a representative member of each AAV was tested under both laboratory (maize) and field (maize and groundnut, in three diverse agroecological zones) conditions (Agbetiameh et al.). As a result, four-four well-preforming isolates were selected and incorporated into two biocontrol products, Aflasafe GH01 and Aflasafe GH02, for use in maize and groundnut cropping systems in Ghana (Agbetiameh et al.). Importantly, each isolate has a unique simple sequence repeat (SSR) signature based on 17 SSR loci, which makes the tracking of each active ingredient possible under field conditions

(Agbetiameh et al.). Fast and reliable tracking and quantification of active ingredients of biocontrol products in crops are also required by farmers, the regulatory community and crop end-users (Shenge et al.). An array of quantitative pyrosequencing-based assays was developed and successfully applied in maize-associated fungal populations to monitor frequencies of SNPs characteristic of non-aflatoxigenic *A. flavus* isolates included in the Aflasafe product in Nigeria (Shenge et al.).

Biopesticides and Biostimulants

The use of biological agents and biostimulants for the control of *A. flavus* is a prerequisite for creating an Integrated Pest Management (IPM) in order to protect plants and related products prone to aflatoxins contamination. Commercial biopesticides could offer an economically effective solution that may contribute to the exclusion of aflatoxigenic fungi from maize plants and minimize the mycotoxin production. The efficiency evaluation of these biopesticides *in vitro* assay is crucial for a future IPM system friendly and sustainable for the environment (Lagogianni and Tsitsigiannis; Lagogianni and Tsitsigiannis).

PRE-HARVEST AND POST-HARVEST MANAGEMENT OF MYCOTOXIGENIC *ASPERGILLI* AND THEIR MYCOTOXIN PRODUCTION

Regulation of Mycotoxin Biosynthetic Gene Clusters

The remarkable complexity of aflatoxin biosynthesis has been revealed in aflatoxigenic fungi and both biotic and abiotic factors contribute to the fine-tuning of mycotoxin production in these microorganisms (Peles et al.; Pfliegler et al.). For example, abiotic oxidative stress stimulates aflatoxin biosynthesis in *A. flavus* (Peles et al.), and 3.5% ethanol exposure of *A. flavus* colonies not only significantly down-regulated the great majority of the aflatoxin cluster genes but also up-regulated important elements of the oxidative stress defense system including the *Cat*, *Cat1*, *Cat2*, *CatA,* and *sod1* genes as well as the oxidative stress response regulator genes *ap-1* and *msnA* (Ren, Jin et al.). These transcriptional changes coincided with a nearly complete inhibition of aflatoxin B_1 production (Ren, Jin et al.).

The ceaseless demand for the development of novel mycotoxin control technologies also requires further research to be performed on the regulation of mycotoxin biosynthetic gene clusters (Peles et al.). Wang et al. reported on the pivotal role the velvet complex (consisting of LaeA, VeA, and VelB proteins) of *Aspergillus ochraceus* plays in the maintenance of vegetative growth, asexual sporulation, virulence (on pears), and ochratoxin A production of the fungus and, therefore, elements of the velvet complex seem to be attractive targets for future ochratoxin A control technologies. It is worth noting that LaeA extensively regulates the secondary metabolism of *A. ochraceus* (Wang et al.). Another study by Barda et al. shed light on the remarkable importance of the pH-responsive transcription factor AcPacC on the regulation of fungal growth at neutral/alkaline pH, asexual sporulation, spore germination, gluconic and citric acid productions, ochratoxin A production (also on grapes and nectarine fruits) and virulence of *Aspergillus carbonarius*. Importantly, glucose oxidase encoded by *Acgox* was demonstrated to be a virulence factor of *A. carbonarius* and its production was also under a strict AcPacC control (Barda et al.).

Plant Bioactive Compounds Against Mycotoxigenic Fungi

Bioactive metabolites of plants like phenolic compounds, terpenes and nitrogen-containing compounds may also possess antifungal activities *via* interfering cell wall and cell membrane biosynthesis, mitochondrial functions, and important enzyme activities in fungi (Loi et al.). In addition to controlling pre-harvest and post-harvest growths of the *Aspergilli*, the direct inhibitory effects of plant bioactive compounds on aflatoxin biosynthetic pathway seem to be exploitable as well (Loi et al.). Various plant extracts containing low molecular weight active ingredients and enzymes have also been shown to be effective in aflatoxin degradation (Loi et al.). Cinnamaldehyde, a widely used α,ß-unsaturated aldehyde food additive, appears to be especially promising in control of aflatoxigenic *A. flavus* e.g., on corn (Qu et al.). Considering the antifungal mechanism of action of this compound, cinnamaldehyde triggers a series of apoptotic events in *A. flavus*, including elevated intracellular Ca^{2+} and reactive oxygen species levels, various mitochondrial dysfunctions, metacaspase activation, phosphatidylserine externalization, DNA damage and nuclear fragmentation as well as up-regulation of apoptosis-related genes (Qu et al.). A methanolic pomegranate peel extract (PPE) acted synergistically with the azole fungicide prochloraz (PRZ) in controlling the growths of the mycotoxigenic fungi *A. flavus* and *Fusarium proliferatum* (Sadhasivam et al.). PPE+PRZ combined treatments delayed conidial germination and hyphal elongation in both fungi, and the combined application of sub-inhibitory doses of PPE and PRZ blocked aflatoxin B1 production by *A. flavus* (Sadhasivam et al.). Such combined antifungal treatments may help us to decrease the applied doses of potentially harmful synthetic fungicides like the azole drugs in the agriculture (Sadhasivam et al.).

Fungal Biomass as Aflatoxin Biosorbent

Adsorbent materials mixed with the feed may protect animals by binding efficiently mycotoxins including aflatoxins. The adsorbents reduce the bioavailability of mycotoxins in the gastro-intestinal tract and thus the diffusion into the bloodstream and transport to the target organs. The use of microbial biomasses as adsorbent seems very promising since less expensive though effective, and environmentally friendly materials (Haidukowski et al.). The characterization of the bio-sorbent properties of the mycelium of the king oyster mushroom (*Pleurotus eryngii*) as for Aflatoxin B_1 binding capability and the effects of physical and chemical conditions on the binding efficiency is strategic for a sustainable mycotoxin risk minimization in animal health (Haidukowski et al.).

AUTHOR CONTRIBUTIONS

All authors served as co-editors to the Research Topic and also contributed to, critically read and discussed and approved this Editorial.

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REVIEW
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Toxicological and Medical Aspects of *Aspergillus*-Derived Mycotoxins Entering the Feed and Food Chain

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Due to Earth's changing climate, the ongoing and foreseeable spreading of mycotoxigenic *Aspergillus* species has increased the possibility of mycotoxin contamination in the feed and food production chain. These harmful mycotoxins have aroused serious health and economic problems since their first appearance. The most potent *Aspergillus*-derived mycotoxins include aflatoxins, ochratoxins, gliotoxin, fumonisins, sterigmatocystin, and patulin. Some of them can be found in dairy products, mainly in milk and cheese, as well as in fresh and especially in dried fruits and vegetables, in nut products, typically in groundnuts, in oil seeds, in coffee beans, in different grain products, like rice, wheat, barley, rye, and frequently in maize and, furthermore, even in the liver of livestock fed by mycotoxin-contaminated forage. Though the mycotoxins present in the feed and food chain are well documented, the human physiological effects of mycotoxin exposure are not yet fully understood. It is known that mycotoxins have nephrotoxic, genotoxic, teratogenic, carcinogenic, and cytotoxic properties and, as a consequence, these toxins may cause liver carcinomas, renal dysfunctions, and also immunosuppressed states. The deleterious physiological effects of mycotoxins on humans are still a first-priority question. In food production and also in the case of acute and chronic poisoning, there are possibilities to set suitable food safety measures into operation to minimize the effects of mycotoxin contaminations. On the other hand, preventive actions are always better, due to the multivariate nature of mycotoxin exposures. In this review, the occurrence and toxicological features of major *Aspergillus*-derived mycotoxins are summarized and, furthermore, the possibilities of treatments in the medical practice to heal the deleterious consequences of acute and/or chronic exposures are presented.

Keywords: mycotoxin, aflatoxins, ochratoxins, fumonisins, sterigmatocystin, food poisoning, carcinogenic, secondary metabolites

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INTRODUCTION

Each mycotoxin is a secondary metabolite produced by fungi, but not all secondary metabolites are toxic (Bennett and Klich, 2003; Richard, 2007). Apart from mycotoxins, other secondary metabolites are often produced by fungi, e.g., plant growth regulators, pharmaceutically useful compounds, and pigments (Richard, 2007). These biological compounds usually play a part in the survival of fungi and, concomitantly, are disadvantageous for their surroundings as well

(Bennett and Klich, 2003; Keller, 2019). Various types of environmental stress may trigger the production of these deleterious compounds, suggesting their protective role, e.g., under oxidative stress (Reverberi et al., 2010). Hence, the production of mycotoxins may facilitate the successful adaptation of fungi to a broad spectrum of environmental stress conditions (Schmidt-Heydt et al., 2009), which are raised, e.g., by the changing environment and climate (Van der Fels-Klerx and Camenzuli, 2016; Medina et al., 2017). Mycotoxin production may help fungi in competition with other microorganisms (Hymery et al., 2014) or to resist against grazing by insects (Rohlfs, 2015). In host – phytopathogenic fungus interactions, mycotoxins may inhibit the germination of seeds and may also contribute to the invasion of plant tissues *via* eliciting versatile apoptotic and necrotic cell death processes (Pusztahelyi et al., 2015).

Since 1962, when almost 100,000 turkeys died in an unusual veterinary crisis in London, the field of mycotoxin research has become a relevant scientific issue. That particular "turkey X disease" was linked to peanut meals, which were contaminated by aflatoxins (Sweeney and Dobson, 1998; Smith et al., 2016). This specific new field of knowledge was called mycotoxicology, which includes all areas of research related to mycotoxins; meanwhile, the term mycotoxicosis covers all animal and human diseases caused by mycotoxins. Mycotoxins can be classified according to their chemical structures, origin of biosynthesis, and characteristic symptoms assigned to the particular toxins. In this paper, we aim at summarizing the medical risks of consuming food contaminated by *Aspergillus*-derived mycotoxins. Additionally, we included a brief overview on some socioeconomic and environmental impacts of mycotoxin food and feed contaminations, possibilities for prevention, and the available decontamination methods and medical treatments.

During the past 60 years, it has become clear that the world has to deal with mycotoxin exposure (see **Figure 1**). Agricultural commodities are often contaminated with mycotoxins, which results in either visible, acute effects or chronic, long-term hidden health damages (Souers et al., 2013; Smith et al., 2016; Udovicki et al., 2018; Rushing and Selim, 2019). As maize, rice, and wheat are among the most important crops, the presence of mycotoxins in these feed and foodstuffs entails a high public health risk of chronic exposure to mycotoxins (Jard et al., 2011; Rodrigues et al., 2011; Smith et al., 2016; Udovicki et al., 2018). The food shortage typical of mainly developing countries resulted in necessary negligence of the mycotoxin content of food and feed. The lack of knowledge about mycotoxins and their effects, safety regulations and enforcement, infrastructure to monitor and quantify the mycotoxin content, and the lack of political will all contribute to mycotoxin exposures. These regrettable circumstances led to the continuous risk of mycotoxin poisoning and the worsening of living conditions in the affected regions, especially in the case of children. Although mycotoxicoses mainly occur in developing regions of the world, recent years showed that industrialized countries in the moderate climate belt also have to face the risks of *Aspergillus*-derived toxin exposure (Cleveland et al., 2003; Udovicki et al., 2018). The occurrence and spread of molds depend on several factors, including environmental, social, and economic conditions (Omotayo et al., 2019). Grain producers and exporters in the world encountered the challenging problem of how mycotoxin contents in food and feed should be somehow regulated (Cleveland et al., 2003; EC 1881/2006, 2006; Udovicki et al., 2018). Although industrial countries are mostly located in the moderate continental climate belt and malnutrition is rare there, toxigenic *Aspergillus* species are moving constantly north due to climate change (see **Figure 1**; Battilani et al., 2016; Udovicki et al., 2018). Even nowadays, mycotoxin contaminations and mycotoxicoses are taken mainly as the problem of the Third World (**Figure 1**). Africa, South America, and other tropical countries have already been combating the ever-growing threat of mycotoxins for a long time. Even there, the types and the amounts of mycotoxins in the feed and food will be altered with the changing climate. To make things even worse, non-prioritized toxins can also emerge as new risks with unforeseeable effects and interactions. Unfortunately, big nations, organizations, or countries, like the World Health Organization (WHO), United States, China, or the European Union (EU) have different limiting values for mycotoxins (EC 1881/2006, 2006; Marasas, 1995), which makes any concerted actions by them quite difficult. During the last few years, several economical, health, and agricultural studies opened the question: what kind of pre- and post- harvest conditions and prevention methods would be manageable and safe for human and animal health (Shephard, 2008; Hamid et al., 2013; Mitchell et al., 2016)? As humans are on the top of the food chain, accumulation of mycotoxins clearly depends on animal consumption as well, so feed contamination should also be taken into account and thoroughly controlled. Nowadays, the globalization of food production systems can easily lead to accidental exposures of the consumers to multiple mycotoxins because (i) various mold infestations can affect the same crop concomitantly, (ii) additional infestations can occur during food processing, and (iii) customers can buy and consume contaminated foodstuffs bearing different mycotoxin contaminants. Importantly, all the above events can be separated both spatially and temporally. These palpable tendencies should raise the need for complex analytical and interdisciplinary studies in the future, especially when the changing climate represents a new global challenge to the food production and food safety regulatory systems (Gruber-Dorninger et al., 2019).

FOOD TOXICOLOGY AND MOLECULAR MECHANISM OF MYCOTOXINS

Food toxicology is the field of science which deals with the toxicological effects of food components (Hussein and Brasel, 2001). Not surprisingly, food and feed also contain the most complex mixture of low-molecular-weight xenobiotics to which humans and animals are exposed. Because of the growing amount of evidence on the presence of mycotoxins in the feed and food chain, food toxicology should be considered seriously as an important discipline in combating mycotoxicoses (Shaw, 2014; Dellafiora et al., 2018).

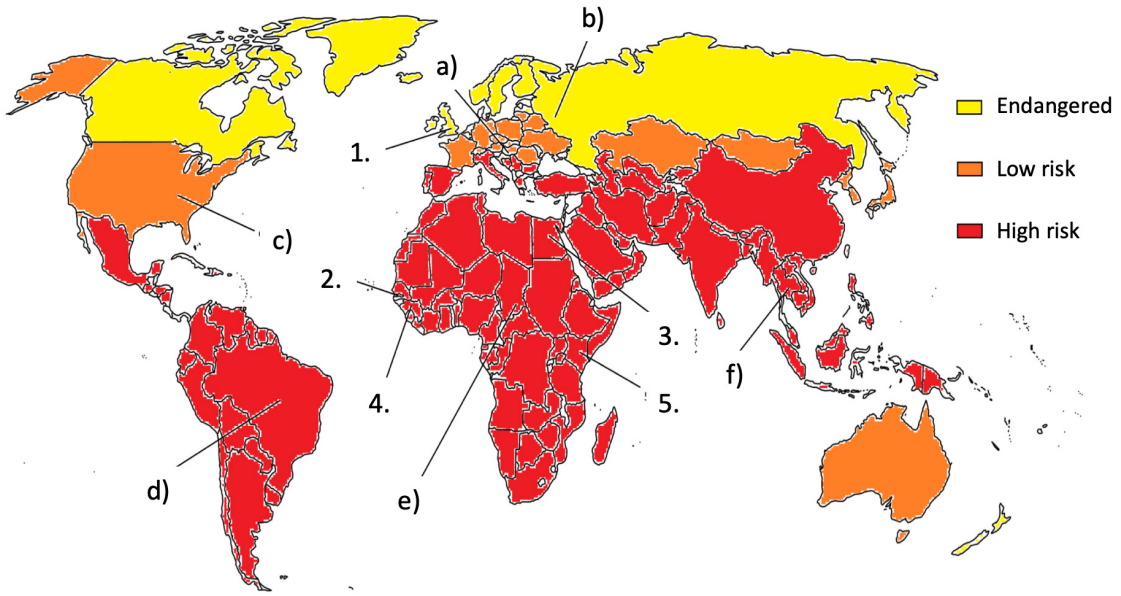

FIGURE 1 | The risk of mycotoxin exposure. Milestones in mycotoxicology: (1) In 1962, mycotoxins are identified as cause of turkey "X" disease; (2) Aflatoxin outbreak in Gambia in 1988, No. subjects: 391; (3) Aflatoxin poisoning in Egypt in 1992, No. subjects: 19; **(4)** Aflatoxin outbreak in Guinea in 1999, No. subjects: approx. 600; and (5) Aflatoxin outbreak in Kenya 2004, No. subjects: approx. 100; **(a)** Due to the climate change and increasing mean temperature, mycotoxin-producing fungi spread to the north. **(b)** Monoculture farming is sensitive to mold infestation. **(c)** Strict federal regulation can prevent the spread of mold. **(d)** The strict regulation of import and export are important to minimalize mycotoxin contaminations. **(e)** Prevention of mycotoxin infestation is of primary importance. Without sufficient education and up-to-date methods, it is hard to store, process, transport, or even analyze properly and safely food and feed. **(f)** Mycotoxins have serious economic and financial consequences (see references in the text).

The dose – response relationship specifies the magnitude of the response of an organism to exposure to a given chemical stimulus after a certain exposure time. Acute mycotoxicoses could be described with a rapid onset and a general response (Marroquín-Cardona et al., 2014). The relationship between the concentration of mycotoxins in food and the concentration of toxicologically active substances at the site of action could be characterized by toxicokinetics. The relationship between the concentration of toxicants at the site of action and the toxic effect at the level of molecules, tissues, or organs is determined by toxicodynamics (Dellafiora et al., 2018). All *Aspergillus* species can produce a wide range of mycotoxins, although each species has one predominant, characteristic toxin in many cases (Sweeney and Dobson, 1998). Because of the multivariate nature of mycotoxins and their co-occurrence in food and feed, co-ingested mycotoxins give rise usually to mixed symptoms coming from additive and synergistic effects (Marroquín-Cardona et al., 2014; Flores and González-Peñas, 2016; Dellafiora and Dall'Asta, 2017; Dellafiora et al., 2018). Brief toxicological aspects of *Aspergillus*-derived mycotoxins are described in the following.

Aflatoxins

More than 20 types of aflatoxins (AFs) and their derivatives occur in nature, but mainly four, B1, B2, G1, and G2, are proved to be dangerous for humans and livestock (Wu et al., 2013; Smith et al., 2016; Udovicki et al., 2018; Rushing and Selim, 2019). AFs are furanocoumarins and are produced by various strains of *Aspergillus*, including *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus nomius*, and *Aspergillus pseudotamarii* as main AF producers (**Figure 2**; Council for Agricultural Science and Technology, 2003). Immunotoxic, carcinogenic, and mutagenic effects are mainly attributed to the presence of the lactone ring and the difuran ring (Vanhoutte et al., 2016). Aflatoxin B1 (AFB1) is the most carcinogenic and best-studied AF. Aflatoxin M1 (AFM1) is the 4-hydroxy derivative of AFB1, formed in the liver and excreted into the milk by the mammary glands of both humans and lactating animals that have been fed with AFB1-contaminated diet (Benkerroum, 2016; Cherkani-Hassani et al., 2016; Alshannaq et al., 2017). As it is also excreted in the urine, it is used frequently as a biomarker after AF exposure. AFB1 is metabolized in the liver by the cytochrome P450 enzyme system (CYPs) and a potent carcinogen derivative is aflatoxin B1-8,9-epoxide (AFB0), which has an *exo* and an *endo* isomer (Rushing and Selim, 2019). Primarily, the CYP3A4 and CYP1A2

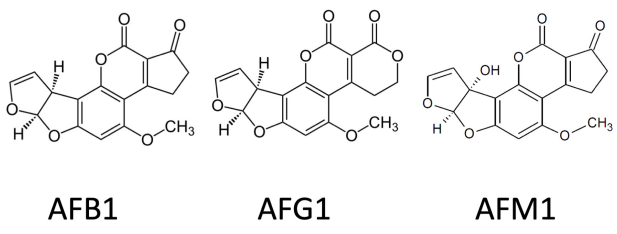

FIGURE 2 | Chemical structure of some carcinogenic aflatoxins.

are responsible for AF biotransformation, and mainly the *exo* isomer is formed, which has a highly electrophilic nature, perfect for spontaneous reactions with biological amines in nucleic acids and proteins (Rushing and Selim, 2019). In the case of DNA, AFB0 binds covalently to the N_7 position on guanine, forming AFB1-N_7-guanine adduct. The *endo* isomer has lower affinity than the *exo*, so AFB1-*exo*-8,9-epoxide is thought to be the major carcinogenic metabolite. Aflatoxicol (AFL) is the only metabolite that could go through the placenta and which is formed by the placenta itself. AFL is often found in the cytosolic fraction of liver preparations and thought to be a reservoir for AFB1, because it could be enzymatically converted back into AFB1, using the cytosolic NADPH system. That mechanism could be responsible for the AF-caused growth impairment, observed mainly in developing countries (Rushing and Selim, 2019).

Acute aflatoxicosis results in death while chronic exposure results in cancer, immunosuppression, and slowly manifesting pathological conditions (Phillips et al., 2002; Dharumadurai et al., 2011; **Figure 4**). Chronic aflatoxin poisoning leads to impaired DNA duplication in the bone marrow, which causes low leukocyte levels (Corrier, 1991; Fink-Gremmels, 1999; Benedict et al., 2016), which in turn gives rise to immunodeficiency and various infections. AFs also have a non-specific, cell multiplication inhibiting effect on other cell types (Bennett and Klich, 2003; Khlangwiset et al., 2011). This effect is the most prominent in the gastrointestinal tract, where an intact cell cycle is essential for the proper function of the digestive system (Liew and Mohd-Redzwan, 2018). The lethal dose (LD_{50}) values for AFs are within the range of 0.5–10 mg/kg, depending on the chemistry of the derivative (Hymery et al., 2014). The primarily affected organ is the liver, and patients suffer from bile duct proliferation, centrilobular necrosis, hepatic lesions, and fatty acid infiltration, which often ends in liver cancer (Wu and Santella, 2012; Hymery et al., 2014; Saha Turna and Wu, 2019).

The International Agency for Research on Cancer (IARC) has classified aflatoxins, including AFB1, AFB2, AFG1, AFG2, and AFM1 as carcinogenic to humans, i.e., as GROUP 1 carcinogens (International Agency for Research on Cancer, 2012; Ostry et al., 2017). AFs and the metabolites produced by the hepatic CYP enzymes showed an interference with nucleotide pairing, which can lead to different genetic changes, large-scale chromosomal aberrations, or even to DNA strand breaks (Wild and Gong, 2009). The G→T transversion in codon 249 of the p53 gene causing an Arg249Ser mutation on p53 protein is one of the most common mutations found in human hepatocytes exposed to AFB1. Arg249Ser mutation enhances cell growth and clonal expansion and inhibits wild-type p53 activity and apoptosis (Dumenco et al., 1995; Forrester et al., 1995; Rushing and Selim, 2019). Glutathione conjugation catalyzed by GST (glutathione-S-transferase) of AFBO is a major route of detoxification, forming an inert metabolite that is not able to react with the DNA (Rushing and Selim, 2019). That conjugate is then converted into a mercapturic acid adduct *in vivo* and is then excreted in the urine (Moss et al., 1985; Rushing and Selim, 2019). Glutathione-*S*-transferase expression is higher in mouse than in other animals, which could be a reason why these rodents are more resistant to AFB1 exposure.

Aflatoxins can also damage the hepatocytes directly or through changing the expression of lipid metabolism connected genes (*Cpt1a*, *Lipc*, *Lcat*, *Scarb1*, and *Ahr*). The elevated cholesterol, triglyceride, and lipoprotein production can cause the deterioration of hepatocytes because of the increased metabolic need and anaerobic cell metabolism (Rotimi et al., 2017). The elevated lipid fraction and the changed HDL–LDL ratio in the blood can increase the possibility of coronary heart diseases. The death of hepatocytes will lead to acute hepatitis, which can cause liver failure and death or lower the chance of survival (Hamid et al., 2013). Patients with hepatitis have an impaired metabolism, which can result in malnutrition (Nurul Adilah et al., 2018). The lack of nutrients also leads to the depletion of reducing agents like glutathione and thus to the overall reduction of antioxidative capacity in hepatocytes. In the absence of nutrients, the hepatic tissue repair and regeneration cannot function properly and the liver failure is almost inevitable (Magnussen and Parsi, 2013).

Ochratoxins

Ochratoxin A (OTA) was first described in 1965, and it is one of the most important mycotoxins (Heussner et al., 2015), which is produced mainly by *Aspergillus ochraceus*, *Aspergillus carbonarius* and *Aspergillus niger* as well as by *Penicillium verrucosum* (Ostry et al., 2013; Bui-Klimke and Wu, 2015). OTA is a pentaketide compound derived from a dihydrocoumarin family derivative coupled to β-phenylalanine (Zhu et al., 2017). IARC has classified OTA as a Group 2B carcinogen, which means that it is possibly carcinogenic to humans. OTA has also been reported as nephrotoxic, hepatotoxic, embryotoxic, teratogenic, neurotoxic, immunotoxic, and genotoxic (Pfohl-Leszkowicz and Manderville, 2007; Bui-Klimke and Wu, 2015). The symptoms of OTA poisoning are dose-dependent, and its carcinogenic properties are already well known in a variety of animal species.

The human aspects of OTA poisoning are not yet fully understood, although OTA in humans can cause kidney damage, cancer, or kidney failure, according to previous studies (**Figure 4**; Heussner et al., 2015). A well-reported case was the so called Balkan Endemic Nephropathy (BEN) (Barnes et al., 1977). Several various human nephropathies reported in countries as Bulgaria, Romania, Serbia, Croatia, Bosnia, Herzegovina, Slovenia, Macedonia, and Montenegro could be related to OTA (Reddy et al., 2010). African countries such as Congo, South Africa, Tunisia, Morocco, and Egypt struggled with similar cases. These effects of OTA were, however, not conclusive under laboratory conditions. Both the monitoring of OTA and the diagnosis of OTA-induced mycotoxicosis in humans rely on blood and urinary OTA levels. The BEN cases could not be related to the genetic background of the patients but, instead, to environmental factors like the mold-contaminated local grain (Pfohl-Leszkowicz and Manderville, 2007; Bui-Klimke and Wu, 2015). Surprisingly, chronic exposures to low OTA doses could even be more harmful than acute high-dose exposures (Pfohl-Leszkowicz and Manderville, 2007; Reddy et al., 2010). The most frequent way of OTA exposure is dietary intake (Reddy et al., 2010). Naturally and after biotransformation in the human body, more than 20 OTA derivatives exist. Importantly, OTA

forms covalent DNA adducts through radical and benzoquinone intermediates. In addition, the OTA hydroquinone (OTHQ) metabolite can undergo an autoxidative process to generate the quinone electrophile OTA quinone (OTQ) that also reacts with DNA. Furthermore, the formation of OTQ or phenoxy and aryl radicals can result in increased reactive oxygen species (ROS) production that is responsible for its cytotoxicity. The mechanisms leading to OTA nephrotoxicity as well as its hepatotoxicity and immunotoxicity can be linked to the inhibition of protein synthesis, lipid peroxidation, and the modulation of the MAP kinase cascade, in a way similar to the exposure to pentachlorophenol derivatives (Heussner et al., 2015; Malir et al., 2016; Zhu et al., 2017).

Emerging and Other Mycotoxins

Beside the toxins discussed above, *Aspergillus* species can also produce other toxic compounds that are not in the focus of food toxicology yet. They are, nonetheless, important and form an emerging branch of mycotoxicology and are already the objects of complex medical research projects in many cases.

Gliotoxin

Gliotoxin (GTX) is often referred to as a virulence factor. It is produced mainly by *Aspergillus fumigatus*, although *A. terreus*, *A. flavus*, and *Aspergillus niger* are also able to synthesize it (Kwon-Chung and Sugui, 2008). GTX is a dipeptide and has a disulfide bridge across the piperazine ring, being a member of epipolythiodioxopiperazines (ETPs; **Figure 3**; Trown and Bilello, 1972). This molecular feature could function in cross-linking with cysteine residues in proteins, which results in the generation of ROS through redox cycling reactions. The outcome of these deleterious molecular processes is immunosuppression and necrosis. GTX also alters the tight junction structures by an unknown molecular mechanism and has a cytotoxic effect on astrocytes (Patel et al., 2018).

Gliotoxin, like AFs, has an immunosuppressive effect, but the molecular mechanism is different. GTX in lower concentrations can inhibit the activation of inflammatory cells, the signaling and communication pathways between the leukocytes, the phagocytosis of macrophages, or the oxidative agent production of neutrophils and macrophages (**Figure 4**; Corrier, 1991). In higher concentrations (>250 ng/ml) GTX can induce apoptosis in leukocytes (Lewis et al., 2005). The GTX-producing human pathogenic fungi like *A. fumigatus* can evade the immunological responses. Other immunodeficiencies, as AIDS, chronic steroid treatment, alcohol abuse, and malnutrition can also be enhanced by GTX poisoning.

Fumonisins

Fumonisins are a group of related polyketide-derived, non-fluorescent mycotoxins. More than 53 different fumonisins have been reported so far (Marasas, 1995; Månsson et al., 2010; Nair, 2017). They can be divided into four main series (A, B, C, and P) but research has focused on the B series, mainly FB1, FB2, and FB3, which are the most abundant in nature (Mogensen et al., 2009). Fumonisin B compounds consist of a long hydroxylated hydrocarbon chain, which are decorated by tricarballylic acid and amino and methyl groups. FB2, FB3, and FB1 have different hydroxylation patterns (Kouzi et al., 2018). Fumonisins are structurally similar to cellular sphingolipids and, not surprisingly, they have been shown to inhibit sphingolipid biosynthesis at ceramide synthase (Marasas, 1995). The primary amino and tricarballylic acid groups of the toxin are responsible for the reaction with ceramide synthase. Fumonisin-induced toxicity often results in apoptosis, alteration in cytokine expression, or generation of oxidative stress (Kouzi et al., 2018). IARC has been classified FB1 in toxicity Group 2B as probably carcinogenic for people. *Aspergillus* species belonging to *Aspergillus* section *Nigri* are widely occurring species, and one of them, *A. niger*, is a highly important industrial organism in citric acid production. Black *Aspergilli* including *A. niger* and *A. welwitschiae* can be responsible for the FB2 (and FB4) contents observable in some foods and feeds as grapes, raisins, wine (Mogensen et al., 2009, 2010), onions (Varga et al., 2012; Gherbawy et al., 2015), and maize (Logrieco et al., 2014). However, *Fusarium verticillioides*, *Fusarium proliferatum*, and other *Fusarium* spp. cause higher fumonisin contaminations with FB1 (Frisvad et al., 2007; Kamle et al., 2019). Co-occurrence of fumonisin producing *Fusaria* and black *Aspergilli* in the kernels of maize may influence the observed FB1/FB2 ratios (Logrieco et al., 2011; Susca et al., 2014). Studies indicate that the fumonisins could be responsible for esophageal cancer in South Africa and have been shown to cause leukoencephalomalacia in horses and pulmonary edema in pigs (Kouzi et al., 2018). Fumonisins are also responsible for other diseases including neural tube defects, leukoencephalomalacia, pulmonary edema, hepatotoxicity, nephrotoxicity, or renal carcinogenesis (Nair, 2017; **Figure 4**). As sphingolipids are vital in regulating various cellular processes and they are a large family of metabolically linked signaling molecules, the acute and chronic toxicities of fumonisins are the result of the disruption of the sphingolipid metabolism and, as a result, the affected organs are very diverse. Recent findings also showed increased ROS production after fumonisin exposure, which may result in DNA damage and other enzyme defects but more research is needed to clarify the molecular backgrounds of these effects (Kouzi et al., 2018).

Sterigmatocystin

More than 50 fungal species can produce sterigmatocystin (STC), which, similar to AFs, is a polyketide mycotoxin. *A. flavus*, *A. parasiticus*, and *Aspergillus* section *Nidulantes*, subclade *Versicolores* are the most common source. Biosynthetic pathways of AFs and STC share many biosynthetic enzymes (Díaz Nieto et al., 2018). Since *A. nidulans* and *A. versicolor* are apparently unable to biotransform STC into O-methylsterigmatocystin, the direct precursor of AFB1 and AFG1, substrates colonized by these fungi can contain high amounts of STC. On the other hand, substrates invaded by *A. flavus* and *A. parasiticus* contain only low amounts of STC as most of it is converted into AFs (EFSA, 2013). According to different animal models and cell culture experiments, STC can also induce tumors; therefore, IARC classifies it in the Group 2B as possible human carcinogen (EFSA, 2013). In spite of this classification, the maximum acceptable levels of STC in food are not regulated worldwide. The acute oral

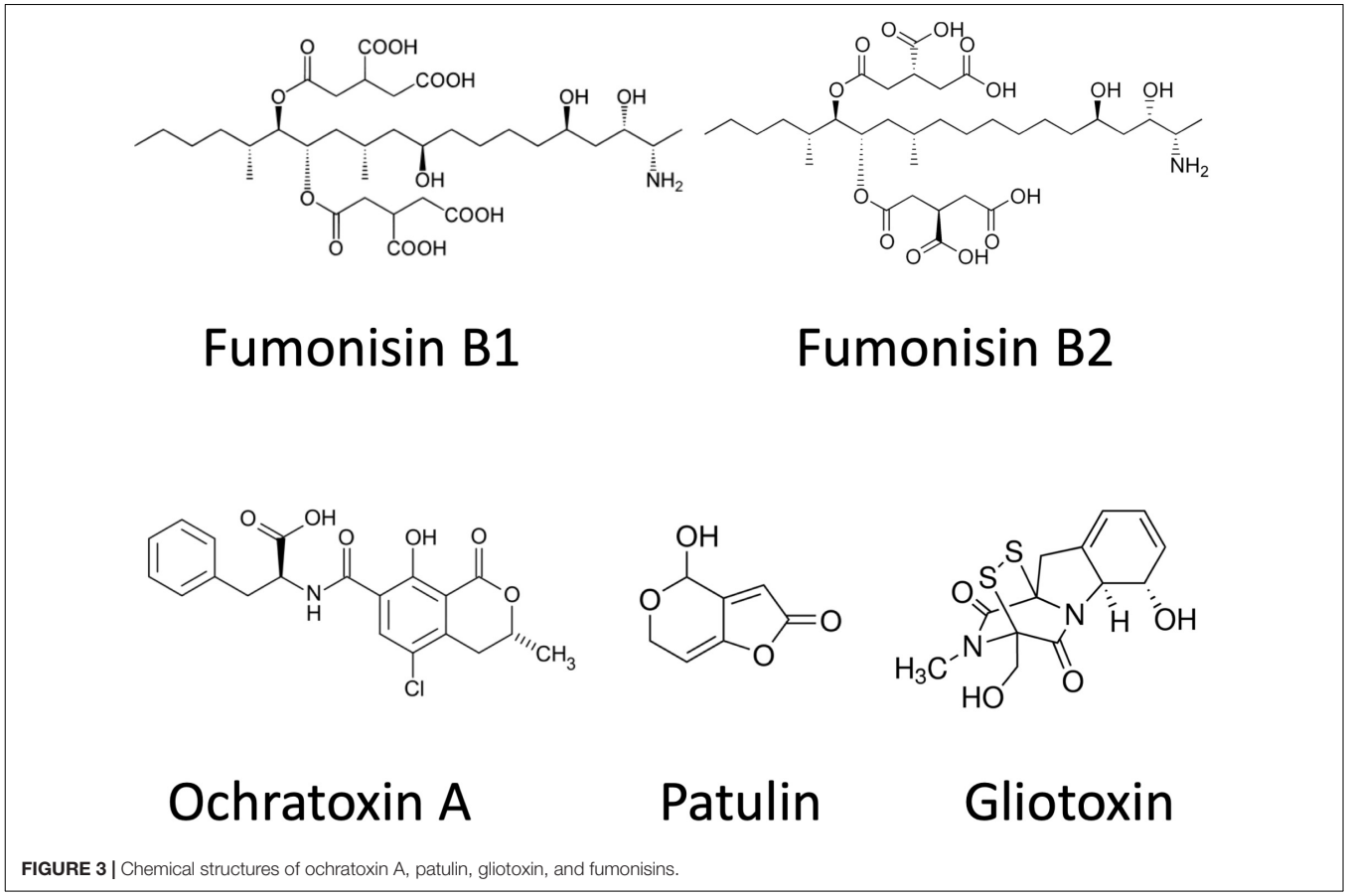

FIGURE 3 | Chemical structures of ochratoxin A, patulin, gliotoxin, and fumonisins.

toxicity of STC is relatively low, with LD_{50} values varying between 120 and 166 mg/kg bw. After oral exposure, premalignant and malignant lesions, such as hepatocellular carcinomas and angiosarcomas in the brown fat, have been reported. STC is genotoxic and carcinogenic, although the carcinogenic potency of STC is approximately three orders of magnitude lower than that of AFB1. STC is metabolized in the liver and lung by various CYP enzymes into different hydroxy metabolites (Díaz Nieto et al., 2018), and STC-metabolites are excreted in the bile and the urine (EFSA, 2013). The mutagenicity of STC is due to these reactive epoxi-adducts, which can covalently bind to DNA and generate the STC-N_7-guanine adducts. Another mechanism was also proposed by Pfeiffer et al. (2014), who suggested that the hydroxylation of the aromatic ring generates a catechol, which could react with DNA. This was based on the finding that in liver microsomes of humans and rats the catechol was mainly formed while the epoxide was formed in smaller amounts. Intensive research has been launched recently on the role of STC in human esophageal and gastric cancers (**Figure 4**). *In vivo* experiments were performed in a rat model system, and these findings confirmed the conclusions previously drawn from experiments on human-derived cell lines (Tong et al., 2013; Díaz Nieto et al., 2018). It has been demonstrated in a human immortalized bronchial epithelial cell line that STC could induce DNA double-strand breaks, which may lead to adenocarcinomas.

Patulin

Patulin (PAT) is produced by many different molds, predominantly by *Penicillium* spp. (Puel et al., 2010; Frisvad, 2018; Vidal et al., 2019) but, occasionally, by some *Byssochlamys* (Sant'Ana et al., 2010; Frisvad, 2018) and *Aspergillus* spp., including *A. giganteus*, *A. longivesica*, and *A. clavatus* (Varga et al., 2007; Pal et al., 2017; Frisvad, 2018) as well. Chemically, PAT is a water-soluble, colorless, polyketide lactone (**Figure 3**), which is thought to exert its toxicity through reacting with thiol groups (cysteine, glutathione, thiol moieties of proteins) in the cytoplasm (Pal et al., 2017). In addition to its antibacterial, antiviral, and antiprotozoal activities, PAT was also reclassified as a mycotoxin.

Because PAT also possesses acute toxicity, teratogenicity, and mutagenicity properties at the same time (Puel et al., 2010), the emerging symptoms of PAT mycotoxicoses are typically non-specific but mostly connected to the enzyme inhibitions (Pal et al., 2017). The affected enzymes usually take part in digestion, metabolism, and energy production. Intestinal disorders, decreased food intake, decreased weight, together with altered lipid metabolism could be observed in many animal models. PAT can also compromise the immune system and modify the different response mechanisms of the host (Corrier, 1991), and also inhibits transcription, translation, and DNA synthesis in leukocytes (Mahfoud et al., 2002; **Figure 4**). *In vitro* studies have demonstrated that PAT inhibits macrophage functions like reduced rate of protein synthesis of lysosomal

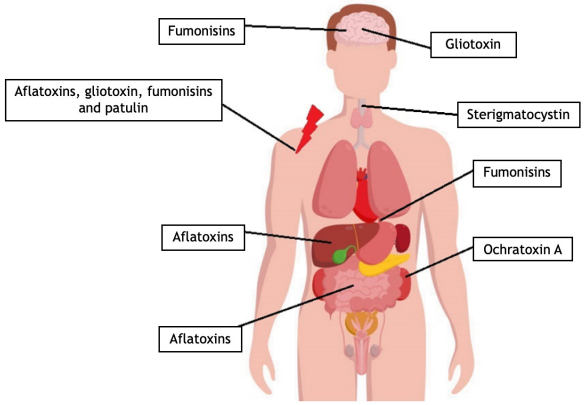

FIGURE 4 | Toxicological effects of mycotoxins in the human body. Fumonisins can alter the sphingolipid metabolism, and it has an effect on the membrane of different cells like neurons. Fumonisins may increase the possibility of esophageal cancer formation. With different molecular pathways, aflatoxin, gliotoxin, fumonisin, and patulin can suppress several immunological mechanisms. Aflatoxins affect the pairing of nucleotides. Mutations of proto-oncogenes or tumor suppressor genes can cause liver cancer. Aflatoxin metabolites produced by the hepatic CYP enzymes can lead to chromosomal DNA strand breaks. Aflatoxins can inhibit cell proliferation. In the gut, mycotoxins can interfere with the regeneration of the gastrointestinal tract forming cells. Gliotoxin can penetrate the blood–brain barrier, and due to its cytotoxicity, it can damage the astrocytes. Sterigmatocystin may cause esophageal cancer. Ochratoxin A is nephrotoxic and can cause kidney damage, cancer, or renal failure. OTA was recently connected to Balkan Endemic Nephropathy (BEN) kidney disease and chronic interstitial nephropathy (for references, see the text).

enzymes and cytokines, altered membrane functions, and significantly decreased ROS production, defects in phagosome–lysosome fusion, and phagocytosis (Wichmann et al., 2002).

OCCURRENCE OF *Aspergillus*-DERIVED MYCOTOXINS IN THE FEED AND FOOD CHAIN

Several studies have been carried out in order to set appropriate food safety regulations and recommendations (see **Table 1**). These regulatory actions, however, must pursue reasonable trade-offs to avoid unreasonable food wasting and to regulate trade economic effects (Marroquín-Cardona et al., 2014; Dellafiora et al., 2018). About 20–25% of the harvested fruits and vegetables are lost due to various post-harvest diseases primarily caused by molds even in developed countries, and this loss can even be more severe in developing countries (Medeiros et al., 2012). The average annual economic loss attributable to mycotoxin contamination is about 1 billion USD in the United States alone (Amaike and Keller, 2011). AFs are leading the list of the most harmful mycotoxins when economic losses as well as agricultural and health threats are considered and evaluated (Amaike and Keller, 2011). The European Union (EU) Rapid Alert System for Food and Feed (RASFF) was created in 1979, which is currently based on the Regulation 178/2002 (European Parliament and of the Council, 2002). The EU members can exchange information on hazards in food through the Alarm System. Six types of notifications are in use: alerts, information, information for attention, information for follow-up, border rejections, and news; however, the last one is not available for AFs. When a toxin-containing food appears on the market, rapid action, like product recall, is necessary and an alert notification is sent to RASFF as well. Nearly 90% of the reported risks come from outside of the EU (**Figure 5**); thus, border rejections are sent to all external border posts of the EU to secure that the contaminated product does not enter through other entry points (Pigłowski, 2018).

January 2009 the most commonly infected plants are cereal crops, like maize and wheat, as well as cotton, soybean, and different forms of nuts, especially groundnuts (Jelinek et al., 1989; Dharumadurai et al., 2011). Fungal growth and toxin contamination are the consequence of interactions among fungi, the host, and the environment. As mentioned above, animals can act as transmitting agents, as meat, milk, or eggs can pass AFs to species in the food chain (Völkel et al., 2011; **Figure 6**). Food processing can increase or decrease the concentration of AFs. For instance, AFM1 is associated with protein fractions of the milk. It is worth noting that AFM1 is heat-stable and binds to casein and, hence, tends to accumulate in cheese (Sengun et al., 2008; Busman et al., 2015; Benkerroum, 2016). Milk products like different types of cheese can have three to five times higher concentration compared to bulk milk, while butter or yogurt processing can significantly decrease the concentration (Govaris et al., 2001; Iha et al., 2013). Another group reported that cocoa butter transmitted no infection from the originally infected cocoa beans (Turcotte et al., 2013). Tropical and Mediterranean climates facilitate the production of AFs, as toxin production of *A. flavus* and *A. parasiticus* is reported between 28 and 35°C (average, 30°C), but some fungi stop the synthesis of AFs above 36°C (**Table 2**; Yu et al., 2008). These factors mean that ingredients from these regions have higher risk of AFs contamination (Battilani et al., 2016).

Not surprisingly, data for dietary intake of mycotoxins are available in many countries for different age cohorts including children and infants (Marin et al., 2013). The physiological effects of mycotoxins and the assessed health risks for children and infants are different from those of adults (Sherif et al., 2009; Raiola et al., 2015). A recent study on Gambian infants revealed an effect of AF exposure on the growth of infants (Watson et al., 2018). Although further research is needed, AF content of baby food might cause growth impairment in children. Even though the WHO designated AFB1 and AFM1 as Class 1 carcinogens, some levels of consumption can be tolerated. The safe content of the derivatives of AFs depends on the foodstuffs. Limits in the EU are between 2 and 8 μg/kg AFB1 in foodstuffs dedicated for adults and 0.1 μg/kg AFB1 in baby foods for infants and toddlers. Regarding AFM1, the limits are lower, particularly 0.025 μg/kg in dairy products, including infant formula. The overall content of AFB1, AFB2, AFG1, and AFG2 in different foodstuffs is not allowed to be higher than 15 μg/kg (EC 1881/2006, 2006). Risk assessment analysis indicated that the hazard index for children under the age of 3 years was considerably higher than that for adults, which supports the need for more effective mycotoxin risk assessment and self-control

TABLE 1 | *Aspergillus*-derived mycotoxins and *Aspergillus* spp. that produce them, high-risk foods, maximum levels in EU, FDA levels, and guidance values by WHO.

Mycotoxin	Producing fungi	High-risk foods	EU Maximum Level	FDA levels	Guidance value by WHO
Aflatoxins (AFB1, AFB2) (AFG1, AFG2, AFM1)	*A. flavus, A. parasiticus* *A. parasiticus*	Maize, wheat, barley and other cereals, peanuts and oil seeds, cottonseed, coffee and cocoa beans, figs and dried fruits, spices, milk and dairy products	AFB1 2–8 μg/kg sum of AFs 4 15 μg/kg AFM1 0.025–0.050 μg/kg baby and infant foods 0.10 μg/kg	Foods 20 μg/kg Milk 0.5 μg/L	PTWI is not established
Fumonisins (FB2, FB4)	Predominantly *Fusarium*-derived mycotoxins, but also produced by *A. welwitschiae* and *A. niger*	Maize, wheat, barley, rice, millet, oats, coffee beans, grapes	Unprocessed maize 4 mg/kg maize-based foods 1 mg/kg cereals and snacks 800 μg/kg baby and infant foods 200 μg/kg	2–4 mg/kg	PMTDI 2 μg/kg bw
Ochratoxin A (OTA)	*A. ochraceus, A. carbonarius, A. niger*	Maize, wheat, barley, legumes, oil seeds, peanuts, coffee beans, cocoa beans, dried fruits, grape juice and wine, spices, meat products	Unprocessed cereals 3 μg/kg coffee beans 5 μg/kg dried fruit 10 μg/kg juice and wine 2 μg/L dried spices 15 μg/kg baby and dietary foods 0.5 μg/kg	No level set (Mitchell et al., 2016)	PTWI 112 ng/kg bw
Patulin (PAT)	Predominantly *Penicillium*-derived mycotoxin, occasionally also produced by *A. clavatus* infestation of feed and food stuffs	Apples, grapes, many fruits, juice, cider, tomatoes	Fruit juice and cider 50 μg/L baby foods 0.10 μg/kg	Apple juice 50 μg/L	PMTDI 0.4 μg/kg bw
Sterigmatocystin (STC)	*A. versicolor,* *A. nidulans*	Maize, wheat, peanuts, oil seeds, coffee beans, milk and dairy products	No data	No data	PTWI is not established
Gliotoxin (GTX)	*A. fumigatus*	Cattle feed, mussel	No data	No data	PTWI is not established

PMTDI, provisional maximum tolerable daily intake; PTWI, provisional tolerable weekly intake. European Union (Regulation 1881/2006), US FDA–chemical contaminants, metals, natural toxins, and pesticides guidance documents and regulations, JECFA–Joint FAO/WHO expert committee on food additives.

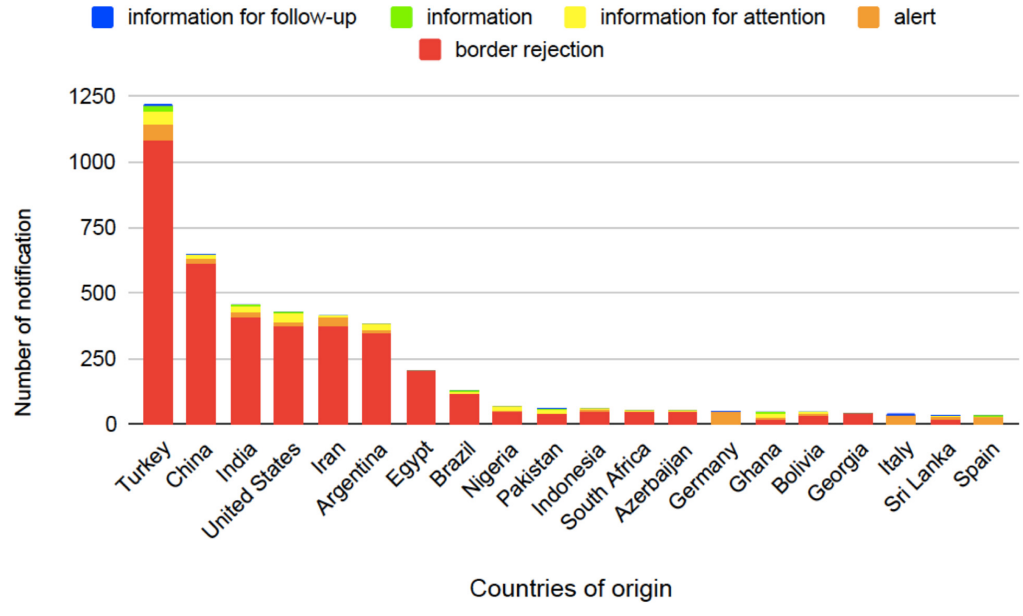

FIGURE 5 | Countries of origin for aflatoxin-related notifications in food based on the European Union (EU) Rapid Alert System for Food and Feed (RASFF) database from 1st January 2009 until 27th June 2019 (European Parliament and of the Council, 2002).

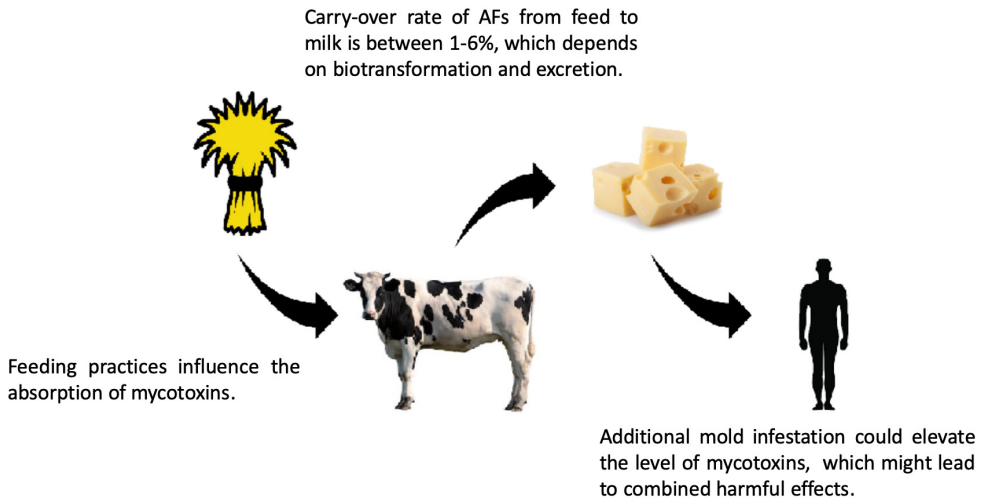

FIGURE 6 | Risk of mycotoxin exposure in the feed and food chain. Mycotoxins, like aflatoxins (e.g., AFB1), go through biotransformation in the livestock and different metabolites are produced, such as AFM1, which can be excreted into the milk, where AFM1 can bind to casein. After digestion, AFM1 is released from the casein – AFM1 complexes. The consumption of high amounts of dairy products contaminated with AFM1 can lead to acute mycotoxicosis (for references, see the text). The carry-over rates of mycotoxins show seasonal differences, and there are other diverse factors influencing the prevalence of carry-over, e.g., the quantity of mycotoxins in the feed and the excreted amount of toxin in the milk. The geographical location and feeding practice could also affect the carry-over rates, which could be even 6%, regarding AFs (Völkel et al., 2011).

TABLE 2 | Growth conditions of some *Aspergillus* species and their optimum temperature for mycotoxin production.

Fungi	Mycotoxins	Growth temperature	Optimal toxin production temperature	Optimal growth pH	Water activity	References
A. flavus	AFB1, AFB2	25–30°C	28–35°C	5–6	0.94–0.97	Lahouar et al., 2016; Stein and Bulboacă, 2017; Frisvad et al., 2019
A. parasiticus	AFB1, AFB2, AFG1, AFG2	15–33°C	28–35°C	5	0.95–0.99	Mannaa and Kim, 2017; Stein and Bulboacă, 2017; Frisvad et al., 2019
A. niger	FB2	24–37°C	25–30°C	5	0.97–0.99	Mogensen et al., 2009; Passamani et al., 2014
A. versicolor	STC	30°C	23–29°C	3.1–10.2	Min. 0.76	Veršilovskis and De Saeger, 2010; Stein and Bulboacă, 2017
A. ochraceus	OTA	24–37°C	31°C	3–10	Min. 0.8	Reddy et al., 2010
A. clavatus	PAT	24–26°C	25°C	4.7	0.87	Zutz et al., 2013
A. fumigatus	GTX	under 42°C	37°C	7.35–7.45	0.92–0.97	Alonso et al., 2016

These representative data could be influenced by different environmental circumstances.

strategies in the milk industry (Farkas et al., 2014; Trevisani et al., 2014; Kerekes et al., 2016; Ortiz et al., 2018).

The ochratoxins produced by strains of *A. ochraceus*, *A. carbonarius*, and *A. niger* are often present together in food. OTA can be found in a variety of agricultural products, especially in cereals, grapes, and related products (Streit et al., 2012; Tsitsigiannis et al., 2012). This mycotoxin occurs naturally and is widespread around the world, but mainly in the Mediterranean Basin, including Italy, Spain, and Greece (Covarelli et al., 2012; Somma et al., 2012; Perrone et al., 2013; Arroyo-Manzanares et al., 2019), and furthermore, in several African countries like Cameroon, Senegal, Benin, and Nigeria (Rodrigues et al., 2011; Tang et al., 2019). OTA can also be considered as a potentially emerging mycotoxin in Central Europe due to the climate change (Tóth et al., 2013; Pleadin et al., 2017; Udovicki et al., 2018). The most common types of food bearing OTA are cereal grains, oil seeds and tree nuts, wine, wine grapes and dried fruits, spices, herbs and herbal teas, cocoa powder, and coffee beans. Ochratoxins are food-borne mycotoxins, and this post-harvest contamination can appear if crop-drying practices are suboptimal and delayed (Reddy et al., 2010; Bui-Klimke and Wu, 2015). Analysis of several food and feed samples were performed with enzyme immunoassays, which gave detection limits of 0.5 to 5 μg/kg. Intoxicated dry beans could bear 5–30 μg/kg, whereas maize can bear 10–50 μg/kg, and green coffee beans contain 18–48 μg/kg. Even as low as 0.16 μg/L and 0.24 μg/L of OTA could be detected in South African white and red wines, where the detection limit was above 0.01 μg/L (Reddy et al., 2010). Ordinary food processing is not able to eliminate or substantially reduce the quantity of OTA in foods and beverages. Furthermore, processed food products such as sausages and bread were also found to contain OTA since it is a chemically very stable compound. The EU set the maximum permissible levels of OTA in unprocessed cereals at 3 μg/kg, in roasted coffee beans at 5 μg/kg, in

dried fruits at 10 μg/kg, in fruit juice and wine at 2 μg/kg, in dried spices at 15 μg/kg, and in dietary and baby foods at 0.5 μg/kg (EC 1881/2006, 2006). The United States Food and Drug Administration (FDA) has not set maximum regulatory limits for OTA in food (Mitchell et al., 2016). Based on a detailed WHO risk assessment, including hazard identification, hazard characterization, exposure assessment, and risk characterization, the Joint Expert Committee on Food Additives (JECFA) issued an official statement on OTA and have set provisional tolerable weekly intake at 112 ng/kg bw, which was later rounded down to 100 ng/kg bw. The limit was set based on various dose–response studies on animals. The average weekly OTA intake in Europe is 8–17 ng/kg bw, being well below the advised limit (Bui-Klimke and Wu, 2015).

Patulin is produced by many different molds, which need special, e.g., dirty, wet environments for spreading (Puel et al., 2010; Ioi et al., 2017). Although mostly *Penicillium* spp. have been isolated from food with PAT contamination in the moderate climate belt, some recent studies have provided us with new insights into PAT occurrence in food, which are mainly connected to climate change. Inadequately stored cereals, e.g., under high moisture conditions, can lead to the colonization by *A. clavatus*, which is also responsible for the PAT contents of food in tropical and sub-tropical regions. It is hard to estimate the contribution of these molds to the PAT contents of foods and feeds precisely but the role of *Aspergillus* spp. in global PAT exposures should not be underestimated. Furthermore, *A. clavatus* may also colonize malted barley and wheat, which might also contribute to the appearance of PAT in the feed and food chain (Lopez-Diaz and Flannigan, 1997; Loretti et al., 2003; Sabater-Vilar et al., 2004). Different food products, like vegetables, rotting apples, grains, and fruits may contain primarily *Penicillium*-derived PAT (Puel et al., 2010; Wright, 2015; Frisvad, 2018; Vidal et al., 2019). As this is a quite stable secondary metabolite, it can withstand various harsh processing steps, such as milling and heating. Apples and apple derivatives have the highest concentration of PAT, and a maximum of 16 mg/kg has been reported so far (Pal et al., 2017). Although the incidence of PAT contamination is fairly high worldwide (Schatzmayr and Streit, 2013) commercial apple juices normally contain less than 10 μg/kg of PAT (Pal et al., 2017). Because PAT remains stable during apple processing, PAT detection is often used as a quality control parameter, indicating whether or not moldy apple was processed (Karlovsky et al., 2016). During ethanol fermentation, *Saccharomyces cerevisiae* can destroy PAT and, hence, ciders and other fermented fruit drinks will not contain this toxin (Yu et al., 2008), except when fresh fruit juice is added to the cider after fermentation. Due to its toxicity and potential harm to human health, according to JECFA, the provisional maximum tolerable daily intake of PAT is 0.4 μg/kg bw. PAT contaminations present in different food products are mainly hazardous for special age cohorts, such as infants and elderly people and also for gravidae. Since 2006, the European Commission and China have set the maximum limit for PAT to 50 μg/kg in fruits, while for products dedicated to younger people, the limit has been set to 10 μg/kg (EC 1881/2006, 2006; Ji et al., 2017).

Fumonisins are among the most significant agricultural toxin. Although these mycotoxins are mainly produced by *Fusarium* species, like *F. verticillioides* and *F. proliferatum*, this paper focuses mostly on fumonisins produced by *Aspergillus* species (Kamle et al., 2019). Fumonisins can cause serious loss to agricultural production of cereals both in the field and during storage and can be dangerous to animals and humans as well (Mudili et al., 2014). It has been shown that *A. niger* can be responsible for the presence of FB2 and FB4 (Varga et al., 2010). Since grapes, wines, dried fruits, and grape-derived products have a significant importance worldwide, the presence of *A. niger* and *A. welwitschiae* in the global grape and wine production chain has a high importance. When the temperature is below 30°C, several molds are responsible for the observed varying mycotoxin exposures, but when the temperature is higher than 37°C, predominantly black *Aspergilli* are responsible for FB2 and FB4 contents of these foods and drinks. The spreading of these species is even faster when the storing conditions are not optimal, and physical damages on the berries also help fungal invasion (Logrieco et al., 2011; Storari et al., 2012; Onami et al., 2018). Other commonly infected food grains are maize, wheat, barley, rice, millet, oats, and rye, but fumonisins are present in coffee beans, too (Palencia et al., 2010; Varga et al., 2010; Mudili et al., 2014). The most endangered species are horses, pigs, and humans through direct ingestion. Importantly, Mediterranean climate supports the spread of FB2 producer black *Aspergilli*, as their optimum temperature for growth lies between 25 and 30°C, with the upper and lower limits of 42 and 12°C (Mogensen et al., 2009).

Fumonisins are recognized by authorities and official limit values have been issued. FDA has set the safe intake limit to 4000 μg/kg for food products containing whole maize grains and 2000 μg/kg for products made with dried milled maize products. Animal feed limits depend on the targeted animal, so the limits can range from 5 to 100 mg/kg (FDA, 2001). JECFA and the European Commission Scientific Committee for Food have set the tolerable daily intake level of FB1, FB2, FB3, or their combination at 2 μg/kg bw. The EU has defined the maximum permissible levels for the sum of FB1+FB2 in unprocessed maize at 4000 μg/kg, in maize-based foods at 1000 μg/kg, and in cereals or snacks at 800 μg/kg. The maximum limit is 200 μg/kg in processed foods for infants and toddlers (Commission of the European Communities, 2006). Similar US regulations set 2000–4000 μg/kg levels for the sum of FB1+FB2+FB3 depending on the foodstuff (Bryła et al., 2017; Zhang et al., 2018).

Sterigmatocystin producing *Aspergillus* species, mostly *A. versicolor*, infect mainly grains and grain products. Part of the STC content in the food and feed are usually converted to AFs by aflatoxigenic species, e.g., *A. nidulans*. The impact of STC may appear smaller than AFs in the case of human intake, but the importance of STC cannot be excluded (EFSA, 2013). The occurrence of STC has been shown in cheese quite often because AF-producing fungi are rarely present there. Previous STC measurements in cheese found toxin levels from 5 to 600 μg/kg (Díaz Nieto et al., 2018). STC occurrence in spices (fennel sample, red pepper, black pepper, and caraway seeds) was also reported from African and Asian countries. For cereals,

STC was reported in barley, wheat, rye, and oat, concentrations being around 10–60 μg/kg from some European countries. As traditional Chinese medicine is based on plants, STC was also reported in these medicinal plant products, too. We cannot state that STC occurrence in cheese is because of the feed as the rate of carry-over of STC into milk when ruminants are exposed to contaminated feed has not been inevitably proved. Moreover, no information is available about the transfer of STC and/or its metabolites into other animal products such as meat and eggs. The exact toxicity of STC in livestock is not clear, as no signs of toxicity were observed in sheep, when a feeding trial at the highest dose were performed (16 mg/kg STC in feed, estimated as equivalent to 0.3 mg/kg bw per day) (EFSA, 2013; Díaz Nieto et al., 2018). As risk characterization is not possible for STC, several international organizations recommend that more accurate data for STC in food and feed across European countries need to be collected. In case of food, methods with an LOQ (limit of quantification) of less than 1.5 μg/kg should be applied, whereas for feed, the available information is insufficient to make a recommendation. The development of suitable certified reference materials and/or proficiency tests to support analytical methodology should be encouraged (EFSA, 2013; Díaz Nieto et al., 2018). As the structure of STC and AFs are similar and metabolites are often common, analytical method development (immunoassays, isotope assays, etc.) and differentiation assays are needed to differentiate between these mycotoxins.

PREVENTION STRATEGIES OF MYCOTOXICOSES

Current possibilities for the treatment of mycotoxin poisoning are still quite limited and are not specific. The best solution is, therefore, to prevent mycotoxins to enter the feed and food chain (Wagacha and Muthomi, 2008; Milićević et al., 2010; Kumar et al., 2017; Ortiz et al., 2018; Arroyo-Manzanares et al., 2019). The completely mycotoxin-free food and feed industry is, most likely, an irrational goal but the minimization of mold infestations and toxin deposition in the different agricultural products may be possible and can effectively prevent mycotoxin poisoning (Udomkun et al., 2017). It is important to state that the mold infestation is not equal to mycotoxin contamination. But the defense against all molds is favorable due to their effect on the economy (Wu and Khlangwiset, 2010; Ehrlich, 2014).

Aspergillus species can enter the food and feed chain at many stations of the industry (Gallo et al., 2015). The complex production systems, climate change, economic processes, and the resilience of the mycotoxins make it difficult to establish secure prevention protocols, sampling methods, and an international pipeline (Wild and Gong, 2009; Tasheva-Petkova et al., 2014; Bandyopadhyay et al., 2016; Paterson et al., 2017). The diverse factors that have an effect on the agricultural products can be divided into two groups: the pre-harvest and post-harvest circumstances (Jouany, 2007). Pre-harvesting factors include the production of crops, growing conditions, and the prevention of mold infestations in crops and other agricultural products (Kabak et al., 2006). Masked mycotoxins may mount an even greater risk to the consumers. It is well known that mold-infected plants may alter the chemical structures of mycotoxins as part of their defense mechanism against xenobiotics (Berthiller et al., 2013). The modified mycotoxins can generate deposits in the plant tissues and may remain hidden for conventional analytics. These masked mycotoxins might pose additional threats to human health and also represent further challenges to both global food safety and the scientific community working in this field. Obviously, to gain reliable and reproducible data on the masked mycotoxins present in feed and food, we need new analytical methods and also novel *in vivo* experiments. To lessen the possibility of mycotoxin exposures, it is important to raise awareness among the food- and feed-producing countries with educational campaigns. There are numerous options to lower the mycotoxin content of crops before harvest (Sundh and Goettel, 2013; Mahuku et al., 2019). Preventing mold infestations, limiting the spread of molds to other plants, or neutralizing the mycotoxins already at pre-harvest are all good examples and may hold great potentials. Competitive but atoxigenic mold species and variants can supersede toxin-producing *Aspergillus* species and, hence, are suitable candidates in the elaboration of various biocontrol strategies (Kagot et al., 2019). Large-scale monoculture farming is highly prone to mold infestations, and this tendency may strengthen further with changing climate. Cultivating more diverse crop variants with different harvest dates on smaller areas can effectively mitigate the risks of subsequent mold infestations. There are possibilities to reduce the mycotoxin production even if the mold infestation is present in crops (Pfliegler et al., 2015). Co-cultivating the crops with genetically modified plants or microorganisms might alter the chemical structure of mycotoxins *via* changed metabolic pathways as part of the defense against xenobiotics (Berthiller et al., 2013). Vitamin C may regulate the genes of mycotoxin production, inhibiting the expression of toxin-producing enzymes (Akbari Dana et al., 2018). With polyculture farming on timed planting and with modern methods like environmental stressors to prevent the infestation, the economical and medical effects of the mold contamination and mycotoxins could be minimized (Abramson et al., 1997; Atehnkeng et al., 2014). Before the time of harvest, an extensive examination of the crops should precede any other procedures (Cleveland et al., 2003), since after harvest it is much harder to reveal the contamination. The infected field should be decontaminated by immediate harvesting and discarding the contaminated crops to prevent further spreading.

The largest part of the threats is the post-harvest factors. These include the harvesting criteria, the transporting circumstances, the storage conditions before, and, after the processing steps, the sampling methods, the inspections and toxin detection protocols, and the international pipelines and regulations about the amount of the mycotoxins contained in the foodstuffs (Zain, 2011). The circumstances of storage are also crucial. Sorting before the storage of crops is essential (Fandohan et al., 2005) since in large storage facilities the mold infestation can spread more easily between the different portions of the harvested crops (Hell et al., 2000; Williams et al., 2014). On the other hand, the correct cleanliness of the storage buildings is also critical (Adda

et al., 2011), since if the storage conditions are not correct or even favorable for the growing and spreading of mold, it could lead to huge economical and financial losses or even medical crises (Hussein and Brasel, 2001; Khlangwiset and Wu, 2010). Inappropriately chosen storage parameters like concomitantly high temperature and humidity can propagate mold infestations. Therefore, ingredients should be dried and/or cooled to prevent or at least limit fungal growth.

International pipelines and regulations have already been put into operation to find the occurring mold infestation and mycotoxin contamination as early as possible, but not everyone keeps the rules. In the current ecological situation, the ingredients of a product may come from all over the world. In the case of such multifactorial systems, it is even more difficult to control every aspect and, therefore, the ingredients should be investigated individually.

If the mold infestation remained undetected and the mycotoxin deposits are already formed, there are still possibilities to lower the toxin levels (Yang et al., 2014; Udomkun et al., 2017; Omotayo et al., 2019). Here, we outline the advantages and disadvantages of some mycotoxin decontaminating methods currently used in the agriculture and food industry and also aim to evaluate some foreseeable future tendencies in this field. Even though the toxins are heat-stable in a 150–200°C temperature range, their amount can still be lowered effectively by heating (Herzallah et al., 2008). This amount of heat can be problematic; in the case of heat-sensitive substances, the administered heat has therefore to be limited. Because of the remarkable heat stability of the *Aspergillus*-derived toxins and the high thermal sensitivity of some valuable nutrients and vitamins, any possibility for decontamination by heating should be considered with care. Under mild conditions, the efficiency of mycotoxin decomposition might be low because most mycotoxins are heat-resistant within the range of usual food-processing temperatures (80–121°C) (Bullerman and Bianchini, 2007; Kabak, 2009; Karlovsky et al., 2016).

Ionized radiation produced by gamma rays can also be used to lower the toxin levels (Ghanem et al., 2008; Jalili et al., 2010). As the large-scale application of this technique, it is quite difficult and it is usually applied as the last step in the food production, when the commodities have already been packed. In 2015, the International Atomic Energy Agency (IAEA) in partnership with the Food and Agriculture Organization of the United Nations (FAO) released the manual of good practice in food irradiation aiming at improving food irradiation practices worldwide, with a focus on developing countries (Di Stefano and Pitonzo, 2014). The dose for package sterilization is set between 10 and 20 kGy while the different foodstuffs like dried materials or spices are irradiated with 30–50 Gy. Any overdosing on gamma rays is contraindicated because it may induce the degradation of valuable nutrients and the formation of other toxic compounds. While this is a good method to lower the toxin content, in the case of rural food production when the crops are harvested for strictly personal use, it is not perfect. However, portable food-irradiation machines are accessible, although problems with financing and operating difficulties limits their usage (Roberts, 2016). Although irradiation tools may be quite complicated and may require a more advanced technical background, irradiation may represent a reliable and safe alternative for the decontamination of *Aspergillus*-derived mycotoxins in the future. There is also a more complex side of the reduction of mycotoxins by gamma irradiation. Especially in the case of high starting toxin concentration, radiolytic mycotoxin forms may be generated due to irradiation (Wang et al., 2011; Yang, 2019). Although the toxicological effects of the intact toxins and their radiolytic decomposition derivatives were compared, the radiolytes had significantly less impact on human health; the possible toxicological effects of the latter need further investigations. In the future, the foreseeable increases in the mycotoxin contents of different food commodities could bring the effects of these radiolytic mycotoxin degradation products into the spotlight.

Ozonation can also be an effective and reliable detoxification method. In the case of ozonation, oxygen radicals are generated through splitting of reactive ozone molecules, which then affect different contaminants. The application of ozone can be in both gas and liquid forms. One downside of this method is that the effective ranges of these radicals are short, and, hence, they cannot penetrate deeply into the different substances. The treatments must be used on a large surface, which is only achievable in the end of food production, just before the packaging. This protocol has the same disadvantage as irradiation does, as it cannot be applied on large quantities of foodstuff at the same time.

Besides physical toxin reductions, there are chemical substances available to change the properties of the mycotoxins and lower their physiological activities (Bryła et al., 2017). These methods are very popular due to the fact that most of the effective chemical components like citric, lactic, tartaric, hydrochloric, succinic, acetic, and formic acids are already in use in the food industry (Méndez-Albores et al., 2005, 2009; Wu and Khlangwiset, 2010). The chemical treatment can be acidification, ammonization, or ozonation (Karaca and Velioglu, 2014). Every procedure can be accelerated with increased temperature; otherwise, these methods would take days.

There are also biological methods to prevent and neutralize mycotoxins in food and feed stuffs (Komala et al., 2012; Quiles et al., 2015; Sultana et al., 2015). Biocontrol methods can give rise to the most effective prevention techniques in the future, and some methods have already been used with promising results. These protocols use different biocontrol agents (BCAs), which can modulate mycotoxin contaminations in various ways. These agents can be different microorganisms like other, atoxigenic but highly competitive, fungi, which can limit the spreading of the mycotoxin producer strains. One possibility is the inoculation of different microorganisms like *Lactobacillus* or *Saccharomyces* into the toxin-contaminated foodstuff (Tsitsigiannis et al., 2012). In addition, the application of yeasts in various technological processes may have a direct inhibitory effect on toxin production of certain molds, which is independent of their growth suppressing effect (Pfliegler et al., 2015). Other genetically modified BCAs can produce different substances like Vitamin C, which can silence the gene clusters responsible for mycotoxin productions. Furthermore, different enzymes obtained from various *Bacillus* species showed high

efficiency, but they have not been tested on a large scale. Plant extracts with various enzymes might also be effective. The different methods work synergistically, through the degradation of the toxin, decreasing the active form of the mycotoxin or just binding to the toxin and reducing the free toxin ratio. These procedures are fairly effective, but their timescale is too long (48–72 h) and the methods are difficult to apply on large quantities, which mostly excludes them from industrial applications.

The combination of different methods can lead to reliable protocols that can be used to reduce the mycotoxin levels in the contaminated food and feed (Udomkun et al., 2017). The combinations can also be effective in cases when the properties of the target material limit the use of some toxin-decreasing procedures. The detoxification methods are essential in the fight against the mycotoxins but the wide array of toxin types and their different effects and physical and chemical properties make it difficult to find a universal solution (Omotayo et al., 2019). The best solution is to minimize the occurrence of mycotoxins in the food and feed industry.

MEDICAL ASPECTS OF *Aspergillus*-DERIVED MYCOTOXINS

Despite prevention methods and strict regulations, mycotoxins are still present in the feed and food chain, and the diseases caused by dietary toxic fungal exposures are called mycotoxicoses (Peraica et al., 1999). The processes of mycotoxin poisonings have been partially cleared, but due to the multivariate nature of the food and feed contaminations and to their not yet fully understood metabolisms, the human side of poisoning needs further investigations. The medical data presented here are mainly acquired from large-scale toxin exposures as those recorded in the acute poisoning outbreak in Kenya in 2004 with 125 deaths (Probst et al., 2007), in Tanzania during 2016 with 68 affected individuals, or in the former members of Yugoslavia (Klarić et al., 2013). While the mycotoxins can enter the body through the skin or the respiratory system, the most common entry point is the gastrointestinal tract (Hedayati et al., 2007). The manifested symptoms depend on the type and form of digested mycotoxins, the amount of intake, the duration of poisoning, age, sex, genetic background, and the health status of the patients (Marroquín-Cardona et al., 2014; Dellafiora et al., 2018; Keller, 2019). The absorption of the different forms of the toxins depends on several factors (Gallo et al., 2015). In the human body, the toxins undergo a detoxification process and may form deposits mostly in the liver, but other tissues could also store them. The mycotoxin derivatives formed *in vivo* in humans and domestic animals may still have pathological effects. As mentioned earlier, mycotoxins can have nephrotoxic, genotoxic, teratogenic, carcinogenic, and cytotoxic properties but are also capable of affecting tumor development due to their antineoplastic potential (Pócsi et al., 2018).

Mycotoxicosis like most types of poisoning can be acute or chronic. Acute poisoning has a rapid onset and characteristic toxicity symptoms, like gastrointestinal discomfort, general malaise and fatigue, or diarrhea due to the damage of the enterocytes. Acute poisoning may occur when large quantities of mycotoxin are consumed in a short period of time. The incidence of acute mycotoxicosis is sporadic. In acute poisoning, the type of mycotoxin exposure can change the mechanism of the disease. The most frequent symptom being acute hepatitis elicited by the toxins. The occurrence of mycotoxin-inflicted hepatitis depends on many factors, e.g., Kwashiorkor, where the resistance of the affected individual to harmful stressors is generally decreased (Shephard, 2008). Other hepatotoxic conditions such as viral hepatitis infections, heavy metals, or alcohol and drug use can propagate the emergence of hepatitis (Saha Turna and Wu, 2019). The chronic mycotoxin poisoning is a worldwide problem. Compared to acute poisoning, the incidence is higher, even so that not all chronic mycotoxicoses are documented. Chronic poisoning is usually a consequence of a low-dose exposure over a long time period, which might result in irreversible effects such as neoplastic diseases (Wu and Santella, 2012; Magnussen and Parsi, 2013). Several factors influence the chronic toxicity of mycotoxins or the occurrence of the first noticeable symptoms. These include the dosage, route of exposure, and the overall health of the affected individual. During chronic mycotoxin exposure, the effects are extensive. The abovementioned basic molecular malfunctions are distinguished but the clinical appearances are varied. The symptoms are slow to appear and hard to connect to a specific disease. This is even more difficult when the mycotoxin exposure is irregular, the nutritional status is not stable, and other factors may alter the overall medical status.

It is not easy to distinguish between acute and chronic toxicities in mycotoxicoses because these diseases can easily be mistaken for other common illnesses with similar symptoms. The current understanding of *Aspergillus*-derived mycotoxins still relies on some case studies (Smith et al., 2016; Udovicki et al., 2018). There are possibilities to measure the mycotoxin levels in the patient's urine and blood, but without knowing any intake ratio, it is hard to interpret these pieces of information (Escrivá et al., 2017). Although there are some data recorded in larger mycotoxin outbreaks in the third world, any connection between the mycotoxin levels and the severity of the symptoms is difficult to establish. On account of the individual differences, patients with no detectable toxin levels showed symptoms, but these findings could be the consequences of other unrelated diseases. Furthermore, affected individuals with the same mycotoxin urine concentrations had different symptoms (Peraica et al., 1999). There is a well-documented case when a young woman tried, but failed to commit suicide with purified AFB1 (Willis et al., 1980). She took 5.5 mg of AFB1 over 2 days, and a half year later, a total amount of 35 mg in a 2-week period. Different diagnostic methods like X-ray and ultrasound of the liver or urine and blood tests showed no pathological results throughout the years. The lack of symptoms or any other abnormalities in physiological parameters can be explained by her good physical condition and nutritional status.

Combined Effects of Mycotoxins

Multiple mycotoxicoses may also occur because the human diet is a complex mixture of various ingredients. Simultaneous

spoiling of food by more than one toxigenic fungus has been reported many times. Moreover, some fungi are able to produce a broad spectrum of mycotoxins, and it is confirmed that combined physiological effects of mycotoxins are as relevant as the toxicity of a single mycotoxin. The harmful effects of simultaneous exposures to mycotoxins cannot be predicted solely relying on their individual toxicities. Additive, synergistic, or less than additive toxic effects have been proven among different mycotoxins. For example, interactions were shown between OTA, AFs, and their metabolites in a dose-dependent manner, and in lower concentration ranges, their effects were additive. The explanation resides in the fact that both toxins affect DNA pairing and duplication so they could induce carcinogenic malformations. At higher concentrations, the combined effect was less than additive, but it cannot be called antagonistic. The different physiological effects were explained by the fact that AFs and OTA went through the same bioactivation routes by CYP enzymes in the liver; thus, the amount of bioactivated, potent toxin forms was less compared to the separated experiments (Klarić et al., 2013). Combined effects of AFs, OTA, and fumonisins are "hot topics", but ongoing and future research should put more effort into the combinations of other emerging mycotoxins as well.

In order to understand the combined effects of different mycotoxins, researchers have developed various model systems. Although these experiments are still in their infancy, we aim at presenting some possible methods on how to analyze these effects. Most of the combined mycotoxin tests were done using binary or tertiary systems, and some of them are summarized in **Table 3**. Intestinal cell lines (e.g., Caco-2 or IPEC-J2) or gastric cell lines (e.g., NCI-N87) are widely used in cytotoxicity and transportation assays because the first host defense barrier against *per os* mycotoxin exposure is the gastrointestinal wall (Wang et al., 2018; Assunção et al., 2019). In order to describe the chronic-combined toxicological effects more accurately, further experimental data are needed, where sub-toxic mycotoxin concentrations should also be tested to simulate real food consumption habits. Obviously, all *in vitro* studies have their own limitations, but a 2- to 3-week-long mycotoxin treatment may represent suitable models of organ-dependent toxicities. Animal models are an efficient alternative to perform toxicity experiments owing to the known genetic background and strictly regulated diet (Alassane-Kpembi et al., 2017).

Although AFs, OTA, and FBs are all among those mycotoxins that have been already regulated worldwide, a regulation of the co-occurring different mycotoxins is still missing. This lack of regulations could be explained by several factors. For example, when a foodstuff is deemed to be contaminated by, e.g., AFs, it is not analyzed further, so other contaminations may remain hidden. However, this approach is favorable in terms of food safety and is financially acceptable as well, because the AF-affected food will be discarded anyway. The co-occurrence of different mycotoxins could be the consequence of either pre-harvest or post-harvest technologies. It has been shown that AFs and OTA can be found together mainly in cereals but herbs, spices, and dried fruits are also on the lists of potentially contaminated foods (Almeida et al., 2012; Smith et al., 2016). Furthermore, *A. niger* and *A. carbonarius* have been isolated frequently from grapes grown in Australia, South America, or Europe, and they are responsible for FB2 and OTA content of grape wine (Logrieco et al., 2011; Storari et al., 2012), and these two toxins could be responsible for several neoplastic changes in humans.

However, to set a rational limit for combined mycotoxin exposures, the exact concentrations of co-occurring mycotoxins should be determined, even when the individual concentrations are in the sub-toxic ranges. This will be an important goal for further research in this field because people may consume mycotoxins in sub-toxic concentrations without any detectable symptoms, but the combinations of these sub-toxic exposures may be deleterious (Anninou et al., 2014). An example for chronic-combined effects of mycotoxins could be when they

TABLE 3 | Some representative combination of different mycotoxins and their interaction types.

Mycotoxin couples	Doses	Model system	Exposure	Interaction type	Assays	References
PAT + OTA	PAT: 0.7–100 μM OTA: 1–200 μM	Caco-2 cell line	24 h	Synergism (Lower IC_{50} level) Less than additive (High IC_{50} level)	MTT, TEER	Assunção et al., 2019
AFB1 + OTA	AFB1: 5–25 μM OTA: 2.5–50 μM	Caco-2 cell line and HepG2 cell line	72 h	Synergism and nearly additive (effects were concentration dependent)	MTT	Sobral et al., 2018
STC + OTA	pM to μM	Hep3B cell line	24–48 h	Synergism and Less than additive (Concentration ratio dependent)	MTT, SCE	Anninou et al., 2014
STC + PAT	PAT: 5–30 μM STC: 0–35 μM	*T. pyriformis*	24 h	Synergism and Less than additive (Concentration ratio dependent)	Inhibition of cell proliferation	Mueller et al., 2013
STC + GTX	STC: 0–30 μM GTX: 0–3.5 μM	*T. pyriformis*	24 h	Synergism and Less than additive (Concentration ratio dependent)	Inhibition of cell proliferation	Mueller et al., 2013
PAT + GTX	PAT: 5–30 μM GTX: 0–3.5 μM	*T. pyriformis*	24 h	Synergism	Inhibition of cell proliferation	Mueller et al., 2013
AFB1 + GTX	AFB1: 0.5–128 μg/ml GTX: 2–500 ng/ml	HCE cell line	24–72 h	Synergism	Cell impedance, MTT	Bossou et al., 2017

TEER, transepithelial electrical resistance; SCE, sister chromatid exchange; MTT, cell viability assay.

target the same physiological pathways. Complex biological systems, like the immune system, where every aspect of the mechanisms is essential and strictly regulated, are very sensitive to multiple mycotoxin exposures. The production of leukocytes could be impaired due to the genotoxic properties of the mycotoxins and this can decelerate the division of the progenitor cells and, furthermore, the function of the differentiated leukocytes can also be inhibited. As some toxins can negatively affect the protein synthesis of leukocytes, signaling pathways, phagocytosis, and the differentiation of progenitors, the overall result might be a large-scale immunosuppression.

The Risks of Mycotoxins at Different Stages of Life

Mycotoxicosis can occur at every stages of life, and it can affect the individuals differently according to their age. The harmful effects of mycotoxins on cell division can lead to drastic consequences, which are even more severe during intrauterine life. There are some data on mycotoxicoses in children, infants, and even in embryonic stage, but these topics definitely need additional attention from the scientific community. Using human embryonic stem cells (hESCs), a research group showed the dose dependency of OTA toxicity (Erceg et al., 2019). More data could give us a clearer view on how different mycotoxin exposures affect the differentiation of hESC cells.

Mycotoxins mount variable challenges to humans at different stages of life (**Figure 7**). They can influence the production of gametes and thus the success of impregnation, because the cytotoxic effect of the mycotoxins could hamper the division and differentiation of the gametes and thus may cause infertility by interfering with, e.g., spermatogenesis (El. Khoury et al., 2019). Mycotoxins can damage the body of the mother, in the abovementioned ways, and can cause nutrition deficit in the embryo, but mycotoxins could also have more direct impacts. AFL, a derivative of AFB1, can go through the placenta and affect the embryo. This phenomenon was already documented in humans, but the adverse effects of this has not yet been fully investigated (IARC, 2015). From animal experiment, it is known that mycotoxins can increase the possibility of stillborn (Kanora and Maes, 2009). During lactation, AFM1 can also be excreted within the breast milk. These circumstances show the additional risks of mycotoxin poisoning in pregnant or breastfeeding women (Ortiz et al., 2018; Rushing and Selim, 2019). According to the above, mycotoxin poisoning is a significant risk to human development. The complex nature of mycotoxicosis can cause various symptoms, which are mostly connected to the cytotoxic and genotoxic properties of the mycotoxins. In newborns and young children, the symptoms may be more severe due to the fact that the mycotoxins like AFs and OTA have a general negative effect on cell multiplication. During development, the lack of adequate cell division may lead to a delay or retention in growth, mental retardation, and severe immunosuppression (Khlangwiset et al., 2011; Watson et al., 2018). *In vitro* studies showed that GTX can alter the connections between astrocytes and neurons, which could affect the formation of the brain and ruin cognitive development (Patel et al., 2018). As fumonisins inhibit sphingolipid biosynthesis, it is cited as a possible cause of neuronal tube defects (Lumsangkul et al., 2019). In children, the not yet fully developed and/or damaged gastrointestinal tract cannot perform its task and the pathways of nutrient absorption are compromised (Herrera et al., 2019). Malnutrition is thus a well-known adverse factor in mycotoxicosis since without sufficient nutrient intake, the body cannot cope with the damage caused by the toxins or any other external factors. Furthermore, the impaired digestive capability makes the treatment even more difficult and less effective (Lombard, 2014). For example, in developed countries, where apple juice is a popular beverage among children, PAT content of such soft drinks should be seriously regulated even though the

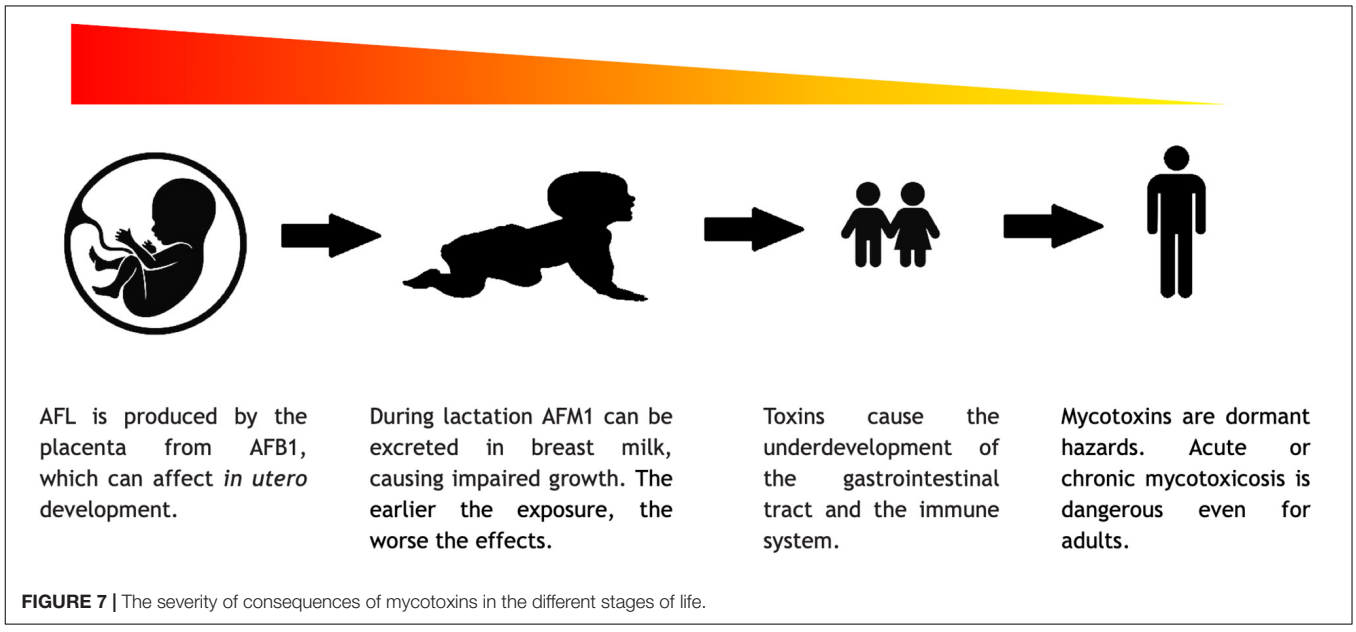

FIGURE 7 | The severity of consequences of mycotoxins in the different stages of life.

long-term deleterious physiological effects of PAT have remained yet to be fully understood (Pal et al., 2017). As mentioned earlier, it is hard to distinguish between the effects of malnutrition and mycotoxin exposures, although in the past two decades, governments tried to put effort in a more thorough mycotoxin monitoring. Blood, urine, and maternal milk specimens were mostly analyzed for AFs, OTA, and FB1 (Chen et al., 2018). From these data, the conclusion is that children are typically at high risk under mycotoxin exposures. However, to get a better insight into this matter, an international standard protocol should be introduced (Al-Jaal et al., 2019).

Although healthy adults are endangered as well, their detoxification system can usually handle an acute mycotoxin exposure. However, different environmental factors, like drug abuse, alcoholism, and malnutrition could act as a synergistic factor in mycotoxicoses. In the case of chronic exposures in adults, developmental disorders are not as significant as in childhood. However, tissues where high cell division rate is essential for their function are affected more by the harmful mycotoxins. Hematopoiesis, the function of the enterocytes, or the immune system requires sufficient cell-multiplication where the xenobiotics, like mycotoxins, could have a drastic impact on the cell cycle (Omotayo et al., 2019; Sobral et al., 2019). It is undeniable that the carcinogenic properties of the mycotoxins affect adults as well, and prolonged exposures may cause complex neoplastic diseases (Alshannaq et al., 2017). Throughout the aging process, the adaptive capabilities of the body are decreasing, which could propagate the manifestation of the abovementioned negative effects even earlier. These affected individuals need a complex and life-saving therapy, e.g., liver transplantations or immune therapies, which could take its toll on the global health system.

Therapeutic Procedures

It is undeniable that mycotoxicoses represent a serious threat to general health. The symptoms are treatable, although to set appropriate differential diagnosis is quite difficult. When investigating food poisoning, mycotoxins as the underlying factor may usually emerge only when there are no other possibilities left and all other contingencies such as viral gastrointestinal infections or bacterial enterotoxins have been excluded. In acute mycotoxin poisoning, the source of the exposure can easily be recognized as the contaminated foodstuff can be analyzed relatively easily and the given mycotoxin can be identified. However, there are no specific and effective treatments for the different mycotoxicoses until now (Hope, 2013). In the case of acute poisonings, merely the symptoms are usually treated, but these non-specific methods are rarely sufficient. The termination of the exposure to mycotoxins and an appropriate diet could better diminish the symptoms than any other medical procedure. Nevertheless, below, we list the most commonly used current methods to counteract mycotoxin poisonings (see also **Figure 8**).

Sequestering agents are non-absorbable materials that can bind and neutralize mycotoxins in the gastrointestinal tract (Phillips et al., 2002; Jard et al., 2011). These substances have a large surface-to-volume ratio and, hence, they have a large absorptive capacity. Activated carbon (charcoal) is a non-specific

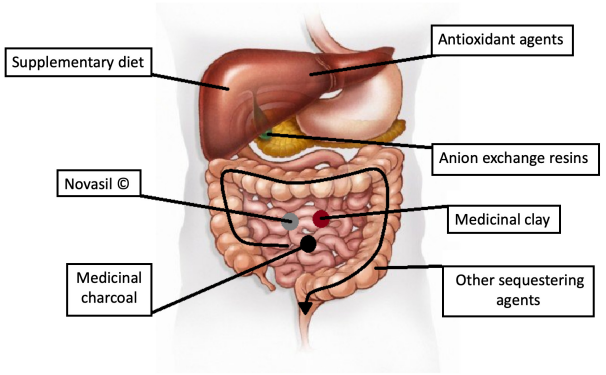

FIGURE 8 | Possible target points for therapy.

absorbent, and it is a useful agent in multi-mycotoxin poisoning. Clay is a widely studied material, especially in reducing the toxicity of AFs. Novasil® is a frequently studied agent and Phase II clinical trials showed the safety of this material. It has been shown that a 3-month-long treatment decreased the urinary concentrations of AFB1 adducts and reduced the AFM1 level in urine. Other trials were based on Novasil® delivery in capsule, in food, or added to water. Even a daily uptake of 3 g of this absorbent is safe for adults, but a trial in Ghana showed that 0.75 g/day is safe even for children (Hope, 2013; Watson et al., 2018). Cholestyramine (CSM) is an anion exchange resin and acts as a bile sequestering agent. It could reduce the enterohepatic recirculation of fat-soluble mycotoxins. *In vitro* studies showed that CSM has a higher affinity to OTA than to bile salts, and some animal experiments using CSM resulted in decreased plasma and urine levels of OTA but also in an elevated OTA secretion in feces (Hope, 2013).

The boosting of glutathione system may help in the detoxification process. As the detoxification capacity of liver varies with age, sex, and other factors, only boosting this detoxification system is not enough in the neutralization of mycotoxins. Other substances, like Vitamin C, E, D, or Q10 with zinc, could also help to prevent the harmful effects of ROS. Unfortunately, these materials are not specific, and their mechanism of action is based on the reduction of free radicals (Rea et al., 2009; Hope, 2013). Dialysis and other supplementary procedures to aid and protect the affected organs like the liver and bone marrow may also help.

The efficiency of the diagnosis of chronic mycotoxicoses is still low. The symptoms are non-specific and can be easily mistaken for other diseases. Without further clinical investigations, it is thus hard or nearly impossible to differentiate between mycotoxicoses and other diseases. The course of the disease may be modified when Kwashiorkor or other harmful effects such as alcohol are present or the mycotoxin intake is fluctuating. In chronic poisoning, the identification of the different mycotoxins in any feed and food is also difficult, because of the unknown time window of the poisoning. The termination of mycotoxin intake from the food chain and an

adequate nutrition can reduce the symptoms considerably in a short period of time. Complementary medical procedures should aid the damaged organs (Yilmaz et al., 2017), and liver or bone marrow transplantation may also be taken into consideration, when the affected organ is completely destroyed. The direct administration of T leukocyte cultures has been hypothesized to have a significant effect (Rea et al., 2009). However, the above mentioned possibilities of treatment are hard to propagate due to their complexity and high costs.

CONCLUDING REMARKS

The presence of mycotoxins in the feed and food chain has been a widespread problem since the beginning of human history. In the past, our possibilities to prevent or treat mycotoxin poisonings were rather limited. Today, with our current knowledge and technical capabilities, we are able to select and use highly efficient, verified methods to mitigate the deleterious effects of mold infestations. However, there are some newly emerging difficulties on the horizon of a mycotoxin free agriculture. The geographical border for harmful mold species like the toxigenic species in the *Aspergillus* genus is moving north as a clear-cut consequence of climate change. The possibility of mold infestations will rise and the size of the affected territory will drastically increase in the near future. To minimize agricultural, economical, and medical risks set by spreading mycotoxins, it will be essential to find a solution for the early detection and the prevention of mycotoxin contaminations. Obviously, there are possibilities to respond to these challenges adequately, but the ideal long-term solution would be a pipeline, which is accepted and followed with independent authorities worldwide to regulate and synchronize the joint community efforts in combating mycotoxins.

Genetically engineered crops could help us to fight off mold infestations even before mycotoxin contaminations have started. Cheap and reliable analytical methods are needed for the early and reliable detection of mycotoxins. The risk of mycotoxins on human health and economy could be neutralized with low-cost detoxification protocols, the implementation of which would require a minimal technical background. The continuous monitoring of storage and processing facilities is also a necessity. The production of foodstuffs with ingredients from different countries should be checked not only in the country where the primary commodity was produced but also in the destination countries once the final product is released into the market. Exposures to multiple mycotoxins may lead to unforeseen toxicological consequences and symptoms, which are currently not known or not investigated yet.

In summary, mycotoxicoses may be a much bigger threat to human health than they currently seem to be. For example, the exact number of people suffering from any kind of acute or chronic mycotoxin poisonings is almost impossible to calculate, and the long-lasting adverse effects of chronic mycotoxin exposures on human health have not been fully realized yet. The carcinogenic effects and developmental disorders might not be the most dangerous features of the *Aspergillus*-derived mycotoxins because they can contribute considerably to human infertility as well. Hence, the impacts of mycotoxins on future generations can be even more significant than we thought before. The increasing frequency of mycotoxin contaminations and the astonishing complexity and variability of multiple mycotoxin exposures might have severe and, at least in part, still hidden effects on public health. Nevertheless, the best time to act should be well before the mycotoxin-related problems have become uncontrollable because prevention is always better and cheaper than to cure an already manifested disease.

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All authors contributed to writing different sections of the manuscript, worked on compiling the figures, and selecting the appropriate references.

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REVIEW
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Adverse Effects, Transformation and Channeling of Aflatoxins Into Food Raw Materials in Livestock

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Aflatoxins are wide-spread harmful carcinogenic secondary metabolites produced by *Aspergillus* species, which cause serious feed and food contaminations and affect farm animals deleteriously with acute or chronic manifestations of mycotoxicoses. On farm, both pre-harvest and post-harvest strategies are applied to minimize the risk of aflatoxin contaminations in feeds. The great economic losses attributable to mycotoxin contaminations have initiated a plethora of research projects to develop new, effective technologies to prevent the highly toxic effects of these secondary metabolites on domestic animals and also to block the carry-over of these mycotoxins to humans through the food chain. Among other areas, this review summarizes the latest findings on the effects of silage production technologies and silage microbiota on aflatoxins, and it also discusses the current applications of probiotic organisms and microbial products in feeding technologies. After ingesting contaminated foodstuffs, aflatoxins are metabolized and biotransformed differently in various animals depending on their inherent and acquired physiological properties. These mycotoxins may cause primary aflatoxicoses with versatile, species-specific adverse effects, which are also dependent on the susceptibility of individual animals within a species, and will be a function of the dose and duration of aflatoxin exposures. The transfer of these undesired compounds from contaminated feed into food of animal origin and the aflatoxin residues present in foods become an additional risk to human health, leading to secondary aflatoxicoses. Considering the biological transformation of aflatoxins in livestock, this review summarizes (i) the metabolism of aflatoxins in different animal species, (ii) the deleterious effects of the mycotoxins and their derivatives on the animals, and (iii) the major risks to animal health in terms of the symptoms and consequences of acute or chronic aflatoxicoses, animal welfare and productivity. Furthermore, we traced the transformation and channeling of Aspergillus-derived mycotoxins into food raw materials, particularly in the case of aflatoxin contaminated milk, which represents the major route of human exposure among animal-derived foods. The early and reliable detection of aflatoxins in feed, forage and primary commodities is an increasingly important issue and, therefore, the newly developed, easy-to-use qualitative and quantitative aflatoxin analytical methods are also summarized in the review.

Keywords: aflatoxin, *Aspergillus*, storage conditions, mitigation strategies, livestock

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INTRODUCTION

Mycotoxins are harmful secondary metabolites produced by a variety of mold species that represent serious health risks to both humans and household animals (Beardall and Miller, 1994) and, not surprisingly, they cause both acute and chronic diseases called mycotoxicoses. The chronic pathological conditions develop over a longer period of time through the consumption of both cereals and animal products, e.g., milk, meat, and eggs. They represent a risk factor to human health directly in the food chain and through biological transformations as well. Mycotoxinogenic fungi are present mainly in small grains like wheat, barley, rye, rice, triticale, and corn (Miller, 2008; Gacem and El Hadj-Khelil, 2016; Udovicki et al., 2018) and also in different feedstuffs. In fact, aflatoxins were first discovered following a severe livestock poisoning incident in England involving turkeys (e.g., Amare and Keller, 2014; Keller, 2019). In addition, aflatoxins may also occur in peanuts, figs, pistachios, Brazil nuts and cottonseeds.

A number of *Aspergillus* spp. belonging to sections *Flavi*, *Ochraceorosei* and *Nidulantes* have the ability to produce the harmful, carcinogenic difuranocoumarin derivatives called aflatoxins (Varga et al., 2015; Chen A.J. et al., 2016; Niessen et al., 2018; Frisvad et al., 2019). *Aspergillus flavus*, *Aspergillus parasiticus,* and *Aspergillus nominus* are the most often detected aflatoxigenic Aspergilli in feed (**Table 1**). Aflatoxin producer Aspergilli are of paramount importance because the aflatoxins synthesized by them are among the strongest naturally occurring carcinogenic substances (Kumar et al., 2008). Considering their chemical structures, aflatoxins are furanocoumarin derivatives (**Figure 1**), of which aflatoxin M_1 (AFM_1), a hydroxylated derivative of aflatoxin B_1 (AFB_1), occurs in milk and in various dairy products (Prandini et al., 2009; Giovati et al., 2015). AFM_1 is a distinguished target in on-going mycotoxin-related research, because AFM_1 consumption may be exceptionally dangerous for children especially at younger ages (Udomkun et al., 2017; Rodríguez-Blanco et al., 2019; Ojuri et al., 2019).

The risks associated with mycotoxins have an enormous economic impact, which heavily supports the need for further research in this field (Gnonlonfin et al., 2013). The scope of future mycotoxin-linked studies should be broadened and should focus more on the prevention of mycotoxin production and the reduction of their deleterious effects. One of the major objectives of current investigations is the breeding and cultivation of novel plant varieties/hybrids more resistant to infections by mycotoxin producer fungi. Another major goal focuses on the accuracy of the storage of crops and crop products, especially silage, to control the production of mycotoxins more tightly (Driehuis et al., 2018; Ogunade et al., 2018; Glamočić et al., 2019). A further important step in mycotoxin control would be to make feeding practices more rigorous to prevent mycotoxins from entering the body of animals in the first place (Aslam et al., 2016; Shanakhat et al., 2018). Furthermore, countermeasures may also include the application of various mycotoxin binding agents mixed with the feed (De Mil et al., 2015; Vila-Donat et al., 2018). Besides agricultural and technological approaches combating aflatoxins successfully, we also need to develop more sensitive and more reliable analytical methods (Kos et al., 2016).

TABLE 1 | Aflatoxin producer *Aspergillus* species detected in feed.

Country	Type of feed	Isolated *Aspergillus* spp.	References
Argentina	Maize silage, corn grains, cotton seed, finished feed	*A. flavus, A. parasiticus*	Alonso et al., 2009
Argentina	Maize silage	*A. flavus, A. parasiticus*	González Pereyra et al., 2011
Brazil	Concentrated feed and maize silage	*A. parasiticus*, *A. nomius*	Variane et al., 2018
Egypt	Maize silage	*A. flavus*	El-Shanawany et al., 2005
France	Maize silage	*A. parasiticus*	Garon et al., 2006
Ghana	Corn grain	*A. flavus*	Dadzie et al., 2019
Indonesia	Maize of livestock feed	*A. flavus*	Sukmawati et al., 2018
Iran	Silage, concentrate, hay, TMR	*A. flavus*	Davari et al., 2015
Malaysia	Corn grains	*A. flavus*	Zulkifli and Zakaria, 2017
Malaysia	Wheat and barley	*A. flavus*	Reddy and Salleh, 2010
Pakistan	Feed samples	*A. flavus, A. parasiticus*	Usman et al., 2019
Saudi Arabia	Animal feedstuff samples	*A. flavus, A. parasiticus, A. nomius*	Gherbawy et al., 2019
Serbia	Corn, wheat, barley, soybean and sunflower grains	*A. flavus*	Lević et al., 2013
Spain	Barley grains	*A. flavus, A. parasiticus*	Mateo et al., 2011
Tanzania	Corn grains	*A. flavus*	Manoza et al., 2017

To eradicate or at least to decrease mycotoxins considerably in the feed and food chain is undoubtedly a high-complexity and highly prestigious aim, which absolutely requires the effective cooperation of experts working in different fields. Such expanding co-operations will hopefully help on-going research obey the "from farm to fork" principle more. In this case, this concept means that we need to deal not only with production, storage and processing issues but also their impacts on human health as well (Fink-Gremmels, 2008b; Ogunade et al., 2014; Asemoloye et al., 2017).

In this review, we focus on special parts of the feed and food chain like silage production and mitigation of mycotoxins by microbial products. A special attention will be paid to novel findings, which may help the feed management in animal husbandry to prevent and alleviate aflatoxin contamination. Other major issues tackled by this review include new pieces of information on the deleterious physiological effects of aflatoxins on domestic animals, which help us further in proper risk assessment and management. Moreover, up-to-date analytical tools and methods to measure aflatoxins precisely both on farms and analytical laboratories will also be covered. We hope that shedding light on the high-complexity relations between aflatoxin producer Aspergilli, aflatoxin contaminations in feeds and feeding practices in animal husbandry will also give us new

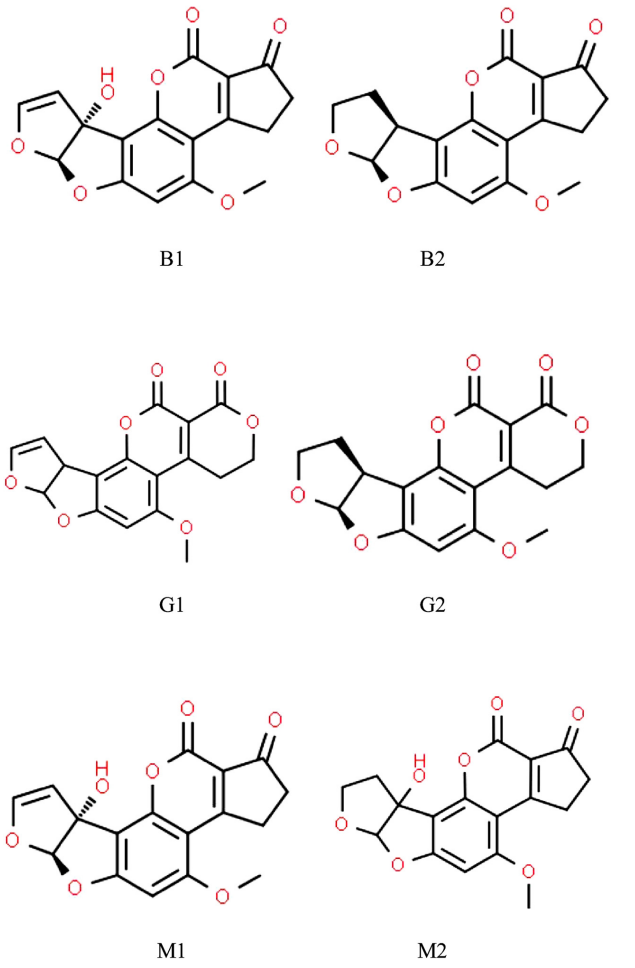

FIGURE 1 | Chemical structures of aflatoxins most frequently found in animal husbandry.

hints on the efficient control of aflatoxin contaminations in feeds and minimizing the carry-over of these harmful myctotoxins to humans through the food chain.

AFLATOXIN PRODUCTION IN FUNGI: BIOSYNTHESIS AND REGULATION

Considering the aflatoxin biosynthetic pathway acetate molecules are converted to norsoloinic acid at first by two fatty acid synthases, a polyketide synthase and a monooxygenase (Ehrlich et al., 2010; Yu, 2012; Roze et al., 2013). The biosynthesis proceeds through the intermediates averantin, averufin, versiconal and branches at versicolorin B to give rise to aflatoxin B_1 and G1 *via* the versicolorin A/sterigmatocystin and to aflatoxin B_2 and G_2 *via* the versicolorin B/dehydrosterigmatocystin pathways, respectively (Yu, 2012). The letters B and G stand for the blue and green fluorescence of these compounds observable under ultraviolet light, when separated by thin-layer chromatography (Yu, 2012). The aflatoxin biosynthetic gene cluster is sophisticatedly regulated by both local (AflR and AflS) and global (Velvet Complex) regulatory elements (Amaike and Keller, 2011; Alkhayyat and Yu, 2014; Amare and Keller, 2014; Gil-Serna et al., 2019; Keller, 2019). Environmental factors like the availability of carbon and nitrogen sources, changing pH, temperature and light conditions as well as variations in the redox status of the fungal cells all have their impacts on aflatoxin production (Alkhayyat and Yu, 2014). Among environmental stresses, oxidative stress seems to play a pivotal role in the initiation of aflatoxin production (Reverberi et al., 2010; Hong et al., 2013; Roze et al., 2013; Amare and Keller, 2014). Plant–fungus interactions also affect the biosynthesis of aflatoxins e.g., through oxylipin production, which have been reviewed e.g., by Pusztahelyi et al. (2015). Undoubtedly, a deeper understanding of the elements and regulation of the aflatoxin biosynthetic gene clusters operating in aflatoxigenic fungi is an important prerequisite for the development of novel and successful mycotoxin control strategies in the future (Alkhayyat and Yu, 2014; Gil-Serna et al., 2019).

FUNGAL ACTIVITY AND AFLATOXIN PRODUCTION IN STORED GRAINS

Aflatoxin-producing Aspergilli (Varga et al., 2015; Chen A.J. et al., 2016; Niessen et al., 2018; Frisvad et al., 2019) may originate from crop fields but post-harvest infections have also been reported (Gachara et al., 2018). Aflatoxin production cannot be linked strictly to any specific phase of growth or processing status although poorly managed post-harvest conditions during drying and storage can result in rapid increase in mycotoxin concentrations (Hell et al., 2010; Chulze, 2010). Grain drying is costly but selecting a variety or hybrid optimal for a given crop field can help farmers to harvest cereals with lower than 13–15% kernel moisture contents, which is required for safe storage (Magan and Aldred, 2007) (**Figure 2**). Nevertheless, artificial drying is unsurmountablein most cases.

Obeying quality regulations, the recommended drying temperature is less than 65°C for most feed cereals and below 90°C for corn (Hellevang, 2013). Of course, these high drying temperatures will also have on impact on the *Aspergillus* spp., which contaminate grains. *A. flavus* has an outstandingly high heat tolerance in comparison to other fungi with an upper tolerance limit of 40°C (Neme and Mohammed, 2017). Prencipe et al. (2018) also found that while the growth of *A. flavus* was suboptimal above 40°C this relatively high temperature resulted in the most intensive aflatoxin synthesis on chestnut. Hawkins et al. (2005) found that 60°C drying temperature still had no adverse effect on *A. flavus* thriving on corn kernels but raising the temperature up to 70°C significantly decreased fungal infection. Favorable effects of high drying temperature in the restriction of fungal growth were also reported for rice (Hell and Mutegi, 2011).

Unfortunately, the aflatoxin molecules are highly heat-stable as their decomposing temperature is 268–269°C (Peng et al., 2018). As a result, simple drying technologies cannot decrease aflatoxin concentrations significantly in stored grains. On the

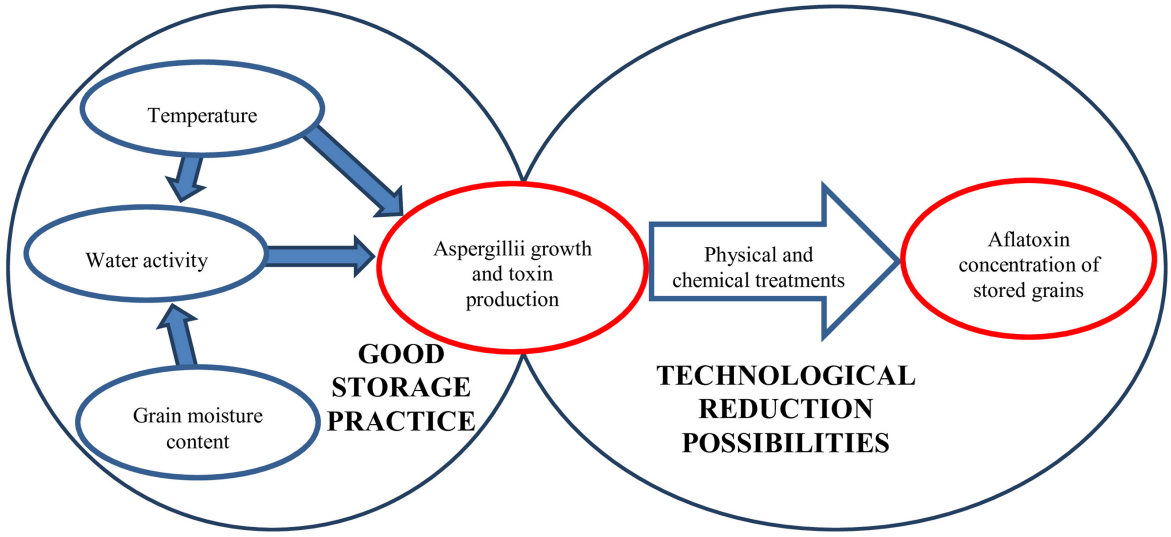

FIGURE 2 | Factors influencing the aflatoxin content of grains during storage.

other hand, elongated high-temperature treatments may have beneficial effects (Lee et al., 2015).

The temperature, kernel moisture content and relative humidity during storage all influence the physiological processes of fungi. As demonstrated, 18 – 19°C temperature and 12 – 13% moisture content were the limiting factors for the growth and activity of the Aspergilli (Villers, 2014; Mwakinyali et al., 2019), although lower temperature (8 – 10°C) may also be permissive for growth and mycotoxin production when the grain moisture content is higher (Mannaa and Kim, 2017). Although these values are accepted widely in good storage practices under continental climatic conditions the relative humidity of grain silos are higher during the cold months, which results in higher water binding by the grains. Nevertheless, the lower temperature hinders increases in microbial activity, and the tolerable water activity is 0.70 for the different *Aspergillus* species (Mannaa and Kim, 2017). It is important to note that 'hot spots' can develop in grain heaps because of insects or increased grain physiological activity and the released heat and moisture can support fungal growth. Therefore, maintaining good hygienic practice and controlling the temperature of the grain heaps are adequate and necessary measures during storage (Magan et al., 2003; Peng et al., 2018).

There are several procedures applicable to decrease fungal infection and mycotoxin production in kernels during storage (**Table 2**). Size separation by sieving and density separation by gravity table are useful measures as the lighter, smaller and broken kernels and the small components of heap may be infected or damaged by fungi and, therefore, they can be starting points for further deterioration. Not surprisingly, their removal significantly decreases aflatoxin contamination (De Mello and Scussel, 2007; Shi et al., 2014; Peng et al., 2018).

Hand sorting based on visible fungal infections is a very useful tool to decrease the aflatoxin B_1 (AFB_1) content of corn kernels but obviously this is not a viable option in industrial scale (Matumba et al., 2015). Another possibility is optical sorting because *A. flavus* contaminated corn kernels emit bright greenish-yellowish light when illuminated by UV light enabling separation using suitable optical equipment. Unfortunately, such light emission does not occur in each case and hidden, internal fungi contaminations have no visible effects either. Nevertheless, a sorting method based on the evaluation of red and green light reflectance was also developed to separate aflatoxin containing peanuts and another one for cleaning pecans, based on fluorescence (Pasikatan and Dowell, 2001). It is noteworthy that a low cost multi-spectral analyzer was manufactured to screen single corn kernels at nine distinct wavelengths in the 470 – 1550 nm region for qualitative use (Stasiewicz et al., 2017). Although fluorescent optical techniques have higher sensitivities and specificities than near infrared spectroscopy-based and hyperspectral imaging methods near infrared spectroscopic evaluations seem to have greater capabilities to reveal both aflatoxin and fungal contaminations. Most importantly, these techniques have already been applied in automatic sorters (Tao et al., 2018). Color analyses can be combined easily with other visible properties. For example, the Raspberry Pi optical analytical equipment (Vasishth and Bavarva, 2016) is able to sort peanuts based on their color, size, edge length and area of kernel with more than 40 kg/h sorting capacity. It is foreseeable that recent improvements in computing techniques will open new ways for visual analyses in combating both fungi and their mycotoxins.

Dehulling, the removal of external layers of kernel surface, can be an effective tool to decontaminate grains from toxigenic fungi and significantly decreases the aflatoxin content of grains (Siwela et al., 2005; Peng et al., 2018). This beneficial effect could be improved further by floating and washing before application (Fandohan et al., 2005; Mutungi et al., 2008; Matumba et al., 2015; Hadavi et al., 2017). Polishing rice kernels is also effective to reduce aflatoxin and, hence, more than nine-fold decrease in contamination was recorded (Castells et al., 2007).

TABLE 2 | Summary of possibilities and examples for the reduction of the aflatoxin content of stored grains.

	Method	Effect	References
Removal	Cleaning and sorting by size and density	Only small Brazil nuts (smaller than 36.6 mm length and 6.3 g weight) contained AFB_1	De Mello and Scussel, 2007
		Corn particles passed 5.16 mm sieve contained 46 times higher more toxin than the over fraction and lower density kernels contain 50 times higher aflatoxin	Shi et al., 2014
	Sorting by color	UV light, fluorescent and multi spectral analysis can be used to detect contaminated kernels	Pasikatan and Dowell, 2001; Vasishth and Bavarva, 2016; Stasiewicz et al., 2017; Tao et al., 2018
	Removal of contaminated part by dehulling and polishing	Dehulling removed 92% of the initial aflatoxin content from corn kernel	Siwela et al., 2005
		Aflatoxin residuals in corn after crushing and dehulling was almost negligible	Fandohan et al., 2005
		Dehulling decreased AF content of corn by 5.5–70%	Mutungi et al., 2008
		Dehulling and whitening of rice kernel resulted 96% decrease in AF content in polished broken grains and 79% in polished whole kernels	Castells et al., 2007
		Dehulling of corn kernels resulted in 88 and 92% reduction in AFB_1 and AFB_2 levels	Matumba et al., 2015
Reduction, destruction	Thermal treatment for a long time	Heating at 100 and 150°C for 90 min decreased the AFB_1 content of soybean 41.9 and 81.2%, respectively	Lee et al., 2015
	Oxidation by ozone	2.8 and 5.3 mg/l ozone concentration applied for 4 hours resulted 76–84% decrease in AFB_1 content of poultry feed	Torlak et al., 2016
		66–95% AFB_1 reduction in peanut, corn and wheat kernel	Ismail et al., 2018
	Irradiation by ionizing and non-ionizing radiation	25 kGy gamma irradiation resulted 43% decrease, microwave heating for 10 min at 1.45 kW resulted 32% decrease, direct solar irradiation for 3–30 h resulted 25–40% decrease in AFB_1 content of poultry feed	Herzallah et al., 2008
		4, 6, and 8 kGy gamma irradiation doses resulted 15–56% reduction in aflatoxin content for corn, wheat and rice kernels	Mohamed et al., 2015
		5 and 10 kGy irradiation doses resulted in 69.8 and 94.5% decreases in AFB_1 content, respectively	Markov et al., 2015
		Pulsed light treatment (0.52 J/cm^2/pulse in spectrum of 100–1100 nm with a xenon flash lamp) resulted 75–90% decreases in AFB_1 and AFB_2 contents of rice and rice bran	Wang et al., 2016
		6 and 10 kGy gamma irradiation doses resulted 90 and 95% reduction in AFB_1, respectively	Serra et al., 2018
		In peanuts, 5–9 kGy gamma irradiation doses result 20–43% decrease in aflatoxins, microwave radiation at 360, 480, and 600 W resulted 59–67% decrease, combined treatments have higher than 95% efficiency	Patil et al., 2019
	Destruction by cold plasma	Hazelnuts, peanuts, and pistachio nuts treated with air gases plasma for 20 min resulted 50% decrease in total aflatoxins, SF_6 plasma application resulted only 20%reduction	Basaran et al., 2008
		Atmospheric plasma generated with 400–1150 W power for 1–12 min resulted 46–71% decrease in AFB_1 in peanuts	Siciliano et al., 2016
		High voltage atmospheric cold plasma applied for 1 and 10 min resulted 62 and 82% reduction in AFs levels of corn.	Shi et al., 2017
		Atmospheric and low pressure cold plasma reduced the AFB_1 content of hazelnut by 72–73%	Sen et al., 2019

Application of ozone during cereal storage is a relatively new method to improve storage conditions, based on the combined antifungal and insecticide effects of this reactive gas (Isikber and Athanassiou, 2015). Importantly, ozone treatments reduce mycotoxin contaminants without any negative effect on the quality of the grains (Tiwari et al., 2010; Zhu, 2018), and eliminate aflatoxins with high efficiency (66–95% of the original toxin concentration) in cereal grains and flours, as well as in soybean and peanut (Torlak et al., 2016; Ismail et al., 2018).

Another physical method to reduce aflatoxin contaminations is irradiation. Several radiation sources have been evaluated thus far and many of them were found to be effective. For example, the advantageous effects of UV in liquid phase (Patras et al., 2017), gamma irradiation in corn (Markov et al., 2015; Serra et al., 2018), in other cereal kernels (Mohamed et al., 2015), in peanuts (Patil et al., 2019) and in poultry feed (Herzallah et al., 2008) have been reported in a number of publications. Direct sunlight was also effective in aflatoxin reduction in poultry feed (Herzallah et al., 2008) and, in addition to exposures to direct light, the applicability of pulsed light has also been tested and evaluated, and it has already been employed in new decontamination technologies (Moreau et al., 2013). Meanwhile exposure to pulsed light was effective in liquid medium (Moreau et al., 2013) pulsed polychromatic light applied with a simple xenon flash lamp also resulted in significant decreases in the aflatoxin content in cereal kernels (Wang et al., 2016).

Cold plasma treatment is another possible physical treatment against pathogens and fungal toxins. Cold plasma is generally a result of atmospheric dielectric discharge, and the effects of pressure (atmospheric or vacuum), air composition, humidity and flow rate, discharging power and treatment time are under continuous evaluation nowadays in different cereals and nuts (Basaran et al., 2008; Siciliano et al., 2016; Shi et al., 2017; Misra et al., 2019; Sen et al., 2019). Cold plasma treatments are cost effective, ecologically neutral and have only a negligible effect on the quality of the grains when compared to classical detoxification methods (Hojnik et al., 2017).

FUNGAL ACTIVITY AND AFLATOXIN PRODUCTION IN SILAGE

Climate change has a major impact on agriculture in many ways and, thereby, many studies have already been published on the effects of climate change on the growth, spread and toxin production of mycotoxigenic fungi on economically important crops (Magan et al., 2011; Paterson and Lima, 2011; Wu et al., 2011; Battilani et al., 2012, 2016).

Aflatoxin contaminations of maize, wheat, etc. have become a major safety issue in the European agricultural industry (Battilani et al., 2016), and aflatoxin producer *Aspergillus* spp. have also been detected in temperate Europe (Dobolyi et al., 2013). As a consequence, mycotoxins including the *Aspergillus*-derived harmful aflatoxins may also contaminate European agricultural products – a foreseeable threat, which we should by no means neglect (Magan et al., 2011; Battilani et al., 2012, 2016; Dobolyi et al., 2013).

Maize silage, one of the most important components in the feeding of dairy cows in Europe and worldwide, can be contaminated by several mycotoxin-producer fungi entering the feed production chain at various stages (Ogunade et al., 2018). Not surprisingly, aflatoxin contaminations can be detected occasionally both before and after ensiling (Storm et al., 2014; Gallo et al., 2015; Ogunade et al., 2018; Peng et al., 2018). Therefore, the rigorous control of the growth of aflatoxigenic fungi is of pivotal importance, if the production of aflatoxin-free silage is to be guaranteed (Borreani and Tabacco, 2010; Ogunade et al., 2018).

Although microaerophilic conditions and low pH, which are typical features of silage fermentations, may prevent the growth of the majority of molds, some species of the genera *Aspergillus*, *Byssochlamys*, *Monascus*, *Penicillium,* and *Trichoderma* are able to survive even under ensiling conditions (Mansfield and Kuldau, 2007; Pereyra et al., 2008). To make things even worse, the aflatoxigenic capacity of the *Aspergillus* section *Flavi* strains derived from silage samples is remarkable. For example, del Palacio et al. (2016) demonstrated that 27.5% of these strains produced AFB_1, 17.5% of them aflatoxin G_2 (AFG_2) and 10% synthesized aflatoxin G_1 (AFG_1). Interestingly, only 5% of the strains produced AFB_2 (del Palacio et al., 2016). In another study concomitantly performed in Pakistan (Sultana et al., 2017), *A. niger*, *A. flavus, A. fumigatus, A. ochraceous,* and *A. terrus* were identified in both fresh fodder and corn silage. Importantly, the authors also found AFB_1 in 37.5% of the fresh fodder and in 41.7% of the corn silage samples with average AFB_1 concentrations of 9.5 and 8.4 μg/kg, respectively, meanwhile AFB_2 was present in only two samples (1.2 and 1.3 μg/kg), and none of the analyzed samples was contaminated by AFG_1 or AFG_2 (Sultana et al., 2017). In Southern Brazil, aflatoxigenic *A. parasiticus* and *A. nomius* strains have been detected in the tested silage and concentrated feed samples (Variane et al., 2018).

Considering the world-wide occurrence of aflatoxin contaminations (**Table 3**), AFB_1 has been reported in corn silage in Argentina (González Pereyra et al., 2008, 2011), in Brazil (Keller et al., 2013; Schmidt et al., 2015), and in France (Richard et al., 2009). Total aflatoxin contaminations have also been determined in silage samples collected in Iran (Hashemi et al., 2012) and in Uruguay (del Palacio et al., 2016).

MICROBIAL BIOCONTROL AND MICROBIAL DETOXIFICATION PRODUCTS FOR MYCOTOXIN MITIGATION IN ANIMAL HUSBANDRY

In recent decades, several feasible and cost-effective strategies have entered the market aiming to mitigate the effects of feed mycotoxin contamination in animal husbandry, especially in the dairy industry. Technologies to reduce the incidence

TABLE 3 | Worldwide occurrence of aflatoxins in silage.

Country	Mycotoxin	Sample	No. of samples	No. of positive sample (Incidence%)	Mean concentration (μg/kg)	Range (μg/kg)	References
Argentina	AFB_1	Corn silage	35	6 (17.0%)	–	1.4 – 155.8	González Pereyra et al., 2008
Argentina	AFB_1	Trench silo	43	6 (14.0%)	–	1.0 – 190.0	González Pereyra et al., 2011
Argentina	AFB_1	Silo bag	35	19 (54.3%)	–	5.8 – 47.4	González Pereyra et al., 2011
Brazil	AFB_1	Corn silage	116	15 (13.0%)	33.0	2.0 – 61.0	Keller et al., 2013
Brazil	AFB_1	Corn silage	327	3 (0.9%)	3.0	1.0 – 6.0	Schmidt et al., 2015
France	AFB_1	Corn silage	–	–	28.0	7.0 – 51.3	Richard et al., 2009
Iran	Total AF	Silage	42	7 (16.7%)	1.24	1.1 – 27.3	Hashemi et al., 2012
Uruguay	Total AF	Wheat silage	220	–	17.0	6.1 – 23.3	del Palacio et al., 2016

–, not evaluated data.

of mold and mycotoxin contaminations of silages can be employed in one of the three main phases (preharvest, harvest, ensiling) of silage production. During the preharvest phase, the appropriate agronomic practices may rely on (i) the use of crop varieties or hybrids, which are resistant to fungal infections, (ii) the application of pesticides and fungicides, (iii) adequate management of weeds and crop residues, (iv) the use of appropriate crop rotation, tillage, fertilization and irrigation and (v) the application of biocontrol agents, e.g., bacteria, yeasts, or atoxigenic strains of *A. flavus* or *A. parasiticus* (Gallo et al., 2015; Pfliegler et al., 2015; Ogunade et al., 2018; Peng et al., 2018). During the harvest phase, the most important factors that should be taken into consideration are proper harvest timing (maturity stage) and cutting height (to minimize soil contamination), as well as immediate storage of harvested feeds (Gallo et al., 2015; Ogunade et al., 2018; Peng et al., 2018).

Pre-harvest biocontrol microbes represent a promising and already widely applied method to lower mycotoxin risks in food and feed by protecting plants from pathogens and inhibiting the growth of molds during postharvest conditions. They both reduce economic loss caused by fungal infections and lower toxin levels in products (e.g., Pfliegler et al., 2015). Biocontrol agents compete for nutrients and space, may secrete antifungals or even parasitize molds, and can also stimulate host plant resistance (Liu et al., 2013) and, thereby, they mitigate the risk of plant infections and their undesirable consequences. Regarding Aspergilli infection and aflatoxin contamination, non-aflatoxigenic biocontrol *Aspergillus flavus* strains are most commonly applied to crops (Ehrlich, 2014; Weaver and Abbas, 2019), while biocontrol yeasts species are also effective, such as the 2-phenylethanol producing *Wickerhamomyces anomalus* (Hua et al., 2014). These biocontrol agents are mostly applied to protect plants directly used in food production but may exert their effects on plant parts that are to be ensiled for feed production concomitantly.

In the ensiling phase, attention must be payed to adequate particle size, proper silo size, immediate rapid filling, proper compaction, complete sealing (to maintain strictly anaerobic conditions), and the use of acid-based additives or microbial inoculants, e.g., lactic acid bacteria (Gallo et al., 2015; Ogunade et al., 2018; Peng et al., 2018). Some specific strains in the *Lactobacillus* (*L. buchneri, L. fermentum, L. hilgardii, L. plantarum, L. reuteri, L. rhamnosus*), *Lactococcus* (*L. lactis*), *Leuconostoc*, and *Pediococcus* (*P. pentosaceus*) genera can inhibit or can even prevent completely the growths of various mycotoxigenic molds and their mycotoxin productions as well (Dalié et al., 2010; Cavallarin et al., 2011; Queiroz et al., 2012; Dogi et al., 2013; Ahlberg et al., 2015; Ma et al., 2017; Gallo et al., 2018; Zielińska and Fabiszewska, 2018; Ferrero et al., 2019). It is noteworthy that there is a wide spectrum of environmental factors which influence the antifungal activity of LAB, including the type of the matrix and culture medium, the availability of nutritional compounds, the incubation time and temperature (Dalié et al., 2010; Ahlberg et al., 2015; Leyva Salas et al., 2017). In addition, some biological (e.g., the natural microbiota), and chemical (e.g., pH, water activity) parameters will also affect the antifungal activity in a species-specific manner (Dalié et al., 2010; Ahlberg et al., 2015; Leyva Salas et al., 2017). Species- and strain-specific factors are noteworthy, for example both *L. rhamnosus* and *L. plantarum* were efficient against *A. parasiticus* only *L. rhamnosus* reduced the AFB1 levels produced by *A. parasiticus* (Dogi et al., 2013). Quite unexpectedly, the *A. parasiticus – L. plantarum* interaction even stimulated aflatoxin B_1 production, which makes the use of *L. plantarum* undesirable as a silage inoculant.

In another study, a mixture of *P. pentosaceus* and *L. buchneri* reduced the adverse effects of rust infestation during ensiling and also decreased aerobic spoilage and aflatoxin production in maize silages with high levels of southern rust infestation (Queiroz et al., 2012). Importantly, *L. buchneri* increased the aerobic stability of the silage as well (Cavallarin et al., 2011). Inoculation of corn silage with a combined inoculant of *L. buchneri* and *Lactococcus lactis* improved the aerobic stability of the silage, and the higher silage density increased the stability further (Gallo et al., 2018). The interaction of *L. buchneri*, *L. reuteri*, *L. plantarum,* and *L. fermentum* strains reduced the AFB_1 level, improved the stability and, furthermore, the microbiological and chemical purity of maize silage (Zielińska and Fabiszewska, 2018). In a most recent study by Ferrero et al. (2019), the authors examined the effect of *L. buchneri*, *Lactobacillus hilgardii*, and their combination on *A. flavus* contaminants and their aflatoxin production in maize silage. The results showed that the inoculation of corn silage with *L. buchneri* and *L. hilgardii* increased the aerobic stability and delayed the beginning of aerobic microbial degradation of maize silage, and indirectly reduced the risk of *A. flavus* emergence and aflatoxin B_1 level after silage opening.

Ma et al. (2017) examined the AFB_1 binding capacity of various silage bacteria including *L. plantarum*, *L. buchneri*, *P. acidilactici,* and *P. pentosaceus* and found that high concentration of silage bacteria could bind the AFB_1 content of maize silage but population, strain, viability, and medium acidity have all affected the efficacy of binding.

Antifungal compounds produced by LAB also reduce the mycotoxin production of molds (Ahlberg et al., 2015). These LAB-produced compounds cover organic acids (e.g., acetic, lactic, and propionic acid), carboxylic acids, phenolic compounds, including phenolic acids (gallic acid, tannins, benzoic acids, phenyllactic acid, hydroxyphenyllactic acid), fatty acids (caproic acid, decanoic acid, 3-hydroxydecanoic acid, coriolic acid, ricinoleic acid), volatile compounds (e.g., diacetyl, acetoin), cyclopeptides [*e.g.,* cyclo(Phe-Pro), cyclo(L-Leu-L-Pro), cyclo(L-Met-L-Pro), cyclo(L-Tyr-L-Pro)], hydrogen peroxide, ethanol, reuterin, and proteinaceous compounds (Dalié et al., 2010; Li et al., 2012; Crowley et al., 2013; Le Lay et al., 2016; Leyva Salas et al., 2017).

Considering the mechanisms of actions of these antifungals, the dissociated forms of organic acids can decrease the intracellular pH within the cells, can increase the permeability of the cytoplasmic membrane, and finally can lead to the death of the fungal cells (Leyva Salas et al., 2017). In addition, H_2O_2 oxidizes directly the cellular proteins and the lipid components of the cellular membranes (Dalié et al., 2010). Nevertheless, the mechanisms of the antifungal actions of hydroxy fatty acids and

TABLE 4 | Antifungal activity of lactic acid bacteria (LAB).

LAB	Strain	Effect	References
Lactobacillus buchneri	NCIMB 40 788	Decreased mold count, decreased AFB_2 and increased aerobic stability of the silage	Cavallarin et al., 2011
Lb. buchneri	40788	Decreased the population of spoilage fungi, and aflatoxin production in silages	Queiroz et al., 2012
Lb. buchneri	R1102	Bound AFB_1	Ma et al., 2017
Lb. buchneri	LB1819	Enhanced the fermentation and aerobic stability of maize silage	Gallo et al., 2018
Lb. buchneri	A KKP 2047 p	Reduced mold count and decreased AFB_1 amount	Zielińska and Fabiszewska, 2018
Lb. buchneri	NCIMB 40788	Reduced the risk of *Aspergillus flavus* outgrowth and AFB_1 production after silage opening	Ferrero et al., 2019
Lactobacillus fermentum	N KKP 2020 p	Reduced mold count and decreased AFB_1 amount	Zielińska and Fabiszewska, 2018
Lactobacillus hilgardii	CNCM I-4785	Reduced the risk of *Aspergillus flavus* outgrowth and AFB_1 production after silage opening	Ferrero et al., 2019
Lactobacillus plantarum	RC009	Reduce *Aspergillus parasiticus* growth rate	Dogi et al., 2013
Lb. plantarum	PT5B	Bound AFB_1	Ma et al., 2017
Lb. plantarum	K KKP 593 p, S KKP 2021 p	Reduced mold count and decreased AFB_1 amount	Zielińska and Fabiszewska, 2018
Lactobacillus reuteri	M KKP 2048 p	Reduced mold count and decreased AFB_1 amount	Zielińska and Fabiszewska, 2018
Lactobacillus rhamnosus	RC007	Reduce *Aspergillus parasiticus* growth rate	Dogi et al., 2013
Lactococcus lactis	O224	Enhanced the fermentation and aerobic stability of maize silage	Gallo et al., 2018
Pediococcus pentosaceus	12455	Decreased the population of spoilage fungi and aflatoxin production in silages	Queiroz et al., 2012
Pediococcus acidilactici	R2142, EQ01	Bound AFB_1	Ma et al., 2017

proteinaceous compounds have remained yet to be elucidated (Dalié et al., 2010).

Silage decontamination may also be applied if measures to avoid contamination were proven ineffective. Such strategies are primarily based on the adsorbents. Advantages of using adsorbent feed additives over decontamination of the final product, e.g., milk, are their safety and inexpensiveness, and that they may simply be mixed into animal feed to achieve the desired effect. These products may lower the bioavailability of mycotoxins and can help to decrease toxic effects, as well as the amount of toxin detectable in the final product (meat or milk). Such strategies may involve the use of live microbial (LAB or yeast) cultures (usually termed microbial enterosorption, biosorption), microbial or plant extracts, other organic/inorganic materials such as activated carbons or charcoals, hydrated sodium calcium aluminosilicates, and various clay-based products (Kutz et al., 2009; Giovati et al., 2015). LAB can not only inhibit the growth of molds but are also able to bind aflatoxins in different matrices (**Table 4**; Ahlberg et al., 2015; Muck et al., 2018), thereby reducing the health risks of aflatoxins. Environmental conditions have a great impact on the aflatoxin binding capabilities of LAB (Dalié et al., 2010; Ahlberg et al., 2015; Ma et al., 2017), which is highly species-specific (Gomah et al., 2010; Dogi et al., 2013; Ahlberg et al., 2015). Some studies demonstrated that non-viable LAB cells had better binding capability for aflatoxin than viable LAB cells (Ahlberg et al., 2015; Damayanti et al., 2017; Ma et al., 2017). On the contrary, Liew et al. (2018) reported on a higher binding efficiency by living cells. Regardless of alive or dead bacterial cells, the aflatoxin binding seems to be reversible and the bound mycotoxins are released slowly over time (Verheecke et al., 2016).

Based on various microbe species, sources, manufacturers, and formulations, live yeast products include several categories: yeast probiotics, *Saccharomyces cerevisiae* fermentation products (SCFP), dried yeast products (DY or DYP), brewery yeasts (BY), and active dry *S. cerevisiae* (ADSC) (Pizzolitto et al., 2012; Poppy et al., 2012; Gonçalves et al., 2017). Compared to live bacteria-based products, these yeast products are considered and employed as direct feed additives in most cases and are not applied at the ensiling phase (Giovati et al., 2015). Some bacterial species, e.g., *Nocardia corynebacteroides* (NC) are also added as direct feed additives for poultry (Tejada-Castañeda et al., 2008). Microbe-derived feed additive products are also based on yeasts, and include autolyzed yeast (AZ), inactivated yeast cells (IY), distillery yeast sludge, and yeast cell wall (YCW) products (Gonçalves et al., 2017; Plaizier et al., 2018).

Live yeast or bacterial cells intended to colonize the gastrointestinal tract (GIT) of humans, or in some cases, poultry or laboratory rodents, are occasionally termed probiotics (Śliżewska and Smulikowska, 2011; Pizzolitto et al., 2012; González Pereyra et al., 2014). However, especially in the case of ruminants, the use of live cells may not necessarily result in gastrointestinal colonization. The rumen's own microbiota is also to be taken into account, as it can contribute to aflatoxin detoxification and degradation (e.g., biotransformation to aflatoxicol) (Upadhaya et al., 2009; Jiang et al., 2012). Aflatoxin B1 is absorbed in the rumen mainly at acidic pH (Pantaya et al., 2014), and the degradation of aflatoxins in rumen depends on both the animal species and feed type (Upadhaya et al., 2009). However, it must be noted that rumen colonization by *A. flavus* has also been recorded, leading to toxin production in rumen liquor (Nidhina et al., 2017).

The products SCFP, DY, BY, and ADSC consist of yeast cells, the nutrient medium on which the yeasts were grown, and the metabolites produced by the yeasts and have been shown to increase DMI, milk yield, as well as milk fat and protein yield in

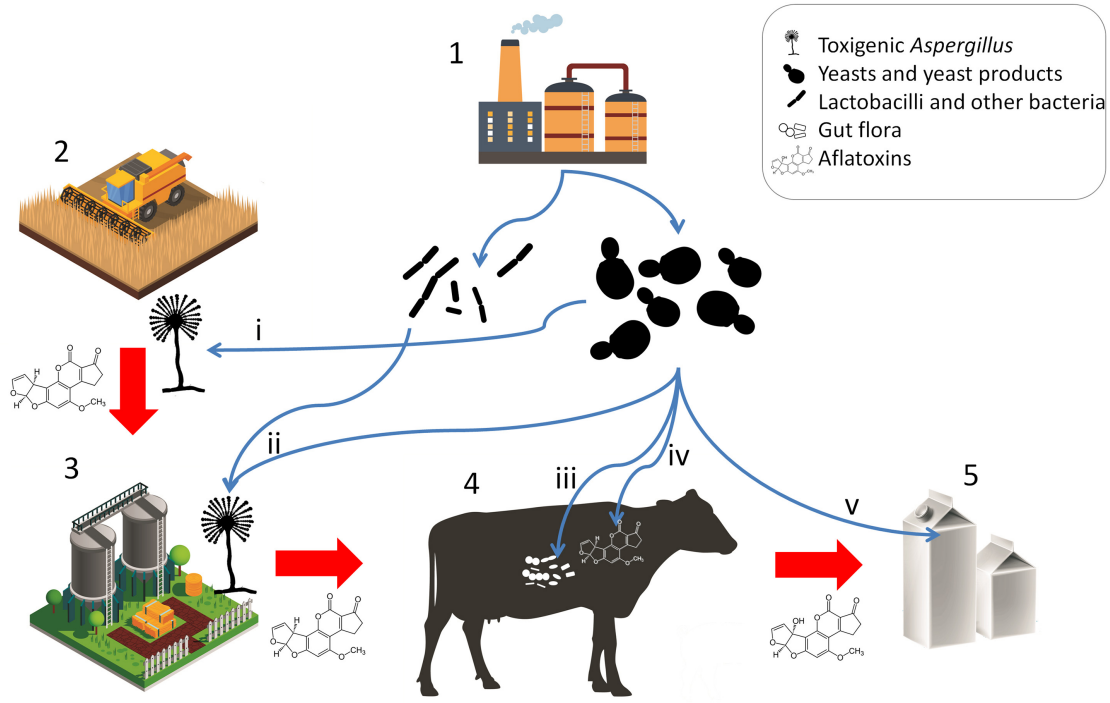

FIGURE 3 | Microbial products for mycotoxin mitigation in animal husbandry and their applications. Red arrows represent potential carry-over of mycotoxins or toxigenic *Aspergilli*. Blue arrows represent applications of microbes and microbe-derived products. (1) Fermentation and animal feed supplement industries; (2) crop production; (3) preparation and storage of silage and other feedstuffs; (4) livestock; (5) product; (i) pre-harvest biocontrol; (ii) antagonism in silage and feed; (iii) host gut microbiota and immune modulation, probiotic effect; (iv) enterosorption; (v) bioadsorption from product (milk). [Stock image credits: Freepik, macrovector, and vectorpocket].

lactating dairy cows (Poppy et al., 2012). However, these positive effects are attributed to adsorption of toxic substances and the modulation of the gut (prokaryote) microbiota, not to long-term gut colonization by the yeasts. Yeasts in fact are thought to play a negligible role in the microbiome of ruminants, although they may survive gastrointestinal conditions and retain their aflatoxin B_1 binding ability under gastrointestinal conditions (Dogi et al., 2011). Various studies have shown the effects of these live yeast products on the microbiota of the cows, however, uncovering the underlying mechanisms and a holistic understanding of dairy cow gastrointestinal health still requires further research (Zhu et al., 2017; Huebner et al., 2019). Interestingly, YCW has also been shown to positively modulate the gut health in broiler chicken challenged with AFB_1 or with *Clostridium* infection (Liu et al., 2018). These observations raise the possibility that yeast products, whether live or not, generally contribute to animal health both as bioadsorbents and as modulators of the gut prokaryote microbiota, as well as the immune status of the animal. Such positive effects may not only prevent toxicosis but result in increased feed intake and production (Pasha, 2008). In poultry feedstuff, *S. cerevisiae* strains have been tested and made commercially available as a probiotic microbe. It must be noted though, that the intended effect of the yeasts is not necessarily gut colonization and microbiome modulation, but aflatoxin adsorption (Śliżewska and Smulikowska, 2011; Pizzolitto et al., 2012), a role, which yeasts can effectively fulfill. The applications of microbes and microbial products for mycotoxin risk mitigation are summarized in **Figure 3**.

Yeast cell wall β-D-glucans, glucomannans and mannan-oligosaccharides are responsible for the mycotoxin binding abilities of these products (Pfliegler et al., 2015). Some purified cell wall components have been tested in animal husbandry, such as mannan-oligosaccharides supplemented into the diet of Japanese quails affected by aflatoxicoses (Oguz and Parlat, 2005). However, no direct correlation between the amount of individual components and toxin binding are evident (Joannis-Cassan et al., 2011). Structural integrity and amount of the yeast cell wall is crucial in binding efficacy, while viability is not: heat-treatment can even increase adsorption capacity (Bueno et al., 2007; Joannis-Cassan et al., 2011). Toxin binding can reach saturation rapidly and is reversible, and mycotoxins are not modified chemically during the process (Bueno et al., 2007). It must be noted that some yeasts (reviewed by Pfliegler et al., 2015) and bacteria (Wang Y. et al., 2018) are known to be able to enzymatically degrade mycotoxins if applied in viable form.

A novel approach for the microbiological detoxification of animal feed is the screening of isolates from various environmental sources (Intanoo et al., 2018), instead of using the most widespread species, *S. cerevisiae*. Various bacteria and yeasts may exhibit toxin-binding or even toxin-degrading

abilities, as well as biocontrol effects on toxigenic molds (Pfliegler et al., 2015) and these may be directly applied to supplement animal feed (Intanoo et al., 2018). Novel yeast species in this field include members of the genera *Kluyveromyces* and *Pichia*, both related to the widely used *Saccharomyces*. *P. kudriavzevii* has been successfully applied as a bioadsorbent feed additive to ameliorate the negative effects of AFB_1 contamination on broiler chicken performance (Magnoli et al., 2017). Novel isolates of *K. marxianus* have also been proposed as bioadsorbents based on *in vitro* characterization (Intanoo et al., 2018). However, Battacone et al. (2009) found no evidence for AFB_1 detoxification in ewes fed with *Kluyveromyces lactis* DYP, highlighting the need for rigorous testing of novel strains in different setups and with multiple animal species.

Apart from novel microbial strains, combined treatments of microbial and inorganic products constitute a promising strategy in ameliorating mycotoxin contamination. Recently, Jiang et al. (2018) found that both dietary clay and clay + SCFP reduced transfer of dietary AFB_1 to milk as well as milk aflatoxin M_1 (AFM_1) concentration, while the combined treatment was the only one that also prevented the decrease in milk yield caused by AFB_1. Thus, the potent adsorbing capability of inorganic products may act synergistically with the adsorbent, gut health-promoting and immunomodulatory effects of yeast products.

MICROBIAL DETOXIFICATION PRODUCTS TO COUNTERACT AFLATOXIN CONTAMINATION IN DAIRY PRODUCTS

Some studies have explored microbial aflatoxin decontamination strategies in dairy products, taking advantage of the high efficacy and relative ease of utilizing LAB and yeast, recently been reviewed by Assaf et al. (2019). Briefly, such microbial decontamination approaches rely on heat-killed or immobilized cells, and promising results were obtained when both LAB and yeasts were applied simultaneously. Heat-treatment of bacterial cells was found to improve binding capabilities in some studies (Pierides et al., 2000; Bovo et al., 2015; Assaf et al., 2018), while no such effect was reported by Kabak and Var (2008). Bacteria tested in the aforementioned studies include members of the genera traditionally considered probiotics and/or important in food production, as *Bifidobacterium*, *Lactobacillus*, or *Pediococcus*, and oddly, a potential pathogen, Enterococcus.

In UHT skim milk, both LAB and yeasts showed promising results (Corassin et al., 2013), and the binding of toxins to microbial cell walls was shown to be rapid, enabling short incubation times in potential industrial applications. Yeasts of the genera *Saccharomyces* and *Kluyveromyces* have been tested by Abdelmotilib et al. (2018), where the higher efficacy of heat-killed cells was also demonstrated for yeasts.

There are certain limitations on applying yeasts and bacteria for the decontamination of dairy products (Assaf et al., 2019), such as the need for their subsequent removal, reversibility of binding, or even legislations on tolerated number of live or dead microbial cells in products. Nevertheless, the high toxin binding capability and the safety of heat-killed cells toward consumers compared to chemical methods makes microbial decontamination a promising strategy.

AFLATOXIN METABOLISM IN LIVESTOCK

The toxicity of AFB_1 is strictly related to the bioactivation and detoxification pathways operating animals *in vivo* (**Figure 4**). Indeed, AFB_1 is a "pro-carcinogen" that is activated biologically by cytochrome P450 (CYP450), a microsomal enzyme of phase I detoxification (oxidation) to the extremely reactive and electrophilic AFB_1-8,9-epoxide (AFBO). This harmful AFB_1 derivative is able to covalently bind to macromolecules such as DNA and proteins, thereby forming adducts, which cause acute and chronic cytotoxicity, DNA mutations and eventually expressing carcinogenic activity (Diaz et al., 2010; Deng et al., 2018).

Moreover, AFBO can be hydrolyzed to AFB_1-8,9-dihydrodiol (AFB_1-dhd) by an epoxide hydrolase. AFB_1-dhd is able to react with proteins causing cytotoxicity or, alternatively, AFBO can be metabolically detoxified *via* conjugation with glutathione (GSH) by glutathione S-transferase (GST), a phase II detoxification enzyme. This pathway is considered as one of the main routes of AFBO detoxification (Diaz et al., 2010; Deng et al., 2018). Microsomal epoxide hydrolase (mEH) and aflatoxin-aldehyde reductase (AFAR) can also transform reactive AFB_1 to AFB_1-dialcohol, a real detoxified AFB_1 derivative, which can be excreted in urine (Guengerich et al., 2001; Deng et al., 2018).

It is important to note that several isoenzymes belonging to the CYP450 supergene family metabolize AFB_1 through oxidative reactions, producing various metabolites with different carcinogenic potential.

In addition to the highly reactive and toxic AFBO, the main AFB_1 metabolic pathways described in animals can also give rise to relatively less toxic metabolites such as aflatoxicol (AFL) by ketoreduction or AFM_1 by hydroxylation and non-toxic metabolites such as AFB_{2a} or aflatoxin Q_1 (AFQ_1) by hydroxylation or aflatoxic P_1 (AFP_1) by demethylation (**Figure 4**; Dohnal et al., 2014; Deng et al., 2018).

Current literature data indicate that the rates of the bioactivation and detoxification of AFB_1 contribute greatly to the manifestation of its toxic effects. Furthermore, the metabolism of aflatoxins shows considerable interspatial differences and also significant variations among individuals belonging to the same species, depending for example on the age (Dohnal et al., 2014).

In conclusion, the largely different sensitivities of different animal species to the toxic effects of aflatoxins could be explained mainly with the remarkable variability of the metabolic pathways and enzymes that contribute to the bioactivation and detoxification of aflatoxins (Dohnal et al., 2014).

Poultry

Poultry are generally very sensitive to AFB_1 and adverse health effects have been reported in turkeys, quail, chickens, and ducks

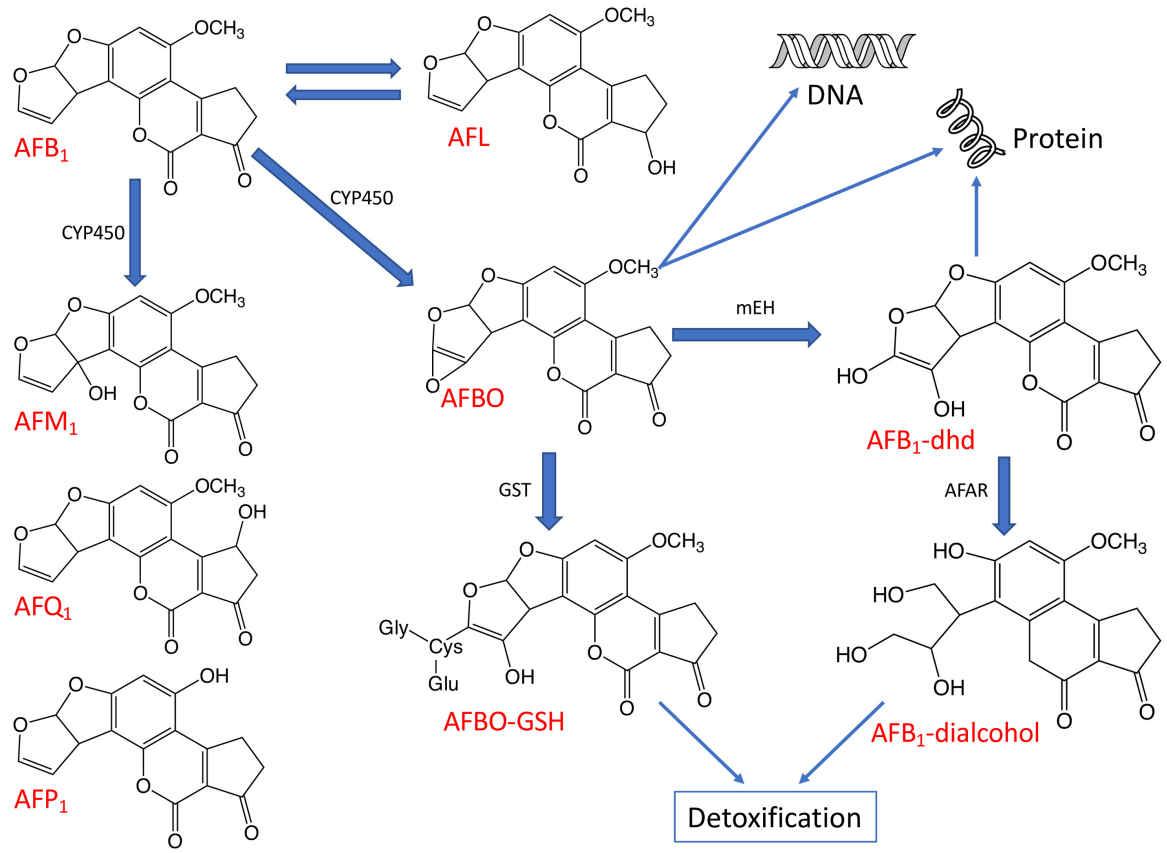

FIGURE 4 | The major metabolic pathways of AFB_1. The aflatoxin derivatives presented here include aflatoxin B_1 (AFB_1), aflatoxin M_1 (AFM_1), aflatoxin Q_1 (AFQ_1), aflatoxin P_1 (AFP_1), AFB_1-8,9-epoxide (AFBO), AFB_1-8,9-dihydrodiol (AFB_1-dhd) and aflatoxicol (AFL). Some enzymes taking part in the biotransformation and detoxification of aflatoxins are also indicated including cytochrome P450 (CYP450), glutathione-*S*-transferase (GST), microsomal epoxide hydrolase (mEH), and aflatoxin-aldehyde reductase (AFAR).

but there is a great variability in species-specific sensitivities to aflatoxins (Klein et al., 2000; Diaz et al., 2010; Rawal et al., 2010). Several toxicological studies pointed at the existence of a sensitivity scale starting from the less resistant young duck and turkey, followed by quails, up to the more resistant chickens. Obviously, species-specific differences in the AFB_1 biotransformation pathways, e.g., in AFB_1 modifying hepatic microsomal enzymes, could explain the varying susceptibilities of the species (Lozano and Diaz, 2006). It has been reported in some works that the microsomal liver fractions produced only AFBO in avian species (**Figure 4**), unless these animals were stimulated with CYP450 inducers (Lozano and Diaz, 2006). However, the ability of poultry species to metabolize AFB_1 to AFM_1 was reported in other works, in which the AFM_1 was detected in different tissues (Madden and Stahr, 1995; Wang H. et al., 2018). Lozano and Diaz (2006) reported that turkey microsomes produced 1.8–3.5 times more AFBO than quail and chicken microsomes. Furthermore, Diaz et al. (2010) suggested that the higher resistance of chicken to AFB_1 in comparison to quail could be due to a lower activation rate of AFB_1 to AFBO in chicken and also to a lower affinity for AFB_1 of the chicken microsomal enzymes.

The high susceptibility of poultry to AFB_1 appears to be a consequence of both the high activity of phase I microsomal detoxification enzymes to form AFBO, and to a low GST efficiency as well to conjugate AFBO with GSH (phase II detoxification). Some works reported that the partial or complete lack of GST-dependent detoxification of AFBO was the major reason for the exceptionally high susceptibility of poultry including turkeys to AFB_1 (Klein et al., 2000; Rawal et al., 2010).

Another metabolic pathway that may contribute to the extreme susceptibility of poultry to aflatoxins could be the reduction of AFB_1 to AFL *via* cytosolic reductase because the cytosolic metabolite AFL is produced in larger quantities in turkey and duck than in quail and chicken. This fact underlines that the formation of AFL cannot be regarded as a real detoxification pathway in these birds, moreover, microsomal dehydrogenase may oxidize AFL back to AFB_1, increasing the physiological half life of AFB_1 (Lozano and Diaz, 2006). Other aflatoxin metabolic pathways may also be involved in the manifestation of the high cytotoxicity of AFB_1 in poultry species.

Furthermore, it has also been reported that AFB_1 susceptibility correlated with age in both turkeys and broiler chickens. When livers obtained from 9, 45, and 61 day-old turkeys

were compared, microsomes from younger birds were more active in AFB_1 bioactivation than those from older ones (Klein et al., 2002). Moreover, Wang H. et al. (2018) underlined the efficient bioactivation of AFB_1 by CYP enzymes and the deficient detoxification by GST enzymes in younger 7-day old broilers.

Aflatoxin residues were detected in various tissues mainly in liver, kidney, the organs where AFB_1 is metabolized, but also in reproductive organs, in gizzard, breast and in legs (Herzallah et al., 2014). The metabolites AFB_1, AFQ_1, and AFL were excreted as such or as glucuronyl conjugates from bile in feces (Yunus et al., 2011). Some of these metabolites (AFM_1 and AFL) have been found in liver, kidneys and thigh muscles (Micco et al., 1988). The concentrations of AFB_1 residues decreased in the livers and muscles of all the birds after the suspension of mycotoxin feeding, and the elimination of AFB_1 from tissues was faster in older than in younger birds (Yunus et al., 2011). The dietary exposure to aflatoxin of hens, even at low concentrations, may also cause contamination of eggs. AFB_1 residues appeared in eggs after 5 days following the administration of AFB_1 contaminated feedstuffs, and they accumulated in line with protracted feeding with contaminated grain (Hassan et al., 2012). However, the amount of mycotoxin contaminants was below 0.1% of the AFB_1 intake owing to the AFB_1 metabolism in the birds. Few works also demonstrated the presence of hydroxylated AFB_1 derivatives (AFM_1 and AFQ_1) in eggs (Anfossi et al., 2015).

Pigs

Pigs are considered relatively susceptible to AFB_1. Tulayakul et al. (2006) studied AFB_1 metabolism in liver of different species in relation to the susceptibility to the toxic effects. The piglet's liver showed a relatively lower cytosolic GST activity to convert AFB_1-epoxide to AFB_1-glutathione conjugate product, thus favoring the formation of AFB_1-DNA adducts.

The metabolism and tissue distribution of AFB_1 in pigs were studied by Lüthy et al. (1980), and the major excretory route was found to be the feces (51–65% of the dose administered) but also urine was also an important excretory route. Actually, both AFM_1 and AFB_1 were detected in pig urine samples and AFM_1 was always found at higher concentrations in all studies (Thieu and Pettersson, 2009). In fact, urinary AFB_1 and AFM_1 are often used as biomarkers for aflatoxin exposure in pigs.

Ruminants

Ruminants are generally more resistant to the toxic effects of mycotoxins than monogastric animals, which could be explained mainly by AFB_1 degradation or bioconversion by rumen microorganisms. Controversely, some studies reported on that aflatoxins were generally poorly bioconverted in the rumen, with an overall decrease of only 10% (Westlake et al., 1989). Moreover, AFB_1 was incubated with intact rumen fluid or fractions of rumen protozoa and bacteria from sheep and cattle in the presence or absence of milled feed and the result clearly indicated that rumen fluid had no effect on AFB_1 (Kiessling et al., 1984). Another study showed that AFB_1 metabolism in rumen fluid was influenced by the animal species and the type of feed. In fact, rumen microbes from Korean native goats exhibited a greater degradation capacity for AFB_1 in comparison to Holstein steers. These diverging observations might be the consequence of varying rumen microbe profiles (Upadhaya et al., 2009).

AFM_1 is the most prominent metabolite formed in bovine hepatocytes within the first hours of incubation whereas AFB_1-dhd becomes determinative after a prolonged incubation. These two metabolites are mainly formed by CYP1A and CYP3A hepatic monooxygenase activities (Kuilman et al., 2000). According to Larsson et al. (1994), several extrahepatic tissues of sheep can also bioactivate AFB_1 very efficiently and can conjugate the bioactivated AFB_1 with GSH as well.

Following the channeling of AFB_1 in ruminants, the ingested aflatoxins may be degraded, at least in part, to AFL, AFM_1 and many other hydroxylated metabolites by certain rumen microbes or may be sequestered by some rumen fluid components such as chlorophyllin structures as well as bacterial and yeast cell walls (Gallo et al., 2015). The remaining fraction is rapidly adsorbed in the gastro-intestinal tract by passive diffusion and then is extensively metabolized in the liver to AFM_1, which enters the systemic circulation or is conjugated to glucuronic acid, and afterward excreted *via* bile, urine or milk (Kuilman et al., 2000; Rodrigues et al., 2019). Obviously, different levels of feed contamination may lead to different carry-over rates, which are also influenced by other physiological factors such the health status of animals including the status of the liver and its enzymatic activities. AFB_1, AFM_1 and AFL have been detected in liver, kidney and muscle tissue of bovine (Kuilman et al., 1998). AFM_1 is excreted *via* urine at a greater extent than through milk but the physiological factors regulating the relative uptake by kidneys and mammary glands are still unknown (Rodrigues et al., 2019). AFM_1 has been detected in both the milk and urine of cattle and dairy ewes 6 h after AFB_1 ingestion (Helferich et al., 1986; Battacone et al., 2003), and its concentration decreased rapidly after withdrawal of aflatoxin from diets (Rodrigues et al., 2019). Fecal excretion of FB_1 results from a lack of absorption by the GIT or a highly efficient elimination by the biliary system in the form of conjugated metabolites (Yiannikouris and Jouany, 2002; Jouany et al., 2009).

Goats were administrated with [^{14}C]-AFB_1, and urine, milk and feces were collected after 120 h. AFM_1 was found in milk at the highest concentration meanwhile AFQ_1 and AFL were found only in trace quantities in milk (Helferich et al., 1986). Other studies on goats also indicated that the absorption of AFB_1 in the GIT of adult ruminants was very fast, as was its hydroxylation to AFM_1 and release into the blood (Battacone et al., 2012). The short interval between AFB_1 administration and the detection of its metabolite in milk confirmed that the absorption of the toxin took place already in the rumen in goats.

AFLATOXINS IN FOODS OF ANIMAL ORIGIN

Aflatoxins are generally considered as the most important mycotoxins due to their carcinogenic properties, their persistence in food commodities once formed, and the wide range of food commodities that may be contaminated by them (Fink-Gremmels and van der Merwe, 2019). Aflatoxins contaminating

feeds pose a direct threat to livestock health and, indirectly, also affect human nutrition and health by reducing livestock productivity and *via* transfer from feed to foods of animal origin, namely milk, meat and eggs, even if milk is the only food of animal origin with relevant aflatoxin carry-over (Frazzoli et al., 2017).

Aflatoxins, particularly AFM_1, are of public health concern because they are efficiently excreted into milk, even if they may also contaminate other foods of animal origin at low levels and, therefore, the associated risks are considered to be minor (Fink-Gremmels and van der Merwe, 2019). Not surprisingly, many countries have set maximum levels of aflatoxins (AFB_1 or total aflatoxins, AFM_1) in food commodities and animal feeds, with the main aims to protect animal health and to prevent aflatoxin contamination of animal-derived foods. This review does not provide a systematic overview on aflatoxins in foods of animal origin but summarizes the discussions on the potential public health concerns specifically related to aflatoxins residues in these food commodities. In livestock animals, the best estimate transfer factors for mycotoxins in kidney, liver, muscle, fat, milk and egg were reported by MacLachlan (2011), and they clearly showed that no significant residues coming from aflatoxin contaminants of livestock feed are present in meat and eggs.

In the case of human dietary exposure from dairy products, aflatoxins are considered the most important mycotoxins and, based on data belonging to Food and Feed Safety Alert, 93% of the overall mycotoxin notifications referred to aflatoxins, whereas dioxins, dioxin-like polychlorinated biphenyls and AFM_1 were the most frequently reported chemical issues in dairy products (van Asselt et al., 2017). When ruminants were fed with contaminated feed, the AFB_1 consumed by the animals was partly degraded by the forestomach before reaching the circulatory system, and the remaining part was transformed by the liver into monohydroxy derivative forms, mainly to AFM_1, and, in smaller quantities, also to AFM_2, AFM_4 and AFL. Afterward, AFM_1 was secreted into the milk through the mammary glands (Frazzoli et al., 2017). AFM_1 has only from 2 to 10% of the carcinogenic potency of AFB_1 but it possesses the same liver toxicity. The ability of ruminants to convert the AFB_1 ingested with feedstuff to AFM_1 and to excrete this derivative in milk varies within broad limits in large and small ruminants and ranges between 0.35 and 3% in cows, 0.018 and 3.1% in goats and between 0.08 and 0.33% in sheep (Virdis et al., 2014). This remarkable variability in AFB_1 biotransformation observed in these species can be explained with differences in the activity of hepatic enzymes involved in the biotransformation and detoxification processes considering both their expression and catalytic activity (Becker-Algeri et al., 2016). The average conversion value was 2.5% (Veldman et al., 1992) in high yielding dairy cows, which produced a daily amount of about 40 L of milk, were tested. Importantly, Veldman et al. (1992) found a direct relationship between the carry-over rate and the milk yield with a maximal 6.2% carryover rate. AFM_1 is the most commonly detected aflatoxin in milk and the excretion of AFM_1 depends on a range of factors including diet composition, rumen degradation and liver biotransformation capacities, the duration of lactation (Fink-Gremmels and van der Merwe, 2019) as well as on the animal breed and udder health status (Masoero et al., 2007). In dairy cows ingesting AFB_1 contaminated feedstuffs, the excretoin of AFM_1 occured in 12 – 24 h and up to 2 – 3 days in milk, whereas the AFM_1 clearance in milk depended on several factors, mainly on the amount of ingested AFB_1 and the duration of mycotoxin consumption with an excretion for a variable period of about 5 – 7 days from the ending of AFB_1 assumption by cows (Masoero et al., 2007).

Well-reported variations in AFM_1 contamination were observed in milk worldwide, which were dependent on several factors like geographical area, environmental and climatic conditions including seasons and weather, as well as on the diversity and level of development of farming systems and the consumption of feed concentrates and green forage (Becker-Algeri et al., 2016). In recent years and independently of the type of commodity, the occurrence of AFM_1 in milk and dairy products was lower in Europe (for example in Italy, Portugal, Turkia, and Croatia) than in Asia or South America, where higher mycotoxin frequencies up to 100% were reported (Filazi and Sireli, 2013; Becker-Algeri et al., 2016). In Europe, low levels of AFM_1 contamination were reported in milk, and only 0.06% of the analyzed samples were above the European limit of 0.05 μg/kg milk. Nevertheless, when such incidents occur a widespread AFM_1 contamination of milk may develop, which has to be taken into account and adequately considered and controlled (van Asselt et al., 2017). In addition, risk managers should also consider that aflatoxin concentrations in milk may vary within the year and may also depend on the geographical location and climatic conditions. Finally, AFM_2 has also been investigated in milk with different outcomes varying from its absence to a not negligible occurrence in powdered, UHT and pasteurized milk samples (Becker-Algeri et al., 2016).

The AFM_1 contamination of dairy products is classified as an indirect contamination. For example, when the milk used in cheese-making was contaminated by aflatoxins, AFM_1 unevenly distributes between whey and curd, because AFM_1 prefers to bind to milk proteins, first of all to casein. For this reason, AFM_1 is more concentrated in the curd and cheese than in the milk itself, which was used for cheese-making (Anfossi et al., 2012). Therefore, AFM_1 levels were 3 – 8 times higher in certain dairy products than in the milk, and stable AFM_1 residues were detected in the final dairy products like milk powder even after heat processing. In addition, the total amount of AFM_1 does not change significantly during the cheese-making and cheese maturation processes but these steps influence the AFM_1 and protein concentration ratios as a result of skimming and water loss (Anfossi et al., 2012). Although many studies on the contaminations of dairy products by AFM_1 are available (Anfossi et al., 2012; Becker-Algeri et al., 2016) only few of them present any data estimating concentration factors for AFM_1 in different cheeses. However, 2.5 – 3.3 and 3.9 – 5.8 times higher concentrations of AFM_1 calculated on a weight basis were recorded in soft and hard cheeses, respectively, than those AFM_1 concentrations found in the milk, from which these cheeses were made (Filazi and Sireli, 2013). In Europe, the food business operator has to justify and provide the specific concentration or dilution factors for AFM_1 in the processed

foodstuffs during official controls performed by the competent authority (EC Regulation, 1881/2016).

In this context, AFM_1 contaminating milk should be unremitting to our attention and we should also take a special care of infants avoiding their exposures to AFM_1 *via* milk and infant formulas (Fink-Gremmels and van der Merwe, 2019). Kerekes et al. (2016) emphasized the importance of regular control of produced milk and also the introduction of an appropriate action limit in combination with immediate corrective actions at the farm level. In fact, feed producers have to manage and control the feed ingredients intended for the production of feed for the lactating animals for risk mitigation. Feed ingredients should be selected based on their quality characteristics, whereas farmers, when the AFM_1 content of milk exceeds the legal limit, have to withdraw milk consignments and also have to remove contaminated feedstuffs (Trevisani et al., 2014).

As far as the aflatoxin residues detected in edible tissues of bovine, pigs and poultry are concerned, these AFB_1 entry routes do not contribute significantly to human aflatoxin exposures (Fink-Gremmels and van der Merwe, 2019). Nevertheless, data on the aflatoxin contents in the edible tissues of bovine species are scarce and it is generally assumed that aflatoxins are partly degraded in the rumen and they are rapidly metabolized in the liver after absorption from the intestines. The transfer rates of aflatoxins into the edible tissues of pigs are very low owing to the rapid pre-systemic and hepatic metabolisms, and the aflatoxin residues in pork are therefore not considered as of public health concern. Similarly, poultry with low levels of aflatoxin contaminations do not seem hazardous to humans although the presence of aflatoxin-residues in poultry liver is well-documented (Fink-Gremmels and van der Merwe, 2019). Importantly, a rapid decrease in AFB_1 residues was observed in poultry muscles and liver after 3–7 days of uncontaminated dietary, significantly reducing the risk for human health (Filazi and Sireli, 2013). However, AFL is the main component of total AF residues in poultry with highest contents in liver (Frazzoli et al., 2017). In the case of laying hens, aflatoxins and their metabolites, particularly AFB_1 itself and AFL, can also be carried over to eggs but very discrepant transmission ratios were reported in this case. Recent studies demonstrated very low amounts of aflatoxin residues in eggs, merely between 0.01% (Herzallah, 2013) and 0.07% (Hassan et al., 2012) of the aflatoxin intake. AFB_1 residues appeared in eggs after 5 days of feeding with contaminated feedstuffs and the amount of AFB_1 depended on the duration of feeding with contaminated grain. Similar to dairy products, the presence of aflatoxins in eggs may be indicative of the aflatoxins contamination of the feed.

AFLATOXICOSES AND ANIMAL SUSCEPTIBILITY

In general, mycotoxicosis refers to syndromes appearing after ingestion, skin contact or inhalation of toxic secondary metabolites produced by toxigenic molds belonging to the genera *Aspergillus*, *Fusarium,* and *Penicillium* as well as to some other fungal taxa (Gallo et al., 2015). Within mycotoxicoses, aflatoxicosis refers to any disease caused by the consumption of foods and feeds contaminated with aflatoxins. It is well-known that AFB_1 is a potent mutagenic, carcinogenic, teratogenic, and immunosuppressive fungal secondary metabolite and all these effects may be linked to the interference of AFB_1 and its derivatives with the synthesis of proteins, the inhibition of various metabolic pathways or to the onset of oxidative stress. All these disadvantageous physiological effects will lead consequently to damages in various organs, especially in the liver, kidney, and the heart.

Aflatoxicoses may emerge in any livestock but literature reports on outbreaks mostly in poultry, pigs, equine, sheep, and cattle. The exposure of domestic animals to AFB_1 mainly occurs through the ingestion of contaminated feeds, however, skin contacts or inhalation exposures might also contribute (Gallo et al., 2015). It is well-known that ruminants are among the least susceptible animals to the negative effects of mycotoxins in comparison to monogastrics. However, the rumen has a saturable capacity of detoxifying aflatoxins by microflora, depending on (i) variations in the diet, (ii) the consequences of metabolic diseases, such as rumen acidosis, (iii) rumen barrier alterations as a result of animal diseases, and also (iv) the actual concentrations of aflatoxins present in the animal feed (Fink-Gremmels, 2008a). Consequently, clinical manifestations of aflatoxicoses in ruminants are associated typically with aflatoxins that are not degraded at all or not completely degraded by the rumen microflora.

Most of the data we have already had in our hands on mycotoxin toxicity are coming from experimental studies with purified compounds in otherwise healthy animals, which knowledge may help us with the early and reliable diagnosis of mycotoxicoses. However, when natural episodes of mycotoxicoses occur, versatile signs of disease could appear depending on the environmental conditions and also on several other features of the animals involved, including nutrition, sex and breed. For this reason, the diagnosis of mycotoxicoses is often difficult but it should rely on observing the clinical symptoms on the affected animals and also on analyzing the feed involved in the intoxication (Council for Agricultural Science and Technology [CAST], 2003). Given aflatoxins could act in synergy with other mycotoxins and also with other disease-provoking agents and, therefore, additional apparently unrelated pathological symptoms and even diseases are observed and reported in the affected animals. Furthermore, most mycotoxicoses including aflatoxicoses may present non-pathognomonic features and, consequently, there are no definitive diagnostic symptoms to orient farmers and veterinarians to assign aflatoxin exposures unequivocally to the death of animals. Obviously, even other otherwise unrelated diseases may trigger similar responses in the domestic animals to those of aflatoxins (Richard, 2008).

Aflatoxins do not affect all animals uniformly. Some animal species are inherently more resistant, such as sheep, goats and cattle, whereas other animals are more susceptible like swine, chickens, turkeys, and ducklings. In addition, considerable breed differences are documented within a given species (Richard, 2008), and the physiological responses to the adverse effects of

aflatoxins are also influenced by age (young animals are usually more sensitive than elder ones and, in particular, piglets and chicks), sex, diet, and weight, exposure to infectious agents, and the presence of other mycotoxins or other pharmacologically active substances (Zain, 2011). In addition, when mycotoxins are present simultaneously, some interactive effects, classified as additive, antagonistic or synergistic, could also occur (Gallo et al., 2015).

Animal Exposure to Aflatoxins

The exposure of animals to aflatoxins may trigger biological reactions that could be classified as acute, overt diseases with high morbidity and mortality, or, as it is usually the case, chronic, insidious disorders that impairs animal productivity (Bryden, 2012; Pierron et al., 2016). When livestock ingest aflatoxins the health effects could be acute, with severe consequences and evident signs of disease or even may be lethal when these toxins are abundantly consumed, even if this event is rare under farm conditions (Gallo et al., 2015). The timing of the proper diagnosis is a crucially important factor because the suspicious contaminated feed is likely consumed well before it can be tested (Council for Agricultural Science and Technology [CAST], 2003). The earliest clinical signs and lesions observed in turkey "X" disease, hepatitis "X" of dogs, and similar cases of acute aflatoxicoses were anorexia, lethargy, hemorrhages, hepatic necrosis, and bile duct proliferation (Miller and Wilson, 1994). Furthermore, the aflatoxins' impact on animals should not be limited to the extreme effects of aflatoxicoses because it is related mainly to the chronic toxicity caused by the consumption of sublethal doses and to the fact that low levels of chronic exposures may result in cancer.

Considering the chronic effects of aflatoxins, hidden pathological alterations with reduced ingestion, productivity and fertility were implied, including lowered milk, meat, and egg productions, decreased weight gains and/or unclear changes in animal growth, feed intake reductions or feed refusals, alterations in nutrient absorption and metabolism, various typologies of damages to vital body organs, disadvantageous effects on the reproduction and endocrine systems and also suppression of the immune system with subsequently increased disease incidence. The economic consequences of chronic aflatoxicoses are many times larger than those of the rare acute cases with immediate morbidity and lethality (Council for Agricultural Science and Technology [CAST], 2003).

Hepatotoxic, Carcinogenic and Mutagenic Effects

Among the major devastating effects of aflatoxins on animals, these harmful metabolites specifically target the liver and, hence, are proved to be primarily hepatotoxic. In acute aflatoxicosis, the emerging clinical symptoms of acute hepatic injury include coagulopathy, increased capillary fragility, hemorrhage and prolonged clotting times. Gross liver changes are caused by hemorrhage, centrilobular congestion, and fatty changes in surviving hepatocytes. Death of the poisoned animal may occur within hours or a few days after exposure. In broiler chicks, hemorrhagic anemia syndrome develops as characterized by massive hemorrhagic lesions in major organs and musculature even if the anemia could be considered as a secondary effect of severe hypoproteinemia caused by primary liver damage (Council for Agricultural Science and Technology [CAST], 2003). However, changes in extrinsic coagulation factors as determined by increased fibrinogen concentration were also reported in lambs (Zain, 2011). In addition, in broiler chicks, other reported clinical signs of aflatoxicosis were glomerular hypertrophy, hydropic degeneration of tubuler epithelium in kidneys and increases in the number of mesengial cells, as well as atrophy and lymphoid depletion in the thymus and bursa of Fabricius (Ortatatli and Oguz, 2001).

Even in chronic aflatoxicosis, most of the effects can be attributed to hepatic injury but with milder symptomes and icterus can also be observed. The pathological alterations in the liver mostly consist of degenerative changes and circulatory disturbances and also include a yellow to brassy color, enlarged gall bladder, diluted bile, histological signs of fatty changes in the hepatocytes, bile duct proliferation and periportal necrosis. In chronic aflatoxicosis, the signs are so protean that the episode may go undiagnosed for long periods of time (Pier, 1992). Because aflatoxins metabolized in the liver, the histological changes are observed primarily within this organ. Not surprisingly, centrilobular hepatic necrosis or hepatocellular vacuolar change and bile duct proliferation are consistent lesions in cow, sheep, goat and swine. Hepatic fibrosis has been reported in all species when the animals did not die from acute aflatoxicosis (Miller and Wilson, 1994). In Piedmontese calves, an outbreak of hepatic encephalopathy consequent to aflatoxin intoxication is to be mentioned: neurological signs varying from comatose or depressed mental status, spinal hyporeflexia, wasting and proprioceptive deficits, and compulsive behavior characterized by anteropulsion and right circling in large circles (D'Angelo et al., 2007).

Aflatoxins are also carcinogenic in animals and aflatoxin B_1 is the most powerful liver carcinogen known for rats. AFB_1 and AFG_1 possess an unsaturated bond at the 8,9 position on the terminal furan ring (**Figures 1**, **4**), and epoxidation at this position results in a reactive species, which induces oxidative stress of tissues, depletes antioxidants, forms DNA adducts and, hence, initiates malignant transformations. AFB_2 and AFG_2 are relatively less toxic unless they are metabolically oxidized first to AFB_1 and AFG_1 *in vivo*. Chronic exposure to low doses of aflatoxins is one of the major risk factors in the etiology of hepatocellular carcinoma, and all animal models exposed to AFB_1 have developed this type of cancerous desease thus far. Aflatoxins have been reported to cause other malignancies as well, including adenomas of esophagus, trachea, kidney and lungs, carcinoma of the pancreas and osteogenic sarcomas (Yilmaz et al., 2018). However, the carcinogenicity in farmed animals cannot be detected because of the relatively short period of time, in which the animals are fed prior to marketing (Richard, 2008). In addition, the chronic form of aflatoxicosis includes teratogenic effects in animals, which are associated with congenital malformations and, in the fetuses, multiple skeletal anomalies as incomplete ossification of skull

bones and failure of ossification of long and flat bones, as well as delay in the intramembranous ossification process, defects in the vertebrae formation or their reduction in size. Other mutagenic effects of aflatoxins cover mutations in genes, alterations of DNA by chromosomal breaks, rearrangement of chromosome pieces or even acquisition or loss of entire chromosomes (Fetaih et al., 2014).

Immunotoxic Effects

Although aflatoxins are primarily known as hepatotoxins and hepatocarcinogens, they have notable immunotoxic effects as well making animals more susceptible to many bacterial, viral, fungal and parasitic infections, as well as to the reactivation of chronic infections or reductions in vaccine and therapeutic efficacies (Oswald et al., 2005). Poultry (chickens and turkeys), pigs and in particular lambs are susceptible to induced immunosuppression due to aflatoxin exposure. Aflatoxins could impair both the cellular and humoral immune systems. *In vitro* and *in vivo* studies have demonstrated that AFB_1 is immunotoxic, exerting its action particularly on cell-mediated immunity through (i) reducing the number of circulating lymphocytes, (ii) the inhibition or suppression of lymphocyte blastogenesis, (iii) impairing both cutaneous delayed-type hypersensitivity and graft versus host reaction and (iv) the modification of the activities of natural killer cells and of macrophage functions through the inhibition of phagocytosis, the expression and secretion of cytokines (TNF-α, IL-1β, IL-6, IL-10, and IFN-γ), and also by reducing intracellular killing as well as the spontaneous production of oxidative radicals (Council for Agricultural Science and Technology [CAST], 2003; Oswald et al., 2005; Meissonnier et al., 2008). The general mechanism of the immunosuppressive effects of AFB_1 appears to be directly associated with the impairment of the synthesis of proteins. In fact, AFB_1 is transformed *in vivo* into metabolites, which are able to bind actively DNA and RNA, to impair the activity of DNA-dependent RNA polymerase and also to inhibit the synthesis of both RNA and proteins. These inhibitory mechanisms have direct and indirect effects on the proliferation and differentiation of the lymphoid system cells and on the synthesis of cytokines involved in the regulation of the immune system (Oswald et al., 2005). An alteration of the inflammatory responses with a reduced synthesis of pro-inflammatory cytokines and an increase of anti-inflammatory cytokines was reported in weanling piglets fed for 4 weeks with low doses of aflatoxin (Marin et al., 2002). The effects of aflatoxins on humoral immunity are not so clear as their effects on cell-mediated immunity, and these differences are hardly recognizable between the different animal species unless higher doses of aflatoxins were introduced (Council for Agricultural Science and Technology [CAST], 2003; Meissonnier et al., 2008). Suppression of humoral immunity has also been recorded after observing decreases in lymphocyte infiltration, hemagglutination and in serum protein levels (Rushing and Selim, 2019). In pig, no major effects on humoral immunity were observed after AFB_1 exposure but delayed and decreased ovalbumin-specific proliferation, suggesting an impaired lymphocyte activation (Pierron et al., 2016). However, a biphasic effect of AFB_1 was shown in piglets and broiler chicks, with immunosuppressive effects observable during acute exposures and with inflammatory response with stimulatory effects depending on the doses, more precisely, low doses of AFB_1 caused immunosuppression meanwhile high doses of it stimulated the immune system (Marin et al., 2002; Yunus et al., 2011). In details, piglets showed decreased leukocyte counts when exposed to low AFB_1, and an increase in leukocytes with a high dose (Marin et al., 2002). This immunotoxic effect has significantly disadvantageous consequences on the health of farmed animals, via increasing both the susceptibility and the severity of infections like coccidiosis, salmonellosis and *Cryptosporidium bailey* infections in chicken, *Erysipelothrix rhusiopathiae*, *Brachyspira hyodysentariae,* and *Escherichia coli* infections in pigs, the reactivation of chronic infection by *Toxoplasma*, and the impairment of vaccination efficacies for *Bordetella bronchiseptica* and *E. rhusiopathiae* or with the model antigen ovalbumin in swine (Oswald et al., 2005; Pierron et al., 2016), as well as for fowl cholera and Mareck disease in chickens and/or turkeys (Oswald et al., 2005).

Nephrotoxic Effect

Renal damages have also been reported after long-term administration of aflatoxins with the symptoms of inflammation, cell necrosis, and toxicosis, which may increase the weight of kidneys and may induce congestion in renal sinusoids. The kidneys are one of the target organs of aflatoxins, and their toxicity is activated by oxidative stress that alters the expression of proline dehydrogenase reducing the proline levels, which induces downstream apoptotic cell death. Moderate focal to diffuse necrosis in the renal tubules and increased renal tubular cells, which may be filled with bile pigments, hyaline, and lipid, with occlusions of their lumens with local edematous changes were reported in the kidneys of aflatoxin-exposed rats (Li et al., 2018). In poultry, the toxic effects exerted by AFB_1 on renal functions included reduced concentrations of calcium, inorganic phosphate, sodium, and potassium and increased levels of urea, creatinine and uric acid (Yilmaz et al., 2018). In addition, AFB_1 was reported to cause severe heart damage with tachycardia, tachypnea and even death, although the exact mechanism of cardiotoxicity has not been completely known.

Reproductive Effects

Not so long time ago, the harmful effects of aflatoxins on animals did not include any direct impairment of reproduction but indirect effects through other physiological systems have been considered. Nevertheless, more recent animal studies suggested that aflatoxins should also induce direct reproductive toxicity in both male and female animals based on adverse effects to both spermatozoa and oocytes. Following aflatoxin exposure *in utero*, monitoring growth parameters in baby animals indicated growth retardation, reduced fetal or egg weights and reduced fetal lengths of the offspring animals. In piglets exposed to maternal aflatoxicosis, growth retardation, thymic involution and impaired peripheral immune efficiency were events frequently reported and leading to early death (Mocchegiani et al., 1998), whereas broiler hens exposed to aflatoxin resulted in embryonic mortality and lowered the

immunity in the progeny chicks (Rawal et al., 2010). In addition, aflatoxins also possess spermatotoxic effects, which have an impact on the morphology and physiology of spermatozoa: AFB_1 affects the male reproduction system altering spermatogenesis as well as epididymal and Leydig cell functions, and also reducing the production of testosterone and the fertility in rats, birds and cattle (Agnes and Akbarsha, 2003). In females, AFB_1 reduces the fertility of oocytes by the disruption of oocyte maturation through epigenetic modifications as well as oxidative stress, excessive autophagy and apoptosis (Liu et al., 2015). In addition, in poultry, worsening egg production and quality, together with the deposition of aflatoxins residues in the eggs are described in both acute and chronic aflatoxicoses. The lowered egg production was attributed to the aflatoxins' effect on liver metabolism and function as well as liver lesions in layers, to the inhibited synthesis of proteins and lipogenesis, and to decreased feed intake and digestibility (Jia et al., 2016). It is well-known that aflatoxin causes alterations in the carbohydrate metabolism and impairments of the lipid transport, which effects result in decreased glucose levels and reduced lipid accumulations within hepatocytes, as well as pathological alterations in serum biochemistry and of most coagulation factors have been described in poultry, pigs, cattle and rabbits.

Gastrointestinal Dysfunctions

Aflatoxins modulate and affect the GIT in multiple ways, the most important of which are changes in the gut morphology, the digestive ability or activity of digestive enzymes, intestinal innate immunity and gut microbiota (Mughal et al., 2017). Nevertheless, only few reports are available in this field and the presented data are also controversial in many cases, especially for ruminants. The absorption of aflatoxins across the intestinal barrier is maximal in the upper part of the GIT in non-ruminant animals whereas in ruminants, the harmful aflatoxins like AFB_1 are transformed to less toxic compounds (e.g., AFM_1) or to metabolites with similar or even higher toxicity than the parent molecules (e.g., aflatoxicol) (Gallo et al., 2015). Among the overall adverse effects of aflatoxins, the most significant ones are related to the growth of animals and result in reduced performance. Aflatoxins cause reduced feed intake or even feed refusal with a subsequent decrease in body weight gain, which is determined by direct and/or indirect effects of aflatoxins on the nutrient quality, digestibility and/or absorption. During AFB_1 exposure, piglets showed reduced weight gain and Japanese quail have shown a reduction in egg weight (Marin et al., 2002). Reduced absorption of nutrients was reported after aflatoxin exposure and, in cattle, this decreased feed efficiency contributed to the observed compromised ruminal function by reducing cellulose digestion, volatile fatty acid production and rumen motility (Zain, 2011). In relation to nutrient digestibility and metabolizable energy, the presence of aflatoxin in dietary was suggested to reduce the apparent digestibility of crude proteins in ducks, to increase amino acid requirements and to reduce energy utilization in terms of net protein utilization and apparent digestible and metabolizable energy in ducks and chickens. Aflatoxins modulate the activity of digestive enzymes but contradictory effects were reported for amylase, trypsin and chymotrypsin activities in pancreas and duodenum with unchanged level of nutrient digestion in the intestine. However, aflatoxins seem to have only moderate affects on or even sometimes do not affect at all the growths of animals through the alteration or modulation of digestive functions (Grenier and Applegate, 2013), even if, in broiler chicks feed with experimental AFB_1 diet, impaired growth, major serum biochemistry measures, gut barrier, endogenous loss, and energy and amino acid digestibility were reported (Chen X. et al., 2016). The effects of AFB_1 on intestinal epithelium and microbiota were investigated in some *in vivo* studies in broiler chicken and rodents. The density of the whole intestine was reduced in the case of low AFB_1 doses but at higher doses no such changes were recorded, instead the number of apoptotic cells in the jejunum were elevated, jejunal villi presented lower height, and intestinal lesions were observed in duodenum and ileum, with leucocytic and lymphocytic infiltration. Meanwhile, reduced microbial diversity was observed in the colon with adverse effects on lactic acid bacteria *versus* unchanged proportion of *Firmicutes* and *Bacterioidetes* (Robert et al., 2017).

Additional symptoms of aflatoxicosis involved malnutrition. *In vitro* methods and animal models, predominantly, in piglets and broiler chicks, have showed that AFB_1 altered bioavailability and distributions of essential metal ions as zinc, calcium, magnesium and potassium, reduced the activities of lipogenic and amino acid metabolizing enzymes leading to reduced lipogenesis, and reduced serum concentrations of 25-hydroxy vitamin D, 1,25-dihydroxy vitamin D and calcium, consequently altering renal functions and parathyroid metabolism (Rushing and Selim, 2019).

Finally, aflatoxicosis in horses showed non-specific clinical signs, such as inappetence, depression, fever, tremor, ataxia and cough. Meanwhile, at necropsy, yellow-brown liver with centrilobular necrosis, icterus, hemorrhage, tracheal exudates and brown urine were observed (Caloni and Cortinovis, 2011).

QUALITATIVE AND QUANTITATIVE AFLATOXIN ANALYTICAL METHODS – ECONOMIC SIGNIFICANCE OF ANALYSIS

Since the massive death of turkeys (Turkey-X deceases) recorded in England in 1960, a wide spectrum of research has been launched and carried out to shed light on the causes of such high mortality (Büchi and Rae, 1969; Rodricks and Stoloff, 1977). Deciphering the factors leading to Turkey-X disease is a fascinating illustration of how a multidisciplinary approach may help us to solve an important animal health problem. The research covered the development of new analytical tools to measure mycotoxins more precisely, the exploration of the physiological and toxicological effects of these harmful compounds as well as the efficient removal of the toxins and setting up to prevent the manifestation of and to cure the disease itself (Forgacs and Carli, 1962).

Mycotoxins are mainly produced on small grains, cereals such as wheat, barley, oats, rye and triticale or on corn but animal products such as milk, meat, liver or eggs can also be contaminated by mycotoxins at various points of the feed and food chain (Gacem and El Hadj-Khelil, 2016; Udovicki et al., 2018). Because the sampling of feeds and foods for mycotoxin analysis may follow quite different protocols in different laboratories the standardization of these procedures represents a real challenge for analytics. During mycotoxin analysis, extraction and detection are crucially important issues to gain reliable analytical data, which may help us to optimize storage conditions and setting up rules to control mycotoxin production (Yao et al., 2015).

The first step in the analysis is to extract mycotoxins from the sample after correct sampling and sample preparation. The former and traditional extraction methods for aflatoxin analysis gave us a sample matrix in which the HPLC analysis was too complicated to carry out because of the presence of disturbing and interfering components (Kamimura et al., 1985). Later, the clean-up immonoaffinity columns containing gel suspension of monoclonal antibodies gained ground and became popular due its high specificity. The suspension retains the aflatoxin molecules what can be eluted cleanly, free from any disturbing compounds (Borbély et al., 2010; Lai et al., 2014). Another intention is the extraction with different solvents such as carbon tetrachloride (CCl_4), chlorobenzene (C_6H_5Cl), chloroform ($CHCl_3$), and dichloromethane (CH_2Cl_2), methanol and acetonitrile (Sepherd, 2009; Bertuzzi et al., 2012; Sarnoski et al., 2015).

Analytical methods of mycotoxin surveillance are wide-ranging and may vary within broad limits across countries. As a result of a community effort having been made to unify surveillance regulations in the European Union, the European Commission (2006) Regulation (EC) No. 401/2006 laid down the requirements for both recovery and precision in different toxin concentration ranges and gives the methodology for the validation of any analytical procedure, making possible to check if it is acceptable for official analysis (EC No. 401/2006). This covers all characteristics required for an analytical method with such a specific sample background, and the list of characteristics ranges from accuracy to measurement uncertainty through the limit of detection (EC No. 401/2006; Sheppard, 2008; Alshannaq and Yae-Hiuk, 2017; Shanakhat et al., 2018).

An overview on the available analytical methods can be given based on the remarkably abundant literature having been published in this field. We have a plethora of quantitative methods ranging from the different types of Thin Layer Chromatograpy-based to different varieties of HPLC to LC-MS/MS-based methodologies. In addition, we can also find good performance procedures among semi-quantitative methods like ELISA-based or biosensor-based protocols. Emerging technologies include hyperspectral imaging and aptamer-based biosensors (EC No. 401/2006; Sheppard, 2008; Vidal et al., 2013; Alshannaq and Yae-Hiuk, 2017; Shanakhat et al., 2018).

The performance parameters of different aflatoxin analytical methods are summarized in **Table 5**. The different methods can be characterized by several parameters such as accuracy, applicability, reproducibility, limit of detection and so on (Sheppard, 2008; Alshannaq and Yae-Hiuk, 2017; Shanakhat et al., 2018). Trucksess and Zhang (2016) argued that all practically useful analytical methods should meet the basic

TABLE 5 | Analytical methods for aflatoxin measurement.

Type of method	Technique	LOD	References
Quantitative methods	Thin Layer Chromatography combined with scanner	0.1 μg/kg B_2; G_2; M_1, 0.2 μ/kg B_1; G_1;	Kamimura et al., 1985
	High Performance Liquid Chromatography, in combination with fluorescence detector	0.002 μg/kg	Kilicel et al., 2017
	Liquid Chromatography with Mass Spectrometry	0.5 μg/kg	Sirhan et al., 2013
	Liquid Chromatography with tandem Mass Spectrometry	1 μg/kg	Alsharif et al., 2019; Ouakhssase et al., 2019
	Ultra High Performance Liquid Chromatography with fluorescence detector	0.02 μg/kg	Beltrán et al., 2011; Cui et al., 2017
	Capillary electrophoresis	1 μg/kg 0.1 ng/g	Arroyo-Manzanares et al., 2010; Xiao et al., 2018
Semi-quantitative methods	ELISA	1 ng/l	Huybrechts, 2011
	Lateral flow tests LFT	5 μg/kg	Goh et al., 2014,
	Direct fluorescence	5 μ/kg	Wacoo et al., 2014
	Fluorescence polarization immunoassay	30 ng/ml	Maragos, 2009
	Biosensors	0.05 ml 0.005 μg/l	Gurban et al., 2017; Man et al., 2017
Indirect methods	Spectroscopy	4 μg/kg	Wacoo et al., 2014
Emerging technologies	Hyperspectral imaging	10 μg/kg	Wang et al., 2014
	Electronic nose	5 μ/kg	Ottoboni et al., 2018
	Aptamer-based biosensors ECL	0.1 pg/ml	Shim et al., 2014; Castillo et al., 2015; Guo et al., 2016; Jia et al., 2019; Kordasht et al., 2019;

guidelines of reproducibility in different laboratory settings. Based on these premises, protocols that are used in different laboratories from sampling to analysis were compiled, and systems relying on certified material samples (CRMs) are also closely related to this.

Currently, a number of HPLC-MS or MS/MS equipment are used world-wide to gain a detailed overview on the mycotoxin spectra in feeds and foods depending on laboratory capabilities (Berthiller et al., 2018). At the same time, ELISA methods and equipment are used for quick mycotoxin measurements (Christoforidou et al., 2015; Xu et al., 2015; Sineque et al., 2017). New developments in this field have been published in the latest literature (Pennington, 2017; Udomkun et al., 2017; Yan et al., 2017). For example, a novel and promising method has been presented to detect aflatoxin B_1, B_2 and ochratoxin A in rice starting with dispersive liquid–liquid microextraction followed by LC and fluorescence detection (Lai et al., 2014; Adi and Matcha, 2018).

The impact of aflatoxins on human health (Theumer et al., 2018; Omotayo et al., 2019) is far the most important challenge, which we should keep an eye on in the whole feed and food chain (Zheng et al., 2018). This is the reason for why aflatoxin-related research including analytics is flourishing today. Future research should aim at a deeper understanding of the high-complexity and multi-parameter processes influencing the aflatoxin contents of feeds and foods. Novel multilateral approaches are definitely needed to control mycotoxins and their disadvantageous agricultural, health care and economic impacts more effectively (Krska et al., 2016; Stadler et al., 2018).

AUTHOR CONTRIBUTIONS

IP and ZG acquired funding, managed methodology, contributed to writing, reviewing and editing of the manuscript. FP, PS, WP, GP, TG, FG, AS, and ZG contributed to draft preparation, writing, editing and visualization. IP, PS, WP, and FP finalized the manuscript.

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REVIEW
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Aspergillus section *Flavi* and Aflatoxins: Occurrence, Detection, and Identification in Raw Peanuts and Peanut-Based Products Along the Supply Chain

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Aflatoxin contamination in foods is a global concern as they are carcinogenic, teratogenic and mutagenic compounds. The aflatoxin-producing fungi, mainly from the *Aspergillus* section *Flavi*, are ubiquitous in nature and readily contaminate various food commodities, thereby affecting human's health. The incidence of aflatoxigenic *Aspergillus* spp. and aflatoxins in various types of food, especially raw peanuts and peanut-based products along the supply chain has been a concern particularly in countries having tropical and sub-tropical climate, including Malaysia. These climatic conditions naturally support the growth of *Aspergillus* section *Flavi*, especially *A. flavus*, particularly when raw peanuts and peanut-based products are stored under inappropriate conditions. Peanut supply chain generally consists of several major stakeholders which include the producers, collectors, exporters, importers, manufacturers, retailers and finally, the consumers. A thorough examination of the processes along the supply chain reveals that *Aspergillus* section *Flavi* and aflatoxins could occur at any step along the chain, from farm to table. Thus, this review aims to give an overview on the prevalence of *Aspergillus* section *Flavi* and the occurrence of aflatoxins in raw peanuts and peanut-based products, the impact of aflatoxins on global trade, and aflatoxin management in peanuts with a special focus on peanut supply chain in Malaysia. Furthermore, aflatoxin detection and quantification methods as well as the identification of *Aspergillus* section *Flavi* are also reviewed herein. This review could help to shed light to the researchers, peanut stakeholders and consumers on the risk of aflatoxin contamination in peanuts along the supply chain.

Keywords: aflatoxins, *Aspergillus* section *Flavi*, peanuts, peanut supply chain, raw peanuts, peanut-based products

INTRODUCTION

Mycotoxins are toxic secondary metabolites produced mostly by fungi from the genus *Aspergillus*, *Penicillium*, *Fusarium,* and *Alternaria* which are formed pre- and post-harvest (Pitt and Hocking, 2009). The most significant mycotoxins contaminating agricultural commodities and foods are aflatoxins, fumonisins, ochratoxin A, zearalenone, patulin, citrinin, and deoxynivalenol (Afsah-Hejri et al., 2013a). According to Wild and Turner (2002), of these, aflatoxins are the most toxic, and have been extensively studied.

Peanuts (*Arachis hypogaea* L.) are legumes native to the western hemisphere. It is believed that peanut cultivation began in Bolivia and its neighboring countries before traders spread it to Asian and African continents. Peanuts consist of kernels and protective layer of outer shells. Peanuts are a good source of total energy, fats, minerals, vitamins, and proteins (Singh and Singh, 1991). Presently, peanuts are well adapted and widely grown in the tropical and sub-tropical countries such as India, China, Nigeria, Kenya, and the Southeast Asian countries including Malaysia (Archer, 2016). However, peanuts are not the main agricultural commodities in Malaysia, and the people rely on the import of peanuts from other countries such as India, China and Vietnam to fulfill the increasing demand (Afsah-Hejri et al., 2013a).

Recently, the occurrence of *Aspergillus* section *Flavi* and aflatoxin contamination has been reported in the supply chain of peanut-importing countries including Malaysia (Guezlane-tebibel et al., 2013; Norlia et al., 2018b). As a peanut-importing country, Malaysia is more concerned about aflatoxin production and contamination during storage, since Malaysia's tropical weather favors the growth of fungi including that of the aflatoxigenic *Aspergillus* spp. In addition, the precise identification and characterization of aflatoxigenic *Aspergillus* spp. that could survive and proliferate on the imported peanuts are less studied as compared to that on peanuts in the field (Zhang et al., 2017).

AFLATOXINS AND *Aspergillus* section *Flavi*

To date, there are 18 known analogs of aflatoxins with three series being significantly important from a food safety perspective: B-series (AFB_1 and AFB_2), G-series (AFG_1 and AFG_2) and M-series (AFM_1 and AFM_2). *A. flavus* and *A. parasiticus* are the major producers of aflatoxins, whereby the *A. flavus* produce B-series aflatoxins, while *A. parasiticus* produce both B- and G-series. The "B" and "G" refer to the blue and green fluorescence colors produced under UV light, while the subscript numbers indicate major and minor compounds, respectively (Dhanasekaran et al., 2011). Of these, AFB_1 is classified as a Group 1 carcinogen by the IARC (1993) due to the sufficient evidence of its involvement in cancer development in humans. Upon ingestion of the contaminated feeds by the animals, AFB_1 and AFB_2 are then metabolized in the body, thereby causing milk produced by the animals to be contaminated with their hydroxylated derivatives known as AFM_1 and AFM_2 (Dhanasekaran et al., 2011).

Morphological identification of *Aspergillus* section *Flavi* is usually based on the microscopic structures, such as the uni- or biseriate conidial heads, production of dark-colored sclerotia by certain species, and yellow green to brown shades conidia. *Aspergillus* section *Flavi* includes 33 species, and most of them are natural producers of aflatoxins (Frisvad et al., 2019). Members of this section can exist in the soil as sclerotia or conidia, or mycelia in plant tissue. Sclerotia of *A. flavus* (Horn et al., 2009a) and *A. parasiticus* (Horn et al., 2009b) can also be produced naturally in crops by an asexual or sexual stage and are dispersed onto the soil during harvest. Sclerotia can survive under severe environmental conditions in the field and germinate into mycelia, followed by the formation of the conidiophores and conidia when the condition becomes favorable (Horn et al., 2014). The mechanism of *A. flavus* sexual reproduction in a natural environment which includes the fertilization in soil and crops, has been described by Horn et al. (2016). The exchange of genetic materials during sexual recombination results in the high genetic diversity in *A. flavus* population. Thus, the morphology, mycotoxin production and vegetative compatibility groups (VCGs) in *A. flavus* are more diverse as compared to other species in section *Flavi*.

According to Cotty (1989), two morphotypes of *A. flavus* have been designated based on the size of their sclerotia. The large (L) strain and small (S) strain are indicated by sclerotia size of >400 μm and <400 μm in diameter, respectively. The S-type *A. flavus* has been reported to be more toxigenic than the L-type, and it is also more dominant in the West Africa. Probst et al. (2007) revealed that the S-type *A. flavus* was the causal agent of the aflatoxicosis outbreak in Kenya in 2004 due to the consumption of contaminated corn. However, the phylogenetic studies revealed that the S-strain *A. flavus* from Kenya were different from the US and Asian S-type *A. flavus*, but were closer to *A. minisclerotigenes* (Probst et al., 2012).

The accurate identification of *Aspergillus* section *Flavi* requires a triphasic approach which includes the morphological, chemical and molecular approaches as these species are closely related and could not be easily distinguished by morphological characteristics alone (Varga et al., 2011; Frisvad et al., 2019). The information on the production of secondary metabolites such as cyclopiazonic acid (CPA), aspergillic acid, kojic acid, asperfuran, paspalinin, paspaline, nominine, chrysogine, parasiticolides, aflavarins, aflatrems, and aflavinines will strengthen the species identification (Pildain et al., 2008; Varga et al., 2011; Frisvad et al., 2019). According to Lansden and Davidson (1983), CPA can be found either alone or co-occurring with aflatoxins in various crops such as peanuts and corn. During the outbreak of Turkey X disease in England (1960's), about 100,000 of Turkeys and other poultry died due to the consumption of contaminated peanut meal imported from Brazil. It was believed that CPA acted as a co-contaminant with aflatoxins, thereby causing severe aflatoxicosis (Cole, 1986). The co-occurrence of CPA and aflatoxins in stored peanuts has also been reported by Zorzete et al. (2013).

In contrast, *A. sojae* and *A. oryzae*, which are respectively known as the domesticated counterparts of *A. parasiticus* and *A. flavus*, do not produce aflatoxins, although they possess the homologues of the aflatoxin biosynthesis pathway gene. For their safety status, these species are widely used for food fermentation in Asian countries such as *sake*, soy sauce and *miso* (Payne et al., 2006). There are also some cases of *A. flavus* losing their toxigenic properties thus becoming non-aflatoxigenic even though they possess all the necessary genes for aflatoxin biosynthesis in their genome (Yu et al., 2004). It is believed that the genetic variation in the non-aflatoxigenic *A. flavus* strains is caused by the sexual reproduction and genetic recombinant in nature (Horn et al., 2016).

The non-aflatoxigenic *A. flavus* has been previously described and is used as a biological control agent based on the competitive exclusion to reduce the aflatoxigenic species in peanuts (Chulze et al., 2014; Ehrlich, 2014). The conidia of the inoculated non-aflatoxigenic strains will compete with the aflatoxigenic strains naturally present in the soil for growth and essential nutrients from peanuts. The application of non-aflatoxigenic *A. flavus* in the peanut field successfully reduced the aflatoxin contamination in peanut-producing regions in the United States (Dorner et al., 2003) and Northern Argentina (Zanon et al., 2016). In addition, Dorner and Cole (2002) also successfully demonstrated the ability of non-aflatoxigenic strains of *A. flavus* and *A. parasiticus* to reduce the aflatoxin contamination in peanuts during storage. However, there is a limitation on using the non-aflatoxigenic strains as a biocontrol. According to Ehrlich (2014), the application of non-aflatoxigenic strains in the field should be of concern as the outcross with the native population of *A. flavus* in soil could result in the offspring regaining the ability to produce aflatoxins. The global warming that causes the climate change might also be a challenge as the crops can be subjected to damage and further facilitate the fungal infection since the stress on plants could induce the gene expression for mycotoxin production and sexual recombination in *A. flavus*.

FACTORS AFFECTING *Aspergillus* spp. GROWTH AND AFLATOXIN PRODUCTION IN PEANUTS

Temperature, relative humidity and moisture content are the main factors that determine the ability of *A. flavus* to grow during storage (Waliyar et al., 2015a). Relative humidity and water activity (a_w) in foods are interrelated to each other and could be used to determine the ability of fungi to grow. Technically, a_w is defined as the amount of freely accessible water on a substrate which is readily available for microbial growth. The a_w of pure water is 1.00 which equals to 100% relative humidity. Peanuts might be contaminated by aflatoxins if they are not dried immediately and fail to maintain a safe moisture level during post-harvest. According to Dorner (2008), inadequate drying of peanuts favors the growth of aflatoxigenic *Aspergillus* spp. during storage. This is in fact a challenge since peanuts are naturally hygroscopic and tend to absorb moisture from the surrounding storage environment (Waliyar et al., 2015a). Therefore, the source of moisture during storage such as leaking roofs and condensation due to improper ventilation in the warehouse should be avoided in order to maintain low moisture levels during storage. It is recommended to store peanuts with moisture content <7% and <9% for shelled and unshelled peanuts, respectively to avoid fungal growth. These moisture content levels might guarantee safe storage for peanuts for approximately 1 year if the temperature and relative humidity are maintained at 25 – 27°C and 70%, respectively (Torres et al., 2014; Waliyar et al., 2015a). According to Villers (2014), fungi start to grow when the relative humidity exceeds 65% during storage. Temperature and a_w has a significant effect on the growth of *Aspergillus* section *Flavi*, aflatoxin biosynthesis gene expression and the subsequent aflatoxin production (Schmidt-Heydt et al., 2009; Abdel-Hadi et al., 2012; Bernáldez et al., 2017). However, the minimum a_w for growth varies depending on the temperature and nutrient availability in the substrate. The minimum a_w for *A. flavus* growth was reported to be at 0.91 a_w at 25 and 37°C in sorghums (Lahouar et al., 2016), while the minimum a_w in paddy was predicted between 0.83 and 0.85 (Mousa et al., 2011). A similar range of minimum a_w was observed in shelled peanuts (Liu et al., 2017). The authors also demonstrated a lower growth rate when $a_w < 0.85$ or temperature < 20°C, while better growth was observed at a higher a_w and around 28–40°C.

The growth of *A. flavus* might occur over a wider range of temperature and a_w level as compared to the aflatoxin production which occur in a narrower range of conditions (Abdel-Hadi et al., 2012; Liu et al., 2017). According to Abdel-Hadi et al. (2012), the optimum temperatures and a_w level for *A. flavus* was 30 – 35°C and 0.99 a_w. The marginal conditions for the growth were reported at 15 and 40°C at 0.99 a_w. On the other hand, the optimum conditions for AFB_1 production were 30 – 35°C at 0.95 a_w, and 25 – 30°C at 0.99 a_w. Another study by Schmidt-Heydt et al. (2010) reported that the growth of *A. parasiticus* was optimum at 35°C. However, AFB_1 and AFG_1 production were optimum at >37°C and 20 – 30°C, respectively. They also discovered that temperature was the key parameter for AFB_1 production, whereas a_w contributed more to AFG_1. The optimum temperature of *A. flavus* growth on shelled peanut was 37°C while the production of AFB_1 was maximum at 28°C and 0.96 a_w. AFB_1 was not detected at $a_w < 0.90$ when temperature fell below 20°C or $a_w \geq 0.96$ when the temperature was higher than 40°C (Liu et al., 2017).

Drought stress in the field was reported to increase the aflatoxin contamination in peanuts due to over-maturity, reduction of moisture content in seeds and increased risk of insect and pod damage which facilitate the aflatoxigenic *Aspergillus* spp. infection in peanuts (Craufurd et al., 2006; Waliyar et al., 2015b; Sibakwe et al., 2017). A previous study by Sibakwe et al. (2017) reported that severe drought caused poor growth and pod development which increased the susceptibility to *A. flavus* infection. In addition, the growth of *A. flavus* was supported by the exudation of sucrose from roots and peanut pods under the drought stress. Therefore, high levels of *A. flavus* and aflatoxins were recorded during prolonged drought. Another study by Arunyanark et al. (2009) demonstrated that

high soil temperature and low moisture in soil favored aflatoxin production in peanuts. High soil temperature enhanced moisture loss from peanut kernel and subsequently reduced the a_w level. Low a_w in peanut kernels results in the reduction of phytoalexins which are responsible for the defense mechanism against plant pathogens.

PEANUT PRODUCTION AND CONSUMPTION IN MALAYSIA

Peanuts are not the main agricultural product in Malaysia, and the local production was just around 231 tons in 2016 as compared to the main producer countries such as China (16,685,915 tons), India (6,857,000 tons), Nigeria (3,028,571 tons) and the United States (2,578,500 tons). In Southeast Asia, Indonesia, and Vietnam are the main peanut producers, which recorded a total production of 504,912 tons and 427,190 tons in 2016, respectively (FAOSTAT, 2017). Peanut production in Malaysia has declined since 1985 and since then, the import of peanuts has gradually increased and peaked in 2011 (FAOSTAT, 2017). As local peanut production is low, Malaysia needs to import peanuts from other countries in order to meet the local demand.

In Malaysia, peanuts are widely used as the raw material for local dishes and other peanut-based products such as peanut sauces, cookies, roasted peanuts, peanut butter and peanut snacks (Leong et al., 2010; Norlia et al., 2018b). However, from a food safety perspective, peanuts are known as a common food allergen and a carrier for foodborne diseases such as aflatoxicosis and salmonellosis (Chang et al., 2013). The presence of aflatoxins is among one of the crucial aspects that regulate the quality of peanuts other than the physical and chemical properties. Based on Malaysian Food Consumption Statistics (IPH, 2014), the mean daily intake of peanuts among Malaysian were 1.86 g/day (non-frequent eaters) and 4.95 g/day (frequent eaters), respectively. Generally, the Malays recorded the highest intake for both peanuts and peanut butter. Long term intake of aflatoxin-contaminated foods leads to a chronic exposure and hence increases the risk of hepatocellular carcinoma (HCC), commonly known as liver cancer. Several researchers have estimated the dietary exposure of aflatoxins among the Malaysian population (Leong et al., 2011; Chin et al., 2012). For AFB_1, Chin et al. (2012) reported the dietary exposure of 24.3–34.0 ng/kg bw/day. Among 236 food composites tested, peanuts were found to be the main contributor to aflatoxin contamination. Based on this finding, the liver cancer risk among the Malaysian population was estimated to be 0.61 – 0.85% cancers/100,000 population/year which contributed to 12.4 –17.3% of the liver cancer cases.

ADVERSE EFFECTS OF AFLATOXINS TO HUMANS AND ANIMALS

Aflatoxin exposure in humans could be due to direct or indirect consumption of contaminated foods. Direct exposure is when the aflatoxin-contaminated food is directly consumed while the indirect exposure is caused by the ingestion of dairy product contaminated with AFM_1, or consumption of meat product from animals fed with contaminated feed. AFM_1 has also been detected in human breast milk which subsequently exposes the baby to aflatoxins (Dhanasekaran et al., 2011). Aflatoxicosis is a health complication due to the ingestion of aflatoxin-contaminated foods. However, the response depends on the age and health condition, nutritional diet, level and duration of exposure, and environmental factors (Wagacha and Muthomi, 2008). The rapid onset and obvious toxic response are signs of acute toxicity of aflatoxins. Other symptoms of aflatoxicosis might include diarrhea, jaundice, low-grade fever, anorexia, and a decrease in the amount of essential serum protein, which is synthesized by the liver. In severe cases, aflatoxicosis might cause death to humans. Chronic aflatoxicosis results in cancer, immune suppression, stunted growth and malnutrition among children (Lewis et al., 2005; Wild and Gong, 2010).

The liver is known to be the main target for aflatoxin toxicity and carcinogenicity. The lesion could be observed in the affected liver, and this increases the risk of HCC over time (Liu and Wu, 2010). The HCC has been well documented, and the incidence is most likely to occur in a person with chronic hepatitis B virus (HPV) infection. In addition, children chronically exposed to aflatoxin-contaminated breast milk and other dietary foods, especially peanut-based product might develop cirrhosis especially in the malnourished ones (Dhanasekaran et al., 2011).

The consumption of aflatoxin-contaminated feed in animals also results in similar symptoms, and the susceptibility depends on age, species and individual variation. Acute aflatoxicosis may cause depression, weight loss, liver damage and gastrointestinal bleeding in animals while in severe cases, death may occur within several days. Prolonged aflatoxin exposure may reduce the growth rate of young animals and affect the quality of milk and egg due to the contamination of AFM_1. The hepatic pathology in affected animals includes an enlarged gall bladder, changes of fatty acid in the hepatocytes, bile duct proliferation and diluted bile. In addition, AFB_1 has also been reported to reduce the nutrient adsorption and causes immunosuppression in animals (Lizárraga-Paulín et al., 2011; Sarma et al., 2017).

THE OCCURRENCE OF AFLATOXINS IN RAW PEANUTS AND PEANUT-BASED PRODUCTS

The warm temperature (28 – 31°C) and high humidity (70 – 80%) in Malaysia favor the growth of *Aspergillus* spp. and cause the peanuts to be easily deteriorated due to fungal infection when stored under these conditions. The occurrence of aflatoxigenic *Aspergillus* section *Flavi* in a variety of nuts, cocoa beans, coffee, grapes, rice, dried fruits, corn, and small grains has been extensively reviewed by Taniwaki et al. (2018). However, the occurrence of these species does not always result in aflatoxin contamination as they might be present in foods without producing any toxins. In relation to aflatoxins, some authors pointed out that, on average, 50% of the isolated strains were

able to produce aflatoxins in food (Geisen, 1998). Many strategies on the mitigation of aflatoxin in peanuts, including physical, chemical and biological methods, have been discussed and reported (Dorner, 2008; Wagacha and Muthomi, 2008; Torres et al., 2014; Waliyar et al., 2015a). However, none of the method could entirely eliminate aflatoxins in the food commodities.

Aflatoxin contamination occurs during pre-harvest, post-harvest and worsens during storage at the granary. A previous study in Mali indicated that aflatoxin level increased with increasing storage period at the granary (Waliyar et al., 2015b). According to the authors, aflatoxin contamination occurred due to pest damage and the inappropriate storage conditions that favored the growth of aflatoxigenic *Aspergillus* spp. Another study in Malawi also demonstrated a similar trend in aflatoxin contamination during post-harvest (Monyo et al., 2012). Samples were collected from different districts in Malawi, and the results revealed that 21 and 8% of samples in 2008 and 2009 respectively, were contaminated with aflatoxin level higher than 20 ppb. Aflatoxins in peanut-based products have also been reported especially from the African and Asian countries. **Table 1** summarizes the occurrence of aflatoxins in raw peanuts and peanut-based products from different countries. Most of the peanut-producing countries such as Kenya, Haiti, and Indonesia reported very high concentrations of aflatoxins in peanut based-products (Ambarwati et al., 2011; Ndungu et al., 2013; Schwartzbord and Brown, 2015). In contrast, other peanut-importing countries such as Taiwan (Chen et al., 2013) and Korea (Ok et al., 2007) recorded a lower level of aflatoxin concentration in their peanut-based products. A study by Matumba et al. (2015) revealed that aflatoxin levels in peanut-based products on the local market in Malawi were significantly higher as compared to the raw peanuts intended for exports. This crucially indicated that the non-compliant samples for exports were not removed from the domestic supplies probably due to the limited public awareness among the consumers. A similar finding was reported by Schwartzbord and Brown (2015) who found that 94% of the peanut butter samples were heavily contaminated with aflatoxins, with the majority of samples exceeding 20 μg/kg. In contrast, only 14% of the raw peanut samples exceeded the regulatory limit. This might indicate that the contamination occurred more during storage pre-processing as compared to post-harvest. Ezekiel et al. (2012) also reported high aflatoxin contamination level in peanut cakes marketed in Nigeria, with 90% of the samples exceeding 20 μg/kg for total aflatoxins.

In Malaysia however, aflatoxin contamination was mostly reported in raw peanuts as compared to peanut-based products. Abidin et al. (2003) revealed that 92% of raw peanut samples collected from five districts in Perak were contaminated in the range of 0.3 – 762.1 μg/kg. Furthermore, about 42% of raw peanut samples collected from Kuala Terengganu were also contaminated with aflatoxins in the range of 0.2 – 101.8 μg/kg (Hong et al., 2010). In Selangor, Arzandeh et al. (2010) reported that about 78.5% from a total of 84 raw peanut samples collected from the retail market were contaminated, and about 10.7% of the samples exceeded the maximum tolerable limit. The aflatoxin concentrations varied from 2.76 to 97.28 μg/kg. Another study by Farawahida (2018) reported that aflatoxin contamination ranged from 12.8 – 537.1 μg/kg and 5.1 – 59.5 μg/kg in raw peanuts and peanut sauce, respectively. About 38 and 22% of raw peanut samples collected from the retailers and manufacturers in Malaysia respectively, were found to exceed the Malaysian Regulation limit (Norlia et al., 2018b). In addition, the authors reported that aflatoxin contamination in raw peanut samples ranged from <LOD – 1021.4 μg/kg, while peanut-based product samples recorded a lower level of contamination (<LOD – 19.4 μg/kg). However, there was no significant difference in the *Aspergillus* spp. contamination for both types of peanuts, and there was only a moderate relationship (Pearson's r = 0.425, p = 0.00) between AFB_1 and *A. flavus/A. parasiticus* count. According to Martins et al. (2017), the *Aspergillus* spp. count and aflatoxin amount in peanuts does not always positively and strongly correlate especially in processed peanuts. The reduced a_w in the dried peanut-based products reduces the levels of viable aflatoxigenic fungi as they rarely grow below 0.8 a_w. However, the aflatoxins still remain in the products. According to Farawahida et al. (2017), a combination of oil-less frying of chili powder and retort processing of peanut sauces significantly reduced the aflatoxin concentration but could not entirely eliminate them from the products.

Aflatoxins in peanut-based products were also reported in samples collected from the local markets in Malaysia. In Penang, a total of 196 nuts and nut products were tested for aflatoxins, and 16.3% of these were contaminated with aflatoxins ranging from 16.6 to 711 μg/kg (Leong et al., 2010). Coated nut products were found to be the highest contaminated sample in the range of 113.0 – 514.0 μg/kg. Apart from that, a previous study by Ali (2000) also reported high contamination of aflatoxins in peanut butter (0.1 – 35 μg/kg), and a local traditional product called "*kacang tumbuk,*" which was prepared from blended peanut, was found to be the most contaminated product. Similar findings were also reported by researchers from the neighboring country, Indonesia (Ambarwati et al., 2011).

Aspergillus spp. AND AFLATOXIN CONTAMINATION ALONG THE PEANUT SUPPLY CHAIN

A food supply chain describes the processes involved from food production to food consumption which often includes processors, packers, distributors, transporters, retailers, and consumers (Levinson, 2009). For agricultural commodities, an efficient supply chain management is vital since these commodities are naturally susceptible to fungal invasion pre- and post-harvest, and as a result, aflatoxin contamination. The overall peanut supply chain consists of several major stakeholders which include the producers, collectors, shellers, exporters, importers, manufacturers, retailers, and finally the consumers (Archer, 2016). There are several stages for fungal contamination at post-harvest stage such as sun-drying and threshing, shelling, sorting, blanching and roasting. However, the manufacturing process varies depending on the types of its final product. For example,

TABLE 1 | The occurrence of aflatoxins in peanuts from different countries.

Country	Type of peanuts	No. of samples	Aflatoxin level (μg/kg)		*Non-compliant samples (%)	References
			Mean	Range		
[a]Kenya(Nairobi and Nyanza)	Raw peanut	3	18.3	0.0 – 52.4	20	Ndungu et al., 2013
	Roasted peanut	8	54.8	2.4–297.7	50	Ndungu et al., 2013
	Peanut butter	11	318.3	0.0–2377.1	73	Ndungu et al., 2013
	Unsorted peanut	11	111.2	0.0–364.7	74	Ndungu et al., 2013
	Sorted peanut	4	24.0	0.0–82.4	18	Ndungu et al., 2013
[a]Kenya(Eldoret and Kericho)	Raw peanut	78	146.8	37.8–340.2	n.a.	Nyirahakizimana et al., 2013
	Roasted coated	101	56.5	29.4–93.1	n.a.	Nyirahakizimana et al., 2013
	Roasted de-coated	49	19.9	0.0–42.3	n.a.	Nyirahakizimana et al., 2013
Nigeria[b]	Peanut cake	29	200.0	10–2820	90	Ezekiel et al., 2012
Brazil	Raw peanut	48	12.9	n.a.	8.3	Oliveira et al., 2009
	Raw peanut	58	45.3	n.a.	n.a.	Hoeltz et al., 2012
	Peanut product	43	49.8	n.a.	n.a.	Hoeltz et al., 2012
	Ground candy peanut	48	9.0	n.a.	8.3	Oliveira et al., 2009
	Salty roasted peanut	48	1.6	n.a.	–	Oliveira et al., 2009
	Salty dragee peanut	48	3.32	n.a.	2.1	Oliveira et al., 2009
Malawi	Raw peanut (local market)	69	122.3	0–501.0	n.a.	Matumba et al., 2015
	Raw peanut (for export)	27	2.6	0–9.3	–	Matumba et al., 2015
	Peanut butter	14	72.0	34.2–115.6	n.a.	Matumba et al., 2015
[b]Haiti	Raw peanut	21	n.a.	2.0–787	14	Schwartzbord and Brown, 2015
	Peanut butter	11	n.a.	2.0–2720	82	Schwartzbord and Brown, 2015
Korea	Raw peanut	27	4.07	0.1–18.0	n.a.	Ok et al., 2007
	Peanut butter	19	3.6	1.3–6.4	n.a.	Ok et al., 2007
Taiwan	Raw peanut	257	14.9	0.3–107.1	0.8	Chen et al., 2013
	Peanut butter	142	2.8	0.2–32.5	4.9	Chen et al., 2013
[c]Thailand	Raw peanut	20	47.1	n.d.–303.6	5	Kooprasertying et al., 2016
	Raw peanut	28	102	4 - 576	n.a.	Lipigorngoson et al., 2003
	Peanut product	713	n.a.	0.7–3238	n.a.	Songsermsakul, 2015
	Roasted peanut	20	13.5	0.7–41.6	5	Kooprasertying et al., 2016
	Ground peanut	20	68.2	0.9–362.5	9	Kooprasertying et al., 2016
[d]Indonesia	Peanut products	15	8.0	0.4–53.1	13.3	Aisyah et al., 2015
	Roasted peanut	33	43.2	0–316.8	42	Ambarwati et al., 2011
	Flour-coated peanut	33	34.28	0–160	30	Ambarwati et al., 2011
	Pecel/gado-gado sauce	33	17.1	0–197.8	21	Ambarwati et al., 2011
	Siomay sauce	18	4.41	0–39.9	11	Ambarwati et al., 2011
	Peanut sauce	12	23.17	0–198.6	17	Ambarwati et al., 2011
	Roasted peanut	12	n.a.	0–204	n.a.	Razzazi-Fazeli et al., 2004
	Coated peanut	16	n.a.	5–870	n.a.	Razzazi-Fazeli et al., 2004
	Peanut cake	10	n.a.	5–302	n.a.	Razzazi-Fazeli et al., 2004
	Peanut sauce	12	n.a.	7–613	n.a.	Razzazi-Fazeli et al., 2004
	Peanut butter	10	n.a.	7–228	n.a.	Razzazi-Fazeli et al., 2004
[e]Malaysia	Raw peanut	6	146.5	0–537.1	33	Farawahida et al., 2017
	Raw peanut	6	6.1	0.6–19.3	n.a.	Afsah-Hejri et al., 2013a
	Raw peanut	9	2.0	2.2–6.4	-	Khayoon et al., 2012
	Raw peanut	13	4.25	1.47–15.3	n.a.	Reddy et al., 2011
	Raw peanut	77	n.a.	0.1 – > 50	21	Ali, 2000
	Raw peanut	84	11.3	0–103.2	10.7	Arzandeh et al., 2010
	Raw peanut	14	n.a.	17.8–711	n.a.	Leong et al., 2010
	Raw peanut	20	n.a.	0–33.4	n.a.	Hong et al., 2010
	Raw peanut	145	n.a.	0.85–547.5	45	Sulaiman et al., 2007

(Continued)

TABLE 1 | Continued

Country	Type of peanuts	No. of samples	Aflatoxin level (μg/kg)		*Non-compliant samples (%)	References
			Mean	Range		
	Raw peanut	210	n.a.	0.3–762.1	n.a.	Abidin et al., 2003
	Peanut sauce	6	22	0–59.5	33	Farawahida, 2018
	Roasted peanut (in shell)	10	n.a.	29.7–179	n.a.	Leong et al., 2010
	Roasted peanut (shelled)	20	n.a.	40.1–46.0	n.a.	Leong et al., 2010
	Peanut butter	12	n.a.	16.6–67.3	n.a.	Leong et al., 2010
	Coated nut product	20	n.a.	113.0–514.0	n.a.	Leong et al., 2010
	Peanut butter	23	n.a.	0.1–35	17	Ali, 2000
	Other peanut product	74	n.a.	0.1–>50	26	Ali, 2000

*Maximum regulatory limit for total aflatoxins set by respective countries. [a]Kenya Bureau of Standard (KEBS): 10 μg/kg. [b]USDA maximum limit of total aflatoxins: 20 μg/kg. [c]Thai National Bureau of Agricultural Commodity and Food: 20 μg/kg. [d]Indonesian Regulation: 15 μg/kg (raw peanut), 20 μg/kg (peanut product). [e]Malaysian Regulation (1985): 15 μg/kg (raw peanut), 10 μg/kg (peanut product). n.a., data not available. n.d., not detected.

the process might include grinding, pressing, blending, heating, cooling, and packing.

Martins et al. (2017) reported that various fungi, such as *Fusarium* spp., *Penicillium* spp. and *Aspergillus* spp., were isolated from peanuts along the production chain. Drying is the most important step to reduce the a_w in peanuts in order to prevent fungal growth. Interestingly, apart from fungi, aflatoxins were also found throughout the peanut production chain. This indicated that even though the level of fungal contamination could be reduced upon drying, aflatoxins remained in the peanuts. Another study by Guezlane-tebibel et al. (2013) on imported peanuts from China marketed in Algiers reported that the *Aspergillus* section *Flavi* was the highest with 79.3% of the isolates being highly toxigenic. Three strains of *Aspergillus* section *Flavi* (*A. flavus*, *A. minisclerotigenes* and *A. caelatus*) were identified through the polyphasic approach which included morphological, chemical and molecular techniques. These results indicated that these species were able to survive and contaminate the imported peanuts.

Figure 1 illustrates the flow of the peanut supply chain in Malaysia. The supply chain of imported peanut involves several major stakeholders, which are directly accountable and equally involved in handling the peanuts from entry at ports to the manufacturing industry, retailing and finally the consumers. The importers, manufacturers and retailers are the three main peanut stakeholders in the supply chain in Malaysia. To date, there is still lack of reports on the occurrence of aflatoxins in peanuts along the supply chain in Malaysia especially at the importer's and manufacturer's stages. The available data on the occurrence of aflatoxins in foodstuffs are mainly from the samples collected from the retailers, and most of the findings revealed high levels of aflatoxins especially in peanuts and peanut-based products (Ali, 2000; Abidin et al., 2003; Arzandeh et al., 2010; Leong et al., 2010; Reddy et al., 2011; Chin et al., 2012). Therefore, more investigations are required to identify the critical points of aflatoxin contamination along the peanut supply chain in Malaysia. Even though aflatoxin is not easily eliminated from the food supply chain, the information will be useful for use as a database in the development of intervention strategies to further reduce aflatoxins in foodstuffs.

Previous researches were only focusing on the peanut-producing countries especially in the African region (Mutegi et al., 2013; Wagacha et al., 2013). According to Waliyar et al. (2015a), the optimal bulk storage condition for peanut kernels at post-harvest stage was by maintaining the moisture content of <7.5%, relative humidity of 65% and temperature of 10°C. For the unshelled peanuts, higher moisture content (9%), relative humidity (70%), and temperature (25 – 27°C) could prevent the aflatoxigenic fungal growth and ensure a safe storage of peanuts for up to 1 year for export purposes. However, the optimal condition could not be maintained during shipping, transportation, and storage at the manufacturer's or retailer's premises due to the fluctuated temperature, inadequate ventilation and condensation which might occur along such processes (Wagacha and Muthomi, 2008). In this case, there is a possibility for re-emergence of the aflatoxigenic fungi in the peanuts once they reached the importing countries. Thus, it is important to identify and characterize the fungal species that could survive in the importing countries and evaluate their ability to re-produce the aflatoxins.

A recent study on *Aspergillus* spp. contamination and aflatoxins in imported raw peanuts and their products (produced locally using the imported raw peanuts) along the supply chain in Malaysia revealed that aflatoxins were absent in samples collected from the importer (Norlia et al., 2018b). However, the fungal contamination, especially from the *Aspergillus* section *Flavi* were high in these samples and not significantly different from other stakeholders (manufacturers and retailers). In contrast, aflatoxin contamination in raw peanuts was significantly higher in samples collected from the manufacturers and retailers. Their findings indicated that the aflatoxigenic *Aspergillus* spp. could survive in imported peanuts and start to grow and produce aflatoxins when the storage conditions at the manufacturer' and retailer's premises become favorable for their growth. The tropical climate with high temperature and humidity in this country easily deteriorates the stored peanuts and favors the growth of aflatoxigenic *Aspergillus*

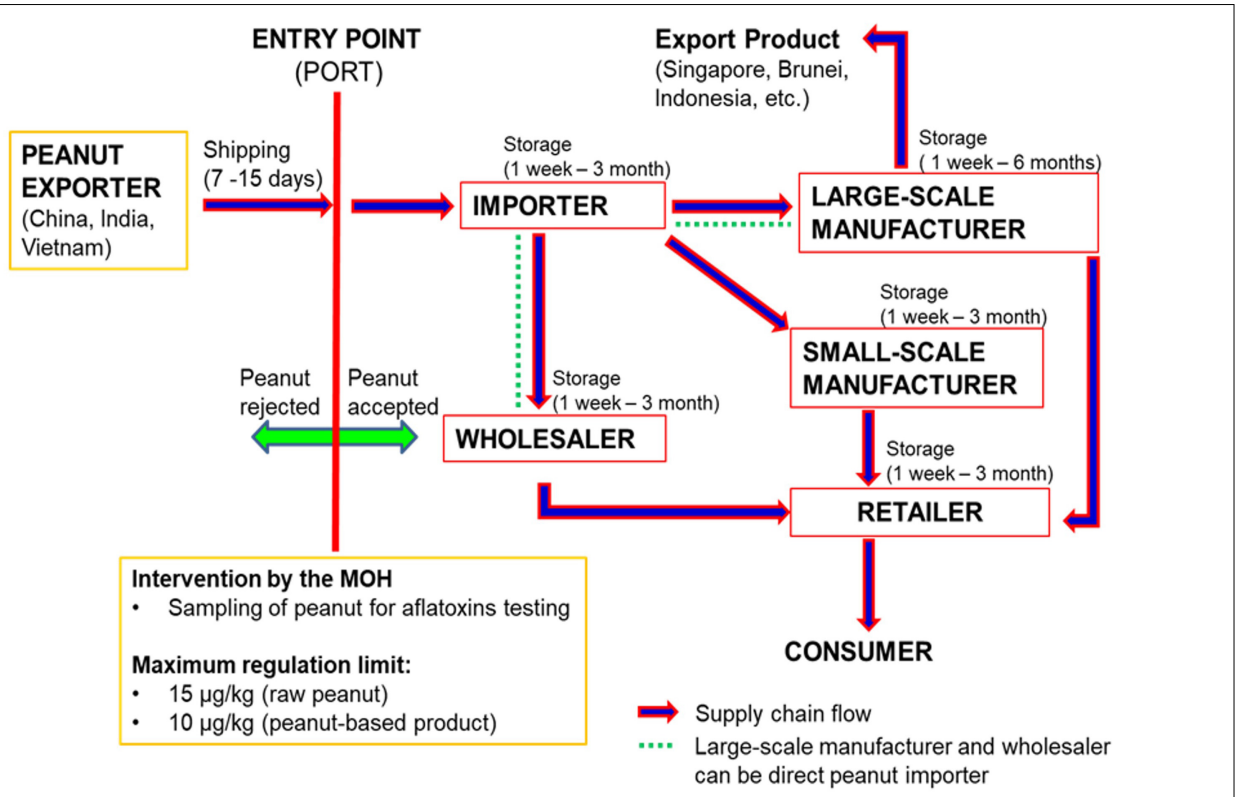

FIGURE 1 | Peanut supply chain Malaysia (Source: Personal Communication with Malaysian Ministry of Health and Peanut's stakeholders).

spp. Further identification and characterization of the isolates using the morphological, chemical and molecular approach confirmed the identity of the aflatoxigenic species as *A. flavus* (Norlia et al., 2018a, 2019).

INTERNATIONAL REGULATIONS OF AFLATOXINS AND THE TRADE IMPACT ON PEANUT SUPPLY CHAIN

Many countries have set the mycotoxin regulations to ensure the safety of the consumers and avoid the harmful effects of mycotoxins. These regulations are enforced by removing the non-compliant food products from the market (van Egmond et al., 2007). Based on the government regulations and guidelines in each country, both consumers and food processors could expect that aflatoxin level in foods should be below the disease-inflicting limits (Anukul et al., 2013). Aflatoxins were the first mycotoxin to be regulated (in the late 1960's), and now the regulations have been set in approximately 100 countries around the world which cover approximately 85% of the world's population (van Egmond and Jonker, 2004). The accessibility of the toxicological data and its incidence, socio-economic problems, and information on the sampling and analysis are the important aspects involved in the decision-making process of setting up the regulation limit (van Egmond and Jonker, 2004).

Internationally, the European Union (EU) regulation, US Food and Drug Administration (FDA) and Codex Alimentarius Commission (CAC) have been accepted as the guidelines for establishing the maximum regulatory limit for aflatoxins. Codex was co-founded by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) in 1963 with the objective to establish the Codex standards, guidelines, and Code of Practice for defending the health of consumers and verifying good practices in food trade. Generally, the aflatoxin regulatory limits are different in each country as shown in **Table 2**. Aflatoxins in peanuts are regulated in most of the countries since this commodity are naturally vulnerable to *Aspergillus* spp. infection and the subsequent aflatoxin contamination. European Union has the strictest regulations which allow only 2 µg/kg and 8 µg/kg of AFB_1 in peanut products for direct human consumption and raw peanuts intended for further processing, respectively [Commission Regulation (EU) No. 165/2010], while Codex sets the maximum limit of total aflatoxins at 15 µg/kg (Codex Stan Cxs 193-1995, 1995). A maximum level of 20 µg/kg of total aflatoxins in peanuts has been enforced by the FDA[1]. Other countries mostly regulate the total aflatoxins in peanuts and peanut based-products with a maximum limit of 10 – 35 µg/kg except for Singapore (5 µg/kg). In this regard, Malaysia has set a maximum limit of 10 µg/kg and 15 µg/kg

[1]https://www.fda.gov/media/72073/download

TABLE 2 | Aflatoxin regulatory limits in different countries.

Country/ Organization	Type of aflatoxins	Type of food	Maximum (μg/kg)
European Union	AFB_1	Peanuts	8
	Total aflatoxins	Peanuts	15
	AFB_1	Peanut products	2
	Total aflatoxins	Peanut products	4
FDA	Total aflatoxins	Peanuts	20
Codex	Total aflatoxins	Peanuts	15
China	AFB_1	Peanut, corn	20
Hong Kong	Total aflatoxins	Peanuts and peanut products	20
India	AFB_1	All food	30
Indonesia	Total aflatoxins	All food	35
	AFB_1	Peanut and corn	15
	Total aflatoxins	Peanut and corn	20
Japan	Total	All foods	10
South Korea	AFB_1	Grains, cereal products	10
Malaysia	Total aflatoxins	Raw peanuts	15
	Total aflatoxins	Peanut products	10
Philippines	Total aflatoxins	All food	20
Singapore	Total aflatoxins	All foods	5
Sri Lanka	Total aflatoxins	All foods	30
Taiwan	Total aflatoxins	Peanut and corn	15
Thailand	Total aflatoxins	All foods	20
Vietnam	Total aflatoxins	All foods	10

Source: Commission Regulation (EC) No. 165/2010 (2010) and Anukul et al. (2013), US Food and Drug Administration (FDA), Codex Stan Cxs 193-1995 (1995), Malaysian Regulation Food Act 1983 (2014).

for total aflatoxins in ready-to-eat peanuts and raw peanuts intended for further processing, respectively (Food Act 1983, 2014). These regulations were established to help protect the consumers against the harmful effects of aflatoxin by preventing the compounds from entering the peanut supply chain in the country. Even though the current maximum regulatory limit was reported to be adequate in protecting Malaysians' health against aflatoxin, the chronic exposure is still a concern (Chin et al., 2012).

Nevertheless, the implementation of strict regulations may neither be a trade barrier nor a catalyst on the improvement of aflatoxin management (Emmott, 2012). This factor was the most important reason as to why sub-Saharan Africa and Malawi were stopped from exporting their peanuts to European countries, back in the late 1990s. These countries were losing their competitiveness and struggled to reach the stringent thresholds put in place. Only 40% of peanuts are directed to the core processing, wholesale and retail markets. Meanwhile, another 60% is locally consumed by farmers or sold directly by the producers on local markets (Emmott, 2012). According to Matumba et al. (2015), there are no other channels for diversion of the grade-outs to be exported and, hence, the peanuts are projected to only local market. Therefore, without proper aflatoxin management and control, this scenario will consequently affect the public which lacks the knowledge on aflatoxins. A survey conducted in Malawi discovered that information concerning aflatoxin was very restricted among the general public especially farmers (Matumba et al., 2015). Besides, the decline of the raw peanut export in most countries including Africa was also attributed to the internal supply side or macroeconomic, climatic shocks, market development, competitive cost, quality and sectoral-specific policies which subsequently reduced producer inducement through direct and indirect taxation (Rios and Jaffee, 2008).

AFLATOXIN MANAGEMENT IN PEANUTS ALONG THE SUPPLY CHAIN

Aflatoxins could not be easily eliminated from peanuts once they are formed. Hence, the aflatoxin management practices are important as the mitigation tools of aflatoxin contamination in the peanut supply chain. Proper prevention and management strategies of aflatoxins in peanuts during pre- and post-harvest stages has been suggested including lot segregation, density segregation, kernel moisture control, blanching, color sorting, and the use of biological control in the field (Dorner, 2008). Aflatoxin management strategies in the field have been described and reviewed extensively (Dorner, 2008; Torres et al., 2014; Waliyar et al., 2015a). Florkowski and Kolavalli (2014) reported on the application of soil amendments including the use of gypsum and compost as one of the strategies to reduce aflatoxins during pre-harvest. However, this method might not be economically feasible for farmers who are unable to commit and in return require higher yields to recover the additional production costs. Pandey et al. (2019) critically reviewed three pre-harvest mitigation alternative methods of aflatoxin by implementing genetic resistance for *in vitro* seed colonization (IVSC), pre-harvest aflatoxin contamination (PAC) and aflatoxin production (AP). The next-generation sequencing (NGS) technologies are believed to accelerate the advancement of genomic resources at a very reasonable cost even for large genome-polyploid crops including peanuts (Varshney et al., 2019).

Wood, bamboo, thatch or mud are commonly used by farmers as the storage structure for harvested peanuts. Poor storage practices is the main factor that leads to aflatoxigenic *Aspergillus* spp. infestation (Florkowski and Kolavalli, 2014). Although the aflatoxin regulation in each country could help to protect the consumers from the risk of aflatoxins in the imported peanuts, the presence of aflatoxigenic fungi might increase the risk of aflatoxin production and accumulation in peanuts during storage, especially at the manufacturer' and retailer's stages. However, the new storage practices including the use of metal or cement bins, polypropylene bags and hermetic packaging have been reported to improve the storage system and reduce aflatoxin contamination (Waliyar et al., 2015a). It is also important to retain low moisture level during storage, transportation and sales (Wagacha and Muthomi, 2008). Besides, the implementation of post-harvest machinery including threshers, dryers and shellers supports higher yield and lessens

post-harvest processing and drying time. Physical separation or sorting also helps to remove the contaminated kernels by observing the physical appearances including color, size and density (Waliyar et al., 2015a).

It is the basic consumers' right to consume safe and nutritious food products. Nevertheless, reports on the aflatoxin occurrence in peanuts on the Malaysian market found that some of the samples exceeded the maximum regulation limit (Arzandeh et al., 2010; Leong et al., 2010; Norlia et al., 2018b). Therefore, the cooperation between regulatory bodies, scientific communities and the industries is of utmost importance to promote and produce safe and quality foods (Anukul et al., 2013). The Malaysian government has enforced a strict regulation on aflatoxins in order to protect the consumers. Imported peanuts are screened for aflatoxins before they can be released to the local markets. The Malaysian Ministry of Health is responsible for conducting the screening of aflatoxins from the peanut consignment at the entry ports. The screening process involves peanut sampling and testing for aflatoxins. Any peanut consignment found to exceed the permissible limit will be rejected.

The involvement of private sectors in peanut-importing countries might also help in the management of aflatoxin issue along the supply chain. A previous study on the peanut stakeholders in Malaysia revealed that the hygiene and training program, knowledge on aflatoxins, storage practices and the quality assurance certification influence the hygiene practices required in minimizing aflatoxin contamination in peanut-based products (Azaman et al., 2016). It was also reported that the stakeholders who attended the training program on aflatoxin management applied better hygiene practices than those that did not attend any training programs. It was also found that the importers and large-scale manufacturers had a better knowledge and understanding of aflatoxin contamination as compared to the small-scale manufacturers and retailers. In Malaysia, most of the large-scale peanut manufacturers are certified with the Good Manufacturing Practice (GMP) and Hazard Analysis and Critical Control Point (HACCP) to ensure the safety of their products (Norlia et al., 2018b). A previous study by Farawahida (2018) revealed that aflatoxins in raw peanuts and peanut sauce samples obtained from the small and medium enterprises (SME's) were more contaminated than the companies certified with GMP and HACCP.

Another study by Azaman et al. (2015) reported that the majority of food industry managers had a better knowledge of aflatoxins, and they recommended to provide relevant trainings to their food handlers and operators in order to further reduce aflatoxin contamination in peanut-based products. In this regard, peanut industries should only buy the raw materials from trusted suppliers which can provide the certification of aflatoxin analysis to ensure the safety of raw peanuts. The manufacturers can also have in-house validation of aflatoxin testing using the commercial aflatoxin testing kits to screen for aflatoxins in peanuts or other ingredients in peanut-based products such as spices. The involvement of the private sector in raising the public awareness on aflatoxin risk through public talks, trainings, fact sheets, social media and radio broadcasts might help to disseminate information and increase the knowledge among the peanut retailers and consumers as the majority of them are unaware of aflatoxin contamination (Sugri et al., 2017).

SAMPLING, DETECTION AND QUANTIFICATION OF AFLATOXINS IN PEANUTS

A proper sampling procedure is crucial to obtain a representative sample that is valid for aflatoxin analysis. The variation in the amount of aflatoxins and the small percentage of contaminated kernels in a lot are the main challenges in sampling (Fonseca, 2002). The EU has published a guideline (Commission Regulation (EC) No. 401/2006, 2006) on the sampling and aflatoxin analysis for official controls of aflatoxins in imported peanuts and other types of nuts. The regulation is in line with the Codex sampling standard (Codex Stan Cxs 193-1995, 1995). In general, an aggregate sample of 20 kg is collected from 10 to 100 incremental samples collected at different sites and locations of the peanut lot. The samples are divided into two equal laboratory samples before grinding it for further analysis. The laboratory samples shall be mixed thoroughly to achieve complete homogenization. The lot will be rejected if the laboratory samples exceed the maximum limit of the permitted aflatoxins level after taking into account the correction for recovery and measurement of uncertainty. For sampling in storage structures (bins, sacks, containers), a suitable probe should be used to get a representative sample collected from different depths of the containers. Samples are taken at three different levels (bottom, middle and top) using a probe. Approximately 1 kg of total aggregate samples are randomly taken from each level, and mixed thoroughly before 1 kg of samples are taken for laboratory analysis (Mahuku et al., 2010).

The detection and quantification of aflatoxins in peanuts are usually based on their absorption and emission spectra. The AFB's and AFG's exhibit blue and green fluorescence at 425 and 540 nm under UV irradiation, respectively (Kumar et al., 2017). Thin Layer Chromatography (TLC) which is based on the visualization of fluorescent spots and their intensities is one of the oldest methods used for aflatoxin detection in peanuts (Younis and Malik, 2003; Bakhiet and Musa, 2011). Nowadays, more recent and advanced methods such as High Performance Liquid Chromatography (HPLC), Ultra-High Pressure Liquid Chromatography (UHPLC) and Liquid Chromatography Mass Spectroscopy (LC-MS) have been widely used in aflatoxin analysis (Afsah-Hejri et al., 2011; Ibáñez-vea et al., 2011; Sameni et al., 2014; Kumar et al., 2017). HPLC equipped with a fluorescence detector and C_{18} analytical column is the most frequent method cited in the literature for aflatoxin analysis in peanuts (Afsah-Hejri et al., 2011). This method, either with pre- or post-column derivatization, requires sample extraction with a mixture of methanol and water or chloroform and phosphoric acid, followed by the purification step using either the liquid-liquid extraction (LLE) (Bakhiet and Musa, 2011), solid phase extraction (SPE) (Khayoon et al., 2012) or immunoaffinity column (IAC). The IAC method is the most

popular purification method for aflatoxins from peanuts used by researchers such as the AflaTest from Vicam (Afsah-Hejri et al., 2013b; Schwartzbord and Brown, 2015; Martins et al., 2017), and AflaPrep® from R-Biopharm Rhone Ltd. (Magrine et al., 2011; Ruadrew et al., 2013).

Aflatoxin derivatization is required for aflatoxin analysis using a fluorescence detector to enhance the detection. Triflouro acetic acid (TFA) is used for pre-column derivatization (Khayoon et al., 2012) while post-column derivatization requires a Photochemical Reactor for Enhanced Detection (PHRED) which is attached adjacent to the HPLC analytical column (Afsah-Hejri et al., 2011). According to Soleimany et al. (2012), tandem mass spectrometry (MS/MS) has a high level of selectivity and could provide a higher degree of certainty in the identification of analytes. Besides, LC-MS or LCMS/MS techniques also enable the simultaneous detection and quantification of multi-mycotoxins at relatively low concentrations in various food products. Recently, UHPLC-MS/MS was used for multi-mycotoxin determination in peanuts (Sameni et al., 2014; Manizan et al., 2018).

Fast and easy-to-use methods for aflatoxin detection are required to facilitate the screening process. Rapid aflatoxin tests are being improved and allow the operators to carry out the test at point of purchase (*in situ*). In this regard, the immunochemical-based method such as Enzyme-Linked Immuno-Sorbent Assay (ELISA) is commonly used for aflatoxin screening in peanuts as the ELISA test kit for commercial application requires only a simple extraction method (Lipigorngoson et al., 2003; Mutegi et al., 2009; Leong et al., 2010; Aisyah et al., 2015). Many researches on the development and optimization of the monoclonal antibody's performance in terms of sensitivity and cross-reactivity have been done to improve the method (Oplatowska-Stachowiak et al., 2016). A precise test kit based on the concept of lateral flow immunoassay can be used during field inspection and gives results within 5–15 min (Chen et al., 2016; Yu et al., 2018). It is very important to acquire high assay sensitivity as well as optimum immune-parameters. These testing kits have the potential to be a commercially viable intervention.

Immunosensor, a type of biosensor, is another alternative method for aflatoxin detection. Biosensor is an analytical instrument which combines the use of biological components (e.g., antibodies, nucleic acids, enzymes, cells, etc.) with a physicochemical transducer (Mosiello and Lamberti, 2011). Based on the same approach of the established analytical methods such as ELISA, many researchers aimed to transfer the method of the immunological assay from microtiter plates into a biosensor format (Azri et al., 2018). The developed electrochemical immunosensor showed a dynamic working range within 0.0001–10 μg/L, and the detection in spiked peanut samples provided a good recovery of between 80 and 127% (Azri et al., 2018).

The screening of aflatoxins might be a barrier to the peanut stakeholders primarily because of the testing cost and the need of a trained analyst to carry out the test. However, there are many other potential savings associated with aflatoxin screening at the point of purchase such as by ceasing the purchase of contaminated peanuts and lowering the processing cost by separating the highly contaminated peanuts from the good ones (Emmott, 2012).

MOLECULAR IDENTIFICATION AND CHARACTERIZATION OF *Aspergillus* section *Flavi*

The traditional method of isolation and cultivation using selective media are frequently used for the detection and identification of aflatoxigenic fungi. However, these methods are laborious, time-consuming and require taxonomical expertise as it is difficult to correctly identify based on morphological characteristics alone, especially those that are closely related (Rodrigues et al., 2009; Reis et al., 2014). Afsah-Hejri et al. (2013b) reported on the occurrence of aflatoxigenic *A. flavus* in peanuts from Malaysia but only based on the morphological identification. Besides, a similar study was reported by Reddy et al. (2011) on the occurrence of *Aspergillus* spp. in various food products marketed in Malaysia based on morphological identification. Morphology alone is insufficient and unreliable to correctly identify and differentiate the closely-related species within *Aspergillus* section *Flavi*. Therefore, the chemical profile of *Aspergillus* spp. is often used to assist the morphological identification (Rodrigues et al., 2009; Baquião et al., 2013). According to Samson et al. (2006), aflatoxins, aspergillic acid and cyclopiazonic acid are the main extrolites that are commonly used for the identification of aflatoxigenic *Aspergillus* spp. from section *Flavi.* **Table 3** shows the common morphology, extrolites, and molecular identification which have been used as the major parameters to differentiate these species.

Nowadays, the molecular approach is widely used to accurately identify and describe the species in the genus *Aspergillus* especially when introducing a new species (Peterson, 2008; Frisvad et al., 2019). DNA sequence analysis of certain regions, such as the internal transcribed spacer (ITS), β-tubulin, calmodulin, and the aflatoxin gene cluster, has been analyzed to get information regarding the phylogenetic relationship among the species in this section (Pildain et al., 2008; Varga et al., 2011). However, none of them used a single approach to solve the identification problem. A polyphasic approach, which includes the morphological, chemical and molecular characteristics, is often used to identify and characterize the *Aspergillus* spp. in this section (Baquião et al., 2013; Reis et al., 2014). Godet and Munaut (2010) successfully identified nine species within the *Aspergillus* section *Flavi* using a six-step of molecular strategy including real-time PCR, RAPD and *SmaI* digestion. The results were validated by the partial sequencing of the calmodulin gene to confirm the identification.

The nuclear ribosomal DNA (rDNA) of the ITS region is the most widely sequenced region and recommended as the DNA barcoding marker for fungal identification at and below the genus level as well as the source of phylogenetic information. It is therefore necessary to include the ITS sequences whenever a new fungal species is described (Schoch et al., 2012). The ITS region is situated between the 18S (SSU) and 28S (LSU) genes in the rDNA repeat unit which includes the ITS1 and ITS 2 regions,

TABLE 3 | Morphology, extrolite production and molecular identification of *Aspergillus* section *Flavi* species.

Species	Morphology	Extrolites	Molecular identification	Origin	References
A. flavus	Yellow-green conidia, small and large sclerotia, orange reverse on AFPA	AFB (+/−), CPA (+/−), aspergillic acid, asperfuran (+/−), paspalinin and paspaline (+/−)	β-tubulin and calmodulin	*Arachis* hypogaea	Pildain et al., 2008
A. parasiticus	Dark-green conidia, orange reverse on AFPA	AFB, AFG, kojic acid, aspergillic acid, parasiticolides, paspalinin and paspaline (+/−)	β-tubulin and calmodulin	*Arachis hypogaea, A. vilosa, A. correntina*	Pildain et al., 2008
A. nomius	Yellow green conidia, orange reverse on AFPA	AFB, AFG, kojic acid, aspergillic acid, nominine	β-tubulin and calmodulin	Wheat	Pildain et al., 2008
A. pseudonomius	n.a	AFB, kojic acid, chrysogine	ITS, β-tubulin and calmodulin	Diseased alkali bees	Varga et al., 2011
A. bombycis	Yellow-green conidia, orange reverse on AFPA	AFB, AFG, kojic acid, aspergillic acid	β-tubulin and calmodulin	Frass in a silkworm rearing house	Pildain et al., 2008
A. tamarii	Dark-brown conidia, dark brown reverse on AFPA	Kojic acid, CPA (+/−),	β-tubulin and calmodulin	*Arachis hypogaea*	Pildain et al., 2008
A. pseudotamarii	Dark-brown conidia, dark brown reverse on AFPA	Kojic acid, AFB, CPA (+/−)	β-tubulin and calmodulin	Soil	Pildain et al., 2008
A. caelatus	Dark-brown conidia, dark brown reverse on AFPA	Kojic acid, CPA (+/−),	β-tubulin and calmodulin	Soil	Pildain et al., 2008
A. pseudocaelatus	n.a	AFB, AFG, kojic acid, CPA	ITS, β-tubulin and calmodulin	*Arachis bukartii*	Varga et al., 2011
A. minisclerotigenes	Yellow-green conidia, small sclerotia, orange reverse on AFPA	AFB, AFG, CPA, kojic acid, aspergillic acid, parasiticolides, aflavarins, paspalinin and paspaline, aflatrems and aflavinines	β-tubulin and calmodulin	*Arachis hypogaea,* soil, and peanut field	Pildain et al., 2008
A. arachidicola	Dark-green conidia, orange reverse on AFPA	AFB, AFG, aspergillic acid, kojic acid, parasiticolides, chrysogine	β-tubulin and calmodulin	*Arachis glabrata*	Pildain et al., 2008
A. toxicarius	n.a	n.a	β-tubulin and calmodulin	*Arachis hypogaea*	Pildain et al., 2008
A. parvisclerotigenus	Yellow-green conidia, orange reverse on AFPA	Kojic acid, AFB, AFG, CPA, aspergillic acid, aflavarins, paspalinin and paspaline, aflatrems and aflavinines	β-tubulin and calmodulin	*Arachis hypogaea*	Pildain et al., 2008
A. korhogoencis	Yellow-green to brown conidia, small sclerotia, orange reverse on AFPA	AFB, AFG, kojic acid, CPA, aspergillic acid, aflatrem, leporins, asparasone, aflavarin, aflavinine, paspalinin, and paspaline	ITS, benA, cmdA, mcm7, amdS, rpb1, preB, ppgA, and preA	*Arachis hypogaea*	Carvajal-campos et al., 2017
A. leporis	Yellow-green conidia	Kojic acid	β-tubulin and calmodulin	dung of *Lepus townsendii*	Pildain et al., 2008
A. oryzae	Yellow-green conidia	Kojic acid, asperfuran, aspirochlorin	β-tubulin and calmodulin	Unknown source, Japan	Varga et al., 2011
A. sojae	Yellow-green conidia	Kojic acid, aspergillic acid, asperfuran, aspirochlorine	β-tubulin and calmodulin	Soy sauce	Varga et al., 2011
A. avenaceus	n.a	Aspirochlorine	ITS, β-tubulin and calmodulin		Varga et al., 2011

n.a, data not available. AFPA, Aspergillus flavus and parasiticus Agar. AFB, Aflatoxin B. AFG, Aflatoxin G. CPA, Cyclopiazonic acid. ITS, Internal Transcribed Spacer. +, present; −, absent.

and separated by the 5.8S gene. Of its three sub-regions, ITS1 and ITS2 are typically species specific and show a high rate of evolution (Nilsson et al., 2009). The entire sequence of the ITS region typically ranged from 450 to 700 bp. The amplification of the entire or part of the ITS region has been done by using various primers with the most commonly used primers were published by White et al. (1990).

Nevertheless, secondary identification markers, such as β-tubulin and calmodulin genes, are still needed to accurately identify *Aspergillus* section *Flavi* as ITS alone is still insufficient for molecular identification purposes (Samson et al., 2014). β-tubulin is a protein-coding gene that encodes for the tubulin protein which can be found in all eukaryotic cells as an elementary sub-unit of the microtubules. It involves in the eukaryotic cellular processes, and represents the main components of the cytoskeleton and eukaryotic flagella (Einax and Voigt, 2003). Calmodulin (CaM) is a calcium-binding protein that involves in the cell proliferation and differentiation in eukaryotic cells. It is highly conserved and serves as the main receptor for intracellular calcium (Ma et al., 2009). These three genes are widely used as the DNA markers for the identification and phylogenetic analysis of *Aspergillus* spp.

A. arachidicola and *A. minisclerotigenes* are the examples of two new aflatoxin-producing species in *Aspergillus* section *Flavi* that have been isolated from different species of peanuts and identified using phenotypic and molecular (β-tubulin and calmodulin gene sequences) characters (Pildain et al., 2008). Another new species in this section, *A. pseudotamarii*, has been described by Ito et al. (2001) by comparing the morphology, mycotoxin production, and divergence in ITS, 28S, β-tubulin and calmodulin gene sequences with the closely related species *A. tamarii* and *A. caelatus*. Besides, Tam et al. (2014) reported that the ITS, β-tubulin and calmodulin gene sequencing had successfully resolved the misidentification of *A. nomius* and *A. tamarii* from clinical isolates which were previously identified as *A. flavus* based on the morphological characteristic. However, this method could not be used to differentiate between the aflatoxigenic and non-aflatoxigenic species of *A. flavus* (Norlia et al., 2019). The aflatoxin biosynthesis gene cluster are present exclusively in the aflatoxigenic *Aspergillus* spp. such as *A. flavus* and *A. parasiticus*. The full cluster of aflatoxin biosynthesis genes has been characterized by Yu et al. (2004) and specific primers can be used to amplify the genes by using the PCR-based detection method (Erami et al., 2007). However, the identification of aflatoxigenic species could not be confirmed by this method as other genes that have not been tested might have defects or mutations that are not detectable by the specific primers. Takahashi et al. (2002) reported that deletion and other genetic flaws might have disrupted the aflatoxin pathway in both species. According to Abdel-Hadi et al. (2012), the gene expression and the aflatoxin production were affected by the temperature and a_w. Therefore, the aflatoxin biosynthesis pathway can either be fully inhibited or activated depending on the environmental factors.

CONCLUSION

Contamination of *Aspergillus* section *Flavi* and aflatoxins could occur at any stage along the peanut supply chain, specifically from the pre- and post-harvest stage at the producing countries to the peanut manufacturers and retailers at the importing countries. The high temperature and humidity in the tropical regions causes the inability to maintain the low moisture/a_w level of peanuts during storage, which subsequently enhances the growth of aflatoxigenic *Aspergillus* spp. especially *A. flavus*. Due to these reasons, the imported peanuts that are initially free from aflatoxins could be re-contaminated during the storage period at the manufacturers' and retailers' premises. Regular screening on the aflatoxins and aflatoxigenic *Aspergillus* spp. in peanuts should be regularly conducted to ensure that the stored peanuts are safe from the risk of aflatoxins. Various methods for aflatoxin and *Aspergillus* spp. screening, detection and quantification have been reviewed herein. The aflatoxin regulation in each country might help in protecting the population from the risk of aflatoxins but it does not guarantee the post-contamination after it enters the importing countries. Thus, aflatoxin management in peanut supply chain is very important and should involve both the government and private sectors. In addition, the awareness and knowledge on aflatoxins should be instilled among the peanut stakeholders and consumers to ensure that good handling and hygiene practices are applied during the storage of peanuts. Besides, the storage facilities, structures and conditions at the importing countries should also be taken into consideration in reducing the risk of aflatoxin contamination.

AUTHOR CONTRIBUTIONS

MN, MN-K, NS, and FA participated in the preparation of the manuscript. SJ and SR critically revised the manuscript and participated in the final editing of the manuscript.

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Aflatoxins in Food and Feed: An Overview on Prevalence, Detection and Control Strategies

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Aflatoxins produced by the *Aspergillus* species are highly toxic, carcinogenic, and cause severe contamination to food sources, leading to serious health consequences. Contaminations by aflatoxins have been reported in food and feed, such as groundnuts, millet, sesame seeds, maize, wheat, rice, fig, spices and cocoa due to fungal infection during pre- and post-harvest conditions. Besides these food products, commercial products like peanut butter, cooking oil and cosmetics have also been reported to be contaminated by aflatoxins. Even a low concentration of aflatoxins is hazardous for human and livestock. The identification and quantification of aflatoxins in food and feed is a major challenge to guarantee food safety. Therefore, developing feasible, sensitive and robust analytical methods is paramount for the identification and quantification of aflatoxins present in low concentrations in food and feed. There are various chromatographic and sensor-based methods used for the detection of aflatoxins. The current review provides insight into the sources of contamination, occurrence, detection techniques, and masked mycotoxin, in addition to management strategies of aflatoxins to ensure food safety and security.

Keywords: human health, outbreaks, aflatoxins contamination, detection, food and feed

INTRODUCTION

Food safety and security are among the major problems in the current climate of increasing population. These are mainly determined by three key aspects viz., (i) enough food availability, (ii) access to safe food and (iii) utilization of the food in terms of quality, nutritional and cultural purposes for a healthy life (FAO, 1996). The failure of any of these aspects leads to food insecurity and malnutrition that further influences human health, in addition to the socio-economic aspect of society. In addition, food and feed contamination by mycotoxins are one of the key factors responsible for creating food insecurity (Udomkun et al., 2017).

As per the Food and Agriculture Organization (FAO), one-fourth of the world's crop is affected by mycotoxins (Wu, 2007; Pankaj et al., 2018). The three main genera of fungi producing mycotoxins are *Aspergillus*, *Fusarium*, and *Penicillium* (Reddy et al., 2010). Among various type of

mycotoxins, aflatoxins (AFs) are highly toxic and are known to contaminate a wide variety of foods such as maize, groundnuts, dried fruits, meat and milk-based products (Mutegi et al., 2009; Perrone et al., 2014; Iqbal et al., 2015). AFs are produced by *Aspergillus* species, namely *A. flavus*, *A. nomius* and *A. parasiticus* (Payne and Brown, 1998), in addition to its production by other species of *Aspergillus* like *A. astellatus* (Reiter et al., 2009). These fungi usually grow in the warm and humid conditions of tropical and subtropical regions (Magan and Aldred, 2007; Battilani et al., 2011). Food processing techniques are not sufficient to eliminate AFs from contaminated food and feed due to their heat resistant nature (Medina et al., 2017b).

The ingestion of AFs from contaminated food and feed has led to serious health complications in humans and animals (Fung and Clark, 2004; Binder et al., 2007; Sherif et al., 2009). Therefore, different countries have implemented strict regulations for AFs in food and feed to maintain the health of individuals (Juan et al., 2012). The safe limit of AFs lies in the range of 4–30 μg/kg for human consumption. The European Union has the strictest standard level with AFB1 and total AFs not beyond 2 μg/kg and 4 μg/kg, respectively, in any product meant for direct consumption (EC, 2007, 2010). Similarly, the maximum acceptable limit set for AFs in the United States is 20 μg/kg (Wu, 2006). Besides this, various innovative technologies and control strategies are applied for pre- and post-harvest management of AFs to enhance sustainable agricultural productivity (Prietto et al., 2015). Though there are numerous publications on AFs in food and feed, the novelty and strength of this review lie with the enlistment of the new methods developed for AFs detection in food and feed with special reference to masked AFs. In addition, the review also focuses on the occurrence, impact of climate change along with the control strategies of AFs in food and feed to ensure food safety and security for healthy living and socio-economic development.

OCCURRENCE OF AFLATOXINS IN FOOD AND FEED

Aflatoxins are chemically difuranocoumarin derivatives with a bifuran group attached to the coumarin nucleus and a pentanone ring (in case of AFBs) or a lactone ring (in case of AFGs) (Schuda, 1980). The four major AFs among the identified 20 are AFB1, AFB2, AFG1 and AFG2. The B-types are produced by *A. flavus* while G-types are produced by *A. parasiticus* (Kumar et al., 2017). The biosynthesis of AFs consists of 18 enzymatic steps with at least 25 genes responsible for producing the enzymes and regulating the biosynthetic process (Yu et al., 2002; Yabe and Nakajima, 2004).

The occurrence of AFs is common in wide varieties of food and feed **(Table 1)**. Some of the most affected food and feed include peanuts, nuts, figs, corn, rice, spices and dried fruits (Martinez-Miranda et al., 2019). It has been shown that among the tested cereals, 37.6% were at least contaminated by any of the AFs (Andrade and Caldas, 2015). Though rice is not the high-risk commodity for AFs contamination, but AFB1 besides other mycotoxins have been detected in rice from China, Egypt, India, Iran, Malaysia, Nepal, Pakistan, Philippines, United Kingdom and United States (Tanaka et al., 2007; Rahmani et al., 2011; Lutfullah and Hussain, 2012). Therefore, AFs pose serious health issues by their ingestion from contaminated food and feed or by carryover AFs in them (Nordkvist et al., 2009; Reiter et al., 2010).

AFB1, as a potent carcinogen to humans, is associated with serious health complications (IARC, 2012). It has been a causal factor for liver cancer and acute hepatitis as well as periodic outbreaks of acute aflatoxicosis leading to death (Azziz-Baumgartner et al., 2005) as reported with lethal aflatoxicosis in Kenya (Probst et al., 2007). AFs are mostly detoxified in the liver which is the reason why liver cancer is rare. After the ingestion of AFB1, a series of metabolic processes converts it to an active intermediate, AFB1-exo-8,9-epoxide, by cytochrome P450 enzymes. The detoxification reaction occurs in conjugation with glutathione transferases (GSTs). The detoxification mechanism of AFB1-exo-8,9-epoxide might be linked to the mechanism that prevents liver cancer, however, it is not yet fully revealed (Guengerich et al., 1998). Unfortunately, on the other hand, the food and feed contamination by AFs is a persistent problem worldwide. The outbreaks due to AFs are more prone in tropical and subtropical areas, with a few in temperate regions (like the United States Midwest). In addition, the Mediterranean zones have become prone to AFs contamination due to shifting in traditional occurrence areas of AFs because of climate change i.e., increase in average temperatures, CO_2 levels and rainfall patterns (Marasas et al., 2008). This has led to an increase in contamination of crops with fungi and AFs worldwide.

CROPS AFFECTED BY AFLATOXINS

Cereals and cereal-based products are the major foods for human consumption worldwide (Temba et al., 2017). Among cereals, rice and corn are mostly contaminated by AFs in natural conditions due to changes in agricultural practices. The AFs are produced both in pre- and post-harvest conditions (Hesseltine, 1974). Filazi and Sireli (2013) reported rice to be more prone to AFs contamination as compared to other cereals. The fungal growth occurs due to improper drying of rice grains retaining higher moisture content (>14%). As a result, these fungi cause discoloration of grain and/or husk along with deteriorating the quality of the grains. Groundnut and beans, on the other hand, are frequently used in many African diets to supplement cereal diets (Soro-Yao et al., 2014). However, these are highly prone to AFs contamination both in field and storage conditions (Lombard, 2014). The extent of fungal growth and AFs production in cereals depends on temperature, moisture, soil type, and storage conditions (Achaglinkame et al., 2017). In addition, spices are susceptible to AFs contamination and are significantly affected by storage and processing conditions. Elshafie et al. (2002) reported the AFs contamination in a wide variety of spices including black pepper, cardamom, cinnamon, clove, cumin, coriander, and ginger in the Sultanate of Oman. Furthermore, Tchana et al. (2010) reported the presence of AFs in eggs collected from a poultry farm and in raw cow milk in

TABLE 1 | Occurrence of Aflatoxins in food and feed around the world.

Country	Food matrix	Aflatoxin	Range (μg/kg)	Detection technique	References
Turkey	Almond	AFB1	1–13	TLC	Gürses, 2006
Turkey	Butter	AFM1	<0.001–0.100	ELISA	Aycicek et al., 2005
Brazil	Cashew nuts	Total AFs	0.60–31.50	ELISA	Milhome et al., 2014
United States	Chilies	AFB1	<2	ELISA and TLC	Singh and Cotty, 2017
Costa Rica	Corn	Total AFs	24	ELISA and HPLC	Granados-Chinchilla et al., 2017
Zimbabwe	Corn	AFB1	0.75–26.6	HPLC	Murashiki et al., 2017
India	Corn	AFB1	48–383	HPLC	Mudili et al., 2014
Serbia	Corn	Total AFs	1.01–86.10	ELISA	Kos et al., 2013
Vietnam	Corn	AFB1	1.0–34.80	ELISA	Lee et al., 2017
Turkey	Cream cheese	AFM1	0.1–0.70	ELISA	Yaroglu et al., 2005
Pakistan	Dried Fruits	AFB1	0.04–9.80	HPLC	Masood et al., 2015
Turkey	Feed	AFB1	0–5	LCMS/MS	Yalcin et al., 2017
Turkey	Figs	Total AFs	0.1–28.20	HPLC	Kabak, 2016
Nigeria	Ginger	Total AFs	0.11–9.52	HPLC	Lippolis et al., 2017
Ethiopia	Groundnuts	Total AFs	15–11,900	HPLC	Chala et al., 2013
Turkey	Hazelnut	AFB1	0.07–43.60	HPLC	Baltaci et al., 2012
Serbia	Infant formula	AFM1	<0.03–0.02	HPLC	Torović, 2015
Turkey	Lentil	AFB1	0.57–1.74	HPLC	Baydan et al., 2016
Turkey	Maize flour	AFB1	0.041–1.12	HPLC	Kara et al., 2015
Egypt	Meat products	Total AFs	0.47–2.10	Fluorimeter	Abd-Elghany and Sallam, 2015
Greece	Milk	AFM1	<0.005–0.02	ELISA	Tsakiris et al., 2013
Iran	Milk (cow)	AFM1	0.006–0.18	HPLC	Bahrami et al., 2016
Brazil	Milk (cow)	AFM1	0.05	HPLC	Picinin et al., 2013
Italy	Milk (cow/buffalo)	AFM1	0.004	HPLC	De Roma et al., 2017
Portugal	Milk (cow)	AFM1	0.005–0.07	ELISA	Duarte et al., 2013
Japan	Nuts	AFB1	0.17–2.59	HPLC, HPTLC	Kumagai et al., 2008
Saudi Arabia	Nuts	Total AFs	1.0–110	HPLC	Neamatallah and Serdar, 2013
Malawi	Nut-based foods	AFB1	0.1–40.60	HPLC	Matumba et al., 2014
Zambia	Peanuts	AB1	0.015–46.60	HPLC	Bumbangi et al., 2016
Taiwan	Peanut products	Total AFs	0.2–513.40	HPLC	Chen et al., 2013
Turkey	Red-chili powder	AFB1	0.025–40.90	ELISA	Aydin et al., 2007
China	Rice	AFB1	0.03–20	HPLC	Lai et al., 2015
India	Rice	AFB1	0.1–308	Indirect competitive (icELISA)	Reddy et al., 2009
Pakistan	Rice	AFB1	0.04–21.30	HPLC	Iqbal et al., 2016
China	Rice	AFB1	0.1–136.80	HPLC	Sun et al., 2011
Tunisia	Sorghum	AFB1	0.4–25.1	HPLC	Ghali et al., 2010
Italy	Spices	AFB1	0.59–5.38	HPLC	Prelle et al., 2014
Malaysia	Spices	AFB1	0.58–4.64	ELISA	Reddy et al., 2011
Tunisia	Wheat	AFB1	0.12–18	HPLC	Ghali et al., 2010
Malaysia	Wheat	AFB1	0.55–5.07	ELISA	Reddy et al., 2011
China	Yogurt	AFM1	0.05	HPLC	Guo et al., 2013
Iran	Yogurt	AFM1	0.006–0.021	HPLC	Bahrami et al., 2016

Cameroon. Hence, the affected crops allow AFs to enter the food chain, which is very much influenced by the climatic conditions.

IMPACT OF CLIMATE CHANGE ON AFLATOXIN PRODUCTION

Climate change significantly impacts on the quality and availability of staple foods for consumption. With the increasing population worldwide, a major emphasis has been put on the safety of food and feed that can address the increasing demand with the increase in the yields by protecting the crops from adverse climatic conditions (Medina et al., 2017a). Aflatoxins contamination has affected millions of hectares of maize and peanut crops in the United States (Robens and Cardwell, 2003). Maize is a staple food for people living in warm climates throughout Asia, Africa, and the Americas, which are prone to the influences of climate change (Lewis et al., 2005). The change in climate simultaneously impacts the complex communities of AF-producing fungi by altering the number

of AF-producers to change its fungal community's structure. Aflatoxins contamination occurs via an initial phase during crop development and a second phase during crop maturation. The contamination is greater in warm, humid, and even hot deserts and drought conditions (Cotty and Jaime-Garcia, 2007).

A. flavus has highly evolved physiological mechanisms to acclimatize to adverse climatic conditions and dominates other fungal species (Nesci et al., 2004; Magan, 2007). Climate change alters the temperature and water activity (a_w) in the environment which further influences the gene expression to produce AFs. The conditions of temperature and a_w regulate the extent of fungal growth and AFs production (Schmidt-Heydt et al., 2009; Schmidt-Heydt et al., 2010). The AF-producing genes are grouped on the genome and express the main regulatory genes (aflR; aflS), as well as structural genes (aflD) which are influenced by the interaction of temperature × a_w conditions. As revealed by Schmidt-Heydt et al. (2010), the expression proportion of aflR/aflS significantly correlates with the amount of AFB1 produced. In addition, the expression of sugar transporter genes was significantly affected by the condition of temperature and a_w (Medina et al., 2014; Medina et al., 2015). Further, Bernáldez et al. (2017) studied the effect of interactions of temperature and a_w on the biosynthetic regulatory gene (aflR) expression and production of AFB1 by *A. flavus* in maize. They observed the optimum growth of *A. flavus* at 30°C/0.99 a_w with no growth at 20°C/0.90 a_w. Both temperature and a_w influenced the relative aflR gene expression and AFB1 production, however, the trends for the production of AFB1 were not in accordance with the gene expression. Further, the effect of temperature (20, 27, and 35°C) and a_w (0.82, 0.86, 0.90, 0.94, and 0.98) on the growth of *A. flavus* and *A. parasiticus* along with the production of AFB1 were investigated on ground Nyjer seeds by Gizachew et al. (2019). The maximum AFB1 production was observed at 27°C/0.90 a_w for both *A. flavus* and *A. parasiticus*. In addition to this, the fungi showed optimum growth on polished rice in the range of 28–37°C/0.92–0.96 a_w. The maximum AFB1 was produced at 33°C/0.96 a_w (Lv et al., 2019). Based on the investigation by Battilani et al. (2016) on the possible emergence of AFB1 in cereals in the European Union as a result of climate change, for every 2°C increase in temperature, there is an increase in AFs risk in the various regions of Spain, Italy, Greece, Portugal, Bulgaria, Albania, Cyprus and Turkey. The risk for AFs contamination in maize is likely to increase in Europe due to favorable climatic conditions for *A. flavus* in the next 30 years (Moretti et al., 2019). Therefore, proper detection methods and control strategies are crucial to combat the burning issues of AFs in food and feed.

DETECTION METHODS

The detection of AFs has been performed by the Association of Official Analytical Chemists (AOAC) official method in food and feed samples (Kumar et al., 2017). Among the most commonly employed methods are chromatographic methods like thin layer chromatography (TLC) (Fallah et al., 2011), high performance liquid chromatography (HPLC) and liquid chromatography mass spectroscopy (LCMS) (McDanell et al., 1988; Samarajeewa et al., 1991; Herzallah, 2009), besides the enzyme-linked immunosorbent assay (ELISA) (Tabari et al., 2011; Andrade et al., 2013; Sulyok et al., 2015). However, the drawbacks of these standard methods are that they are unsuitable for rapid and real-time applications in food and feed samples as they are tedious, time-consuming and require skilled personnel to operate. Therefore, rapid and robust methods like polymerase chain reaction (PCR) and non-destructive methods based on fluorescence/near-infrared spectroscopy (FS/NIRS) and hyperspectral imaging (HSI) have emerged for the quick and easy detection of AFs (Tao et al., 2018).

Hussain et al. (2015) utilized the PCR technique for the molecular detection of AF producing *A. flavus* from peanuts. Similarly, the avfA, omtA, and ver-1 genes encoding the major enzymes in AF-biosynthesis were used as target genes for detecting AFs using multiplex PCR (Yang et al., 2004). Further, PCR was employed to detect AF-producing genes in *Aspergillus* species in Iranian pistachio nuts for their aflatoxigenic effect (Rahimi et al., 2008). In addition, Kim et al. (2014) utilized PCR, ELISA and HPLC for the detection of AFs from *A. oryzae* isolated from different Korean foods. HSI uses the integration of both imaging and spectroscopy to record spatial and spectral characteristics of a given sample (Wu and Sun, 2013; Ropodi et al., 2016; Shrestha et al., 2016; Siche et al., 2016). The visible/near-infrared (VNIR) HSI has been utilized for the identification of maize kernels of different varieties (Zhang et al., 2012; Wang et al., 2016). VNIR or short-wave (SWNIR) HSI techniques are feasible for the detection of AFs as well as identification of different fungal species in maize (Pearson and Wicklow, 2006; Williams et al., 2012; Wang et al., 2015a,b). Later, Kimuli et al. (2018b) used the VNIR-HSI system to detect AFB1 on surfaces of maize kernels from Georgia, Illinois, Indiana and Nebraska of United States. Chu et al. (2017) used short-wave infrared (SWIR) HIS to detect AFB1 in single maize kernels. But as the image quality could not effectively classify AFB1 level qualitatively in individual maize kernels, therefore, to improve this Kimuli et al. (2018a) further combined the SWIR-HSI system with chemometric data analysis for the better detection of AFB1 on the surfaces of maize kernels. Furthermore, the color-encoded lateral flow immunoassay (LFIA) technique has been used for the simultaneous detection of AFB1 as well as fumonisins in a single test line (Di Nardo et al., 2019).

To further enhance the sensitivity and detection of AFs in food and feed, nanoparticles (NPs) based on Au/Ag, carbon (CBNs), magnetic (MNPs), Quantum dots (QDs), up-conversion (UCNPs), metal-organic frameworks (MOFs) as well as hybrid nanostructures have been utilized (Xue et al., 2019). Rui et al. (2019) prepared molecular imprinted polymers (FDU-12@MIPs) using structural analog of AFs. This highly selective surface was used as an extraction sorbent in conjunction with HPLC for the detection of AFs in different food and feed samples. In addition to this, the use of biosensors compared to other spectrophotometric or chromatographic methods allow for higher selectivity, direct detection with minimal sample pretreatment, minimal cost, portability and on-field analysis of mycotoxins (Rotariu et al., 2016). Selvolini et al. (2019) utilized

an electrochemical enzyme-linked oligonucleotide array for easy and quick multi-detection of AFB1 in maize. Furthermore, assays based on aptamer have been developed for the rapid detection of AFB1. Wang et al. (2019) successfully detected the AFB1 spiked in wine, methanol and corn flour samples using the simple aptamer molecular beacon assay, which has the potential for the rapid detection of AFs in the food and feed.

MASKED MYCOTOXINS AS A MAJOR CONCERN IN DETECTION

Masked mycotoxins pose a major concern in food and feed as they are not identified and detected by the usually employed detection techniques (Kamle et al., 2019). These are the mycotoxins produced by fungi but are modified by plant enzymes during the infection stages. They are present in vacuoles in the soluble form or bound to macromolecules, therefore, are unable to be identified by routine analysis processes and referred to as masked mycotoxins (Berthiller et al., 2013). However, the modified AFs can hydrolyze back into the toxic forms during food processing and/or digestion process (Gareis et al., 1990; Nagl et al., 2014; Broekaert et al., 2015). Some of these modified toxins are present in different forms as complexes with matrix compounds, hence also referred to as matrix-associated mycotoxins (Rychlik et al., 2014). The masked mycotoxins have been reported to occur in Asia, Africa, America and Europe. Therefore, a high amount of masked mycotoxins prevailing in various food and feed can pose serious health issues to both humans and animals (Zhang et al., 2019). Therefore, the detection of masked mycotoxins is an essential part to ensure food and feed safety. Masked fumonisins were determined through hydrolysis where modified forms were converted back to their free forms and subsequently analyzed and detected through LC/MS/MS (Dall'Asta et al., 2008; Dall'Asta et al., 2009). The hydrolytic process may involve either alkaline, acidic or enzymatic treatments (Dall'Asta et al., 2009; Beloglazova et al., 2013; Vidal et al., 2018). However, there is less information available on the masked AFs as most of the preference is given for the detection of free AFs in agricultural food and feed. Therefore, methods like *in vitro* digestion and hydrolysis, as applied in case of masked fumonisins, can be carried out for masked AFs in food and feed followed by detection with LC/MS/MS and confirmation by other methods like ELISA to ensure the food and feed safety.

CONTROL STRATEGIES OF AFLATOXINS

Implementation of advanced agricultural technologies, good agricultural practices (GAPs), good manufacturing practices (GMPs) and good storage practices (GSPs) can mitigate the mycotoxins contamination (Kamle et al., 2019). The novel processing techniques involving a microwave, UV, pulsed light, electrolyzed water, cold plasma, ozone, electron beam and gamma (γ) irradiation treatment have the potential for AFs management and preserving and maintaining the quality of agricultural and food products (Jalili et al., 2010; Pankaj et al., 2018). The application of ozone degrades AFs by an electrophilic attack on the double-bonded carbons (C8-C9) of the furan ring resulting in the formation of primary ozonides followed by rearrangement into monozonide derivatives like aldehydes, ketones and organic acids (Jalili, 2016). Further, the detailed mechanism of ozone degrading AFB1 has been discussed by Diao et al. (2013). The application of ozone for the degradation AF is limited in food products due to the cost factor (Womack et al., 2014). Similarly, the mechanism behind the AF degradation by gamma rays lies on the effects of free radicals produced during the radiolysis of water and other components that attacks the terminal furan ring of AFB1 resulting in byproducts of reduced biological activity (Rustom, 1997). The degradation efficiency of gamma irradiation is more effective when combined with other technologies.

In addition to these, several synthetic and natural food additives have been studied for AFs reduction in food and feed. For examples, the use of citric acid in combination with moisture under high temperature (200°C) and pressure (8N) was effective in degrading AFs in extruded sorghum (Méndez-Albores et al., 2009). On the other hand, the efficacy of sodium hydrosulphite ($Na_2S_2O_4$) was enhanced with increased pressure for AFs reduction in black pepper (Jalili and Jinap, 2012). Furthermore, as a part of biological control measures, Anjaiah et al. (2006) reported that inoculation of antagonistic strains of *Pseudomonas, Bacillus* and *Trichoderma* spp. had a significant reduction of *A. flavus* in pre-harvest crops. The non-aflatoxin forming strains of *A. flavus* and other non-toxigenic molds are prominent biological control agents against AFs contamination (Dorner et al., 2003; Udomkun et al., 2017). The application of each technique has its advantages and disadvantages. Therefore, biocontrol measures in synchrony with other physical and chemical methods along with improved packaging materials should be implemented to attain food safety and security.

CONCLUSION

Aflatoxins' contamination of crops at pre- and post-harvest conditions can be controlled to some extent by the implementation of good agricultural practices (GAPs), good manufacturing practices (GMPs) and good storage practices (GSPs). Further, the novel processing technologies involving a microwave, UV, pulsed light, electrolyzed water, cold plasma, ozone, electron beam or gamma (γ) irradiation in combination with either biological, physical, chemical or genetic engineering methods have the potential to improve the efficiency of AFs decontamination as well as to overcome the limitations of any specific technology. However, it is vital to understand the mechanisms of AFs detoxification so that no AF-residues are left behind when these methods are applied in food and feed samples. Furthermore, as there is less information on the masked AFs present in food and feed, it requires in-depth research and understanding with regards to adequate hydrolysis, identification, detection and control strategies. Therefore,

utilization of the novel technologies along with raising public awareness for implementing GAPs, GMPs and GSPs are crucial for controlling AFs contamination in food and feed to ensure food safety and security and to safeguard human and animal health.

AUTHOR CONTRIBUTIONS

PK conceived and designed the manuscript. DM, PK, and MK wrote the manuscript. KL, KD, and SD helped in the editing of the manuscript. PK and SK critically reviewed the manuscript and did the required editing.

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REVIEW
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Detection of Aflatoxins in Different Matrices and Food-Chain Positions

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Aflatoxins, produced mainly by filamentous fungi *Aspergillus flavus* and *Aspergillus parasiticus,* are one of the most carcinogenic compounds that have adverse health effects on both humans and animals consuming contaminated food and feed, respectively. Aflatoxin B1 (AFB1) and aflatoxin B2 (AFB2) as well as aflatoxin G1(AFG1) and aflatoxin G2 (AFG2) occur in the contaminated foods and feed. In the case of dairy ruminants, after the consumption of feed contaminated with aflatoxins, aflatoxin metabolites [aflatoxin M1 (AFM1) and aflatoxin M2 (AFM2)] may appear in milk. Because of the health risk and the official maximum limits of aflatoxins, there is a need for application of fast and accurate testing methods. At present, there are several analytical methods applied in practice for determination of aflatoxins. The aim of this review is to provide a guide that summarizes worldwide aflatoxin regulations and analytical methods for determination of aflatoxins in different food and feed matrices, that helps in the decision to choose the most appropriate method that meets the practical requirements of fast and sensitive control of their contamination. Analytical options are outlined from the simplest and fastest methods with the smallest instrument requirements, through separation methods, to the latest hyphenated techniques.

Keywords: aflatoxins, LOD, LOQ, limits, extraction, clean-up, analysis, detection

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INTRODUCTION

Mycotoxins are secondary metabolites of filamentous fungi and their presence indicates biological contamination. These compounds may enter the human and animal bodies directly by the consumption of contaminated agricultural products or ready-to-eat products or indirectly through the consumption of animal products (mainly milk, eggs, and offal), deriving from animals that consumed contaminated feed (Adányi, 2013).

Aflatoxins are the first known mycotoxin group, described as a result of turkey "X" disease in the 1960s (Blount, 1961; Wannop, 1961). Mycotoxin research has begun worldwide from that time on.

More than ten types of aflatoxins exist naturally, of which AFB1 is the most toxic. AFB1 and AFB2, AFG1, and AFG2 occur in the contaminated feed. AFM1 and AFM2 are present in ruminant milk after the digestion of feed contaminated by AFB1 and AFB2. In order to analyze aflatoxins, various analytical methods are required. Transformation of aflatoxins can be seen in **Figure 1**.

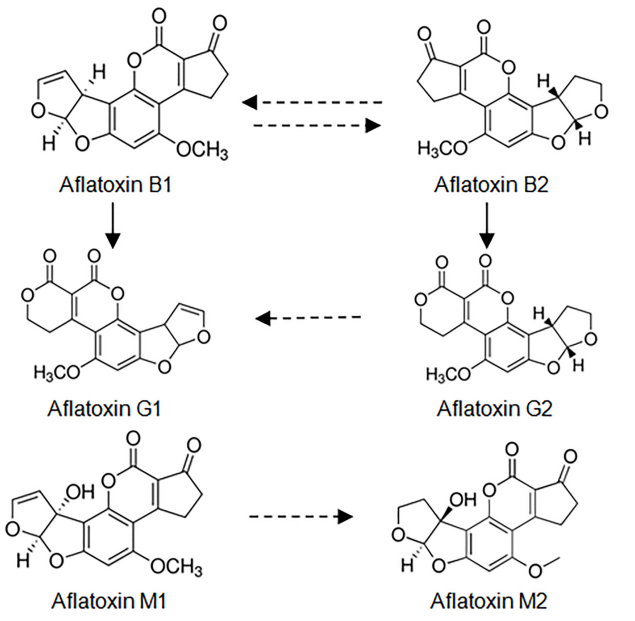

FIGURE 1 | Transformation of aflatoxins.

There is a wealth of scientific information with respect to aflatoxins and their acute and chronic effects and numerous research groups have worked on this topic recently. According to Web of Science, there are nearly 16,000 publications since 1975 to this day in connection with aflatoxins, of which over 7,000 have been published in the last decade. These numbers and legal restrictions across the world regarding the highly carcinogenic aflatoxins indicate the importance of the topic.

This publication gives a complex and transparent summary of the regulatory environment and the diverse measurement techniques of aflatoxins from rapid methods through seemingly simple separation techniques to complex hyphenated techniques. Sample preparation methods associated with the different measurement techniques are also covered.

ANALYTICAL EXPECTATIONS

Free trade of food and feed is getting more and more common around the world. In order to keep the product flow under control, there is a need for harmonized regulation and control systems both in exporting and importing countries. Because of this, many countries have already established common regulations and maximum levels for different contaminants, including aflatoxins. Nonetheless, some non-community countries (**Table 1**) have their own maximum levels for aflatoxins. There are different maximum permitted levels around the world mainly regarding AFB1 and aflatoxins total (AFT) (AFB1, AFB2, AFG1, and AFG2) for food and feed and AFM1 for milk and milk products. Consequently, it is important to be aware of these regulations, among others, for selecting appropriate analytical methods to verify the necessary compliance. Examples for the different regulations regarding aflatoxin levels are shown in **Table 1**.

As **Table 1** shows, the regulatory environment varies greatly in different areas. Therefore, high performance and sensitivity of the analytical methods are not always necessary in the case of controlling the compliance with legal limits. Nonetheless, product control has to be carried out in economically underdeveloped countries as well, where more sophisticated analytical techniques and instruments are rarely available. However, in some cases, where the legal limits are lower (e.g., in the European Union or ASEAN countries), more sensitive methods have to be used (Williams et al., 2004).

In **Supplementary Table 1**, methods for aflatoxin measurement, which will be discussed later, are summarized.

SAMPLE PREPARATION METHODS

Mycotoxins are toxic chemical compounds with low molecular weight (MW < 1000), and due to their diverse chemical structure, there exists no single standard technique for their analysis and/or detection (Turner et al., 2009).

Most of the methods used are based on appropriate extraction and clean-up. Sample preparation is one of the most important steps in the determination of mycotoxins. It may add up to two-thirds of the time of the full analysis and could significantly affect the accuracy and precision of the results. The most commonly used clean-up methods applied in aflatoxin analysis are liquid–liquid extraction (LLE), solid-phase extraction (SPE) and QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) methods. In addition, there are a number of other extraction methods in the literature that are less widely used in routine analysis at present.

Extraction and Clean-Up Methods

Liquid–Liquid Extraction (LLE)

This is a simple and cheap method for the extraction of aflatoxins. It is based on the solubility properties of the toxin in the aqueous or organic phase or in their mixture. The disadvantage of this method is that it does not provide sufficiently clean analyte in all cases. Researchers have tested AFB1, AFB2, AFG1, AFG2, and AFM1 in breast milk with LLE, then high-pressure liquid chromatography (HPLC) with photochemical derivatization (PHRED) and fluorescence detection (FLD). The limits of the quantification (LOQ) were between 0.005 and 0.03 μg/kg (Andrale et al., 2013). Using the same procedure, LOQ of 0.01 μg/kg was obtained for AFB1 in rice and grain samples (Sheeijooni-Fumani et al., 2011; Biancardi et al., 2013) and co-workers got an LOQ of 15 ng/ml in skimmed milk matrix with HPLC/MS-MS measurement after LLE by using sodium chloride and ethyl acetate extraction agents. The average recovery of the method was 95% (n = 24; CV = 4,5%).

Liquid–Solid Extraction (LSE)

Liquid–solid extraction is a simple method for the extraction of aflatoxins from solid matrices of different consistency. The extraction steps include the weighing of homogenized sample

TABLE 1 | Worldwide aflatoxin regulations, allowed maximum levels.

Communities	Countries	Organization	Reference of regulation	Aflatoxin B1 (μg/kg) (food)	Total Aflatoxin (μg/kg) (food)	AflatoxinM1 (μg/kg)	Aflatoxin B1 (μg/kg) (feed)	Total Aflatoxin (μg/kg) (feed)
African Union (AU)	South Africa	South Africa Department of Health		5	10	x	x	x
ASEAN (Association of Southeast Asian Nations)	Brunei	Department of Health Services, Ministry of Health		0	0	0	x	x
	Cambodia			x	x	x	x	x
	Democratic Republic of Laos, Myanmar			x	x	x	x	x
	Indonesia	National Agency of Drug and Food Control (NADFC)		15	0.5–5	0.5–5	x	20–50
	Malaysia	Food Safety and Quality Division, Ministry of Health Malaysia		0.1	5–35	0.025–0.5	x	x
	Philippines	Department of Agriculture		10	10–50	0.5	20	x
	Singapore	Food Regulations	Singapore Government, 2002	0.1–5	5	0.025–0.5	x	x
	Thailand	Bureau of Quality and Safety of Food (BQSF)		x	15–50	x	x	x
	Vietnam	National Institute for Food Control		0.1–12	4–15	0.025–0.5	x	x
CODEX				x	15	x	x	x
Codex GCC (Gulf Cooperation Council)	Bahrain, Yemen, Kuwait, Oman, Qatar, Saudi Arabia, United Arab Emirates			5–12	0.05–20	0.05	x	x
EU (European Union)		European Food Safety Authority	Food: Commission Regulation *European Commission* (EC) No 1881/2006 Feed: EU Directive 2002/32 and EU Recommendation 2006/576/EC	0.1–12	4–15	0.025–0.050	5–20	x
MERCOSUR (Mercado Común del Sur) (Southern Common Market)	Argentina, Paraguay, Uruguay, Venezuela (suspended since 2016)			x	20	x	x	x
	Brazil			0.5–2.5	1–20	0.5–2.5	x	x

(Continued)

TABLE 1 | Continued

Communities	Countries	Organization	Reference of regulation	Aflatoxin B1 (μg/kg) (food)	Total Aflatoxin (μg/kg) (food)	AflatoxinM1 (μg/kg)	Aflatoxin B1 (μg/kg) (feed)	Total Aflatoxin (μg/kg) (feed)
USA (United States of America)		Food and Drug Administration	US FDA, 2019	x	20	0.5	x	x
	Algeria			8		x	x	x
	Australia, New-Zealand	Australian New Zealand Food Standards Code (ANZFA)		x	15	0.02	x	x
	Bosnia and Herzegovina			8–12	10–15	x	x	x
	Canada	Canadian Food Inspection Agency		x	15	x	x	x
	China		USDA, 2014	0.5–20	only for: Chinese medicine: Chen pi, suan zao ren, jiang can, pang da hai, tao ren: 10	0.5–20	≤10–≤50	0.5
	Egypt			0.1–12	4–15	x	x	x
	India	APEDA (Agricultural and Processed Food Products Export Development Authority)		x	10–15	x	x	x
	Japan	Food Safety Commission; Feed: MAFF (Ministry of Agriculture, Forestry and Fisheries)	USDA, 2010	10	10	0.5	10–20	10–1000
	Korea			0.1–10	x	0.1–10	15	0.5
	Mexico			x	20	x	x	x
	Nigeria	National Agency For Food And Drug Administration And Control (NAFDAC)		20	x	x	x	x
	Peru	Codex		x	15	x	x	x
	Russia			5	x	x	x	x
	Turkey			8–12	12–15	x	x	x
	Ukraine			8–12	10–15	x	x	x

of the appropriate particle size, adding the suitable extraction agent and then disintegrating the mixture applying, e.g., shaker, ultra-turrax, blender, vortex, or other methods to extract the components of interest. The extract, before analysis, is filtered and cleaned if necessary. An important step in the process is to select the most effective extraction solvent. The most commonly used extraction agents are mixtures of acetonitrile/water or methanol/water in different ratios (Sheibani and Ghaziaskar, 2009). For instance, the 80% methanol/water mixture proved to be the most optimal for extraction of aflatoxins in the case of nutmeg samples. The choice of methanol for further use (e.g., immunoaffinity chromatography, IAC) is also preferable, because the antibodies better tolerate higher concentrations of methanol than acetonitrile. Methanol was also suitable for chromatographic separation, as aflatoxins were measurable without interference (Kong et al., 2013). The efficiency of extraction is greatly influenced by the sample/solvent ratio, the composition of the extraction agent and the time of extraction. LSE alone is not satisfactory to extract aflatoxins without interference and further selective purification step(s) are usually required.

Ultrasound Extraction

The use of ultrasound can substantially increase the efficiency of LSE. Ultrasound extraction is most often implemented by immersing the vessel (e.g., Erlemeyer flask, centrifuge tube or vial) containing the sample to be extracted and the extraction solvent into an ultrasonic bath that contains water. During a few-minute treatment, the acoustic cavitation induced by the ultrasound significantly increases the transfer of the analytes and matrix components from the sample to the extraction solvent, thereby increasing the efficiency of extraction (Xie et al., 2016). According to Bacaloni et al. (2008) ultrasound treatment over 10 min did not significantly increase the efficiency of extraction in the case of hazelnut samples.

Pressurized Liquid Extraction (PLE)

The PLE procedure, also known as accelerated solvent extraction (ASE), is actually the same as LSE performed under increased pressure and temperature in a suitable pressure-resistant vessel. By selecting a vessel of appropriate size, samples of 1 to 100 g can be extracted. Naturally, in the case of test portions of a few grams, it is important to investigate the magnitude of the random and systematic errors resulting from the reduction of sample size, in order to avoid subsequent inadequate results. The advantages of the procedure are that the extraction process can be automated, and higher extraction efficiency can be achieved in shorter time and with lower amount of extraction solvent (Xie et al., 2016). This extraction method was successfully used in the case of aflatoxin analysis of pistachio samples (Sheibani and Ghaziaskar, 2009). This procedure increases the efficiency of extraction of the analytes from solid samples; nonetheless, it is not widely used because of the high price of the instrument.

Supercritical Fluid Extraction (SFE)

Supercritical fluid extraction uses a supercritical CO_2 fluid for the extraction of the required compound from the matrix. The SFE procedure is mainly used efficiently for the extraction of apolar organic molecules (Anklam et al., 1998). During the extraction of polar aflatoxins with SFE a number of problems have arisen, e.g., low recoveries and high concentrations of co-extracts. Furthermore, lipids may cause difficulties during further clean-up and chromatographic separation (Shephard, 2009). However, the SFE procedure was successfully used in the case of aflatoxin extraction from pepper (Ehlers et al., 2006) and from Ziziphy Fructus, a traditional Chinese medicine (Liau et al., 2007).

Solid Phase Extraction (SPE)

Solid phase extraction is a popular clean-up method before qualitative and quantitative measurements of the components that have already been dissolved. Two types of SPE are used. In the case of the multi-step process (conditioning, sample application, washing, elution), either the measurand or the matrix component(s) is bound or removed from the sample (Yao et al., 2015). Various extenders are used in the SPE columns. Aflatoxins are often analyzed by using C-18 (octadecylsilane) column. The automated version of the procedure has been used for the online SPE ultra-high-performance liquid chromatograph coupled to a triple-quadrupole mass spectrometer (UHPLC-MS/MS) to determine aflatoxins from dried fruits. With this method, 83–103% recovery was achieved with RSD < 8, $n = 3$. These performance parameters are in line with EU requirements for determining mycotoxin levels in foods (Campone et al., 2018).

Special types of SPE procedures are **solid-phase micro extraction (SPME)** and IAC clean-up procedure that are based on the principle of immunoaffinity.

Compared to other extraction techniques, SPME has a number of benefits. Among others, it requires only sorption and desorption steps, it is a method easy to be automated, compatible with chromatographic systems, allows to achieve high enrichment, appropriate specificity can be assured, and it has very small sample requirements. The SPME method has been tested on the extraction of the aflatoxin content of nuts, spices, cereals and dried fruits. The result of the 8-min LC-MS measurement after clean-up with SPME method showed a sensitivity of 2.1–2.8 pg/ml for aflatoxins, which is more than 23 times greater than that achieved by the direct injection method (10 μl injection volume) (Nonaka et al., 2009). SPME was used for the clean-up of various types of cereal flours performed before the liquid chromatography and post-column PHRED-FLD measurements. The LOD and LOQ for aflatoxins were 0.035–0.2 ng/g and 0.1−0.63 ng/g, respectively (Quinto et al., 2009).

A specific application of SPE is the so-called immuno-affinity clean-up columns (IAC). They are applicable for the selective binding of mycotoxins as well. These columns contain selective antibodies produced against the mycotoxin to be analyzed and placed in the gel in the column. Chen et al. (2005) determined AFM1 in Pasteurized milk applying IAC cleanup and HPLC-FLD detection. In normal and low-fat content milks the average recovery and LOD were 78−79% and 0.59−0.66 ng/l, respectively.

Multifunctional clean-up columns (MFC) were designed for the simultaneous extraction of multiple types of mycotoxins (e.g., aflatoxins + zearalenone). The sample extract is pushed through

the column and the lipophilic part of the packing binds fats and other non-polar matrix components, while the polar, ionic sites of the packing bind carbohydrates, proteins and other polar matrix components, while analytes pass through the column (Krska et al., 2008). There are dedicated columns commercially available for mycotoxin (aflatoxin) clean-up, e.g., MultiSep®, MycoSep®, and Myco6in1 column (Tang et al., 2013).

Others combined different IAC columns with hyphenated methods for selective clean-up of rye flour, maize and morning cereal samples (Wilcox et al., 2015). Immunoaffinity-based columns, applicable for multi-mycotoxin clean-up, were developed in recent years as a result of extensive research. Zhang et al. (2016) have developed IAC for AFB1, AFB2, AFG1, AFG2, Ochratoxin A (OTA), Zearalenone (ZEN) and T-2 toxins and tested agricultural products for them. By using acetonitrile/water/acetic acid (80:19:1, v/v/v) extraction, after multi-mycotoxin IAC, the samples were measured with HPLC-MS-MS. The linear ranges were 0.30−25, 0.12−20, 0.30−20, 0.12−20, 0.60−30, 0.30−25, and 1.2−40 μg/kg for AFB1, AFB2, AFG1, AFG2, OTA, ZEN and T-2, respectively. The LOD values were 0.1, 0.04, 0.1, 0.04, 0.2, 0.1, and 0.4 μg/kg, respectively. Hu et al. (2016) have developed immunoaffinity columns sensitive and specific for AFB1, AFB2, AFG1, AFG2, OTA, ZEN and sterigmatocystin T-2 toxins. This method allows the fast, simple and simultaneous determination of the above mentioned toxins in complex feed matrices after UPLC-MS-MS measurement. The LOD and LOQ of the method was 0.006−0.12 ng/ml 0.06−0.75 ng/ml, respectively.

Khayoon et al. (2010) used MFC columns successfully for the clean-up of aflatoxins from feed samples (Berthiller et al., 2017). This method is practical, portable and fast and requires no further clean-up steps (Wilson and Romer, 1991).

Matrix solid phase dispersion (MSPD) is a special type of SPE. It was developed as an alternative for the LLE procedure. Usually aluminum oxide, magnesium silicate or modified silica gel (C8, C18, amino, cyano) supports are used. It is particularly suitable for preparation, extraction and component fractionation of solid, semisolid and rather viscous biological samples (Cavaliere et al., 2007).

Matrix solid phase dispersion clean-up was used for aflatoxin analysis in olive oil samples with liquid chromatography electrospray ionization tandem mass spectrometric (LC/ESI-MS/MS) detection giving LOQ values between 0.04 and 0.12 μg/kg (Cavaliere et al., 2007).

The **Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS)** method, developed for the extraction of pesticides with acetonitrile from vegetable samples, can be considered as a special alternative of the MSPD procedure (Anastassiades et al., 2003). Nowadays, with some modifications, it is widely used for mycotoxin clean-up as well (Xie et al., 2016).

Choochuay et al. (2018) developed a reliable and fast method for AFB1 determination in four feed types (broken rice, peanut, maize and fish feed). Sample preparation has been done by the QuEChERS method, then HPLC, precolumn derivatization and FLD were used. LOD was between 0.2 and 1.2 μg/kg and LOQ range was 0.3–1.5 μg/kg. The validated method was successfully used for the analysis of 120 samples The QuEChERS method has proved to be successful for the clean-up of AFM1, AFB2, AFG1, and AFG2 as well (Sartori et al., 2015).

Turbulent Flow Columns (TFC)

TurboFlow™ technology is an automatic online sample preparation method for mass spectrometric analysis of complex matrices (Liang and Zhou, 2019). TurboFlow™ technology combines the principles of diffusion, turbulence and chemistry in order to remove coextracted compounds from the matrix and capture the analyte rapidly and efficiently from the complex samples. It can be used with low input and high sensitivity in the case of difficult, multi-component samples. TurboFlow™ columns have been tested for AFB1 and AFM1 in milk and milk powder samples. LOD was 0.05 μg/kg and LOQ was 0.1 μg/kg. Recovery of AFB1 and AFM1 was 81.1–102.1% for all samples (Fan et al., 2015).

Magnetic Nanoparticles Based Solid Phase Extraction (MSPE)

Magnetic nanoparticles based solid phase extraction based on the use of magnetic or magnetizable adsorbents can be used for the preconcentration of target analytes from large sample volumes (Safarikova and Safarik, 1999). Due to the diversity of the matrices to be tested, MSPE in itself is not sufficient for the extraction of aflatoxins from test samples, but in combination with other purification steps appropriate results can be achieved. Zhao et al. (2016) developed a two-step extraction technique combining ionic−liquid−based dispersive liquid–liquid microextraction and magnetic solid−phase extraction for the preconcentration and separation of aflatoxins in animal feedstuffs. After sample preparation HPLC-FLD was used for the detection of aflatoxins. Due to the rapid mass transfer associated with the steps of the dispersive liquid–liquid microextraction and the magnetic solid−phase extraction methods, fast extraction could be achieved. The detection limits (LOD) were 0.632, 0.087, 0.422, and 0.166 ng/ml for AB1, AFB2, AFG1, and AFG2, respectively.

SEPARATION TECHNIQUES

Thin-Layer Chromatography (TLC), High-Performance Thin-Layer Chromatography (HPTLC)

At present, TLC is the best-known separation technique, but it may not be the most widely used anymore. Its popularity can be associated with its simplicity and low price, since its instrumental requirements at basic level are small. In preparative chemical laboratories TLC can be used to monitor the progress of reactions, determine the purity of a substance or identify compounds present in a given mixture.

In planar chromatography techniques, the stationary phase is an adsorbent material with different thicknesses through which the liquid mobile phase migrates via capillary forces. The most commonly used porous layers are silica gel, chemically modified silica gel, aluminum oxide (alumina), cellulose, chemically modified cellulose, polymer or ion-exchange resin. According to

the phases we can differentiate between normal-, reversed- or mixed-phase plates.

HPTLC allows more selective and accurate quantitative measurements. The main differences between the techniques (TLC and HPTLC) can be derived from the differences in the particle size of the stationary phases, their sensitivity and data processing methods (Fuchs et al., 2010; Gurav and Medhe, 2018). When quantifying the concentration of aflatoxins on TLC plates coupled with fluorescent densitometry, the detection limit in red paprika, fish, maize and wheat was 0.5 μg/kg (Shephard, 2009). Corn samples spiked at 5 and 50 ng/g levels were measured by TLC separation and densitometric detection in an interlaboratory study. The relative repeatability standard deviation (RSDr) of the AB1 was between 56.6 and 41.7% (Park et al., 1994). Despite the fact, that TLC is still an accepted reference method for the detection of aflatoxins, the quantitative analysis of aflatoxins was replaced by HPLC and UPLC in most cases.

Over-Pressured Layer Chromatography (OPLC)

Over-pressured layer chromatography was developed by Hungarian scientists in the mid-70s (Tyihák et al., 1979; Kalász et al., 1980; Tyihák et al., 1981; Hauck and Jost, 1983).

Over-pressured layer chromatography is carried out on a TLC or HPTLC plate, applying forced flow in a pressurized ultramicro (UM) chamber, based on the principle of liquid chromatography (Tyihák and Mincsovics, 2011).

Over-pressured layer chromatography integrates the advantages of classical TLC and HPLC, namely the possibility of parallel analysis in thin layer chromatography and the application of forced flow used in HPLC (Tyihák et al., 1979).

The applicability of OPLC for aflatoxins was proven in a validation procedure carried out by the scientists who developed the technology. As a result, the following LODs were defined for aflatoxins: 0.018, 0.100, 0.15, and 0.14 μg/kg for AFG2, AFG1, AFB2 and AFB1, respectively (Papp et al., 2000).

High/Ultrahigh Performance Liquid Chromatography (HPLC/UHPLC)

The reference methods for the detection of aflatoxins are based on chromatography, more precisely on HPLC/UPLC. During the determination of aflatoxins HPLC-fluorescent detection (FLD) and HPLC-MS/MS systems can be used in most cases. If the separated components are detected with fluorescent detector, there is a need for post-column derivatization (PCD) in order to increase the natural fluorescence properties of AFB1 and AFG1. This derivatization can be based on electrochemical or photochemical principles. For electrochemical derivatization trifluoroacetic acid (TFA), potassium bromide (KBr) or iodine can be used as reagent.

After MultiSep # 228 column clean-up Akiyama et al. (2001) applied TFA derivatization with LC FLD in red pepper for aflatoxin detection. With this derivatization technique 0.5 μg/kg LOD was measured for red pepper.

Post-column derivatization (PCD) including electrochemical bromination is considered as a widely used method for the analysis of aflatoxins. PCD can be achieved with either pyridinyl hydrobromide perbromide (PBPB) or with an electrochemical cell (KobraCell) where KBr is added to the mobile phase. Both derivatization techniques were used in several laboratories to analyze baby foods. When evaluating the results, no significant differences were found between the two PCD techniques. The recoveries ranged from 92 to 101%. During the laboratory analyses the technique resulted in an LOD of 0.02 μg/kg, LOQ of 0.1 μg/kg for AFB1 in baby food (infant formula) samples (Stroka et al., 2001; Gilbert and Vargas, 2003).

For enhancing the fluorescence properties/response of aflatoxins, PCD using iodine can also be considered as a method for aflatoxin detection. A great disadvantage of PCD using iodine is that the derivatization capability of iodine constantly reduces over time and, consequently, there is a parallel decrease in the sensitivity of the technique. The method yielded reproducible results at 1 μg/kg LOD for peanut butter samples.

Aggressive chemicals (e.g., KBr), however, which shorten the lifespan of instruments and capillaries, can be replaced by PHRED. Significant features of detection of aflatoxins with PHRED and FLD are 0.004 μg/kg (LOD) and 0.015 μg/kg (LOQ) (Rahmani et al., 2013). HPLC with FLD and in-line photochemical reactor is capable of determining aflatoxins separately in low μg/kg concentrations. An advantage of the method is that reagents for the sensitive measurement and substances for derivatization are not needed. The latter is based on the fact, that upon irradiation by 254 nm ultraviolet (UV) light, fluorescent properties of AFB1 and AFG1 components are increasing equivalently to electrochemical derivatization (Papadopoulou-Bouraoui et al., 2002).

There are further possibilities for the fluorescence-based detection of aflatoxins, e.g., HPLC-LIF. Laser-induced fluorescence (LIF) is based on the analysis of fluorescent light emitted during laser irradiation. Sensitivity of the method is 0.1 μg/kg for AFB1 and AFG1, and 1.2 μg/kg for AFB2 and AFG2 (Gan et al., 1989; Gilbert and Vargas, 2003). Its application is not widespread as HPLC-FLD is a cheaper and suitable technique for the detection of aflatoxins. UV detection is often mentioned in the literature besides fluorescence, but this procedure is not widespread in routine analysis. HPLC-UV determination was performed in egg and liver matrices, where the LOD and LOQ for AFB1 were 0.08 and 0.28 μg/kg (Amirkhizi et al., 2015). Aflatoxins can be detected by UV absorption; however, it is not sufficiently sensitive in all cases to reach the μg/kg range. Spectrometric detection will be discussed later.

Derivatization is not needed for the analysis of AFM1 occurring in milk and dairy products, as this component can be analyzed with HPLC-FLD with sufficient sensitivity. AFM1 determination was performed in milk and milk powder samples by using OASIS^{TM} Hydrophilic-Lipophilic Balance (HLB) SPE clean-up column, C-18 reversed-phase HPLC column and FLD detection, which is a simple and not the most expensive method. The detection limit/quantification limit of this method was 0.006/0,026 μg/kg for milk and 0.026/0.087 μg/kg for milk powder (Wang et al., 2012). The recovery was 85.4−96.9%. AFM1

was analyzed in milk, yogurt and cheese matrices with IAC clean-up, reversed phase HPLC separation and FLD detection, where the limit of determination for AFM1 was 0.003 μg/kg in milk, 0.07 μg/kg in yogurt and 0.05 μg/kg in cheese. The recovery was 85.4−96.9% (Yoon et al., 2016).

Electric Driven Techniques

Capillary electrophoresis (CE) is in fact a range of separation techniques based on different separation principles: capillary zone electrophoresis – CZE (based on differences between electrophoretic mobilities of analyses), micellar electro-kinetic capillary chromatography – MEKC (partition of neutral compounds with surface active micelles), capillary gel electrophoresis – CGE (filtration of analytes through a gel network), capillary isoelectric focusing – CIEF (separation of zwitterionic analytes with pH gradient), capillary electrochromatography – CEC (separation of compounds on a column packed with silica gel particles using electric field) (Hancu et al., 2013).

The classic CZE method, which is based on the differences between the electrophoretic mobilities of the analytes, is unfit for the separation of neutral compounds, which migrate with the same rate as the electro-osmotic flow (EOF) (Hancu et al., 2013).

Based on a hybrid method combining chromatographic and electrophoretic separation principles, micellar electro-kinetic capillary chromatography (MEKC) extends the applicability of capillary electrophoretic methods to neutral analytes. In the case of MEKC, surface-active compounds are added to the buffer solution in a concentration exceeding their critical micellar concentration. Consequently, they form micelles, which affect the electrophoretic migration, like any other charged particle. The separation is based on the differential distribution of the analyte between the two phases of the system: the mobile liquid phase and the micellar pseudostatic phase (Hancu et al., 2013). Aflatoxins were measured with the MEKC procedure in the feed of milking cows, including alfalfa, wheat bran and maize grains. Aflatoxins were separated in a silica capillary, and fluorescence was induced by 355 nm UV light. LODs and LOQs were between 0.002–0.075 and 0.007–0.300 μg/kg for the four aflatoxins, with analysis time within 6.5 min. The recovery was 70−108% (Gao et al., 2019). Six mycotoxins were determined with high reproducibility from feed samples, with the use of the MEKC procedure. The LOD/LOQ values were between 0.02/0.12 and 0.06/0.42 μg/kg, the recovery was 80−130% (Peña et al., 2002). Modified methods of MEKC, among others, are reversed-flow micellar electrokinetic chromatography (RFMEKC) and capillary electrokinetic chromatography (CEKC) with multiphoton excited fluorescence (MPE) detection (Gilbert and Vargas, 2003). CEC or CEKC are procedures to be applied for the separation of big molecules; however, no validated method was found. CE and, in particular, MEKC with laser-induced fluorescence detection (MEKC-LIF) appeared to be interesting techniques for determination of aflatoxins for a while, but no applications can be found in routine analysis (Naushad and Khan, 2014). The techniques mentioned above can be coupled with other detection systems, such as MEKC-fiber-optic sensor (SBFOS) (Dickens and Sepaniak, 2000).

Hyphenated Techniques

Hyphenated techniques usually mean separation procedures connected to a mass spectrometer. Of these, LC/UPLC-MS, SFC-MS, CE-MS and Chip-MS techniques have been used to determine aflatoxins. These procedures are presented below.

Liquid Chromatography/Ultra-Performance Liquid Chromatography Mass Spectrometry (LC/UPLC-MS) and Tandem Mass Spectrometry (MS/MS)

Until the early 1990s, thermospray, particle beam and fast atom bombardment interfaces were used for the LC/MS measurement of mycotoxins (Zöllner and Mayer-Helm, 2006). Using these interfaces, however, sensitivity and ionization efficiency problems often occurred. A breakthrough came in the beginning of 1990s, when the first instruments equipped with atmospheric pressure ionization sources (API) appeared on the analytical market. For the past 3 decades, both LC/UPLC-MS and MS/MS systems have become basic apparatus in almost all well-equipped research and routine laboratories of organic analytics. Due to their versatile applicability, these instruments are increasingly used in mycotoxin analytics as the sole qualitative/quantitative methods or as confirmatory methods to accurately determine the mycotoxin content of samples found to be positive at the screening by rapid methods (such as ELISA, Lateral Flow).

It needs to be mentioned, however, that the wider proliferation of these methods is hindered by their high price and the costs of training personnel for their professional operation and method development.

Atmospheric Pressure Ion Sources for the Determination of Aflatoxins by LC/UPLC-MS and MS/MS

LC-MS analysis of aflatoxins is possible with the application of all three commonly used atmospheric pressure ion sources. Review publications reveal that the atmospheric pressure electrospray (ESI) source is used predominantly for the LC-MS determination of aflatoxins (Zöllner and Mayer-Helm, 2006; Li et al., 2013; Yao et al., 2015). One reason for this is that ESI ionization of aflatoxins is very effective and the protonated molecules ($[M + H]^+$) and fragment ions created in the collision zone (CID) in the case of MS/MS can be measured well. Another reason is that users usually don't purchase the atmospheric pressure photoionization source (APPI) for most LC-MS instruments, or in the case of purchase, they don't have sufficient experience with its application. Atmospheric pressure chemical ionization (APCI) has also been successfully used for the sensitive LC-MS determination of aflatoxins (Abbas et al., 2002, 2006; Pacheco and Scussel, 2007; Xie et al., 2016).

If only aflatoxins need to be determined in samples to be tested, APPI can be considered to be the best choice among atmospheric pressure ion sources, as it has considerably lower background noise and ion suppression compared to ESI and APCI. The reason is that in the case of direct photoionization (direct APPI), only components with ionization potential (IP) value below the energy of photons emitted by the vacuum UV lamp of the ion source (10 eV) are ionized in the ion source. In other words, significant portion of matrix components and

potential contaminants in the mobile phase will not give noise during photoionization (signal enhancement/ion suppression). It was found that a mass spectrometer will be 2–3 times more sensitive during aflatoxin measurement, if equipped with APPI instead of ESI ion source (Takino et al., 2004; Cavaliere et al., 2006). It must also be noted, however, that the so called multitoxin methods based on LC-MS/MS are spreading increasingly (Berthiller et al., 2007; Li et al., 2013; Xie et al., 2016; Zhang et al., 2016; Malachová et al., 2018). These methods need to use ESI ion source, being the most effective to measure all mycotoxins, which are officially regulated. Furthermore, most mycotoxins will not give sufficient signal when detected by MS or MS/MS with APPI ion source.

Mass Analyzer Types for the LC/UPLC-MS and MS/MS Determination of Aflatoxins

Leaving the atmospheric pressure ion source, the ionized molecules enter the vacuum chamber of the mass spectrometer, and they reach the actual mass filter/mass analyzer through an iontransporting and focusing region. The mass analyzer can be single-stage or multi-stage (MS/MS) (**Figure 2**).

Due to the lack of collision-induced dissociation (CID), the fragmentation of molecular ions is not possible in mass spectrometers equipped with single-stage mass analyzers (e.g., single quadrupole) (with the exception of in-source CID), which would be prerequisite to the MS/MS spectrum based identification and exact determination of components eluting from the LC/UPLC column. Single-stage type mass analyzers are not compliant with EU requirements of residue analysis, requiring a precursor ion, two product ions and their ratio for the MS identification of a component (EU, 2002). Mass spectrometers equipped with multi-stage mass analyzer are compliant with these conditions. Several mass spectrometers equipped with multi-stage mass analyzer (MS/MS) have been applied for the analysis of aflatoxins: triple quadrupole (QqQ), 3D ion trap, quadrupole-linear ion trap (Q-TRAP), quadrupole-time of flight (Q-TOF), and orbitrap. Moreover, the availability of instruments equipped with these mass analyzers allowed the development of multitoxin procedures mentioned previously.

The most widespread and one of the best solutions for the quantitative determination of organic compounds with hyphenated techniques (e.g., LC/UPLC-MS/MS) is certainly the application of mass spectrometers equipped with triple quadrupole (QqQ) mass analyzer. LC/UPLC-QqQ-MS procedures are the most widespread among multitoxin methods (including aflatoxins, too) (Zöllner and Mayer-Helm, 2006; Herebian et al., 2009; Wei et al., 2013; Zhang et al., 2016; Malachová et al., 2018). Zhang et al. (2016) investigated the occurrence of 7 mycotoxins (including AFB1, AFB2, AFG1 and AFG2) in peanut, maize and wheat samples after IAC clean-up using the multitoxin LC-ESI-QqQ-MS/MS procedure. The LOD/LOQ values of the four mycotoxins were 0.1, 0.04, 0.1, 0.04/0.3, 0.12, 0.3, and 0.12 μg/kg. The recoveries were between 95.3 and 103.3%. Huang et al. (2014) investigated milk samples (row milk, liquid milk, milk powder) with UPLC-ESI-QqQ-MS/MS multitoxin (including aflatoxin) method after SPE. LOD values were 0.001–0.003 μg/kg, while LOQ values were between 0.003 and 0.015 μg/kg with recoveries ranged between 87 and 109%. Wei et al. (2013) elaborated a procedure with IAC clean-up followed by LC-ESI-QqQ-MS/MS for aflatoxin and ochratoxin A analysis in licorice (*Glycyrrhiza uralensis*) samples. For AFB1, AFB2, AFG1, AFG2 the LODs were 0.007, 0.005, 0.003, 0.005 μg/kg; while the LOQs were 0.020, 0.015, 0.010, 0.015 μg/kg, respectively. The recoveries ranges between 72.7 and 123.3%. McCullum et al. (2014) investigated the aflatoxin contamination of red wine samples with MSPE followed by the LC-ESI-QqQ-MS/MS method. The calibration curve was linear in the 0.006–3 ng/ml range. LOD values for AFB1, AFB2 and AFG1 toxins were 0.0012 ng/ml and 0.0031 ng/ml for AFG2. Mass spectrometers equipped with QqQ mass analyzer have excellent sensitivity and selectivity, but in quantitative measurement, usually the third quadrupole is also working in selected ion monitoring (SIM) mode; therefore, the information needed for structural identification is lost (Hernández et al., 2005).

If necessary, this information can be acquired by the application of a hybrid mass spectrometer such as a quadrupole-linear ion trap (QTRAP®) equipment, which enables both quantitative determination and confirmation based on the mass spectrum (Martínez Bueno et al., 2007).

LC-MS/MS having QTRAP® mass analyzer has been applied for multi-toxin measurement of aflatoxins in baby food. LOD and LOQ values ranged between 0.05–0.4 and 0.1–1 μg/l for the four aflatoxins (AFB1, AFB2, AFG1, and AFG2); the recovery was 78% (Rubert et al., 2012). This mass analyzer together with APPI ionization source has also been used for the detection of AFM1 toxin in very low concentrations in milk without observing any significant matrix effect. LOQ values ranged between 0.006–0.035 μg/l; note, however, that LOD values were not reported.

For aflatoxin analysis, LC-MS instruments including the so-called 3D iontrap (IT) mass analyzer have already been used. Cavaliere et al. (2006) determined AFM1 in milk samples. The LOD and LOQ were 1 and 6 ng/kg compared to 3 and 12 ng/kg obtained with ESI ion source. The recovery was between 92 and 98%.

Lattanzio et al. (2007) investigated 11 mycotoxins, including aflatoxins (AFB1, AFB2, AFG1, and AFG2) from maize extracts with multitoxin immunoaffinity sample clean-up followed by LC-ESI-IT-MS/MS procedure. LOD values of 0.3–4.2 μg/kg were found for mycotoxins with average recovery of 79%. Schatzki and Haddon (2002) applied an IT-MS device without clean-up for the screening of aflatoxin content of 65,000 walnut samples. Aflatoxin contamination was found in 120 samples in the concentration range of 250–43,000 ng/g.

Saldan et al. (2018) coupled a quadrupole–time-of-flight (QTOF) mass spectrometer to a liquid chromatograph (LC-QTOF-MS) for the identification of *Aspergillus flavus* strains grown on agar medium, based on chemical markers (secondary metabolites including AFB1, AFG2). LOD and LOQ values ranged between 0.1–0.3 μg/kg and 0.2–0.9 μg/kg for the identified components during the analysis of the culture extracts.

Herebian et al. (2009) combined micro-LC separation with a mass spectrometer containing a linear trap quadrupole (LTQ)-Orbitrap mass analyzer for multitoxin determination, where the

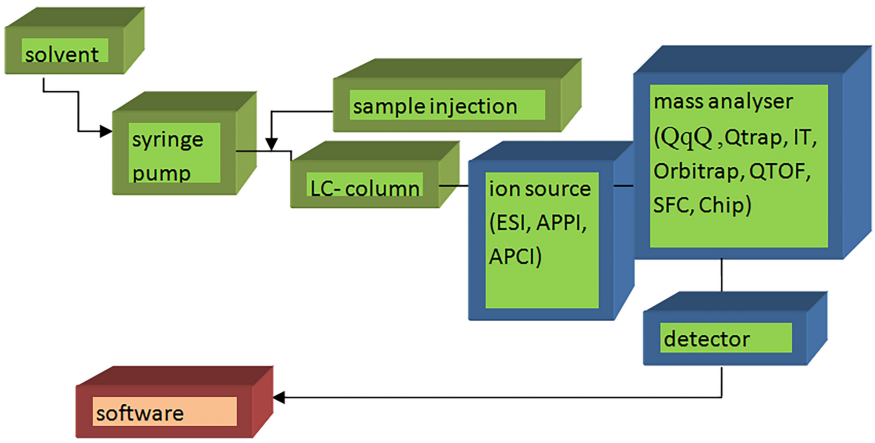

FIGURE 2 | Simple semantic of LC-MS system.

AFB1, AFB2, AFG1, and AFG2 contents of wheat and maize extracts were also analyzed. The measurement was performed in full scan mode by determining the accurate mass of extracted ions. LOD for mycotoxins was between 0.4 and 2000 ng/ml. Specific LOD values for aflatoxins, however, were not reported.

The ion suppression/enhancement caused by the matrix effect can rarely be avoided even by these sophisticated multi-stage mass analyzers, particularly, when the raw sample extract is analyzed by LC/UPLC-MS/MS without clean-up ("extract and shoot" method). To avoid such problems and reduce the LOD/LOQ values, the sample clean-up procedures discussed above are extensively used before the LC/UPLC-MS/MS measurement of mycotoxins, including aflatoxins. Prominent procedures of these are the IAC clean-up (Dragacci et al., 2001; Mazaheri, 2009; Xie et al., 2016; Zhang et al., 2016) and QuEChERS (Anastassiades et al., 2003; Xie et al., 2016) discussed above. It also needs to be mentioned, that to increase the accuracy of quantitative evaluation, at least the so-called external matrix-matched calibration needs to be performed. However, the best solution used currently is to add isotope-labeled internal standards of the mycotoxins by an automatic sample injector to both the matrix-matched calibration samples and samples to be measured (Zöllner and Mayer-Helm, 2006). Obviously, the application of isotope-labeled internal standards, particularly for multitoxin analysis, results in significant cost increase (Li et al., 2013; Šarkanj et al., 2018).

Supercritical Fluid Chromatography Mass Spectrometry (SFC-MS)

The SFC technique combines the numerous advantages of liquid and gas chromatography. Its application is beneficial for non-volatile, heat sensitive, reactive and multicomponent samples. SFC provides results faster than HPLC, because diffusion of the substance is 10 times faster in the supercritical solvent (CO_2) than in liquid phase. The analysis is usually performed in environmentally benign manner without the use of organic solvents; however, MeOH or a 1:2 MeOH:ACN mixture is added to CO_2 as a polar modifier if necessary (Taylor et al., 1997). The separation process takes place at a lower temperature than in the case of GC, and with similar efficiency. Its disadvantage is its very high price; therefore, SFC procedures have been developed for the determination of relatively few compounds.

The SFC procedure combined with a tandem mass spectrometer containing ESI ion source (SFC-MS/MS) has been used for the simple, fast and sensitive determination of aflatoxins in edible oil (Lei et al., 2016). CO_2–methanol gradient elution was used to the baseline separation of the four aflatoxins. Following separation, there was a need to use post-column make-up flow before the introduction into the ESI ion source, to achieve a sensitive SFC-MS/MS determination of the components. The LOD and LOQ values for aflatoxins ranged in order 0.02−0,04 and 0.05–0.12 μg/l, while RSD was lower than 8.5%. Applying internal standard a recovery of 98% was achieved.

Chromatin Interacting Protein-Mass Spectrometry (Chip-MS)

In the first chip-MS-based system for AFB1 determination, a plastic microfluidic chip was used for the automatic affinity dialysis, concentration and subsequent ESI-MS determination of reaction mixtures containing AFB1 antibodies and aflatoxins (Yiang et al., 2001).

For the determination of aflatoxins in peanut products, a procedure was also developed, where a nano LC pump was coupled to a QqQ-MS through a chip-ESI-MS ion source (chip-nano LC) (Liu et al., 2013). Following solvent extraction, immunoaffinity solid-phase clean-up was carried out to reduce the matrix effect. Separation was performed by gradient elution and detection was done using multiple reaction monitoring. Linear dynamic range for the four main aflatoxins was 0.048–16 ng/g. LOD was reported to be between 0.004 and 0.008 ng/g. Accuracy (96.1%-105.7%/95.5%-104.9%) were obtained.

Beside the sensitivity of determination and the low amounts of sample needed, the significance of the chip-MS procedure is its environmentally benign manner resulting from low solvent consumption. Due to decreasing prices of the chips and instruments, the spreading of these methods is to be expected.

Rapid Test Methods

Rapid tests developed for the analysis of aflatoxins are built upon several different technologies. The most common ones are the enzyme-linked immunosorbent assay (ELISA), lateral flow devices (LFD) and chemical methods. Rapid tests are indispensable to provide analytical results within a short time. These procedures enable the analysis to be easily performed with lower prices, even at the location of sampling.

The vast majority of the rapid methods used for aflatoxin measurement are immunoassays based on the reaction of a special antibody and the antigen of the analyte, which can be detected by various markers.

Markers

Many markers have been developed over the years, including enzymes, radioisotopes, fluorophores, gold nanoparticles and other sensitive optical and electrochemical components (Mataboro et al., 2017).

Enzyme label Enzyme-linked immunosorbent assay (ELISA)

The aim of the ELISA technique is the qualitative or quantitative determination of mycotoxins found in the analytical sample, based on the application of antibodies, which are specific to compounds to be analyzed. The method is based on an enzyme-linked color reaction. For the detection of mycotoxins, competitive-type ELISA tests are typically used. Consequently, the measured color intensity is inversely proportional to the concentration of the measured compound (Waliyar et al., 2009; **Figure 3**).

These ELISA analytical systems are excellent screening devices, provide quantitative results in a short time period, and as previously mentioned, they can often be used at the location of sampling, too. However, cross-reactions with molecules very similar to the analyzed substance and matrix effects found during the analysis of different products may influence the results. Naturally, quantitative determination of AFB1, AFT and AFM1 can also be performed with the ELISA technique (Ketney et al., 2017). The producers of the tests have considered the different regulatory limits of different regions. A substantial part of agricultural raw materials can be analyzed with the ELISA technique, according to the guidance provided by the producer, without the application of particular cleaning steps. ELISA analysis of more complicated sample types, like compound feed, however, may provide inaccurate results. In order to avoid this situation, it is recommended to consult the producer of the tests concerning the sample to be analyzed. Alternatively, the process is recommended to be individually validated for the matrices to be tested. However, if the measurement of a complex matrix is needed, which is not on the list of substances validated by the producers, or if the aim is to confirm the result of a rapid test, the sample has to be analyzed with reference methods (Andreasson et al., 2015).

The sensitivity of the ELISA kit depends on the manufacturer. For instance Romer Labs Inc. United States reported an LOD of 0.018 μg/kg and LOQ of 0.025 μg/kg with recoveries ranging between 80 and 120% for the determination of AFM1 in milk.

An improved version of ELISA is **(Tumor Specific Antigen) TSA-ELISA**, where the intensity of the sign generated by ELISA can be increased several folds by the addition of tyramide. Under optimal circumstances, the LOD, IC10 and the half maximum inhibition concentration (IC) (IC50) of TSA-ELISA is 0.004 and 0.039 ng/ml, respectively, in the case of AFB1. The elaborated TSA-ELISA method afforded LOD values 11 times better and IC50 values 6 times better compared to those measured by the traditional ELISA method in the analysis of AFB1 in edible oil samples (Zhang et al., 2018). TSA-ELISA is a satisfactory, sensitive and cheap method with good reproducibility, and a useful alternative for AFB1 detection in edible oil samples.

Radioimmunoassay (RIA)

Radioimmunoassay applies radioactively labeled molecules during the stepwise formation of immunocomplexes. RIA is a highly specific and very sensitive method. In the case of agricultural samples (maize, soybean, wheat and rice), the LOD/LOQ of the method was 0.2/0.5 μg/kg for AFB1. The recovery was between 92 and 107% (Korde et al., 2003).

RIA requires the application of an expensive, special equipment to minimize the adverse effects caused by gamma rays (Waliyar et al., 2009).

For this reason, in order to avoid health risks, other types of marker compounds might be more beneficial for the analysis of aflatoxins (Hemmilä, 1985).

Fluoroimmunoassays (FIA)

Immuno reagents with probes based on fluorescent labeling are already used widely. By combining the highly sensitive fluorescence method with the sensitivity of the measuring instrument, a simple and rapid analytical procedure can be achieved, where the concentration of the analyte can be directly measured in the reaction mixture. The problem with FIA methods was the low sensitivity caused largely by the high background noise of the fluorometric measurement (Hemmilä, 1985). The background has been reduced by continuous improvements, e.g., solid-phase separation systems, new fluorescent probes and new instruments **time-resolved fluoroimmunoassays (TRFIA)**, resulting in a sensitivity, which is suitable to analyze mycotoxins today. It was demonstrated that under optimal analytical conditions, TRFIA was very sensitive and specific to detect AFB1 with an LOD of 0.1 μg/kg in feed samples. TRFIA demonstrated high accuracy during the determination of AFB1 in feed samples. Average recovery ranged between 93.71% and 97.80% with a coefficient of variation of 1.25–3.73%. A very good correlation was found between TRFIA and HPLC methods during AFB1 determination of feeds, which confirmed the reliability of the developed method (Hu et al., 2018). Wang et al. (2016) determined AFB1 toxin from soy sauce with TRFIA technique. The range of the measurement was between 0.3 and 10.0 μg/kg, the LOD value was 0.1 μg/kg. The recovery was between 87 and 113%.

Flow cytometry based competitive fluorescent microsphere immunoassay (CFIA) is a microbead-based competitive fluorescent immunoassay applying monoclonal antibodies of high affinity. It can simultaneously detect six mycotoxins (OTA,

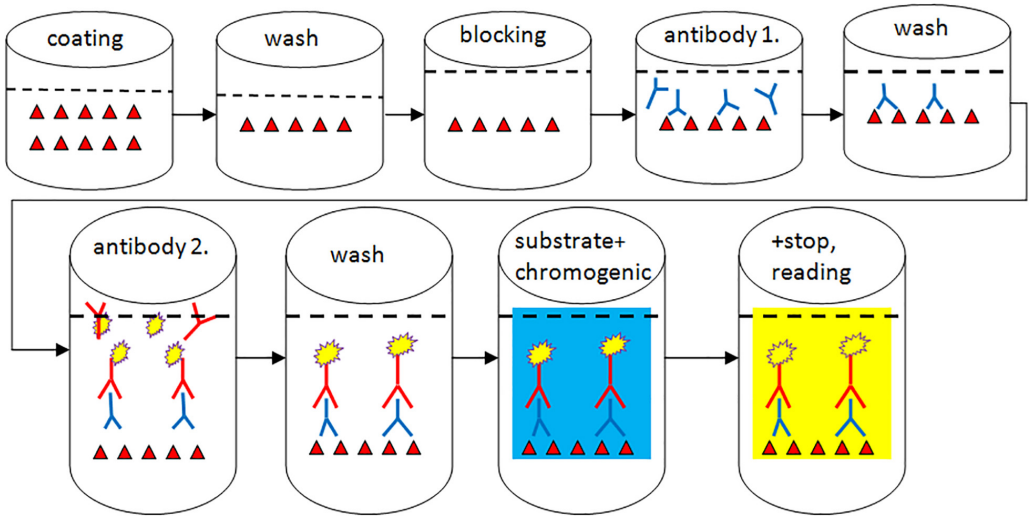

FIGURE 3 | Structure of a competitive ELISA.

AFB1, FB1, DON, T2, ZEA) with increased sensitivity for aflatoxins (0.12 μg/kg) following a simple extraction procedure compared to an ELISA method (Czéh et al., 2012; Czéh, 2014; Bánáti et al., 2017).

Chemiluminescence immunoassay (CLIA)

Chemiluminescence immunoassay is an immunoanalytical technique, where the marker is a luminescent molecule. Luminescence is usually the emission of visible or near visible (λ = 300–800 nm) radiation. The advantage of luminescence in spectrophotometry over absorption is that its signal is absolute, while the latter one is relative. Chemiluminescence methods can be direct, by using luminophores as markers or indirect, by using enzyme markers. Each of them can be competitive or non-competitive. Fang et al. (2011) developed a CLIA technique for the analysis of AFB1 in agricultural products. The method had a LOD of 0.01 ng/g and a linear range of 0.05 to 10 ng/g with 79.8−115.4% recovery.

Other

In some areas of analytics, color label markers (e.g., gold nanoparticles, colored latex) are the most widely used for rapid and qualitative determination. In addition to the above mentioned markers, aflatoxins can also be made fluorescent by irradiation with UV or laser light. However, they may also be derivatized with various chemical agents (e.g., iodine, bromine, etc.) (Li et al., 2009).

Immunological Devices

The most widely used immunological devices are microplate-based immunoassays, lateral flow immunoassays (LFIAs) and different biosensors (immunosensors) (Li et al., 2009).

Microplate−based immunoassays

When analyzing aflatoxins, microtiter plate and reader-based immunoassays allow simultaneous analysis of many samples, since the plates used have multiple wells. Most widely used microplate-based immunoassays are ELISA, fluorescence and chemiluminescence based analyses (Li et al., 2009).

Lateral flow immunoassay (LFIA), (LFA), or lateral flow devices (LFD)

Immunochromatographic dipsticks are another appropriate technology on the market of rapid mycotoxin tests. The basis of the method is the detection of the analyzed component by linking to a specific antibody in the test zone, which is placed on a membrane fixed on the dipstick. In addition to the test zone there is the control zone on the membrane verifying the correct functioning of the test. When the sample extract flows on the membrane, it passes the test and control zones and, depending on the concentration of the toxin, both (test and control) lines or only the control line will become visible. The dipstick can be evaluated visually by the naked eye or with the help of a reading device. When quantitative results are needed, the evaluation is performed by an instrument (reflectance photometer), which measures the intensity of the test and control lines and evaluates the results on the basis of data determined. The immunochromatographic dipstick is a rapid, easy-to-perform technique, which is ideal and cost-effective even for the analysis of a single sample. Similar to the ELISA technique, cross-reactions and matrix effects occurring during the analysis of certain products limit the applicability of the dipstick. For the determination of aflatoxins, qualitative, and quantitative immunochromatographic dipsticks are available (Anfossi et al., 2013). These tests have basically been validated for simple sample matrices; thus, their application is recommended for the screening analyses of raw materials.

However, results are available from the analysis of certain more complex matrices as well. The visual detection limit for AFB1 in this case was 5 μg/kg (Delmull et al., 2005). A decision level of 0.1 μg/kg was achieved with LFIA technique in food samples (Liao and Li, 2010; **Figures 4**, **5**).

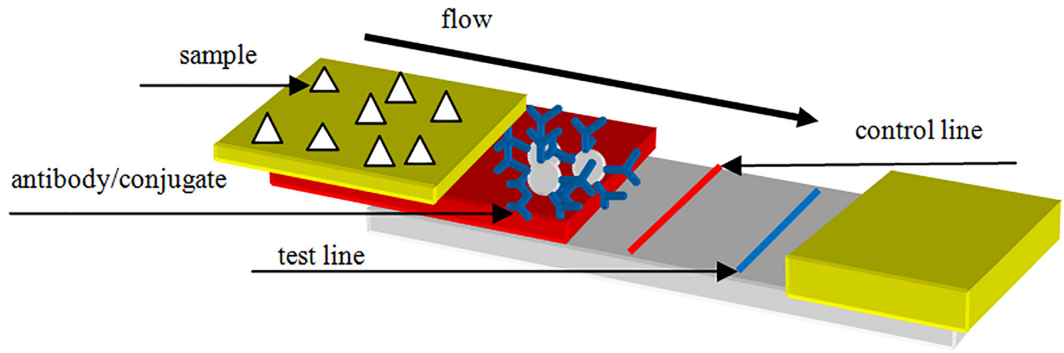

FIGURE 4 | Structure of lateral flow immunoassay.

The detection options of LFIA depends on the type of the marker. In case of color label markers (e.g., gold nanoparticles, colored latex), besides instrumental reader, there is a possibility of visual evaluation, while in case of fluorescence (e.g., quantum dots, ruthenium complexes) or other markers (e.g., enzyme labels or paramagnetic labels), only readers or expensive detectors can be used for quantification (Koczula and Gallotta, 2016).

Chromatographic time-resolved fluoroimmunoassay (CTRFIA)

A portable immunosensor based on chromatographic time-resolved fluoroimmunoassay has been developed for fast on-site sensitive determination of AFB1 in food and feed samples. CTRFIA provides an increased positive signal and low signal-to-noise ratio in time-resolved mode. Zhang et al. (2015) applied the method to various food and feed matrices such as maize (LOD 0.06 μg/kg), peanut (LOD 0.09 μg/kg) and vegetable oil (LOD 0.09 μg/kg). These matrices yielded a recovery of 116.7% from 80.5%. Tang et al. (2015) showed a fast method without sample preparation that can be performed just within 6 minutes. Its LOD for raw milk AFM1 matrix was 0.03 ng/ml, the measurement range was between 0.1 and 0.2 ng/ml and the recovery in case of quantitative determination was 80−110%. Tang et al. (2017) also measured simultaneously AFB1 and ZEN in maize with CTRFIA method. The LOD values of the method were 0.05 ng/ml for AFB1 and 0.07 ng/ml for ZEN.

LFIA is considered as a fast and sufficiently sensitive screening method. The need for the development of multi mycotoxin analysis has arisen in this research area as well, as this method was previously only applicable for one mycotoxin analysis at a time. The publication of Zhang et al. (2018) describes a multicolor-based immunochromatographic strip (ICS) semi-quantification method that is suitable for the simultaneous determination of 3 mycotoxins (AFB1, ZEN, T-2). Maize and cereal-based feed matrices were analyzed. Visual LOD-s estimated by the researchers were 0.5, 2, and 30 ng/ml for the above-mentioned toxins, respectively. The cut-off values were 1, 10, and 50 ng/ml respectively.

Biosensors

Chemical sensors are small-size devices, which convert the chemical information characterizing the composition of the compound into electronic or optical signal by continuous tracking, in real time. Such sensors represent modern analytical devices of our days. They take over the role of traditional analytical methods in several areas, since they can be well miniaturized due to their robust structure, can be integrated in automatic systems, and can be applied in *in situ* analysis as well. Chemical sensors usually lag behind laboratory instruments regarding analytical performance parameters of selectivity, sensitivity and stability. For this reason, the requirements of the area of application should be borne in mind during the development of sensors. Their grouping is usually based on the functioning of the transducer system or on the substance to be measured (e.g., gas-, ionic-, biosensor).

Label based biosensors. Biosensors, a sub-group of chemical sensors, are special selective analytical devices, which are closely linked to or integrated into a physico-chemical transducer (e.g., electrochemical, optical, piezoelectric, etc.) and contain a substance of biological origin (e.g., enzyme, tissue, microorganism, antibody, etc.) or an imitating substance (e.g., molecularly imprinted polymers, MIP) (Sharma et al., 2003).

Detection is based on the linking of the analyte to its specific complementary biological element (bioreceptor), which is fixed on a suitable portable surface (Velasco-Garcia and Mottram, 2003). The rise of biosensor techniques can be explained by their many advantages compared to conventional analytical techniques. The selectivity provided by the biologically based element grounds the development of specific devices, which often facilitate real-time analyses of small amounts of complex samples, with simple sample preparation. The MIP procedure was successfully used for the selective extraction and pre-concentration of AFB1 in infant food sample. The LOD was 0.0275 μg/kg with recoveries of 83.51−90.03% (Semong and Batlokwa, 2017). The sensor developed by Jiang et al. (2015) showed a wide linear range between 1 fg/ml and 1 μg/ml. In the rice sample the LOD of AFB1 was 0.3 fg/ml and the LOQ was 1 fg/ml. Depending on the type of label, highly sensitive and selective analyses include, among others, FIA, RIA and EIA. See Section "Rapid Test Methods."

Label-free biosensors. Techniques based on labeling molecules are increasingly lagging behind in the area of measurement

FIGURE 5 | Practical application of the lateral flow immunoassay.

of interactions between different molecules in biological and biochemical systems. Surface plasmon resonance (SPR) is a distinguished method among label-free analytical methods, which can analyze the interactions near surfaces, based on the SPR phenomenon. It can indicate not only the endpoint, but the whole process can be monitored.

Mass-change-based sensors most often use mechano-acoustic sensors based on the change of resonance frequency, with label-free techniques of quartz crystal microbalance (QCM) and optical waveguide light-mode spectroscopy (OWLS). Similar to other label-free detection methods, OWLS enables the real-time inspection of molecular-level processes at the interface. This can be achieved by the application of the two-part integrated optical waveguide sensor (chip), which is the basis of the technique. A sensitive method could be developed for mycotoxins including aflatoxins from pepper, applying gold nanoparticles of different sizes and origin (Adányi et al., 2018). When analyzing aflatoxins with OWLS, the LOQ for AFB1 in wheat, barley and pepper samples was between 0.001–1 μg/kg, while the LOD was 0.0005 μg/kg with 76.4−108.6% recoveries (Adányi, 2013). Its disadvantage is that although it is sensitive, it is not selective in the case of complex samples. However, the required selectivity can be achieved by prior sample clean-up with immunoaffinity column, providing a clean solution without interferences (Majzik et al., 2015).

Lab-on-a-chip based biosensor (LOC). Lab-on-a-chip is a device, which integrates one or more laboratory functions into one chip, having a size of only a few square centimeters. LOCs are able to manage extraordinarily small amounts of liquid below pico-liter quantities (Volpatti and Yetisen, 2014). LOC systems and MS fit together remarkably well (Oedit et al., 2015).

Biosensors enable real-time detection of AFB1 in foods with a fast, sensitive, completely automated and miniaturized system (Uludag et al., 2016).

Flow injection immunoassays (FI-IA)

Flow injection immunoassays is an automatic method for chemical analyses, where the sample is injected into a flowing carrier solution, which is mixed with the reagents before reaching the detector. The automated system can be combined with several different detectors, e.g., biosensor, spectrophotometer, or even with mass spectrometer. For the determination of AFM1 in milk, a FI-IA method was developed with amperometric detection (Badea et al., 2014). Good potentials were demonstrated, and it was suitable as a rapid method for the screening of the toxin in raw milk. The LOD/LOQ were 0.011/0.02 ng/ml in milk with recoveries 80−120%. It should be noted that there are countries where this sensitivity of detection is not sufficient to meet the requirements of the corresponding legislation (see **Table 1**). Sample preparation is very simple and fast requiring only heating and dilution. Results found with this method were in good correlation with both HPLC and ELISA. The method is capable to analyze many samples in a short time. For sample preparation, the application of Protein G column is needed. The FI-IA system presented here contains low-cost devices with simple handling and it is suitable for automation (Badea et al., 2014).

Other Techniques

Currently, several other analytical procedures are under development, which can be grouped in several ways. Some procedures are exceptions regarding the groupings as they may be allocated into more than one group such as direct analysis in real-time-mass spectrometry (DART-MS), near infrared spectroscopy (NIRS), Luminex xMAP® technology and Biochip Array Technology (BAT) as a new technological direction.

Matrix-Assisted Laser Desorption Ionization-Time of Flight-Mass Spectroscopy (MALDI-TOF-MS)

Since there is no chromatographic or eletrophoretic separation in MALDI-TOF-MS, it is not in the group of hyphenated

techniques. Ramos Catharino et al. (2005) investigated the applicability of MALDI-TOF-MS for the analysis of AFB1, AFB2, AFG1, and AFG2 content of different agricultural crops. α-Cyano-4-hydroxycinnamic acid (Et_3N-α-CHCA) was applied as MALDI matrix and NaCl was added to the matrix in order to increase sensitivity. Even an LOD of 50 fmol could be achieved with this fast method that requires minimal sample processing. The procedure seems to be applicable for high-throughput screening not only of aflatoxins, but of other mycotoxins as well.

Direct Analysis in Real Time-Mass Spectrometry (DART-MS)

The DART-MS procedure includes no *de facto* separation, but the sample is usually put on a TLC or paper plate. The charged helium beam emitted from the DART ion gun is directed to the sample surface at an angle about 45°, inducing the ionization of the analyte, followed by the ESI source focusing the ionized components toward the ion entrance of the mass spectrometer (Cody et al., 2005). Busman et al. (2014) studied the possible quantitative applications of DART-MS for the aflatoxin measurement. They prepared solvent, matrix and matrix calibration standard solutions spiked with internal standard in the 1–250 ng/ml range. For all three types of calibrations, the concentration/detector response correlation was linear in the studied interval. The lowest calibration level (LCL) for AFB1 was found to be 4 μg/kg. The recovery range was 94 110%.

Near-Infrared Spectroscopy (NIRS)

Near-infrared spectroscopy is an innovative technology used in the food-, chemical-, pharmaceutical- and petrochemical industries. Coupled with the development of chemometric techniques, this technology is an efficient, fast, reliable and non-destructive analytical method to measure the qualitative and quantitative characteristics of organic substances. Results of earlier studies showed that the application of the NIRS technique was successful in the detection and to some extent the determination of chemical contaminants, for example mycotoxins (Tripathi and Mishra, 2009). It was observed, however, that the low sensitivity of NIR spectroscopy was not sufficient to quantitate the chemical residues in food substances. We can therefore conclude that the further development of this method is needed in order to ensure the accurate measurement of chemical contaminants found in foods and feeds. This device is able to analyze food products without any kind of preparation, but for the time being, it is considered to be quite basic for the measurement of aflatoxins (Teye et al., 2013). Because of its LOD 15–500 μg/kg, it can be used only for the prescreening of toxin-contaminated samples. More sensitive NIRS instruments are necessary for further quantitative measurements.

Luminex xMAP Technology

The xMAP technology enables the multiplexing of biological tests, and the reduction of time, human resources and costs spent, compared to traditional methods such as ELISA, Western blot or PCR techniques (Luminex, Austin, TX, United States). Microbeads are labeled with a special mixture of dyes, resulting in color-coded microbeads. The different microbead clusters can be mixed. As each microbead carries an individual recognition signal, the xMAP system can detect which microbead belongs to which cluster. With the aid of several lasers or LEDs, a high-speed digital signal processing system reads the processes taking place on the surface of each color-marked microbead. Red laser excites both the red and infrared dyes found in the microbeads, enabling the grouping of the microbead into one of the potential 100 clusters. Green laser induces fluorophore linked to the surface of the microbeads, enabling the determination of the substance contained in the sample. Theoretically, 100 different measurements can be performed in one sample at the same time. Peters et al. (2011) spiked 4 blank feed samples with AFB1 at the 7–23 μg/kg range with inhibition above 90−98% in all samples.

Fiber-Optic (Immuno)Sensor

Maragos and Thompson (1999) investigated fumonisines and aflatoxins with the fiber-optic immunosensor technique in spiked and naturally contaminated maize samples. In contrast with fumonisines, in the case of AFB1, a non-competitive sensor was used. As the fluorescence of AFB1 itself was detectable, the reaction of the sensor was proportional to the concentration of the toxin. The sensor, though could detect 2 μg/kg AFB1 in the solution, was technically not an immunosensor, as the binding of aflatoxin specific antibodies was not necessary. Therefore, this technique is not considered to be an immunochemical test. The applied sensor types are able to rapidly screen the different maize samples, but to achieve real efficiency, the sample needs to be cleaned in a separate preliminary step.

Biochip Array Technology (BAT)

Biochip Array technology is an immunoassay based technology enabling the simultaneous semi-quantitative detection of various mycotoxins from various cereals and cereal based products. The immunoassays define discrete test regions on the biochip surface on which the immunoreactions take place. Applying specific Myco 7 kit, the screening decision levels were for aflatoxin B1 and ochratoxin A (0.25 μg/kg); aflatoxin G1, deoxynivalenol, zearalenone, T2-toxin, fumonisin B1 0.5, 100, 2.5, 5, and 10 μg/kg, respectively. The within laboratory reproducibility was 11.6% and the overall average recovery was 104%. With multiplex Myco arrays, results can be obtained within 3 h, which is comparable to that required when using a single ELISA kit. The chemiluminescence reactions can be monitored with digital picture imaging technology. such as Evidence Investigator. The flexibility of the technology allows extension of analytical profile and implementation of new assays. It should be noted that the cost of the instrument is in the range of HPLC systems, though its operation cost is lower (**Figures 6A–C**).

DISCUSSION

As aflatoxins pose danger to both humans and animals, researchers are continuously searching for analytical methods most suitable for specific tasks. Due to the development of analytical and IT techniques, increasingly faster and

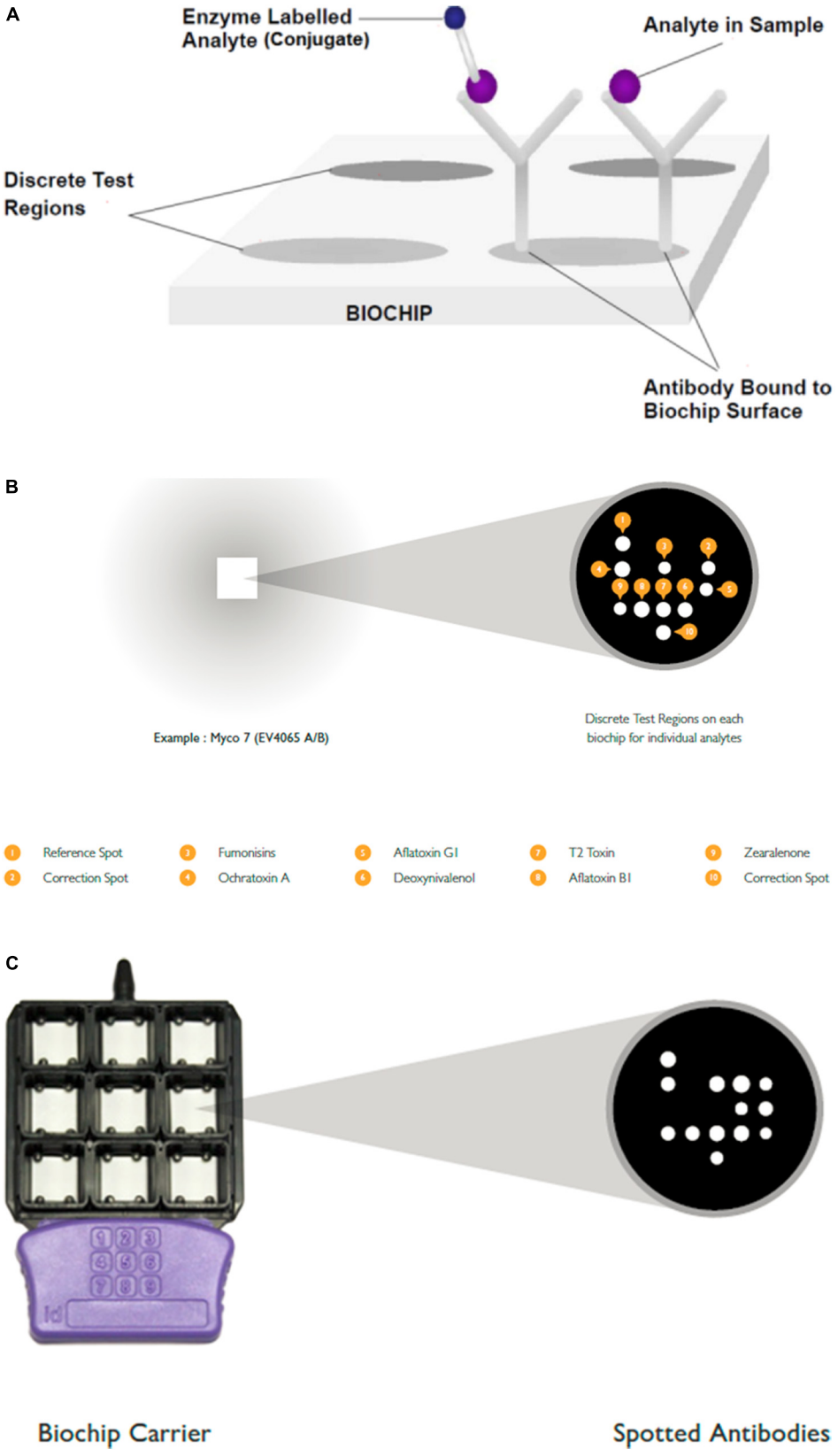

FIGURE 6 | (A–C) shows the principle of Competitive Biochip Assay. Published with the permission of the manufacturer.

more sensitive have come into focus in the last decades, but only a few of them have gained applicability in routine analysis.

Immunoanalytical methods (e.g., LFIA, ELISA) proved to be promising to detect the aflatoxin present in low levels in feed and food. Immunoanalyses with portable devices are simple, fast, sensitive, and cost-effective. Occasionally they are even capable of quantification with the aid of a reader. However, application of these methods provides only informative data on the given analyzed product. Their disadvantage is that despite their general suitability for the analysis of raw materials, interferences may occur at the measurement of more complex matrices. Therefore, the areas of future research are primarily including the removal or compensation of matrix components or compensating their adverse effects, application of nanoparticle technology, specific antibody production, automation and the miniaturization of instruments.

Several immunological methods including ELISA and other fast antibody-based tests can be used for screening purposes. However, confirmatory analyses with more robust methods are needed in these cases as well.

Analytical methods for the accurate quantitative determination of aflatoxins are under constant development. **Supplementary Table 1** provides guidance on the current performance characteristics of various detection techniques and highlights their limitations for practical use. Among the traditional techniques IAC clean-up followed by HPLC/FLD is the most frequently applied combination of methods for the measurement of aflatoxins. It is an excellent technique for routine laboratory analyses to comply with legal limits. Multi-mycotoxin environments (simultaneous occurrence of several mycotoxins) provide a more serious and complex health risk and challenge. Therefore, wider and more extensive monitoring of multi-mycotoxin contaminations has become necessary. At the same time, based on publications of past years reporting mycotoxin co-infections, demand for multiplex analyses is obviously rising. LC-MS/MS is an accurate and highly sensitive technique to analyze multi mycotoxins at present and years to come. It is capable to determine several mycotoxins simultaneously, and now it is considered to be a routine method. Its disadvantage is that it is an expensive technique. The operation and service costs of the instrument can be several orders of magnitude higher than those of classic LC systems. Furthermore, the treatment and maintenance of these instrument systems require a well-trained staff.

Future developments will be directed to lab-on-a-chip miniaturized technologies, chip-based biosensors and multitoxin detection by immuno-based techniques, where some analytical steps will be partly or fully replaced by micro/nanotechnology. An important goal for the research of chip-based technology is to achieve simple, fast and cost-effective methods, which can be combined with other devices and methods (e.g., immunochemical analyses) in a flexible way. It can be expected that methods and technologies, recently or further developed, will be more user-friendly and will provide better results.

Nowadays, ELISA is the most commonly used fast method in the laboratories. Using test strips for solid matrices in the fields is a technology which needs to be developed before practical application. There are many publications regarding this topic. Sample homogenization and extraction needs more development. Under industrial laboratory circumstances, methods based on test strips are mainly used as they provide faster results than ELISA.

For the confirmation of screening methods and the exact quantitative determination of aflatoxins, HPLC-FLD, combined with pre- or post-column derivatization is still the most commonly used procedure.

The best method for the exact, reproducible, qualitative and quantitative determination of aflatoxins today is HPLC-MS-MS technique using triple quadrupole mass analyzer.

However, in industrial and smaller laboratory circumstances, regarding screening tests the future is pointing toward fast and micro methods with low solvent-need, such as immuno flow cytometry.

This publication summarizes the analytical techniques that were or can be used for aflatoxin measurement or detection. The major deficiency of the majority of published methods is that they do not include the processes applied for reduction of large laboratory samples to the few grams of test portions to be extracted. Moreover, the evaluation of repeatability or reproducibility of the results, if reported, was based on a few spiked samples. Materials contaminated naturally have rarely been used to evaluate the performance of the developed methods. Much more attention is needed in the future to characterize the contribution of sample size reduction and test portion size to the overall uncertainty of the results, which are required for the correct interpretation of the measured concentration in relation to the legal limits and estimating the exposure of consumers.

In the future, when methods are evaluated from technical point of view, sources of errors must be indicated, and potential limitations of the performance parameters must be pointed out. The spike levels and the number of replicates applied must be indicated together with the reported repeatability and if possible reproducibility data. Finally, it is a must to indicate, whether repeatability and or reproducibility of mycotoxin concentration was investigated in naturally contaminated samples or not.

AUTHOR CONTRIBUTIONS

GM and TB contributed to create the conception and design of the review. CA, AN, and VK organized the database. GM and TB wrote the first draft of the manuscript. ÁA reviewed the first and revised drafts of the manuscript. ZF, AZ, KK, and ÁJ have done the language verification. GM, TB, and ÁA finalized the manuscript and prepared for publication.

All authors contributed to manuscript revision, read, and approved the version to be submitted.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01916/full#supplementary-material

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Small Molecular Contaminant and Microorganism Can Be Simultaneously Detected Based on Nanobody-Phage: Using Carcinogen Aflatoxin and Its Main Fungal *Aspergillus* Section *Flavi* spp. in Stored Maize for Demonstration

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Simultaneous detection technology has become a hot topic in analytical chemistry; however, very few reports on how to simultaneously detect small molecular contaminants and microorganisms have been in place. Aflatoxins are a group of highly toxic and carcinogenic compounds, which are produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus* from section *Flavi* responsible for aflatoxin accumulation in stored cereals. Both aflatoxins and *Aspergillus* section *Flavi* were used to demonstrate the duplex real-time RCR method of simultaneously detecting small molecular contaminants and microorganisms. The detection of aflatoxins and *Aspergillus* section *Flavi* was carried out depending on the anti-idiotypic nanobody-phage V_{2-5} and aflatoxin-synthesis related gene *nor-1* (=*aflD*), respectively. The quantitative standard curves for simultaneous detection of aflatoxins and *Aspergillus* section *Flavi* were constructed, with detection limits of 0.02 ng/ml and 8×10^2 spores/g, respectively. Naturally contaminated maize samples (n = 25) were analyzed for a further validation. The results were in good agreement between the new developed method and the referential methods (high-performance liquid chromatography and the conventional plating counts).

Keywords: real-time PCR, aflatoxin, *Aspergillus*, nanobody-phage, *Nor-1* gene

INTRODUCTION

Simultaneous detection technology has been becoming a hot topic in analytical chemistry. Many methods have been reported for simultaneous detection of multi small molecular contaminants such as mycotoxins (Li et al., 2013, 2019; Zhang et al., 2016; Wang et al., 2017a), pesticide residues (Bagheri et al., 2016; Wang et al., 2017b), and

veterinary drugs (Taranova et al., 2015; Dasenaki et al., 2016; Zhu et al., 2016). Also, a lot of methods were described for simultaneous detection of multi microorganisms such as pathogenic bacteria (Li et al., 2015; Yoo et al., 2015; Vaisocherova-Lisalova et al., 2016), fungal pathogens (Playford et al., 2006; Priyanka et al., 2015; Rahn et al., 2016), and even varied pathogens that belong to different kingdoms (Leber et al., 2016). However, very few reported on how to simultaneously detect small molecular contaminants and microorganisms. In many cases, small molecular contaminants and food-borne microorganisms may simultaneously occur in an identical sample. In this study, we developed a new method for simultaneous detection of aflatoxin and its major fungi in stored maize to demonstrate the potential to simultaneously detect small molecular contaminants and microorganisms.

Aflatoxins are highly toxic, carcinogenic, and mutagenic small molecular contaminants that can not only cause acute or chronic liver diseases but also seriously damage on other tissue organs (Eaton and Gallagher, 1994; Bennett and Klich, 2003). Aflatoxins B_1, B_2, G_1, and G_2 are the most frequent ones in agricultural products and the most toxic member whereby aflatoxin B1 has been classified as group I human carcinogen by the International Agency for Research on Cancer. In addition, main aflatoxigenic species, namely, *A. flavus* and *A. parasiticus* that belong to *Aspergillus* section *Flavi* (Giorni et al., 2007; Varga et al., 2011) are dominant in infection and colonization of agricultural crops (Desjardins, 2003). *A. flavus* is dominant in invading peanuts, corns, and cottons (Klich, 2007), while *A. parasiticus* contaminates broadly on cereals, oilseeds, spices, and nuts (Reddy et al., 2010). The contaminations triggered by *A. flavus* and *A. parasiticus* result in direct negative effects such as a reduction of production, a loss of nutrition and a diminution of market value, and aggravate environmental especially aqueous pollution and also pose serious threats to the health of animals and humans. The pathogenic *Aspergillus* spp. can cause avian aspergillosis and bovine mycotic abortion, and their spores are associated with human hypersensitivity pneumonitis (Gourama and Bullerman, 1995). Contaminations from aflatoxin and its producing molds usually occur concurrently, which increases a serious dangerousness for people's health as well as significantly reduces economic values of the host plants, agricultural products, feeds and/or foods.

Currently, a number of quantitative techniques for aflatoxin determination have been developed, mainly including High-Performance Liquid Chromatography (HPLC), Liquid Chromatography tandem Mass Spectrometry (LC-MS), rapid immune-chromatographic assays (ICA) and enzyme-linked immune sorbent assay (ELISA). Methods for quantifying *Aspergillus* section *Flavi* involved morphological and molecular technologies, the former of which need microbiologists who have a rich morphological knowledge to complete, whereas the latter have been widely used because of features of speediness, sensitivity, and accuracy. The present study developed a new method that realized a simultaneous run of two different types of PCR: (1) Display Mediated Immuno-polymerase Chain Reaction (PD-IPCR), which helps to determine total aflatoxins, and (2) a conventional real-time PCR (RT-PCR), which serves for determination of the main aflatoxin-producing fungi *Aspergillus* section *Flavi* in stored maize. Through the combination of the two PCRs, a new detection platform was developed, which makes it possible to simultaneously detect small molecular contaminants and microorganisms.

MATERIALS AND METHODS

Materials

The standard mycotoxin powders, the surfactants Tween-20, and the enzyme stabilizer bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO, United States). *Escherichia coli* ER2738 competent cells were purchased from Lucigen Corp. (Middleton, WI, United States). The Universal Probes Supermix was supplied by Bio-Rad (Hercules, CA, United States). DNA polymerase (iTaq), Mg^{2+}, dNTPs, 6× loading buffer, and DNA marker were bought from Takara Bio (Beijing, China). All the other reagents used were of analytical grade or better.

The anti-aflatoxins monoclonal antibody 1C11 (mAb 1C11) and V_{2-5} phage displaying nanobody specific for 1C11 were produced by our team (Zhang et al., 2009; Wang et al., 2013b). *A. flavus* strain 3.4408 producing a high level of aflatoxins B_1 and B_2 was used as a standard strain.

Preparation of Phage for Small Molecular Contaminant Detection

V_{2-5} phagemids, specific for mAb 1C11, previously transferred to *E. coli* ER2738 and stored at −70°C, need to be released and amplified from the *E. coli*, which was carried out as described in Lei et al. (2014). Finally, the phage particles were titrated by determining phage-forming unit (pfu) and stored at −20°C as ready-to-use reagents to prepare additional supplies if needed.

Preparation of Reference Gene for Microorganism Detection

Nor-1 gene, catalyzing the transformation from norsolorinic to averantin, is the first key gene in the pathway of aflatoxins synthesis (Trail et al., 1994; Zhou and Linz, 1999). *A. flavus* strain 3.4408 was used to obtain *nor-1* gene. After the inoculation on Czap ekDox Agar (CDA), the fungus was incubated at 28°C and 90% humidity for 7 days, and then the spores were washed down, counted using a hemocytometer counting chamber, and diluted into 50 ml of potato dextrose broth (PDB) to a final concentration of 1×10^5 spores/ml, followed by a shaking at 180 rpm for 96 h at 28°C using a Thermo Scientific MaxQ 4000 shaker (Danville, CA, United States). Finally, the mycelia were washed three times with double-distilled water, filtered through double-filter papers (Whatman #4, Maidstone, United Kingdom), immediately freeze-dried and stored at −70°C prior to DNA extraction.

DNA was extracted using DNeasy Plant Mini Kit (Qiagen, GmbH, Germany) according to the manufacturer's introductions. After DNA extraction, a conventional PCR was performed essentially as described by Geisen (1996). The larger fragments

(400 bp) of *nor-1* gene were generated with primers: nor1-F, 5′-ACCGCTACGCCGGCACTCTCGGCAC-3′ and nor1-R, 5′-GTTGGCCGCCAGCTTCGACACTCCG-3′. Then, these larger fragments were purified using E.Z.N.A.TM Gel Extraction Kit (Omega Bio-Tek, Norcross, GA, United States) according to the manufacturer's protocols. Concentration of the purified products was determined by measuring the absorbance of samples at 260 and 280 nm, using a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, United States), and the number of copies was calculated.

Optimization of the Duplex Real-Time PCR

The primer/probe systems are shown in **Table 1**. Ph-F, -R primers, and Ph-probe were designed according to the corresponding specific DNA sequences encoding anti-idiotypic nanobody (V_{2-5}) (Lei et al., 2014), while Tq-nor1-F, -R primers, and Tq-probe were designed according to the sequences of *nor-1* gene (Mayer et al., 2003a). The probes were labeled with non-fluorescent BHQ1 at the 3′-end and with reporter dyes of FAM or Hex at the 5′-end. Primer Premier 6.0 (Premier Biosoft International, Palo Alto, CA, United States) was used to ensure the compatibility of primers and probes.

A CFX96^{TM} real-time PCR system (Bio-Rad, Hercules, CA, United States) was used to perform the real-time PCR assay. The duplex real-time PCR consisted of two single-plex amplification systems that separately used V_{2-5} phage DNA and *nor-1* gene as templates. After the separate optimization, the two single-plex PCRs were combined to form a duplex real-time PCR, with an additional 0.25–1.0 U DNA polymerase (iTaq), Mg^{+2} (1–2 mM), and dNTPs (200–400 μM).

Parameters for the optimized system were as follows: V_{2-5} phage (2 μl) and *nor-1* (l μl) were mixed with the PCR working solution containing two primer/probe systems (**Table 1**), iTaq Universal Probes Supermix (5 μl), an additional iTaq (0.75 U), $MgCl_2$ (2 mM), and dNTPs (400 μM). Double-distilled water was added to make up the total volume to 10 μl. After an initial denaturation at 95°C for 5 min, 40 cycles were at 95°C for 10 s and 60°C for 30 s. No template control was used to verify the quality of amplification. All the assays were carried out in triplicate.

To evaluate the amplification efficiency (E), V_{2-5} phage particles were diluted in PBS buffer (10 mM sodium phosphate buffer containing 137 mM NaCl and 2.68 mM KCl, pH 7.4) to a series of final concentrations ranging from 10^9 to 10^2 pfu/ml. The reference *nor-1* gene was 10-fold serially diluted in nuclease-free H_2O to final concentrations of 10^8–10^1 copies/μl. Ct values, corresponding to each dilution, were automatically calculated by the instrument. The efficiency was calculated based on: $E = [10^{1/-\text{slope}} - 1] \times 100\%$, by using logarithm of templates as abscissa and Ct values as ordinate to plot amplification calibration curves.

Immunoreaction for Small Molecular Contaminant Detection

A polystyrene microtiter plate (96-well) was coated with 1.0 μg/ml mAb 1C11 at 37°C for 1 h. Then, the plate was washed with PBST [PBS containing 0.05% (v/v) Tween 20] three times and, then, blocked with a buffer [PBST containing 3% (w/v) skimmed milk] at 37°C for 45 min. The plate was washed three times. Then, the mixture (100 μl) containing 50 μl of V_{2-5} phages (1.0×10^{10} pfu/ml) and the same volume of aflatoxins solution were added into each micro-plate well. After the incubation at 37°C for 1 h, the plate was washed with PBST 10 times. Subsequently, the V_{2-5} phages captured by mAb 1C11 at the bottom of the plate were eluted by Glycine/HCl buffer (100 μl, 0.2 M, pH 2.1, containing 1% BSA) at 37°C for 15 min. Then, the eluent containing the released phages was neutralized using 1 M Tris–base buffer (pH 9.1).

Aflatoxins B_1, B_2, G_1, and G_2 occur in natural samples at different ratios (Kensler et al., 2011). According to their frequencies of occurrence in natural samples, standards (B_1: B_2: G_1: G_2 = 1.0: 0.1: 0.3: 0.03, w/w/w/w) used for total aflatoxin determination were prepared. The standard was threefold serially diluted at serial concentrations (33.3 ng/ml to 1.69 pg/ml). After the immunoreaction as described above, the eluted V_{2-5} phages solution (2 μl) was used for the duplex real-time PCR system. The standard curve was constructed by plotting Ct values versus Log total aflatoxin concentrations (Log 10) by four parameter logistic regression.

Isolation of DNA From Maize Samples

Ten grams of maize was finely milled into particles < 500 μm diameter using a laboratory mill. Subsequently, 0.2 g of the powder was precisely weighed, transferred into a nuclease-free tube, and smashed using an automatic fast-grinding apparatus Tissuelyser-48 (Jingxin Science, Shanghai, China), in the presence of 200 μl of CTAB (hexadecyltrimethyl ammonium bromide) buffer [20 g/L CTAB, 0.1 M Tris–HCl (pH = 8.0), 20 mM EDTA (pH = 8.0) and 1.4 M NaCl] and two steel beads (1.5 mm diameter). Then, an additional 1.6 ml of CTAB buffer was added

TABLE 1 | Primer and probe systems used in the duplex real-time PCR system.

Primer/probe	Sequence (5′ to 3′)	T_m (°C)	Amplicon (bp)	Working concentration (μM)	Target gene
Ph-F	GTGGTAGCACAAACTATG	49.5	131	0.3	Phage DNA
Ph-R	GGCTGCACAGTAATAAAC	50.2		0.3	
Ph- probe	FAM-CCGATTCACCATCTCCAGAGACA-BHQ1	58.2		0.4	
Tq-nor1-F	GTCCAAGCAACAGGCCAAGT	57.4	66	0.2	*Nor-1* gene
Tq-nor1-R	TCGTGCATGTTGGTGATGGT	55.4		0.2	
Tq-probe	HEX-TGTCTTGATCGGCGCCCG-BHQ1	62.2		0.3	

into the tube and immediately incubated in a water bath at 63°C for 2 h. Then, 1 volume of phenol:chloroform:isoamyl alcohol (v:v:v, 25:24:1) was added, gently mixed, and centrifuged at 15,000 *g* for 10 min. After the recovery of the aqueous phase, 1 volume of chloroform:isoamyl alcohol (v:v = 24:1) was added, homogenized, and centrifuged again. The aqueous phase was recovered and 1 volume of chloroform was added. After the centrifugation at 15,000 *g* for 10 min, the aqueous phase was recovered again, followed by an addition of 0.6 volume of isopropyl alcohol (pre-cooled at −20°C for 2 h). After the centrifugation at 15,000 *g* for 15 min, the aqueous phase was discarded. The DNA was cleaned with 70% alcohol, suspended in 70 μl of nuclease-free H_2O, and stored at −20°C.

To generate a standard curve for *Aspergillus* section *Flavi* determination, 0.2 g of the finely milled blank-maize powder was precisely weighed, transferred into a nuclease-free tube, and inoculated with 200-μl spores (obtained from 6-day-old *A. flavus* strain 3.4408) to final concentrations of 8×10^2 to 8×10^8 spores/g. After incubation at 28°C for 1 h, samples were used to extract DNA as described above. DNA products (l μl) were used as templates for the duplex real-time PCR system. Log spores/g was used as abscissa and the corresponding Ct values were used as ordinate to plot the standard curve.

Samples Analysis and Validation

The blank maize samples were purchased at a local market and verified as blank using HPLC and conventional plating counts. The naturally contaminated maize samples ($n = 25$) were gathered from Shandong province of China. Samples (10 g) were finely milled into particles < 500 μm diameter, 0.2 g of which was used to extract DNA for *Aspergillus* section *Flavi* determination. For aflatoxin extraction, 5.0 g of the milled samples was treated with 15 ml of methanol:water (70:30, v/v) under a shaking condition at 250 rpm for 1 h. After a centrifugation (5000 *g* for 10 min at 4°C), samples were filtered with double-filter papers (Whatman #4, Maidstone, United Kingdom) and diluted sevenfold with 4% BSA/PBS (w/v). Dilutions were used directly in the Section "Immunoreaction for Small Molecular Contaminant Detection." After the immunoreaction and DNA extraction, the eluted phages DNA and DNA products extracted from maize samples were amplified simultaneously in the optimized duplex real-time PCR system. Ct values were associated to standard curves to calculate concentrations of aflatoxins and aflatoxigenic fungi.

A validation involved testing of 25 naturally contaminated maize samples, using the newly developed method and the gold standard reference methods (HPLC and conventional plating counts) in parallel. HPLC was carried out as described in Ren et al. (2019). For determination of *Aspergillus* section *Flavi* density by conventional plating counts, colony-forming units (CFUs) were determined using dichloran rose bengal chloramphenicol agar (DRBC) supplemented with 1% NaCl (Passone et al., 2010).

Statistical Analysis

For aflatoxin determination, IC_{10}, IC_{50} (half-maximal inhibition), and IC_{20} - IC_{80} were used to calculate the limit of detection (LOD), sensitivity and linear range, respectively. The statistical analysis and plotting were performed using Microsoft Excel 2007 and OriginPro 9.0 (OriginLab Corporation, Northampton, MA, United States). To assess matrix effects, data were compared according to Student *t*-test using Graph PadInstat 3.0 (GraphPad Software, San Diego, CA, United States).

RESULTS AND DISCUSSION

Optimization of the Duplex Real-Time PCR

At first, two single-plex real-time PCR systems were directly combined without any further optimization. As shown in **Figure 1A**, Ct values corresponding to V_{2-5} phages were much higher, which indicated that the amplification of phage DNA was extremely inhibited, whereas the additional Mg^{+2}, dNTPs (**Figure 1B**), and DNA polymerase (iTaq) (**Figure 1C**) enhanced phage DNA amplification with significantly lower Ct values. Principles defining optimal parameters were intended to ensure that positive Ct values are lower, because the lower Ct values ensured a wider linear range and a lower limit of the detection. Thus, the additional 2 mM $MgCl_2$, 400 μM dNTPs, and 0.75 U iTaq were selected as the optimal conditions. These results suggest that insufficient DNA polymerase, Mg^{+2}, and/or nucleotides can inhibit the amplification of low-abundance templates, which was in accordance with the conclusion of Svec et al. (2015).

Efficiency Assessment of the Duplex Real-Time PCR

Amplification data are shown in **Figures 2A,C**. For V_{2-5} phage and reference *nor-1* gene, the resulting slopes for linear fit were −3.37 (**Figure 2B**) and −3.56 (**Figure 2D**), respectively. Thus, amplification efficiencies were calculated as 98 and 91%, with the lowest detectable concentrations of 10^3 pfu/ml V_{2-5} and 10^2 copies/μl *nor-1*, indicating that the optimized duplex real-time PCR was accurate enough for simultaneous quantification of the both targets.

Matrix Effect, Sensitivity, and Specificity for Total Aflatoxin Determination

To assess matrix effects on total aflatoxin determination, 5 g of blank maize samples were treated with 15 ml of methanol/PBS (70:30, v/v) under a shaking condition (250 rpm for 1 h), centrifuged at 5000 *g* for 10 min, and filtered through double-filter paper, and then the supernatants were diluted sevenfold with distilled-water. Subsequently, total aflatoxin standard was diluted into 10% methanol/PBS (10:90, v/v) buffer and the dilutions of maize extracts to a final concentration of 33.3 ng/ml to 1.69 pg/ml. Maximal and minimal Ct values were obtained at 33.3 ng/ml and 1.69 pg/ml, respectively. As shown in **Figure 3A**, ΔCt (=Maximal Ct - Minimal Ct) had a significant difference ($P < 0.001$, according to the Student *t*-test) between 10% methanol/PBS buffer (ΔCt = 10.8) and maize extracts

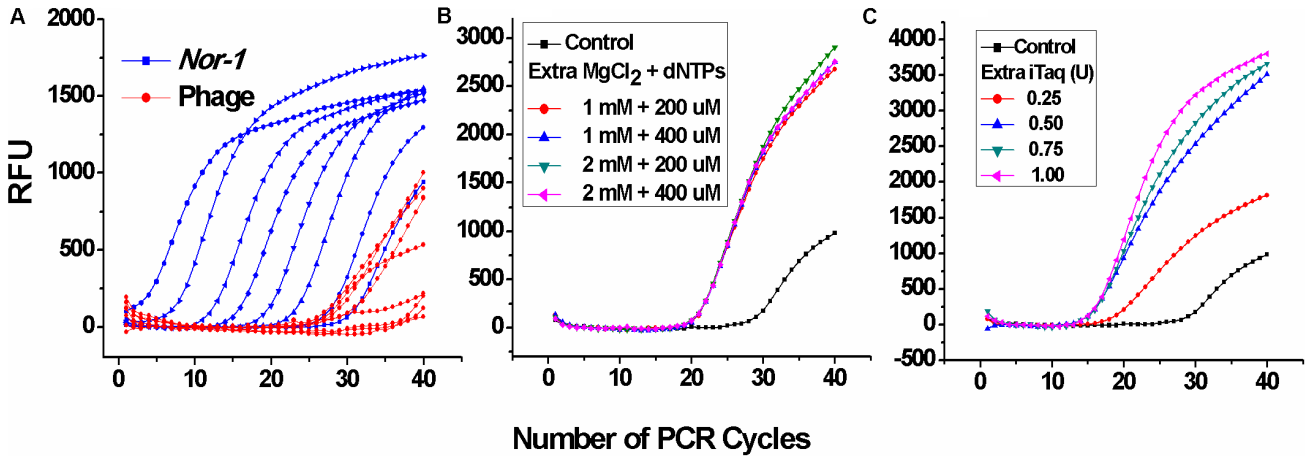

FIGURE 1 | Optimization of iTaq (DNA polymerase), dNTPs, and Mg^{2+} for the duplex real-time PCR. **(A)** Amplification data of the duplex real-time PCR system directly combined by two single-plex systems without any extra reagents. RFU means relative fluorescence units. **(B)** Amplification data of V_{2-5} phage at different concentrations of additional $MgCl_2$ and dNTPs and **(C)** DNA polymerase (iTaq).

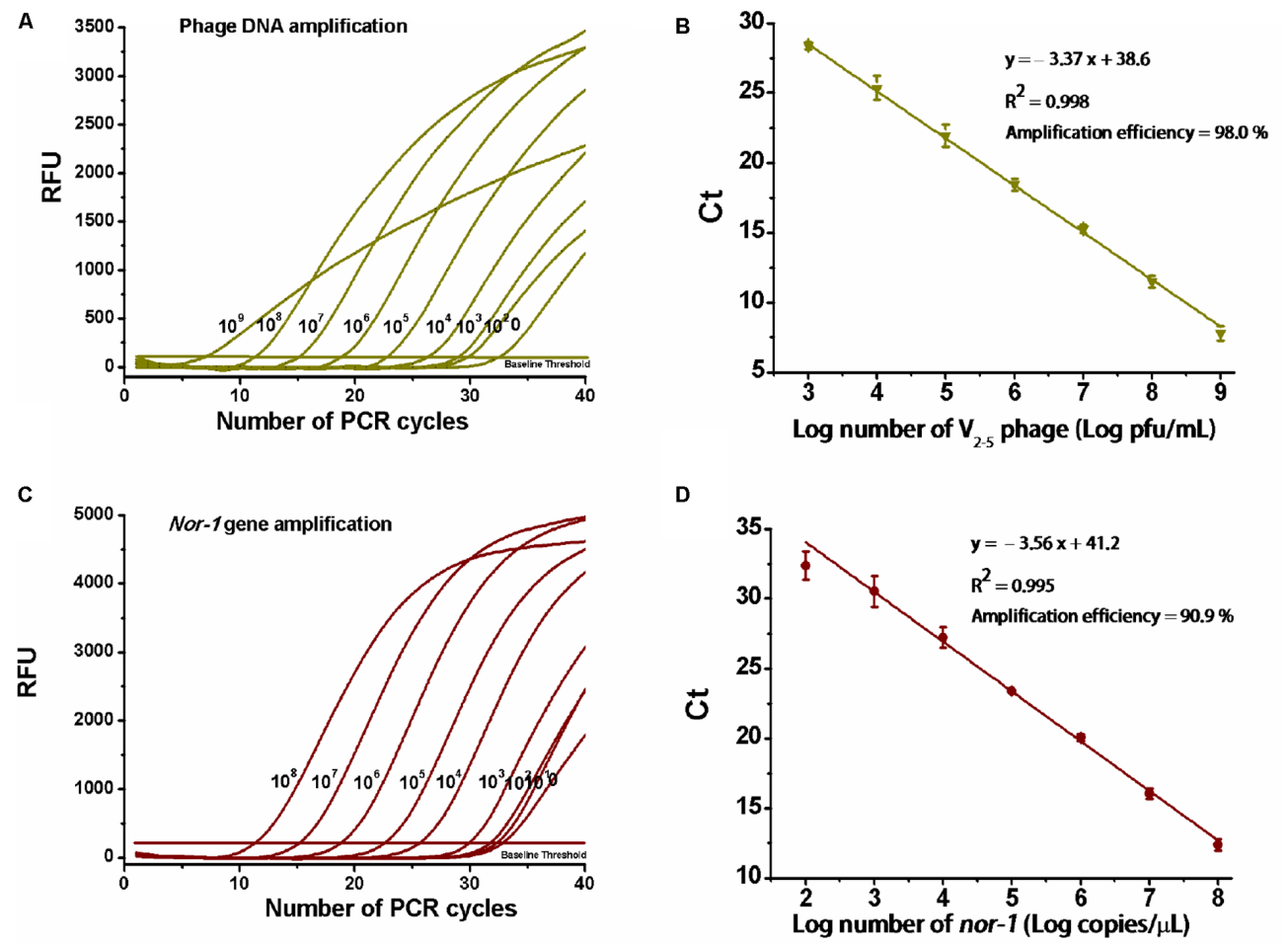

FIGURE 2 | Determination of amplification efficiency of the real-time PCR. **(A)** PCR amplification for 10-fold serial dilutions of V_{2-5} phage particles (10^9,...10^8,...10^7,...10^6,...10^5,...10^4,...10^3,...10^2 pfu/ml) and **(C)** reference *nor-1* gene (10^8,...10^7,...10^6,...10^5,...10^4,...10^3,...10^2,...10^1 copies/μl). RFU means relative fluorescence units. **(B)** Standard curve of amplification efficiency for V_{2-5} phage and **(D)** reference *nor-1* gene. Each data point is the average of three independent measurements.

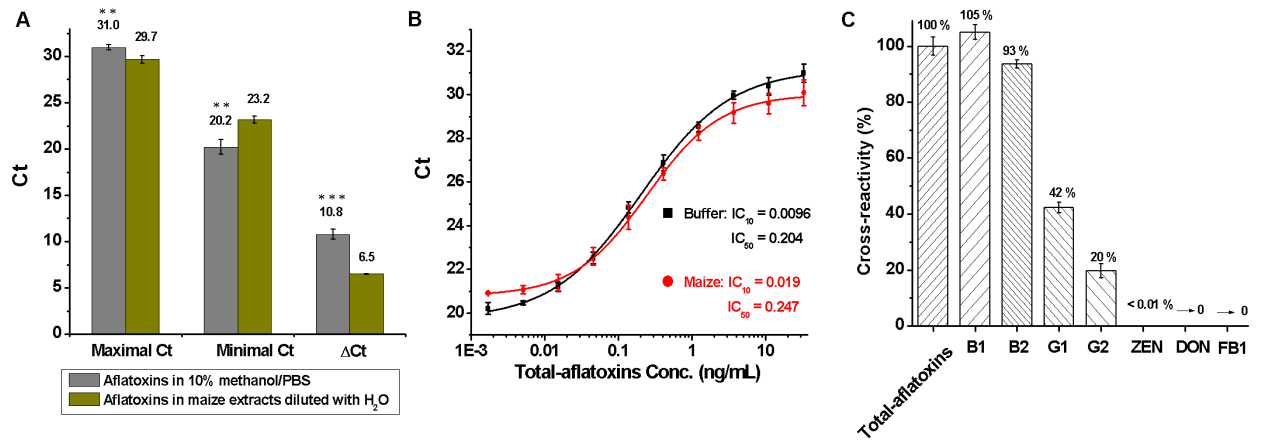

FIGURE 3 | Evaluation of the duplex real-time PCR for total aflatoxins detection. **(A)** Analysis of matrix effects, by comparing the difference of maximal, minimal, and ΔCt values between 10% methanol/PBS buffer and maize extracts that diluted with water; $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ according to the Student *t*-test. **(B)** Standard curves for total aflatoxins in 10% methanol/PBS buffer (black) and maize extracts (red) after the elimination of matrix interference with 4% BSA/PBS (w/v); conc. is the abbreviation for concentration. **(C)** Cross-reactivity (CR) for mycotoxins including total aflatoxins, B_1, B_2, G_1, G_2, zearalenone (ZEN), deoxynivalenol (DON), and fumonisin B_1 (FB_1).

(ΔCt = 6.5), meaning maize matrix had a significant effect on total aflatoxin detection.

To eliminate matrix effects, the maize extracts were diluted sevenfold with 4% BSA/PBS (w/v), which essentially eliminated the matrix interference. Standard curves for total aflatoxins in 10% methanol/PBS buffer and maize extracts that were diluted with BSA/PBS (w/v) are shown in **Figure 3B**. The LOD, sensitivity, and linear range of the method for total aflatoxins in maize were 0.02, 0.25, and 0.05–1.21 ng/ml, respectively. The LOD was much lower than that of immune-chromatographic assays (Li et al., 2013), immunochip (Wang et al., 2012), and HPLC methods (Khayoon et al., 2010) reported previously.

During assessment of specificity, the cross-reactivity (CR) for common mycotoxins was tested and calculated as: % CR = ($IC_{50Total\ aflatoxins}$ /$IC_{50analyte}$) × 100. As shown in **Figure 3C**, higher cross-reactivity against total aflatoxins (100%) and aflatoxins B_1 (105%) and B_2 (93%), lower cross-reactivity toward aflatoxins G_1 (42%) and G_2 (20%), and no cross-reactivity with zearalenone (ZEN), deoxynivalenol (DON), and fumonisin B_1 (FB_1) were obtained, indicating that the method was specific for aflatoxins B_1, B_2, G_1, and G_2.

Matrix Effect, Sensitivity, and Specificity for *Aspergillus* Section *Flavi* Determination

On assessment of matrix effects on *Aspergillus* section *Flavi* determination, spores were diluted in water or inoculated in maize to serial concentrations of 8×10^2 to 8×10^8 spores/ml or spores/g. As shown in **Figure 4A**, maximal, minimal, and ΔCt values had no differences between spores inoculated in maize and in water, indicating no matrix effects. Due to the complexity of food samples, food matrices-associated inhibitors such as protein, polysaccharide, and oleic acid usually interfere with the activities of enzymes and, subsequently, reduce the detection sensitivity (Wilson, 1997; Hanna et al., 2005). Fortunately, no matrix inhibition was discovered in this experiment, probably because of the use of phenol and chloroform during the extraction of DNA, which could not only remove proteins but also eliminate other matrix inhibitors.

Amplification data of *A. flavus* spores that were 10-fold serially diluted in maize are shown in **Figure 4B**. The standard curve for mold detection is shown in **Figure 4C**. A good linear relationship between Ct values and spore numbers was obtained, with detective standard curve: $y = -2.03x + 38.8$ and $R^2 = 0.98$. As shown in **Figures 4B,C**, the linear range for *A. flavus* detection was 8×10^2 to 8×10^8 spores/g, with the lowest detectable concentration of 8×10^2 spores/g.

The specificity of the primer/probe set of *nor-1* has been already demonstrated, using the purified genomic DNA of different food-related fungi (Mayer et al., 2003a; Iheanacho et al., 2014). Their studies showed that *A. flavus* and *A. parasiticus* gave positive results, whereas other tested strains such as different *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp. gave negative results. In our study, the specificity was also tested using strains commonly occurred in maize. As expected, *A. parasiticus* ($n = 3$) and *A. flavus* ($n = 4$, including two aflatoxin non-producing strains) gave PCR amplifications similar to that of *A. flavus* strain 3.4408 (data not shown), indicating that the new method could detect *A. parasiticus* and *A. flavus* including aflatoxin producing and non-producing strains, whereas no PCR amplifications were detected for the other tested strains (*Aspergillus niger*, *Aspergillus nidulans*, *Penicillium oxalicum*, *Fusarium moniliforme*, *Fusarium nivale*, *Alternaria alternate*, *Trichoderma harzianum*, and *Rhizopus nigricans*).

Regarding *nor-1* gene as a biomarker for *A. flavus* and *A. parasiticus* detection has been demonstrated for several times. Mayer et al. (2003b) demonstrated that the tendency of *nor-1* gene copies was the same as that of *A. flavus* CFUs in wheat with the prolonged incubation time (Mayer et al., 2003b).

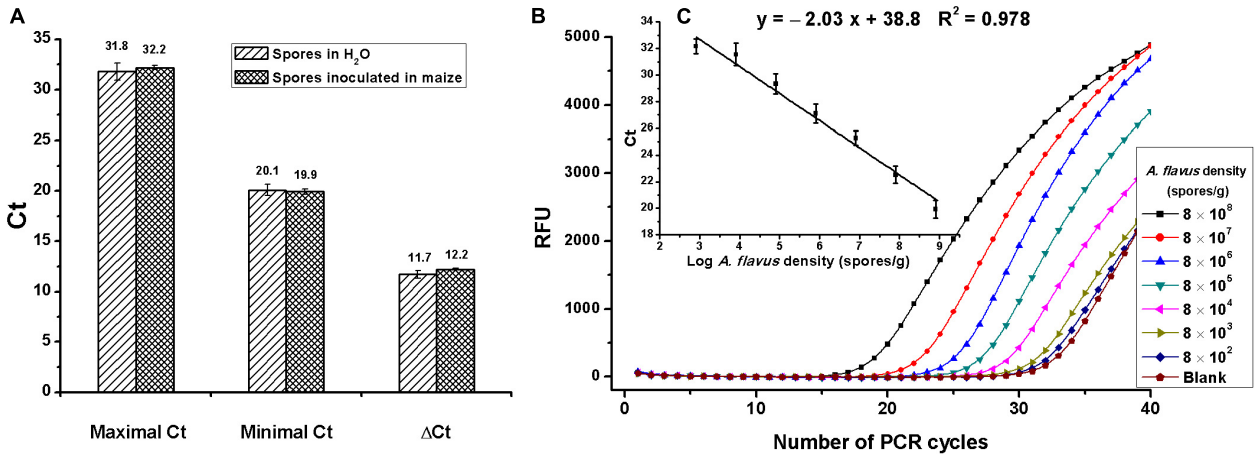

FIGURE 4 | Evaluation of the duplex real-time PCR for *Aspergillus* section *Flavi* determination. **(A)** Analysis of matrix effects, by comparing the difference of maximal, minimal, and ΔCt values between assays that spores diluted in water or inoculated in maize; no difference was found, according to the Student *t*-test. **(B)** Amplification data for serial concentrations of *A. flavus* spores inoculated in maize (8×10^8,...8×10^7,...8×10^6,...8×10^5,...8×10^4,...8×10^3,...8×10^2 spores/g). RFU means relative fluorescence units. **(C)** Standard curves constructed for *Aspergillus* section *Flavi* detection in maize with detection linear range of 8×10^2 to 8×10^8 spores/g.

Additionally, *nor-1* copies were demonstrated to be correlated to CFUs of *A. flavus* in pepper, maize, and paprika (Bagnara et al., 2000; Mayer et al., 2003a). Passone et al. (2010) also developed an analytical method determining *Aspergillus* section *Flavi* based on *nor-1* gene and demonstrated a good correlation ($r = 0.613$; $P < 0.0001$) between *nor-1* copies and CFUs in naturally stored peanut. These results indicated that the PCR system based on *nor-1* gene was specific and accurate for *A. flavus* and *A. parasiticus* determination, which was in accordance with our finding.

At present, some other methods based on PCRs have also been established to detect aflatoxigenic fungi in agricultural products. For example, an analytical method determining CFU values of *Aspergillus* section *Flavi* in stored peanut samples was established, with a detection linear range of 2.5×10^3 to 10^7 cfu/g (Passone et al., 2010), a lower sensitivity compared with that of our method. The method based on omt-1 gene was also proposed to quantify aflatoxin-producing molds, over the range 4 to 1 log cfu/g (Rodriguez et al., 2012). Since mycelial fragments consist of many multinucleate cells (Jennings and Lysek, 1996; Kaminskyj and Hamer, 1998), but give only one colony in a plate, CFU values could not mirror the real density of *Aspergillus* section *Flavi* in samples. Thus, our new method, based on the detection of spores, was more sensitive and accurate.

Recovery of Total Aflatoxins and *A. flavus* Spores

To test the recovery, blank maize samples (10 g) were spiked with total aflatoxin standard (10, 100, and 200 μg/kg) and simultaneously inoculated with fresh spores of *A. flavus* 3.4408 (3, 5, and 8 Log spores/g). Assays were carried out in triplicate on the same day for intra-assay precision evaluation and in five different days for inter-assay precision evaluation. Recoveries for aflatoxins and *A. flavus* spores were 84–111% and 94–107%, respectively, with variable coefficients (CVs) of 0.47–11.2% (**Table 2**), indicating a good repeatability and reproducibility of the method.

Validation

The testing results of 25 natural samples and correlations of the results obtained by different methods are shown in **Table 3** and **Figure 5**. For total aflatoxin determination, results of the new method and HPLC had a good correlation, with a linear regression equation: $y = 0.97x - 4.31$ and $R^2 = 0.99$; for *Aspergillus* section *Flavi*, validation results were also in good

TABLE 2 | Recovery of total aflatoxins and *A. flavus* in maize by the duplex real-time PCR analysis.

Assay	Analyte	Spiked level	Recovered level ± SD	Recovery (%)	CV (%)
Intra-assay	Total aflatoxins	10	8.84 ± 0.30	88.4	3.43
(n = 3)[a]	(μg/kg)	100	92.1 ± 6.12	92.1	6.65
		200	206 ± 5.50	103	2.67
	A. flavus	3	2.83 ± 0.24	94.4	8.59
	(log spores/g)	5	5.10 ± 0.36	102	6.70
		8	8.57 ± 0.37	107	4.29
Interassay	Total aflatoxins	10	8.39 ± 0.04	83.9	0.47
(n = 5)[b]	(μg/kg)	100	43.9 ± 1.29	87.8	2.94
		200	111 ± 3.57	111	3.23
	A. flavus	3	3.04 ± 0.34	101	11.2
	(log spores/g)	5	4.89 ± 0.30	97.8	6.13
		8	8.15 ± 0.38	102	4.60

[a]*Each assay was carried out in triplicate on the same day.* [b]*The interassay was carried out in five different days.*

TABLE 3 | Comparison of results obtained by the duplex real-time PCR and referential methods for total aflatoxins and *Aspergillus* section *Flavi* detection in naturally contaminated maize.

Maize sample	Total aflatoxins concentration		*Aspergillus* section *Flavi* density	
	HPLC (ng/ml)	Duplex RT-PCR (ng/ml)	Plating counts (Log cfu/g)	Duplex RT-PCR (Log spores/g)
1	[a]ND	0.53	ND	ND
2	ND	ND	2.67	3.63
3	ND	ND	ND	ND
4	ND	ND	2.23	3.29
5	ND	0.45	3.00	3.76
6	114	106	6.28	6.75
7	53.6	46.2	6.94	7.33
8	198	185	6.98	7.55
9	111	110	7.01	7.66
10	32.0	25.9	6.86	7.61
11	57.2	47.4	6.88	7.66
12	70.7	66.7	6.88	7.58
13	177	169	7.12	8.29
14	143	127	7.15	8.25
15	651	640	7.19	8.28
16	241	233	6.51	7.03
17	269	237	6.73	7.10
18	261	241	7.02	7.73
19	308	288	6.93	7.22
20	337	321	7.38	8.65
21	318	314	6.61	7.25
22	5.19	6.13	4.74	5.74
23	36.2	38.2	5.10	5.90
24	556	524	5.67	6.39
25	980	965	7.25	8.40

[a]*ND, not detectable. All assays were carried out in five replicates.*

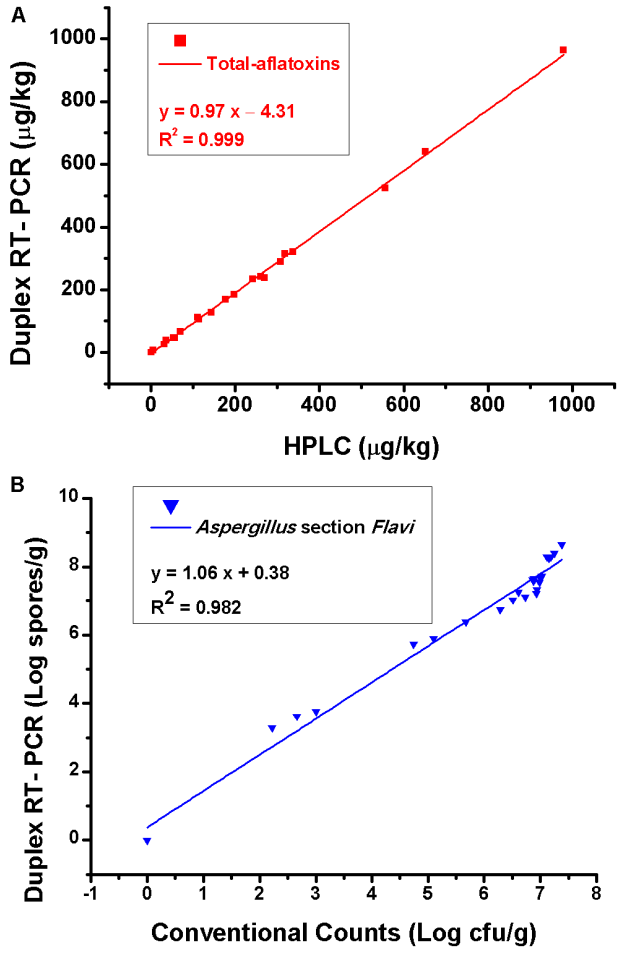

FIGURE 5 | (A) Correlation of total aflatoxins results obtained by the duplex real-time PCR and HPLC and **(B)** results of *Aspergillus* section *Flavi* obtained by the duplex real-time PCR and conventional plating counts. Each data point is the average of five independent measurements.

agreement, with a linear regression equation: $y = 1.06x + 0.38$ and $R^2 = 0.98$ (**Figure 5**).

Application Prospect

According to the sample analysis protocol, aflatoxins in maize samples were 21-fold diluted, meaning the LOD, sensitivity, and linear range for total aflatoxin detection in maize were 0.42, 5.25, and 1.05–25.41 μg/kg, respectively, and linear range for *Aspergillus* section *Flavi* detection was 8×10^2 to 8×10^8 spores/g. Additionally, approximately 2 h was needed for samples preparation, 4 h for the Section "Immunoreaction for Small Molecular Contaminant Detection," 4 h for the Section "Isolation of DNA from Maize Samples," and 1 h for the analysis using real-time PCR instrument. Therefore, approximately 11 h was enough for the whole detection period.

With all of the above performance, this newly developed method was a good demonstration for simultaneous detection of small molecular contaminants and microorganisms in agro-foods. Generally, if nanobody phages specific for small molecular contaminants are available, the simultaneous detection would become not a challenge. Currently, nanobody phages specific for various contaminants such as zearalenone (Wang et al., 2016), ochratoxin A (Liu et al., 2014), deoxynivalenol (Tu et al., 2012), fumonisin B_1 (Shu et al., 2019), synthetic micro-organics (Wang et al., 2013a; Hua et al., 2015; Ding et al., 2017), citrinin (CIT) (Xu et al., 2015), and microcystins (MCs) (Xu et al., 2018) are available. Therefore, using the new method developed here, the simultaneous detection for these small molecular contaminants and their related microorganisms could also be realized.

CONCLUSION

In order to provide an analytical technology to detect small molecular contaminants and microorganisms, the simultaneous detection of aflatoxins and its major fungi (*Aspergillus flavus* and *A. parasiticus*) in maize was developed as an example to demonstrate it. The entire process for the simultaneous detection requires less than 1 day, thus time saving compared

with separate detections. Importantly, this technical platform not only achieved the goal of simultaneous quantifications but also satisfied technical features of high throughput, high sensitivity, and wider linear range. However, the tedious technical procedure might be considered as inefficiency at current stage, especially on the procedure of the DNA isolation. Therefore, simplifying protocols for samples preparation are necessary to be explored, further to save time and improve work efficiency. Overall, this detection platform had a great potential for simultaneous detection of small molecular contaminants and microorganisms, which could, in a significant measure, advance new ideas for the development of detection technologies.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

PL and QZ conceived the research and acquired the funding. QZ, PL, and XR designed the experiments and analyzed the data. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Occurrence of Aflatoxin M1 in Raw Milk Marketed in Italy: Exposure Assessment and Risk Characterization

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The current study is based on the AFM_1 contamination of milk determined from April 2013 to December 2018 in the framework of a self-control plan of six milk processing plants in Italy. These data – together with the consumption data of milk consumers – were evaluated and used for the calculation of the Estimated Daily Intake (EDI), the Hazard Index (HI), and the fraction of hepatocarcinoma cases (HCC) due to AFM_1 exposure in different population groups. Altogether a total of 31,702 milk samples were analyzed, representing 556,413 tons of milk, which is an outstanding amount compared to published studies. The results indicate the monthly fluctuation of AFM_1 levels through a period of nearly 6 years. The EDI of AFM_1 in different population groups was in the range of 0.025–0.328 ng kg^{-1} body weight (bw) per day, based on the average consumption levels and weighted mean contamination of the milk in the study period. Considering average consumptions, in the groups of infants and toddlers, the HI calculation resulted in 1.64 and 1.4, respectively, while for older age groups, it was <1. The estimated fractions of HCC incidences attributable to the AFM_1 intakes were 0.005 and 0.004 cases per 100,000 individuals in the 0–0.9 and 1–2.9-year age groups, respectively, and below 0.004 cases in the other age categories. The monthly average AFM_1 contamination of tested milk consignments ranged between 7.19 and 22.53 ng kg^{-1}. Although the results of this extensive investigation showed a low risk of HCC, the variability of climatic conditions throughout years that influence AFB_1 contamination of feed and consequently AFM_1 contamination of milk justifies their continuous monitoring and update of the risk assessment.

Keywords: Aflatoxin M1, enzyme-linked immunosorbent assay, cow's milk, raw milk, exposure assessment, food safety risk

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INTRODUCTION

Aflatoxins (AFs) are secondary metabolites produced by *Aspergillus flavus*, *Aspergillus parasiticus,* and *Aspergillus nominus* fungi under certain growing and storage conditions (WHO, 1997; Giorni et al., 2007). The AFs consisted of Aflatoxin B_1, B_2, G_1, and G_2 may contaminate food and feed. Maize grains and other feedstuffs such as corn silage, soybean, and press cakes from

oil plants can be commonly contaminated by *Aspergillus* spp. The critical factors facilitating the growth of Aflatoxin-producing molds in corn grains and silage include among others: lack of good agricultural (Kebede et al., 2012), storage practices, and unfavorable climatic conditions (FAO/WHO Codex Alimentarius, 2014; Frazzoli et al., 2017). The risk of Aflatoxin contamination is generally higher in geographical regions with a tropical climate or a subtropical climate (Fakhri et al., 2019a), but an extreme hot and droughty season may promote the growth of *Aspergillus* spp. in crops and increases their AF contamination as reported in the South and Southwestern regions of Europe (Trevisani et al., 2014; Milićević et al., 2017; Udovicki et al., 2019), the United States (Fakhri et al., 2019a), Turkey (Madali et al., 2018), and in other regions (Rama et al., 2015; Rahmani et al., 2018; Pardakhti and Maleki, 2019). The effects of such conditions on the Aflatoxin contamination of maize prevailed in 2003 and 2012 in the Po valley were evaluated in detail by Canever et al. (2004) and Marchetti et al. (2013).

AFM_1 contamination in milk was also reported from a number of countries (EFSA, 2004; Cano-Sancho et al., 2013; Duarte et al., 2013; Tsakiris et al., 2013; Trevisani et al., 2014; Fakhri et al., 2019a,b). In Italy, due to its climatic conditions, the Po valley is considered one of the highest risk areas in this regard, which happens to be the region that produces most of the milk in the country (Frazzoli et al., 2017). Several factors may affect the AFM_1 contamination of milk, for example, environmental conditions (Giorni et al., 2007; Prandini et al., 2009; Kebede et al., 2012; Miliĉeviĉ et al., 2019; Fakhri et al., 2019a), different farming and feeding practices, and the quality and safety control system of the food business operators concordant with the different legislations in force.

The mother's milk may also contain AFM_1 in comparable concentrations to the dairy cow's milk (Kunter et al., 2017; Radonić et al., 2017; Bogalho et al., 2018; Valitutti et al., 2018; Fakhri et al., 2019a,b).

These conditions justify the increased activity in Italy in the field of basic research (Perrone et al., 2014), biological control (e.g., use of non-aflatoxin-producing strains; Mauro et al., 2014, 2018), monitoring of Aflatoxin levels throughout the milk value chain (Anfossi et al., 2011; Kerekes et al., 2016), development and application of different prevention and intervention procedures (Gallo and Masoero, 2010), analytical methods, and validation protocols for the detection of Aflatoxins (Rosi et al., 2007; Bellio et al., 2016).

Abbreviations: AFB1, Aflatoxin B1; 95% CI, 95% confidence intervals; AFM_1, Aflatoxin M1; AQM, Average quality milk (normal and high quality altogether); $BMDL_{10}$, Benchmark dose lower confidence limit for a 10% response; bw, Body weight; EC, European Commission; EDI, Estimated Daily Intake; EFTA, European Free Trade Association; EFSA, European Food Safety Authority; ELISA, Enzyme-linked immunosorbent assay; EU, European Union; FAO, Food and Agriculture Organization of the United Nations; HBV, Hepatitis B virus; HCC, Hepatocellular carcinoma; HI, Hazard Index; HPLC, High Performance Liquid Chromatography; HQM, High quality milk; JECFA, Joint FAO/WHO Expert Committee on Food Additives; LCI, Liver cancer incidence; LCL, Lower confidence limit; MoE, Margin of Exposure; NIST, National Institute of Standards and Technology; NQM, Normal quality milk; OM, Organic milk; SD, Standard deviation; TD50, Dose causing 50% of the animals developing tumor; TDI, Tolerable daily intake; UCL, Upper confidence limit; WHO, World Health Organization; WM, Weighted mean.

If ruminants are fed with contaminated feed, the Aflatoxin B_1 consumed by the animals is partly degraded by the forestomach before reaching the circulatory system. The remaining part is transformed by the liver into monohydroxy derivative forms: mainly AFM_1, and in smaller quantities AFM_2, AFM_4, and other metabolites such as aflatoxicol. Afterward, it is being secreted into the milk through the mammary glands (Frazzoli et al., 2017). In dairy cows, the excretion takes 12–24 h after AFB_1 intake, and the depuration interval is about 2–3 days after the animals are fed with AFB_1-free feed. The excreted amount of toxin through milk varies between 1 and 6% of ingested AFB_1, depending on the variety of dairy cows and the amount of produced milk. The high-yielding breeds have higher carry-over rate (Tsakiris et al., 2013).

The exposure to Aflatoxins – and other mycotoxins – compromises the health of animals and humans as well (Kunter et al., 2017). The International Agency for Research on Cancer (2002) classified AFB_1 to Group 1 of carcinogenic substances for humans. Therefore, no tolerable daily intake (TDI ng AFB_1 kg^{-1} bw day^{-1}) could be set for this substance, and the exposure levels should be kept as low as reasonably achievable. AFM_1 has 2–10% of the carcinogenic potency of AFB_1 but has the same liver toxicity (Hsieh et al., 1984; Cullen et al., 1987).

Milk is a very important food that provides macro- and micronutrients for the growth and development of the body and for the maintenance of human health, but its AFM_1 contamination may impose health risk for the consumers. AFM_1 is heat stable and processing, and storage conditions are ineffective in reducing the concentration of AFM_1 in milk and milk products (Joint FAO/WHO Expert Committee on Food Additives, 2001; Campagnollo et al., 2016).

The presence of AFM_1 in milk and milk products, even in small quantities, represents a concern, mainly because these products are widely consumed by children who are more susceptible to the toxic effects of Aflatoxins, due to their underdeveloped metabolic and immune system (Gonzales-Osnaya et al., 2008; Kunter et al., 2017; Fakhri et al., 2019a).

In view of its hepatotoxicity and potential carcinogenicity, the regulatory agencies established maximum permissible levels for AFM_1 in milk ranging from 10 to 500 ng kg^{-1} (FAO/WHO Codex Alimentarius, 1995; European Community, 2006; USA Guidance levels; Bogalho et al., 2018) following the principle of "As low as reasonably achievable" (ALARA), taking into account the inevitable Aflatoxin contamination of feed.

Quantitative exposure assessment is a methodology developed to evaluate the probable intake of chemical substances *via* food. Aflatoxins are genotoxic and carcinogenic; therefore, there is no intake level, which can be considered risk free (EFSA Scientific Committee, 2007). The safe dose proposed by Kuiper-Goodman (1990) was derived from the dose causing 50% of the animals developing tumor (TD_{50}) divided by a safety factor of 50,000. The suggested value is 0.2 ng kg^{-1} of body weight, which was derived from extrapolation to a risk level of 1:100,000. The risk from exposure to genotoxic and carcinogenic substances found in food and feed can be characterized by the margin of exposure (MoE). The MoE provides an indication of the level of safety concern about

a contaminant's presence in food, but it does not quantify the risk as such. As stated by EFSA Scientific Committee (2012), if it is based on the $BMDL_{10}$ from an animal study, a margin of exposure of 10,000 or higher (in view of uncertainties) considered being of low concern from a public health point of view. Risk characterization, based on the estimated human exposure and available toxicological reference values, provides important information for risk managers on the probability of occurrence and severity of potential adverse health effects to implement appropriate control measures for assuring the safety of food (Leblanc et al., 2005).

The objectives of this study were to evaluate the annual and monthly fluctuation of AFM_1 contamination of milk over a period of 5.5 years, the human exposure, and the potential risk of consumers in different age categories based on the vast amount of AFM_1 contamination data in milk representing a significant proportion produced and marketed in Italy during the study period, and use these results to justify the need for continuous monitoring of AFM_1 contamination in milk.

MATERIALS AND METHODS

To provide baseline data for future evaluation of the change in AFM_1 contamination, the milk collected in six dairy plants from April 2013 to December 2018 in the framework of a self-control plan of the Italian dairy industry is investigated. The milk processing plants, located in Northern, Central, and Southern Italy, collected about 465 million liters of milk per year. Five of them applied the same self-control plan using 40 ng kg^{-1} AFM_1 concentration as action limit (AL), while one plant used a 30 ng kg^{-1} AL. When the AFM_1 concentration of the sample reached the AL, the dairy farms were informed, and corrective measures were applied on the farm level in order to avoid high contamination of the milk. The milk was collected from about 650 dairy farms. The routing of the trucks covering diverse number of dairy farms – depending on the amount of milk produced by each farm – was decided on the basis of logistic optimization. Three types of milk were collected: (1) high quality milk (HQM); (2) normal quality milk (NQM); and (3) organic milk (OM). In case of the truck collected milk from different farms, the milk of the same type was mixed, but the three types of milk (HQM, NQM, and OM) were loaded in different compartments of the truck.

Description of the Self-Control Plan

The self-control plan applied for the control of AFM_1 content starts with sampling of the milk of the truck before unloading its content. If trucks contained different types of milk, the personnel of the milk processing plants collected one sample from each type of milk during the discharge of the tanker. All samples were analyzed immediately by a rapid commercial immunochromatographic test (Charm MRLAFMQ® Charm Science INC, Lawrence, MA, USA) utilizing highly specific reactions between antibodies and AFM_1. It detects AFM_1 at or above 25 ng kg^{-1} in milk and suitable to indicate the compliance with EU ML of 50 ng kg^{-1}. To obtain quantitative data for the AFM_1 as part of a separate program, different milk batches of each collecting zone were also sampled and analyzed at least twice a month with an ELISA kit (Immunoscreen AFM_1, Tecna s.r.l., Trieste, Italy), which was validated within the range of 2.5–100 ng L^{-1} giving linear response up to 80 ng L^{-1} (Rosi et al., 2007). Note that the AFM_1 contamination was reported in some cases from 1 ng kg^{-1}, which is the limit of detection of the ELISA method applied. The ISO (1998) HPLC-FD reference method (LOQ: 8 ng L^{-1}, linearity 3–1,000 ng L^{-1}) was used for confirmation of values higher than 50 ng kg^{-1}. The procedures were performed by the dairy plants as described by Rosi et al. (2007). The performance characteristics of the methods were regularly tested by the plants and periodically verified by the official inspectors according to the HACCP plan of the industries. No further validations of the methods were carried out.

After confirmation that the AFM_1 concentration exceeded the legal limit, the competent authority was informed in accordance with the Italian law (Ministero della Salute, 2013). The plants did not process milk with AFM_1 content higher than 50 ng kg^{-1}. In view of the inevitable uncertainty of detection with CHARM test and the biweekly frequency of analyses with ELISA tests for obtaining the possible most realistic information on the exposure levels, the AFM_1 content higher than 50 ng kg^{-1} determined with HPLC was used to complement the database obtained with ELISA tests, which did not cover all milk consignments. Data of AFM_1 concentration together with the quantity of milk unloaded from each truck were used to calculate the weighted mean AFM_1.

Characterization of Data and Exposure Estimation

Descriptive statistical parameters of the AFM_1 concentrations [mean, weighted mean (weight was assigned according to the quantity of milk loaded from the sampled trucks), standard deviation, median, percentile values, and their confidence intervals] were calculated for HQM, NQM, and OM. The percentile values were calculated with NIST method (NIST/SEMATECH, 2013). The confidence intervals of the mean and percentile values of the three types of milk were overlapping; hence, there was no significant difference between them.

Dietary Exposure Assessment and Risk Characterization

Food consumption data were obtained from the Comprehensive Food Consumption Database of EFSA[1]. The database contained data derived from the Italian National Food Consumption Survey (INRAN-SCAI) conducted from October 2005 to December 2006. It involved 3,322 consumers from 1,329 households located in the four main geographical areas of Italy (North-West, North-East, Centre and South, and Islands;

[1]EFSA, The Comprehensive Food Consumption Database (2018). https://www.efsa.europa.eu/en/food-consumption/comprehensive-database

Leclercq et al., 2009). The exposure assessment is based on the mean and 95th percentile "Cattle milk" consumption data of "consumers only" of each population groups: infants (0–0.9 years), toddlers (1–2.9 years), other children (3–9.9 years), adolescents (10–17.9 years), adults (18–64.9 years), elderly (65–74.9), and very elderly (>75). The proportion of milk consumers of the respective population groups is presented in **Table 1**.

Data used for EDI calculation are summarized in **Table 1**. Since the number of consumers (5) in the infant category was low, these consumption data were substituted by the cattle milk consumption of all available (infant) consumers in the EFSA database in order to provide an approximate estimate for the mean consumption values for the Italian population. The 95th percentile exposure calculations were carried out only on a monthly basis because it is not realistic that such high quantity of milk is consumed over the year.

The estimated daily intakes (EDI: ng kg^{-1} bw day^{-1}) of the population groups were calculated as:

$$\mathrm{EDI} = \frac{\sum\left[\mathrm{WM}_{\mathrm{AFM1}}\ \text{concentration}\left(\frac{\mathrm{ng}}{\mathrm{kg}}\right) \times \mathrm{AC}\left(\frac{\mathrm{kg}}{\mathrm{day}}\right)\right]}{\left[\text{Mean body weight}\,(\mathrm{kg})\right]}.$$

Monthly, yearly, and four-year average EDI values were calculated from the corresponding weighted mean (WM) AFM_1 concentrations unloaded from the tankers in the given period of time and the average (AC) and large portion size (95th percentile – as worst-case scenario calculation) consumption data (kg/day).

In order to calculate hazard indices (HI), the monthly, yearly, and four-year average estimated daily intakes were divided with 0.2 (Kuiper-Goodman, 1990). The same approach was also used in other studies (Shundo et al., 2009; Duarte et al., 2013; Tsakiris et al., 2013; Kerekes et al., 2016).

Because $BMDL_{10}$ value is not available for AFM_1, the $BMDL_{10}$ of AFB_1 (870 ng kg^{-1} bw day^{-1}) was used as a conservative value. MoE was calculated by dividing the benchmark dose for a 10% increase in hepatocellular carcinoma (HCC) incidence ($BMDL_{10}$) by the human exposure (EDI) values. The MoE then was divided by the mean or 95th percentile EDI values for each population groups. The calculation was carried out for each month from April 2013 to December 2018.

The prevalence of carriers of hepatitis B (HBV) in the Italian population is between 1.2 and 2% (Serraino et al., 2003). The risk potency was calculated assuming that 2% of population is HBV+ and using the cancer potencies for AFB_1, which was estimated by JECFA to be 0.01 for hepatitis B surface antigen negative (HBsAg–) individuals and 0.3 for HBsAg+ individuals (JECFA, 1998). Based on the given cancer potencies, the risk potency can be calculated as follows = 0.01 × 98% + 0.3 × 2% = 0.016 HCC/year per 100,000 persons (Cano-Sancho et al., 2013). The proportion of population at risk was estimated by multiplying the risk potency with the $BMDL_{10}$ and then dividing with the MoE considering the mean and 95th percentile of exposure estimation:

$$\text{Population at risk} = \frac{\text{risk potency} \times \mathrm{BMDL}_{10}}{\mathrm{MoE}}$$

RESULTS

Aflatoxin M1 Results

A total of 31,702 milk samples were analyzed for AFM_1, representing 556,413 tons of milk, which comprised 16,107 (304,625,633 kg), 13,726 (222,189,472 kg), and 1,869 (29,598,042 kg) trucks (batches) of HQM, NQM, and OM, respectively, during 2013–2018.

As the confidence intervals of the median values of the AFM_1 contamination in HQM and NQM overlapped, these data were merged into one subset (AQM – average quality milk). The difference between the Northern, Central, and Southern regions was negligible, however, the median values of AQM were statistically different from that of organic milk (OM 8 ng kg^{-1}) collected only in the Northern region. Details of the descriptive statistics of the AFM_1 levels for AQM and OM are reported in **Table 2**. The differences between the number of samples taken in each region should be noted. **Figure 1** demonstrates the changes occurring throughout the years. In 2017 and 2018, the levels of contamination were

TABLE 1 | Mean body weight and cow milk consumption data used for Estimated Daily Intake (EDI) calculation in different age groups.

Population Group	Number of consumers	Percentage of milk consumers[1]	Mean consumption (g day^{-1})	95th percentile consumption (g day^{-1})	Mean body weight (kg)
Infants	2,396[2]	36.61%	131.52[2]	348.13[2]	5.00
Toddlers	33[3]	91.67%	269.01[3]	600.00[3]	12.00
Other children	184	95.34%	205.98	392.50	26.10
Adolescents	208	84.21%	177.80	305.42	52.60
Adults	1,733	74.92%	136.03	275.88	70.00
Elderly	223	76.90%	141.10	266.25	70.10
Very elderly	188	82.46%	177.13	337.19	70.10

[1]*Percentage of population groups consuming milk in Italy. EFSA, The Comprehensive Food Consumption Database (2018).*

[2]*Because the number of consumption data was low (5), the original data were substituted by the calculated European averages: 132 and 348 g* day^{-1}.

[3]*Although the number of consumption data was also low in this category, the data were not substituted, because they were the same as the European averages: 270 and 600 g* day^{-1}.

TABLE 2 | Distribution of Aflatoxin M_1 concentration (ng kg^{-1}) in different milk types and in various geographical areas of Italy during the 5.5-year period.

	OM[1] Northern Italy			AQM[2] Northern Italy			AQM[2] Central Italy			AQM[2] Southern Italy		
Number of samples	1,869			20,574			2,438			6,821		
Confidence intervals	95% CI	(LCL-UCL)		95% CI	(LCL-UCL)		95% CI	(LCL-UCL)		95% CI	(LCL-UCL)	
Mean concentration	10.3	9.9	10.6	12.6	12.5	12.7	13.3	12.9	13.6	11.4	11.3	11.6
SD	7.7			8.5			8.6			7.5		
Median	8	8	9	10	10	11	11	11	11	9	9	10
P0.90	18	17	20	23	23	23	24	24	26	21	20	21
P0.95	24	23	27	28	28	29	30	29	33	26	25	27
P0.975	30	29	33	34	34	35	38	35	40	32	30	33
P0.99	41	36	49	41	40	41	43	41	46	40	38	40
Weighted mean concentration	10.8	10.4	11.1	12.6	12.5	12.6	13.4	13.0	13.7	11.7	11.6	11.9

The percentile values (P0.90–P0.99) were calculated with the NIST method; LCL and UCL are the lower and upper 95% confidence intervals (CI). [1]Organic milk. [2]Average quality milk.

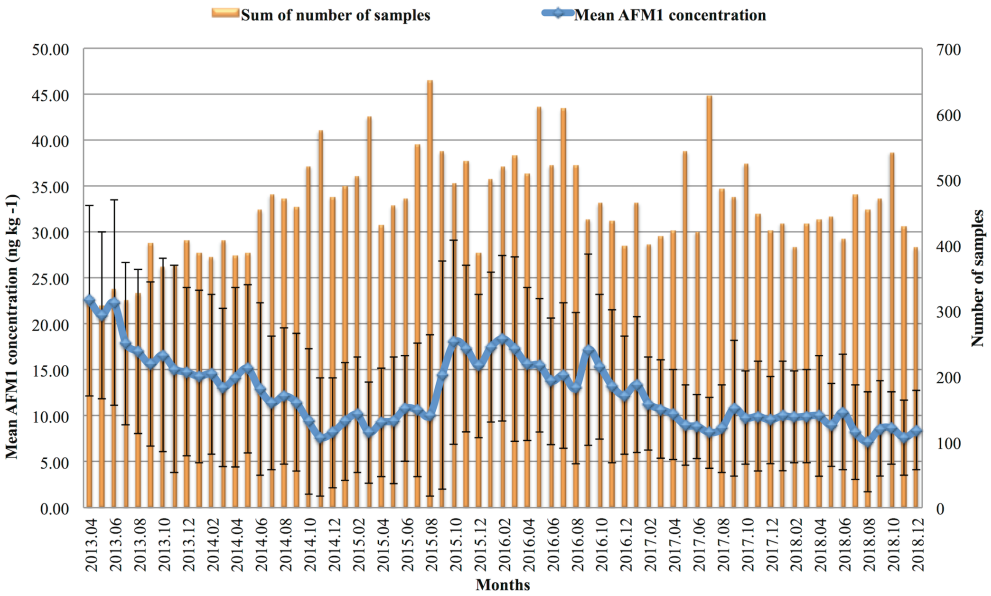

FIGURE 1 | Monthly summary of the total number of samples analyzed and the mean Aflatoxin M_1 (AFM_1) concentration and standard deviation of milk samples in the given month.

about the same as it was observed from December 2014 through August 2015. However, between September 2015 and December 2016, the AFM_1 contamination was nearly as high as in 2013 during the Aflatoxin crisis.

Exposure Assessment

The monthly and yearly averages of EDI, HI, and liver cancer incidence (LCI) values were calculated together with their average values for the whole study period. In **Figure 2**, the results of monthly EDI calculations, based on mean and large portion size consumption (95th percentile) data, are shown for two different age categories: toddlers and the adult population. Among adults, the mean EDI values varied between 0.02 and 0.08 ng kg^{-1} bw day^{-1} during the study period, and for the large portion size consumers, the results were between 0.04 and 0.13 ng kg^{-1} bw day^{-1}. In the population of infants, mean EDI of AFM_1 resulted in the monthly range of 0.19–0.61 ng kg^{-1} bw day^{-1}, and in the range of 0.49–1.62 ng kg^{-1} bw day^{-1} considering the 95th percentile consumption values. Similarly, among toddlers, the mean EDI values varied between 0.16 and 0.52 ng kg^{-1} bw day^{-1}. In case of large portion size consumers, the results ranged between 0.35 and 1.16 ng kg^{-1} bw day^{-1}. Naturally, the EDI patterns throughout the years follow the same pattern as the weighted mean AFM_1 concentrations presented in **Figure 1**.

To facilitate the interpretation of EDI values, the corresponding hazard indices (HIs) were calculated by dividing the (monthly, yearly, or four-year average) EDI with 0.2 (the "safe dose"). The calculation shows the amount of AFM_1 of concern (HI value >1). The results of yearly mean hazard index calculations for each population groups are presented in **Figure 3**.

Over the age of 3 years, the HI was <1 considering mean intake values. However, for infants and toddlers, the observed intake levels resulted in HI values higher than 1 in each year during the study period.

The fraction of incidence of HCC or liver cancer incidence (LCI) attributable to the intake of AFM_1 was taken into account on the basis of MoE considering the estimated mean exposure.

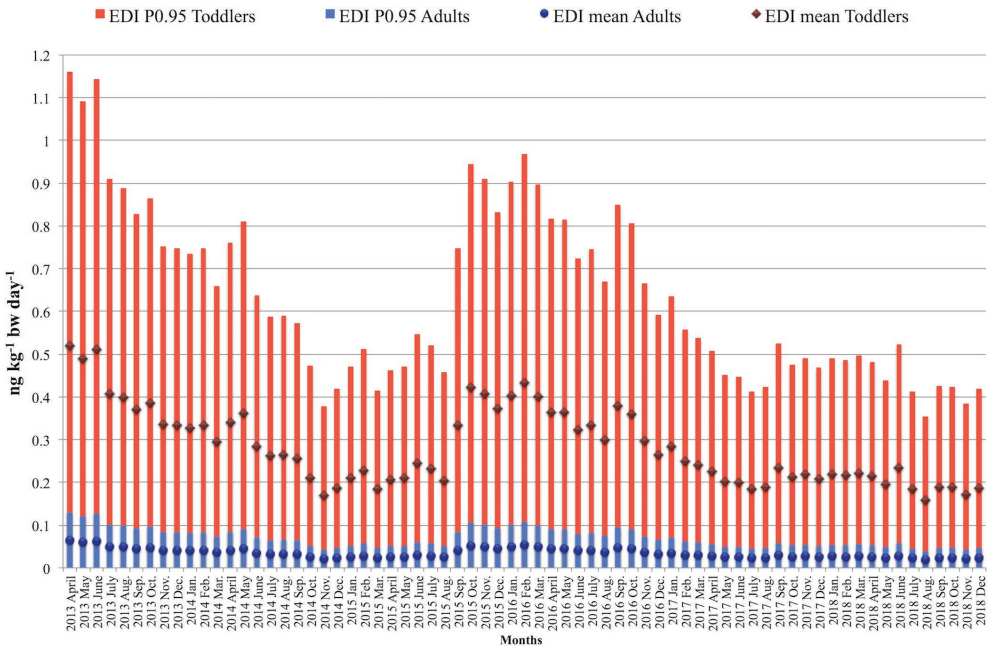

FIGURE 2 | Monthly variation of Estimated Daily Intake (EDI) values of adults and toddlers during the 2013–2018 study period.

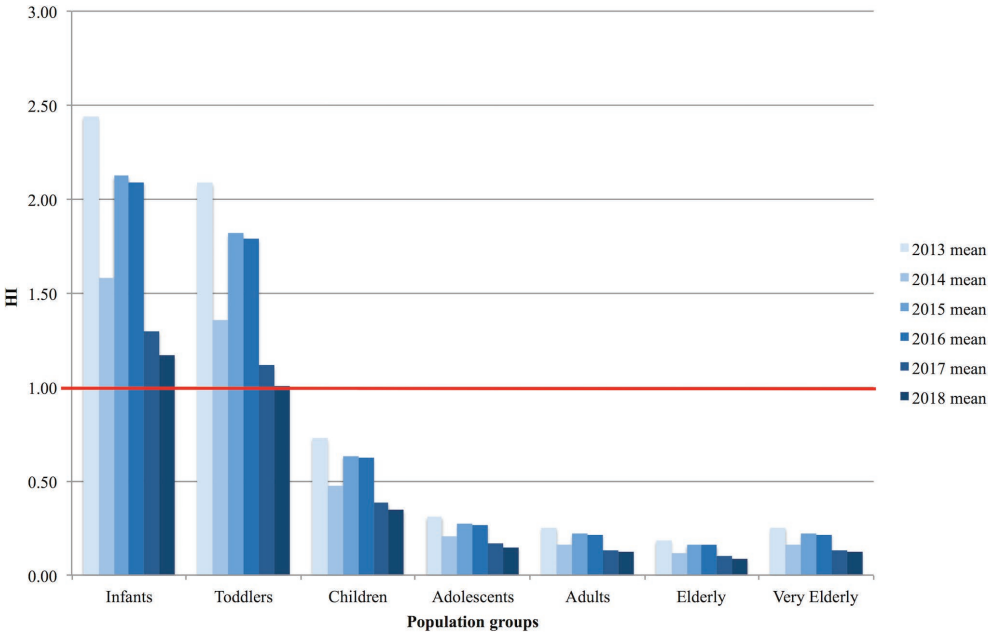

FIGURE 3 | Yearly variation of mean Hazard Index (HI) values in the population groups throughout the 2013–2018 study period.

TABLE 3 | "Heat map" (scale: green-yellow-red) of the estimated yearly average liver cancer incidence (LCI) (cases per 100,000 people) in the Italian population by age groups during 2013–2018.

Year/Age group	Infants	Toddlers	Children	Adolescents	Adults	Elderly	Very Elderly
2013	0.0078	0.0067	0.0023	0.0010	0.0008	0.0006	0.0008
2014	0.0051	0.0043	0.0015	0.0006	0.0005	0.0004	0.0005
2015	0.0068	0.0058	0.0020	0.0009	0.0007	0.0005	0.0007
2016	0.0067	0.0057	0.0020	0.0009	0.0007	0.0005	0.0007
2017	0.0042	0.0036	0.0012	0.0005	0.0004	0.0003	0.0004
2018	0.0038	0.0032	0.0011	0.0005	0.0004	0.0003	0.0004

The yearly average LCI values for the whole study period are reported for the population groups in **Table 3**. The calculation predicted a low additional number of cases in the examined age categories.

Based on the mean consumption and yearly weighted mean AFM_1 concentration, the HCC incidence cases were between 0.0004 and 0.0008, 0.0032 and 0.0067, and 0.0038 and 0.0078 per 100,000 people for adults, toddlers, and infants, respectively. The highest risk group is the infants.

DISCUSSION

The reported concentration of AFM_1 in milk varied widely in recent years worldwide, ranging from non-detects to values up to 48,000 ng kg^{-1} (Shundo et al., 2009; Duarte et al., 2013; Tsakiris et al., 2013; Oluwafemi et al., 2014; Scaglioni et al., 2014; Temamogullari and Kanici, 2014; Flores-Flores et al., 2015; Rahmani et al., 2018; Fakhri et al., 2019a).

In our study, 63 (0.20%) raw milk samples collected from trucks contained AFM_1 higher than 50 ng kg^{-1}. These batches were discarded. The raw milk complying with EC regulation was processed to pasteurize and UHT milk as well as for cheese and other milk-based products. The mean AFM_1 concentrations were between 10.3 ng kg^{-1} in OM and 12.4 ng kg^{-1} in AQM with a weighted mean of 10.9 and 12.5 ng kg^{-1}, respectively. These data are comparable with the mean contamination levels previously reported in other European countries such as Spain (n = 603, mean = 9.69 ng L^{-1} in UHT milk; Cano-Sancho et al., 2013), France (n = 264, mean = 14.3 ng kg^{-1} in raw milk; Boudra et al., 2007), and Portugal (n = 40, mean = 23.4 ng L^{-1} in pasteurized milk; Duarte et al., 2013) except in Serbia (ranging from 5 to 1,260 ng kg^{-1}; mean 71 ± 130; Milićević et al., 2017). The percentages of non-compliant samples were in the lower range of the results (0 and 9.1% in raw milk) reported in previous studies (Roussi et al., 2002; Rodríguez-Velasco et al., 2003; Martins et al., 2005; Boudra et al., 2007; Milićević et al., 2017).

Comparison of the results reported in this study (2013–2018) with data obtained during the mycotoxin crisis (1999–2004) by the same industry shows a clear reduction in AFM_1 concentration. Both the percentage of milk batches containing AFM_1 above the EU limit and the mean AFM_1 concentration decreased (see **Table 4**). The investigations performed during 2005–2010 showed a higher percentage of non-compliant batches than the present investigation. The notable reduction of the ratio of samples over the legal limit is attributed to the regular monitoring of raw milk, and timely advice is given to the dairy farms for corrective measures.

In view of the similar mean AFM_1 concentrations and the lack of data on the different consumption levels of HQM, NQM, or OM among the Italian population groups, the exposure assessment was performed using the combined database of all types of milk and the average daily milk consumption.

The EDI and HI results indicate that – due to the relatively large milk intake compared to their body weights – infants and toddlers are the two most exposed groups of the population to AFM_1. As demonstrated in **Figure 2**, the EDI of the other population groups (adolescents-adults-elderly-very elderly) resulted in a significantly lower range of 0.01–0.18 ng kg^{-1} bw day^{-1}, while infants and toddlers are exposed to 0.35–1.16 ng kg^{-1} bw day^{-1} daily intake levels. The latter data are in line with previously reported mean EDIs of 0.08 ng kg^{-1} bw day^{-1} (n = 40) in Portugal (Duarte et al., 2013), 0.09 ng kg^{-1} bw day^{-1} (n = 16) in France (Leblanc et al., 2005), and 0.18–0.20 ng kg^{-1} bw day^{-1} (n = 1,233) in Serbia (Milićević et al., 2017). The calculated monthly and yearly mean HI values were < 1 in the age groups of adolescents, adults, elderly, and very elderly, but for infants, toddlers, and children, the results are close to or well over 1, which means that the amount of AFM_1 consumed with milk (**Figure 3**) might be a considerable risk. The higher HI values for younger consumers compared to older age groups are in agreement with the results of Tsakiris et al. (2013); however, the results of this study show a higher exposure level. The slight differences in the outcome of the two studies can be explained by the different calculation methods – considering "consumers only" in this study – and the number of samples.

The LCI estimated in other population groups is significantly lower (**Table 3**). The estimated fraction of incidence of HCC in the Italian population that predicted a slight increase in cases due to milk consumption is in line with those reported previously by Trevisani et al. (2006; 0.011–0.057 cases/100,000 people in different age categories).

The results of the current study represent the exposure of people consuming milk. Therefore, the estimates cannot be extrapolated to the whole age groups including non-consumers.

Comparison of our results with the previously reported ones should be made with caution, because the latter ones are based on much fewer samples taken within a short period of time compared to our database. Even the comprehensive review on the presence of mycotoxins in animal milk (Flores-Flores et al., 2015) covering 38 countries during the period of 1991–2012

TABLE 4 | AFM_1 concentration and the ratio of non-compliant samples of raw milk collected in Italy by the same milk processing plants during a 17-year period.

Year(s)	Number of samples	Mean AFM_1 concentration (ng kg^{-1})	95th percentile	Number of samples >50 ppt (%)	Reference
2000–2001	791	27–30**	NA*	23.5	Serraino et al. (2003)
Jan. 2001-July 2004	2,512	29–34	80	NA*	Trevisani et al. (2006)
Sep. 2003-July 2004	4,190	35	80	NA*	Trevisani et al. (2006)
2005	4,886	12–19**	30–40**	0.7–3.1**	Trevisani et al. (2014)
2006	4,718	13–15**	33–40**	0.6–1.7**	Trevisani et al. (2014)
2007	4,354	11–13**	27–29**	0.3–1.1**	Trevisani et al. (2014)
2008	4,195	15–18**	30–38**	1.7–2.5**	Trevisani et al. (2014)
2010	3,740	11–12**	25–28**	0.5–0.7**	Trevisani et al. (2014)
2013–2014	9,017	13–17**	29–35**	0.24	Kerekes et al. (2016)
2013–2018	31,702	10–13**	24–30**	0.20	Present study

NA, data not available.

***Range of AFM_1 contamination detected in different types of milk (i.e. HQM, NQM, AQM, or OM) or in samples collected in different Italian regions.*

includes results obtained based on 3–6,537 samples taken within 1 or 2 years. Our study is the first, which evaluates the monthly variation of AFM_1 exposure, based on 300–650 samples per month totaling 31,702 samples during the period of almost 6 years (69 months), enabling the reliable estimation of the mean AFM_1 concentrations, and the corresponding EDI values, and demonstrates their fluctuations over the years.

CONCLUSIONS

Although the results of this investigation showed a low risk of HCC for the adolescent and adult population attributable to intake of AFM_1 *via* milk consumption during the study period (2013–2018), it should be considered that the present study does not include the AFM_1 intake due to other milk-based products, e.g., cheese and yoghurt, which could add a notable amount to the estimated quantity consumed. Furthermore, it should be taken into account that our EDI calculations could not include the exposure derived from the consumption of mother's milk either, because we had no data on the combined intake of breast milk and cow milk. Breast milk may also contain AFM_1 derived from cow milk as well as from the mother's food contaminated with AFB_1 (Galvano et al., 2008; Radonić et al., 2017). In Italy, the AFM_1 contamination was found in four (5%) breast milk samples [ranging from <7 to 140 ng L^{-1}; mean = 55.35 ng L^{-1} (Galvano et al., 2008)]. Another Italian study revealed that AFM_1 was detected in 37% of samples (mean = 12 ng L^{-1} ± SD = 11 ng ml^{-1}; range = 3–340 ng L^{-1}) taken from patients (n = 30) with celiac disease, while in the healthy control group, the mean AFM_1 concentration levels (9 ± 07 ng L^{-1}; range = 3–67 ng L^{-1}) were lower (Valitutti et al., 2018). The latter results indicate that the exposure of infants can be substantially higher than our estimate depending on the dietary pattern of the mothers. Further investigation is needed to evaluate the total exposure for this contaminant for all population groups.

The previous Aflatoxin crisis due to high AFB_1 contamination of maize has increased the awareness of the food safety risk managers; induced regulatory measures, research, and innovation activities; and reinforced the consciousness of the food business operators. Consequently, they have implemented strict monitoring and regular control along the feed and food chain utilizing the availability of rapid and less expensive detection kits. This self-control and corrective measures at dairy farms resulted in the slow decrease of AFM_1 contamination.

Nevertheless, the variability of climatic conditions throughout years and the number of other factors that may influence AFB_1 contamination of crops and consequently AFM_1 contamination of milk underline the need of continuous monitoring of milk contamination and regular update of the exposure assessments.

DATA AVAILABILITY STATEMENT

The datasets generated for this study will not be made publicly available. The dataset is based on confidential private industry data.

AUTHOR CONTRIBUTIONS

ÁA, AS, and FG contributed to the conception and design of the study. AC and AZ organized the database. PB and KK performed the statistical analysis. AS wrote the first draft of the manuscript. KK, ZF, and ÁA finalized the manuscript and prepared for publication. All authors contributed to manuscript revision, read and approved the submitted version.

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REVIEW
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The *Aspergilli* and Their Mycotoxins: Metabolic Interactions With Plants and the Soil Biota

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Species of the highly diverse fungal genus *Aspergillus* are well-known agricultural pests, and, most importantly, producers of various mycotoxins threatening food safety worldwide. Mycotoxins are studied predominantly from the perspectives of human and livestock health. Meanwhile, their roles are far less known in nature. However, to understand the factors behind mycotoxin production, the roles of the toxins of *Aspergilli* must be understood from a complex ecological perspective, taking mold-plant, mold-microbe, and mold-animal interactions into account. The *Aspergilli* may switch between saprophytic and pathogenic lifestyles, and the production of secondary metabolites, such as mycotoxins, may vary according to these fungal ways of life. Recent studies highlighted the complex ecological network of soil microbiotas determining the niches that *Aspergilli* can fill in. Interactions with the soil microbiota and soil macro-organisms determine the role of secondary metabolite production to a great extent. While, upon infection of plants, metabolic communication including fungal secondary metabolites like aflatoxins, gliotoxin, patulin, cyclopiazonic acid, and ochratoxin, influences the fate of both the invader and the host. In this review, the role of mycotoxin producing *Aspergillus* species and their interactions in the ecosystem are discussed. We intend to highlight the complexity of the roles of the main toxic secondary metabolites as well as their fate in natural environments and agriculture, a field that still has important knowledge gaps.

Keywords: *Aspergillus*, aflatoxin, mycotoxin, plant, insect, microbe, soil, interaction

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INTRODUCTION

The lifestyles of *Aspergillus* species associated with plants range from saprophytes and symptomless endophytes to weak and opportunistic phytopathogens. The shift between these lifestyles is the result of global transcriptome changes, primarily affecting secondary metabolite (SM) production (e.g., Reverberi et al., 2013). The principal and well-known mycotoxins produced by the *Aspergilli* are ochratoxin A (OTA) and aflatoxins (AFs), as well as less-prominent toxins like patulin (Keller et al., 2005). These toxins are found in different agricultural commodities (Varga et al., 2004), and are tightly regulated with different threshold limits depending on the matrix (Cano et al., 2016).

Due to the importance of SMs in plant pathogenesis and animal toxicoses, understanding their regulation and biosynthesis is crucial but still hindered by notable knowledge gaps. The species *A. flavus*, for example, has been predicted to possess 56 SM biosynthesis gene clusters (Keller et al., 2005), but only some secondary metabolites, e.g., AFs (Yu et al., 2004), aflatrem (Nicholson et al., 2009), piperazine (Forseth et al., 2013), asparasone (Malysheva et al., 2014), cyclopiazonic acid (CPA) (Chang et al., 2009), and kojic acid (Terabayashi et al., 2010) have been assigned to a particular gene cluster (Ehrlich and Mack, 2014). *A. flavus* thus might produce metabolites besides well-known mycotoxins that could be underrated contributors to its toxicity to humans and animals.

Initially, it was hypothesized that mycotoxin production helps fungi to compete with other organisms for nutrient sources like fruits or seeds (Janzen, 1977). Mycotoxins are now also known to act as chemical signals between representatives of different kingdoms, e.g., as inhibitors of quorum sensing (QS), virulence factors in pathogens, or as protectors of sclerotia from insect predation (Ciegler, 1983; Wicklow et al., 1994; Desjardins and Hohn, 1997; Rasmussen et al., 2005; Rohlfs et al., 2010).

Due to their economic and public health importance, the research on mycotoxins has so far mostly been focused on animal husbandry, the food chain, and human aspects. However, for a comprehensive understanding of toxigenic molds' ecology and of the evolutionary pressures shaping mycotoxin production, interactions with the micro- and macroflora and fauna in different habitats need to be considered and investigated. The study of the overall role of microbial SMs in natural habitats is a previously mostly neglected, but an emerging field (O'Brien and Wright, 2011).

ASPERGILLUS MYCOTOXINS AND THEIR ECOLOGICAL ROLES

Sterigmatocystin/Aflatoxins

AFs are produced by as much as 16 species (Frisvad et al., 2019), most notably by *A. flavus* and *A. parasiticus*. A wide range of *Aspergillus* spp. produces the AF precursor sterigmatocystin (ST), which is also a carcinogenic compound. The ST/AF polyketide biosynthetic pathways are perhaps the most thoroughly studied ones in fungi (Cleveland et al., 2009; Khaldi et al., 2010).

The most common AF-producing species and the most common member of section *Flavi* is *A. flavus*, which possesses two distinct morphotypes, namely the "L-type" with big sclerotia (with average diameter of >400 μm), and the "S-type" that produces small sclerotia (under 400 μm) (Gilbert et al., 2018). However, several additional and often newly delimited species (*A. aflatoxiformans, A. arachidicola, A. austwickii*, *A. cerealis, A. minisclerotigenes, A. mottae, A. pipericola,* and *A. texensis*) have been characterized by S-type sclerotia. Earlier reports on S-type *A. flavus* may have referred to any of these species, including those that produce both aflatoxin B1 (AFB1) and aflatoxin G1 (AFG1) (so-called SBG strains) (Singh et al., 2018; Frisvad et al., 2019).

While the ecological role of ST is not known in detail, it is presumably antagonistic to organisms competing for resources with ST producers. Both AFs and ST have been reported to be phytotoxic (Stoessl, 1981; McLean et al., 1995). AFs inhibit plant photosynthesis by hindering chlorophyll and carotenoid synthesis (Anjorin and Inje, 2014), leading to virescence or albinism in the contaminated plants (Reiss, 1978). However, in plant pathogenesis, the role of these mycotoxins needs to be investigated as non-aflatoxigenic strains also have the potential to colonize plant hosts, e.g., on cotton bolls (Cotty, 2007), and these types of strains are isolated frequently.

Soil is the natural habitat for *A. flavus*, and AF production is considered to give a fitness advantage in that environment (Drott et al., 2017). Selective forces that maintain the polymorphism of non-aflatoxigenic and aflatoxigenic colonies are mainly unknown. Resource competition among the closely related strains is modulated by factors such as chemical composition and pH of the soil or nutrient and water availability (Ehrlich, 2014). Moreover, competition between aflatoxigenic and non-aflatoxigenic strains is strain-dependent, and it must be noted that non-aflatoxigenic strains are not necessarily atoxigenic, as they may produce toxins other than AFs. Under high fungal density, non-aflatoxigenic strains can outcompete both toxigenic and other non-aflatoxigenic populations (Cotty, 2006). Aflatoxigenic isolates were shown to have lower fitness than non-aflatoxigenic isolates in wide temperature ranges (25–42°C) (Drott et al., 2019). This may explain the success of the latter in competition. The metabolic cost of AF production seems to explain the low fitness as AFB1 itself does not affect the growth of *A. flavus* at concentrations as high as 500 ng g^{-1} (Drott et al., 2019), orders of magnitude higher than what can be measured in soils (0.6–5.5 ng·g^{-1}) (Accinelli et al., 2008). Inoculation of soil with non-aflatoxigenic strains also protects crops from AF contamination during storage (Dorner and Cole, 2002; Bandyopadhyay et al., 2016).

AFB1 is transient in soils with a half-life of approximately 5 days at 28°C; however, it is produced continuously as long as there is a substrate, e.g., corn residues (Accinelli et al., 2008). High *A. flavus* levels (log10 3.1–4.5 cfu·g^{-1}), AFB1 production, and expression of the AF biosynthetic genes (*aflG, aflD, aflP, aflR*, and *aflS*; Ehrlich et al., 2005) have been reported in the former study.

Studies on AFB1 transformation in soil or purified mineral systems have identified AFs B2 (AFB2) and G2 (AFG2) as the primary transformation products using thin-layer chromatography. However, the more sophisticated HPLC-MS technique did not detect these molecules in spiked soils. In an aqueous-soil environment, a new structure, B2a (AFB2a), was detected as the single primary transformation product. AFB2a is a hydrolytic product of AFB1 and the soil acting as an acid catalyst (Starr et al., 2017) (**Figure 1**).

AFs taken up through plant roots can be accumulated, transported to other tissues (e.g., in groundnut seedlings; Hariprasad et al., 2015; Snigdha et al., 2015), degraded, metabolized, or masked, or can be diffused back to the medium (e.g., in maize; Mertz et al., 1980).

Various fungi can inhibit AF accumulation. In an *in vitro* soil environment, *Fusarium oxysporum* was able to inhibit AF production at different temperatures (25 and 30°C) and fumonisins accumulated instead of AFB1 (Falade et al., 2016). On the

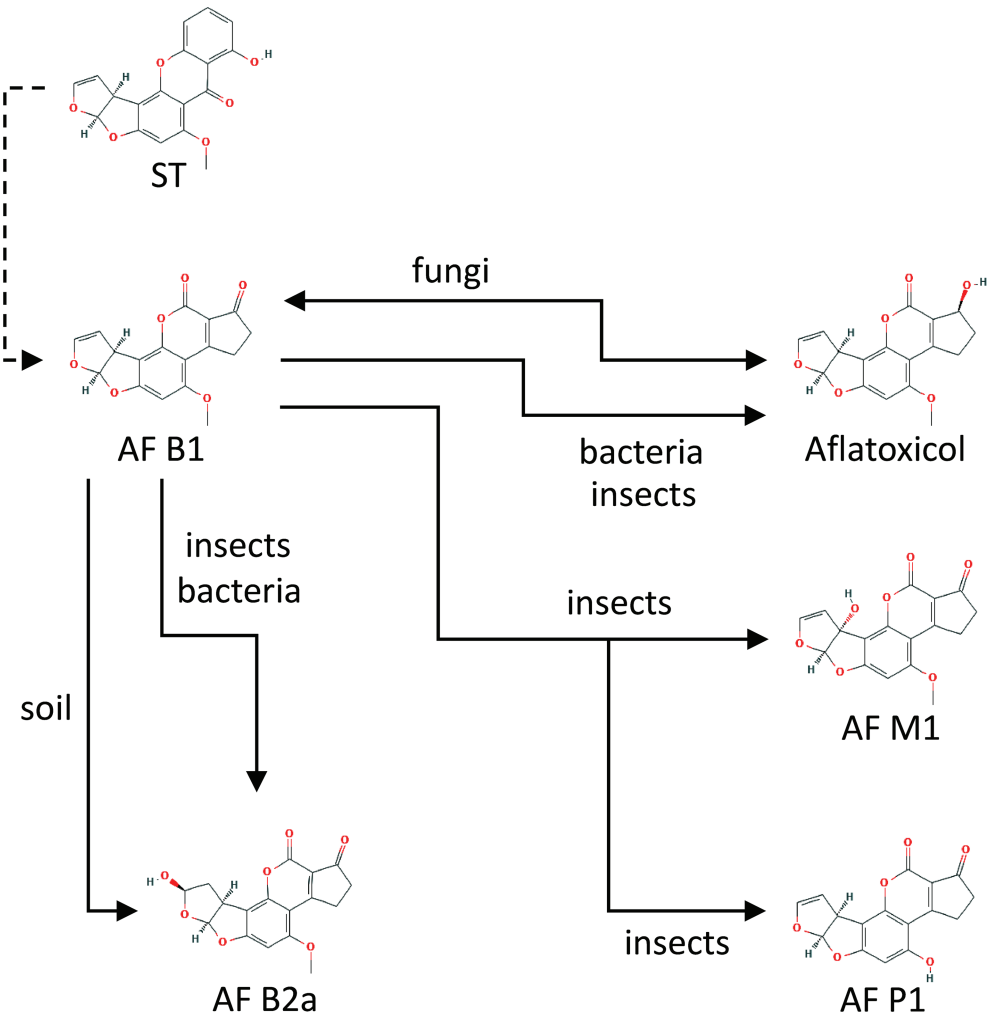

FIGURE 1 | Main chemical conversions of aflatoxin B1 (AFB1) under interaction with different organisms and soil. Sterigmatocystin (ST) is a chemical precursor of aflatoxin B1 (AFB1) in aflatoxigenic fungi. The further conversion processes are explained in details in the text. Source: National Center for Biotechnology Information. PubChem Compound Database (accessed June 6, 2019) (Bolton et al., 2008).

contrary, inhibitory effect by *A. flavus* on *Fusarium oxysporum* f. sp. *niveum* and *Fusarium solani* f. sp. *cucurbitae* has also been described with an inhibition rate exceeding 50 % in *in vitro* and greenhouse experiments. Hyperparasitism of *A. niger, A. flavus*, and *A. terreus* on *F. oxysporum* f. sp. *melonis* was also demonstrated (Boughalleb-M'Hamdi et al., 2018).

Gliotoxin

Gliotoxin (an epipolythiodioxopiperazine) has internal disulfide bridges that conjugate proteins (Spikes et al., 2008). Gliotoxin biosynthesis and regulation are reviewed by Dolan et al. (2015). The compound is implicated in the formation of reactive oxygen species (ROS) by redox cycling and is generally broadly cytotoxic (Gardiner et al., 2005). Therefore, its detoxification is only possible by its biosynthetic enzymes (Scharf et al., 2018). One of the significant gliotoxin producers besides biocontrol *Trichoderma* ssp. is *A. fumigatus*, a saprophyte and an opportunistic animal pathogen. Gliotoxin produced by this fungus acts as a virulence factor mediating systemic mycosis in susceptible vertebrates (Latgé, 2001; Scharf et al., 2016) and presumably in insects (Reeves et al., 2004). *A. fumigatus* possesses a self-protecting system against gliotoxin (Schrettl et al., 2010; O'Keeffe et al., 2014). RNA-seq revealed 164 differentially expressed genes (DEGs) in *A. fumigatus* treated with external gliotoxin, and besides gliotoxin biosynthesis genes, helvolic acid biosynthesis genes, siderophore-iron transport genes showed altered expression (O'Keeffe et al., 2014). High temperature and humidity during crop maturation may favor *A. fumigatus* presence and toxin production. Gliotoxin enters the food chain and reaches the most sensitive farm animals, like horses and poultry (Pena et al., 2010). However, there is no threshold limit for this molecule.

In composted mineral soil with a natural microbiota, the toxin may function as an antibiotic, effectively controlling the damping-off disease of *Zinnia elegans* (zinnia) seedlings caused by the fungus *Rhizoctonia solani* and the water mold *Pythium*

ultimum (Lumsden et al., 1992). A strong correlation between the presence of bacterial peptidoglycan, lipopolysaccharide, or lipoteichoic acid in soil and the gliotoxin secretion of *A. fumigatus* was described by Svahn et al. (2014). This finding was potentially relevant for drug discovery research, and parallelism was found with the increased virulence of *A. fumigatus* in case of bacterial co-infection.

Ochratoxins

Several *Aspergilli* in sections *Circumdati* (such as *A. steynii* and *A. westerdijkiae*), *Flavi*, and *Nigri* (e.g., *A. carbonarius* and *A. niger*; Palencia et al., 2010) are well-known producers of OTA, a mycotoxin teratogenic, carcinogenic, immunosuppressive, and nephrotoxic in animals (Samson et al., 2014). All studied OTA-producing fungi have a consensus OTA biosynthetic pathway with four highly conserved biosynthetic genes in a cluster and a bZIP transcription factor (Wang et al., 2018).

OTA induced necrotic lesions on *Arabidopsis thaliana* leaves *via* induction of an oxidative burst by elevated ROS (hydrogen peroxide and superoxide anion) levels (Peng et al., 2010). Meanwhile, the downregulation of the antioxidant defense enzymes in host plants and up-regulation of lipid peroxidation were detected, along with root growth inhibition of seedlings (Peng et al., 2010). Infiltration of 4-week-old *A. thaliana* leaves with 2 mM and 1 mM OTA solutions *in vitro* resulted in macroscopic lesions (Wang et al., 2012), and the growth of *A. thaliana* was repressed, while cell death was detected with characteristic hypersensitive response-type lesions on the excised leaves. Cell death did not only result in a manifestation of oxidative burst but the deposition of phenols and callose (Peng et al., 2010) as well. McLean (1996) investigated the effect of the toxin on germinating *Zea mays* embryos. Interestingly, there was no linear relationship between the inhibitory effect and the OTA concentrations as 10 $\mu g \cdot ml^{-1}$ OTA was inhibitory, while 5 or 25 $\mu g \cdot ml^{-1}$ OTA was stimulatory for root and shoot growth.

Soil type, in connection with microbial activity, affects OTA half-life. In soils with higher microbial activity, like planted soils, faster degradation could be measured (Mortensen et al., 2006) caused by the microbial biomass (e.g., Barberis et al., 2014). Regulation of OTA biosynthesis can be modulated by volatile organic carbons (VOCs) as observed for *A. carbonarius* and fruit ketones, C-8 alcohols, and trans-nerolidol (Zhang et al., 2017).

Patulin

Patulin is a polyketide mycotoxin produced by *Penicillium* spp. and to a lesser extent, various *Aspergilli* (Zhang et al., 2008). It is frequently found in fresh fruits or fruit juices and jams contaminated with blue mold rot (Logrieco et al., 2003). Like clavatol, patulin inhibits numerous plant pathogenic fungi and water molds *in vitro*, i.e., *Fusarium oxysporum* f. sp. *cucumerinum*, *Botrytis cinerea, Didymella bryoniae, Rhizoctonia solani*, and *Pythium ultimum* (Zhang et al., 2008). Patulin and clavatol produced by *Aspergillus clavatonanicus* endophyte of *Taxus mairei* have been shown to antagonize plant pathogens (Zhang et al., 2008). Interestingly, Botha et al. (2018) reported that *A. clavatus* produced higher concentration of tremorgenic mycotoxins (i.e., tryptoquivaline A, deoxytryptoquivaline A, and deoxynortryptoquivaline) than concomitant patulin and cytochalasin E. Patulin, similarly to penicillic acid has the potential to interfere with bacterial QS communication in soil (Rasmussen et al., 2005), hinting at its potentially manifold ecological roles in microbial communities.

Cyclopiazonic Acid

The neurotoxic CPA is an indole-tetramic acid produced by 13 species in section *Flavi* (Frisvad et al., 2019). It inhibits endoplasmic reticulum calcium ATPases at nanomolar concentrations, and therefore, it is an inducer of cell death in plants (Chang et al., 2009). Usually, CPA and AFs are concomitant mycotoxins. Most *A. flavus* strains synthesize AFs B1 and B2 besides CPA, although some strains also synthesize AFs G1 and G2 (Geiser et al., 2000; Cardwell and Cotty, 2002). In contrast, *A. parasiticus* strains produce all four AFs without CPA biosynthesis (Dorner et al., 1984). Moreover, a "sleeping" CPA cluster was activated by the overexpression of a general secondary metabolism regulator gene (*laeA*) in *A. fumisynnematus* (Hong et al., 2015).

CPA was proposed to modify calcium homeostasis, mitochondria, and cytoplasm membranes based on animal studies (Riley and Goeger, 1992). This mycotoxin serves as a critical pathogenicity factor that enables the saprophytic lifestyle of *A. flavus* (Chalivendra et al., 2017), presumably, through its good iron-chelating characteristics (Riley and Goeger, 1992).

PLANT-FUNGAL INTERACTIONS

Peanut-*Aspergillus flavus* Interaction

It is well-known that multiple mechanisms are involved in host plant defense systems in response to *A. flavus* infection and AF accumulation. Peanut was found to have evolved complex defense mechanisms to resist pathogens, such as blocking the invasion and activating a range of defense responses (Holbrook and Stalker, 2003). Eight hundred forty-two candidate genes were recognized for *A. flavus* resistance in post-harvest seeds (Wang et al., 2016a). Genes involved in defensive responses to *A. flavus* and AF biosynthesis were stimulated in resistant genotype (Wang et al., 2016b).

The plant cell wall, the first line of defense against microbial pathogens, is primarily made up of polysaccharides cellulose, hemicellulose, and pectin. While opportunistic fungi usually infect plants through wounds (e.g., mechanical or pest damages), pathogenic ones actively penetrate cell walls, often through the secretion of a range of polysaccharide-degrading enzymes such as pectinesterase, arabinofuranosidase, mannosidase, and galacturonidase along with amylases or proteases (Whitehead et al., 1995; Bellincampi et al., 2014; Wang et al., 2016b). In peanuts resistant to *A. flavus* infection, feruloyl esterase, pectinesterase, arabinofuranosidase, mannosidase, polygalacturonase, and galacturonidase fungal activities were significantly downregulated compared to the sensitive plants (Wang et al., 2016a). Resistance to *A. flavus* infection is naturally the most critical factor in

avoiding AF exposure to consumers. Pod infection, seed invasion, and AF production in the cotyledon are the crucial steps to be considered (Nigam et al., 2009). The first interaction between the plant and the mold is at the pod shell, where the pathogen resistance depends on the shell structure. The second barrier is the undamaged seed coat. Upon a successful invasion, *A. flavus* colonizes the seed cotyledon and produces AFs. In a proteomic study, a total of 29 seed proteins showed differential expression between the resistant and susceptible peanut cultivars under drought stress in response to *A. flavus* (Wang et al., 2010). Under drought stress, AF production was consistent in peanut pods even if roots of those plants were well watered. Meanwhile, AF was not produced in well-watered peanuts pods, while roots were under drought stress (Sanders et al., 1993).

The data suggest that drought stress is the most critical factor in the interaction of the plant and the fungal agent. Therefore, watering of the fields is crucial along with the improvement of the plant's resistance by genetic modification or selection.

Maize-*Aspergillus flavus* Interaction

Pathogenesis in maize depends on environmental factors (e.g., Payne and Widstrom, 1992; Kebede et al., 2012; Fountain et al., 2014), metabolic state of the kernels (Chen et al., 2010; Jiang et al., 2011), physiological state of the fungus (Jayashree and Subramanyam, 2000), and time elapsed following infection (Scott and Zummo, 1994; Betrán and Isakeit, 2004). Vitreous compared to softer dent type endosperm was positively correlated with AF contamination and resistance to ear rot (Betrán and Isakeit, 2004; Llorente et al., 2004).

Since maize is a favorable host for the *Aspergilli*, especially for *A. flavus*, and the plant's resistance is genetically determined, much effort was invested worldwide to develop resistant maize genotypes. Recent breeding investigations focused on quantitative trait loci (QTL) for AF resistance (Kelley et al., 2012; Fountain et al., 2015), and the studies demonstrated that the resistance to *A. flavus* is highly quantitative and is not conferred by a single gene. Any given QTL was found to account for a rather low level of phenotypic variance explained regarding AF resistance. Resistance thus has a polygenic nature with a combination of multiple traits being involved in the resistant phenotype (Fountain et al., 2014; Yin et al., 2014). Maize inbred lines were found also to vary in their tolerance to CPA (Chalivendra et al., 2017). Moreover, CPA tolerance of the root was in a significant correlation to silk resistance under fungal colonization (Mideros et al., 2012).

During infection, mycelia were detected inside the scutellum, exhibiting a biofilm-like formation at the endosperm-scutellum interface (Dolezal et al., 2013). This biofilm-like structure bears resemblance to the biofilm of *A. fumigatus* in the human lung (Loussert et al., 2010). *In situ* hybridization of RNA showed the expression of the pathogenesis-related protein gene in the aleurone and scutellum of maize seed (PRms) during *A. flavus* infection (Shu et al., 2015). Transcripts of the maize sucrose synthase-encoding gene (shrunken-1; Sh1) were detected in the embryo in non-infected kernels, but the gene was up-regulated in the aleurone and scutellum under *A. flavus* infection. Moreover, the transcripts of PRms and Sh1 showed accumulation in the seeds before infection (Shu et al., 2015).

A recent study was conducted on expression profiling of 267 unigenes (mostly genes of metabolism, stress response and disease resistance) in a mapping population derived from a cross between susceptible and resistant parent plants (Dhakal et al., 2017). It revealed that many genes involved in the synthesis and hydrolysis of starch and sugar mobilization and others related to energy production and/or precursors of lignin and phytoalexins used in the defense response were highly expressed (Dolezal et al., 2014; Shu et al., 2015; Dhakal et al., 2017).

Apart from *Fusarium* infection (Mesterházy, 2008), *A. flavus* causes the most economic loss on cornfields. However, co-infection by these genera is not investigated in detail, and only some aspects are known like the inhibitory effect on AFB1 production by *Fusarium* (Falade et al., 2016), and inhibitory and hyper-parasitic effect of *A. flavus* on Fusaria (Boughalleb-M'Hamdi et al., 2018). Moreover, the physiological effects of the co-produced mycotoxins like CPA and AFs or the effect of the co-infection on mycotoxin productions is rarely investigated (e.g., Marín et al., 2001; Giorni et al., 2016).

Cotton-*Aspergillus flavus* Interaction

Cottonseed can be contaminated pre-and postharvest by *Aspergilli*. A comparative transcriptome analysis was performed investigating the genes expressed differentially in corn, peanut, and cotton under aflatoxigenic *A. flavus* infection (Bedre et al., 2015). Only 26 common genes were identified as candidate *A. flavus* resistance genes in all the three plants. Six of these genes coded for Fe(II)-dependent oxygenase superfamily proteins and 2-oxoglutarate. In response to both non-aflatoxigenic and aflatoxigenic strains, genes encoding alcohol dehydrogenase, UDP glycosylation transferase, and helix loop helix protein were induced (Bedre et al., 2015). Upregulation of primary metabolism modulated signal transduction cascades that were essential to plant defense responses (Rojas et al., 2014). In the pericarp, sucrose and starch metabolism besides glycerolipid metabolism were upregulated under infection with non-aflatoxigenic *A. flavus*. The metabolic pathways activated by the presence of non-aflatoxigenic *A. flavus* in the plant pericarp and seeds compared to aflatoxigenic *A. flavus* activated pathways can lead to possible target genes to develop fungal stress tolerance and resistance in cotton (Bedre et al., 2015).

Phytohormone Guided Interactions

Phytohormones are well-known mediators of fungus-plant interactions with different roles. The abscisic acid (ABA) (Hauser et al., 2011; Xin et al., 2012), salicylic acid (SA) (Janda and Ruelland, 2014), and ethylene (ET) (Bleecker and Kende, 2002; Ton et al., 2002) phytohormonal pathways in plants can act against *A. flavus* and AF production by mediating and channeling many stress-response genes (Bari and Jones, 2009). Transcriptomic analysis revealed DEGs of phytohormone production and signaling in response to AF production in peanut (Wang et al., 2016a). Moreover, DEGs concerning ABA production and signaling showed higher expression in a sensitive peanut genotype than in the resistant plants (Wang et al., 2016b).

Determining the roles of ET is challenging as disease symptoms seem to be either reduced or enhanced or not affected depending on the pathogen-host interaction (Bleecker and Kende, 2002). It inhibits AF biosynthesis in *A. flavus* through alleviation of oxidative stress (Huang et al., 2009). However, DEGs involved in ET production were downregulated in response to AF production, and most of them were also repressed in the resistant genotype. Wang et al. (2016b) concluded that ET might suppress resistance to AF production, and later Wang et al. (2017) found that ET emitted by infected seed facilitated the colonization by *A. flavus* but not AF production in maize, potentially opening up biotechnological applications.

Contrary, SA is suppressive for some fungi (Seyfferth and Tsuda, 2014). SA inhibited mycelial growth and mycotoxin formation of *A. flavus in vitro*, and the *in vivo* evaluation resulted in more significant inhibitory effects for the intact treated pistachio fruit as for injured ones (Panahirad et al., 2014).

Jasmonates are lipid-derived signals compounds in plant growth and development in response to stresses like pathogen attack or drought (Wasternack, 2014). Jasmonic acid (JA) and its metabolites, members of the oxylipin family, are synthesized in the alpha-linolenic acid pathway. Many of them modify gene expression in a regulatory network with synergistic and antagonistic effects concerning other plant hormones such as SA, auxin, ET, and ABA (Wasternack, 2007). Metabolism of alpha-linolenic acid was upregulated in pericarp under both non-aflatoxigenic and toxigenic *A. flavus* infection in comparison to seeds. Similarly, the alkaloid biosynthetic pathway was more intensively upregulated in the pericarp under both non-aflatoxigenic and toxigenic *A. flavus* infection than in the seed. In tobacco host plants, the alkaloid biosynthesis was increased in response to insect foraging and application of JA (Todd et al., 2010). Therefore, it was suggested that the JA-regulated defense response is also stimulated as an answer to *A. flavus* infection (Bedre et al., 2015).

Furthermore, in the case of the aflatoxigenic *A. flavus* infection, upregulation of arachidonic acid (AA) metabolism was detected in seeds, exceeding that under non-aflatoxigenic infection in the pericarp. AA has a role in plants as a signaling compound, and it stimulates plant defense responses through fatty acids. Meanwhile, pathogen AA triggers plant innate immunity resulting in defense responses and programmed plant cell death (Savchenko et al., 2010).

Pathogenesis-Related (PR) Proteins

PR proteins are disease resistance proteins induced in the host plant in response to pathogen infection (Bravo et al., 2003; Luo et al., 2011). Identification and characterization of such plant genes have importance in reducing fungal pathogenicity. In maize, PR-protein genes included PR-1, PR-4, PR-5, PR-10, and chitinase (Dhakal et al., 2017).

The plant hydrolytic enzymes like β-1,3-glucanases and chitinases show antifungal activity owing to the degradation of fungal cell wall components (Cordero et al., 1994; Dolezal et al., 2014). Plant chitinases also have lysozyme activity and are active in preventing mycelial development (Collinge and Slusarenko, 1987; Collinge et al., 1993). The gene expression of chitinase 2 and PR-10 was reported to be upregulated in maize seeds during fungal infection (Cordero et al., 1994). *In vitro* PR-10 protein possessed antifungal activity against *A. flavus*, and its production was upregulated upon *A. flavus* infection in a resistant maize hybrid but not in a susceptible one (Chen et al., 2006). RNAi gene silencing driven repression of PR-10 resulted in an increased susceptibility to *A. flavus* and AF production (Chen et al., 2010). Moreover, overexpression of chitinase genes (Cletus et al., 2013) resulted in resistance against fungal infection in rice (Baisakh et al., 2001) and peanut (Rohini and Sankara Rao, 2001; Prasad et al., 2013).

Besides chitinases (Singh et al., 2015), lectins are also involved in the plant defense mechanisms (Dang and Van Damme, 2015) and probably play an essential role in inhibiting AF production (Hawkins et al., 2015). In resistant and sensitive plant genotypes, chitinase showed different expression levels (Wang et al., 2016a). Eleven chitinase encoding transcripts were expressed differentially in pericarp and seed during infection by both aflatoxigenic and non-aflatoxigenic strains in cotton (Bedre et al., 2015), while in maize seven chitinase genes were associated with the increased *in vivo* resistance to *A. flavus* infection and AF accumulation (Hawkins et al., 2015).

Production of the PR maize seed protein, ZmPRms, was recently shown to be involved in resistance to *A. flavus* and other pathogens in a seed-specific RNA interference study (Majumdar et al., 2017). *A. flavus* infection increased significantly on corn kernels with downregulated *ZmPRms* with a concomitant 4.5–7.5-fold higher accumulation of AFs, presenting the protein's role in evading infection and toxin accumulation (Majumdar et al., 2017).

Plants also produce cell wall polygalacturonase-inhibiting proteins to counteract the activity of fungal polygalacturonases (Kalunke et al., 2015), enzymes that catalyze the hydrolysis of the α-(1–4) linkages between the D-galacturonic acid units in homogalacturonan resulting in cell separation in the plant tissues. The interaction between polygalacturonases and inhibiting proteins promoted the formation of oligogalacturonides, which evoked further defense responses (Federici et al., 2006). In peanut, Wang et al. (2016b) showed that all six DEGs of polygalacturonase-inhibiting proteins were upregulated to a much higher level in a resistant genotype than in a sensitive one.

Oxylipins

Plant's linoleic acid and 9- and 13-hydroperoxy fatty acids (9S- and 13S-HPODE oxylipin products) have a substantial effect on the differentiation processes of *Aspergillus* spp. Both 9S- and 13S-HPODE alter secondary metabolism in *A. parasiticus* and *A. nidulans* (Gardner, 1995; Burow et al., 1997). They also increase the production of the conidiospores in *A. nidulans* and *A. flavus*, and, in *A. nidulans*, elevate cAMP levels (Calvo et al., 1999; Affeldt et al., 2012). Additionally, *A. flavus* infection of peanut seeds promoted linoleate 9-LOX expression and 9S-HPODE accumulation. 13S-HPODE producing lipoxygenase alleles (PnLOX2 and PnLOX3) were highly expressed in mature seed, but these genes were repressed between 5-fold and 250-fold during *A. flavus* infection. The outcomes of these investigations proposed that 9S-HPODE is a susceptibility, while 13S-HPODE

is a resistance factor during *Aspergillus* spp. infection (Tsitsigiannis et al., 2005). Similarly, linoleic acid host-derived oxylipins were also suggested to drive mycotoxin synthesis (Burow et al., 1997; Brodhagen et al., 2008; Reverberi et al., 2010). 13S-HPODE repressed expression of ST and AF biosynthetic pathway genes at concentrations of 10 and 100 μM and, in this way, significantly reduced ST and AF production in both *A. nidulans* (ST producer) and *A. parasiticus* (AF producer) *in vitro* (Burow et al., 1997). The maize ZmLOX3-mediated pathway acted as a root-specific suppressor of all three major defense signaling pathways (Gao et al., 2008a,b).

The oxylipin-driven processes are complicated further by fungal oxylipin production. *A. flavus* single lipoxygenase produced oxylipins influence host responses. Reverberi et al. (2010) found that a lox-like gene mutant *A. ochraceus* was not only failed to produce 13S-HPODE, but a sharp decrease was detected in its OTA production. The conidium formation was also delayed, and the sclerotium production was increased in the cultures. Moreover, seeds infected with the *A. ochraceus* mutant could not produce normal 9S-HPODE levels or induce the defensive PR1, suggesting the importance of the fungal 13S-HPODE in the regulation of host defense response. The oxylipin profile of the maize kernels inoculated with wild type and *lox* mutant *A. flavus* strains showed elevated levels of HPODE and diHODES, also suggesting that the fungal Lox produces compounds that suppress plant oxylipin production. The *ΔAflox1* mutant strain was able to produce AF only on kernels, but not in axenic culture (Scarpari et al., 2014), revealing the complexity of the metabolic interactions.

PSIB α oxylipins derived from linoleic acid in *A. nidulans* were also reminiscent of those produced from seed fatty acids, and the infected seeds were able to influence the fungal development imitating and interfering with signals controlling conidiogenesis (Prost et al., 2005).

Antioxidants

Oxidative stress is a critical factor that can stimulate the synthesis of AF and other SMs (Reverberi et al., 2010, 2013). H_2O_2 and other oxidative agents (Fanelli et al., 1985; Jayashree and Subramanyam, 2000; Narasaiah et al., 2006) activate AF biosynthesis in *Aspergillus* sect. *Flavi* (Reverberi et al., 2008). At the plant-pathogen boundary, ROS production is an essential feature that contributed to *Aspergillus* virulence besides SM production (Reverberi et al., 2013). In seeds contaminated with Aspergilli, a burst of H_2O_2 was detectable within a few hours of infection (Lamb and Dixon, 1997; Kachroo et al., 2003; Reverberi et al., 2008; Peng et al., 2010). For *A. flavus*, it appeared that lowering H_2O_2 levels in the corn embryo helps to prevent *A. flavus* infection and AF accumulation (Magbanua et al., 2007).

Among the stress-related transcripts, the presence of fungal superoxide dismutase in the dent samples indicated oxidative stress, known to be coupled to the production of AFs (Jayashree and Subramanyam, 2000; Fountain et al., 2015, 2016). It is arising that oxidative stress in fungi plays an essential role not only in SM biosynthesis but also in plant-fungal interactions. Within plant tissues, environmental stresses, e.g., drought and heat stress, may also result in the accumulation of ROS and play an essential role in communication between plants and the Aspergilli (Fountain et al., 2014).

In various plant seeds (e.g., maize, sunflower), the processes of lipoperoxidation induce a change in the ratio of oxidants and antioxidants, in favor of ROS accumulation in fungal cells and stimulating synthesis of AFs in *A. flavus* and *A. parasiticus* (Fabbri et al., 1983; Burow et al., 1997; Reverberi et al., 2008; Gao and Kolomiets, 2009). The SM production may be considered as the result of fungal cell response to incomplete scavenging of ROS (Reverberi et al., 2008; Hong et al., 2013).

At the plant's side, DEGs and antioxidant transcripts of glutathione S-transferase, ferredoxin, copper amine oxidase, ascorbate peroxidase, and peroxidase involved in ROS processing and scavenging showed amplified activity during infection with both non-aflatoxigenic and toxigenic *A. flavus* (Bedre et al., 2015). Plant peroxidases also contributed to the response to AF production. DEG peroxidases showed a significantly higher expression in an *A. flavus* resistant peanut genotype than in a sensitive one, indicating better management of ROS in the former during fungal infection (Wang et al., 2016a).

Genes of the phenylpropanoid biosynthetic pathway that produce antimicrobial phytoalexins, phenolic substances, and lignin in plants (Collinge and Slusarenko, 1987; Lawton and Lamb, 1987) were found to show higher expression and more rapid activation in an *A. flavus* resistant maize genotype than in a sensitive one. Moreover, biosynthesis genes of phenylpropanoids, flavonoids, stilbenoids, diarylheptanoids, and gingerol were enriched only in the resistant maize genotype (Wang et al., 2016a). DEGs analysis in cotton inoculated with aflatoxigenic and non-aflatoxigenic *A. flavus* also revealed some significant variances in the expression rates of the genes taking part in the defense mechanisms. For instance, in the pericarp, the phenylpropanoid pathway was enriched at a higher level under aflatoxigenic strain infection than under non-aflatoxigenic infection (Bedre et al., 2015).

The flavonoid pathway is essential in the production of several antifungal compounds and, therefore, it is related to defense reactions (Treutter, 2005). In seeds, the flavonoid biosynthesis pathway was the utmost upregulated under non-aflatoxigenic *A. flavus* infection exceeding the pericarp (Bedre et al., 2015). Numerous studies illustrated the potential impact that flavonoids could exert on SM production. Rutin (quercetin-3-rutinoside) was demonstrated as an effective inhibitor of AFB1 production (Chitarrini et al., 2014). Naringin (flavanone-7-O-glycoside), hesperidin (3′,5,7-trihydroxy 4′-methoxy flavanones 7-rutinoside), and some plant glucosides were characterized for their capacity to restrain mycotoxin production (e.g., patulin by *Penicillium expansum*, *A. terreus*, and *Byssochlamys fulva;* Salas et al., 2012). Similarly, the growth of *A. parasiticus* and its AFB1 production were repressed by methanolic extracts of *Ephedra major* roots (Bagheri-Gavkosh et al., 2009). The inhibition of the growth and AFB1 production of *A. parasiticus* was attributed to quercetin and *p*-coumaric acid flavonoid compounds. In peanut, some stilbenoids (arachidin-1, arachidin-3, and chiricanine A) caused changes in growth rate, mycelial morphology, and spore germination of *A. flavus* (Sobolev et al., 2018). Moreover, a significant decrease or almost complete suppression of AF production was revealed in *A. parasiticus, A. flavus* and *A. nomius* (Sobolev et al., 2018).

Similarly, plants with high concentrations of other antioxidants like β-carotene, β-cryptoxanthin, and total provitamin A also had a reduced amount of AF contamination than hybrids with low carotenoid contents (Suwarno et al., 2019). The relative ease of plant breeding for increased provitamin A as compared to breeding directly for AF resistance suggested novel approaches to suppress AF contamination.

Masked Mycotoxins

Plants metabolize xenobiotic compounds such as mycotoxins as part of their defense mechanisms. In plants, similar to animals, phase I metabolism (enzymatic transformation such as oxidation, reduction, or hydrolysis), phase II process (sulfatation, glucosidation, glucuronidation) (Coleman et al., 1997; Berthiller et al., 2009), and phase III detoxification (sequestration of compounds conjugated to glucose or glutathione into a vacuole or their permanent attachment to the plant cell wall) (Berthiller et al., 2013) can be differentiated. The chemical transformations in phase I are typical for lipophilic compounds, and most of the hydrophilic compounds are not affected by this phase. In phase I, oxidations are catalyzed by the cytochrome P-450 system, while the hydrolysis is catalyzed by esterases and amidases (Coleman et al., 1997).

Plant-metabolized mycotoxins have been identified mostly for *Fusarium* toxins (HT-2 toxin, T-2 toxin, nivalenol, fusarenon-X, deoxynivalenol, zearalenone, fusaric acid; Berthiller et al., 2013) or insecticidal destruxins from *Metarhizium anisopliae* (Pal et al., 2007). The metabolism of some *Alternaria* toxin derivatives and *Aspergillus* mycotoxins was studied using plant cell cultures (Ruhland et al., 1996) and germinating cereals and vegetables (Ruhland et al., 1997). The same OTA derivatives were isolated from all the tested plant species, and the conversion was nearly complete (Berthiller et al., 2013). However, the quantitative distribution strongly depended on the plant species. In addition to ochratoxin α, the main derivatives were (4R)- and (4S)-4-hydroxy-ochratoxin A and β-glucosides of both isomers were detected. Ochratoxin α is considered as a non-toxic molecule, whereas hydroxy-ochratoxin A is as potent immunosuppressant as OTA (Berthiller et al., 2013).

The lack of current studies on plant-modified and masked *Aspergillus* mycotoxins calls for attention to a considerable gap in the understanding of mycotoxins' fate and ecological roles, especially in the case of toxins produced by plant pathogens, such as *A. flavus*.

INTERACTIONS OF THE *ASPERGILLI* AND THEIR MYCOTOXINS WITH SOIL MICRO- AND MACROBIOTA

The possible interactions of fungi in the genus *Aspergillus* with the micro- and macrobiota of the soil can be very diverse ranging from direct physical contact, through non-contact biochemical/enzymatic interactions (e.g., *via* biotransformation), up to volatile organic compounds (VOCs) exerting their effects without physical contact between competing organisms.

Aspergilli and Their Mycotoxins Versus Soil Microbiome

Actinomycetes (e.g., Verheecke et al., 2014), Lactobacilli (e.g., Romanens et al., 2019), Bifidobacteria (e.g., Ghazvini et al., 2016), and Bacilli (Siahmoshteh et al., 2017) are the best-studied groups from these aspects. Several studies have conducted screening on microbial collections to find potential biocontrol isolates that inhibit mold growth, testing (1) bacteria ranging from endophytes and rhizosphere species (Wang et al., 2013); (2) traditional fermentation products (Ahlberg et al., 2017); (3) various other samples where natural interactions with toxigenic molds are far less plausible, as in halophilic soils (Jafari et al., 2018) or fish intestines (Veras et al., 2016). The effects on toxin production and the underlying mechanisms of growth and toxigenic nature are, similarly to yeasts, less understood and often not attempted to uncover. OTA biodetoxification was reviewed by Chen et al. (2018) in detail. Microbes can affect OTA concentration by degradation or absorption and at gene regulation level. OTA biosynthesis genes (*acpks, acOTApks*, and *acOTAnrps*) and the general SM regulator *veA* of *A. carbonarius* were downregulated upon co-culturing with *Streptomyces* isolates, with a concomitant decrease in OTA production (El Khoury et al., 2017). While *acOTAnrps* and *acOTApks*, along with *laeA*, a general regulator of fungal secondary metabolism, were found to be downregulated by *Lactobacillus plantarum* (Lappa et al., 2018).

Close physical interaction between bacteria and fungi induced otherwise silent biosynthesis genes in *A. nidulans* (Schroeckh et al., 2009). These are from a wide range of gene clusters known as silent or non-expressed ones of merely predicted SMs (Keller et al., 2005). For example, the direct physical interaction between *A. nidulans* and actinomycetes resulted in orsellinic acid and lecanoric acid production *via* chromatin remodeling (Netzker et al., 2015) of the fungal culture (Schroeckh et al., 2009). Intimate interaction was also described for plant root-*Bacillus subtilis*-*A. niger* interactions, where *B. subtilis* attached on the surface of the plant root and onto fungal mycelia. Transcriptomic data revealed that both the fungus and the bacterium modified their metabolism during the interaction. The antifungal and antibacterial defense mechanisms of both *B. subtilis* and *A. niger* were reduced upon attachment of bacteria to the mycelia (Benoit et al., 2015). Furthermore, bacterial-fungal interaction can also affect plants negatively, for example, *Salmonella enterica subsp. enterica* serovar. Typhimurium established biofilm on *A. niger* hyphae, where the bacterial growth was promoted, while the bacterial biofilm protected the fungus in a mutualistic relationship (Balbontín et al., 2014). Regarding the maize plant, the co-colonization has more adverse consequences on plant growth than colonization by either microbe individually.

Mycotoxins in soil are subjects of microbial biotransformation, detoxification, or degradation. A wide variety of microorganisms can biotransform mycotoxins (reviewed by Verheecke et al., 2016). Most studies were conducted with AFB1 due to its high toxicity and carcinogenicity. Several bacteria and fungi, including *Rhizopus* sp. (Cole et al., 1972), *Hypomyces rosellus* (*Dactylium dendroides*), and *Corynebacterium rubrum* (Mann and Rehm, 1976) convert AFB1 to aflatoxicol (**Figure 1**) reducing its C-3 keto on the cyclopentanone ring. AFB1 degradation

of *Nocardia corynebacteroides* (*Flavobacterium aurantiacum*) was reported first by Ciegler et al. (1966). However, AFB1 was only metabolized partially and mostly adsorbed to *N. corynebacteroides* cells (Line and Brackett, 1995).

Bacteria can reduce the amount of AFB1 by forming AFB2 with lower toxicity, and by making other compounds (AFG2, aflatoxicol) undetectable. *Myxococcus fulvus* reduced AFB1 by 80.7% (Guan et al., 2010). Teniola et al. (2005) studied *Rhodococcus erythropolis*, and a remarkable reduction (70%) of AFB1 was observed with cell-free extracts, and an almost total (over 90%) degradation was detected within 4 h. *Nocardia asteroides* was also able to transform AFB1 to another fluorescent product (Arai et al., 1967).

Among fungi, *Rhizopus* species, such as *R. arrhizus* (Cole et al., 1972), *R. oryzae* (Knol et al., 1990; Faraj et al., 1993; Varga et al., 2005) and *R. oligosporus* (Kusumaningtyas et al., 2006) have been described as being able to degrade AFB1, whereas several other *Rhizopus* species (Cole et al., 1972) also have been shown to remove AFG1. Non-aflatoxigenic *A. flavus* isolates, *Rhizopus* sp., *A. niger*, and *A. glaucus* (*Eurotium herbariorum*) converted AFB1 to aflatoxicol (**Figure 1**) and vice versa (Nakazato et al., 1990). *Alternaria* sp., *Phoma* sp., *Trichoderma* sp., and *Sporotrichum* sp. have been found to lower AFB1 to 65–99% of the original concentrations (Shantha, 1999). Other fungi, such as *Hypomyces rosellus* (*Dactylium dendroides*) (Detroy and Hesseltine, 1968), *Mucor ambiguous*, *Trichoderma viride* (Mann and Rehm, 1976), *Armillaria tabescens* (Liu et al., 1998), *Phoma* sp. (Shantha, 1999), *Pleurotus ostreatus* (Motomura et al., 2003), and *Trametes versicolor* (Zjalic et al., 2006) have also been described to lower AFB1 concentrations. OTA degradation was demonstrated when applying *Bacillus licheniformis* (Petchkongkaew et al., 2008), *Brevibacterium* species (*B. linens, B. iodinum, B. epidermidis, B. casei*) (Rodriguez et al., 2011), *Acinetobacter calcoaceticus* (Hwang and Draughon, 1994), and *Phenylobacterium immobile* (Wegst and Lingens, 1983). Cell-free supernatants of *Pseudomonas putida* reduced OTA concentration by 8.45–25.70% (Rodriguez et al., 2011). The dimorphic fungus *Apiotrichum mycotoxinivorans* (*Trichosporon mycotoxinivorans*) also degraded OTA (Molnar et al., 2004). *Aspergillus* species such as *A. niger*, *A. fumigatus*, *A. japonicus,* and section *Nigri* species were also able to remove OTA from liquid media (Varga et al., 2000; Abrunhosa et al., 2002, 2014; Bejaoui et al., 2006). Patulin degradation was rarely demonstrated. However, for example, the yeast *Rhodosporidium kratochvilovae* was shown to decrease patulin concentration, whereas the concentration of desoxypatulinic acid increased with time (Castoria et al., 2011). Another possible detoxification mechanism is done by PGUG enzyme from yeast *Meyerozyma guilliermondii* (Chen et al., 2017) or by oxidoreductase from bacteria *Gluconobacter oxydans* (Ricelli et al., 2007). Besides the antagonistic effects of yeasts on mycotoxin production, the cytotoxic and inhibitory effects of the toxins on yeasts (summarized in **Figure 2**) have also been investigated in some cases (reviewed by Pfliegler et al., 2015). In these studies, the well-known model organisms *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* have been studied. The toxic effects of AF and OTA, among other mycotoxins, negatively affected the yield of maize mash fermentation processes (Kłosowski et al., 2010), suggesting considerable toxicity. The mechanism of the AF toxic action was shown to be a DNA replication block (Fasullo et al., 2010). Mutagenic effects were detected after ST exposure (Kuczuk et al., 1978). Furthermore, patulin was found to induce oxidative stress and DNA damage both in fission and budding yeasts (Horváth et al., 2012; Papp et al., 2012; Ianiri et al., 2013), with an additional effect of fluidization of the cytoplasm membrane in *S. pombe* (Horváth et al., 2010).

Yeasts utilize general and oxidative stress response pathways along with potential degradation mechanisms to resist mycotoxin exposure (Iwahashi et al., 2006; Ianiri et al., 2013); thus, variation in sensitivity to mycotoxins is not a surprise. Indeed, *Hanseniaspora uvarum*, *S. cerevisiae*, and *Kluyveromyces marxianus* were all found to be resistant to AF and OTA (Angioni et al., 2007). *Aspergillus* mycotoxin toxicity to bacteria is far less understood. Madhyastha et al. (1994) found *Bacillus* and *Brevibacillus* spp. to be highly susceptible to AFB1, but mostly resistant to OTA (except for *B. brevis* and *B. cereus*). Tested strains of *Pseudomonas, Salmonella, Listeria*, and *Escherichia* were usually unaffected by mycotoxins. Additionally, Kuczuk et al. (1978) demonstrated the mutagenic effects of ST on *S.* Typhimurium.

Biodegradation techniques with higher effectiveness may be developed based on existing data and novel research, by further identifying microorganisms capable of biodegrading mycotoxins, by confirming non-toxicity of degradation compounds, by improving both their toxin tolerance and their degradation abilities, and by testing various modes of application.

Volatile Organic Compounds in Soil Interactions

Fungi interact with plants through VOCs. This phenomenon could play an essential role in fungal pathogenesis. VOCs released by pathogenic fungi could influence plants before any physical interaction between the two organisms. Some VOCs (fatty acid derivatives, terpenoids, phenylpropanoids) are lipophilic; they are small (less than 300 Da) and have high vapor pressure (0.01 kPa or higher at 20°C) and are well known as signal molecules among various organisms. Some of the VOCs (e.g., $C_{15}H_{24}$) were found to be unique to aflatoxigenic *A. flavus* (Zeringue et al., 1993). Different fungal-bacterial interaction leads to the specific initiation of fungal SM genes. The two-way volatile interaction between *A. flavus* and *Ralstonia solanacearum*, a similarly widespread and economically crucial soil-borne pathogenic bacterium of peanut, was studied by Spraker et al. (2014). *R. solanacearum* decreased the production of its major virulence factor extracellular polysaccharide in response to *A. flavus* VOCs, while *A. flavus* responded to the bacterial VOCs by reducing conidiospore production and by increasing AF production on peanut. Arbuscular mycorrhizae are also affected by the Aspergilli. *Funneliformis mosseae* (*Glomus mosseae*) decreased the saprobic *A. niger* population through its effect on the plant, whereas *A. niger* inhibited *F. mosseae* in its extramatrical stage through the production of soluble substances or VOCs (McAllister et al., 1995).

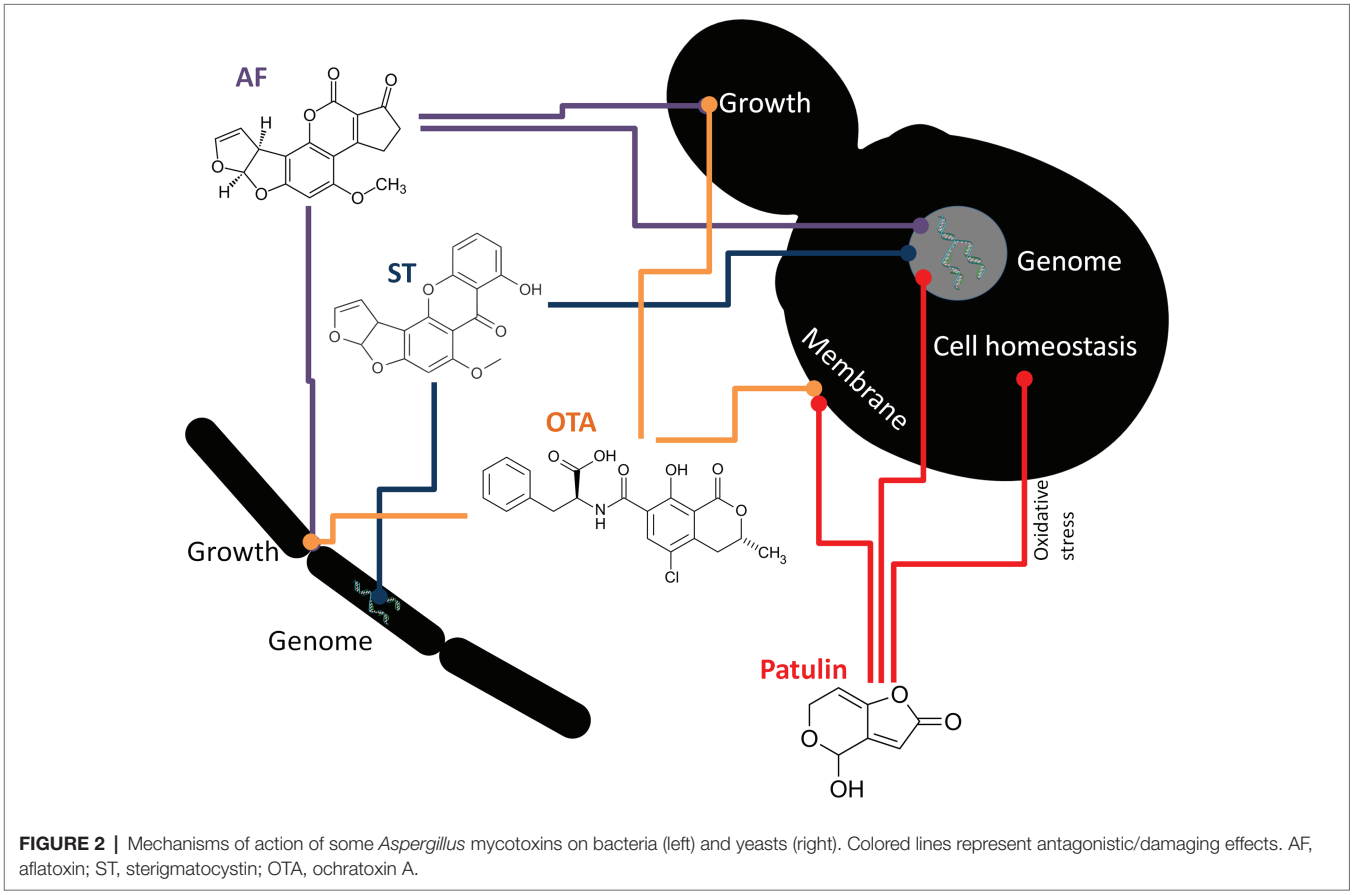

FIGURE 2 | Mechanisms of action of some *Aspergillus* mycotoxins on bacteria (left) and yeasts (right). Colored lines represent antagonistic/damaging effects. AF, aflatoxin; ST, sterigmatocystin; OTA, ochratoxin A.

Application of some special yeasts may cause a direct inhibition of mycotoxin production of filamentous fungi, independently of their growth suppressing effect (Petersson et al., 1998; Hua et al., 2014). However, the effect on toxin production is rarely separated from the growth-inhibiting effect due to methodological constraints. *Wickerhamomyces anomalus* (*Pichia anomala*) is the best-characterized yeast species from this aspect. Hua et al. (2014) recognized 2-phenyl ethanol (2-PE), a volatile compound produced by *W. anomalus* as both growth and AF biosynthesis inhibitor in *A. flavus*. AF biosynthesis genes *aflR* (a positive regulator), *aflC* (polyketide synthase, an early gene in the AF pathway), *aflS* (transcription enhancer), *aflK* (versicolorin B synthase), and *aflO* (O-methyltransferase B) were downregulated more than 10,000-fold following 2-PE treatment. Altered expression patterns were also observed for chromatin-modifying genes (MYST1, MYST2, MYST3, *hdaA, gcn5, rpdA*), influencing mold growth negatively (Hua et al., 2014). On the contrary, a subsequent characterization of the temporal transcriptome response of *A. flavus* to smaller, subinhibitory 2-PE concentration revealed inhibition of CPA and AF biosynthesis genes that can be attributed to stimulating active growth of the mold, a condition that does not favor SM production (Chang et al., 2015). These results highlighted the complexity of fungus-fungus interactions depending on the metabolic state and VOC concentration as delicately controlled as the production of mycotoxins (**Figure 3**).

Streptomyces isolates decreased AF levels when co-cultured with *A. flavus*, and this effect was also linked to suppressing AF regulator gene expression (Verheecke et al., 2015). Subsequently, *S. alboflavus* VOCs (mainly dimethyl trisulfide and benzenamine) were shown to play a critical role in this effect, downregulating genes involved in AF biosynthesis in addition to growth inhibition (Yang et al., 2019). Along with *W. anomalus*, *Hanseniaspora uvarum* and *Pichia kluyveri* yeasts were also found to produce VOCs (most notably 2-PE) that hindered the growth and OTA production of *A. ochraceus* (Masoud et al., 2005; Masoud and Kaltoft, 2006). A follow-up study showed that 2-PE inhibition of OTA production byin *A. carbonarius* and *A. ochraceus* isolates was also inhibited by 2-PE, though was caused by the downregulation of their non-ribosomal peptide synthase, polyketide synthase, and monooxygenase genes (Farbo et al., 2018) and the regulatory *veA* and *laeA* genes (Amaike and Keller, 2009).

Another VOC, ethylacetate, was involved in the biocontrol effects of *Saccharomyces*, *Metschnikowia,* and *W. anomalus* yeasts against various molds, including *A. carbonarius* (Oro et al., 2018). VOCs were also responsible for the biocontrol effect of *Candida friedrichii, Candida intermedia, Lachancea thermotolerans*, and *Cyberlindnera jadinii* (Fiori et al., 2014). However, this effect was species-specific. Only *C. friedrichii* reduced mold growth significantly, while the others only inhibited the fungal sporulation.

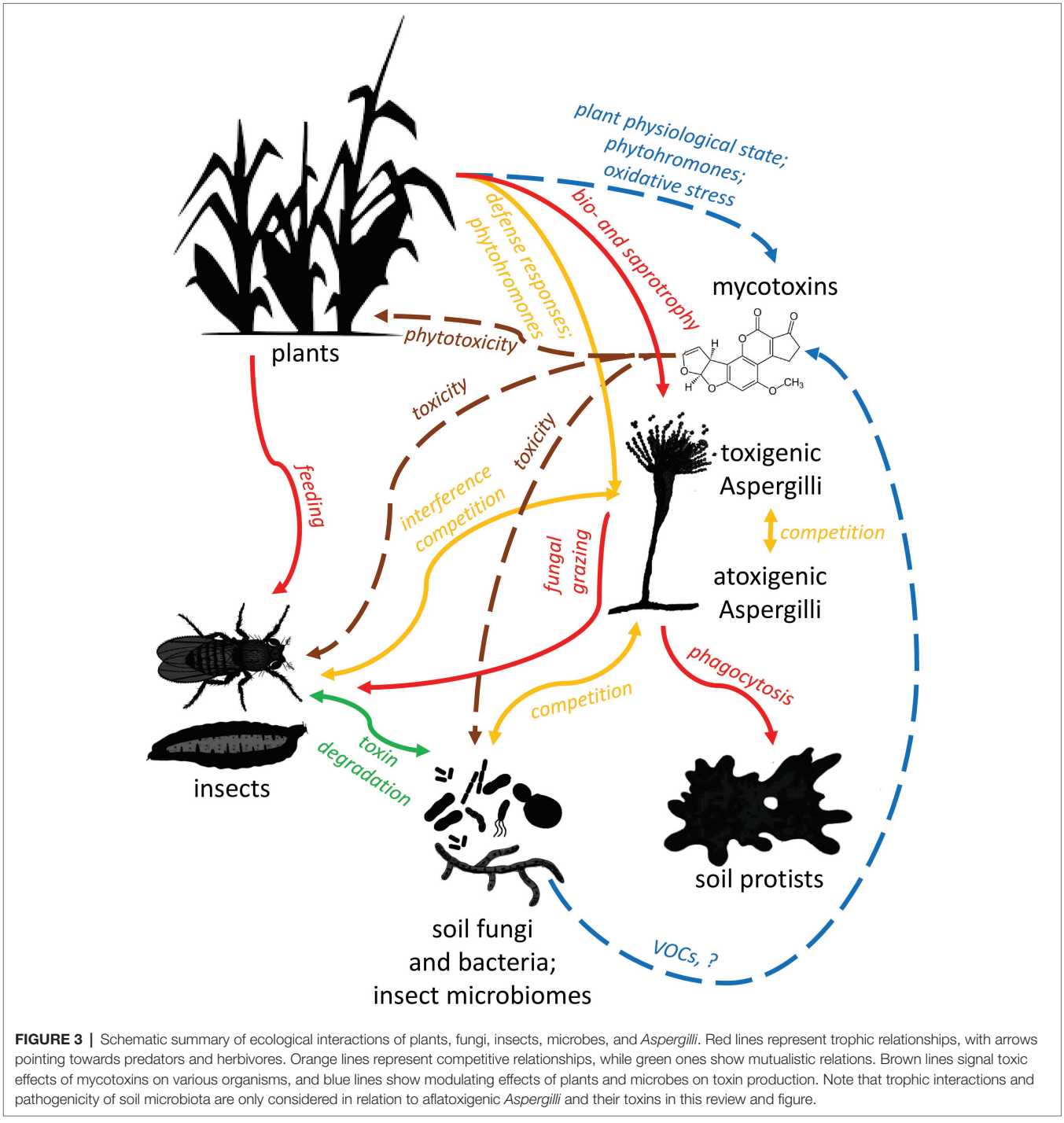

FIGURE 3 | Schematic summary of ecological interactions of plants, fungi, insects, microbes, and *Aspergilli*. Red lines represent trophic relationships, with arrows pointing towards predators and herbivores. Orange lines represent competitive relationships, while green ones show mutualistic relations. Brown lines signal toxic effects of mycotoxins on various organisms, and blue lines show modulating effects of plants and microbes on toxin production. Note that trophic interactions and pathogenicity of soil microbiota are only considered in relation to aflatoxigenic *Aspergilli* and their toxins in this review and figure.

Finally, it should be noted that yeast-mold, and bacteria-mold interactions through VOCs and other factors, including growth inhibition mechanisms and the mechanisms of gene expression alterations in mycotoxin gene clusters, mostly have been tested in solid and liquid co-cultures, i.e., isolated from the plant host. Studies based on results of the last decades thus should focus on disentangling the interplay among microbes *in vivo*, both to understand the microbial ecology of mycotoxin production in crops and to evaluate the utilization strategies.

The *Aspergilli* and Their Mycotoxins Versus Protists

Secretion of mycotoxins and escape from phagocytosis are strategies evolved in molds to counter predation in the natural environment. *A. fumigatus* and free-living amoebal species are both abundant soil organisms with antagonistic relationships. Mechanisms of *A. fumigatus* to avoid ingestion by amoebae were modeled with *Acanthamoeba castellanii* (Van Waeyenberghe et al., 2013). Intra-amoebal passage left a fraction of the

consumed conidia viable. These spores were able to escape the food vacuoles after phagocytosis and germinated intracytoplasmatically, resulting in amoebal death. Interactions with mammalian and avian macrophages and *A. fumigatus* have been compared to these processes, leading to the hypothesis that the ability of the fungus to kill and escape macrophages is a pre-adoptive trait developed in their original ecological niche, namely the soil (Van Waeyenberghe et al., 2013).

Similarly, the slime mold *Dictyostelium discoideum* efficiently consumed fungal spores upon contact with *A. fumigatus,* but the ingestion was more intensive when conidia contained lower amounts of the green spore pigment dihydroxy naphthalene (DHN) melanin (Hillmann et al., 2015). Conidia could survive phagocytosis, and the intracellular germination began only after some hours of co-incubation, which leads to a fatal disruption of the predatory cell. Furthermore, both organisms secreted cross-inhibitory factors that could block fungal growth or induce amoebal aggregation (caused by fungal gliotoxin) with subsequent cell lysis, respectively (**Figure 3**). *A. fumigatus* and related ascomycetes produced the above mentioned DHN melanin in their spores. However, *A. terreus* is a DHN-melanin synthesis deficient fungus and, instead, had a tyrosinase (TyrP), and an unusual NRPS-like enzyme (MelA) expressed under conidiation. MelA produced aspulvinone E, which is stimulated for polymerization by TyrP. The new pigment, Asp-melanin, in addition to its usual function conferring resistance against UV radiation, hindered phagocytosis by soil amoeba. Contrary to DHN melanin, Asp-melanin did not prevent acidification of phagolysosomes. Therefore, it is probable that it contributes to the endurance of *A. terreus* conidia in an acidic environment (Geib et al., 2016).

Furthermore, the antibiotic compound fumagillin produced by *A. fumigatus* is active against microsporidia and several amoebae but is also poisonous when administered to mammals (Stevanovic et al., 2008). However, this substance was widely used in apiculture against amoebal disease (Bailey, 1955).

The *Aspergilli* and Their Mycotoxins Versus Arthropods

Recently, roles of fungal SMs in the ecosystem have been demonstrated by toxicological, behavioral, and experimental evolutionary setups with a still limited number of arthropod species. Using fruit fly larvae (*Drosophila*), the role of AF in protection from fungivores is linked to its role in interference competition (Drott et al., 2017), supporting Janzen's (1977) old and not universally accepted hypothesis (Sherratt et al., 2006). Janzen postulated a fitness advantage of AF production in the presence of soil microbes, vertebrates, or arthropods with which the fungus engages in interference competition. Recent experiments have shown that deterring arthropods indeed confers a fitness advantage to the fungus colonizing nutrient-rich sources (e.g., decaying fruits, seeds, dung, and carrion) (Drott et al., 2017), in addition to the more straightforward and previously described (Caballero Ortiz et al., 2013; Doll et al., 2013) deterring effect on fungal grazers. Mycotoxin production by colonizing fungi may create an adverse environment for arthropods competing for these nutrition sources (Rohlfs and Churchill, 2011). The fact that arthropods, especially insects, are not only competitors of the Aspergilli, but their feeding may predispose the plant or the harvested plant product upon which it feeds to *Aspergillus* infection (Beti et al., 1995; Niu et al., 2008; Ni et al., 2011) further illustrates the complicated tripartite ecological interactions of these molds with plants and arthropods (summarized in **Figure 3**).

Naturally, the production of AFs may exert selective pressure on exposed arthropods to evolve resistance or tolerance mechanisms that can manifest in detoxification mechanisms or active antagonism towards the fungus. Arthropods are very diverse in their interactions with toxigenic molds, ranging from high susceptibility to remarkable tolerance, presumably, resulting from the variable nature of this evolutionary pressure across habitats. Variation in susceptibility to AF and other mycotoxins has been detected by various studies focusing on mycophagous mites (Racovitza, 2009), *Drosophila* species (Rohlfs and Obmann, 2009), soldier fly larvae (*Hermetia illucens*) (Bosch et al., 2017; Camenzuli et al., 2018), the maize weevil (*Sitophilus zeamais*) (Drott et al., 2017), the yellow and lesser mealworms (*Tenebrio molitor* and *Alphitobius diaperinus*) (Bosch et al., 2017; Camenzuli et al., 2018), the navel orangeworm (*Amyelois transitella*) (Niu et al., 2009), the cabbage looper (*Trichoplusia ni*) (Zeng et al., 2013), or the corn earworm (*Helicoverpa zea*) (Zeng et al., 2006; Niu et al., 2008, 2009). It is plausible that species feeding on highly contaminated food sources are selected towards higher tolerance. Maize weevils are remarkable from this aspect: no mortality increase was observed among these pests even when their food sources contained up to 30,000 μg kg^{-1} AFB1 (Drott et al., 2017).

Additionally, using *Drosophila melanogaster* as a model organism, within-species variation in tolerating mycotoxins has also been observed (Rohlfs, 2006). This intraspecific variation may enable populations to adapt to increased fungal competition and mycotoxin exposure, as demonstrated with the same fly species and *A. nidulans* in an experimental evolutionary setup (Trienens and Rohlfs, 2011). The authors concluded that evolved lineages were more tolerant both to fungal and to purified ST exposure without increased resistance, i.e., without increased ability to impair fungal growth. At the same time, grazing by *D. melanogaster* larvae induced resistance in *A. nidulans*. Grazing activated the expression of many putative resistance genes of the fungus, along with *laeA*, the key SM regulator gene (Amaike and Keller, 2011). The reaction to the fungivores co-occurred with gene expression changes in signal transduction, epigenetic regulation, and SM biosynthesis. Reciprocal insect-fungus interactions may select the Aspergilli for inducible resistance resulting in higher fitness in habitats with a high abundance of fungivores (Caballero Ortiz et al., 2013).

Feeding by *D. melanogaster* larvae induced synthesis of methyl farnesoate and juvenile hormone-III in *A. nidulans* upon expressing a heterologous regulatory protein (Nielsen et al., 2013). It indicates the probable importance of juvenile hormone biosynthesis in fungal-insect antagonistic relationships while also raising possibilities in insecticidal strategies, given the developmental and metabolic importance of juvenile hormones in arthropods (Nielsen et al., 2013). Vice versa, insects may also develop behavioral adaptations to respond to toxic fungal competitors.

For example, *Drosophila* larvae have been shown to aggregate around aflatoxigenic *A. nidulans* colonies suppressing fungal growth, improving the chance of larval survival to the adult stage in natural habitats (Rohlfs, 2005; Trienens et al., 2017).

Another fungal-bacterial-insect interaction was described with the connection of an endophytic herbivore, *Dendroctonus rufipennis* (spruce beetle), which is accompanied by an invasion of its galleries by several fungal species (e.g., *A. fumigatus*, *A. nomius*, *Leptographium abietinum*, *Trichoderma harzianum*) (Cardoza et al., 2006). *Trichoderma* and Aspergilli significantly decreased the survival and reproduction of spruce beetle in controlled circumstances. Adult spruce beetle insects exuded an oral secretion, which inhibited the growth of tested fungi except for *A. nomius* or disrupted the fungal morphology in a dose-dependent way. Oral secretions on microbiological media revealed presence of bacteria responsible for the antifungal activity. The isolated bacteria belonged to the Actinobacteria, Firmicutes, Betaproteobacteria, and Gammaproteobacteria taxa that showed species-specific inhibitory activities (Cardoza et al., 2006).

Tolerance requires effective detoxification of food-derived AFs, mechanisms of which have recently been uncovered, but so far only in a few species. *H. zea* has been shown to predispose the plant upon which it feeds to *Aspergillus* infection and concomitant AF contamination, and this pest insect was shown to be able to efficiently metabolize AFB1 into the less toxic AFP1 (**Figure 1**) using cytochrome P450 monooxygenases (Niu et al., 2008). However, the action of these monooxygenase enzymes is not yet fully understood, as some results indicate that bioactivation, not detoxification may also result from their activity in insects (Zeng et al., 2006, 2013). Larvae of *A. transitella,* a significant pest of almonds and pistachios have been shown to metabolize AFB1 into three biotransformation products, mainly aflatoxicol, and to negligible amounts of AFM1 and AFB2a (**Figure 1**). The relatively high production of aflatoxicol may reflect a detoxifying adaptation arising from the often mold-infected habitats of the *A. transitella* (Lee and Campbell, 2000). The codling moth *Cydia pomonella*, a pest infecting walnuts and pome fruits, produced none to low levels of AFB1 biotransformation products, suggesting a lower level of detoxification capability (Lee and Campbell, 2000).

A further aspect of insect mycotoxin tolerance and indirect mold-microbiome interactions may also be relevant: the effects of insect symbionts during mycotoxin exposure (**Figure 3**). Insect microbial symbionts are ubiquitous, incredibly diverse, and their interactions with their hosts are far from being wholly understood (e.g., Dowd and Vega, 2004). At least one symbiotic yeast-like species, *Symbiotaphrina kochii*, can enzymatically detoxify and utilize mycotoxins as carbon sources (along with plant allochemicals and insecticides, even as sole carbon sources) (Shen and Dowd, 1991). More recently, Rohlfs and Kürschner (2010) reported that increased diversity of dietary yeast species benefited *Drosophila* larvae competing with, and exposed to the toxins of *A. nidulans*, by apparently ameliorating the effects of the toxins. These works call attention to the highly under-researched interactions of invertebrate gut microbiotas and toxins. It is plausible that the microbiome of insects and other arthropods, especially of those that are fungal grazers or face interference competition from molds, is an essential factor contributing to the observed variation in resistance to AF and other mycotoxins, and hence the ability of certain arthropods to compete with highly toxigenic molds.

Finally, the application of entomopathogenic fungi is a capable alternative to chemical control of insects, e.g., mosquitoes. *Aspergillus clavatus* from *Oedaleus senegalensis* (Senegalese locust) was highly pathogenic against *Culex quinquefasciatus*, *Aedes aegypti*, and *Anopheles gambiae* mosquito larvae. Application of *A. clavatus* using spore concentrations ranging between 4.3 and 21 $\times$ 10^7 ml^{-1} resulted in 11–68% mortality against *C. quinquefasciatus*, and 37–100% against *A. aegypti* (Seye et al., 2010). Moreover, also in pheromone production, a possible biotechnological application is hiding. The VOC spiroketal (E)-conophthorin (7-methyl-1,6-dioxaspiro[4.5]decane) (Beck and Higbee, 2015) and the isomeric chalcogran are recognized as semiochemicals of some scolytid beetles. Conophthorin is produced by both insects and plants and widely known as a non-host plant VOC from the bark of angiosperm species. Interestingly, VOC production was tested as a response to primary fatty acids of the host plants by non-aflatoxigenic and aflatoxigenic *A. flavus,* as well as *A. niger, A. parasiticus*, *Penicillium glabrum*, and *Rhizopus stolonifera*. On linoleic acid, these fungi formed both spiroketals, while those on linolenic acid emitted only chalcogran. Conversely, no production was detected on palmitic and oleic acid, which also adds a new level of insect-plant-*Aspergillus* VOC interaction (Beck et al., 2012).

Non-aflatoxigenic knockout and low toxin-producing strains of *Aspergillus* are less capable of antagonizing insect populations (Regulin and Kempken, 2018). In addition to balancing selection on mycotoxin production, it must be noted that insect adaptation to mold competition seems to favor tolerance instead of resistance (Trienens and Rohlfs, 2011). Thus, selective pressure on fungi competing with insects is less likely to fuel co-evolutionary arms races or Red Queen dynamics (Rabajante et al., 2015) that would clearly favor more toxigenic strains.

CONCLUSIONS

Because of their economic and public health importance, research on fungal SM mycotoxins has mostly been focused on animal husbandry, the food chain, and human aspects. However, genome data analyses of numerous fungi and the analytical measurements revealed that most of the predicted SM-associated clusters are silent, demonstrating that fungi continue to be a yet undiscovered resource of biologically active molecules. It was also concluded that *A. flavus* might produce metabolites besides well-known mycotoxins that could be underrated contributors to the toxicity to humans and animals. By changing the culture conditions or the genetic regulation to activate silent clusters, new molecules may be discovered that later can be available for medicine or selective biocontrol of fungi or higher eukaryotes.

For a comprehensive understanding of toxigenic molds' ecology and the evolutionary pressures shaping mycotoxin production, interactions with the micro- and macroflora and

fauna in different habitats need to be considered and investigated. The study of the overall role of microbial SMs in natural habitats is now an emerging field. However, the lack of current studies on plant-modified and masked *Aspergillus* mycotoxins calls for attention to a considerable gap in our understanding of mycotoxins' fate and ecological roles.

Some interaction research revealed new levels of regulations of SM gene expressions through chemical interactions even without direct physical contact. Metabolomic studies at the level of VOCs can boost our knowledge to solve the puzzle of the interactions.

Microbial symbionts of insects are ubiquitous and incredibly diverse; however, their interactions with their hosts are far from being wholly understood. The review also calls attention to the highly under-researched interactions of invertebrate gut microbiotas and mycotoxins. The microbiome of insects and other arthropods is an essential factor contributing to the observed variation in resistance to AF and other mycotoxins, and, hence, in the ability of certain arthropods to compete with highly toxigenic molds.

Recently developed and applied plant protection or soil fertilization agents also should be studied focusing on their effects on interkingdom interactions in soil, or on plants and in plant tissues. In connection with this, the recently approved non-aflatoxigenic *A. flavus* strains and fungal preparations are also a subject for further research on interactions of the soil macro- and microbiota. Studying metabolic pathways in pericarp and seeds that are activated differentially by non-aflatoxigenic and aflatoxigenic *A. flavus* may help to identify possible target genes to increase plant tolerance and resistance and to fight AF contamination. Mycotoxin biodegradation techniques with higher effectiveness may also be developed based on the existing data and novel research by identifying further microorganisms capable of biodegrading mycotoxins, by improving both their toxin tolerance and their degradation abilities, and by modification of the application.

This article also wanted to attract attention to the fact that most of the direct and indirect yeast-mold and bacteria-mold interactions have been tested only in *in vitro* conditions. Such studies targeted fungal growth inhibition mechanisms and the gene expression alterations in SM gene clusters. Therefore, studies initiated by the results of the last decades should focus on disentangling the interplay *in vivo*, both to understand the microbial ecology of mycotoxin production in crops and to evaluate the utilization strategies. Therefore, greenhouse or microplot experiments should be applied for the extended data collection.

AUTHOR CONTRIBUTIONS

IP encouraged TP and WP to investigate the literature on interaction. IP and ZG supervised the writing of this work. TP took the lead in writing the manuscript. WP prepared the figures and wrote sections about yeast-fungal, insect-fungal interactions. TP prepared the sections considering microbial, plant, and soil interactions. ZG prepared the section about masked mycotoxins. All authors discussed the review and contributed to the final manuscript.

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REVIEW
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Functional Biology and Molecular Mechanisms of Host-Pathogen Interactions for Aflatoxin Contamination in Groundnut (*Arachis hypogaea* L.) and Maize (*Zea mays* L.)

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Aflatoxins are secondary metabolites produced by soilborne saprophytic fungus *Aspergillus flavus* and closely related species that infect several agricultural commodities including groundnut and maize. The consumption of contaminated commodities adversely affects the health of humans and livestock. Aflatoxin contamination also causes significant economic and financial losses to producers. Research efforts and significant progress have been made in the past three decades to understand the genetic behavior, molecular mechanisms, as well as the detailed biology of host-pathogen interactions. A range of omics approaches have facilitated better understanding of the resistance mechanisms and identified pathways involved during host-pathogen interactions. Most of such studies were however undertaken in groundnut and maize. Current efforts are geared toward harnessing knowledge on host-pathogen interactions and crop resistant factors that control aflatoxin contamination. This study provides a summary of the recent progress made in enhancing the understanding of the functional biology and molecular mechanisms associated with host-pathogen interactions during aflatoxin contamination in groundnut and maize.

Keywords: *Aspergillus flavus*, aflatoxin contamination, host-pathogen interactions, molecular mechanisms, QTLs, groundnut, maize

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INTRODUCTION

Aflatoxins are teratogenic, carcinogenic and immunosuppressive secondary metabolites produced by several *Aspergillus* section Flavi species (Frisvad et al., 2019). The most common aflatoxin-producing species is *A. flavus* (Amaike and Keller, 2011) but, *A. parasiticus*, *A. nomius*, and other species may be important causal agents of contamination in some areas/years (Diedhiou et al., 2011; Probst et al., 2014; Kachapulula et al., 2017; Kumar P. et al., 2017). Aflatoxin-producing fungi contaminate several agricultural commodities such as groundnut, maize, cottonseed, wheat, rice, tree nuts, and chili peppers (Doster et al., 2014; Khan et al., 2014; Kumar P. et al., 2017; Sarma et al., 2017; Ezekiel et al., 2019).

Aflatoxin remains in food and feed even after cooking and drying of the crop because of its heat and freeze stable nature. There are four major types of aflatoxins, namely, AFB_1, AFB_2, AFG_1, and AFG_2 which are discernible based on their blue and green fluorescence under UV light and migration rate. AFB_1, the most potent and toxic, is associated with hepatocellular carcinoma (Liu and Wu, 2010). Consuming contaminated commodities may have chronic and/or acute effects that may lead to mortality (Sarma et al., 2017). In addition to the large array of negative health effects of the toxins, the contamination of crops results in large economic losses to farmers and to countries because of produce rejected by markets seeking aflatoxin-compliant crops (Wild and Gong, 2010; Bryden, 2012). For instance, India could export only 800,000 tons each year despite being 2nd largest groundnut producer in the world, and aflatoxin contamination being one of the major reason behind low export (Suneja, 2019). In semi-arid and arid regions of the United States, and tropical and sub-tropical Asia and Africa, aflatoxin contamination of agricultural products occurs frequently (Cotty et al., 2008; Razzaghi-Abyanehed, 2013; Bandyopadhyay et al., 2016). In such affected areas, mitigation of contamination is necessary to protect the health of consumers, maintain crop competitiveness, and to harness the full potential of crops to ensure food and nutritional security.

Deploying pre- and post-harvest genetic resistance in new crop varieties together with good agricultural practices may provide a permanent solution to this problem (Ayalew et al., 2017; Meseka et al., 2018). In this context, it is imperative to explore and deploy all possible resistance mechanisms/methods to control aflatoxin accumulation in the field followed by best practices in the entire value chain. In the case of groundnut, three different types of resistance mechanisms, namely *in vitro* seed colonization (IVSC), pre-harvest aflatoxin contamination (PAC), and aflatoxin production (AP) have been reported, which are inherited independently (Nigam et al., 2009). In addition, genetic resistance is modulated by high soil temperature and moisture stress which promote higher rates of fungal infection and contamination. To achieve stable genetic resistance against *A. flavus* infection, we believe all three mechanisms should be examined and integrated to effectively provide resistance under field conditions, during harvest, and throughout storage (see Pandey et al., 2019).

Groundnut and maize are among the most aflatoxin-prone crops. Both are commonly exposed to *Aspergillus* infection during pre- and post-harvest stages (Guo et al., 2008). For example in Ghana, these two crops that are considered as staples are frequently infected by *Aspergillus* species, with unsafe aflatoxin levels (Samson et al., 1981; MoFA, 2011; Agbetiameh et al., 2018). In Ghana, as in any other country, aflatoxin-resistant varieties are not commercially available. In addition, farmers typically do not follow good agricultural practices; so contamination begins in the field and may continue until the crops are consumed. Therefore, farmers and traders must receive training and information on good agricultural practices such as timely sowing and irrigation, ensuring adequate dry field conditions before harvest, timely harvesting, and post-harvest management strategies to limit aflatoxin contamination (Dorner, 2004; Jaime-Garcia and Cotty, 2004; Hell et al., 2008; Florkowski and Kolavalli, 2013; Bandyopadhyay et al., 2016). Although some success has been achieved, good management practices are neither very cost effective nor always practical for the resource-poor farmers, or are not effective in reducing aflatoxin content below tolerance thresholds if not used as part of a holistic aflatoxin management strategy. Climate change and frequent extreme weather events, hot and dry conditions, and erratic rainfall have become more pronounced, allowing aflatoxin-producing fungi to thrive, exacerbating the frequency and severity of contamination events (Chen et al., 2015). Heat and drought stresses are the most important abiotic stresses that predispose crops to *Aspergillus* infection and also affect crop productivity.

A promising strategy is the field application of atoxigenic *A. flavus* strains to reduce aflatoxin content in crops. In the United States and several African countries, driven primarily by USDA-ARS and IITA, respectively, the application of carefully selected atoxigenic *A. flavus* strains as biocontrol agents has consistently reduced aflatoxin contamination in commercially produced crops and allowed farmers to enter domestic and international premium markets (Cotty et al., 2007; Dorner, 2009; Mehl et al., 2012; Doster et al., 2014; Bandyopadhyay et al., 2019; Ortega-Beltran and Bandyopadhyay, 2019; Schreurs et al., 2019; Senghor et al., 2019). When applied at the right stage, treated crops accumulate over 80% less and sometimes even 100% less aflatoxin than non-treated adjacent crops. In addition, when biocontrol is used as a centerpiece of a holistic aflatoxin management strategy, lower aflatoxins accumulate in treated crops at harvest and throughout storage (Bandyopadhyay et al., 2019). Research groups in Italy, Argentina, China, Thailand, and Australia have conducted extensive work on biocontrol in addition to the United States and Africa (Alaniz Zanon et al., 2013, 2016; Mauro et al., 2015; Pitt et al., 2015). Although significant progress has been made, there are many countries where the biocontrol technology has not yet been developed and in the meantime other aflatoxin management strategies need to be employed.

In rainfed areas where farmers are subjected to unavoidable biotic and abiotic stresses that influence aflatoxin accumulation, it is paramount to conduct comprehensive genetics and genomics studies for a better understanding of the genetic behavior, genetic

architecture, and molecular mechanisms that govern different types of aflatoxin resistance in groundnut and maize. Several genetic mapping studies conducted in both groundnut and maize have concluded that aflatoxin resistance is a quantitative trait and has complex genetic behavior with high G × E interaction (Chen et al., 2015; Pandey et al., 2019). Hence, by dissecting host-pathogen interactions during fungal infection by aflatoxin producers and aflatoxin contamination, important host-specific, resistance-related genes/proteins/pathways/resistant factors can be characterized in both groundnut and maize. This study focusses on the current status of resistance and molecular mechanisms in these two major crops using different omics approaches such as genetics, genomics, transcriptomics, and proteomics in addition to emphasizing on host-pathogen interactions. We also discuss the research gaps in global efforts to understand resistance mechanisms and translational genomics in developing aflatoxin-resistant groundnut and maize varieties to provide safe products to consumers as well as safeguard the multibillion-dollar industries associated with both crops.

GENERAL CHARACTERISTICS OF AFLATOXIN-PRODUCING FUNGI

Aspergillus is a diverse genus of fungi that contains more than 200 species (Samson, 1992). Among those that produce aflatoxin, the agriculturally important species belong to section Flavi (Frisvad et al., 2019). Within section Flavi, *A. flavus* and *A. parasiticus* are the most common causal agents of aflatoxin contamination and are associated with a large number of crops (Pildain et al., 2008; Probst et al., 2014). *A. flavus* produces B aflatoxins and *A. parasiticus* produces both B and G aflatoxins. Some *A. flavus* strains cannot produce aflatoxin due to deletions or defects in the aflatoxin biosynthesis gene cluster (Chang et al., 2005; Adhikari et al., 2016). *A. flavus* strains may also produce other toxic compounds such as sterigmatocystin, cyclopiazonic acid, kojic acid, β-nitropropionic acid, aspertoxin, aflatrem, gliotoxin, and aspergillic acid (Hedayati et al., 2007); however, their incidence and frequency in field crops and toxicity to humans and animals are not clear.

Based on sclerotia size, *A. flavus* can be classified into two groups, L and S morphotypes. L morphotype produces few, large sclerotia (>400 μm), abundant conidia, and variable aflatoxin levels while S morphotype produces few conidia, abundant small sclerotia (<400 μm), and consistently high aflatoxin levels (Cotty, 1989). Some L morphotype strains do not produce aflatoxin due to lesions in the aflatoxin gene cluster and are known as atoxigenic (Chang et al., 2005; Adhikari et al., 2016). In nature, *A. flavus* produces primarily asexual spores (conidia) (Amaike and Keller, 2011). The fungus lives in the soil as conidia and the sclerotia, aggregates of hyphae that serve as survival structures that germinate to form saprophytically growing mycelia. Conidia are carried by wind or insects to host tissues, where they germinate and infect both aerial and subterraneanly grown organs of agronomically important crops (Cotty, 2001; Amaike and Keller, 2011); hence, insects may act as vectors during crop infection. Sclerotia allow aflatoxin producers to survive in extreme environmental conditions (Wicklow et al., 1993; Payne, 1998). Certain strains of *A. flavus* – both aflatoxin producers and atoxigenic strains – have higher adaptation and increased competitiveness in diverse cropping systems (Mehl and Cotty, 2011; Atehnkeng et al., 2016; Agbetiameh et al., 2019). Further, sexual reproduction has been reported to occur in *A. flavus*, *A. parasiticus*, and *A. nomius* under highly artificial laboratory conditions (Horn et al., 2009a,b) and also in the field after the release of *A. flavus* sclerotia incubated for 6 months (Horn et al., 2014). However, the significance of sexual reproduction in nature needs further studies.

FACTORS AFFECTING TOXIGENICITY AND AFLATOXIN CONTAMINATION

Different biotic factors such as fungal virulence, host susceptibility, insect damage, and abiotic factors such as soil moisture, temperature, high humidity, and mechanical damage while attempting inter-cultivation practices significantly influence *A. flavus* invasion and aflatoxin accumulation in groundnut (Asis et al., 2005). In maize, hot and dry environments (>32°C and >70% RH), drought conditions and damage to kernel seed coat compromise predispose the crop to aflatoxin contamination. Under drought conditions, drought-tolerant varieties accumulate lower aflatoxin levels compared to non-drought-tolerant varieties. High grain moisture increases post-harvest molding and aflatoxin contamination. Hence, proper drying of grains after harvest to 7% moisture level in groundnut and 12% moisture level in maize is ideal to prevent fungal growth (Liang et al., 2009). Temperature is also an important factor as *A. flavus* thrives well in a wide range of temperatures between 10 and 40°C. However, the optimum temperature range for high AP by *A. flavus* is 25–30°C (Gqaleni et al., 1997). Storage conditions largely influence aflatoxin in crops. Storing pods/grains in jute bags provides favorable conditions for *A. flavus* growth. Jute bags can easily absorb moisture because of high porosity which favors rapid growth and multiplication of molds. Purdue Improved Crop Storage (PICS) bags that rely on the principle of hermetic storage have been used to prevent *A. flavus* infestation and aflatoxin contamination during storage (Sudini et al., 2015; Danso et al., 2018, 2019; Walker et al., 2018). Although aflatoxin contamination is more severe in the field during pre-harvest stage, contamination may increase during post-harvest if management practices such as transportation and storage are deficient. Hence, integrated management of aflatoxin contamination during pre-harvest, post-harvest and storage is necessary to reduce aflatoxin contamination and aflatoxin exposure.

GENETICS OF RESISTANCE MECHANISMS

The mechanisms of resistance to infection and reduced AP are quantitative in nature (Warburton and Williams, 2014). In groundnut, the mechanisms include resistance to infection in

the pod wall, resistance to seed invasion and colonization of seed coat, and resistance to AP in cotyledons. At the time of infection, aflatoxin producers have to penetrate the pod wall and then the seed coat to reach the cotyledons, from which they derive nutrients and produce aflatoxin. In groundnut, resistance to pod infection is attributed to pod shell structure, while resistance to seed invasion and colonization are mostly physical and attributed to seed coat thickness, density of palisade cell layers, and the presence of wax layers (Upadhyaya et al., 2002). In the case of maize, resistance mechanisms include good husk coverage, presence of proteins inhibiting fungal growth (Moore et al., 2004; Chen et al., 2010) wax, and cutin layers (Russin et al., 1997; Gembeh et al., 2001). Maize with kernel integrity intact and a living embryo typically accumulates less aflatoxin (Brown et al., 1993).

Generation mean analysis in maize has shown that additive and dominant gene action are important for resistance to AP (Campbell et al., 1997; Busboom and White, 2004). Diallele mating designs were used to study the inheritance of resistance to both *Aspergillus* ear rot and aflatoxin accumulation. These two studies reported that general combining ability had a greater effect on aflatoxin resistance in maize than specific combining ability, suggesting that additive gene effect is more important than dominant gene effect (Darrah et al., 1987; Gorman et al., 1992).

A resistant inbred of maize Oh516 was developed from the cross (B14 × L97) × B14 at Ohio State University and the hybrid derived from testcross Oh516 × B73 showed resistance to *A. flavus* infection and low aflatoxin concentration in grain (Campbell and White, 1995). The resistant inbred lines from testcross Oh516 × B73 were not significantly different from the inbred lines developed from the testcross Tex6 × B73 (Paul et al., 2003). F_1 crosses developed with inbred lines Oh516 or Tex6 had lower aflatoxin concentration in grain than crosses without Oh516 or Tex6. The F_1 cross Oh516 × Tex6 had the lowest aflatoxin concentration in grain of all F_1 crosses. These findings indicate that the resistance mechanism is quantitative in nature and may be governed by multiple genes.

Types of Resistance Mechanisms

Groundnut has three types of resistance mechanisms, i.e., IVSC, PAC, and AP (Nigam et al., 2009; **Figure 1**). Similarly, in maize, the resistance is a sum of (1) prevention of fungal infection; (2) prevention of subsequent growth of the fungus after infection; and (3) inhibition of aflatoxin biosynthesis after infection (Williams et al., 2015). The extent of aflatoxin contamination varies with geographical location, cultural and agronomic practices, storage and processing period.

In groundnut, the majority of contamination occurs in the field. Hence in the context of developing aflatoxin-resistant groundnut cultivars, host resistance for PAC is a preventive approach that is economical and easy to disseminate. Such strategy does not require extra resources for farmers, leaves no chemical residues as a result of fungicide usage, and is an alternative for areas/nations where atoxigenic biocontrol measures are not available (Garrido-Bazan et al., 2018). ICRISAT has been deploying genetics and genomics approaches to understand resistance mechanisms and identify resistant genes/haplotypes to amalgamate all the three resistance mechanisms into a single genetic background in groundnut using genomics-assisted breeding (GAB) (Pandey et al., 2019). In addition to genetic resistance in groundnut and maize, reduced aflatoxin accumulation will require multidisciplinary approaches such as the use of biocontrol agents, good harvesting practices, appropriate drying, and optimal post-harvest storage (Logrieco et al., 2018). In the long run, the development of new breeding lines using introgression of validated quantitative trait loci (QTLs), single nucleotide polymorphism (SNPs) associated with resistance at the pre-harvest and/or post-harvest stages, optimized markers for marker-assisted selection (MAS), marker-assisted recurrent selection (MARS), and genomic selection (GS), can help the farming community grow crop varieties that may accumulate less/minimal aflatoxin.

Physical and Chemical Barriers to Infection

In groundnut, seed coat thickness and its permeability confer resistance against *A. flavus* infection as a seed coat is the outermost layer that acts as a physical barrier (LaPrade et al., 1973). Smaller hila, a more compact arrangement of palisade-like layer of testa, and thicker waxy surface contribute to resistance against *A. flavus* infection (Taber et al., 1973). It has been reported that higher wax and cutin deposits in groundnut lead to resistance to *A. flavus* invasion and AP in resistant genotypes than in susceptible genotypes (Liang et al., 2003b). Hence, the seed coat, wax, and cutin are effective physical barriers to pathogen invasion and colonization. Groundnut testa is a rich source of tannins that inhibit *A. flavus* infection. 5-7-dimethoxyisoflavone (Turner et al., 1975) and tannins (Sanders and Mixon, 1979) have been reported as important inhibitors of *A. flavus* infection. In groundnut, tannins inhibit *A. parasiticus* growth by arresting mycelial growth and reducing AP (Sanders and Mixon, 1979). The basic composition of testa also contributes to the resistance to invasion. A study on protein profiling in a panel of 15 groundnut genotypes revealed that resistant genotypes had higher trypsin content and activity than susceptible genotypes (Liang et al., 2003a).

In maize, trypsin, ribosome-inactivating protein (RIP), and zeamatin act as inhibitors to the infection of *A. flavus* and *A. parasiticus,* and many other fungi (Chen et al., 1998). Resistance to colonization results from a variety of physiological, biochemical, and molecular factors at different levels of infection. Elevated levels of chitinases pCh2 and pCh11 were reported in the aleurone layer of maize in damaged grains colonized by *A. flavus* (Moore et al., 2004). Hence, breeding to strengthen physical features such as thick testa and chemical barriers such as thick cutin and lignin layers can inhibit *A. flavus* infection and aflatoxin contamination. Similarly, improving the aleurone layer of maize with high chitinase and trypsin inhibitor can reduce aflatoxin accumulation.

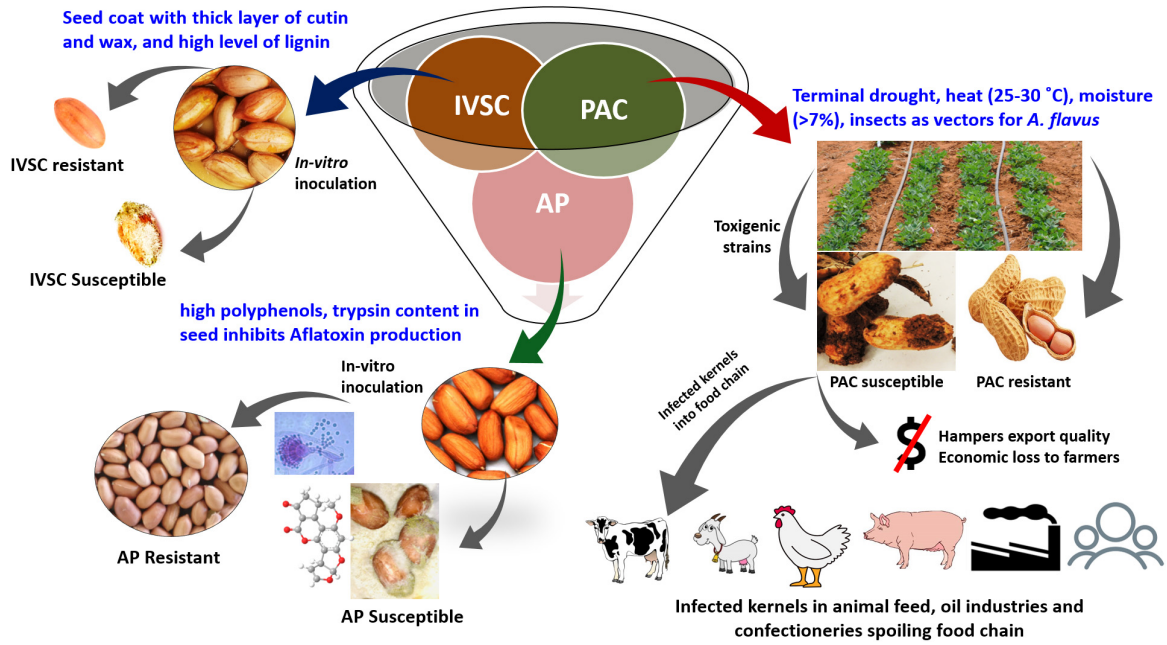

FIGURE 1 | Aflatoxin resistance mechanisms in groundnut. IVSC, *in vitro* seed colonization; PAC, pre-harvest aflatoxin contamination; AP, aflatoxin production.

Constitutive and Induced Resistance Mechanisms

Host plant resistance to biotic stresses has been characterized into two categories, i.e., constitutive and induced resistance. Phytoanticipins confer constitutive resistance while phytoalexins contribute to induced resistance (VanEtten et al., 1994). Secondary metabolites are known to be involved in controlling several immune responses, e.g., callose deposition and programed cell death (Piasecka et al., 2015). Phytoanticipins are antimicrobial metabolites (Pedras and Yaya, 2015). For instance, the groundnut plant produces a variety of phenylpropanoids, such as *p*-coumaric acid, caffeic acid, ferulic acid, methoxycinnamic acid, and mucilagin A, a phenylpropanoid-polyketide-isoprenoid. These metabolites have been known to have antifungal activities against both *A. flavus* and *A. parasiticus* (Sobolev et al., 2006). These phenylpropanoids are likely to function as phytoanticipins in specific groundnut plant tissues (Pedras and Yaya, 2015). Phenylalanine ammonia lyase (PAL) which is a precursor of lignin and phytoalexins, has increased rapidly and reached maximum levels in resistant groundnut genotypes than in susceptible ones (Liang et al., 2001). In the case of membrane lipid peroxidation, the level of malondialdehyde (MDA) increased by 8-fold 2–3 days after inoculation (DAI). Moreover, the generation of O_2^-, H_2O_2, and lipoxygenase (LOX) also increased markedly at the early stage after infection in groundnut (Liang et al., 2002). Resveratrol is an antifungal secondary metabolite or phytoalexin compound found in groundnut seeds (Wang et al., 2015). In resistant genotypes, resveratrol levels increased by 30-fold on the third DAI (Liang et al., 2006). In contrast, the resveratrol level remained unchanged even on the 4-DAI in susceptible genotypes. Plants have several inducible defense responses to pathogens, such as lignification, cell wall cross-linking, phytoalexins, hypersensitive response, production of reactive oxygen species (ROS), and pathogenesis-related (PR) proteins (Liang et al., 2006).

In maize, the first line of defense in response to *A. flavus* results in the activation of expression of transcriptional factors such as WRKY that confer resistance against pathogens (Skriver and Mundy, 1990). WRKY transcription factors were found to be significantly upregulated by *A. flavus* infection in developing maize kernels of resistant maize line TZAR101 (Fountain et al., 2015). ZmWRKY53 is highly expressed in response to a necrotrophic pathogen and also regulates chitinase and peroxidase gene expression. Lignin cross-linking in the cell wall contributes to the resistance to *A. flavus* infection. For instance, less *A. flavus* growth was observed in Mp313E, a maize line that has high cross-linked lignin compared to the susceptible line SC212 (Magbanua et al., 2013). For breeding aflatoxin resistance, the genetic transformation or introgression of resistance genes and transcription factors such as WRKY, PAL, and LOX genes can improve groundnut and maize varieties and reduce the burden of aflatoxin contamination.

GENOMIC REGIONS CONTROLLING AFLATOXIN RESISTANCE

Several QTL mapping studies have been performed leading to discovery of genomic regions for aflatoxin resistance in groundnut and maize (**Table 1**). Each QTL mapping experiment in groundnut has had at least one QTL with phenotypic

TABLE 1 | Key bi-parental QTL mapping and GWAS studies for discovery of genomic regions controlling aflatoxin contamination in groundnut and maize.

Population	Trait	No. of QTLs/MTAs	LOD/*p*-value range	PVE% range	References
Groundnut (*Arachis hypogaea*)					
Bi-parental QTL mapping					
Zhonghua 10 × ICG 12625 (RIL population)	PSII	2	3.1–5.0	8.0–13.0	Yu et al., 2019
	AFB_1	7	3.1–6.4	7.3–17.9	Yu et al., 2019
	AFB_2	5	3.5–8.8	8.3–21.0	Yu et al., 2019
Yueyou 92 × Xinhuixiaoli (RIL population)	Resistance to *A. flavus*	2	2.9–10.5	5.2–19.0	W. Zhuang (personal communication)
***Genome-wide association study* (GWAS)**					
ICRISAT Reference Set 300	Resistance to *A. flavus*	1	9.68×10^{-7}	24.7	Pandey et al., 2014
Maize (*Zea mays*)					
Bi-parental QTL mapping					
M53 × RA ($F_{8:9}$ RIL population)	Resistance to *A. flavus*	8	2.2–5.4	3.6–9.9	Yin et al., 2014
Mp313E × Va35 ($F_{2:3}$ population)	Aflatoxin content	20	2.4–8.0	0.2–21.6	Willcox et al., 2013
Mp715 × T173 ($F_{2:3}$ population)	Aflatoxin content	12	1.8–11.5	2.7–18.5	Warburton et al., 2011
NC300 × Mp717 ($F_{2:3}$ population)	Aflatoxin content	12	–	1.0–11.0	Warburton et al., 2009
B73 × Mp313E ($F_{2:3}$ population)	Aflatoxin content	13	2.9–7.8	5.0–18.4	Brooks et al., 2005
Tex6 × B73 (BC_1S_1)	Aflatoxin content	2	3.8–4.2	16.1–17.8	Paul et al., 2003
Tex6 × B73 ($F_{2:3}$)	Aflatoxin content	3	2.5–5.2	6.7–15.1	Paul et al., 2003
RA × M53 (RIL population)	Amount of Aflatoxin (AA)	1 major QTL (*qAA8*)	8.42	18.23	Zhang et al., 2016
		6 epistatic QTLs	5.0–5.4	14.05–22.6	Zhang et al., 2016
B73 × CML322 (F_2S_5) RIL population	Afl, ICS, IFS, KSP, and SSP	10	2.6–6.2	6.0–16.0	Mideros et al., 2014
B73o2/o2 × CML161 RIL population	Aflatoxin accumulation	9	3.0–4.0	8.0–11.0	Mayfield et al., 2011
B73o2/o2 × CML161 RIL population	Aflatoxin accumulation	9	2.7–3.9	7.8–11.3	Bello, 2007
***Genome-wide association study* (GWAS)**					
Maize inbred lines (346 line)	Aflatoxin resistance	6	5.1–5.5	4.8–6.1	Farfan et al., 2015
Inbred lines (300 line)	Resistance to aflatoxin accumulation (RAA)	107	9.8×10^{-6} to 2.9×10^{-10}	5.4–16.0	Warburton et al., 2015
Maize inbred lines (437 lines)	Amount of aflatoxin (AA)	3	1.1×10^{-8} to 2.1×10^{-7}	6.7–10.4	Zhang et al., 2016
	Resistance to *A. flavus* infection (RAI)	22	3.7×10^{-22} to 8.7×10^{-6}	6.4–26.8	Zhang et al., 2016
Maize inbred lines (287 lines)	Grain aflatoxin levels	298 Maize Cyc pathways	2.9×10^{-10} to 1.0	6.4×10^{-14} to 0.3	Tang et al., 2015

BC_1S_1, selfed backcross population; PSII, percent seed infection index; AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; IVSC, in vitro seed colonization; RIL, recombinant inbred lines; Chr, chromosome; LOD, logarithm of odds; ICS, infection on silk tissue; IFS, infection frequency on silk tissue; KSP, sporulation on developing kernels; SSP, sporulation on silk tissue; Afl, aflatoxin accumulation.

variation explained (PVE) > 10% and reaching up to >20% in some cases. Interestingly in maize, some QTLs were mapped on same genomic regions in different mapping populations which indicated that there are some genes underlying similar function in different studies (Warburton and Williams, 2014; Parish et al., 2019).

In groundnut, very few genetic mapping studies have been reported for aflatoxin resistance. Individual QTLs were identified for AFB_1, AFB_2, and (percent seed infection index; PSII) using a recombinant inbred line (RIL) population Zhonghua 10 × ICG 12625 by Yu et al. (2019). The study identified two QTLs for PSII, one on chromosome A03 with 8.0% PVE and another on chromosome A10 with 13.0% PVE. Seven QTLs were identified for AFB_1 (Aflatoxin B_1) resistance, of which two major QTLs were detected on chromosomes A07 and B06 with 17.9 and 16.3% PVE, respectively. Similarly, five QTLs were identified for resistance to AFB_2, of which chromosomes A07, B05, B06, and B07

recorded higher PVEs of 12.2, 11.1, 21.0, and 14.5% PVE, respectively. Two consistent QTLs for AFB_1 (Aflatoxin B_1) and AFB_2 (Aflatoxin B_2) and one for PSII were identified (Yu et al., 2019). Genetic mapping using a groundnut RIL population Yueyou 92 × Xinhuixiaoli for IVSC identified two major QTLs on chromosomes A03 and B04 with LOD of 10.5 and 2.9 and 19.0 and 5.1% PVE, respectively (W. Zhuang, personal communication). Similarly, genome-wide association studies using a groundnut reference set identified a marker associated with IVSC explaining 24.7% PVE (Pandey et al., 2014). One groundnut MAGIC population using eight genotypes possessing resistance to *Aspergillus* infection and reduced aflatoxin accumulation has been developed at ICRISAT for genetic dissection of component traits.

In the case of maize, major effect QTLs were identified in crosses Tex6 × B73 ($F_{2:3}$) and Tex6 × B73 (BC_1S_1) on chromosomes 3, 4, 5, and 10 with 6.7–17.8% PVE (Paul et al., 2003). Another study (Brooks et al., 2005) conducted in $F_{2:3}$-derived maize populations reported two major effect QTLs for aflatoxin resistance in B73 × Mp313E population that were significant across environments. Other studies in maize have identified one stable QTL in NC300 × Mp717 population which was stable across years. Warburton et al. (2009), three major effect QTLs explaining PVE ranging from 12.1–21.6% in Mp313E × Va35 population (Willcox et al., 2013); small effect QTLs in M53 × Mo17 population (Yin et al., 2014), and single QTL explaining 18.5% PVE in Mp715 × T173 population (Warburton et al., 2011). Similarly, QTL for log aflatoxin accumulations were detected on chromosomes 1, 3, 4, and 9, explaining a total of 17% PVE; while QTL for aflatoxin were detected on chromosomes 3, 4, and 8, explaining a total of 15% PVE in RIL population B73o2/o2 × CML161 (Mayfield et al., 2011). In fact, the same population (B73o2/o2 × CML161) was used earlier (Bello, 2007). QTLs affecting aflatoxin from both parents; however, the favorable alleles for the QTL detected by Bello (2007) were derived mainly from CML161 (Mayfield et al., 2011). In earlier aflatoxin QTL studies, Brooks et al. (2005) evaluated their germplasm in four environments, Paul et al. (2003) used two environments, and Warburton et al. (2009) used four environments. All these studies reported few significant QTLs detected in more than one environment. Warburton et al. (2009) reported the most, with one QTL present in all four environments and one QTL detected in two environments. However, Mayfield et al. (2011) reported three QTLs one on each of chromosomes 1, 4, and 9, across multiple years and environments. In another study by using the B73 × CML322 population, ten QTLs with 6.0–16.0% PVE were found using two QTL mapping methods, six of which were located on the same chromosome segments using both approaches (Mideros et al., 2014). By using various sources of near-isogenic lines (NILs) for selected loci, the resistance QTL located in bin 4.08 was confirmed using a NIL pair. Furthermore, the meta-analysis of QTLs using data from 12 populations indicated that the QTL in bin 4.08 has been reported in four mapping populations. The study showed that the largest-effect QTL, located in bin 4.08, is a good candidate for further characterization and use.

In addition to bi-parental QTL mapping studies, many diverse association panels have been used for genome-wide association study (GWAS) leading to the identification of markers/genomic regions for aflatoxin resistance in maize. For instance, Farfan et al. (2015) identified 6 MTAs for aflatoxin resistance with 4.79–6.06% PVE. In another study (Warburton et al., 2015), GWAS analysis using 300 maize inbred lines identified 107 SNPs associated with aflatoxin accumulation in one or more environments in the association panel. Similarly, in another study using an association panel of 437 maize inbred lines, Zhang et al. (2016) identified 3 MTAs for AA and 22 MTAs for resistance to *A. flavus* infection (RAI). In a comprehensive GWAS analysis undertaken by Tang et al. (2015), 298 maize Cyc pathways were reported to be associated with resistance mechanisms, 17 of the pathways reported high enrichment scores of false discovery rate (FDR) < 0.2, of which the jasmonic acid biosynthesis pathway seems to be a major one for aflatoxin resistance. While these studies are informative, comprehensive efforts are required to perform high resolution GWAS in maize and especially in groundnut so that candidate genomic regions/genes can be identified and validated for breeding applications.

MOLECULAR BASIS OF AFLATOXIN RESISTANCE MECHANISMS

Identification of Resistance-Associated Proteins

Proteomics approaches have identified several plant proteins involved in host-pathogen interaction and in controlling resistance to fungal invasion and toxin production in both groundnut and maize. For instance, in groundnut, a 2D-based proteomics study identified pathways/proteins including resistance-associated proteins (RAPs) which were associated with pre-harvest aflatoxin resistance under drought stress conditions (Wang et al., 2010). That study highlighted the role of iso Ara-h3, oxalate oxidase, PII protein, trypsin inhibitor, SAP domain-containing protein, CDK1, L-ascorbate peroxidase, RIO kinase, and heat shock proteins in reducing aflatoxin accumulation at pre-harvest aflatoxin resistance. Later, Wang et al. (2012) identified several RAPs in groundnut which were key controllers of pathways such as immune signaling, PAMP perception, cell wall responses, and detoxification. The study on effect of H_2O_2-derived oxidative stress on *A. flavus* isolates discovered a sub-set of genes that control fungus pathogenicity, mycelial development, and manage ROS production (Fountain et al., 2018).

In maize, several proteomic approaches have been used to understand the molecular mechanisms involved in host-pathogen interaction and resistance to AP. For instance, RIP and zeamatin were present in higher concentrations in germinating maize kernels and led to decreased aflatoxin levels in susceptible maize kernels and thereby inhibited the growth of *A. flavus* under imbibed conditions (Guo et al., 1997). A similar study has indicated the importance of fungal cell wall degrading enzymes,

particularly isoforms of beta-l,3-glucanase and chitinase, which are induced in maturing kernels in response to *A. flavus* infection and also in maturing uninfected kernels (Lozovaya et al., 1998; Ji et al., 2000). Importantly, antifungal proteins chitinase and zeamatin appear to be associated with the host first and second layer of resistance (Guo et al., 1997), and their constitutive expression in maize can provide resistance against *A. flavus*. Grains of resistant maize genotypes can accumulate inhibitory proteins such as 22 and 28kDa which restrict the growth of the fungus as they are associated key resistant proteins like PR-5 thaumatin-like proteins and zeamatin (Huang et al., 1997; Moore et al., 2004). In another study, the proteome analysis of resistant maize genotypes identified a constitutive expression of 14-kDa trypsin inhibitor that can cause spore rupture and abnormal hyphal development in *A. flavus* (Chen et al., 1998). Also, the trypsin inhibitor produced by maize can inhibit fungal-amylase activity that limits pathogen access to the host food resource (starch) which in turn restrict fungus mycelial growth and sclerotia development (Woloshuk et al., 1997; Chen et al., 1998, 1999).

A proteomic examination of maize seeds has identified several groups of proteins associated with the embryo and endosperm that were significantly upregulated upon *A. flavus* infection. These proteins were grouped into four categories: storage proteins, water stress-related proteins, PR proteins, and antifungal proteins (Chen et al., 2002, 2004b, 2006, 2007, 2012). Storage proteins globulin 1 and 2, water stress responsive related proteins WSI18, aldose reductase, late embryogenesis abundant (LEA; LEA3 and LEA14) and heat stress related proteins (HSP16.9) impart kernel resistance (Chen et al., 2002). Further, glyoxalase I (GLX-I; EC 4.4.1.5), a stress-related protein, directly controls methylglyoxal levels, an aflatoxin inducing substrate, thereby contributing to lower aflatoxin levels in resistant maize genotypes (Chen et al., 2004b). The RAP involves maize PR-10, which exhibits ribonucleolytic and antifungal activities (Chen et al., 2006, 2007); and the genes of encoding PR proteins are usually highly expressed in resistant genotypes (Chen et al., 2007). A United States–Africa collaborative project identified resistant maize inbred lines (Menkir et al., 2006, 2008; Meseka et al., 2018). The project reported the development of 52 BC_1S_4 lines from crosses between five African maize inbreds and five temperate aflatoxin-resistant lines followed by the identification of RAPs related to antifungal, stress-related, storage or regulatory protein categories (Chen et al., 2012). Resistant inbred lines of maize are known to express higher levels of chitinase and proteins associated with phenylpropanoid metabolism pathways (Peethambaran et al., 2009; Pechanova et al., 2011).

Using multiple approaches in groundnut and maize have led to the identification of several moderate/low/high resistant lines for *A. flav*us infection and reduced aflatoxin contamination. These advances have facilitated the development of aflatoxin-resistant transgenic groundnut (Sharma et al., 2018) and maize (Thakare et al., 2017); and it is expected that in the coming years, farmers may have access to superior and aflatoxin-resistant varieties. However, the release of transgenic cultivars is dependent on their acceptance by regulators in the target countries. To date, the use of transgenic maize is accepted only in South Africa and Sudan in Africa. A summary of different proteomic studies in maize and groundnut is provided in **Table 2**. Cumulatively, these studies enhance our knowledge of target proteins in order to identify protein encoding resistance genes in response to aflatoxin contamination in these crops.

Identification of Candidate Genes

Functional genomics provides new insights into a wide number of candidate genes associated with resistance to aflatoxin contamination in both groundnut and maize (**Table 3**). In the case of groundnut, transcriptomics studies have identified candidate genes, pathways, and the regulatory networks for the three resistance mechanisms of aflatoxin accumulation (IVSC, PAC, and AP). Earlier efforts to identify resistance/differentially expressed genes in groundnut were based on EST or microarray-based techniques (Luo et al., 2005; Guo et al., 2011; Wang et al., 2013). The gene expression profiling approach was deployed by Luo et al. (2005) in A13 drought-tolerant and pre-harvest aflatoxin-resistant groundnut genotypes in which a cDNA microarray containing 384 unigenes was selected from two cDNA libraries. Overall, the microarray-based screening approach identified defense responsive (Kunitz-type trypsin inhibitor, auxin repressed protein, cystatin-like protein), signaling component (ethylene-responsive protein, calcium-binding protein), ion-proton transporter (aquaporin 1), stress proteins, and secondary metabolites (lipoxygenase 1) resistance genes in groundnut in response to *A. parasiticus* infection under drought stress (Luo et al., 2005).

To understand the molecular mechanism of host-mediated resistance, a separate study was conducted in *Aspergillus* resistant (GT-C20) and susceptible (Tifrunner) genotypes of groundnut which identified 52 highly and 126 moderately expressed genes (Guo et al., 2011). This study reported several important genes including lipoxygenase, lea-protein 2, proline-rich protein, cupin//Oxalate oxidase, among others, in response to *A. flavus* infection. Some studies have suggested the possible involvement of LOX pathway in the production of jasmonic acid which plays hormone-like regulatory and defense-related roles in plants (Royo et al., 1996; Kolomiets et al., 2001; Yan et al., 2013; Ogunola et al., 2017).

Studies have reported that LOX genes also play a major role in plant defense mechanisms, growth, and developmental processes (Kolomiets et al., 2001, 2018; Gao et al., 2008; Park and Kolomiets, 2010). In this emerging field, more investigations are needed on host-pathogen cross-talk communication that fungi use to exploit the plant host in order to meet their biological needs (Christensen and Kolomiets, 2011). Some LOX genes have been shown to play an important role in plant defense resistance and in mediating fungal colonization and toxin production (Battilani et al., 2018).

A microarray study representing 36,158 unigenes was used to identify genes associated with aflatoxin resistance in groundnut (Wang et al., 2013), providing insights into the co-regulation of multiple pathways such as host defensive

TABLE 2 | List of key proteins and their functions associated with resistance to aflatoxin contamination in groundnut and maize.

RAPs	Function	References
Groundnut		
Oxalate oxidase	Seed storage protein	Wang et al., 2010
Trypsin inhibitor	Antifungal compound	
SAP domain-containing protein	Abiotic stress tolerance protein	
L-ascorbate peroxidase	Regulates antioxidant metabolism	
Iso Ara-h3	Seed storage protein	
Heat shock protein precursor	Regulates heat shock factors	
LRR receptor serine/threonine kinase	PAMPs perception	Wang et al., 2012
Protein phosphatase 2A regulatory B subunit	Dephosphorylation	
Pentatricopeptide repeat-containing protein	RNA stabilization	
Esterase_lipase	Lipid metabolism	
Cytochrome P450	Degrades toxins	
Maize		
Zeamatin	Antimicrobial, fungicide	Guo et al., 1997; Huang et al., 1997; Chen et al., 2002
Ribosome-inactivating protein (RIP)	Protein synthesis inhibitor	Guo et al., 1997
Chitinase	Hydrolytic enzymes that degrade chitin	Guo et al., 1997; Ji et al., 2000; Chen et al., 2002
Glucanase	Destroys cell wall of fungi	Guo et al., 1997
Beta-1,3-glucanase	PR-2 family protein, antifungal	Lozovaya et al., 1998; Ji et al., 2000
PR-5 thaumatin-like protein	PR protein	Huang et al., 1997
Globulin-1,2	Seed storage proteins	Chen et al., 2001, 2002, 2006, 2012
Endochitinase	Degrades chitin molecule at random point	Huang et al., 1997
14-kDa trypsin inhibitor	Spores rupture and cause abnormal hyphal development	Chen et al., 1998, 1999
LEA3,14	Stress responsive proteins	Chen et al., 2002, 2006, 2012
WSI18 and aldose reductase	Osmo-stress responsive and oxidative stress responsive proteins	Chen et al., 2002
HSP16.9 (Heat stress related)	Stress responsive protein	Chen et al., 2002
Glyoxalase I	Controls methylglyoxal level as it stimulates the expression of *aflR*, an aflatoxin regulatory gene	Chen et al., 2004a
PR-10	Disease resistance	Chen et al., 2006
Stress-related-peroxiredoxin antioxidant (PER1)	Antioxidants proteins that protect against oxygen species	
Heat shock proteins (HSP17.2)	Stress responsive proteins	
Antifungal trypsin inhibitor protein (TI)	Inhibits *A. flavus* growth	
Cold-regulated protein (COR)	Inhibits germination of *A. flavus* conidia and mycelial growth	Chen et al., 2006, 2012
Superoxide dismutase	Enhances oxidative stress tolerance	Chen et al., 2012
Peroxiredoxin	Enhances oxidative stress tolerance	
Cupindomain-containing proteins	Seed storage protein	
Putative lipid transfer protein	Stress responsive	
Eukaryotic translation initiation factor 5A	Plays a role in plant growth and development	
Abiotic stress responsive proteins	PR protein and stress responsive	Peethambaran et al., 2009
PRm3 chitinase	Fungal cell wall degradation and stress resistance	
Chitinase 1	Defense mechanism in response to biotic stress	
Chitinase A	Suppresses fungal growth	
Phenylpropanoid metabolism	Secondary metabolite production	Pechanova et al., 2011

responses including carbohydrate biosynthesis/metabolism, transmembrane transport, coenzyme A biosynthesis, oxidation-reduction, proteolysis metabolism, etc., during aflatoxin resistance. Modern approaches such as RNA-seq have been used to identify host resistance associated pathways in different crops including maize and groundnut. For instance, in case of groundnut, an integrated IVSC and RNA-seq approach that analyzed the four different stages of infected seed samples from J11 (resistant) and JL24 (susceptible) identified 4,445 differentially expressed unigenes (DEGs) that were involved in multiple pathways such as defense-related, PR or metabolic pathway targeting genes provided a more solid understanding of cross-talk between host-pathogen interactions (Nayak et al., 2017).

TABLE 3 | A summary of some transcriptomics studies to identify candidate genes involved in aflatoxin contamination in groundnut and maize.

Candidate genes	Functions of candidate genes	References
Groundnut		
Seed maturation protein LEA 4	Stress responsive protein	Guo et al., 2008
Serine protease inhibitor	Involved in inflammatory responses	
Cu/Zn superoxide dismutase II	Antioxidant defensive protein	
Serine protease inhibitor	Involved in inflammatory responses	
Lipoxygenase	Regulates jasmonic acid signaling pathway	Guo et al., 2011
Proline-rich protein	Stress responsive protein	
Cupin//Oxalate oxidase	Seed storage protein	
LEA-protein 2	Stress responsive protein	
Brassinosteroid Insensitive 1-associated Receptor kinase 1	Defense response	Wang et al., 2013
3-ketoacyl-CoA synthase	Fatty acid biosynthetic process	
Em protein	Stress responsive	
TIR	Defense response	
Defensin	Defense response	
Mitogen-activated protein kinase	Signaling cascade gene	Wang et al., 2016
PR proteins	Disease resistance	
Nucleotide-binding site-leucine-rich repeat proteins	PAMPs perception	
Polygalacturonase inhibitor proteins	Inhibit polygalactouronase produced by the fungal pathogen	
Abscisic acid insensitive5	Participates in ABA signaling pathway	Clevenger et al., 2016
BLH1	Modulates seed development	
Respiratory burst oxidase homolog	Regulates numerous plant cell responses	
13S-lipoxygenases	Lipid metabolism	
PR-2	Disease resistance in plants	
Deoxy-chalcone synthase	Synthesizes phytoalexins	
Resveratrol synthase	Biosynthesis stilbene type-phytoalexins	Nayak et al., 2017
Chalcone synthase	Involved in the flavonoid biosynthesis pathway	
Epoxide hydrolase	Detoxification of reactive epoxide	
Receptor-like kinases	Cell wall signaling	
9s-LOX	Lipid metabolism	
WRKY genes	Transcriptional regulators; regulates plant development	Korani et al., 2018
Toll/Interleukin-1 receptor-nucleotide-binding site leucine-rich repeat (TIR-NBS-LRR)	Defense responsive	
α-linolenic acid metabolism	Lipid metabolism	
Hevamine-A	Defense protein	Zhao et al., 2019
PR proteins	Disease resistance	
Chitinase	Hydrolytic enzymes that degrade chitin	
Maize		
Kunitz-type trypsin inhibitor	Serine protease inhibitor activity	Luo et al., 2005
Auxin repressed protein	Regulates growth and disease resistance	
Cystatin-like protein	Defense mechanism	
Lipoxygenase 1	Regulates the jasmonic acid pathway	
Ion-proton transporter (Aquaporin 1),	Accelerates oxidative stress and cell signaling	
Glutathione S-transferase	Antioxidant	
Heat shock protein	Defense mechanism; regulates heat shock factors	
PR protein 1	Disease resistance	
ADP glucose pyrophosphorylase	Starch metabolism	Luo et al., 2009
1-acyl-glycerol-3-phosphate acyltransferase	Lipid metabolism	
Lipoxygenase	Regulates the jasmonic acid pathway	
Oleosin 17	Oil body formation and storage protein	
Abscisic acid inducible gene	Defense-related genes	
Chalcone synthase C2	Involved in the flavonoid biosynthesis pathway	
Glutathione transferase	Antioxidant gene	Luo et al., 2011
Leucine-rich repeat-like protein	Biotic stress-related gene	

(Continued)

TABLE 3 | Continued

Candidate genes	Functions of candidate genes	References
ABI3-interacting protein 2	A transcription factor of the abscisic acid signal transduction pathway that plays a role in seed development	
Beta-1,3-glucanase	Classified in PR-2 family of PR proteins, antifungal	
Zeamatin-like protein	Antimicrobial, fungicide	
PR genes	PR genes	
Phosphoglycerate dehydratase 1	Plays a role in catalysis	Luo et al., 2010
Heat shock protein 90	Signal transduction and stress responsive	
Glycine−rich protein	Stress responsive and signaling	
Cytochrome P450	Degrades toxins	
Ethylene-responsive element binding factor	Regulates jasmonic acid signaling pathway	
9-oxylipins	Suppresses aflatoxin biosynthesis pathway	Fountain et al., 2013
Lipoxygenase-3 (LOX3)	Regulates jasmonic acid signaling pathway	
PR proteins	Disease resistance	
NUP85-like genes	Transports RNA, R-proteins and macromolecules from the nucleus to the cytoplasm	Kelley et al., 2012
Heat shock protein (HSP101)	Molecular chaperone protein	
Molecular chaperones	Plays a role in protein folding	
Cinnamoyl-CoA	Synthesizes lignin compounds	
PR-4	Antifungal proteins play a role in pathogenicity	Dhakal et al., 2017
Leucine-rich repeat family protein	Highly conserved region for disease resistance genes	
DEAD-box RNA helicase	Defense-related signaling	
Fructose-1,6-bisphosphatase	Carbohydrate metabolism	
Plant receptor protein kinases (RPK)	Senses pathogen signals and accelerates defense	
Cysteine proteinase inhibitor	Stress responsive	
PR-1, PR-4, PR-5, PR-10	Disease resistance-related genes	
CC-NBS-LRR	Conserves disease resistance genes	Shu et al., 2017
LRR-RLK	Conserves disease resistance genes	
Thaumatin- like protein	Regulates host defense mechanism	
Chitinase	Hydrolytic enzymes that degrade chitin	

Likewise, an RNA-seq-based approach was deployed in groundnut to identify genes that confer resistance during PAC (Clevenger et al., 2016). The study was able to associate the role of abscisic acid (ABA) signaling pathway during drought stress-induced aflatoxin contamination and/or PAC, and also revealed the role of genes from the fatty acid metabolism, cell wall restructuring and morphology, sugar metabolism and nitrogen metabolism pathways during *A. flavus* contamination in soil. Recently, Zhao et al. (2019) suggested the role of hevamine-A protein in groundnut during PAC resistance. Hevamine-A protein is an enzyme with chitinase activity that is also coordinated with PR proteins and can directly inhibit the growth of *A. flavus* (Zhao et al., 2019).

Post-harvest aflatoxin contamination can take place during drying, storage or transportation due to increase in humidity and/or insect damage, thereby promoting *A. flavus* infection. To understand the post-harvest resistance mechanism, Wang et al. (2016) performed global transcriptome profiling in the grains of resistant (Zhonghua 6) and susceptible (Zhonghua 12) genotypes of groundnut and identified 30,143 DEGs, of which 842 were defense-related genes, including mitogen-activated protein kinase, PR proteins, leucine-rich repeat receptor-like kinases transcription factors, nucleotide-binding site-leucine-rich repeat proteins, polygalacturonase inhibitor proteins, and ADP-ribosylation factors in response to AP by *A. flavus*. A recent study by Korani et al. (2018) provides new insights into post-harvest resistance mechanism in response to *A. flavus* infection by comparing the seed transcriptome of resistant (ICG 1471) and susceptible (Florida-07) groundnut cultivars. The study identified 4,272 DEGs and showed the importance of WRKY TFs, heat shock proteins and TIR-NBS-LRR in providing resistance. Further, this study also showed the altered expression of genes associated with protein processing in the endoplasmic reticulum, spliceosome mediated protein degradation and α-linolenic acid metabolism.

In maize, gene expression analysis of inbred line Tex6 identified 8,497 positive array spots including genes related to disease resistance (chitinase, zeamatin-like protein, endochitinase B precursor, PR-1;4;5), stress responsive (heat shock proteins, auxin responsive factor-1, D-type cyclin), ROS scavenger (glutathione S-transferase, superoxide dismutase), and defense-related genes, as well as storage protein genes and lipid metabolism genes (Luo et al., 2009). Further, Luo et al. (2010) have shown that jasmonate and abscisic acid biosynthetic and signaling pathways play crucial roles in drought-induced *A. flavus* infection and accumulation of aflatoxin in maize. The transcriptomic study of resistant maize (Eyl25) with susceptible (Eyl31) lines identified 530 DEGs

including defense-related genes; beta-1,3-glucanase, zeamatin-like protein, trypsin inhibitor, and PR genes (Luo et al., 2011). Fountain et al. (2013) have highlighted the role of WRKY TFs in conferring resistance to *Aspergillus* infection and subsequently in reduced PAC in maize genotype. The transcriptomic study of maize kernels in two resistant inbred lines (Mp313E and Mp04:86) and two susceptible inbred lines (Va35 and B73) under artificial inoculation conditions identified NUP85-like genes in resistance (Kelley et al., 2012). The NUP85-like protein is a major part of nuclear pore complex (NPCs) and is involved in the transportation of RNA, R-proteins, and other macromolecules from the nucleus to the cytoplasm (Cheng et al., 2009; Garcia and Parker, 2009). A few more genes like heat shock protein (HSP101), metallothionein-like protein (MTLP), lecithin cholesterol acyltransferase (LCAT)-like gene, Prenylated Rab PRA1 proteins, molecular chaperones, and detoxification proteins were found to be highly expressed in resistant maize inbred line Mp313E. Some genes including a nuclease-phosphatase domain superfamily protein, a cinnamoyl-CoA, a heat shock protein HSP18a, and few significantly mapped genes like lysine-rich RNA binding domains, large and small ribosomal units had significantly higher expression in susceptible line Va35 than in resistant line Mp313E (Kelley et al., 2012).

Climate change has a devastating impact on mycotoxin production and fungal infection. Functional genomics tools have shown the impact of elevated CO_2 levels on *aflR* gene (an aflatoxin biosynthetic regulatory gene) in *A. flavus* (Gilbert et al., 2016). A cDNA library of Mp715 (resistant inbred) and B73 (susceptible inbred) was designed to differentiate expression patterns for aflatoxin accumulation in maize, and those cDNA clones were mapped onto the maize genome by *in silico* mapping (Dhakal et al., 2017). This study identified 267 unigenes related to stress tolerance, metabolism, disease resistance, PR-4, and leucine-rich repeat family protein. A comparative study of maize kernels infected with *A. flavus* and *F. verticillioides* identified several candidate genes such as PR-1, 10,4,5,10.1; chitinase, CC-NBS-LRR, LRR-RLK, and Thaumatin-like proteins that showed temporal expression patterns during infection/stress (Shu et al., 2017). Several environmental/external factors affect the expression of transcripts, thus influencing the colonization of *A. flavus* and subsequently toxin production. For instance, the antifungal fumigant benzenamine affects aflatoxin biosynthesis, development, and virulence in *A. flavus* by downregulating the *LeaA* regulatory factor, thus acting as a fumigant against *A. flavus* (Yang et al., 2019).

Transgenic Approaches for Resistance to *A. flavus* Infection and Aflatoxin Contamination

Several transgenic approaches including expressing protein/enzyme that can reduce fungal infection or degrade the toxin have been deployed in groundnut and maize to mitigate aflatoxin contamination (**Table 4**). In groundnut, very few reports on transgenic approaches are available substantiating the importance of host genes like *PR* and *defensin* (Xie et al., 2013; Arias et al., 2015). A study (Sharma et al., 2018) has shown that the overexpression of *Medicago* defensin genes-*MsDef1 and MtDef4.2* reduced *Aspergillus* infection as well as AP in susceptible groundnut variety JL 24. The study also demonstrated a host-induced gene silencing (HIGS) mediated silencing of aflatoxin biosynthetic pathway regulatory genes *aflM* and *aflP* to inhibit AP. Notably, both OE−Def and HIGS lines showed remarkably reduced levels of aflatoxin B_1 ranging from 1 to 20 ppb compared to the wild type cultivar that accumulates up to > 4,000 ppb.

Various studies on maize provide insights into using transgenic approaches and the knowledge of precise engineering strategies to improve food safety. A key approach is RNA interference (RNAi), a technology that limits the transcription of a target gene. This approach has been deployed to silence RAP genes (PR-10, GLXI, TI) in maize to identify the key role of RAPs in host resistance mechanism against *A. flavus* infection (Chen et al., 2004a, 2010). RNAi Pr10 silencing construct was introduced in maize plants showing increased susceptibility to *A. flavus* colonization and aflatoxin accumulation (Chen et al., 2010). Notably, PR-10 was involved in enhancing plant stress tolerance and severe suppression of their PR protein encoding genes drastically increased susceptibility to *A. flavus* infection (Xie et al., 2010; Majumdar et al., 2017). Recently, *aflC* and *aflR* genes were targeted that encode the enzyme in *Aspergillus* aflatoxin biosynthetic pathway to develop aflatoxin-free transgenic kernels (Masanga et al., 2015; Thakare et al., 2017). Also, thanatin, a growth inhibitor of *A. flavus*, was overexpressed in maize, reducing aflatoxin contamination and increasing resistance by three to four-fold resistance (Schubert et al., 2015).

In a recent study, expression analyses of polyamine (PA) metabolism/transport genes during *A. flavus*-maize interaction showed significant increase in the expression of arginine decarboxylase (*Adc*) and *S*-adenosylmethionine decarboxylase (*Samdc*) genes in the maize host and PA uptake transporters in the fungus (Majumdar et al., 2018). This study suggested that future studies targeting spermidine biosynthesis in *A. flavus*, using RNAi-based host-induced gene silencing approaches, may be an effective strategy to reduce aflatoxin contamination in maize and possibly in other susceptible crops. In contrary, Gressel and Polturak (2018) report that RNAi technology can't help post-harvest AP as it may have only limited utility when the grain has been dried. However, the dormant state of seeds is usually alleviated during post-harvest storage conditions or under low moisture conditions and cannot accelerate the production of hpRNAs/siRNAs (Majumdar et al., 2017). Even in the post-transcriptional state, RNAi negatively regulates gene expression and does not produce any protein or enzyme in the host plant (Majumdar et al., 2017). Fakhoury and Woloshuk (1999) produced a mutant strain (101) of *A. flavus* which was defective in the α-amylase activity. The α-amylase enzyme is crucial in *A. flavus* as it is involved in the degradation of the host's carbohydrate reservoir which is an essential energy source for fungus growth and reproduction, as well as AP. Therefore, an α-amylase inhibitor protein (AILP) that inhibits α-amylase activity was expressed in the host; this reduced fungus growth and subsequent AP (Fakhoury and Woloshuk, 2001; see Chen et al., 2015). Recently, a transgenic maize line expressing *AGM182*

TABLE 4 | A summary of some overexpression, RNAi and host-induced gene silencing studies in groundnut and maize.

Gene	Source	Approach	Promoter	Outcome	References
Groundnut					
ARAhPR10	*A. hypogaea*	Overexpression	CaMV35S	Transgenic lines showed both reduced infection and less aflatoxin production	Xie et al., 2013
aflR; aflS; aflJ; aflep; aflC/pksA/pksL1, pes1	*A. flavus*	RNA interference gene silencing technology	CaMV35S	Transgenic lines showed up to 100% reduction in aflatoxin content	Arias et al., 2015
MsDef1; MtDef4	*M. sativa; M. truncatula*	Overexpression	FMV35S	OE-*Def* lines showed a significant reduction in aflatoxin content (up to 99%) HIGS lines showed a significant reduction in aflatoxin content (up to 99.9%)	Sharma et al., 2018
aflM; aflP	*A. flavus*	Host-induced-gene silencing approach	CaMV35S		
Maize					
ZmPR10	*Z. mays*	RNA interference gene silencing technology	CaMV35S promoter	Downregulation of *PR-10* caused increased susceptibility and aflatoxin contamination	Chen et al., 2010
Thanatin	*Podisus maculiventris*	Heterologous expression	Ubiquitin-1 promoter	Cloning of thanatin (an antimicrobial synthetic peptide) improved resistance and reduced aflatoxin content (up to 68%)	Schubert et al., 2015
aflR	*A. flavus*	Host-induced-gene silencing approach	Ubiquitin promoter	Transgenic lines showed up to 14-fold less aflatoxin concentration compared to the wild type	Masanga et al., 2015
aflC	*A. flavus*	RNA interference	γ-zein endosperm-specific promoter	Transgenic lines showed up to 100% reduction in aflatoxin content	Thakare et al., 2017
ZmPRms	*Z. mays*	RNA interference based gene silencing	Zein promoter	Downregulation of *ZmPRms* gene caused increased susceptibility and aflatoxin contamination	Majumdar et al., 2017
AGM182	*Tachypleus tridentatus*	Overexpression	Ubiquitin-1 promoter	Overexpression of *AGM182* (an antimicrobial peptide) caused suppression of *A. flavus* growth and subsequently aflatoxin production (up to 98%)	Rajasekaran et al., 2018

which encodes a tachyplesin1-derived synthetic peptide (an antimicrobial peptide) was developed that exhibited reduced fungal growth and a significant reduction in aflatoxin level (76–98%) compared to the control (Rajasekaran et al., 2018). Characterization of these candidate genes through a transgenic approach would be important in safeguarding food commodities.

Managing Aflatoxin Contamination: Similarities Between Groundnut and Maize

Pre- and post-harvest management strategies largely predict the extent to which *Aspergillus* fungi invade seeds and exacerbate AP (Hell et al., 2008). Most post-harvest management practices like rapid drying of groundnut in-shell and maize ears coupled with appropriate storage conditions are crucial for reducing infection and toxin accumulation. During initiation stage, host-pathogen interactions occur in the cell wall where NBS-LRR receptors, oxylipins, and elicitors play an important role. This is followed by a change in ion flux across the plasma membrane and the activation of a number of genes that lead to changes in the plant's cell wall. It activates various PR-related proteins, phytoalexins-like compounds and TFs which play an important role in defense mechanism. In addition, at the environmental level, PAC is largely exacerbated by drought stress and insect damage in groundnut and maize (Guo et al., 2008; Hell et al., 2008). Attempts to characterize resistance due to the physical barriers suggested that pod shell may serve as a barrier to *A. flavus* infection when the kernels are stored in-shell in the case of groundnut (Liang et al., 2006; Nigam et al., 2009). Similarly, in maize, a tight husk and non-upright ear act as a barrier to the entry of spores and keep the ear dryer, resulting in an unfavorable environment for fungal growth (Warburton and Williams, 2014). Such physical barriers are considered non-desirable traits since they pose serious challenges while threshing or dehulling.

In groundnut and maize, cross-talk communication between the pathogen and host plant is the first critical step toward the rapid activation of defense mechanisms in host plants. Functional and biological composition of resistance mechanisms in maize and groundnut using integrated approaches have led to the elucidation of the roles of several genes, PR-10, chitinase, 14-kDa trypsin inhibitor, zeatin and beta-1,3-glucanase, lipoxygenase, ROS, and stress responsive proteins (such as late embryogenesis abundant protein (LEA14), catalase, glutathione S-transferase, superoxide dismutase, heat shock proteins) which play a vital role in regulating resistance and in cross-kingdom interactions between host plants and *Aspergillus* species in groundnut (Luo et al., 2005; Chadha and Das, 2006; Liang et al., 2006; Wang et al., 2010; Guo et al., 2011; Kumari et al., 2011; Nayak et al., 2017) and maize (Guo et al., 1997; Chen et al., 1998, 1999, 2001, 2002, 2004b, 2006, 2007, 2012; Lozovaya et al., 1998; Ji et al., 2000; Moore et al., 2004; Magbanua et al., 2007; Pechanova et al., 2011; Pegoraro et al., 2011; Roze et al., 2013; Fountain et al., 2014, 2016; Hawkins et al., 2015; Ogunola et al., 2017).

METABOLOMICS UNDER *A. flavus* INFECTION AND AFLATOXIN RESISTANCE

Metabolomics is an emerging field that represents the complete set of metabolites in a biological cell, tissue, organ or organism. It provides an instantaneous snapshot of the "physiological state" of an organism (Ramalingam et al., 2015; Kumar R. et al., 2017). Metabolites are small molecules that are directly involved in growth, development, and reproduction processes.

To understand the aflatoxin resistance mechanism at the metabolite level, some metabolome studies in response to *A. flavus* infection have been conducted in maize. For instance, metabolome profile under *A. flavus* infection showed significant induction and higher expression of polyamine (PA) biosynthesis genes in maize-resistant lines TZAR102, MI82 than in susceptible line SC212. Higher expression of spermidine (Spd), spermine (Spm), and diamine putrescine (Put) along with their increased catabolism in the resistant lines than in the susceptible line indicate that polyamines play an important role in *A. flavus* resistance (Majumdar et al., 2019). In addition, higher concentrations of amino acids such as glutamate (Glu), glutamine (Gln), and γ-aminobutyric acid in susceptible maize line SC212 showed that these amino acids favor *A. flavus* infection. In a similar study by Falade et al. (2018), metabolites were analyzed at R3 (milk), R4 (dough), and R5 (dent) stages of cob development under *A. flavus* infection (4 doses). The study showed that grain colonization decreases with increasing kernel maturity from milk-, dough-, and dent-stage kernels, with approximately 100%, 60%, and 30% colonization, respectively. However, aflatoxin levels increase with increased doses at dough and dent stages. This shows that initial stages of cob development (milk and dough) are more susceptible than the maturity stage (Falade et al., 2018). A study on aflatoxin accumulation in grains of 120 maize hybrids showed that higher concentrations of beta-carotene (BC), beta-cryptoxanthin (BCX), and total provitamin A had significantly less aflatoxin accumulation compared to that in hybrids with lower carotenoid concentration. Hence, breeding for increased carotenoid concentration can increase aflatoxin resistance in maize to help combat aflatoxin contamination as well as malnutrition (Suwarno et al., 2019). In short, metabolites significantly influence *A. flavus* infection and can be used as biomarkers for screening resistant and susceptible maize genotypes.

MOLECULAR BIOLOGY OF *A. flavus* FOR AFLATOXIN PRODUCTION AND RESISTANCE

The genome of the toxigenic strain of *A. flavus* contains ∼12,000 genes involved in the synthesis of secondary metabolites, with more than 56 gene clusters contributing to the production of secondary metabolites, including aflatoxin (Rokas et al., 2007). The aflatoxin biosynthesis gene cluster includes 25 genes spanning approximately 70 kb of DNA (Yu et al., 2004). The aflatoxin gene cluster resides on chromosome 3, next to the telomeric region comprising of pathway-specific regulatory genes as well as surrounded by four sugar-utilization genes at the distal end (Yu et al., 2000). Some regulatory genes (e.g., *aflR* and *aflS*) are reported to be essential for the production of aflatoxin after infection, and they work in conjunction with several other regulators/factors such as *VelB*/*VeA*/*LaeA* complex, *CreA* transcription factor, among others. While the *aflR* gene encodes

a DNA binding Zn-cluster protein that binds to DNA binding-domains of aflatoxin pathway genes, *aflS* is an aflatoxin pathway-specific regulatory gene required to mediate *aflR* transportation to/from the nucleus and assist in *aflR* localization (**Figure 2**; Ehrlich et al., 2012).

Aspergillus flavus can hijack the host machinery to facilitate the uptake of resources required for AP. For instance, the fungus requires the spermidine synthase (a polyamine biosynthetic gene) for AP and can utilize the host substrate to enhance polyamine (PA) biosynthesis and AP (Majumdar et al., 2018). In susceptible maize kernel, the expression of the PA biosynthetic/metabolism genes *S-adenosylmethionine decarboxylase* (*Samdc*) and *arginine decarboxylase* (*Adc*) significantly increased; this was followed by the upregulation of PA transporters in the pathogen (Majumdar et al., 2018). Maize's hypersensitivity and susceptibility to *A. flavus* involve a gene encoding glycine-rich RNA binding protein 2 which is associated with hormone and pathogen stress (Kelley et al., 2012), through salicylic-mediated defense signal transduction and HR reactions (Naqvi et al., 1998; Singh et al., 2011). The NPCs which transport RNA and other macromolecules are highly expressed in resistant maize cultivars and suppress *A. flavus* infection (Kelley et al., 2012). In Arabidopsis, a defect in *MOS7* (an NPC encoding gene) suppresses the accumulation of R-protein in the nucleus that causes a defect in both basal and systemic acquired resistance and R-protein-mediated immunity (Cheng et al., 2009). The infection induces higher expression of ethylene-responsive protein (ETHRP) in resistant maize cultivars suggesting the role of the ethylene signaling pathway in aflatoxin accumulation resistance. ETHRP is a universal stress protein and a key regulator of stress responses, and confers stress survival (Kelley et al., 2012). Further, fungal infection induces the production of several antifungal proteins such as 14-kDa trypsin inhibitor, 18 kDa ribosome-inactivating-protein, 28, 38 and 100 kDa protein, non-specific lipid transfers proteins, 2 S storage proteins, and zeamatin (Liang et al., 2006). An infection can also induce lipid peroxidation, which facilitates resistance to AP in groundnut (Liang et al., 2002).

Aspergillus infection also involves a dynamic network of transcription factors that coordinate the expression of the target biosynthetic genes of the pathogen and the suppression of the host's immune responses. This may involve the suppression of key gene *WRKY,* a transcription factor that modulates the expression of several genes involved in detoxification of ROS as well as aflatoxin (Korani et al., 2018), including NBS-LRR; its suppression is linked to aggravated accumulation of aflatoxin in plants such as groundnut (Nayak et al., 2017). Further, these TFs are also associated with PR proteins, which play a major role in resistance after infection (Pierpoint et al., 1981; Van Loon, 1985; Szerszen, 1990; Van Loon and Van Strien, 1999). In groundnut, WRKY and other key TFs such as ERF and NAC function in a coordinated fashion (Nayak et al., 2017; Korani et al., 2018); their modulation has a substantial impact on antioxidant biosynthetic, PR proteins, chitinase, and beta-1,3-glucanase genes. Modulation of these TFs in the host severely affects the transcription of ROS detoxifying genes such as catalases, superoxide dismutase, glutathione-S-transferase, and antioxidant biosynthesis genes like resveratrol synthase, PAL, chalcone synthase, chitinase, and beta-1,3-glucanase (Nayak et al., 2017; Korani et al., 2018). These genes protect host plants from oxidative damage, increase the levels of secondary metabolites involved in lignin biosynthesis, and restrict fungal invasion as well as its growth. In resistant groundnut genotypes, the activity of PAL enzyme that catalyzes the metabolism of phenolic compounds such as phytoalexin and lignin precursors, increases significantly (Nayak et al., 2017; Korani et al., 2018).

Resveratrol is a potent phytoalexin induced up to 30-fold in resistant genotypes of groundnut seeds upon infection (Liang et al., 2006). In wild groundnut species, the pod shell and seeds are rich in lignin content that prevents aflatoxin contamination (Guimarães et al., 2012). Notably, in maize, exposure to drought severely reduces PAL enzyme activity and phytoalexin production due to reduced moisture content in the kernel, resulting in fungal invasion and toxin production (Gholizadeh, 2011). Although, studies spanning 15 years have identified several gene clusters regulating host-pathogen interactions and AP, the characterization of individual genes is crucial to design strategies toward mitigation of aflatoxin contamination.

CHALLENGES AND OPPORTUNITIES

Aspergillus flavus infection and subsequent aflatoxin contamination is highly influenced by environmental parameters such as high soil temperature, moisture stress, and relative humidity which often outsmart the low levels of genetic resistance available in groundnut and maize genotypes. This could be one of the key reasons in making this trait very complex and limited progress has been made under field conditions as compared to controlled environment. Even under controlled environmental conditions, most studies are targeted at understanding host-pathogen interactions using a single toxigenic *A. flavus* strain and its interaction with the host (groundnut or maize). However, under field conditions, the reality is different. Often, many species of *Aspergillus* group of fungi such as *A. flavus* and *A. parasiticus* are involved in causing aflatoxin contamination. The population dynamics of toxigenic *Aspergillus* in soils and possible shifts in toxigenic and non-toxigenic strains could be an important area to focus on while studying host-pathogen interactions. Also required is a knowledge of the soil composition of toxigenic *A. flavus* group of fungi and the ambient environment in a crop production region that drives *Aspergillus* population levels and other competing and co-existing pathogens. Similar conditions can be created/simulated under a controlled environment to facilitate the easy adoption and translation of results from laboratory conditions to the field. The lack of consistency in host-pathogen-toxin interactions inhibits the understanding of the precise genetic behavior of resistance in groundnut and maize. Despite a sequencing revolution in the last decade, genetic and gene discovery efforts have not led to solutions to aflatoxin reduction because of inconsistent phenotyping results. Devising novel phenotyping techniques to assay AP at different steps is a way forward. Dissecting components of resistance using known

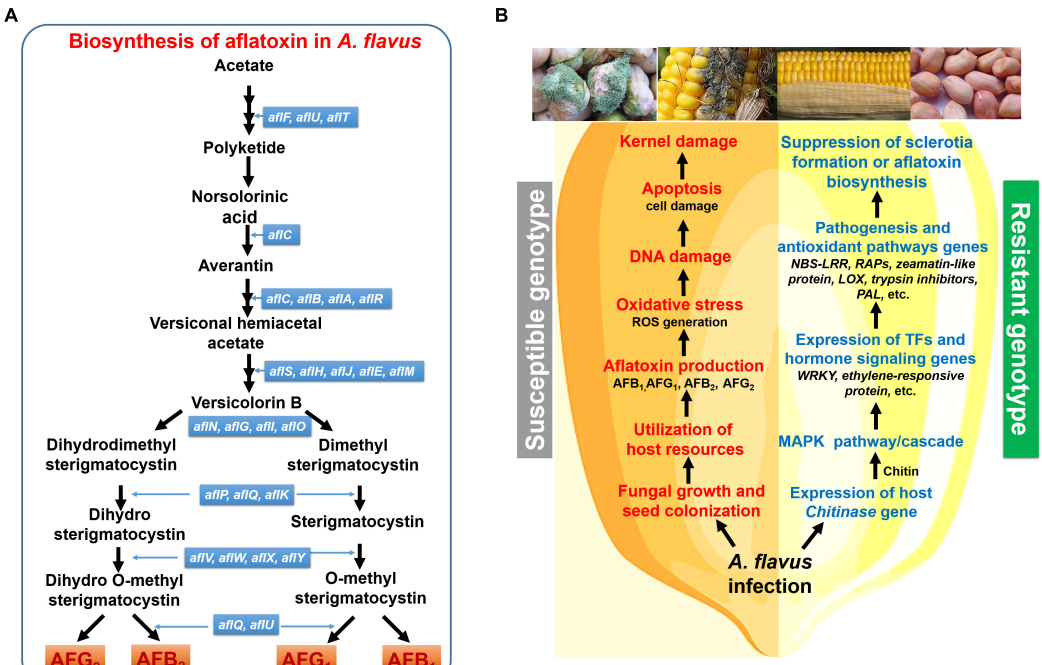

FIGURE 2 | A simplified representation of the aflatoxin biosynthesis pathway and the defense response mechanism in groundnut or maize. **(A)** Aflatoxin biosynthesis in *A. flavus*; **(B)** the aflatoxin biosynthesis pathway involve multiple genes which co-express together for the formation of toxin secondary metabolites. In the susceptible genotype infection leads to the *A. flavus* seed colonization and production of aflatoxin which causes suppression of host defense mechanism results in ROS generation and DNA damage causing cell death (apoptosis). In contrast, in resistant genotypes infection causes induction of host defense mechanism that include MAPK pathway which induces WRKY TF expression which is a key regulator of pathogenesis and antioxidant related genes involved in the suppression of aflatoxin biosynthesis pathway or detoxification of toxin.

pre-harvest resistant sources of groundnut and maize may be an interesting area of research. In this context, studying the biochemical composition of the seed coat could lead to a better understanding of host-pathogen interactions.

Another key challenge as well as an opportunity would be to understand the impact of soil and its environment on AP. Plants growing in unhealthy soils are bound to be more stressed, and this might increase aflatoxin contamination. While most studies have concentrated on the physical and chemical components of soil, the biological component remains unexplored. An analysis of the phytobiome, the microbial component that surrounds the plant, from the leaves down to the roots, is another emerging area of research. A phytobiome that negatively impacts plant health would influence aflatoxin contamination. Insights into the phytobiomes of groundnut and maize would certainly influence our understanding of host-pathogen interactions, especially in complex traits such as aflatoxin contamination.

SUMMARY

While discussing the progress made in understanding the resistance mechanisms of aflatoxin contamination in groundnut and maize using multidisciplinary approaches, the paper elaborates on several QTLs, genes, pathways and complex genetic architecture of the target trait. The paper has also reviewed the potential of different approaches in better understanding the complexities of candidate genes identified after the genome sequencing of host and pathogen. Various cultural and biological methods have been reported to prevent/sustainably manage aflatoxin contamination in groundnut and maize. The development of varieties/hybrids or transgenics with resistance to both fungal infection and aflatoxin contamination remains a challenge. To date, aflatoxin management strategies have centered around the use of good agricultural practices during pre-and post-harvest stages, including the use of biocontrol agents (particularly of non-toxigenic strains of *A. flavus*) in countries where they are available to farmers. Omics studies in the last couple of decades provide an array of genetic and genomic resources and expand the knowledge base on *Aspergillus* infection and aflatoxin reduction mechanisms, host-pathogen interactions, toxigenicity of the fungi, mechanism of aflatoxin biosynthesis, and inhibitors targeting the aflatoxin biosynthetic genes. Promising genomics and transgenic approaches have provided complimentary beneficial effects by integrating genes, peptides/antifungal proteins, and even silencing key genes for *Aspergillus* growth and aflatoxin biosynthesis in susceptible varieties to enhance resistance levels. These integrated approaches comprising of functional and structural genomics, together with NGS platform will provide more information on candidate genes to facilitate the development of molecular

markers for use in molecular breeding. Conventional and modern breeding tools need to be deployed to develop aflatoxin-resistant maize and groundnut varieties that will lead to food safety, poverty reduction and boosting the industry and market.

AUTHOR CONTRIBUTIONS

RV together with MP and PS conceptualized the idea after receiving the invitation from the journal. PS together with SG, AO-B, RK, SP, HS, YL, XN, DH, JF, SN, GM, TR, WZ, BG, BL, PSi, MP, and RB wrote the manuscript. PS, MP, and RV finalized the manuscript.

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Biocontrol Strains Differentially Shift the Genetic Structure of Indigenous Soil Populations of *Aspergillus flavus*

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Biocontrol using non-aflatoxigenic strains of *Aspergillus flavus* has the greatest potential to mitigate aflatoxin contamination in agricultural produce. However, factors that influence the efficacy of biocontrol agents in reducing aflatoxin accumulation under field conditions are not well-understood. Shifts in the genetic structure of indigenous soil populations of *A. flavus* following application of biocontrol products Afla-Guard and AF36 were investigated to determine how these changes can influence the efficacy of biocontrol strains in reducing aflatoxin contamination. Soil samples were collected from maize fields in Alabama, Georgia, and North Carolina in 2012 and 2013 to determine changes in the population genetic structure of *A. flavus* in the soil following application of the biocontrol strains. *A. flavus* L was the most dominant species of *Aspergillus* section *Flavi* with a frequency ranging from 61 to 100%, followed by *Aspergillus parasiticus* that had a frequency of <35%. The frequency of *A. flavus* L increased, while that of *A. parasiticus* decreased after application of biocontrol strains. A total of 112 multilocus haplotypes (MLHs) were inferred from 1,282 isolates of *A. flavus* L using multilocus sequence typing of the *trpC*, *mfs,* and AF17 loci. *A. flavus* individuals belonging to the Afla-Guard MLH in the IB lineage were the most dominant before and after application of biocontrol strains, while individuals of the AF36 MLH in the IC lineage were either recovered in very low frequencies or not recovered at harvest. There were no significant ($P > 0.05$) differences in the frequency of individuals with *MAT1-1* and *MAT1-2* for clone-corrected MLH data, an indication of a recombining population resulting from sexual reproduction. Population mean mutation rates were not different across temporal and spatial scales indicating that mutation alone is not a driving force in observed multilocus sequence diversity. Clustering based on principal component analysis identified two distinct evolutionary lineages (IB and IC) across all three states. Additionally, patristic distance analysis revealed phylogenetic incongruency among single locus phylogenies which suggests ongoing genetic exchange and recombination. Levels of aflatoxin accumulation were very low except in North Carolina in 2012, where aflatoxin levels were significantly ($P < 0.05$) lower in grain from treated compared to untreated plots. Phylogenetic analysis showed that Afla-Guard was more effective than AF36 in shifting the indigenous soil populations

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of *A. flavus* toward the non-toxigenic or low aflatoxin producing IB lineage. These results suggest that Afla-Guard, which matches the genetic and ecological structure of indigenous soil populations of *A. flavus* in Alabama, Georgia, and North Carolina, is likely to be more effective in reducing aflatoxin accumulation and will also persist longer in the soil than AF36 in the southeastern United States.

Keywords: aflatoxin, *Aspergillus* section *Flavi*, biological control, lineage, mating type

INTRODUCTION

Aspergillus flavus and *Aspergillus parasiticus* are considered the most important aflatoxin-producing species within *Aspergillus* section *Flavi* (Klich, 2007). Aflatoxin production by these two *Aspergillus* species contaminates major food crops and tree nuts and thus, consumption of contaminated products poses a health hazard to humans and animals globally (Williams et al., 2004). Aflatoxins are classified as a group 1 carcinogen by the International Agency for Research on Cancer (IARC, 2002). In humans, chronic exposure to aflatoxins can result in suppression of the immune system, teratogenicity and retardation of growth in children (Richard and Payne, 2003; Paulussen et al., 2016). In maize, aflatoxins can form in kernels during crop development if the crop is stressed by heat or drought or if the crop is damaged by insects. Accumulation of aflatoxins can also occur after crop maturation when the crop is exposed to temperature and moisture conditions that are conducive to infection by *A. flavus* post-harvest and in storage (Payne, 1992). Due to the food safety concerns associated with aflatoxin contamination, more than 100 countries including the United States, have set stringent regulatory levels for quantities of aflatoxin in food and feed. The economic impact from aflatoxin contamination in the United States is primarily due to market loss and is estimated to be several hundred million dollars (Wu and Guclu, 2012).

Pre-harvest strategies such as planting resistant cultivars, good cultural practices, and biocontrol control are some strategies that are being investigated to control aflatoxin contamination (Ojiambo et al., 2018). Plant breeding efforts over the last 25 years have not provided adequate levels of resistance to aflatoxin accumulation in maize (Warburton and Williams, 2014). Environmental conditions drive aflatoxin accumulation in several crops by simultaneously affecting the population structure and virulence of *A. flavus* and the susceptibility of the host crop (Munkvold, 2003). These environmental factors continue to pose huge challenges in breeding for aflatoxin resistance due to the large genotype-by-environment interaction (Warburton and Williams, 2014; Bhatnagar-Mathur et al., 2015), an observation that has greatly limited the utility of any available resistant germplasm for the control of aflatoxin accumulation in maize. Of all the above pre-harvest strategies, biocontrol involving the application of non-aflatoxigenic strains of *A. flavus* at high densities in the field, offers the greatest potential in the mitigation of aflatoxin accumulation especially in the near-term (Dorner, 2004; Bhatnagar-Mathur et al., 2015). Non-aflatoxigenic strains are usually applied in the field using inoculated or coated cereal grains but other sprayable formulations that utilize bioplastics instead of grains, have also been developed (Abbas et al., 2017). The type of formulation used for the biocontrol product can also affect the quantity of inoculum applied on the crop (Accinelli et al., 2016). Through competitive exclusion, biocontrol strains exclude native, aflatoxigenic strains from the crop, thereby reducing aflatoxin accumulation (Dorner, 2004). Application of non-aflatoxigenic strains of *A. flavus* as biocontrol strains has reduced aflatoxin contamination in maize, cotton, and peanut by 67–95% (Cotty and Bayman, 1993; Dorner, 2008; Atehnkeng et al., 2014; Mauro et al., 2018). In the United States, Afla-Guard and AF36, are two commercial biocontrol products containing non-aflatoxigenic strains of *A. flavus* that have been approved by the U.S. Environmental Protection Agency for biocontrol of aflatoxin accumulation in peanut, maize, and cottonseed. The non-aflatoxigenic strain in Afla-Guard is NRRL 21882, which was originally isolated from a naturally infected peanut in Georgia (Dorner, 2004). The non-aflatoxigenic strain in AF36 is NRRL 18543, which was isolated from cottonseed in Arizona (Cotty, 1989). The *A. flavus* strain in Afla-Guard does not produce aflatoxins or cyclopiazonic acid (CPA) and belongs to the IB lineage, which is also composed of *A. flavus* L-strains that do not produce or are low producers of aflatoxins and strains of *A. oryzae* (Geiser et al., 2000). Unlike the Afla-Guard strain, the AF36 strain has a full aflatoxin gene cluster with one defective gene and a functional CPA cluster and belongs to the IC lineage that is composed of both aflatoxigenic and non-aflatoxigenic members (Geiser et al., 2000; Moore et al., 2009).

The logic behind the effectiveness of biocontrol using non-aflatoxigenic strains of *A. flavus* is based on the assumption that these strains are predominantly asexual, genetically stable and thus, unable to recombine with native aflatoxigenic strains (Ehrlich and Cotty, 2004; Abbas et al., 2011a). However, subsequent studies have provided unequivocal evidence for recombination within the aflatoxin gene clusters in *A. flavus* and *A. parasiticus* populations (Horn et al., 2009a,b; Moore et al., 2009) within the same field. Such a process could result in reduced or increased efficacy of the non-aflatoxigenic *A. flavus* due to the production of novel *A. flavus* phenotypes, resulting in greater diversity in the field (Fisher and Henk, 2012). The presence of high population densities of *A. flavus* during deployment of biocontrol strains can also increase opportunities for sexual recombination and re-assortment of genes that could further influence the competitiveness between strains and their capacity to produce aflatoxin (Olarte et al., 2012). This is particularly important where the biocontrol strain is genetically different from the predominant local populations of *A. flavus* in the soil.

Field populations of *A. flavus* are highly diverse (Ehrlich et al., 2015) and the genetic structure of *A. flavus* differs greatly across the United States. For example, the population in North Carolina is predominately clonal with a high frequency of the IB lineage, while that in Texas has a high frequency of the IC lineage (Horn and Dorner, 1999; Jaime-Garcia and Cotty, 2010). Afla-Guard has been reported to significantly reduce aflatoxin accumulation to a greater extent than AF36 in Mississippi (Abbas et al., 2011a,b). Similarly, Afla-Guard was found to be more effective than AF36 in reducing aflatoxin accumulation on maize in North Carolina (Meyers et al., 2015). In contrast, AF36 seems to be more effective than Afla-Guard in reducing aflatoxin accumulation in Texas (Outlaw et al., 2014). Although statistically significant differences between these two biocontrol strains in their ability to reduce aflatoxin accumulation has not been observed in all locations tested, prevailing evidence suggests that the relative effectiveness of the two biocontrol strains depends on the location where they are applied. Our working hypothesis is that the genetic composition of the indigenous soil population of *A. flavus* dictates the relative effectiveness of biocontrol strains in reducing aflatoxin contamination. This implies that understanding the genetic structure of *A. flavus* soil populations will enable the selection of biocontrol strains most similar, genetically, to the predominant indigenous multilocus haplotype (MLH) and thus, improve the efficacy of biocontrol (Ehrlich, 2014; Ehrlich et al., 2015).

Application of non-aflatoxigenic biocontrol strains that are genetically similar to local *Aspergillus* soil communities in the soil is not only considered efficacious, but maximizes the potential for sexual recombination. A non-aflatoxigenic strain that is genetically similar to native strains should increase the efficacy of biocontrol and minimize the risk of aflatoxin contamination (Bhatnagar-Mathur et al., 2015; Molo et al., 2019). The overall goal of this study was to establish the impact of the genetic structure of *A. flavus* populations in the soil on the efficacy of biocontrol of aflatoxin accumulation in maize. The specific objectives of this study were to: (i) characterize the temporal distribution of species of *Aspergillus* section *Flavi* following application of either Afla-Guard or AF36 in the field, (ii) determine the dynamics and shifts in predominant MLHs of *A. flavus* in soil treated with Afla-Guard or AF36, and (iii) inform selection of biocontrol strains and infer their effectiveness based on shifts in the frequency of indigenous MLHs of *A. flavus* in the soil. Insights in how well biocontrol strains establish in a field relative to indigenous populations of *A. flavus* can be useful in the selection of the most effective non-aflatoxigenic strains that will result in sustainable biocontrol of aflatoxin accumulation (Ehrlich et al., 2015).

MATERIALS AND METHODS

Description of Field Sites

Field experiments were conducted during the maize growing season in 2012 and 2013 in the southeastern United States in Alabama, Georgia and North Carolina. In 2012, trials were located at the Gulf Coast Research and Extension Center in Fairhope, Alabama, and in Ben Hill County, Georgia. In 2013, trials were conducted at the Prattville Agricultural Research Unit in Prattville, Alabama and at the Coastal Plain Experiment Station in Tifton, Georgia. In North Carolina, the 2012 and 2013 field experiments were conducted at the Upper Coastal Plain Research Station in Rocky Mount. In Alabama, the maize hybrids Pioneer 31P42 and DKC 67-88 were used in 2012 and 2013, respectively, while in Georgia, the maize hybrids Pioneer 33M52 and DK 66-94 were used in 2012 and 2013, respectively. The maize hybrid DKC 64-69 was used in 2012 and 2013 in North Carolina. Standard field plots measuring 51 m wide × 69 m long with 1.5 m borders were adopted in all the three states in both years. The northern-most location of field plots in Georgia was at 31° 25′ 50″ N, −83° 32′ 10″ W, in Alabama at 32° 27′ 30″ N, −86° 34′ 36″ W, and in North Carolina at 35° 53′ 59″ N, −77° 40′ 31″ W.

Treatments and Experimental Design

Two commercially available biocontrol products, Afla-Guard and AF36, were evaluated in this study to determine how the dynamics of dominant MLHs of *A. flavus* in the soil can influence the efficacy of biocontrol in reducing aflatoxin contamination in maize. Afla-Guard contains *A. flavus* strain NRRL 21882 as the active ingredient and is labeled for use on peanuts and maize in the United States (U.S. Environmental Protection Agency, 2013). The *A. flavus* strain in AF36 is NRRL 18543 and the product is labeled for use on maize in Arizona and Texas and on cotton in Arizona, California, and Texas (U.S. Environmental Protection Agency, 2011). Afla-Guard and AF36 were evaluated in North Carolina in 2012 and 2013 and in Alabama in 2013, while only Afla-Guard was evaluated in field plots in Alabama in 2012 and Georgia in 2012 and 2013.

Field plots were established on 21 March 2012 and 2 April 2013 in Alabama, on 10 July 2012 and 1 May 2013 in Georgia. In North Carolina, plots were planted on 3 April 2012 and 11 April 2013. Fertilization and weed control practices were used at each field site according to standard management practices for maize growers in each state. Afla-Guard and AF36 treatments were applied mechanically or manually by broadcasting the biocontrol product at recommended label rates on top of the plant canopy at the VT growth stage. In 2012, treatments were applied on 24 May, 11 May, and 16 May in Alabama, Georgia, and North Carolina, respectively. Treatment application dates in 2013 were 26 June, 8 June, and 21 June, in Alabama, Georgia, and North Carolina, respectively. Based on the number of biocontrol products, three treatments (Afla-Guard, AF36, and untreated control) were evaluated in North Carolina in both years and in Alabama in 2013. Two treatments (Afla-Guard and untreated control) were evaluated in Alabama in 2012 and Georgia in 2012 and 2013. In all states, the experiment was laid out in a randomized complete block design with three to four replications. Weather data at each experimental site during the study period were obtained from the nearest state weather station or from the national weather database at the NC State Climate Office in Raleigh, North Carolina (http://www.nc-climate.ncsu.edu/cronos).

Soil Sampling in Experimental Fields

From each field, 20 soil samples (∼100 g each) were collected using sterile plastic scoops from 20 georeferenced points at approximately equal distances along two diagonals of the field.

During the study, soil samples were taken at three sampling periods: (1) prior to application of biocontrol treatments, (2) 1–2 weeks after application of biocontrol treatments, and (3) at harvest. In North Carolina, soil samples from the three sampling periods were collected on 23 May, 12 July, and 17 September 2012, respectively, while in 2013 the samples were collected on 26 June, 5 July, and 5 September 2013. In the 2012 trial in Alabama, soil samples were collected on 24 May, 18 June, and 7 September, while soil samples were collected on 2 July, 23 August, and 20 September in 2013. In Georgia, soil samples were collected on 18 May 2012 and 15 June in 2012 and no samples were collected at harvest due to flooding of the field. In the 2013, soil samples were collected from two time periods: before application of treatments on 28 May 2013 and after harvest on 21 Feb 2014. After each sample collection, soils were placed in doubled-layered brown paper bags and dried on a laboratory bench for 1–2 weeks. Soil samples collected from Alabama and Georgia were then shipped to NC State University in Raleigh and refrigerated at 4°C until further processing.

Fungal Isolation, Identification, and Determination of Colony Forming Units

Each soil sample was first homogenized manually by shaking the contents in the sampling bag for 1 min. A sample of 33 g of soil was taken from each paper bag and added to 100 mL of 0.2% water agar and the mixture was carefully shaken for 1 min. The soil-water agar suspension was then plated on modified dichloran Rose Bengal (mdRB) medium as described by Horn and Dorner (1998). Briefly, aliquots of 200–400 μl of the soil-agar suspension were spread on the surface of mdRB medium in 100 × 15 mm diameter Petri dishes and the dishes were incubated at 37°C for 3 days. The actual volume of soil solution plated on the mdRB plates varied between samples of soil-agar suspension, so the appropriate aliquot volume was determined by experimenting with the soil to 0.2% water agar ratio (data not shown).

Total colony counts were recorded as described previously (Horn and Dorner, 1998) based on five replicate plates of each soil sample. Colonies of *Aspergillus* were identified at the species level based on conidial color along with the colony shape and colony morphology (Klich and Pitt, 1988; Cotty, 1989). Confirmation of the identity of the species of isolated colonies was determined using NCBI Standard Nucleotide BLAST search tool based on sequenced DNA fragments at the *trpC* locus (Olarte et al., 2012). Final colony-forming units (CFU) per gram of soil were corrected for soil moisture content and expressed on a dry weight soil basis. At each soil sampling period, single spores of 20 isolates of *A. flavus* were randomly picked from 20 soil dilution plates, transferred onto 60 × 15 mm Petri dishes containing mdRB medium and incubated for 5 days. This resulted in 400 isolates of *A. flavus* from each field at each sampling period in each state. A total of 6,400 isolates of *A. flavus* were obtained across the study and subjected to genetic and molecular characterization as described below. Isolates were subjected to short-term storage on mdRB medium at 4°C, while a suspension of spores in a 40% glycerol was stored at −80°C for long term storage.

DNA Extraction and Multilocus Sequence Typing

DNA was extracted using the CTAB method (He et al., 2007) from spores harvested directly from single-spore culture colonies of 6,400 isolates of *A. flavus* grown on mdRB medium. Using PCR amplification, 80–90 *A. flavus* isolates from each sampling period in each state for both years were randomly selected for MLH diversity analysis using multilocus sequence typing (MLST). Genome-wide variation was examined using MLST based on variation at three loci; microsatellite marker AF17 on chromosome 2 (Grubisha and Cotty, 2009), major facilitator superfamily *mfs* gene on chromosome 3, and tryptophan synthase (*trpC*) gene on chromosome 4. Multilocus sequence typing was conducted for both clone corrected and uncorrected mating-type (*MAT)* data (Olarte et al., 2012). Sequences of oligonucleotide primers (*trpC*, *mfs*, AF17, *MAT*) and thermocycler conditions used in this study were adopted from those previously described by Carbone et al. (2007) and Olarte et al. (2012). Reactions were run 5 min at 94°C followed by 40 cycles for 30 s at 60°C for *mfs*, 58°C for *trpC*, *MAT1-1,* and *MAT1-2*, and 57°C for AF17, ending with 1 min at 72°C. Multiplex-PCR was used to determine the mating-type of each isolate using the *MAT1-1* and *MAT1-2* primers (Ramirez-Prado et al., 2008). All the sequencing work was performed at the NC State University Genomic Sciences Laboratory in Raleigh, North Carolina.

DNA sequences were aligned and manually adjusted using Sequencher Version 4.7 (Gene Codes Corporation, Ann Arbor, MI, USA). Alignments were exported as NEXUS files into the Mobyle SNAP Workbench (http://snap.hpc.ncsu.edu/), a web-based analysis portal deployed at NC State University (Monacell and Carbone, 2014). The SNAP Convert tool (Aylor et al., 2006) implemented in Mobyle SNAP workbench was used to convert NEXUS files into PHYLIP format. Multiple sequence alignments for each locus were combined using SNAP Combine (Aylor et al., 2006) and collapsed using SNAP Map for inference of MLHs. For maximal MLH resolution, collapsing into MLHs was performed with the option of recoding insertions/deletions (i.e., indels).

Population Genetics, Structure, and Phylogenetic Analyses

Population summary statistics per locus were generated to infer different genetic aspects of populations of *A. flavus* isolates collected at different sampling periods in this study. These statistics included: (1) number of segregating sites (*s*), (2) average pairwise difference between sequences, π, based on Nei and Li (1979), and (3) Watterson's θ (Watterson, 1975) as implemented in ARLEQUIN v3.5 (Excoffier and Lischer, 2010). Tajima's *D* (Tajima, 1989) and Fu's F_S (Fu and Li, 1993) were used as tests of neutrality and population size constancy. Input files for calculating these population summary statistics were generated using SNAP Map excluding indels and assuming an infinite-sites model of DNA sequence evolution. The phylogenetic relationship of 1,282 isolates was examined for each locus separately and for the combined multi-locus dataset using maximum likelihood analysis implemented in RAxML (Stamatakis et al., 2008) through the CIPRES RESTful

application programming interface (API) (Miller et al., 2015) implemented in the SNAP Portal. Confidence limits on branches in phylogenies were based on 1,000 rapid bootstrap replicates and monophyletic groups were identified as branches having at least 70% bootstrap support. Phylogenetic trees were visualized using the Tree-Based Alignment Selector (T-BAS) v2 toolkit (Carbone et al., 2017, 2019).

Multilocus sequence variation was further subjected to analysis of molecular variance (AMOVA) to test the null hypothesis that populations were not genetically differentiated over the multiple hierarchical spatial scales or among distinct sampling periods. AMOVA was used to estimate the genetic variance components at different hierarchical levels of population structure (Excoffier and Lischer, 2010) and the pairwise fixation index (F_{ST}) was calculated to quantify genetic differentiation within and among *A. flavus* populations. Significance of F_{ST} analyses was determined using 1,000 permutations in ARLEQUIN v3.5. Structure was also examined using principal component analysis (PCA) and the methods described in Patterson et al. (2006) implemented in the Mobyle SNAP Workbench. Principal components were normalized to sum to 1, and the number of significant axes of variation (i.e., principal components or eigenvectors) was determined using the Tracy–Widom statistic (Tracy and Widom, 1994). The optimal number of clusters for *k*-means was determined using the cluster center initialization algorithm that centers on randomly chosen observed points (Khan and Ahmad, 2004). Clusters were evaluated using the Calinski–Harabasz index (Calinski and Harabasz, 1974), which identifies the best cluster based on the average between and within cluster sum of squares. Significant principal components and clusters were displayed graphically using the SCATTERPLOT3D package in R (Ligges and Mächler, 2003). We used Fisher's exact test implemented in the Mobyle SNAP Workbench to determine if there were non-random associations between cluster and state, year, or sampling period.

Phylogenetic incongruence across *trpC, mfs,* and AF17 was examined using patristic distances displayed as a heat map in outer rings (one per locus) in T-BAS v2.1 (Carbone et al., 2019). For each separate locus phylogeny, a matrix of patristic distances, normalized to a maximum value of 1, was generated for all pairs of sequences representing individual isolates. The distances from different loci were compared to identify incongruences in tree topologies that suggest genetic exchange and recombination. Alternatively, congruent distances across topologies suggest clonal transmission and adaptation. Patristic distances from Afla-Guard or AF36 were displayed in T-BAS to compare patterns of phylogenetic incongruence between *trpC, mfs,* and AF17.

Mating-Type Distribution of *A. flavus* Isolates

Clone correction was performed using MLST to eliminate accidental sampling of the same individual multiple times (Moore et al., 2013). In this study, the null hypothesis was that there is no significant difference between the frequencies of *MAT1-1* and *MAT1-2* individuals at each sampling time period in each state and experimental year, which would indicate frequency-dependent selection consistent with sexual reproduction (Linde et al., 2003). This hypothesis was tested using a two-tailed binomial test on clone corrected and clone uncorrected data sets for variation at three MLST loci, *trpC*, *mfs*, and AF17, using the binomial option in PROC FREQ in SAS (version 9.4; SAS Institute, Cary, NC). A significant difference in the frequency of the two mating-types before and after clone correction would indicate a primarily asexual population. In contrast, a significant difference in the frequency of the two mating-types before clone correction and a lack of no significant difference after clone correction, or a lack of significant difference for either the uncorrected or corrected population, would suggest that the fungal population is predominantly undergoing sexual reproduction (Leslie and Klein, 1996; Linde et al., 2003).

Quantification of Aflatoxin in Harvested Grain

At each location, a subsample of about 2.5 kg of harvested grain dried to 15–17% moisture content was randomly selected for enumeration of aflatoxin contamination. Due to logistic and environmental constraints, harvesting was not conducted in Georgia in 2012 and thus, no data on aflatoxin contamination in the field was obtained. Aflatoxin was quantified in harvested grain in Georgia and North Carolina using the VICAM column system as described by Truckness et al. (1991) and the detection limit for the VICAM method is 5 ppb. The Veratox aflatoxin kit (Neogen Corporation, Lansing, MI), which has a detection limit of 2 ppb, was used according to kit instructions to quantify aflatoxin in harvested grain in Alabama as described by (Bowen et al., 2014).

Analysis of Soil Population Densities and Aflatoxin Contamination in Grain

Based on preliminary data analyses, data for soil population densities recorded as colony forming units per g of soil (CFU/g) and aflatoxin concentration (ppb) in harvested grain were analyzed separately for each state and year. Means CFU were calculated at each sampling period and the range was used to depict the soil population densities of various members of *Aspergillus* section *Flavi* at different sampling periods within each state. Means of aflatoxin concentration from each treatment plot were subjected to analysis of variance using the PROC GLM of SAS. Fisher's LSD test ($\alpha = 0.05$) was used to separate means of aflatoxin concentration between biocontrol treatments evaluated in each state.

RESULTS

Weather Conditions

Weather factors recorded during the study period varied between years and experimental sites. In both years, temperatures during the growing season increased from April to July at all experimental sites (**Table 1**). In 2012, the highest temperatures were recorded at Rocky Mount in North Carolina that had a maximum temperature of 34°C with a mean temperature of 32°C between April and July. In 2013, the highest temperatures were recorded at Prattville, Alabama with a maximum temperature of

TABLE 1 | Summary of weather variables recorded at experimental sites in a study conducted to assess the impact biocontrol strains on genetic structure of *Aspergillus flavus* in the field.

	2012			2013		
Variable/month	**North Carolina**	**Alabama**	**Georgia**	**North Carolina**	**Alabama**	**Georgia**
MEAN MAX/MIN TEMPERATURE (°C)						
April–May	28/12	28/16	26/14	24/12	26/13	26/14
June–July	35/19	31/22	32/20	31/20	31/21	30/22
Mean[a]	32/16	30/19	29/17	28/16	29/17	28/18
RAINFALL (mm)						
April–May	259	193	88	141	145	181
June–July	236	399	212	378	465	384
Total[b]	495	592	300	519	610	565

[a]*Mean temperature recorded from April to end of July.*
[b]*Total amount of rain recorded from April to end of July.*

31°C and a mean temperature of 29°C between April and July. The lowest maximum temperatures in 2012 were recorded at Ben Hill in Georgia with a mean temperature of 29°C from April to July, while the corresponding lowest temperatures in 2013 were recorded at Rocky Mount in North Carolina and Tifton in Georgia with a mean of 28°C (**Table 1**).

Rainfall amounts during the season were lower in 2012 than in 2013, with the Ben Hill in Georgia being the driest site in 2012 with 300 mm from April to July, while Rocky Mount in North Carolina was the driest site in 2013 with 519 mm. The wettest sites in 2012 and 2013 were Fairhope and Prattville both in Alabama with 592 and 610 mm, respectively, being recorded from April to July (**Table 1**).

Soil Population Densities of *Aspergillus* Section *Flavi*

Soil densities of *Aspergillus* section *Flavi* in the soil increased over time following the application of biocontrol treatments in both years across the three states except in Georgia in 2012 (**Table 2**). Densities were lowest prior to the application of treatments and highest at harvest in Alabama, Georgia (in 2013), and North Carolina, with the densities at the pre-application sampling period being intermediate. For example, the mean soil population densities at pre-application, post-application, and harvest in North Carolina in 2012 were 38, 237, and 986 CFU/g, respectively, while the corresponding populations in 2013 were 157, 240, and 250 CFU/g, respectively. In 2012, the lowest minimum population density was 3 CFU/g in soil samples from Alabama prior to the application of biocontrol treatments, while the highest maximum population density of 3,019 CFU/g was observed in Alabama at harvest. In 2013, the lowest minimum population density was 1 CFU/g in soils from Georgia prior to treatment application, while the highest maximum soil density of 1,406 CFU/g was observed at harvest in Alabama (**Table 2**).

Application of biocontrol treatments also impacted the densities of *A. flavus* in the soil. This impact was more pronounced in 2012 than in 2013 and at harvest than at post-inoculation (**Table 2**). In addition, this impact was also observed in Alabama and North Carolina in 2012 and Georgia in 2013. For example, the change in soil populations following the application of biocontrol (i.e., ΔCFU) in North Carolina at post-application was about 4-fold higher in 2012 compared to 2013. This pattern was observed across all three states for both years except 2012 in Georgia, where ΔCFU decreased at post-application. In North Carolina, ΔCFU at harvest was about 4- and 1.1-fold higher than at post-application in 2012 and 2013, respectively. This same pattern was also observed in Alabama but with much higher values in both years (**Table 2**).

Frequency of Species Within *Aspergillus* Section *Flavi*

Within *Aspergillus* section *Flavi*, *A. flavus*, *A. parasiticus*, *A. caelatus*, *A. nomius,* and *A. tamarii* were recovered from soil collected from the study sites across three states. However, the incidence of individual species varied between states, with the diversity within section *Flavi* being higher in Alabama compared to Georgia and North Carolina (**Table 3**). In addition, the incidence of members within *Aspergillus* section *Flavi* in each state was fairly consistent in both years of the study. Across all the states, *A. flavus* was the dominant species with a frequency of 61–100%. In addition, all *A. flavus* isolates sampled in Alabama, Georgia and North Carolina belonged to the L-strain morphotype. The highest proportion of *A. flavus* across sampling periods was observed in Georgia (97.9–100%), followed by North Carolina (84.9–96.8%) and Alabama (61.0–98.0%; **Table 3**).

Aspergillus parasiticus was the second most abundant species observed across all states. As with *A. flavus*, *A. parasiticus* was found at all sampling periods in every state, except in Georgia in 2013 (**Table 3**). In contrast to *A. flavus*, the maximum incidence of *A. parasiticus* was highest in Alabama (35.1%) and lowest in Georgia (2.1%), with incidence in North Carolina (15.1%) being intermediate. The incidence of *A. parasiticus* was always highest prior to application of the biocontrol but decreased after the application of the biocontrol treatments with the lowest levels being observed at harvest. The only exception to this trend was in Alabama in 2012, where the incidence of *A. parasiticus* was lower at pre-application (4.3%) than at post-application (35.1%) of the biocontrol treatments. *A. caelatus, A. nomius*, and *A.*

TABLE 2 | Population densities of *Aspergillus* section *Flavi* in soil from fields in the southeastern United States treated with Afla-Guard and AF36 biocontrol strains.

		Colony forming units (CFU) at sampling period[a]			ΔCFU[b]	
Year	**State**	**Pre-application**	**Post-application**	**Harvest**	**Post-application**	**Harvest**
2012	Alabama	33 (3–189)	151 (7–679)	516 (33–3,019)	4.6	15.6
	Georgia	413 (4–1,906)	220 (9–888)	–[c]	−0.5	–[c]
	North Carolina	38 (11–113)	237 (6–1786)	986 (21–1,005)	6.2	25.9
2013	Alabama	106 (16–212)	111 (42–227)	376 (48–1,406)	1.1	3.5
	Georgia	20 (1–103)	–[c]	173 (16–432)	–[c]	8.6
	North Carolina	157 (6–509)	240 (3–1,009)	250 (3–926)	1.5	1.6

[a] *Soil densities (i.e., CFU) are means per gram of soil based on 20 samples collected from each field in a state. Numbers in parenthesis represent the range (minimum to maximum) of CFU. AF36 and Afla-Guard were evaluated in both years in North Carolina. In Alabama, Afla-Guard was evaluated in both years, while AF36 was evaluated only in 2013. In Georgia, only Afla-Guard was evaluated in both years.*

[b] *ΔCFU refers to change (– or +) in CFU relative to CFU prior to application of biocontrol strains. ΔCFU = (x/y), where x = CFU at post-application or harvest, and y = CFU at pre-application of biocontrol strains.*

[c] *Soil samples were not collected at this time period and no data is available.*

TABLE 3 | Frequency of members within *Aspergillus* section *Flavi* isolated from soil in fields in southeastern United States treated with Afla-Guard and AF36 biocontrol strains.

				Incidence (%)				
State[a]	**Year**	**Soil sampling period**	**Number evaluated**	***A. flavus***	***A. parasiticus***	***A. caelatus***	***A. nomius***	***A. tamarii***
Alabama	2012	Pre-application	94	95.7	4.3	0.0	0.0	0.0
		Post-application	154	61.0	35.1	3.2	0.7	0.0
		Harvest	106	82.1	13.2	4.7	0.0	0.0
	2013	Pre-application	105	82.9	16.2	0.9	0.0	0.0
		Post-application	97	90.7	7.2	0.0	0.0	2.1
		Harvest	100	98.0	2.0	0.0	0.0	0.0
Georgia	2012	Pre-application	96	97.9	2.1	0.0	0.0	0.0
		Post-application	94	97.9	2.1	0.0	0.0	0.0
		Harvest	–[b]	–[b]	–[b]	0.0	0.0	0.0
	2013	Pre-application	93	97.8	2.2	0.0	0.0	0.0
		Post-application	–[b]	–[b]	–[b]	0.0	0.0	0.0
		Harvest	94	100.0	0.0	0.0	0.0	0.0
North Carolina	2012	Pre-application	106	84.9	15.1	0.0	0.0	0.0
		Post-application	94	96.8	3.2	0.0	0.0	0.0
		Harvest	94	96.8	3.2	0.0	0.0	0.0
	2013	Pre-application	105	93.3	6.7	0.0	0.0	0.0
		Post-application	94	95.7	4.3	0.0	0.0	0.0
		Harvest	97	94.8	5.2	0.0	0.0	0.0

[a] *Afla-Guard and AF36 were evaluated in both years in North Carolina. In Alabama, Afla-Guard was evaluated in 2012 and 2013, while AF36 was evaluated only in 2013. In Georgia, only Afla-Guard was evaluated in both years.*

[b] *Soil samples were not collected at this time period and no data is available.*

tamarii were the other species within *Aspergillus* section *Flavi* that were isolated in this study. *A. caelatus*, *A. nomius,* and *A. tamarii* were isolated in soils collected only from Alabama. The incidences of these three species ranged from 0 to 4.7% and were considerably lower than those observed for either *A. flavus* or *A. parasiticus*. The incidence of *A. nomius* was about 1%, while that of *A. caelatus* was about 5% of the total population across the three sampling periods. *A. tamari* was detected only in 2013 in Alabama with an incidence of 2.1%. None of these three species were isolated in soil collected at harvest (**Table 3**).

Genetic Diversity in Response to Application of Biocontrol Strains

To assess shifts in the genetic structure of populations of *A. flavus* following treatment application, MLST was used to determine the number of MLHs at each soil sampling period. The number of unique MLHs varied between sampling period, states and growing seasons (**Table S1**). In general, the number of MLH was greater before and after the application of treatments, but lower at harvest (**Table 4**). A total of 112 unique MLHs were inferred in this study based on 1,282 isolates of *A. flavus* that

TABLE 4 | Number of unique multilocus haplotypes (MLHs) inferred from populations of *Aspergillus flavus* in soil from maize fields in southeastern United States treated with Afla-Guard and AF36 biocontrol strains in 2012 and 2013.

	Alabama[a]		Georgia[a]		North Carolina[a]	
Sampling period	2012	2013	2012	2013	2012	2013
Pre-application	16	37	21	23	23	18
Post-application	22	34	19	–[b]	29	17
Harvest	16	36	–[b]	3	19	17
Total[c]	37	73	30	25	38	32

[a]*AF36 and Afla-Guard were evaluated in both years in North Carolina. In Alabama, Afla-Guard was evaluated in both years, while AF36 was evaluated only in 2013. In Georgia, only Afla-Guard was evaluated in both years.*

[b]*Soil samples were not collected at post-application or harvest in 2013 and 2012, respectively, and no data is available.*

[c]*Totals are the number of unique MLHs in each year in each state. The number of unique MLHs were examined within each sampling period of each year at each location.*

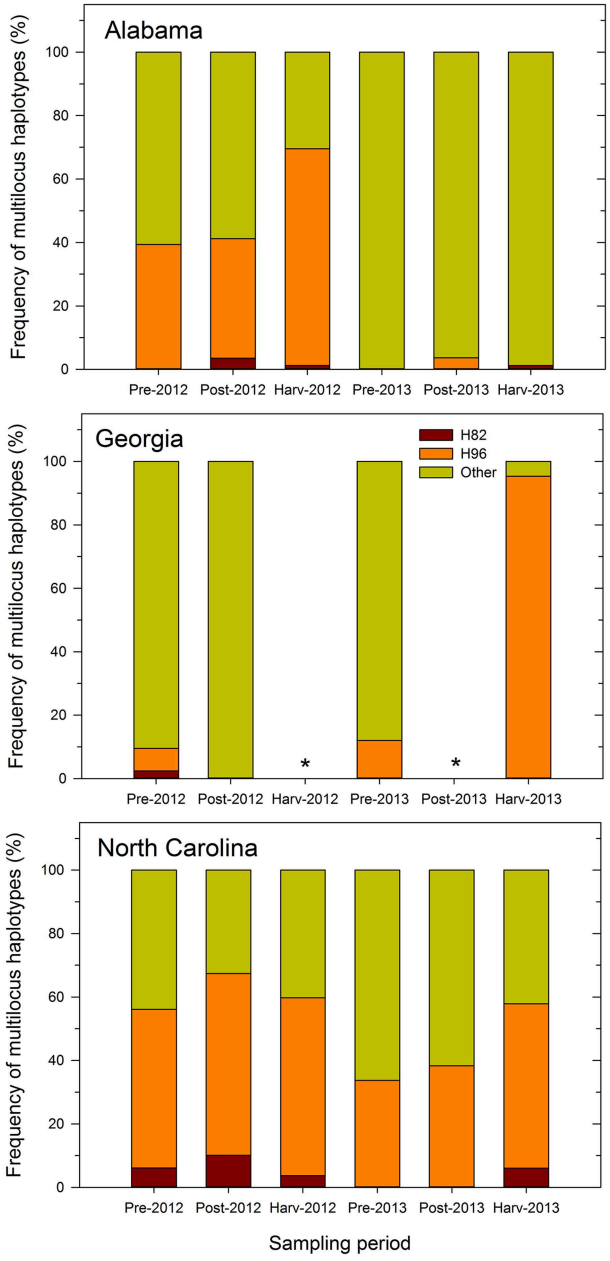

FIGURE 1 | Frequency of multilocus haplotypes (MLHs) recovered (as a proportion of the total number of MLHs observed) at each sampling period from maize fields in Alabama, Georgia, and North Carolina in 2012 and 2013 using combined MLST loci (*trpC*, AF17, and *mfs*) sequence data. Pre- and post-denotes sampling time before and after application of Afla-Guard and AF36. MLHs are designated as belonging to either the Afla-Guard MLH (H96), AF36 MLH (H82), or neither of these two MHLs (Other). The asterisk (*) indicates that soil samples were not collected at harvest in 2012 and at post-application of the biocontrol in 2013 in Georgia and there is no corresponding MLH frequency data.

were characterized. The highest number of unique MLHs was observed in Alabama with 73, while the number of MLHs in Georgia and North Carolina were much lower with 30 and 38, respectively (**Table 4**). The number of MLHs at different sampling periods in Alabama ranged from 16 in 2012 at harvest to 37 at pre-application in 2013. In North Carolina, number of MLHs ranged from 17 in 2013 at harvest to 29 in 2012 post-application of biocontrol treatments. Generally, the number of MLHs was higher in Georgia than either Alabama or North Carolina, with numbers ranging from 3 to 23 in the 2013 growing season (**Table 4**). Only 22 of the 112 unique MLHs were common in all three states, while MLHs unique to a specific state were highest in Alabama with 40 MLHs and considerably lower in Georgia and North Carolina that had only 7 and 16 MLHs, respectively. Sequences used for MLST (AF17, *mfs,* and *trpC*) were submitted to GenBank under accession numbers 2232583, 2233208, and 2233307.

The proportion of inferred individuals that was similar to the MLH of Afla-Guard strain (H96) was higher than that of individuals similar to the MLH of AF36 strain (H82) (**Figure 1**). Further, the recovery of individuals belonging to the two MLHs varied by state and sampling period. For example, the proportion of individuals at different sampling periods that belonged to either H82 or H96 was less consistent across growing seasons in either Alabama or Georgia in 2012 and 2013. However, the proportions of individuals belonging to either H82 or H96 MLH prior to application of biocontrol treatments and at harvest were consistent in 2012 and 2013 in North Carolina. For example, 50 and 56% of isolates recovered in North Carolina prior to biocontrol application and at harvest, respectively, belonged to H96 in 2012. Similar levels were observed in 2013 where 34 and 52% of the isolates recovered prior to biocontrol treatment and at harvest were of the H96 MLH (**Figure 1**).

In Alabama, the proportions of individuals that matched either H82 or H96 varied between growing seasons. In 2012, individuals matching H96 increased over the sampling periods and ranged from 39% prior to application of the biocontrol treatments to 68% at harvest (**Figure 1**). In contrast, individuals belonging to H82 were fewer in 2012 and ranged between 0 and 1%. In 2013, very few individuals (1–4%) belonged to either H82 or H96. The proportion of individuals in Georgia belonging to

either H82 or H96 was very low in 2012 compared to 2013. In 2012, only 2% of the recovered individuals matched the H82 and 7% of the recovered individuals belonged to H96. In 2013, no individuals recovered in Georgia belonged to the H82 haplotype, while 12 and 95% of the individuals before application of treatments and at harvest, respectively, were of the H96 MLH (**Figure 1**).

Recovery of *A. flavus* individuals belonging to either H82 or H96 was more consistent over the two growing seasons in North Carolina compared to either Alabama or Georgia (**Figure 1**). Individuals belonging to H82 and H96 were recovered in both years and at all sampling periods in North Carolina, except during the 2013 pre- and post-application periods. In 2012, most individuals recovered from the field in North Carolina belonged to H96 and they ranged from 50% at the pre-application period to 57% at the post-application period with 56% at harvest). The corresponding number of individuals belonging to H82 ranged from 6% at the harvest period to 10% at the post-application period. A similar pattern for the recovery of individuals similar to H96 in North Carolina was observed in 2013, with numbers ranging from 34% at the pre-application to 38% post-application and 52% at harvest. Individuals belonging to H82 that were recovered only at harvest in 2013 in North Carolina, accounted for only 6% of the total number of MLHs. Across the entire study, the proportion of the recovered individuals with the H96 MLH ranged from 4 to 95%, while that of individuals with H82 MLH ranged from 1 to 10% after application of treatments (**Figure 1**).

TABLE 5 | Frequency and distribution of mating-type (*MAT*) genes among isolates of *Aspergillus flavus* in soil from maize fields in southeastern United States treated with Afla-Guard and AF36 biocontrol strains in 2012.

State	Sampling period[a]	Genetic scale[b]	Mating-type frequency[c]		*P*-value[d]
			MAT1-1	*MAT1-2*	
Alabama	Pre-application	Corrected	80.8 (21)	19.2 (5)	0.0025
		Uncorrected	36.4 (32)	63.6 (56)	0.0138
	Post-application	Corrected	52.8 (19)	47.2 (17)	0.8679
		Uncorrected	38.8 (33)	61.2 (52)	0.0503
	Harvest	Corrected	59.1 (13)	40.9 (9)	0.5235
		Uncorrected	24.4 (20)	75.6 (62)	0.0001
Georgia	Pre-application	Corrected	55.2 (16)	44.8 (13)	0.7111
		Uncorrected	75.0 (63)	25.0 (21)	0.0001
	Post-application	Corrected	62.1 (18)	37.9 (11)	0.2649
		Uncorrected	69.8 (60)	30.2 (26)	0.0001
	Harvest	Corrected	–[c]	–[c]	–
		Uncorrected	–[c]	–[c]	–
North Carolina	Pre-application	Corrected	41.9 (13)	58.1 (18)	0.4731
		Uncorrected	25.6 (21)	74.4 (61)	0.0001
	Post-application	Corrected	34.6 (9)	65.4 (17)	0.1686
		Uncorrected	14.6 (13)	85.4 (76)	0.0001
	Harvest	Corrected	39.3 (11)	60.7 (17)	0.3449
		Uncorrected	23.2 (19)	76.8 (63)	0.0001

[a] *Denotes when soil samples were collected from the field in relation to the application of the biocontrol agents. Afla-Guard and AF36 were evaluated in both years in North Carolina. In Alabama, Afla-Guard was evaluated in both years, while AF36 was evaluated only in 2013. In Georgia, only Afla-Guard was evaluated in both years.*

[b] *Mating-type designation based on either uncorrected or clone corrected multilocus haplotype data.*

[c] *Numbers presented in parentheses refer to number of isolates examined. Soil samples were not collected at harvest in Georgia.*

[d] *Probability from a two-tailed exact binomial test performed under the null hypothesis of no significant difference in the frequency of isolates with MAT1-1 and MAT1-2 genes.*

Frequency and Distribution of Mating Type Genes Among Haplotypes

Based on MLH corrected data, all populations of *A. flavus* in Alabama, Georgia, and North Carolina in 2012 (**Table 5**) and 2013 (**Table 6**) did not significantly ($P > 0.05$) deviate from the 1:1 mating-type ratio except for pre-application populations in Alabama in 2012 ($P = 0.0025$) and 2013 ($P = 0.0031$). The pre-application population in Alabama in 2012 was skewed toward *MAT1-1*, while the pre-application population in 2013 was skewed toward *MAT1-2*.

Unlike with the MLH corrected data, *A. flavus* populations in Alabama significantly ($P < 0.05$) deviated from a 1:1 mating-type ratio except at the post-application population ($P = 0.0503$) when uncorrected data were analyzed using the exact binomial test (**Table 5**). Similar results with MLH uncorrected data were also observed for populations in Georgia and North Carolina, where all populations significantly ($P < 0.05$) deviated from a 1:1 mating-type ratio except the pre-application population ($P = 0.6609$) in 2013 in Georgia and the 2013 pre-application ($P = 1.0000$) and post-application ($P = 0.7407$) populations in North Carolina (**Table 6**).

Population Genetics, Structure, and Phylogenetic Analyses

Nucleotide diversity (π) was low across the three MLST loci and estimates were similar within sampling periods in each state and ranged from 0.0002 at harvest in North Carolina to 0.0116 in Alabama prior to application of biocontrol treatments (**Table 7**). Tajima's D and Fu's F_S used to test the hypothesis of neutral mutation did not show significant ($P > 0.05$) deviations from neutrality except for a single population at harvest in North Carolina that showed significant ($P < 0.05$) deviation from neutrality based on the *mfs* locus (**Table 7**). This significant value indicates the presence of divergent alleles and balancing selection on aflatoxigenicity and non-aflatoxigenicity in the aflatoxin cluster.

The population-scaled mean mutation rate, θ, averaged across all loci was similar in magnitude within and between state (Alabama, Georgia, North Carolina), sampling period (pre-application, post-application, harvest) and year (2012, 2013). At the state level, θ was slightly higher in in Alabama ($\theta = 3.747$) and lower in Georgia ($\theta = 2.343$) with values for North Carolina being intermediate ($\theta = 2.653$). Similarly, θ differed between seasons and was 36% higher in 2013 ($\theta = 3.681$) than in 2012 ($\theta = 2.710$). However, no differences in θ were observed between sampling periods, where the mean θ was about 3.166. The similarity in estimates of π and θ indicates a lack of significant underlying differences in mutation rates and population genetic structure.

TABLE 6 | Frequency and distribution of mating-type (*MAT*) genes among isolates of *Aspergillus flavus* in soil from fields in the southeastern United States treated with Afla-Guard and AF36 biocontrol strains in 2013.

State	Sampling period[a]	Genetic scale[b]	Mating-type frequency[c]		*P*-value[d]
			MAT1-1	*MAT1-2*	
Alabama	Pre-application	Corrected	19.4 (13)	80.6 (54)	0.0031
		Uncorrected	22.5 (18)	77.5 (62)	0.0001
	Post-application	Corrected	53.1 (17)	46.9 (15)	0.8601
		Uncorrected	27.7 (23)	72.3 (60)	0.0001
	Harvest	Corrected	38.6 (17)	61.4 (27)	0.1742
		Uncorrected	25.9 (21)	74.1 (60)	0.0001
Georgia	Pre-application	Corrected	40.5 (15)	59.5 (22)	0.3240
		Uncorrected	53.0 (44)	47.0 (39)	0.6609
	Post-application	Corrected	–[c]	–[c]	–
		Uncorrected	–[c]	–[c]	–
	Harvest	Corrected	25.0 (1)	5.0 (3)	0.6250
		Uncorrected	1.2 (1)	98.8 (85)	0.0001
North Carolina	Pre-application	Corrected	56.7 (17)	43.3 (13)	0.5847
		Uncorrected	50.0 (40)	50.0 (40)	1.0001
	Post-application	Corrected	65.7 (23)	34.3 (12)	0.0895
		Uncorrected	47.6 (39)	52.4 (43)	0.7407
	Harvest	Corrected	56.0 (14)	44.0 (11)	0.6900
		Uncorrected	32.5 (26)	67.5 (54)	0.0023

[a]*Denotes when soil samples were collected from the field in relation to the application of the biocontrol agents. Afla-Guard and AF36 were evaluated in both years in North Carolina. In Alabama, Afla-Guard was evaluated in both years, while AF36 was evaluated only in 2013. In Georgia, only Afla-Guard was evaluated in both years.*

[b]*Mating-type designation is based on either uncorrected or clone corrected multilocus haplotype data.*

[c]*Numbers presented in parentheses refer to number of isolates examined. Soil samples were not collected at post-application of the biocontrol agent harvest in Georgia.*

[d]*Probability from a two-tailed exact binomial test performed under the null hypothesis of no significant difference in the frequency of isolates with MAT1-1 and MAT1-2 genes.*

An overall F_{ST} of 0.0089 ($P < 0.0001$) revealed very little genetic structure among sampling locations in North Carolina, Alabama, and Georgia. PCA and Tracy-Widom of MLST data identified 16 significant axes of variation. The optimal number of clusters for *k*-means ranged from 2 to 8 and the Calinski–Harabasz index found $k = 2$ as the best cluster count (**Figure 2**). The clusters were identified as lineages IB and IC based on sequence similarity of MLHs with previous studies (Moore et al., 2009, 2017; Olarte et al., 2012). Both the Afla-Guard (H96) and AF36 biocontrol (H82) strains were clustered in lineage IB. A two-sided Fisher's exact test showed no significant association of lineage with state ($P = 0.07685$) and year ($P = 1.0000$), but there was a significant association between lineage and sampling period ($P < 0.00001$).

The multilocus phylogenetic tree exhibited a high degree of homoplasy with low bootstrap values (<70%) for many internal branches (**Figure 3**). Although unsupported by bootstrap analysis, two distinct clades were apparent. A large clade with short branch lengths comprising seven MLHs (H1, H92, H95, H96, H98, H106, and H111) included the Afla-Guard strain (H96) and other isolates predominantly in lineage IB (**Figure 3**). The other major clade with long and short branches included isolates that belonged to IB and IC lineages where the long branches are indicative of inter-lineage recombination; the AF36 biocontrol strain (H82) was in this clade. Patristic distances from the Afla-Guard reference isolate showed extensive clonality within IB (patristic distances close to 0 across the three loci) and recombination between IB and IC (incongruent patristic distances across the three loci; **Figure 3**). In *trpC*, both Afla-Guard and AF36 had a patristic distance of 0 which points to identical sequences at this locus; *mfs* showed the greatest sequence divergence from Afla-Guard for some isolates with patristic distances close to 1.

Aflatoxin Contamination in Harvested Grain

Aflatoxin levels varied widely between states and were very low throughout the study. The only exception was in North Carolina in 2012, where the highest level of contamination was 103.8 ppb in the untreated plot (**Table 8**). Contamination levels in the remaining growing season-by-location combinations were very low at <12 ppb except in the untreated plots in Alabama in 2012. Significant differences ($P < 0.05$) in contamination between treated and untreated plots were observed only in North Carolina in 2012, while differences in the remaining growing season-by-location combinations were non-significant. Further, levels of aflatoxin contamination were lower in plots treated with Afla-Guard compared to plots treated with AF36, although these differences were not significant. For example, aflatoxin contamination was 2.75 and 4.75 ppb in plots treated with Afla-Guard and AF36, respectively, in North Carolina in 2012. A similar trend was also observed in 2013 in North Carolina, where aflatoxin contamination was 1.25 and 5.08 ppb in plots treated with Afla-Guard and AF36. Levels of aflatoxin contamination in Alabama in 2013 were below the minimum detection limit (**Table 8**).

DISCUSSION

Biocontrol using non-aflatoxigenic strains of *A. flavus* is considered the most successful option currently available to mitigate aflatoxin contamination of agricultural produce (Bhatnagar-Mathur et al., 2015; Ehrlich et al., 2015). Strains of *A. flavus* within a population vary in their ability to produce aflatoxins, ranging from individuals that do not produce the toxin (non-aflatoxigenic strains), to those that are potent producers of aflatoxins (Horn and Dorner, 1999). The non-aflatoxigenic chemotype is fairly common for the L-strain morphotype of *A. flavus* and the inability to produce the aflatoxins is the result of various deletions in the aflatoxin gene cluster (Chang et al., 2009). Application of non-aflatoxigenic strains that are capable of competitively excluding aflatoxigenic strains has been shown to be effective in reducing aflatoxin accumulation in maize in the United States (Dorner, 2009; Abbas et al., 2011b), Africa (Bandyopadhyay et al., 2016; Ayalew et al., 2017), and Europe (Mauro et al., 2018). However, neither of the non-aflatoxigenic strains in commercially available biocontrol products such as Afla-Guard or AF36, persist in soil and require annual applications to maintain their efficacy. As such,

TABLE 7 | Neutrality based on Fu (F_S) and Tajima (D) tests and nucleotide diversity estimates (π) for the three multilocus sequence typing loci for populations of *Aspergillus flavus* collected from fields in the southeastern United States treated with Afla-Guard and AF36 biocontrol strains.

State	Sampling period	*trpC*[a]			*mfs*[a,b]			AF17[a]		
		F_S	D	π	F_S	D	π	F_S	D	π
Alabama	Pre-application	−1.0786	−0.7180	0.0011	−1.6917	−0.7877	0.0058	−0.4803	−0.5979	0.0116
	Post-application	−0.9824	−0.6453	0.0011	−2.0735	−0.9052	0.0048	0.9633	0.9390	0.0114
	Harvest	−1.3830	−0.7248	0.0017	−3.6820	−0.8408	0.0054	2.3024	1.0782	0.0099
	2012	−1.1333	−0.7239	0.0003	−3.7406	−0.9739	0.0042	0.2268	0.5271	0.0092
	2013	−3.0801	−1.0600	0.0020	−3.3376	−0.7285	0.0055	0.5519	−0.7717	0.0085
Georgia	Pre-application	−1.8387	−0.8155	0.0017	−1.6175	−0.3810	0.0042	−0.0881	−0.3469	0.0050
	Post-application	−1.6738	−0.8295	0.0011	−2.5014	−0.7743	0.0041	−0.3985	−0.1441	0.0044
	Harvest	−1.9450	−0.9182	0.0010	−4.0554	−1.1048	0.0036	0.1461	0.0804	0.0038
	2012	−1.2246	−0.6330	0.0013	−2.3542	−0.9817	0.0033	−0.4891	−0.4299	0.0052
	2013	−1.1754	−0.6162	0.0013	−2.6525	−0.6877	0.0046	0.1260	0.0405	0.0034
North Carolina	Pre-application	1.4768	0.6758	0.0046	−0.4811	−0.0224	0.0042	2.8917	1.7240	0.0100
	Post-application	1.5741	0.9971	0.0057	0.1346	−1.0536	0.0017	1.4757	1.3664	0.0063
	Harvest	−0.9691	−0.7874	0.0002	−1.1847	−1.5218*	0.0005	−1.1684	−1.2295	0.0012
	2012	2.5226	1.4506	0.0060	−2.0405	−1.0387	0.0023	1.8450	0.9399	0.0079
	2013	−2.7494	−1.3204	0.0011	−0.7275	−0.2841	0.0033	0.0532	−0.2678	0.0046

[a] *F_S measures departure from neutrality based on Fu (1997), where negative values are evidence for an excess number of alleles and suggest recent population growth, while positive values are evidence for a deficiency of alleles from a recent bottleneck; D measures departure from neutrality based on Tajima (1989), where negative values suggest rapid population growth, while positive values indicate population contraction; Nucleotide diversity (π) is based on (Nei and Li, 1979).*

[b] *The asterisk (*) denotes values with significant ($P < 0.05$) deviation from neutrality based on either the F_S or D test.*

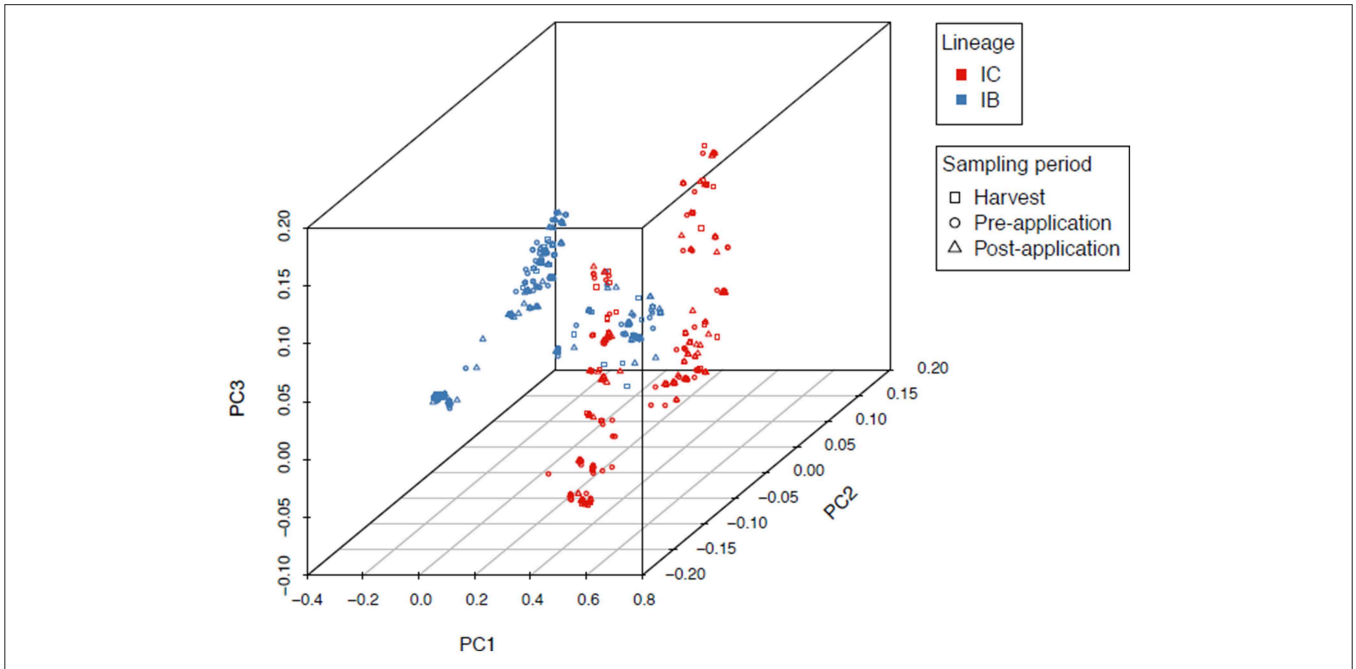

FIGURE 2 | Principal component analysis of 1,282 *A. flavus* isolates showing two distinct clusters identified as lineages IB and IC based on MLST loci (*trpC*, AF17, and *mfs*). Admixture between IB and IC is indicated as a mix of red and blue lineage colors in the middle of the first principal component axis (PC1). There was a significant ($P < 0.00001$) association between lineage and sampling period.

there has been considerable interest to understand factors that influence the efficacy of biocontrol treatments in an effort to develop biocontrol strategies that reduce aflatoxin accumulation at a greater rate but still persist in multiple years and generations of *A. flavus*. Haplotype diversity, mating type frequency and shifts in the populations of *A. flavus* were examined to assess the impact of applying biocontrol products, Afla-Guard and AF36, on the genetic structure of indigenous

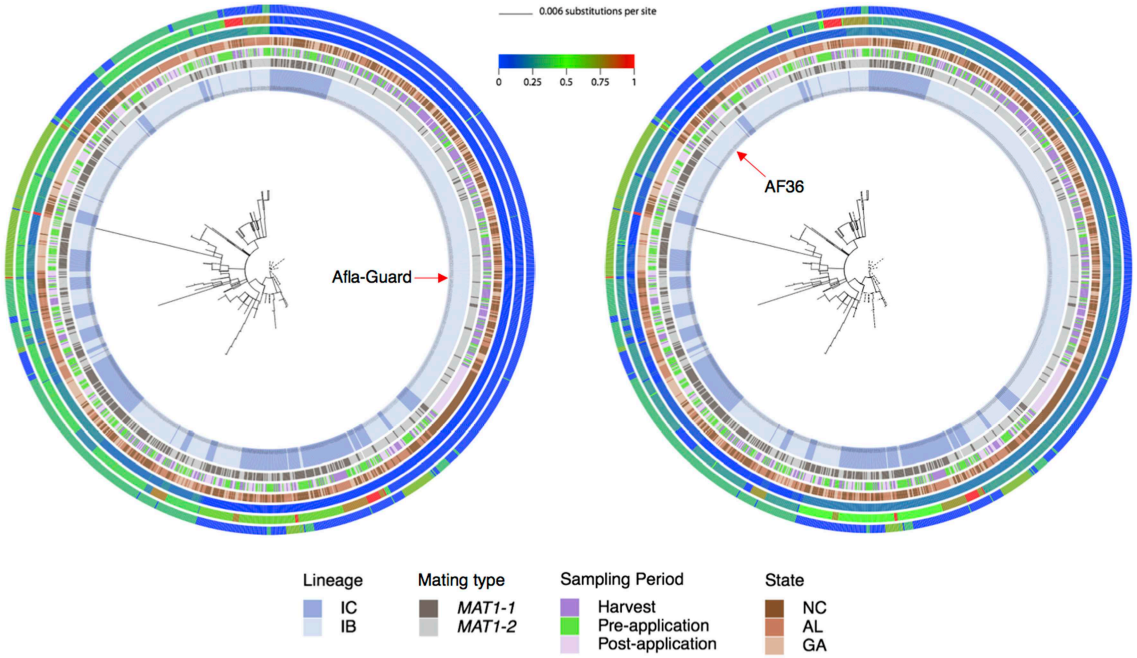

FIGURE 3 | Phylogenetic relationships showing patristic distances of 1,282 *A. flavus* isolates to the Afla-Guard strain (radial tree on left) or the AF36 strain (radial tree on right). In the center of each radial ring is the best maximum likelihood tree for the combined MLST loci (*trpC*, AF17, and *mfs*) with branches drawn to scale (scale bar is shown at the top). The four innermost rings represent *A. flavus* lineage as inferred from principal component analysis, mating type, sampling period, and state, respectively. The three outermost rings represent patristic distances for AF17, *mfs*, and *trpC*, respectively. The distance of each isolate from Afla-Guard or AF36 as a reference is shown using a heat map, where a value of 0 (blue) indicates high genetic similarity of the strain to the reference and a value of 1 (red) is high genetic dissimilarity.

populations of *A. flavus* in maize fields in the southeastern United States.

Aspergillus flavus was the most frequently recovered species within *Aspergillus* section *Flavi* across all states before and after application of Afla-Guard and AF36, with all *A. flavus* isolates belonging to the L-strain morphotype. *A. parasiticus* was the second most recovered species with *A. caelatus*, *A. nomius,* and *A. tamarii* being recovered in very low frequencies only in Alabama. The high frequency of *A. flavus* relative to *A. parasiticus* or other species within section *Flavi* also has been reported in the southern United States (Horn and Dorner, 1998) and in Texas (Jaime-Garcia and Cotty, 2004), South America (Nesci and Etcheverry, 2002), and Africa (Hell et al., 2003; Atehnkeng et al., 2008). The predominance of *A. flavus* is due to its greater competitiveness and ability to survive better on crop debris than *A. parasiticus* or other species within *Aspergillus* section *Flavi* (Zummo and Scott, 1990). Warmer ambient air temperatures during this study were also more conducive for *A. flavus* that grows optimally at 37°C than for *A. parasiticus* that grows optimally at 25°C (Horn, 2005). This ecological niche adaptation explains why *A. parasiticus* is frequently associated with peanut pods in soil compared to above-ground crops such as maize and cotton. The high diversity in Alabama is consistent with reports of increased diversity within *Aspergillus* section *Flavi* in fields near 90° longitude in the southeastern United States and this diversity has been attributed to a combination of crop histories and crop response to environmental factors (Horn and Dorner, 1998). Generally, the frequency of *A. flavus* increased, while that of *A. parasiticus* decreased following application of biocontrol treatments. The increase in the densities of *A. flavus* may be due to other ecological factors rather than a simple dose-response to the introduction of biocontrol strains since 56–60% of individuals recovered after the biocontrol treatments were neither of the Afla-Guard nor the AF36 MLH.

Factors underlying shifts in the MLH diversity observed in this study are not known but could be related to sexual recombination within populations. *A. flavus* L is heterothallic with each individual strain having a single *MAT1-1* or *MAT1-2* mating type gene (Ramirez-Prado et al., 2008). In this study, *A. flavus* L populations exhibited a mating distribution consistent with ongoing sexual reproduction in as little as 2 weeks after biocontrol application. The only exceptions were two populations of *A. flavus* L in Alabama prior to biocontrol application in which individuals were significantly skewed toward *MAT1-1* in 2012 and *MAT1-2* in 2013. However, the mating-type distribution in these two populations in Alabama reverted to a 1:1 distribution of *MAT1-1:MAT1-2* at harvest. Thus, populations of *A. flavus* L in the southeastern United States are mainly sexual in nature as postulated earlier in a study

TABLE 8 | Aflatoxin concentration in harvested grain and dominant multilocus haplotypes (MLHs) of *Aspergillus flavus* in soil from fields in the southeastern United States treated with Afla-Guard and AF36 biocontrol strains.

Year	State	Treatment	Aflatoxin concentration (ppb)[x]		Dominant MLH[z]
			Afla-Guard[y]	AF36[y]	
2012	Alabama	Treated	5.96a	–	H96
		Untreated	27.85a	–	
	Georgia	Treated	–	–	H96
		Untreated	–	–	
	North Carolina	Treated	2.75a	4.75a	H96
		Untreated	103.75b	103.75b	
2013	Alabama	Treated	2.20a	1.28a	H96
		Untreated	2.04a	2.04a	
	Georgia	Treated	5.00a	–	H96
		Untreated	9.00a	–	
	North Carolina	Treated	1.25a	5.08a	H96
		Untreated	11.43a	11.43a	

[x]*Aflatoxin concentrations followed by the same letter are not significantly different at* $\alpha = 0.05$.
[y]*Afla-Guard was not evaluated in Georgia in 2012, while AF36 was not evaluated in Alabama in 2012 and in Georgia in 2012 and 2013.*
[z]*H96 is the Afla-Guard MLH and belongs to lineage IB.*

that examined *A. flavus* populations from a peanut field in Georgia (Ramirez-Prado et al., 2008). Further evidence of sexuality in populations is indicated by the lack of a geographic structure between Alabama, Georgia, and North Carolina, which suggests gene flow and a largely panmictic population of *A. flavus* L. In addition, several strains with the genetic background of the Afla-Guard strain had either one of the two mating-types suggesting that either the Afla-Guard strain is recombining with the indigenous population of *A. flavus* or that the indigenous population is primarily of the IB lineage and is outcrossing. The proliferation and persistence of lineage IB isolates in soil suggests that it is possible to shift soil populations to the more non-aflatoxigenic IB lineage.

Sexual reproduction increases the diversity of aflatoxin profiles creating new vegetative compatible groups and sexuality is also associated with higher recombination rates in the aflatoxin cluster and less pronounced chemotype differences within the populations (Moore et al., 2009). Aflatoxin production in our sampled strains was not determined but an approximate *MAT1-1:MAT1-2* ratio of 1 in each state reported here suggests that populations of *A. flavus* L in the southeastern United States would exhibit variability in aflatoxin concentrations. The potential of a biocontrol strain to recombine with predominantly aflatoxigenic native strains is greater when the *A. flavus* population has equal distribution of *MAT1-1* and *MAT1-2* (Moore et al., 2013) and this has direct implications in selection of non-aflatoxigenic strains. Sexual crosses result in a higher frequency of aflatoxigenic progeny strains when the AF36 strain is the parental strain and a lower frequency of aflatoxin producing progeny strains when the Afla-Guard strain is the parent (Olarte et al., 2012). Unlike the Afla-Guard strain, the AF36 strain has a full aflatoxin gene cluster and replacement with a functional *pskA* can promote synthesis of aflatoxin in AF36 progeny strains. Thus, non-aflatoxigenic strains that lack the cluster gene such as the Afla-Guard strain and similar members within lineage IB, that are likely to recombine with predominant aflatoxigenic strains will be preferable in enhancing the efficacy and sustainability of biocontrol of aflatoxin accumulation.

While clone corrected populations showed a near 1:1 distribution of the two mating types, the frequency of uncorrected *MAT1-1* and *MAT1-2* individuals was significantly skewed toward *MAT1-2* in Alabama and North Carolina and toward *MAT1-1* in Georgia. This skewed distribution to one mating-type can partly be explained by clonal reproduction of a specific vegetative compatibility group that has an advantage over others during vegetative propagation (Leslie and Klein, 1996). The enrichment of either *MAT1-1* or *MAT1-2* in the population also may be due to differences in female fertility or fitness associated with either mating-type (Leslie and Klein, 1996; Moore et al., 2013). Dominance of a specific mating-type suggests that *A. flavus* L populations can be predominantly clonal despite the presence of sexual reproduction, as reported in the pathogenic fungus *Penicillium marneffei* (Henk et al., 2012). The skew toward either *MAT1-1* or *MAT1-2* though not significant after clone correction, can inform selection of non-aflatoxigenic strains in the design of sustainable biocontrol strategies to mitigate aflatoxin accumulation. For example, if a population is predominantly *MAT1-1* as observed in the clonal population of *A. flavus* in Argentina (Moore et al., 2013), then a *MAT1-2* biocontrol strain would be better because there would be more opportunities for sex. While a high frequency of female sterility can ultimately drive a sexually recombining population to clonality (Hornok et al., 2007), the frequency of *MAT1-1* or *MAT1-2* individuals in field populations examined in the present study was approximately equal after clone correction. This suggests that female fertility in *A. flavus* populations was sufficiently high to achieve mating type equilibrium across all three states. Sex can contribute to making biocontrol more sustainable by spreading determinants of non-aflatoxigenicity to subsequent *A. flavus* generations.

Genotyping *A. flavus* field populations before and after biocontrol treatments provides valuable information on the availability and fitness of the biocontrol strain during the growing season and its impact on changing the composition of indigenous populations of *A. flavus* in the soil. Frequently recovered biocontrol strains are likely to persist in soil and be more effective in reducing aflatoxin accumulation over several generations of *A. flavus*. In this study, most of the *A. flavus* L strains recovered after application of treatments belonged to the same MLH as Afla-Guard strain, while very few strains belonged to the same MLH as the AF36 strain. The Afla-Guard haplotype H96 belongs to the IB lineage, while the AF36 MLH H82 belongs to the IC lineage (Geiser et al., 2000). Our data also indicated that both intra- and inter-lineage recombination generates extensive diversity in *A. flavus* with many MLHs sampled only once. This is not surprising given that soil population densities increased several fold over

the course of the season. These results are consistent with a recent study that identified two distinct *A. flavus* populations that were widespread in the United States, where one of the populations was highly clonal and another was more diverse (Drott et al., 2019). While the use of microsatellite markers precluded conclusive evidence of recombination and genetic lineage structuring (Drott et al., 2019) it is clear from the present study that *A. flavus* L populations are structured by lineage (IB and IC) and undergoing intra- and inter-lineage recombination. For example, the results from patristic analysis showed that Afla-Guard (a member of IB) and AF36 (a member of IC) are identical for sequence variation in *trpC,* which was reported previously (Moore et al., 2009). This is expected with ongoing genetic exchange and recombination in field populations and indicates the need to examine more genetic markers to fully determine levels of admixture in populations. Specifically, studies examining single nucleotide polymorphisms from more loci and genome-wide (Geiser et al., 1998, 2000; Taylor et al., 1999; Moore et al., 2009, 2013, 2017; Okoth et al., 2018) are necessary for ultimately tracking the fate of released *A. flavus* biocontrol strains and their potential to shift the relative frequencies of IB and IC lineages.

The complete MLH data from North Carolina allows us to examine the competitiveness and survival of *A. flavus* L individuals in lineages IB and IC between study years. At the end of 2012, 62% of isolates were identical to the Afla-Guard MLH, while only 2% were identical to the AF36 MLH. Prior to biocontrol treatments in the 2013, 15% of the isolates were identical to Afla-Guard haplotype but none were identical to the AF36 haplotype. This suggests that *A. flavus* L individuals in the IB lineage may be more competitive and survive better than those in the IC lineage in the geographical region sampled. These findings indicate that Afla-Guard is more effective than AF36 in shifting the indigenous soil population of *A. flavus* toward the IB lineage. The ability of the Afla-Guard strain to shift soil populations toward the IB lineage could be because the strain is more viable and sexually fertile than the AF36 strain such that both asexual and sexual reproduction results in individuals with a MLH that is similar to that of Afla-Guard. The lower fertility or viability of the AF36 strain seems to be supported by the observation that only 2 of the 16 strains with the AF36 MLH H82 were *MAT1-1*.

Non-aflatoxigenic strains of *A. flavus* in the IB lineage with a MLH similar to that of Afla-Guard strain are expected to be more effective than those in the IC lineage with the AF36 MLH in reducing aflatoxin accumulation in the southeastern United States. Non-aflatoxigenic strains within lineage IB may further be maintained by balancing selection acting to maintain the non-aflatoxigenic phenotype in *A. flavus* populations (Moore et al., 2009; Drott et al., 2017). Thus, use of non-aflatoxigenic strains in lineage IB such as the Afla-Guard strain is expected to be more effective in reducing aflatoxin accumulation over several generations of *A. flavus*. Our prediction of Afla-Guard to be more effective than AF36 in the southeastern United States is supported by previous studies in the region (Abbas et al., 2011a,b; Meyers et al., 2015). Given that the Afla-Guard strain was isolated in Georgia, it is also highly possible that the strain is well-adapted in the region compared to the AF36 strain, which would also partly explain why the AF36 MLH was either recovered in very low frequency or not recovered at harvest. Use of locally or regionally adapted non-aflatoxigenic strains is also desirable as it would favor sexual recombination with indigenous aflatoxigenic strains and result in more a sustainable biocontrol strategy.

The low levels of aflatoxin contamination observed in this study do not allow for a direct assessment of the impact of the shifts in the genetic structure of *A. flavus* on the levels of aflatoxin in maize. In maize, aflatoxin contamination is often associated with heat and drought stress (Windham et al., 2009) especially during reproductive growth with temperatures of 37°C being optimum for the fungus. Here, variations in temperature and rainfall appeared to correlate with levels of aflatoxin. The highest level of contamination in 2012 in North Carolina was primarily due to the high temperature during the reproductive period of maize. Similarly, little to no contamination was observed in 2013 due to the high precipitation and comparatively lower temperatures. Field trials involving large-scale plots where biocontrol treatments are separated by larger buffer zones under conditions that favor aflatoxin accumulation over several seasons will be needed to better assess this impact. In addition, aflatoxin production will need to be determined for sampled strains and lineages to fully understand the relationship between *A. flavus* aflatoxin producing potential and population genetic structure. Ultimately, population genetic data will need to be combined with data on the ecological adaptation of the selected non-aflatoxigenic strains from different environments and crop production systems. While increasing the efficacy of biocontrol of aflatoxin accumulation in maize is important, it is apparent that the population biology of *A. flavus* in the soil will play a critical role in the design of more sustainable biocontrol strategies.

DATA AVAILABILITY

The datasets generated for this study can be found in the Sequences used for MLST (AF17, *mfs*, and *trpC*) were submitted to GenBank under accession numbers 2232583, 2233208, and 223307.

AUTHOR CONTRIBUTIONS

IC and PO conceived the experiments. IC, PO, and GP designed the experiments. ML performed the experiments, collected, and analyzed data. KB, AH, RK, and RH performed experiments and collected data. JL assisted with data analysis. ML, IC, JL, GP, KB, AH, RK, RH, and PO contributed to writing and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01738/full#supplementary-material

Table S1 | Identity metadata of isolates of *Aspergillus flavus* used to study shifts in *A. flavus* soil populations following application of biocontrol strains.

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Potential of Atoxigenic *Aspergillus flavus* Vegetative Compatibility Groups Associated With Maize and Groundnut in Ghana as Biocontrol Agents for Aflatoxin Management

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Increasing knowledge of the deleterious health and economic impacts of aflatoxin in crop commodities has stimulated global interest in aflatoxin mitigation. Current evidence of the incidence of *Aspergillus flavus* isolates belonging to vegetative compatibility groups (VCGs) lacking the ability to produce aflatoxins (i.e., atoxigenic) in Ghana may lead to the development of an aflatoxin biocontrol strategy to mitigate crop aflatoxin content. In this study, 12 genetically diverse atoxigenic African *A. flavus* VCGs (AAVs) were identified from fungal communities associated with maize and groundnut grown in Ghana. Representative isolates of the 12 AAVs were assessed for their ability to inhibit aflatoxin contamination by an aflatoxin-producing isolate in laboratory assays. Then, the 12 isolates were evaluated for their potential as biocontrol agents for aflatoxin mitigation when included in three experimental products (each containing four atoxigenic isolates). The three experimental products were evaluated in 50 maize and 50 groundnut farmers' fields across three agroecological zones (AEZs) in Ghana during the 2014 cropping season. In laboratory assays, the atoxigenic isolates reduced aflatoxin biosynthesis by 87–98% compared to grains inoculated with the aflatoxin-producing isolate alone. In field trials, the applied isolates moved to the crops and had higher ($P < 0.05$) frequencies than other *A. flavus* genotypes. In addition, although at lower frequencies, most atoxigenic genotypes were repeatedly found in untreated crops. Aflatoxin levels in treated crops were lower by 70–100% in groundnut and by 50–100% in maize ($P < 0.05$) than in untreated crops. Results from the current study indicate that combined use of appropriate, well-adapted isolates of atoxigenic AAVs as active ingredients of biocontrol products effectively displace aflatoxin producers and in so doing limit aflatoxin contamination. A member each of eight atoxigenic AAVs with superior competitive potential and wide adaptation across AEZs were selected for further field efficacy trials in Ghana. A major criterion for selection was the atoxigenic

isolate's ability to colonize soils and grains after release in crop field soils. Use of isolates belonging to atoxigenic AAVs in biocontrol management strategies has the potential to improve food safety, productivity, and income opportunities for smallholder farmers in Ghana.

Keywords: aflatoxin, biocontrol, strain selection, efficacy trials, safer food

INTRODUCTION

Following its discovery nearly 60 years ago, aflatoxin contamination of key staple, economically important crops has attracted global attention (Wu, 2015). Developed nations have stringent aflatoxin standards for food/feed crops, milk, and their derived products (Cheli et al., 2014). This allows protecting consumers from health risks associated with aflatoxin exposure (JECFA, 2018). Aflatoxin contamination not only threatens public health but also curtails trade and economic opportunities from farm enterprises when crops exceed tolerance thresholds (Dzirasah, 2015; Kraemer et al., 2016). In contrast, although aflatoxin standards exist in many developing countries such as Ghana (GSA, 2001, 2013), these are poorly enforced. Maize and groundnut in Ghana are prone to aflatoxin contamination. A recent study mirrored the high prevalence of aflatoxin contamination reported frequently over 50 years with concentrations, in most cases, far exceeding the 15 and 10 ppb acceptable threshold for maize and groundnut, respectively, set by the Ghana Standards Authority (Agbetiameh et al., 2018). The two crops constitute major staple and cash crops for millions with per-capita consumption of 44 (US$ 15) and 12 kg (US$ 25) per annum for maize and groundnut, respectively (MoFA, 2011). Consequently, aflatoxin exposure is common and widespread across Ghana. Exposure begins in the unborn child in the uterus and throughout life (Lamplugh et al., 1988; Kumi et al., 2015). Several studies have documented the myriad of health problems associated with aflatoxins in Ghanaians (Shuaib et al., 2010; Jolly et al., 2013; Afum et al., 2016; UNICEF, 2017).

Aflatoxins are produced by fungi belonging to *Aspergillus* section Flavi (Frisvad et al., 2019). *A. flavus*, the most common aflatoxin-producing species worldwide (Klich, 2007), can be subdivided into two distinct morphotypes, the L and S morphotypes (Cotty, 1989). The S morphotype produces numerous small sclerotia (avg. dia <400 μm), few conidia, and consistently high B aflatoxin levels (Cotty, 1989). The L morphotype produces fewer, larger sclerotia (avg. dia >400 μm), numerous conidia, and variable levels of B aflatoxins. There are L morphotype genotypes that lack the ability to produce aflatoxins (i.e., atoxigenic) due to deletions, inversions, or defects in one or more of the aflatoxin biosynthesis genes (Adhikari et al., 2016). *Aspergillus* fungi can be further subdivided into vegetative compatibility groups (VCGs). Members of a VCG descend from the same clonal lineage and therefore are isolated subpopulations (Leslie, 1993; Grubisha and Cotty, 2010, 2015). Diversity among VCGs can be assessed using simple sequence repeat (SSR) markers. Closely related SSR haplotypes in most cases belong to the same VCG (Grubisha and Cotty, 2010, 2015).

Across the globe, several lineages resembling the *A. flavus* S morphotype have been detected with some of them producing copious amounts of both B and G aflatoxins (Probst et al., 2014; Singh and Cotty, 2019). In West Africa, fungi with S morphotype producing both B and G aflatoxins were known as unnamed taxon S_{BG} (Cardwell and Cotty, 2002; Atehnkeng et al., 2008; Donner et al., 2009; Probst et al., 2014). Unknown taxon S_{BG} fungi may be any of the recently described species *A. aflatoxiformans*, *A. austwickii*, *A. cerealis*, or *A. minisclerotigenes* (Pildain et al., 2008; Frisvad et al., 2019). Here we refer as S_{BG} strains to all fungi with S morphotype producing both B and G aflatoxins.

Interactions between atoxigenic and aflatoxin-producing fungi are complex and coupled with other factors determine the extent of crop aflatoxin content (Cotty and Jaime-Garcia, 2007; Mehl et al., 2012; Atehnkeng et al., 2016). In regions where atoxigenic *A. flavus* have been detected, such genotypes have become valuable active ingredients in biocontrol formulations to mitigate crop contamination (Cotty et al., 2007; Atehnkeng et al., 2008; Abbas et al., 2011; Probst et al., 2011; Tran-Dinh et al., 2014; Mauro et al., 2015; Zhou et al., 2015; Alanis Zanon et al., 2016; Bandyopadhyay et al., 2016). Displacement of toxigenic fungi from the crop environment by the deployment of carefully selected atoxigenic *A. flavus* genotypes results in drastic aflatoxin reductions. This has been demonstrated in various crops grown commercially in the United States, Nigeria, Kenya, Senegal, The Gambia, and Italy (Cotty et al., 2007; Dorner, 2010; Doster et al., 2014; Bandyopadhyay et al., 2016; Mauro et al., 2018). This intervention is highly cost-effective in reducing aflatoxin contamination, curtailing aflatoxin-related diseases, and increasing access to local and international premium markets (Wu and Khlangwiset, 2010; Mehl et al., 2012).

In Ghana, aflatoxin management techniques have focused largely on traditional postharvest interventions (Florkowski and Kolavalli, 2013) and more recently on hermetically sealed bags (Paudyal et al., 2017; Danso et al., 2019). In many cases, postharvest technologies are insufficient in curtailing aflatoxin content to safe levels because crop infection and contamination often begins in the field (Mahuku et al., 2019). Once crops become contaminated, aflatoxins cannot be completely removed (Grenier et al., 2014). The aflatoxin biocontrol strategy that targets the source of infection and contamination, the aflatoxin-producing fungi, has not been developed for the farming system in Ghana. However, several atoxigenic *A. flavus* isolates are associated with both maize and groundnut grown across diverse agroecological zones (AEZs) in Ghana (Agbetiameh et al., 2018). The potential of atoxigenic isolates native to Ghana to competitively displace aflatoxin producers and limit crop aflatoxin content has not been investigated.

Atoxigenic biocontrol products are applied during crop development in a formulation (e.g., sterile wheat, sorghum, barley) that gives the active ingredient fungi reproductive advantages over the fungi naturally residing in the treated soils (Mehl et al., 2012). Spores of the beneficial fungi reproduce on the grain, colonize other organic matter substrates in the field, and then become associated with the treated crop during its development (Mehl et al., 2012; Bandyopadhyay et al., 2016). Criteria to select atoxigenic biocontrol agents include wide distribution of the atoxigenic AAV to which they belong over the target nation and superior ability to limit aflatoxin contamination when challenged with highly toxigenic genotypes (Probst et al., 2011; Atehnkeng et al., 2016). It is also necessary to select genotypes with superior abilities to both out-compete other fungi while in the soil and to efficiently move to the crop to provide the intended protection.

The objectives of this study were to: (i) evaluate 12 native atoxigenic *A. flavus* isolates belonging to genetically diverse atoxigenic AAVs for their abilities to reduce aflatoxin production in laboratory assays; (ii) assess comparative abilities of the 12 isolates to establish in soil and crop (maize and groundnut) niches across three AEZs; (iii) determine the extent of aflatoxin reduction by experimental biocontrol products constituted with the candidate isolates; and (iv) select isolates of superior atoxigenic AAVs for use as active ingredients in biocontrol formulations for crop aflatoxin mitigation in Ghana. Native, ecologically adapted atoxigenic AAVs with wide distribution across several AEZs, and with potential as biocontrol agents were detected. Ability to disperse from soil and establish in grains in the field as an ecological criterion for selection of biocontrol active ingredients is a novelty of this study. The identified atoxigenic AAVs are biological resources that can be used to formulate biocontrol products for aflatoxin mitigation. Use of the representative isolates of the selected AAVs may allow for enhanced crop value and food safety and reduce aflatoxin exposure in humans and livestock.

MATERIALS AND METHODS

Microsatellite Genotyping

In a previous study, 4,736 *A. flavus* L morphotype isolates were examined for their aflatoxin-production potential and it was found that 847 isolates lacked aflatoxin-producing abilities (Agbetiameh et al., 2018). We characterized the 847 atoxigenic isolates using SSR markers developed for *A. flavus* (Grubisha and Cotty, 2009). DNA extraction, multiplex-PCR, and microsatellite genotyping were conducted following previously described protocols (Grubisha and Cotty, 2009, 2010; Callicott and Cotty, 2015; Islam et al., 2018). Over 20% of isolates were subjected to at least three independent PCR and genotyping assays for all loci. This allowed to assess consistency of the data.

Population Genetic Analyses

After genotyping, isolates were manually assigned to haplotypes defined by identity across 17 SSR markers (Grubisha and Cotty, 2009). Haplotype frequency was calculated following sample correction, such that a haplotype was only counted once per individual sample. Frequencies were then calculated on a per sample basis (data not shown). Twelve atoxigenic isolates were chosen (**Table 1**) for testing based on a combination of per sample haplotype frequency, presence in other West African countries, and similarity to atoxigenic biocontrol active ingredients already in use in other West African countries (**Figure 1**). Frequently encountered haplotypes were assumed to be already well adapted to Ghana. Isolates belonging to AAVs already selected as active ingredients of biocontrol products have a known ability to reduce aflatoxins when properly applied to crops.

Simple sequence repeat data were re-coded from amplicon size to the number of repeats prior to assessing genetic relationships among all haplotypes. Phylogenetic relationships among the 12 selected genotypes and other registered biocontrol genotypes were assessed with Genodive (Meirmans and Van Tienderen, 2004) after which SplitsTree 4.14.6 (Huson and Bryant, 2005) was used to create a NeighborNet tree (**Figure 1**).

Atoxigenic *Aspergillus flavus* L Morphotype Isolates

The population genetic analyses revealed 12 dominant atoxigenic SSR haplotypes widely distributed across different locations of Ghana (**Table 1**). The origin and distribution of atoxigenic and aflatoxin-producing genotypes is summarized in **Table 1**. Tester pairs of VCGs were developed for 11 of the 12 SSR haplotype groups following previously described protocols (Cove, 1976; Bayman and Cotty, 1991). It was not possible to obtain a complementary pair of *nit* auxotrophs for isolate GHG183-7. The concordance between SSR haplotype and VCG for 11 of the 12 groups was then tested using vegetative compatibility analyses. These VCGs were termed as AAVs.

Laboratory Competition Assays

Representative isolates of the 12 SSR haplotypes were evaluated for their ability to limit aflatoxin accumulation when challenged with *A. flavus* isolate GHG040-1, a potent aflatoxin producer native to Ghana, in laboratory competition assays as described by Probst et al. (2011).

To prepare inocula, single-spored isolates, maintained for long-term storage on silica grains, were grown on 5–2 agar [(5% V-8 juice (Campbell Soup Company, Camden, NJ, United States), 2% Bacto-agar (Difco Laboratories Inc., Detroit, MI, United States), pH 6.0)] at 31°C for 7 days (Cotty, 1989). Spore suspensions of each isolate were prepared in 0.1% TWEEN 80® and adjusted to 10^6 spores ml^{-1} using a turbidimeter (Atehnkeng et al., 2014). A 1-ml spore suspension of the individual atoxigenic isolates and the aflatoxin producer, and mixtures of each atoxigenic/aflatoxin-producing isolate (ratio = 1:1) were separately inoculated on 10 g of autoclaved maize grains. Maize inoculated with 1-ml sterile distilled water served as negative control. Inoculated grains, five replications per treatment, were incubated for 7 days (31°C, dark). The experiment was conducted twice (test 1 and test 2). In test 1, all except atoxigenic isolate GHG083-4 was evaluated.

Following incubation, aflatoxins were extracted from maize fermentations as previously described (Agbetiameh et al., 2018). Briefly, fermentations were combined with 50 ml 70%

TABLE 1 | Origin of a toxigenic isolate and one atoxigenic isolate each of 12 haplotypes of *Aspergillus flavus* used in the current study.

Isolate name[a]	Crop	AEZ[b]	Location[c]	Community[d]	Incidence[e]
GHM001-5	Maize	DS	Nsawam-Adoagyiri	Nsawam	11
GHM017-6	Maize	HF	Ejisu-Juaben	Hwereso	22
GHG079-4	Groundnut	DS	Atebubu-Amantin	Ahotokrom	5
GHG083-4	Groundnut	DS	Atebubu-Amantin	Ahotokrom	5
GHM109-4	Maize	HF	Ejura-Sekyedumase	Teacher Krom	2
GHM173-6	Maize	HF	Wenchi	Nyamebekyere	6
GHM174-1	Maize	HF	Wenchi	Nyamebekyere	14
GHG183-7	Groundnut	DS	Bole	Carpenter	2
GHM287-10	Maize	SGS	Wa West	Varempere	8
GHG321-2	Groundnut	SGS	Nabdam	Asonge	2
GHG331-8	Groundnut	SGS	Talensi	Pwalugu	10
GHM511-3	Maize	DS	Central Tongu	Bakpa-Ajane	14
GHG040-1[f]	Groundnut	HF	Mampong	Sataso	–

*[a]Each isolate belonged to a distinct haplotype which corresponded to a unique African Aspergillus flavus vegetative compatibility group. Haplotype refers to multilocus haploid genotypes based on allele calls at each of 17 SSR loci (Grubisha and Cotty, 2009; **Table 2**). [b]AEZ, agro-ecological zones; DS, derived savanna; HF, humid forest; SGS, southern Guinea Savanna. [c]Administrative district where a community is located. [d]Name of community where household from which maize or groundnut sample containing atoxigenic/toxigenic isolate was found. [e]Number of isolates with similar haplotype encountered among the 847 isolates genotyped. [f]GHG040-1 is an aflatoxin-producing A. flavus isolate. All others are atoxigenic genotypes.*

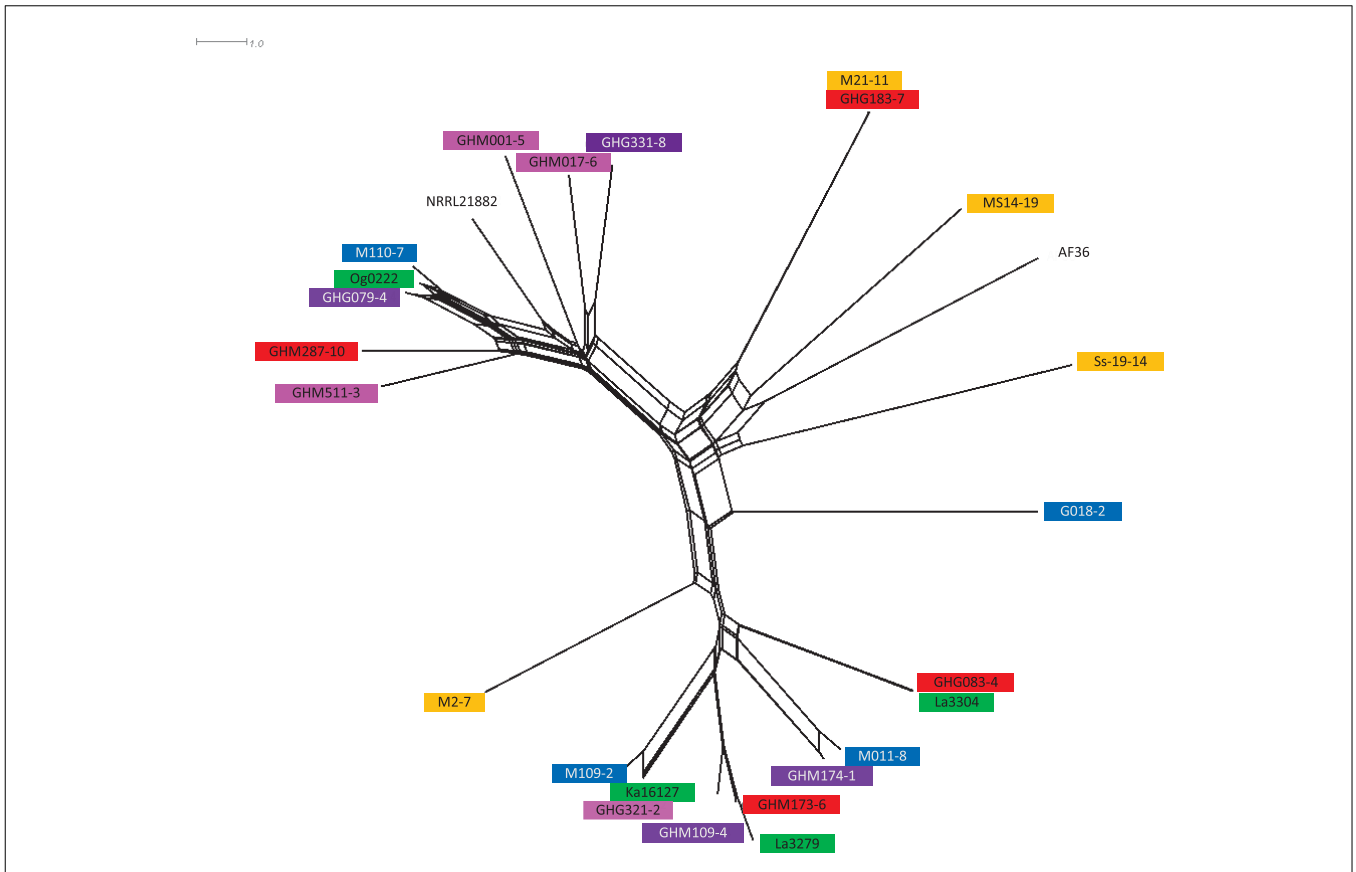

FIGURE 1 | NeighborNet splitstree of 12 selected atoxigenic *Aspergillus flavus* haplotypes from Ghana with other active ingredients of registered aflatoxin biological control products in West Africa. La3279, La3304, Ka16127, and Og0222 are the active ingredients of Aflasafe™ (used in Nigeria, in green); G018-2, M011-8, M109-2, and M110-7 are the active ingredients of Aflasafe BF01 (used in Burkina Faso, in blue); M2-7, M21-11, MS14-19, and Ss19-14 are the active ingredients of Aflasafe SN01 (used in Senegal and The Gambia, in orange); AF36 is the active ingredient of AF36 Prevail™; and NRRL21882 is the active ingredient of Afla-Guard™ (both registered for use in the United States) (Ortega-Beltran and Bandyopadhyay, 2019). Isolates composing experimental product A are in purple, isolates composing experimental product B are in red, and isolates composing experimental product C are in pink. Length of branches are proportional to distances between isolates.

methanol. Suspensions were shaken on a Roto-Shake Genie (Scientific Industries, Bohemia, NY, United States) for 30 min at 400 rpm and filtered through Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, United Kingdom). Filtrates were collected in 250 ml separatory funnels, combined with 5 ml distilled water, and extracted with 15 ml methylene chloride. The methylene chloride phase was filtered through a bed of 25 g anhydrous sodium sulfate contained in fluted Whatman No. 4 filter paper, combined, and evaporated to dryness in a fume hood (Cotty and Cardwell, 1999). Residues were dissolved in 1 ml methylene chloride, spotted (4 μl) alongside aflatoxin standards (Supelco, Bellefonte, PA, United States) on thin layer chromatography (TLC) Aluminum (20 cm × 10 cm) Silica gel 60 F_{254} plates (Merck, Darmstadt, Germany) and developed with diethyl ether–methanol–water (96:3:1) (Probst and Cotty, 2012). Aflatoxins were quantified directly on TLC plates with a scanning densitometer (CAMAG TLC Scanner 3) and quantification software (winCATS 1.4.2, Camag, AG, Muttenz, Switzerland) (Agbetiameh et al., 2018).

Formulation of Experimental Biocontrol Products

Three experimental biocontrol products (named A, B, and C) were composed each with four representative atoxigenic isolates of different haplotypes and manufactured in Ibadan, Nigeria (**Table 2**). To prepare each product, spores of the four atoxigenic isolates were obtained from 5-day-old cultures grown on 5–2 agar to prepare inoculum in bulk. Spores were dislodged and suspended in 0.1% TWEEN 80® and adjusted to 10^6 spores ml^{-1} as above. Spores of each atoxigenic isolate were independently reproduced in glass bottles containing sterilized sorghum grain as follows. Prior to inoculation, sorghum grain was pre-conditioned in sterile 1-L plastic bottles. Moisture content of sorghum grain was increased to 30% by adding sterile distilled water and bottles were rolled for 4 h on a 240 Vac Benchtop Roller (Wheaton, Millville, NJ, United States). Thirty grams of pre-conditioned grain were added to 250-ml glass bottles along with two Teflon balls (1/2″ diameter) and autoclaved (20 min, 121°C). Each cooled bottle containing sorghum was independently inoculated with 4 ml of spore suspension of each atoxigenic isolate. After incubation (7 days, 31°C), 125 ml sterile 0.1% TWEEN® 20 was added to each bottle to harvest spores. Bottles were placed on a Roto-Shake Genie reciprocal shaker (Scientific Industries, Bohemia, NY, United States) at 200 rpm for 20 min. The Teflon balls facilitated dislodging spores from sorghum grains. For each atoxigenic strain, a suspension was adjusted to 4×10^7 spores ml^{-1} as above. To prepare 100 kg of each experimental product, a spore suspension (1 l, 4×10^7 spores ml^{-1}) of the constituent atoxigenic genotypes was individually combined with 150 ml of a polymer (Sentry™, Precision Laboratories, Waukegan, IL, United States) and 200 ml of a blue non-toxic dye (Prism™, Milliken and Company, Spartanburg, SC, United States) and coated on roasted, sterile sorghum grain with a seed treater (Bandyopadhyay et al., 2016). Following phytosanitary certification by the Nigeria Plant Quarantine Service and the issuance of import permit by the

TABLE 2 | Composition of experimental aflatoxin biocontrol products, each containing a mixture of four atoxigenic *Aspergillus flavus* vegetative compatibility groups represented by their type isolates.

Product	Isolate	SSR locus													
		AF28	AF13	AF43	AF22	AF31	AF53	AF34	AF42	AF8	AF16	AF54	AF17	AF11	AF66	AF64	AF63	AF55
A	GHM174-1	113	145	390	192	349	134	301	159	171	169	161	359	141	261	169	127	180
	GHG331-8	119	148	379	144	312	131	296	146	166	169	161	385	126	269	163	127	172
	GHG079-4	119	128	379	144	312	131	296	150	166	169	161	353	132	269	161	127	180
	GHM109-4	135	145	385	192	346	134	301	181	171	169	161	356	141	261	169	127	178
B	GHM173-6	135	145	385	192	346	134	301	181	189	169	161	356	141	261	169	127	180
	GHG083-4	131	135	385	192	315	134	323	159	171	169	161	359	141	255	169	127	184
	GHM287-10	119	141	399	144	312	131	298	150	166	169	161	368	135	271	161	127	180
	GHG183-7	119	145	411	188	325	144	314	168	180	175	172	353	150	269	195	127	172
C	GHM017-6	119	145	426	144	312	131	296	146	174	169	161	374	129	269	163	127	178
	GHM511-3	119	128	399	144	312	131	296	150	174	169	161	368	132	269	159	127	174
	GHG321-2	135	145	385	192	367	134	301	159	160	169	161	362	141	261	169	127	184
	GHM001-5	119	128	387	144	312	131	296	143	168	169	161	374	138	271	161	125	178

For each isolate, the amplicon size at each SSR locus is given.

Plant Protection and Regulatory Services Directorate (PPRSD) of Ghana's Ministry of Food and Agriculture (MoFA), the three experimental products were transported to Ghana for evaluation in farmer field trials.

Field Sites, Plots, and Trial Establishment

Field trials were conducted in 2014 during the major cropping season in Northern Ghana and minor season in the Middle Belt. The trials were conducted in five regions located in three AEZs. In each region, the fields were distributed in two districts. The two cropping seasons and the AEZs' characteristics have been described previously (Agbetiameh et al., 2018). Farmers and their field selection was done in collaboration with Agricultural Extension Agents from the Department of Agriculture of MoFA in the respective districts following stakeholder sensitization and training workshops. In each district, five maize and five groundnut fields (size $\geq$ 2 ha) were selected. Farmers grew their crops according to their own agronomic practices. Each field was divided into four equal-sized plots separated by 5 m from each other. Assignment of plots to treatments across field locations was done using a randomized complete block design (RCBD). Three plots within a block were assigned treatment to one of the three experimental products. The remaining plot was left untreated and served as control. In each district, treatments were replicated five times. When field sizes were <2 ha (mostly groundnut fields), individual fields in a group of four nearby fields were considered as plots. Experimental products were broadcasted by hand (10 kg ha^{-1}) to field soils 2 weeks before flowering and following weeding and/or fertilizer application by farmers. From each plot, before product application and also at harvest, soil samples (up to 2.5 cm depth) were taken randomly from at least 15 different spots resulting in a composite sample of about 150 g (Atehnkeng et al., 2014). Grain samples comprising 25 maize ears and approximately 1-kg groundnut (in-shell) were collected at harvest.

Analysis of *Aspergillus* Section *Flavi* in Soils and Grains

Soil samples were dried in a forced-air oven (50°C, 48 h). Samples with clods were pulverized and sieved through 2 mm wire mesh to remove gravel and large particles. Grains were manually shelled, and 500 g were milled using a laboratory blender (Waring Commercial, Springfield, MO, United States) for 1 min in a 250 ml stainless steel blending jar (MC-2). Milled samples were stored at 4°C before aflatoxin and microbial analyses. The blending jar was washed between samples with 80% ethanol to prevent microbial and aflatoxin cross contamination. *Aspergillus* section *Flavi* fungi in soil and grains were isolated using dilution plate technique on modified rose Bengal Agar as described previously (Atehnkeng et al., 2014). Plates were incubated for 3 days (31°C, dark). From each sample, 12 discrete *Aspergillus* species colonies were sub-cultured on 5–2 agar (31°C, 7 days) and then assigned to their corresponding species based on macroscopic and microscopic characteristics (Pitt and Hocking, 2009). Sporulating cultures of each isolate were saved as agar plugs in 4 ml vials containing 2 ml sterile distilled water until further characterization.

Aflatoxin Determination in Grain Samples

Aflatoxin levels in maize and groundnut sampled at harvest were examined to determine the extent of contamination in grains from treated and control plots. Aflatoxins were extracted from maize by combining 20 g ground sample with 100 ml of 70% methanol (Atehnkeng et al., 2008). For groundnut, 20 g of ground sample was combined with 100 ml of 80% methanol (Cole and Dorner, 1993). Aflatoxins were extracted, combined, separated on TLC plates, and quantified as described above.

Incidence of Atoxigenic Genotypes

Frequencies of *A. flavus* belonging to the applied AAVs of the three experimental products were examined in soils and grains. Nitrate non-utilizing (*nit*) auxotrophs were generated for all recovered *A. flavus* L morphotype isolates (Grubisha and Cotty, 2010). Briefly, a spore suspension of each isolate (approximately 1,000 spores in 15 µl) was seeded into a well at the center of a plate containing mutant selection medium (Czapek-dox broth, 25 g l^{-1} $KClO_3$, 10 ml l^{-1} rose Bengal, 2% Bacto-agar, pH 7.0). Seeded plates were incubated at 31°C for 7–30 days. Spontaneous auxotrophic sectors were transferred to a purification medium (Czapek-dox broth, 15 g l^{-1} $KClO_3$, 2% Bacto-agar, pH 6.5) for 3 days to clean up and stabilize *nit* mutants. A mutant sector was subsequently transferred onto 5–2 agar, and incubated for 5 days at 31°C. Plugs of sporulating mutants were stored in 4 ml glass vials containing 2 ml sterile distilled water for use in complementation assays. Assignment of mutants of isolates to an AAV was based on pairing the isolate auxotroph with complementary tester auxotrophs of each applied AAV (Grubisha and Cotty, 2010). A single complementation test was performed on starch agar (36 g l^{-1} dextrose, 3 g l^{-1} $NaNO_3$, 2% Bacto-agar, 2% soluble starch, pH 6.0) (Cotty and Taylor, 2003) where three wells (3 mm dia, 1 cm apart) were made in a triangular pattern at the center of the plate. Two wells were each seeded with 15 µl of either of the tester pair while the third well was seeded with the isolate auxotroph being characterized. Plates were incubated for 5–10 days at 31°C. Auxotrophs forming a stable heterokaryon with one or both tester auxotrophs of an applied AAV were assigned to that AAV and were considered to be the applied genotype. In all, a total of 47,520 vegetative compatibility tests were conducted.

Data Analysis

All statistical tests were performed with SAS (version 9.4, SAS Institute Inc., Cary, NC, United States). Prior to data analysis, all response variables were log-transformed to stabilize variances. Means of the response variables were subjected to analysis of variance (ANOVA) and separated with Fisher's protected least significant difference (LSD) test ($\alpha = 0.05$). Pairwise comparison means of response variables from treated and control plots were conducted using Student's *t*-test ($\alpha = 0.05$). Applied AAVs were ranked separately by their incidence in soil and grain samples across different geographical locations. To calculate the rank, the proportion of the number of (i) AEZ ($n = 3$), (ii) regions ($n = 5$), (iii) districts ($n = 10$), and (iv) samples ($n = 30$) where the AAV was detected and (v) the proportion of isolates of the

AAV detected (n = 360) was summed. Higher the sum, higher (1 = highest, 11 = lowest) the rank. For example, AAV GHM287-10 in maize was detected in the 3 AEZ (3/3 = 1.0), 5 regions (5/5 = 1.0), 8 districts (8/10 = 0.8), 17 samples (17/30 = 0.57), and 75 isolates were detected (75/360 = 0.21) for a total of 3.58.

RESULTS

Identification of Dominant Atoxigenic Genotypes

Out of the 847 atoxigenic *A. flavus* L morphotype isolates identified previously (Agbetiameh et al., 2018), there were 454 unique and diverse haplotypes. Among those haplotypes, 12 were widely distributed across Ghana (**Table 1**) but not closely related (**Figure 1**). AAV grouping of 11 of the 12 groups concurred with the grouping revealed by SSRs (data not shown). Mutants of isolate GHG183-7 did not complement with tester pairs of any of the 11 AAVs. Therefore, GHG183-7 was considered another AAV.

The SSR signatures for identifying the representative isolates of AAVs constituting the experimental products are reported in **Table 2**. None of the locus was monomorphic among the examined isolates. The number of alleles per locus ranged from 2 to 7 (**Table 2**).

Aflatoxin Inhibition Potential of Atoxigenic Genotypes in Competition Tests

When inoculated individually, none of the 12 atoxigenic isolates produced aflatoxins on maize grains (LOD = 0.1 μg kg^{-1}), as in the previous study (Agbetiameh et al., 2018). The aflatoxin-producing isolate GHG040-1 produced high aflatoxin B_1 levels (>51.0 mg kg^{-1}) on maize grains in both tests, as expected. Marked variations ($P < 0.01$) were detected in the aflatoxin inhibition potential of atoxigenic isolates when co-inoculated with the aflatoxin producer. Aflatoxin reductions ranged from 92.8 to 98.7% (**Table 3**). In test 1, atoxigenic isolates GHM173-6 and GHM511-3 significantly ($P < 0.0001$) reduced aflatoxin accumulation by the aflatoxin producer to <1.0 mg kg^{-1}, the lowest level among all combinations. GHG183-7 had the least aflatoxin inhibition potential (5.59 mg kg^{-1}). However, that level was also significantly ($P < 0.0001$) lower than in grains inoculated solely with the aflatoxin producer. GHG083-4 was not selected when test 1 was conducted, hence no aflatoxin inhibition data were generated in test 1 (**Table 3**).

Similar results were observed in test 2. Aflatoxin reductions ranged from 87.3 to 98.2% (**Table 3**). The lowest toxin inhibition (6.47 mg kg^{-1}) was by GHG183-7, as in test 1. GHM174-1 reduced aflatoxin the most (0.90 mg kg^{-1}).

Quality Control of the Experimental Products

All carrier grains of all batches of the experimental products were colonized only by *A. flavus*. Other microorganisms were not recovered in any of the grains. The recovered *A. flavus* fungi were solely composed of the active ingredient AAVs composing the experimental products. Other AAVs of *A. flavus* were not detected in any of the batches. In each experimental product, each of the four active ingredient AAVs was found on 25 ± 3% carrier grains of the examined batches. Each gram of product contained, on average, 3500 ± 300 colony forming units (CFUs) of the active ingredient fungi.

TABLE 3 | Aflatoxin B (B_1 + B_2) content of maize in μg/kg during co-inoculation of atoxigenic isolates and an aflatoxin-producer.

Isolate	Test 1		Test 2	
	Aflatoxin B (mg kg^{-1})[a]	Reduction (%)[b]	Aflatoxin B (mg kg^{-1})[a]	Reduction (%)[b]
GHM001-5	1.22 ab	98.4	2.51 abc	95.1
GHM017-6	2.81 d	96.4	2.86 abc	94.4
GHG079-4	1.49 ab	98.1	1.61 ab	96.8
GHG083-4[C]	–	–	4.77 cd	90.6
GHM109-4	1.55 ab	98.0	1.32 ab	97.4
GHM173-6	0.98 a	98.7	1.23 ab	97.6
GHM174-1	1.83 bc	97.6	0.90 a	98.2
GHG183-7	5.59 f	92.8	6.47 d	87.3
GHM287-10	1.57 ab	97.9	1.34 ab	97.4
GHG321-2	2.59 d	96.7	2.85 abc	94.4
GHG331-8	4.52 e	94.2	3.35 bc	93.4
GHM511-3	0.99 a	98.7	2.66 abc	94.8
GHG040-1[d]	77.56	–	51.05	–

[a]Aflatoxin B values having a common letter are not significantly different according to Fischer's Least Significant Difference (LSD) test (α = 0.05). [b]Percent aflatoxin B reduction was calculated as [1 – (aflatoxin B content in maize co-inoculated with both toxigenic and atoxigenic isolate/aflatoxin B content in maize inoculated with the aflatoxin-producing isolate alone)] × 100. [c]No data were generated for the atoxigenic isolate GHG083-4 in the first test. [d]GHG040-1 is an aflatoxin-producing isolate.

Aflatoxin Concentration in Crop Samples

Field trials were conducted in 2014 in 10 districts from five regions located in three AEZs in Ghana (**Figure 2**). Across all AEZs, substantially ($P < 0.05$) less aflatoxins accumulated in grains from plots treated with the experimental products, compared to untreated grains. Treated groundnut contained 70.5–99.7% less aflatoxins than those untreated. Across AEZs, aflatoxin levels in treated groundnut ranged from 1 to 61 μg kg^{-1} with those from humid forest (HF) containing safe levels. Aflatoxin content in untreated groundnut ranged from 58 to 302 μg kg^{-1} (**Table 4**). In maize, up to 100% reduction was detected in treated crops. Aflatoxin concentration was below 0.1 μg kg^{-1} in treated maize while it ranged from 0.8 to 7.8 μg kg^{-1} in control plots (**Table 4**).

Aspergillus Fungal Communities in Soils and Grains

Four main members within *Aspergillus* section *Flavi* (*A. flavus* L morphotype, S_{BG} strains, *A. parasiticus*, and *A. tamarii*) were recovered from soil before application and at harvest, and on grain collected at harvest. In all substrates, *A. flavus* L morphotype dominated the communities with frequencies

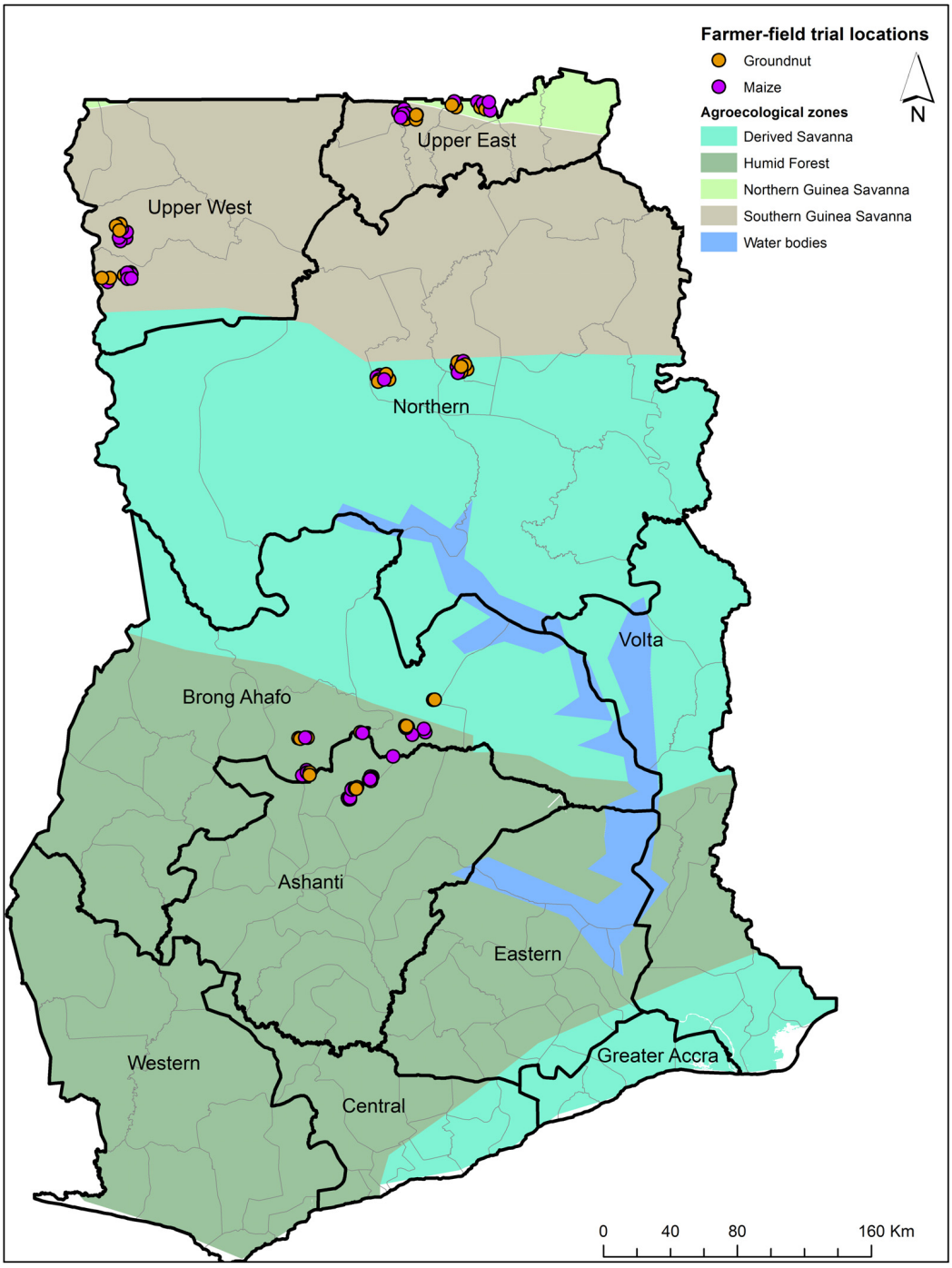

FIGURE 2 | Map of Ghana indicating locations where field trials were conducted in maize and groundnut during 2014.

greater than 83% (**Table 5**). Prior to application of experimental products, incidence of L morphotype in field soils ranged from 87.7% in HF to 99.1% in derived savannah (DS). Frequencies of S_{BG} strains, *A. parasiticus*, and *A. tamarii* were low (range = 0–9.9%; **Table 5**).

Frequencies of *A. flavus* L morphotype in treated soils at harvest ranged from 97.2% in HF to 100% in DS. Across AEZs, in control plots, relatively lower L morphotype frequencies were detected in soil at harvest compared to soil before application. Significantly ($P < 0.05$) higher L morphotype frequencies were observed across treated plots in both DS and HF. Generally, incidences of S_{BG} strains, *A. parasiticus*, and *A. tamarii* were lower in treated soils at harvest, compared to soil before application of experimental products. At harvest in DS, the

TABLE 4 | Aflatoxin content (μg kg^{-1}) in groundnut and maize kernels at harvest from treated and control fields across three agroecological zones (AEZs) in Ghana during 2014 cropping season.

AEZ[a]	N[b]	Treatment[c]	Aflatoxin concentration (μg kg^{-1})			
			Groundnut		Maize	
			Mean	Reduction (%)[d]	Mean	Reduction (%)[d]
DS	9	A	40.2	86.7	0	100
	9	B	0.9	99.7	0	100
	9	C	1.5	99.5	0.4	50.0
	9	Control	302.0		0.8	
HF	9	A	8.3	85.7	0	100
	9	B	3.2	94.5	0.4	94.9
	9	C	0.4	99.3	0.2	97.4
	9	Control	57.9		7.8	
SGS	12	A	45.6	78.1	0.1	96.6
	12	B	13.7	93.4	0.2	93.1
	12	C	61.3	70.5	0.1	96.6
	12	Control	208.0		2.9	

*[a]DS, derived savanna; HF, humid forest; SGS, southern guinea savanna. [b]Number of treated and control plots. [c]Experimental products used in the current study (**Table 2**). Each experimental product (A, B, and C) contained four atoxigenic isolates each representing a unique atoxigenic African Aspergillus flavus vegetative compatibility group. Control refers to plots to which no experimental product was applied. [d]Percent aflatoxin reduction = [1 – (mean aflatoxin content of grains from treated plots/mean aflatoxin content of grains from control plots)] × 100.*

proportions of S_{BG} strains were significantly ($P < 0.05$) higher in untreated soils than in treated soils (**Table 5**). *Aspergillus* communities in treated maize kernels across all three AEZs were entirely composed of the L morphotype. In control maize, the L morphotype dominated and minor frequencies of S_{BG} strains (up to 1.4%) were found (**Table 5**). A similar trend in frequencies of L morphotype, S_{BG} strains, *A. parasiticus*, and *A. tamarii* was observed in soils from groundnut fields and groundnut kernels, except that communities in treated groundnut, in addition to the L morphotype, harbored minor proportions of S_{BG} strains (up to 0.7%) (**Table 6**).

Incidence of Applied Atoxigenic AAVs in Grains After Treatment

The individual atoxigenic AAVs composing the applied experimental products showed varying abilities to disperse from treated soils and establish in the grain of treated and control plots. Each AAV was assigned a rank based on their incidence across AEZ, regions, districts, samples, and number of AAV individuals detected. For instance, 75 isolates belonging to AAV GHM287-10 were recovered from 17 maize samples from 8 out of 10 districts in all five regions across all three AEZs, thus being the most dominant applied AAV in treated grains (rank = 1, **Table 7**). The same AAV was also frequently isolated from control grains (rank = 2, in control grains). Barring a few exceptions (e.g., AAV GHM511-3), most AAVs with high post-release incidence in grains from treated plots also had relatively high incidence in control plots. In contrast, AAV GHM173-6 was the least frequently isolated from grains of both treated and control plots (**Table 7**).

Abilities of the applied AAVs to move into groundnut kernels also varied. Generally, incidence of applied AAVs was relatively lower in groundnut than in maize (**Table 8**). The most prevalent applied AAV was GHG083-4 with 52 member isolates found in 15 samples from all 10 districts in all the regions of the three AEZs. On the other hand, no isolate of AAV GHM173-6 was recovered in groundnut from any field (**Table 7**).

There were some AAVs with high ranking positions in both crops. For example GHM287-10 was the 1st and 2nd ranked AAV

TABLE 5 | Community structure of *Aspergillus* section *Flavi* in soils and maize samples from control and treated plots across three agroecological zones (AEZs) in Ghana.

AEZ[a]	Treatment[b]	N[c]	*Aspergillus* species/strain distribution[d] (%)											
			Soil before application				Soil at harvest				Grain			
			L	S_{BG}	*P*	*T*	*L*	S_{BG}	*P*	*T*	*L*	S_{BG}	*P*	*T*
DS	A	9	97.8	2.2	0	0	100*	0*	0	0	100	0	0	0
	B	9	93.4	3.2	0.8	2.6	99.3*	0.7*	0	0	100	0	0	0
	C	9	99.1	0.9	0	0	99.3*	0.7*	0	0	100	0	0	0
	Control	9	98.4	1.6	0	0	82.7	17.3	0	0	98.6	1.4	0	0
HF	A	9	91.4	2.8	2.9	2.9	97.9*	1.4*	0	0.7	100	0	0	0
	B	9	87.7	9.9	1.5	0.9	99.3*	0.7*	0	0	100	0	0	0
	C	9	94.1	1.6	1.3	3.0	97.2*	2.1	0.7	0	100	0	0	0
	Control	9	90.1	7.7	0	2.2	88.2	8.4	1.4	2	99.3	0.7	0	0
SGS	A	12	97.4	0.6	1.3	0.7	98.4	1.6	0	0	100	0	0	0
	B	12	95.0	0	3.2	1.8	97.9	1.0	0	1.1	100	0	0	0
	C	12	94.8	0.6	1.7	2.9	97.9	2.1	0	0	100	0	0	0
	Control	12	95.6	0	0.6	3.8	89.6	6.8	0	3.6	100	0	0	0

*[a]DS, derived savanna; HF, humid forest; SGS, southern Guinea savanna. [b]Biocontrol experimental products used in the current study (**Table 2**). Control refers to plots to which no experimental product was applied. [c]Number of plots analyzed. [d]L, A. flavus L morphotype; S_{BG}, S_{BG} strains; P, A. parasiticus; T, A. tamarii. An asterisk indicates significant difference in strain/species incidence between a treated plot and control plot within an AEZ by Student's t-test (α = 0.05).*

TABLE 6 | Community structure of *Aspergillus* section *Flavi* in soils and groundnut samples from treated and control plots across three agroecological zones (AEZs) in Ghana.

AEZ[a]	Treatment[b]	N[c]	*Aspergillus* species/strain distribution[d] (%)											
			Soil before application				Soil at harvest				Grain			
			L	S_{BG}	*P*	*T*	*L*	S_{BG}	*P*	*T*	*L*	S_{BG}	*P*	*T*
DS	A	9	97.4	0.9	0.9	0.8	99.3	0	0	0.7	100	0	0	0
	B	9	94.3	1.6	0.8	3.3	98.6	1.4	0	0	99.3	0.7	0	0
	C	9	99.1	0.9	0	0	98.6	1.4	0	0	100	0	0	0
	Control	9	98.3	0.9	0	0.8	95.8	3.5	0.7	0	100	0	0	0
HF	A	9	97.0	2.3	0.7*	0	100*	0*	0	0	100	0	0	0
	B	9	89.5	6.7	3.8	0	99.3*	0.7*	0	0	99.3	0.7	0	0
	C	9	95.5	1.6	2.9	0	95.8*	4.2	0	0	99.3	0.7	0	0
	Control	9	93.6	0	6.4	0	75.7	16.7	6.9	0.7	91.0	9.0	0	0
SGS	A	12	98.7	0	1.3	0	99.5	0	0.5	0	100	0	0	0
	B	12	97.5	1.9	0	0.6	99.5	0	0.5	0	99.5	0.5	0	0
	C	12	99.4	0.6	0	0	99.0	0.5	0	0.5	100	0	0	0
	Control	12	96.9	0.7	1.8	0.6	91.2	6.3	1.0	1.5	99.5	0	0	0.5

*[a]DS, derived savanna; HF, humid forest; SGS, southern Guinea savanna. [b]Biocontrol experimental product used in the current study (**Table 2**). Control refers to plots to which no experimental product was applied. [c]Number of plots analyzed. [d]L, A. flavus L morphotype; S_{BG}, S_{BG} strains; P, A. parasiticus; T, A. tamarii. An asterisk indicates significant difference in strain/species incidence between a treated plot and control plot within an AEZ by Student's t-test ($\alpha = 0.05$).*

TABLE 7 | Rankings[a] of isolates belonging to atoxigenic African *Aspergillus flavus* vegetative compatibility groups (AAVs) in soils and grain from both maize and groundnut plots treated with three experimental products and their corresponding controls in three agroecological zones (AEZs) in Ghana.

Experimental product	Isolate	Plot	Soil		Grain		Average
			Maize	Groundnut	Maize	Groundnut	
A	GHG331-8	Treated	1	9	5	8	5.75
	GHG331-8	Control	9	5	4	8	6.5
	GHG079-4	Treated	3	8	9	10	7.5
	GHG079-4	Control	6	10	7	9	8.0
	GHM109-4	Treated	5	1	4	5	3.75
	GHM109-4	Control	10	9	3	2	6.0
	GHM174-1	Treated	9	3	2	6	5.0
	GHM174-1	Control	11	8	1	5	6.25
B	GHM173-6	Treated	2	10	11	11	8.5
	GHM173-6	Control	1	10	7	9	6.75
	GHG083-4	Treated	4	4	8	1	4.25
	GHG083-4	Control	8	7	5	6	6.5
	GHM287-10	Treated	11	2	1	2	4.0
	GHM287-10	Control	7	3	2	7	4.75
C	GHM017-6	Treated	8	7	7	9	7.75
	GHM017-6	Control	2	4	8	9	5.75
	GHM511-3	Treated	6	6	3	4	4.75
	GHM511-3	Control	3	1	6	1	2.75
	GHG321-2	Treated	7	11	10	7	8.75
	GHG321-2	Control	5	6	8	3	5.5
	GHM001-5	Treated	10	5	6	3	6.0
	GHM001-5	Control	4	2	7	4	4.25

[a]Applied isolates were ranked separately by their incidence in grain samples across different geographical locations. To calculate the rank, the proportion of the number of (i) AEZ (n = 3), (ii) regions (n = 5), (iii) districts (n = 10), and (iv) samples (n = 30) where the AAV was detected, and (v) the proportion of isolates of the AAV detected (n = 360) was summed. Higher the sum, higher (1 = highest, 11 = lowest) the rank. For example, AAV GHM287-10 in maize was detected in the 3 AEZ (3/3 = 1.0), 5 regions (5/5 = 1.0), 8 districts (8/10 = 0.8), 17 samples (17/30 = 0.57), and 75 isolates were detected (75/360 = 0.21) for a total of 3.58, which was rounded to 4.0.

TABLE 8 | Selected isolates belonging to atoxigenic African *Aspergillus flavus* vegetative compatibility groups composing two biocontrol products for further evaluation in Ghana.

S/N	Isolate	Product
1.	GHG079-4	Aflasafe GH01
2.	GHG083-4	Aflasafe GH01
3.	GHG321-2	Aflasafe GH01
4.	GHM174-1	Aflasafe GH01
5.	GHM511-3	Aflasafe GH02
6.	GHM109-4	Aflasafe GH02
7.	GMH001-5	Aflasafe GH02
8.	GHM287-10	Aflasafe GH02

in treated maize and groundnut, respectively (**Table 7**). However, also in treated grains, GHG083-4 was the 1st and 8th ranked AAV in groundnut and maize, respectively. Success of establishment of an AAV in one crop was not always associated with success in the other crop.

Selected Isolates of Atoxigenic AAVs for Aflatoxin Biocontrol in Ghana

Based on incidence of the candidate AAVs in maize and groundnut grains following their release across locations, regions, and AEZs, and SSR data (**Table 2**), one representative atoxigenic isolate of eight AAVs with widest distribution and with superior ability to reduce aflatoxin contamination in grains were selected as active ingredients of two biocontrol products (**Table 8**).

DISCUSSION

In the current study, 12 atoxigenic AAVs native to Ghana were identified and a representative isolate of each AAV were evaluated for their potential as biocontrol agents for aflatoxin mitigation of both maize and groundnut grown across various AEZs. The 12 evaluated isolates successfully inhibited aflatoxin production (range = 87.3–98.7% less) when co-inoculated with a potent aflatoxin-producing *A. flavus* isolate native to Ghana in laboratory tests. Aflatoxin reduction levels were comparable to those detected in elite atoxigenic *A. flavus* isolates endemic to the United States (Cotty and Bayman, 1993; Ortega-Beltran et al., 2019), Nigeria (Atehnkeng et al., 2008), Kenya (Probst et al., 2011), Italy (Mauro et al., 2015), and China (Zhou et al., 2015). In sub-Saharan Africa, specifically Nigeria, Kenya, Senegal, The Gambia, and Burkina Faso, similar evaluations resulted in identification and selection of unique AAVs for the development of atoxigenic products tailored to each country (Atehnkeng et al., 2008; Probst et al., 2011; Bandyopadhyay et al., 2016). To our knowledge, the current work is the first published study of selection of active ingredients of an aflatoxin biocontrol product supported by information on their ability to disperse to crops from a formulated product applied on soil.

For over two decades, atoxigenic aflatoxin biocontrol has been demonstrated as the most effective and sustainable strategy to reduce crop aflatoxin content (Cotty, 1994; Dorner, 2004, 2010; Cotty et al., 2007; Mehl et al., 2012; Atehnkeng et al., 2014; Doster et al., 2014; Grubisha and Cotty, 2015). This strategy is based on the deployment of native atoxigenic isolates of VCGs that both competitively displace aflatoxin-producers and inhibit aflatoxin biosynthesis. Isolates belonging to atoxigenic VCGs locally adapted to specific AEZs and cropping systems, and with superior competitive ability to exclude aflatoxin producers from the target crop or environment are used in aflatoxin management programs (Cotty et al., 2007; Dorner, 2010; Abbas et al., 2011; Mehl et al., 2012; Doster et al., 2014; Bandyopadhyay et al., 2016). In keeping with this paradigm, 12 isolates belonging to genetically diverse SSR haplotypes/AAVs with wide distribution across Ghana (Islam et al., 2015) were identified from 847 atoxigenic isolates described previously (Agbetiameh et al., 2018) using 17 SSR loci (Grubisha and Cotty, 2009).

Mehl et al. (2012) emphasized that VCG analyses provide insights into the diversity of fungal communities including aflatoxin production and inhibition potentials. Indeed, variation in aflatoxin inhibition among representative isolates of the 12 atoxigenic AAVs was expected. GHG183-7 was least effective at inhibiting aflatoxin contamination in laboratory assays. This suggests that GHG183-7 is a poor competitor during host colonization (Mehl and Cotty, 2010) compared to the other evaluated isolates. Atehnkeng et al. (2008) emphasized that reduced competitiveness in laboratory conditions may provide an early signal of low competitiveness during crop development and, subsequently, less efficacy in practice. Furthermore, Atehnkeng et al. (2008) suggested exclusion of atoxigenic isolates with considerably less competitive abilities prior to expensive, time consuming field studies. Apart from being the least competitive isolate, we were unable to obtain a complementary pair of *nit* auxotrophs for this isolate. Whether this isolate is self-incompatible as reported in studies of *Aspergillus* and other genera (Correll et al., 1987; Krnjaja et al., 2013) needs to be clarified. Consequently, frequencies of AAV GHG183-7 were not evaluated even though an isolate of that AAV was a constituent of experimental product B.

Use of native AAVs in biocontrol programs offers better adaptation to target agroecosystems (Probst et al., 2011) and long-term establishment of *A. flavus* communities with low aflatoxin-producing potential (Mehl et al., 2012). Genetic variability among *A. flavus* individuals results in differential adaptation to various agroecological niches (Cotty and Mellon, 2006; Mehl and Cotty, 2013). Indeed, this phenomenon was expected among the 12 atoxigenic isolates evaluated in the current study. Studies of adaptive potentials of these isolates across three AEZs suggest extents of adaptation of their corresponding AAVs to the conditions of the three evaluated AEZs. For instance, the atoxigenic isolate GHM173-6 was the most effective at reducing aflatoxin concentrations in laboratory assays (**Table 3**) and was also one of the isolates most commonly found in treated and untreated maize soil (**Table 7** and **Supplementary Table 1**). This notwithstanding, GHM173-6 was the least encountered in maize grain from all field locations across regions and was never recovered from groundnut (**Table 7** and **Supplementary Tables 2–4**). On the contrary, GHM511-3 exhibited both high aflatoxin inhibition potential and high

recovery on both maize and groundnut across regions and all three AEZs (**Tables 3**, **7**). These observations support both competitiveness and crop adaptation as important criteria for selection of active ingredient AAVs for biocontrol formulations.

A major objective of the field evaluations of multiple isolates was to detect atoxigenic isolates belonging to AAVs with superior ability to establish in the crop after introduction in formulated product on the soil (**Table 7**). Apart from aflatoxin reduction of the experimental products, this portion of the research allowed identification of AAVs with greatest abilities to compete in the presence of both other atoxigenic isolates and aflatoxin producers under field conditions. Similarly in Nigeria, one of the four constituent AAVs of the initial experimental product established poorly in field evaluations (Atehnkeng et al., 2014) and hence was not included as an active ingredient of the final multi-AAV biocontrol product Aflasafe®.

Bandyopadhyay et al. (2016) underscored the importance of distribution and incidence of AAVs with potential as aflatoxin biocontrol agents as proxies for adaptation, competitiveness, and fitness in target environments. However, superior adaptation should also reflect increased efficacy in the target crop (Mauro et al., 2015). We report substantial reductions in aflatoxin concentrations in both groundnut (70–100% less) and maize (50–100% less) from plots treated with mixtures of atoxigenic isolates belonging to genetically diverse AAVs across all three AEZs. Lower than expected aflatoxin levels were also detected in maize from control plots across AEZs and may reflect the effect of drift of atoxigenic fungi from treated plots to adjacent control plots due to the relatively short separation distance (5 m). Indeed, most AAVs of the applied isolates were detected in control crops (**Table 7**). Conidia of *A. flavus* are common constituents of air currents dispersed over short and long distances (Bennett, 2010). Thus, a distance of at least 500 m between a treated and a control plot is necessary to avoid inter-plot interference (Bock et al., 2004; Atehnkeng et al., 2014).

Atehnkeng et al. (2014) demonstrated that mixtures of atoxigenic isolates are effective at reducing aflatoxin contamination in maize. Our results suggest that atoxigenic isolates mixtures belonging to distinct AAVs can be strategically designed for aflatoxin reduction in both maize and groundnut cropping systems in Ghana. Eight atoxigenic isolates belonging to atoxigenic AAVs were selected as active ingredients of two biocontrol products for aflatoxin mitigation and subsequently registered with Ghana's Environmental Protection Agency (**Table 8**). Six of the eight selected isolates had total or partial deletions in the cyclopiazonic acid (CPA) gene cluster while two produced undetectable amount of CPA (unpublished data). For the selection of the active ingredient AAVs, we considered their frequency of occurrence (**Table 1**), the competitive potential against aflatoxin producers (**Table 3**) and the relative adaptation in the evaluated maize and groundnut treated and control soils and crops (**Table 7**). This systematic evaluation protocol offered the opportunity to select the best possible combinations of active ingredients among the evaluated AAVs. However, all experimental products evaluated in the current study were efficient in reducing aflatoxin contamination of both maize and groundnut and each of the 12 AAVs were able to disperse to and increase frequency on the target crops. The selection strategy provides a basis for use of the most detected AAVs. However, even use of the most poorly adapted isolates examined here would provide better crop protection and increased food safety than failure to use atoxigenic strain-based biocontrol.

Application of atoxigenic *A. flavus* isolates on a target crop is a deliberate action to reshape fungal community composition in favor of the applied atoxigenic isolates due to founder events and competitive exclusion resulting in displacement of aflatoxin producers (Cotty and Bayman, 1993; Cotty et al., 2007; Mehl et al., 2012). Effective displacement of resident aflatoxin producers is achieved through proper timing of biocontrol applications during critical crop developmental stages (2–3 weeks before crop flowering) prior to the natural increase of the local *Aspergillus* population (Bandyopadhyay et al., 2016). Timed applications offer atoxigenic genotypes the advantage of becoming the founding population (Cotty and Mellon, 2006; Cotty et al., 2007) to quickly multiply and disperse to other nutrient sources and the target crop so that aflatoxin producers become less frequent (Bandyopadhyay et al., 2016).

In the current study, substantial displacement of aflatoxin producers from soils and crops occurred in treated plots across all three AEZs. The displacement was observed also in the non-treated crops. The *A. flavus* L morphotype largely dominated communities of *Aspergillus* section *Flavi* in soils collected before treatment, soils at harvest, and grains from both treated and control plots. The L morphotype is recognized as the most successful colonizer of soil and other substrates including grains in similar studies (Alanis Zanon et al., 2013; Atehnkeng et al., 2014; Doster et al., 2014). Frequencies of *A. parasiticus* were low (<1%), as reported previously in Ghana (Agbetiameh et al., 2018). Factors leading to low frequencies of this species in groundnut in West Africa remain unknown. Similarly, *A. parasiticus* is not common in portions of the Middle East (Lisker et al., 1993). In other regions of Southern Africa and North America, *A. parasiticus* is an important causal agent of groundnut aflatoxin contamination (Horn and Dorner, 1998; Kachapulula et al., 2017).

CONCLUSION

Twelve atoxigenic African *A. flavus* vegetative compatibility groups (AAVs) commonly occurring across Ghana were characterized. The potential of a representative member of each AAV to inhibit aflatoxin contamination of maize grains was assessed in laboratory assays. AAV adaptation in maize and groundnut cropping systems in three AEZs in Ghana was assessed. The results formed the basis for selection of eight superior atoxigenic *A. flavus* isolates, each belonging to an unique AAV, as active ingredients of two biocontrol products, Aflasafe GH01 and Aflasafe GH02, for use on maize and groundnut in Ghana (**Table 8**). The unique SSR patterns of the eight atoxigenic isolates (**Table 2**) can serve as a resource for identification of the active ingredients of each of Aflasafe GH01 and Aflasafe GH02 after field application. Use of the identified atoxigenic AAVs offers a sustainable management option for aflatoxin mitigation

in maize and groundnut for smallholder farmers in Ghana providing an inexpensive opportunity for improved food safety, productivity, and income.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

DA, JA, PC, and RB contributed to the conception and design of the experiments. DA and JA conducted the experiments and field studies, and collected and analyzed the data. AO-B, RA, PC, and RB provided the guidance. KC, M-SI, and PC conducted the molecular studies. DA, AO-B, KC, PC, and RB drafted the manuscript. All authors read, reviewed, and approved the final version of the manuscript. RB and AO-B secured funds for the study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02069/full#supplementary-material

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Monitoring *Aspergillus flavus* Genotypes in a Multi-Genotype Aflatoxin Biocontrol Product With Quantitative Pyrosequencing

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Aflatoxins pose significant food security and public health risks, decrease productivity and profitability of animal industries, and hamper trade. To minimize aflatoxin contamination in several crops, a biocontrol technology based on atoxigenic strains of *Aspergillus flavus* is commercially used in the United States and some African countries. Significant efforts are underway to popularize the use of biocontrol in Africa by various means including incentives. The purpose of this study was to develop quantitative pyrosequencing assays for rapid, simultaneous quantification of proportions of four *A. flavus* biocontrol genotypes within complex populations of *A. flavus* associated with maize crops in Nigeria to facilitate payment of farmer incentives for Aflasafe (a biocontrol product) use. Protocols were developed to confirm use of Aflasafe by small scale farmers in Nigeria. Nested PCR amplifications followed by sequence by synthesis pyrosequencing assays were required to quantify frequencies of the active ingredients and, in so doing, confirm successful use of biocontrol by participating farmers. The entire verification process could be completed in 3–4 days proving a savings over other monitoring methods in both time and costs and providing data in a time frame that could work with the commercial agriculture scheme. Quantitative pyrosequencing assays represent a reliable tool for rapid detection, quantification, and monitoring of multiple *A. flavus* genotypes within complex fungal communities, satisfying the requirements of the regulatory community and crop end-users that wish to determine which purchased crops were treated with the biocontrol product. Techniques developed in the current study can be modified for monitoring other crop-associated fungi.

Keywords: pyrosequencing, aflatoxin, Aflasafe, biocontrol, atoxigenic, monitoring, maize, Nigeria

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INTRODUCTION

Several species of *Aspergillus* section *Flavi* produce immunosuppressive, hepatotoxic and carcinogenic aflatoxins (Liu and Wu, 2010; Liu et al., 2012) in maize and other crops cultivated in warm environments (Cotty et al., 1994; Cotty and Jaime-Garcia, 2007; Bandyopadhyay et al., 2016). At high concentrations, aflatoxins may cause acute hepatotoxicity,

hemorrhagic liver necrosis, and death (Probst et al., 2007, 2010, 2011). For this reason, levels of aflatoxins in foods and feeds are strictly regulated in more than 100 countries across the world (FAO, 2004; EU, 2010; Matumba et al., 2017; Singh and Cotty, 2017), and aflatoxin management strategies, including biological control, are used for mitigating aflatoxin exposure. Commercial biological control products directed at aflatoxin mitigation have beneficial strains of *A. flavus* that do not produce aflatoxins as active ingredients.

Aspergillus flavus consists of many genetically distinct groups, called vegetative compatibility groups (VCGs), that primarily reproduce clonally (Grubisha and Cotty, 2010, 2015; Ortega-Beltran et al., 2016; Islam et al., 2018) and differ widely in several characteristics, including ability to produce aflatoxins. Aflatoxin-producing potential varies more between VCGs than within them (Bayman and Cotty, 1991); all members of certain VCGs lack the capacity to produce aflatoxins (Cotty et al., 2008; Grubisha and Cotty, 2015; Ortega-Beltran et al., 2016) and are referred to as atoxigenic. Adoption of biocontrol strategies utilizing indigenous atoxigenic genotypes to displace aflatoxin producers in crop-associated fungal communities (Atehnkeng et al., 2008a,b; Mehl et al., 2012) are becoming widespread across the world, due to proven efficacy, low cost, and area-wide benefits (Cotty and Bayman, 1993; Cotty and Bhatnagar, 1994; Dorner, 2008; Atehnkeng et al., 2014; Mauro et al., 2015; Bandyopadhyay et al., 2016). This strategy alters compositions of crop-associated fungal communities through founder effects, competitive displacement and other mechanisms (Cotty and Mellon, 2006; Ortega-Beltran and Cotty, 2018), increasing frequencies of atoxigenic active ingredients and decreasing incidences of aflatoxin-producers through displacement (Abbas et al., 2011; Chang et al., 2012; Atehnkeng et al., 2014). However, after application, monitoring active ingredient genotypes in the *A. flavus* community is necessary to assess influences of various practices on displacement of aflatoxin producers by applied atoxigenics. Monitoring is also required to verify use of the biocontrol products on crops where use is rewarded, as with farmers supplying crops under the Nigeria Aflasafe™ Challenge Project (AgResults, 2019; Schreurs et al., 2019).

As part of post-application monitoring, vegetative compatibility analyses (VCA) are frequently performed to determine displacement efficacy and residual effects (Cotty and Bayman, 1993; Atehnkeng et al., 2014; Mauro et al., 2015). VCA involves generation of nitrate non-utilizing auxotrophs for individual isolates, pairing of auxotrophs with *cnx*$^{-}$ and *niaD* tester pairs, and classification of the complementing fungi as a member of the VCG defined by the tester pair (Bayman and Cotty, 1991; Grubisha and Cotty, 2010). A test must be performed for each isolate and limitations on the assays are imposed by both the isolation process and the number of isolates that can practically be classified. This process is laborious, expensive, and time-consuming, frequently talking over a month to complete. Pyrosequencing assays can reduce costs and increase speed and accuracy of post-application biocontrol monitoring, quantitative pyrosequencing assays targeting specific *A. flavus* isolates have been developed (Das et al., 2008; Mehl and Cotty, 2010, 2013). However, none of these have been successful at monitoring multiple genotypes, and none have been used to monitor commercially significant quantities of samples.

The current study aimed to develop multi-genotype quantitative pyrosequencing assays for quantification of *A. flavus* genotypes. This was predicated on previous success with single-genotype assays (Das et al., 2008; Mehl and Cotty, 2011, 2013) and the need for rapid verification of biocontrol-use on maize. Aflasafe is a commercially available biocontrol product with four endemic *A. flavus* genotypes (as active biological ingredients) isolated from Nigeria for reducing aflatoxin contamination in maize (Atehnkeng et al., 2008a,b, 2014; Bandyopadhyay et al., 2016). Proper use of this biocontrol in fields before flowering leads to occurrence of significant frequency of the active biological ingredients. This initiative, a project under the AgResults multilateral initiative (AgResults, 2019) aims to provide incentives for aflatoxin-mitigation through increased adoption of biocontrol through a performance payment per unit of maize (∼30 tons) that is verified to contain significant frequencies of the active ingredients. The active ingredients must be detected rapidly and precisely to enable accurate and timely implementation of one of the project objectives for paying the incentive. The current work describes efforts to meet these needs with pyrosequencing.

Quantitative pyrosequencing is an advanced sequence-based technology that enables accurate quantification of frequencies of DNA sequence variants in complex microbial populations. Pyrosequencing relies on light generation after nucleotides are incorporated in a growing DNA strand, converting the emitted light into a pyrogram. Pyrogram peaks correspond to light generation, and is proportional to nucleotide incorporation (Siqueira et al., 2012). Pyrosequencing produces a large number of sequence reads in a single run, resulting in enormous sampling depth (number of sequences per sample) that permits detection of both dominant and rare individuals within mixed and complex microbial populations by several orders of magnitude higher than previous technologies allowed (Sogin et al., 2006; Kunin et al., 2010; Mehl and Cotty, 2010). Greater sampling makes pyrosequencing especially suitable for ecological studies, such as monitoring changes in *A. flavus* population structure (Das et al., 2008; Mehl et al., 2012) or incidences of single nucleotide polymorphism (SNP)-based fungicide resistance (Zhou and Mehl, 2019). The current study developed quantitative pyrosequencing assays for quantifying frequencies of active ingredients of Aflasafe in complex microbial populations. The biocontrol product consists of equal proportions of four atoxigenic *A. flavus* isolates (Ka16127, La3279, La3304, and Og0222) (Atehnkeng et al., 2014). Assays were based on SNPs in the genomes of each genotype, and were for specific detection of the target genotype. Multi-isolate assays targeting more than one active ingredient were based on SNPs shared by the target genotypes.

MATERIALS AND METHODS

Detection of Single Nucleotide Polymorphisms in Target *Aspergillus flavus* Genomes

Sequenced reads from the target isolates (Ka16127, La3279, La3304, and Og0222), and control isolates (AF13, MS14-19, and Ss19-14) were mapped to *A. oryzae* RIB40 genome (Machida et al., 2005) using Bowtie v1.1.1 (Langmead et al., 2009). The resulting BAM files were used as input to SAMTools v0.1.16 (Li et al., 2009). SNP positions were identified using mpileup function in SAMTools. SNPs with minimum mapping quality (-Q) below 20 and minimum read coverage below 20× were filtered out. SNPs specific to one isolate, or shared by multiple isolates, were identified using a custom Perl script, and polymorphic regions were validated by checking alignment of the target and control isolates with the reference. Regions containing putative SNPs were annotated using BLAST (Altschul et al., 1990) against non-redundant databases. Target genomic regions with polymorphisms were aligned with sequence from other *A. flavus* isolates reported previously (Adhikari et al., 2016) to ensure that target genomic regions were highly conserved, increasing the potential that allele quantification with the pyrosequencing assays would more accurately reflect the diversity in fungal populations within the communities being assayed. Further checks were done to ensure that target SNPs were not within genes located in the aflatoxin biosynthesis cluster. Since many biocontrol *A. flavus* isolates have lost all or part of the aflatoxin biosynthesis cluster (Adhikari et al., 2016) this genomic region would not be a suitable target for *A. flavus* population studies.

Extraction and Amplification of Target DNA

Total DNA was extracted from maize grain by suspending 10 g of ground maize in 50 ml of 0.1% TWEEN®80. After shaking for 20 min at 175 rpm, the suspension was transferred into a funnel lined with a 4 × 4 in piece of Miracloth (EMD Millipore, Billerica, MA) and vacuum-filtered into a 50 ml centrifuge tube. The flour residue was autoclaved and discarded. After centrifuging the filtrate for 10 min at 4,000 × g, the supernatant was removed, using a 10 ml serological pipette, and discarded. The precipitate was vortexed at 15,000 rpm for 15 s, after which 1 ml, containing maize starch and fungal propagules, was transferred to a 1.5 ml microfuge tube and centrifuged at 8,000 × g for 5 min. The supernatant was again removed, using a 1,000 µl pipette, 450 µl of Lysis Buffer (30 mM Tris, 10 mM EDTA, 1% SDS, pH 8.0) was added, and the tube was vortexed to suspend the precipitate. The tube was then placed in a Thermomixer for 60 min at 60°C and 8,000 rpm, after which it was centrifuged at 14,000 × g for 30 min, and 370 µl of the supernatant transferred to a new 1.5 µl microfuge tube to which 370 µl of 4 M ammonium acetate (NH_4OAc) was also added. After mixing the suspension by inverting it several times, 740 µl of ice-cold ethanol was added. The microfuge tube was incubated at −20°C for 30 min, centrifuged at 14,000 × g for 5 min, the supernatant was removed, and the DNA pellet dried by placing the tube upside-down on a paper towel for about 60 min. The DNA was re-suspended in 25 µl of sterile water and quantified using a NanoDrop™ ND-3300 Fluorospectrometer (NanoDrop Technologies, Inc., Bancroft, DE).

Target *A. flavus* DNA was amplified using nested PCR (Dufour, 1977; Sun et al., 2012), with sequential DNA amplifications. The first amplified a relatively large section (400–580 bp) surrounding the SNP. Outer primers were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Inner primers, were designed using PyroMark Assay Design Software v2.0.1.15 (Qiagen, Valencia, CA) to amplify 90–180 bp containing the target SNP, within the larger outer amplicon. Inner primers were purified using high-performance liquid chromatography (HPLC), and either the forward or reverse primer was tagged with biotin at the 5′ end for biotinylation of the inner PCR amplicon. Biotinylation allows subsequent attachment of the amplicon to Streptavidin Sepharose beads during the pyrosequencing reaction. PCR amplifications used *AccuPower* Hotstart PCR PreMix tubes (Bioneer, Inc., Alameda, CA). Each tube contained a pre-mix of one unit of HotStart DNA polymerase, 1× PCR Buffer and 250 µM of each dNTP. The 20 µl reaction mixture in each tube included 0.5 µl each of the forward and reverse primer, 17 µl of deionized water and 2.0 µl of the DNA template, diluted to 5 ng/µl concentration. Amplicons from the outer reaction served as templates for inner PCR. Amplification conditions were DNA denaturation (94°C, 5 min) followed by 38 cycles of melting at 94°C for 20 s, primer annealing at 56°C for 30 s, extension at 72°C for 30 s, and a final extension step at 72°C for 10 min. Amplicons were visualized with GelRed (Biotium Inc., Fremont, CA), using a G:Box Chemi HR 16 Bio Imaging System (Syngene/Synoptics Ltd., Cambridge, UK), after separation on 1.0% agarose gel via electrophoresis at 110 volts for 15 min. Proportions of reagents in the reaction mix and amplification conditions were the same for outer and inner PCR amplifications.

Outer amplicons were quantified using a Qubit 3.0 Fluorimeter (Thermo Fisher Scientific, Waltham, MA) for initial standardization of the PCR protocol. Serially diluted amplicons, up to 10^{-3}, were used as DNA template for inner reactions to determine optimal dilutions. Total DNA extracted from ground maize was a mixture of DNA from *A. flavus*, maize, and environmental organisms. Therefore, the amplicon size and brightness on the gel was used as a guide to determine the quantity of PCR products to be used as template for the inner PCR reaction.

Confirmation of Predicted SNPs

Design of outer primers for quantitative pyrosequencing assays was based on SNP prediction by computational analyses. Actual presence of the predicted SNPs was confirmed by sequencing amplicons at the University of Arizona Genetics Core (UAGC) facility, using Applied Biosystems 3730XL DNA Analyzer (Life Technologies Corporation, Carlsbad, CA). Sequence data were aligned to reference genomes using Geneious® 9.0.2 (Kearse et al., 2012). Design of inner and sequencing primers followed SNP confirmation.

TABLE 1 | Targets for differentiating atoxigenic *Aspergillus flavus* active ingredients of Aflasafe identified with whole genome analyses and confirmed with amplicon sequencing.

Assay[#]	Target *A. flavus* isolate*	Variable Sequence (Bold letter = SNP)	SNP	IUPAC ambiguity code	Amino acid change	Location on *A. oryzae* genome[#]	Polymorphic site annotation
1Ka1	1	TTCCGGTAT**G**TGCAAAGCGG	A → G	R	Y → C	Chr. 1, SC009	Polyketide synthase
1Ka2	1	TAGCGATTG**C**GCGGCCCCGC	T → C	Y	V → A	Chr. 5, SC113	DNA repair protein Nse1
1Ka3	1	TCGTTCAAT**A**CAATCAAGTA	G → A	R	C → Y	Chr. 5, SC113	Hypothetical protein AOR_1_1238094
1Ka4	1	GCCTGCCTA**T**TTGCCAATGA	C → T	Y	C → T	Chr. 5, SC113	Haloacid dehalogenase
1La791	2	CGTTACATG**C**GAATCAATAA	G → C	S	G → R	Chr. 1, SC009	Polyamine transporter 3
1La792	2	TTGGCAAGC**A**CCGGCGGAGC	G → A	R	R → H	Chr. 2, SC003	Unnamed protein product
1La793	2	AGCCACTTG**T**TCGATCTTCT	C → T	Y	C → Y	Chr. 1, SC009	Unnamed protein product
1La794	2	AGGGCCCCA**C**GACCAGCATA	A → C	M	R → R	Chr. 1, SC009	Hypothetical protein
1La795	2	GGCTGGACG**T**TTCGGCAACC	C → T	Y	A→ V	Chr. 1, SC009	Unnamed protein product
1La796	2	TGTGGAGTT**T**ATGTTTCGTC	C → T	Y	S → L	Chr. 1, SC009	3-ketosteroid-delta-1-dehydrogenase
1La041	3	TTACTGGTG**T**GATCGCTGCG	G → T	K	L → L	Chr. 6, SC020	Guanine nucleotide exchange factor
1La042	3	GGTGGACCA**T**ACGGGATGAA	C → T	Y	H → Y	Chr. 6, SC020	No significant similarity found
1La043	3	GACGCCACC**T**GGTCTCCAGG	C → T	Y	P → L	Chr. 6, SC020	20S cyclosome subunit (APC1/BimE),
1La044	3	GCAGGCACT**C**AAATCTCACC	T → C	Y	* → Q	Chr. 6, SC020	Putative anucleate primary sterigmata (ApsB)
1Og1	4	CAATACCCG**C**ATTATCTTCA	T → C	Y	Y → H	Chr. 3, SC023	Unnamed protein product
1Og2	4	GCCCAAGTG**G**TTCTGGCTAC	C → G	S	C → W	Chr. 3, SC023	Fungal alpha-L-arabinofuranosidase
2KaLa2	1 + 3	ACAACACGG**G**CTTCCAGGAG	A → G	R	G → G	Chr. 5, SC113	Hypothetical protein AFLA70_215g002270
2La9K2	1 + 2	AGCCGGGTC**C**TCCTCTGTGT	A → C	M	H → P	Chr. 8, SC010	Acetylcholinesterase
3La94K1	1 + 2 + 3	TGACTCGAC**T**ATCTTGCTTA	C → T	Y	G → Y	Chr. 8, SC010	Endo-1,4-beta-mannosidase
3La94K2	1 + 2 + 3	CAGGAGCGC**G**TCTCTAAGCT	A → G	R	H → R	Chr. 8, SC010	AMP-binding enzyme
3La94K3	1 + 2 + 3	AAAACGGGC**A**GCATGATGAT	G → A	R	P → K	Chr. 6, SC020	Unnamed protein product
3La94K4	1 + 2 + 3	ACGGCCGAA**C**GAGTCGCTCG	T → C	Y	V → P	Chr. 6, SC020	Unnamed protein product
3La94K5	1 + 2 + 3	TGGCTACTC**T**AAGGTTCTCG	C → T	Y	S→ S	Chr. 6, SC020	Hypothetical protein Z518_10119
3La94K6	1 + 2 + 3	AAAAGCGGT**G**CCAAAGGCG	A → G	R	Y → C	Chr. 6, SC020	Hypothetical protein AFLA_104000

[#]*Numbers preceding letters in Assay names indicate the number of A. flavus isolates targeted by the assays. Where there is more than one assay per A. flavus isolate, letters in the assay name are followed by the serial number of the assay.*

**1 = Ka16127, 2 = La3279, 3 = La3304, 4 = Og0222, 1 + 2 = Ka16127 + La3279, 1 + 3 = Ka16127 + La3304, 1 + 2 + 3 = Ka16127 + La3279 + La3304.*

Quantitative Pyrosequencing Assays

Quantitative pyrosequencing assays were designed using PyroMark Assay Design Software v2.0.1.15 (Qiagen, Valencia, CA) and performed on a PyroMark Q24 Pyrosequencer (Qiagen, Valencia, CA). Twenty-four assays were developed (**Table 1**), consisting of forward and reverse pairs of outer and inner primers and a sequencing primer (**Table 2**). Six of the assays (1Ka1–1Ka6) were for Ka16127, six were for La3279 (1La791–1La796), four were for La3304 (1La041–1La044) and two were for Og0222 (1Og1 and 1Og2) (**Tables 1**, **2**). Two assays (2KaLa2 and 2La9K2) were designed for simultaneous quantification of Ka16127+La3304 and La3279 + Ka16127, respectively, while the remaining six assays (3La94K1–3La94K6) were for simultaneous quantification of Ka16127 + La3279 + La3304. Template DNA preparation for pyrosequencing analysis was done following procedures described by Das et al. (2008).

Pyrosequencing Assay Refinement

Quality of pyrosequencing assays was checked initially with PyroMark Q24 v2.0.7 software, after which the assays were refined by assaying each target DNA after serial dilution with DNA from the non-target *A. flavus* genotype AF13. Target

TABLE 2 | Oligonucleotide primer sets for quantitative pyrosequencing assays directed at polymorphisms described in **Table 1** for estimation of frequencies of *Aflasafe* active ingredients in fungal communities associated with maize produced in Nigeria.

Assay name[#]	Outer primers	Inner primers	Sequencing primer	Target biocontrol isolate*	Amplicon size (bp)[§]
1La041	tctgctgcgtacctcattcg/aggctctgaattgcgaacga	CTCAAGCTCGACGTGGCTTAC/ACGGTAGAGGTCAGGTTCTGC	GCCGGCGCAGCGATC	3	514/188
1La042	ACCACCCATATTTAGCGCATCCT/CGAAGCGCGCAGTTGTTAGC	CCTATCGTCGACCATTTAAGGTAA/AAATCCCTAGCCAAAGACGC	GGCACGTTCATCCCG	3	539/169
1La043	CATCGTGTGGCCTTCGACGC/TTTTCGAGGACCAGCGCGC	TTCAAAAGCAGAGACTCCCACTTC/CTGCGCAAACCACTCGGA	GGACAATAAATGGTTCGAT	3	511/103
1La044	AAGGAGGAGGCGCGGAAACT/CAGTCCGGTCCACACATCGC	TGCGCTACTTGAGAGCCACG/CCGAGATGCTTGGTGGTGAG	CGTTTGAGCAGGCAC	3	542/130
1Ka1	GAGCTGTGATCTACGCGACA/ACAAGAAGGTACGACGCGTT	GGTCCCATCAACCCAGTTAC/GATAATCTTCCCCATGTGCTG	GTGCTGTCCGCTTTG	1	509/93
1Ka2	CAGTCACGGTTACCACAACG/TTCCTTTATCAAGCGCATCC	TACCGTTTCCGCTTGAGACAT/ATCGTCCGGAGATGCAAGT	GAGACATGCTTAGCGA	1	463/97
1Ka3	AGTCAGTGGGTCGAAAAAGG/ACAGCGAAGGTTTGACTGCT	TTCATGTTAACGACATCCGTGATC/GGTGGGACAGTTCTTCATGTTGC	GCTGCCAGATACTTGATT	1	414/98
1Ka4	AACAACAGGTGCCAAGTGTG/CTTTGCATTTGCCGGATAAC	TTCGCCAAGAGTGCTCCT/GATCCCATTTAGCCTATGTCTGAG	GGGCGGTCATTGGCA	1	482/96
1Og1	GTGTCAATCTCCTCCATCAT/CCGATCTGACAACTCAAATA	GAAGCGCATCAGCACTCC/CGCCTGCATCCCTTTACC	GCCAAGCCTGAAGAT	4	702/102
1Og2	GAGTCACAGAAAACCAAACC/GTGAAGTCAAAAGCCTCATT	CCTGTACTTGAGACCGACACTCAT/TTCCCCCGGGTGGAGTAT	CTGTCGAGGCATATAGC	4	616/122
1La791	AGCACGTAAAGATGCTGGCT/CCGTCACTCTCGATGCTTGA	TGGACGAGCTTATCAAGTTAACAA/CGCCAGCACAATTTACAACA	ACAGAGTTAAAGGTCGTTAC	2	502/106
1La792	TCGACGTCGATGCAGTTGAA/AAAACCCCCAGAAAATGCGC	GGTAGTACTGCTGACGGTAGTTCG/GAGGGCCTGTTTGTAACGAGA	AACTCCTGCTCCGCC	2	480/109
1La793	AATGGGAGTCAACGAACCGT/CGAAGGATCTCGCCTATCGC	TGCAGCTCAAGGTATCGTATTTCG/TGCAACGGTAGTACTCGGAGTGAT	GTCAGGGCTGAGCCAC	2	407/105
1La794	GCTACGTCATCGACTCCCAG/ACTATGCCCGGTTGCAATCA	ATGGAATACAGAAGTCGGAGAGG/ACGCGGAAAATTCGTTTG	CAGAGAGTACTGATATGCTG	2	510/112
1La795	TGGTAGGTGGTCTCTAGGCC/GCGTATACTCGGCATCCACA	ACATTGCGAGAGGTTTCCA/GACGGACAACGAAGTTTCAGTA	CAGGATATCTGGCTGG	2	451/106
1La796	GAGTTTTGCGAGCGTTGGTT/TGTGCAGGGACACCGATAAC	TTCGAGAAGCCGGTTCGC/TACACCGATGACAGCACCAGTAGA	CACCTGACGACGAAA	2	510/111
2KaLa2	ACATGACCCTCCTTGGTGTC/GAGTCTTCCAACCAGCGAAG	GGTATCATGTCACTGGCTTATGGA/CGACCATATCTTGCCACTCCTG	CAATCAAGAACAACACG	1 + 3	513/97
2La9K2	GCGGTAGTATCGCCATTTGT/TGGGAATCTGAAACCCATGT	GGCCAAGTCCAGCAACAATC/GGGCATTTGTTGAGTTCACGAGT	ACCTACCAGGACACAGA	1 + 2	474/123
3La94K1	ACGGGTGTCATGCCTAGTTC/CGTCATCTCTCCCCAAACTC	ACGCCTGTCTCAACATTTCCTG/GCTCCGCTCTTGATCCAGAA	CCTGCAATCTGACTCG	1 + 2 + 3	481/112
3La94K2	GCGGTAGTATCGCCATTTGT/TGGGAATCTGAAACCCATGT	GGGATCGGTTTCGGGACT/ACAGAAGGCTCGGGAAGCTTA	GCTCGGGAAGCTTAGA	1 + 2 + 3	474/112
3La94K3	TCAGACAAGCTGCAAACACC/CCAAGGGAGAAAGTTGGTCA	CACCAGCATCTGGAAACGTAC/AGCCTCCGAATAATCAACGA	TCTCCTGATGATCCATT	1 + 2 + 3	511/110
3La94K4	CTACGGTCCATCCCTCAGAA/CTTTGAGCTTGCCGAAAATC	GGATGGCTTTCCCAGAGCTAAAC/GCGACGATAGCCCATGATG	CTTGGCTCATGGCCT	1 + 2 + 3	540/122
3La94K5	CCCGGTTATTTCGGTAAGGT/CCTCCTTGATCTTCCGTTCA	CTGAGCAGCGTGACGCCTAC/ATGGGGATCTCGGGAATGC	CGGGAATGCGGCCCT	1 + 2 + 3	472/118
3La94K6	TGCTTCCATTGTGCATTGTT/TTTTAGTGGCCTTCCACAGC	TTGGGTTGGAAGACTAAGATTCCT/TATGACGCCATTCTTAACGTCGA	GACTTATTCAGCAATGTCTC	1 + 2 + 3	541/132

[#]*Numbers preceding letters in Assay names indicate the number of A. flavus isolates targeted by the assays. Where there is more than one assay per A. flavus isolate, letters in the assay name are followed by the serial number of the assay.*

**1 = Ka16127, 2 = La3279, 3 = La3304, 4 = Og0222, 1 + 2 = Ka16127 + La3279, 1 + 3 = Ka16127 + La3304, 1 + 2 + 3 = Ka16127 + La3279 + La3304.*

[§]*Outer amplicon size/inner amplicon size.*

DNAs were tested at five percentages (100:0, 75:25, 50:50, 25:75, and 0:100). The first and the last treatments served as positive and negative controls, respectively. Proportions of target alleles quantified by pyrosequencing assays at each dilution were fitted into polynomial regression models and compared with actual proportions of target DNA. Each experiment was performed twice, with a completely randomized design and three replications. Goodness of fit for each assay model was determined using the coefficient of determination for the model. Data were subjected to ANOVA and regression models in SAS v9.2 (SAS Institute Inc., Cary, NC).

Use of Pyrosequencing to Determine Successful Application

Validation of biocontrol application was required to meet contractual requirements and to provide a scientific basis for performance payments intended only for maize confirmed to have been treated. Collection and sampling of maize was previously described (Bandyopadhyay et al., 2019). Briefly, maize from groups of farmers were aggregated by middle men that assisted with training, distribution of the biocontrol product, and marketing of the harvested grain. Thirty kilogram composite samples were taken from ∼30 ton lots of aggregated maize by randomly sampling 100 g of maize from each of 300 bags. A 5 kg subsample was taken from each 30 kg sample after homogenization. Subsamples were transported to IITA, Ibadan, Nigeria, where they were milled, homogenized and stored until use.

DNA extracted from maize harvested from fields purported to have been treated were analyzed for the proportion of the *A. flavus* in the maize samples composed of the active ingredients. Proportions were determined using genotype-specific and multi-genotype pyrosequencing assays. Two hundred and ninety-two (292) pelleted DNA samples extracted from the ground maize subsamples were analyzed. DNA extraction was performed at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, following protocols described above, and shipped to the USDA-ARS Lab in Tucson, AZ for pyrosequencing analyses. In Tucson, the DNA samples were re-suspended in 100 μl of purified, autoclaved water, vortexed for 10 s and centrifuged at 14,000 × g for 5 min. Thereafter, 50 μl aliquots of the supernatant were transferred to new 1.5 ml centrifuge tubes as working sub-samples. The remainder of the diluted DNA was stored at −20°C.

To compensate for DNA of reduced quality, 5–10 μl of template DNA was used for both outer and inner amplifications (dependent on ability to visualize PCR products in agarose gel). Outer PCR products were used as template for the inner reaction, after reacting with ExoSAP-IT (Thermo Fisher Scientific Inc., Waltham, MA) to remove unincorporated nucleotides. Biotinylated amplicons from the inner amplifications served as templates for quantitative pyrosequencing, regardless of amplicon quality.

A three-step approach was used to validate application. First, all 292 samples were assayed using 3La94K1, a multi-genotype assay targeting a combination of Ka16127 + La3279 + La3304. Samples with ≥70% of the target active ingredient genotypes were passed, and no further processing of the passed samples was done. Samples with frequencies of the target alleles below 70% were assayed with 1Og2 targeting Og0222. If the sum of Og0222 and Ka16127 + La3279 + La3304 (from 3Ka94K1) was ≥70%, the samples were passed, and no further assays were done on the passed samples. Samples with <70% of the target genotypes, after processing with 1Og2 were analyzed with the second three-isolate assay (3La94K2), and the output added to that 1Og2. If the sum of output from both assays was ≥70%, the samples were passed, otherwise they were deemed to have failed the validation test.

RESULTS

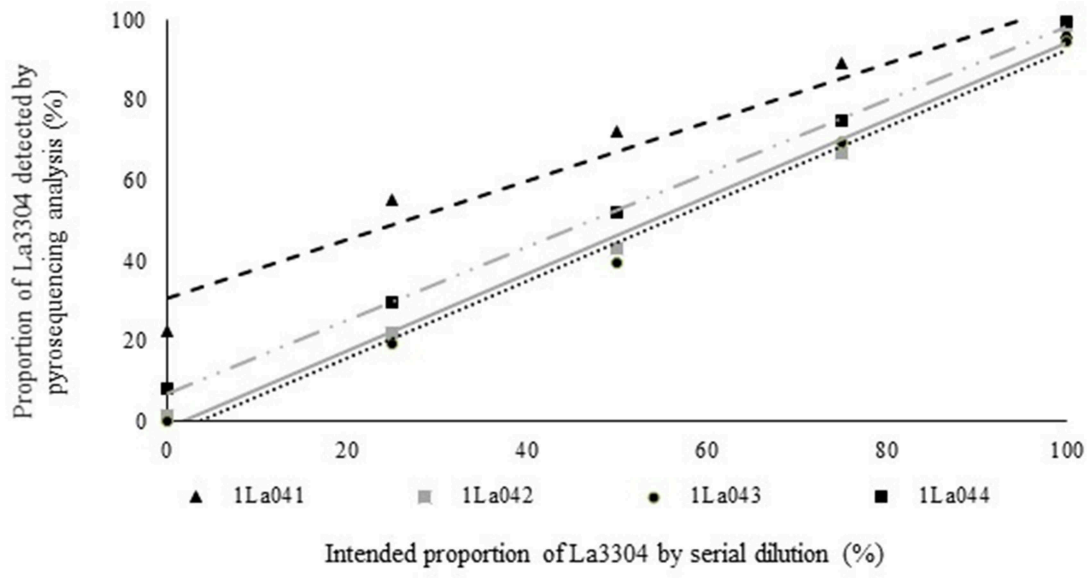
Pyrosequencing Assay Refinement

Quantitative pyrosequencing assays were designed targeting SNPs identified with the outlined bioinformatic approach and residing in a variety of regions of the genomes of the active ingredients. Although, all the developed assays were at least

TABLE 3 | Refinement of single-isolate quantitative pyrosequencing assays using mixtures of target and non-target *Aspergillus flavus* isolates.

Percent of target DNA	Percent of target DNA detected by the quantitative pyrosequencing assay								
	1Ka1	1Ka2	1La791	1La792	1La793	1La794	1La795	1La796	1La041
100	100.00	99.83	99.78	97.87	94.71	100.00	97.92	99.46	96.77
75	79.10	77.50	88.01	82.38	82.38	85.92	81.58	98.92	89.43
50	56.14	54.17	60.83	67.26	60.83	67.26	61.95	80.94	72.41
25	34.17	30.84	48.61	42.32	39.28	48.61	42.32	62.97	55.39
0	12.21	7.50	22.69	1.60	7.44	6.26	1.42	10.34	22.46
AF36	ND	ND	5.18	1.18	0.65	1.65	0.59	8.60	ND
SS19-14	9.92	2.13	1.56	0.99	0.00	0.00	0.00	7.93	0.78
MS14-19	0.00	0.00	ND	ND	ND	ND	ND	ND	0.00
Water	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Critical value of studentized range	5.304	5.304	5.304	5.304	5.304	5.304	5.304	5.304	5.304
Minimum significant difference	5.523	4.709	7.251	12.662	70.096	6.926	11.725	4.000	57.287

ND, not determined.



Measure	1La041	1La042	1La043	1La044
Y	$-0.0098x^2 + 1.6652x$	$0.0028x^2 + 0.6854x$	$0.0024x^2 + 0.7153x$	0.0007x2 + 0.8371x
Intercept	26.062	1.90	-0.290	8.1757
R^2	0.9679	0.9999	0.9978	0.9999

FIGURE 1 | Intended proportion of Aflasafe active ingredient La3304 mixed in varying proportions with *A. flavus* AF13 DNA vs. frequency of La3304 detected using pyrosequencing assays 1La041, 1La042, 1La043, and 1La044.

partially effective at quantifying frequencies of the target SNPs in pools of DNA, characteristics of the response curves differed. Linear curves from regression models for the two Ka16127-specific single-genotype assays showed an excellent linear relationship ($R^2 = 0.9998$) between the proportion of target DNA detected by the assays in serial dilutions with AF13 DNA and the intended proportion. Relationships were consistent across all levels of the serial dilution (**Table 3**). Analysis of data from the six La3279-specific pyrosequencing assays revealed either linear or polynomial relationships to be optimal between proportion of the target DNA detected by the pyrosequencing assays and the intended proportion. Coefficients of determination (R^2 values) for all models were excellent ($R^2 > 0.9$), suggesting useful predictive value. However, regression curves intercepted the ordinate axis between 6.3 and 56.0. The most useful assay across the range of target DNA was 1La795 (**Table 3**). Similarly, the four La3304-specific assays produced response curves with excellent R^2 values ($R^2 > 0.9$) and good predictive value across the assayed percentages of target DNA, but with considerable variation in the Y intercept (**Figure 1**). The two Og0222-specific assays had excellent coefficients of determination ($R^2 > 0.9$) (**Figure 2**). All the multi-genotype pyrosequencing assays similarly had excellent coefficients of determination ($R^2 > 0.9$) with polynomial regression models, indicating that most variance in the data was accounted for by the models (**Figure 3**). The two assays selected to monitor treatment of maize in Nigeria had interception points between two and four on the ordinate axis with excellent curve fit across all the serial dilution range (**Figure 3**). Output from single-genotype assays, when summed and compared with multi-isolate assays for the target isolates showed high degrees of similarity by *t*-tests at $P < 0.05$. This suggests similar accuracy and sensitivity between multi-isolate and single isolate assays.

The two selected multi-isolate assays, 3La94K1, and 3La94K2, were equally effective at detecting either any of the three targeted active ingredients (La3304, La3279, and Ka16127) or mixtures of the three (**Figure 4**). Furthermore, the assay had low sensitivity to five other *A. flavus* L morphotypes genotypes (57-L, AF36, SS19-14, AF13, and MO11-8). However, assay 3LA94K2 was sensitive to *A. aflatoxiformans* isolate BN008R (Singh and Cotty, 2018; Frisvad et al., 2019) providing a response significantly higher than the baseline. Assay 3LA94K1 was not sensitive to BN008R (**Figure 4**).

Analysis of Samples With Quantitative Pyrosequencing Assays

Quantitative pyrosequencing assays specific for the active ingredients detected and quantified the active ingredient genotypes associated with maize samples. Of the 292 samples assayed, 172 (59%) passed the validation tests with either assay 3La94K1 or assay 3La94K2 alone. These two assays quantify three of the four active ingredients. The number of passed samples

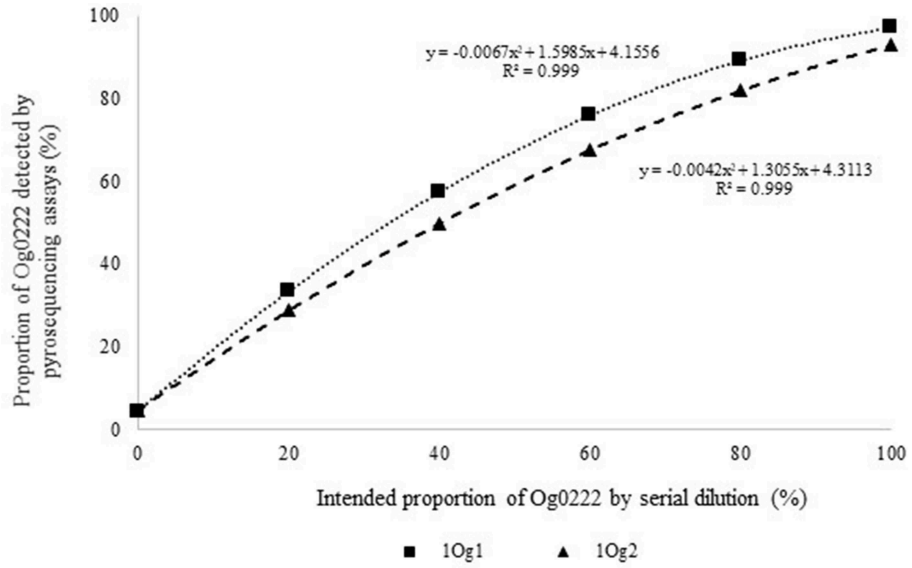
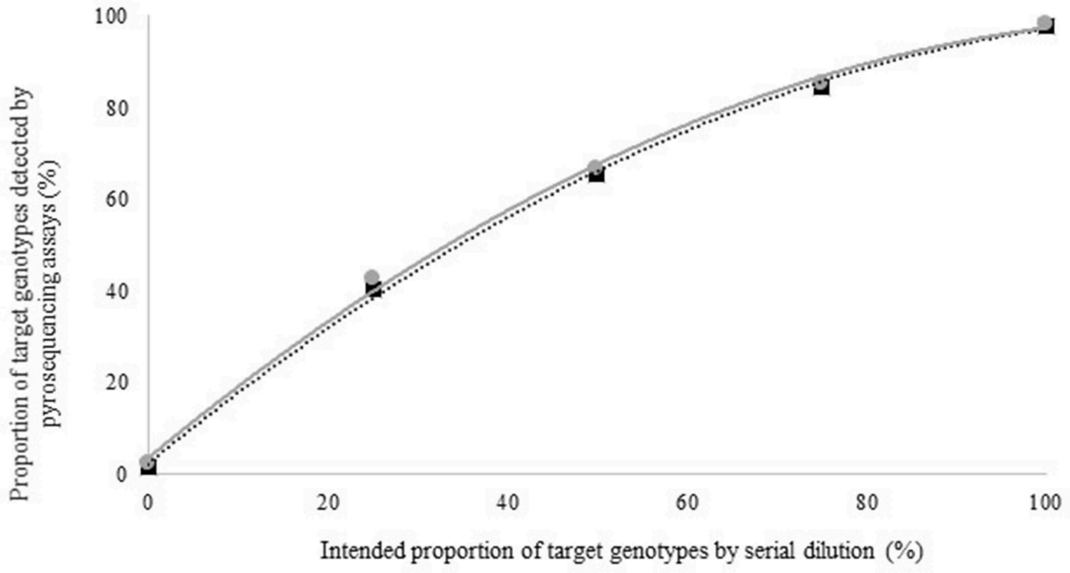


FIGURE 2 | Intended proportion of Aflasafe active ingredient Og0222 DNA mixed in varying proportions with *A. flavus* isolate AF13 vs. frequency of Og0222 detected using pyrosequencing assays 1Og1 and 1Og2.



	3La94K1	3La94K2
y	-0.0066x2 + 1.6038x	-0.0069x2 + 1.6246x
Intercept	2.320	3.471
R^2	0.999	0.9978

FIGURE 3 | Intended proportion of Aflasafe active ingredients Ka16127, La3279, and La3304, mixed in varying proportions with *A. flavus* isolate AF13 vs. frequency of Ka16127 + La3279 + La3304 detected using pyrosequencing assays 3La94K1 and 3La94K2.

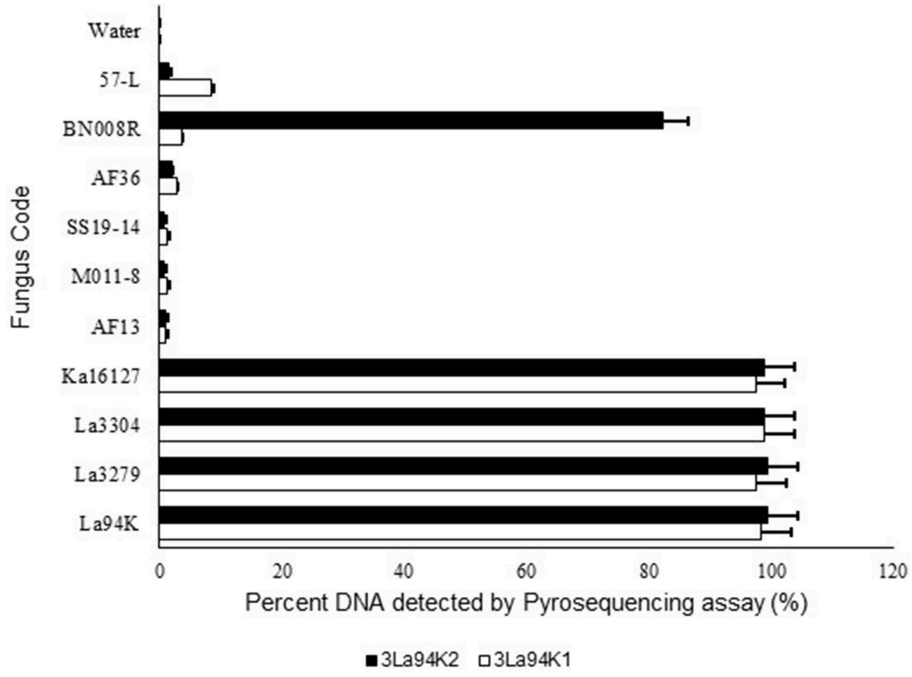


FIGURE 4 | Response of pyrosequencing assays designed to simultaneously detect three active ingredients (Ka16127, La3304, La3279) of Aflasafe to DNA from the three targeted active ingredients and several related *Aspergillus* section *Flavi* fungi.

increased to 274 (94%) when results from assay 1Og2, which quantifies the fourth active ingredient, were added. Eighteen (18) samples (6.2%) were considered to be maize from fields that were not treated properly because the pyrosequencing assays indicated that the four active ingredients composed <70% of the *A. flavus* associated with the maize.

DNA Extraction From Ground and Whole Maize

The DNA extraction protocols resulted in DNA adequate for the developed pyrosequencing assays. Washing both whole and ground maize samples with 0.1% TWEEN®80 resulted in up to a 20% increase in the frequency of assays which passed the PyroMark Q24 internal quality controls on the first attempt.

DISCUSSION

Biocontrol products with atoxigenic *A. flavus* active ingredients are inexpensive effective tools farmers use to reduce crop aflatoxin content (Cotty and Bayman, 1993; Mehl et al., 2012; Atehnkeng et al., 2014; Bandyopadhyay et al., 2016; Abbas et al., 2017). Treatments are effective at reducing the likelihood that crops have unacceptable aflatoxin content. Industries benefit from use of atoxigenics because these products reduce risk associated with the highly heterogeneous nature of contamination, year to year fluctuations in aflatoxin incidences, and impacts of weather events on severity of contamination (Cotty and Jaime-Garcia, 2007; Medina et al., 2014; Bandyopadhyay et al., 2016). For this reason, some purchasers of crops, including processors, dairies, flour mills and market development projects require or recommend that crops brought to them be treated with an atoxigenic *A. flavus* biocontrol product during crop development. In some cases, biocontrol treatments are required to ensure long-term benefits and to make continuing improvements to the aflatoxin vulnerability in areas from which end-users traditionally draw crops. Also, end-users may seek other advantages, including post-harvest protection during silage operations, storage, and animal feeding operations (Cotty and Mellon, 2006; Prandini et al., 2009; Wu and Khlangwiset, 2010; Alonso et al., 2013; Bandyopadhyay et al., 2016).

Although on average, crops treated with atoxigenic biocontrol products have less aflatoxins than untreated crops, aflatoxin contamination is highly variable among fields and, as a result, aflatoxin content alone cannot be used to indicate treatment. Indeed, there are no rapid visual or chemical assays to indicate a crop was properly treated with an atoxigenic biocontrol product. One approach is to isolate individual *A. flavus* from crops, and to characterize each by either VCA (Cotty and Bayman, 1993; Cotty et al., 1994; Ehrlich and Cotty, 2004), or DNA fingerprinting (Grubisha and Cotty, 2015; Islam et al., 2018). However, such methods have significant sampling errors and require trade-off between costs associated with the number of individuals assayed and the desired accuracy. The approach described here allows detection of genetic variants in pools of DNA from millions of individuals, reducing costs and sampling errors associated with culturing and characterizing individuals. Quantitative

pyrosequencing is particularly well-suited to determine the percent of the *A. flavus* community containing a target SNP (Das et al., 2008) and allows resolution of small differences not achievable with other methods (Sogin et al., 2006; Siqueira et al., 2012; Harrington et al., 2013; Zhou and Mehl, 2019). In the current study, quantitative pyrosequencing proved a very useful tool for rapidly determining farmer compliance in Nigeria with a market that requires application of a biocontrol product. In this case, the market is one created by the AgResults Initiative (AgResults, 2019), a large multilateral endeavor that uses a pay-for-results model to incentivize private sector adoption of innovative solutions to problems of smallholder farmers. The Nigeria project is the first time such incentivization has been applied to adoption of a plant disease biocontrol product.

In Nigeria, most farmers are small holders with <2 ha planting area and poor yield of <2 tons/ha. This results in single farm total crop value insufficient to support costs of proper crop sampling, sample preparation and aflatoxin analyses. If tests detect unacceptable aflatoxin levels, the farmer has few options to recover both costs of analyses and crop value. For many farmers, the cost of using an atoxigenic strain-based product is less than the cost of performing per field aflatoxin analyses and treatments are invariably associated with reduced aflatoxins (Bandyopadhyay et al., 2016). Low costs of atoxigenic strain-based biocontrol products give small holder farmers a practical alternative to reduce aflatoxin exposure (Ayedun et al., 2017; Johnson et al., 2018, 2019). Proper treatments result in atoxigenic strain active ingredients composing >80% of the crop-associated *A. flavus* population. High frequencies of the atoxigenic-strain active ingredients on a crop is the most reliable indicator of proper treatment (Cotty and Bayman, 1993; Atehnkeng et al., 2014).

From 2014 to 2018, assays described in the current report were used to determine presence of atoxigenic-strain active ingredients on harvested maize and, in so doing, verify proper use of biocontrol by participating farmers. In total 4,288 maize samples from 48,513 farmers who treated 61,645 ha with biocontrol were analyzed with 91% having sufficient incidences of active ingredients to confirm proper use. Verification of proper use resulted in a performance payment (AgResults, 2019).

Quantitative pyrosequencing is highly precise, accurate, and rapid (Mehl and Cotty, 2010, 2013). However, this technology has been underutilized in plant pathology and only recently has been applied to monitoring frequencies of resistance to fungicides (Zhou and Mehl, 2019). Single-genotype pyrosequencing assays provide accurate and rapid quantification of target *A. flavus* genotypes in crop associated populations (Das et al., 2008; Mehl and Cotty, 2011). The current study utilized whole genome analyses to design twenty-four quantitative pyrosequencing assays developed for rapid and simultaneous quantification of multiple *A. flavus* genotypes in maize associated fungal populations. The use of whole genome sequence analyses in the current study also allowed development of assays for simultaneous quantification of multiple genotypes. Use of pyrosequencing with assays similar to those developed here may allow long-term monitoring of *A. flavus* populations and associated design of low cost, area-wide programs to prevent dangerous concentrations of aflatoxins. This results from characteristics of this technology to precisely quantify frequencies of DNA sequence variation in complex microbial populations (Ronaghi, 2001; Mehl and Cotty, 2010; Siqueira et al., 2012). The high throughput and relatively low cost of the pyrosequencing method provides adequate sampling depth to facilitate detection of both dominant and rare individuals within complex microbial populations (Sogin et al., 2006; Kunin et al., 2010; Siqueira et al., 2012), making it also suitable for ecological studies on environmental influences to *A. flavus* population structure. However, no technology is sufficiently inexpensive to allow frequent economic monitoring of individual small holder crops. Therefore, application of this and similar technologies will likely rely on composite samples from multiple fields as performed in the current study to determine carryover, dispersal, and long-term efficacy of management programs.

Previous studies (Das et al., 2008; Mehl and Cotty, 2010) developed assays for known SNPs in a few specific genes. Whole genomes were scanned in the current study for useful SNPs. The utilized whole genome searches were not able to find SNPs shared by all four target isolates because of divergence of *A. flavus* Og0222 from Ka16127, La3279, and La3304. However, the assays developed for targeting the three latter fungi demonstrate the concept of simultaneous monitoring of multiple genotypes with a single assay. With the multi-genotype assays, a single instrument could simultaneously quantify the target genotypes from up to 240 crop samples per day after DNA extraction, an endeavor that would take several months and greatly increase labor with VCA (Mehl and Cotty, 2010). As increasing numbers of genomes of closely related fungi become available, and sufficient computing power is widely distributed, similar pyrosequencing assays may be developed to monitor incidences of any genotype independent of mutations that influence phenotype. As in the current study, nesting of PCR can be applied to increase specificity and yield of rare genotypes independent of the phenotypes of possible adaptive significance. Indeed specificity of assays in the current study is derived from five primers: Two for the outer PCR; two for the inner PCR, and the primer used to initiate the sequence by synthesis reaction during pryrosequencing. Such specific assays may allow dissection of population genetics and dispersal independent of selected for adaptations.

In the current study, pyrosequencing assays targeting the same Aflasafe active ingredients differed in sensitivity and accuracy. The observed differences were probably the result of variation in regions flanking the SNP. Actual frequencies of SNPs in the assayed populations and presence in non-target genotypes could also affect ability of assays to discriminate between targets and non-targets. The current study used non-target genotypes to identify useful SNPs. However, it was considered prudent to call multiple SNPs for each Aflasafe genotype, and to test several multiple-genotype assays to select the best-performing assays for deployment. The prudence of this approach was affirmed by observed variation in performance of pyrosequencing assays targeting the same Aflasafe active ingredient genotype (**Figure 1**). All developed assays readily detected variation in incidence of the targeted active ingredients. However, preferred assays allowed detection that most closely approximated linear response

curves, had the highest coefficient of determination (R^2), and a Y intercept most approximate to zero (**Figure 1**). The DNA extraction protocols and standardized nested PCR and pyrosequencing methods in the current work can be used for assays for targets beyond those examined here.

CONCLUSION

Pyrosequencing assays provide a flexible and robust tool for assessment of efficacy of biocontrol technologies directed at altering the *A. flavus* community structure. These assays can be used to confirm proper use of biocontrol products in a timeframe of potential value to commercial agriculture. Rapid simultaneous monitoring of multiple genotypes in complex crop-associated *A. flavus* populations may be useful for monitoring the environmental fate of active ingredients and cumulative benefits accrued from varying patterns of biocontrol product use.

DATA AVAILABILITY STATEMENT

The whole genome *Aspergillus flavus* sequence data analyzed in this study and unique to this study are deposited at DDBJ/ENA/GenBank under BioProject PRJNA565924, SUBID SUB6303482, accession VYXL00000000 for Ka16127, VYXK00000000 for La3279, VYXJ00000000 for La3304, and VYXI00000000 for Og0222.

AUTHOR CONTRIBUTIONS

RB and PC designed the overall project and secured funds for the studies from which the data are derived. BA performed the whole genome analyses. AA organized and supervised the farmer training and sampling of the crops. KS, BA, KC, RB, and PC collaborated on designing the analysis pipeline. KS, AA, JA, AO-B, and PK performed the sample preparation, DNA isolation, and pyrosequencing. KS analyzed the data and drafted the original manuscript. PC and RB edited the manuscript. All authors read, reviewed, and approved the final manuscript.

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Conflict of Interest: The authors receive no direct financial benefit from the marketing of the biocontrol technology described in this work. Initial patents for use of atoxigenic strains to prevent aflatoxin contamination were filed in 1988 and awarded by the US patent office to the US Department of Agriculture in 1992 and 1994 with PC as the inventor. The patent protection has expired. The manufacturing process for and the compositions of Aflasafe, the biocontrol product discussed in the current work, are not patented and are used for several atoxigenic strain-based products that differ primarily in the active ingredient genotypes. In addition to the Nigeria Aflasafe product, these products include other products bearing the Aflasafe name (e.g., Aflasafe SN01), AF36 Prevail in the US, and AF X-1 in Italy (Mauro et al., 2015; Bandyopadhyay et al., 2016; Ortega-Beltran and Bandyopadhyay, 2019). The fungal isolates used as active ingredients of Aflasafe Nigeria are considered a portion of the bioresources of Nigeria and, as such, are not patented. However, atoxigenic genotypes suitable for biocontrol applications have been found in all regions where active ingredients have been sought (Bandyopadhyay et al., 2016). The Aflasafe name is a Trademark of the International Institute of Tropical Agriculture (IITA). During the course of this study, IITA manufactured and marketed Aflasafe. Manufacturing and distribution has been transferred to the private sector during 2019. IITA charges a licensing fee to manufacturers for use of the Aflasafe name and associated technology transfer. RB, AO-B, JA, PK, and AA were employed by IITA.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effective Biopesticides and Biostimulants to Reduce Aflatoxins in Maize Fields

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The presence of ear rots in maize caused by *Aspergillus flavus* that are also associated with the production of aflatoxins has evolved into an increasing problem over the last few years. Since no commercial biological control products are still available to control *A. flavus* in maize in Europe, this study targets to the evaluation of six biopesticides/biostimulants (Botector®, Mycostop®, Serenade Max®, Trianum®, Vacciplant®, and zeolite) for the control of *A. flavus* and the derived aflatoxins in *in vitro* and maize field bioassays. Mycostop®, Serenade Max®, Vacciplant®, and zeolite reduced significantly *A. flavus* conidia production by 38.8–63.1%, and most of them were able to reduce aflatoxin B1 (AFB1) production in laboratory studies. Mycostop®, Trianum®, and Botector® were effective in reducing AFB1, *in vitro*. In the field, Mycostop® and Botector® treatments resulted in significant reduction of the disease severity (16.5 and 21.9%, respectively) and decreased significantly AFB1 content in maize kernels by 43.05 and 43.09%, respectively. For the first time, these results demonstrated the potential of commercial non-chemical products to suppress disease symptoms and aflatoxin content caused by *A. flavus* in maize under laboratory and field conditions.

Keywords: *Aspergillus* ear rot, aflatoxins, *Aspergillus flavus*, biological control, mycotoxins

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INTRODUCTION

Mycotoxins are toxic metabolites of low molecular weight that are produced by several species of mycotoxigenic fungi. A plethora of mycotoxins which are differing in their chemical structure have been identified, but all of them have the same common characteristics; they contaminate food and animal feed causing chronic toxicity and lead to more than 25% of agricultural products that are discarded annually (Bennett and Klich, 2003; CAST, 2003). One of the most common mycotoxigenic fungi is *Aspergillus flavus*, a predominant plant pathogen of maize (*Zea mays* L.) causing destructive plant diseases commonly known as ear rots and capable of contaminating maize kernels with aflatoxins (AFs). Toxigenic strains of *A. flavus* produce primarily the AFB1 and AFB2, although other mycotoxins (AFG1, AFG2, cyclopiazonic acid) can also be produced by the same species (Dorner and Cole, 2002; Dorner and Horn, 2007). AFs are worldwide one of the major threats to food

quality and safety of the population feed. They are in first place (44%) as a reason for rejecting imports of various products in EU (RASFF/Rapid Alert System For Food and Feed for the European Union, 2008). Infection of maize by aflatoxigenic strains of *A. flavus* is favored by hot climatic conditions and the risk of aflatoxin biosynthesis is increased due to the dry and warm climate conditions combined with inappropriate storage conditions (Chulze, 2010).

Various strategies including chemical and biological control, development of tolerant varieties and control of insects that favor *Aspergillus* infection have been investigated in the effort to manage aflatoxins (AFs) in crops and agricultural products. Among them, biological control appears a very promising approach to control AFs at pre- and post-harvest level (Udomkun et al., 2017). In maize, the most susceptible stage for infection is during anthesis. Consequently, the most appropriate stage for application of biological or chemical plant protection products is this stage of ear development not only to protect wounds or plant surfaces, but also to give the biocontrol agents the ability to compete plant pathogens for space and nutrients (Vaughan et al., 2005; Dimakopoulou et al., 2008; Ponsone et al., 2011).

Numerous microorganisms including bacteria, yeasts, and non-toxigenic fungi of *A. flavus* have been evaluated for their ability to manage AF contamination in crops including maize, intending to reduce the impact of aflatoxigenic species (Yin et al., 2008; Ponsone et al., 2011; Mauro et al., 2018). Dorner (2004) and Atehnkeng et al. (2014) reported the efficacy of atoxigenic *A. flavus* strains in preventing AF contamination in maize field. Over time, several other effective non-toxigenic fungal strains have been commercialized like AF-X1® in Italy for aflatoxin management in maize (Mauro et al., 2018). In another study, it was reported the efficacy of two *Bacillus* strains in the control of *A. parasiticus* and aflatoxins production on pistachio (Siahmoshteh et al., 2017). Moreover, Chourasia and Sah (2017) pointed out the successful control of *A. flavus* and AF production with geocarposphere bacteria in peanuts in greenhouse experiments. In addition, Sivparsad and Laing (2016) showed that pre-harvest silk treatment with *Trichoderma harzianum* reduced disease severity and AF contamination caused by *A. flavus* in sweet corn, in greenhouse, and field experiments.

The use of biological agents and biostimulants for the control of *A. flavus* is a prerequisite for creating an Integrated Pest Management (IPM) in order to protect maize from AF contamination. Commercial biopesticides could offer an economically effective solution that will contribute to the exclusion of aflatoxigenic fungi from maize plants and the restriction of mycotoxin production with the help of an IPM system that will be friendly and sustainable for the environment. Mycotoxin control and reduction is crucial for food safety, animal welfare, human health reasons, and production economics (Bennett and Klich, 2003; CAST, 2003; Bosco and Mollea, 2012). In spite of the high contamination risk of maize by mycotoxigenic fungi and mycotoxins, biological control studies conducted on this particular crop are limited and most of them refer to *in vitro* results. This study suggests a biocontrol strategy based on commercial plant protection products to reduce AF contamination in maize fields. Therefore, the aim of the present study was: (1) to test the efficacy of six biopesticides/biostimulants, to inhibit conidiogenesis and aflatoxin production *in vitro*, and (2) to evaluate the potential of the most efficient products to reduce *A. flavus* infection and aflatoxin contamination of maize under field conditions.

MATERIALS AND METHODS

Fungal Strains and Culture Conditions

Three *A. flavus* isolates were used in the experiments: A 6.10, D 1.3, and 12S. The isolates A 6.10 and D 1.3 originate from maize fields and pistachio orchards, respectively, in Greece and held in the culture collection of the Laboratory of Plant Pathology, Department of Crop Science, Agricultural University of Athens, whereas 12S originate from a cotton field in the USA. The isolates were mixed with glycerol (AppliChem, Darmstadt, Germany) to a final glycerol concentration of 25% (v/v) and stored at −20°C. The molecular characterization and the determination of aflatoxigenic efficacy of A 6.10, D 1.3, and 12S strains are described in our previous study (O'Donnell, 2000; Lagogianni and Tsitsigiannis, 2018).

Biocontrol Products – Biopesticides/ Biostimulants

Six products containing microorganisms or inorganic components with various modes of actions against a range of plant pathogens (**Table 1**) were used in bioassays: (1) zeolite, a microporous aluminosilicate mineral with special physicochemical properties, (2) Trianum®, a commercial product that contains the fungus *Trichoderma harzianum* and acts by inhibiting the infection and colonization of pathogenic fungi and inducing the plant defense system, (3) Botector®, a commercial product that

TABLE 1 | Commercial biopesticides and biostimulants used in the present study, active ingredients and applied doses according to manufacturer's instructions and company.

Product name	Active ingredient/ biological agent	Applied dosage[a]	Company
Botector®	*Aureobasidium pullulans* strains	1 g L^{-1}	BIO-FERM®
Trianum®	*Trichoderma harzianum*	3 g L^{-1}	Koppert®
Mycostop®	*Streptomyces griseoviridis*	0.5 g L^{-1}	Verdera®
Serenade Max®	*Bacillus subtilis* QST 713	4 g L^{-1}	BASF®
Zeolite®	Mineral	10 g L^{-1}	Olympos®
Vacciplant®	Laminarine	2 g L^{-1}	GOEMAR®

[a]*Highest recommended dosage according to manufacturer's instructions.*

contains yeasts of *Aureobasidium pullulans* with proven activity against *Botrytis cinerea* in grapes, (4) Mycostop®, a biological fungicide developed from the naturally occurring bacterium *Streptomyces griseoviridis* that provides biological protection against root infecting pathogenic fungi, (5) Serenade Max®, a commercial product that contains the bacterium *Bacillus subtilis* strain QST 713 with bio-fungicide/bio-bactericide action that stimulates natural plant defense mechanisms and demonstrates increased plant growth effects, and (6) Vacciplant®, which bases its action on activating the plant defenses thanks to the action of laminarine, a storage glucan from *Laminaria digitata*. All the above mentioned agents were initially tested *in vitro* and the most efficient were further evaluated in 2-year experiments under field conditions.

In vitro Evaluation of Biopesticides and Biostimulants on *Aspergillus flavus* Sporulation and Aflatoxin Production

The effect of the tested biopesticides and biostimulants on *A. flavus* sporulation and AFs production was initially studied *in vitro*. To conduct the bioassays, 40 g corn seeds (maize line N9, House of Agriculture Spirou, Athens, Greece) were surface-sterilized by immersing them in 10% NaClO for 10 min, washed briefly with sterile distilled water (SDW), placed in 70% ETOH for 3 min, and washed again with SDW for each biological product. The surface-sterilization of the seeds was carried out to avoid contamination from the seed surface saprophytes and keep the corn kernels alive. The seeds were not autoclaved to avoid the inactivation of the natural seed tolerance/resistance to *Aspergillus* infection provided by the plant immune system. Then, seeds for each treatment were placed into 250 ml capacity flasks containing each commercial product at the appropriate concentration according to the dose recommended by manufacturer's instructions (**Table 1**). The flasks were shaken at 250 rpm for 30 min, then the solutions were discarded and corn seeds were kept at room temperature for 24 h. Then, seeds were artificially inoculated by adding in each flask 50 ml of *A. flavus* conidial suspension (10^6 conidia ml^{-1}) and shaking at 250 rpm for 30 min (Lagogianni and Tsitsigiannis, 2018). The suspension was removed and the flasks were placed at 28°C in the dark for 13 days to let the fungus produce AFB1. The presence of AFB1 in each sample was determined with thin layer chromatography (TLC) method, according to the following procedure: the seeds were grinded and 3 g of the fine powder were transferred into 50 ml falcon tubes, where 5 ml Tween 80 (0.01%) and 5 ml acetone were consecutively added. The samples were shaken at 150 rpm for 10 min and kept still for 5 min at room temperature; 5 ml chloroform were added and further shaken at 150 rpm for 10 min. The samples were passed through a filter paper and the flow-through collected into a new tube. The flow-through was centrifuged for 10 min at 3,000 rpm and the lower phase transferred into a new tube and kept overnight at room conditions to dry-out. Finally, 100 μl methanol were added and 10 μl of the sample spotted on a TLC plate (TLC Silica gel 60, Merck, Germany). TLC plate development and AFB1 detection were determined as mentioned above (Scott, 1995). The AFB1 that used as standard was purchased from Sigma-Aldrich.

To study the effect of the different tested biopesticides/biostimulants on the sporulation of *A. flavus*, each product was applied on corn seeds as described above and the seeds were placed in sterilized petri dishes (10 seeds per plate). Twenty-four hours later, one droplet of conidial suspension (10 μl of a 10^6 conidia ml^{-1}) of each *A. flavus* isolate (A 6.10, D1.3, and 12S) was deposited on each seed in the plate. Five days post inoculation, the 10 seeds of each plate were transferred in a new 50 ml falcon tube and 10 ml of sterilized distilled water was added. The samples were vortexed vigorously for 30 s and then the numbers of conidia were measured under a light microscope with the use of a Neubauer hemocytometer. The experiment was repeated three times, with 30 replicated maize seeds per treatment.

Maize Field Experiments

Two-year experiments were carried out in the same experimental field of Agricultural University of Athens, Greece, in 2014 and 2015 crop seasons. Corn seeds (maize hybrid N9, House of Agriculture Spirou, Athens, Greece) were sown in the soil in April 2014 and 2015. Vacciplant® and zeolite were applied once whereas Mycostop®, Trianum®, and Botector® were applied twice by using a nozzle sprayer: the first application was carried out at the beginning of the flowering stage whereas the second one 7 days later. The applied dosages for each product are presented in **Table 1**, while no additional adjuvant or surfactants were used. The artificial inoculation was performed according to Lagogianni and Tsitsigiannis, 2018. Briefly, 5 ml of conidial suspension of *A. flavus* strain A6.10 (10^6 conidia ml^{-1} in sterile ddH_20 containing 0.05 g L^{-1} Tween 80) were injecting in maize ears using a 10 ml capacity syringe with a needle. Three milliliter of the inoculum was injected through the silk into the top of each maize ear and 2 ml through the husk into the middle of the ear at each of four points. Both inoculated and mock inoculated ears were immediately covered with paper bags for 48 h to maintain high humidity and favor *Aspergillus* infection (Zummo and Scott, 1989). The experiments were performed with a factorial randomized block design with three blocks and six experimental units (Control+, Botector, Trianum®, Vacciplant®, Mycostop®, and zeolite) per block. Each experimental unit consisted of 30 replicated plants.

Disease Assessment and AFB1 Analysis

Disease symptoms were assessed at the end of each growing season (60 days post inoculation), in September 2014 and 2015. Disease severity index was based on a visual scale from 1 to 7, considering the percentage of symptomatic kernels per ear (1 = healthy, 2 = 1–3%, 3 = 4–10%, 4 = 11–25%, 5 = 26–50%,

6 = 51–75%, and 7 = 76–100%) of infected kernels, respectively (Reid et al., 1999). Maize cobs were harvested and their kernels were detached and placed in a drying oven until their humidity reached 15–18%. Then kernels were homogenized using a grinder and 40 g of the fine powder were used for AFB1 analysis, following the Agra-Quant aflatoxin 4-40ppb ELISA kit protocol (Romer-Labs).

Statistical Analysis

All experimental data were analyzed with SPSS statistical software (SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was used to determine the effects of replication, treatment, year and their interaction on disease severity and AFs production in field experiments. In laboratory experiments, ANOVA was used to determine the effects of replication, treatment, and *A. flavus* isolate on conidia production. When a significant *F*-test was obtained for treatments ($p \leq 0.05$), the data were subjected to means separation by Tukey's honestly significant difference (HSD) test (**Table 2**).

RESULTS

Effect of Biological Products and Biostimulants on *Aspergillus flavus* Sporulation and Aflatoxin Production *in vitro*

Among treatments, Vacciplant® was the most efficient in decreasing *A. flavus* sporulation *in vitro*, followed by Serenade Max®, Mycostop®, and zeolite leading to a reduction of conidia production by 63.1, 55.4, 48.2, and 52.1%, respectively. Botector® and Trianum® did not result in any significant reduction of fungal sporulation (**Figure 1**). Analysis of variance revealed that *A. flavus* isolates differed significantly in terms of sporulation (df = 2, $F = 3.23$, $p < 0.05$). Moreover, treatments effected significantly sporulation of the fungus *in vitro* (df = 6, $F = 7.14$, $p < 0.001$).

The capacity of the biological products and biostimulants to eliminate the aflatoxin production was also evaluated. AF was extracted from infected maize seeds and the extracts were developed by TLC. TLC tests showed that Mycostop®, Trianum®, and Botector® were very effective in reducing aflatoxin biosynthesis *in vitro*, produced by each toxigenic strain, whereas zeolite, Vacciplant®, and Serenade Max® did not provide a constant significant reduction in aflatoxin production (**Figure 2**).

Based on these results, Mycostop® contributed to the inhibition of the conidiogenesis and to a significant reduction in the AFB1 content for all the three tested *A. flavus* strains. Trianum® inhibited AF production but did not have any statistically significant effect to the conidia production. Vacciplant®, Serenade Max®, and zeolite did not lead to any

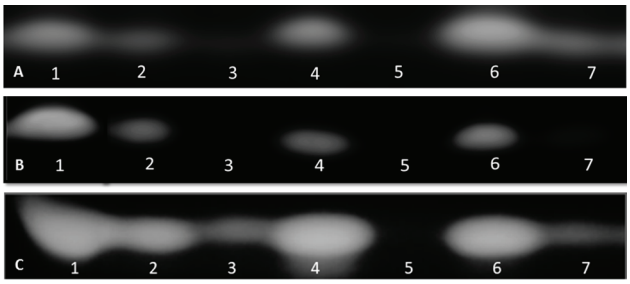

FIGURE 2 | TLC detection of AFB1 in maize seeds treated with various biopesticides/biostimulants 13 days post their artificial inoculation with the toxigenic isolates D1.3 **(A)**, 12S **(B)**, and A6.10 **(C)** of *A. flavus* (1: non-treated seeds that served as positive control, 2: seeds treated with Vacciplant®, 3: seeds treated with Botector®, 4: seeds treated with Serenade Max®, 5: seeds treated with Mycostop®, 6: seeds treated with zeolite, and 7: seeds treated with Trianum®).

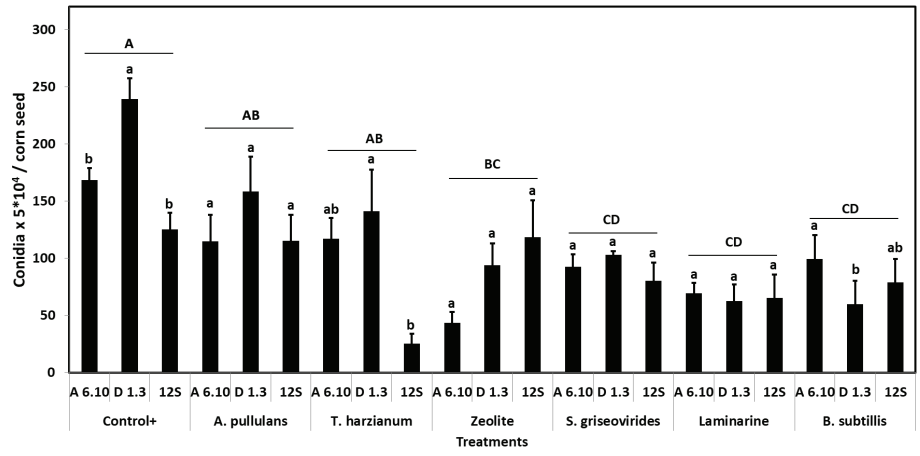

FIGURE 1 | Mean numbers of conidia production of *A. flavus* by the strains A6.10, D1.3, and 12S in maize seeds treated with different commercial biopesticides/biostimulants. Within each treatment, columns with different lower-case letters differ significantly according to Tukey's HSD test ($p \leq 0.05$). Different upper-case letters indicate significant differences between treatments according to Tukey's HSD test ($p \leq 0.05$). Each column represents the mean of three measurements per isolate and vertical bars indicate standard errors of the means.

reduction in the AFB1 content (**Figure 2**) but the inhibition of *A. flavus* conidia production was significant in the case of zeolite and Vacciplant®. Among the three strains, the conidiogenesis of D1.3 was not influenced significantly by the presence of the tested bioproducts except for the case of Serenade Max®.

Suppression of Ear Rot Disease Symptoms and AFB1 Production in the Field by the Use of Biopesticides/Biostimulants

The toxigenic A6.10 maize strain, an isolate from Northern Greece, was used for the 2-year field experiments. Since Serenade Max® did not have a constant reduction of AFB1 in *in vitro* experiments, was not included in the field experiments. ANOVA revealed that neither experimental year nor the interaction between year and other experimental factors affected disease severity and AFB1 quantity significantly (**Table 2**). Therefore, data from 2-year experimentation (2014 and 2015) were combined and presented in **Figure 3**.

In order to evaluate the disease severity of infected maize ears under field conditions after the application of the commercial biopesticides/biostimulants, a scale of 1–7 was used (Reid et al., 1999). The disease severity index in plants treated with Mycostop® and Botector® was significantly lower compared to the Control+ plants (by 16.5 and 21.9%, respectively), a fact that demonstrates the suppressive effect of the above mentioned products under field conditions (**Figure 3**). The observed decrease in symptom severity, in Mycostop® and Botector® treated plants was also associated with significantly lower AFB1 content in maize kernels, by 43.05 and 43.09%, respectively (**Figure 4**). Trianum® and Vacciplant® treated plants did not provide any statistically significant reduction on the AFB1 content, but offered a reduction in the disease severity whereas zeolite did not have any influence on either the disease severity or the AF content of maize ears.

TABLE 2 | Analysis of variance for disease severity and aflatoxin (AFB1) quantity in maize plants artificially inoculated with *A. flavus* isolate A6.10, treated with commercial biopesticides based on *A. pullulans* (Botector®), *S. griseoviridis* (Mycostop®), Zeolite®, *laminarine* (Vacciplant®), and *T. harzianum* (Trianum®) or not (positive control), under field conditions in 2014 and 2015.

Source	df[b]	*F* values[a]	
		Disease severity	AFB1
Replication	2	1.66	—
Treatment	5	23.30***	19.95***
Year	1	0.13	0.28
Replication × Treatment	10	3.02*	—
Replication × Year	2	0.28	—
Treatment × Year	5	1.40	0.29
Replication × Treatment × Year	10	0.26	—

[a]*Symbols: * and *** indicate significance at $p \leq 0.05$ and 0.001 levels, respectively, according to the F test.*
[b]*Degrees of freedom between groups.*

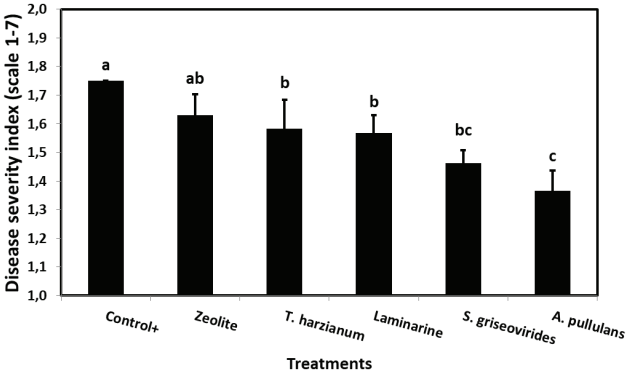

FIGURE 3 | Mean *Aspergillus* ear rot severity indices on field grown maize plants treated with different commercial biopesticides/biostimulants and artificially infected by *A. flavus* maize strain A6.10. Columns followed by different letters are significantly different ($p \leq 0.05$), according to Tukey's HSD test. Vertical bars indicate standard errors of the means. The results represent the average *Aspergillus* ear rot severity for 2014 and 2015.

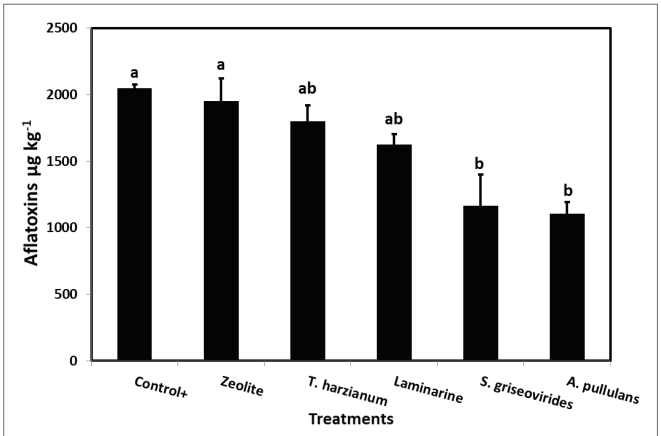

FIGURE 4 | Mean AFs content (μg kg^{-1}) in maize kernels from field grown plants treated with different commercial biopesticides/biostimulants and then artificially inoculated with *A. flavus* strain A6.10. Columns accompanied by different letters are significantly different ($p \leq 0.05$) according to Tukey's HSD test. Vertical bars indicate standard errors of the means. The results represent the average AFs content for 2014 and 2015.

DISCUSSION

Mycotoxins, especially aflatoxins, are one of the major worldwide threats to food quality and safety of the population feed. The public concern of pesticides and their residues as an emerging threat in food and environment have increased the interest in alternative methods for disease control both at pre- and post-harvest stages. In Europe, there is a lack of commercial products (biological or chemicals) to prevent AFs in maize despite the fact that EU sets very strict rules for the maximum limits of AFs in foods. Based on several studies and the impact of the climate conditions in the life cycle of mycotoxigenic fungi and mycotoxin production (Edlayne et al., 2009; Chulze, 2010; Russell et al., 2010;

Battilani et al., 2012), a "biological" solution seems to be the only promising solution for the aflatoxin reduction combined with good agricultural practices, sustainable IPM strategies and agricultural precision technologies.

The use of certain bacteria, yeasts, and other antagonistic fungi to reduce AF contamination has been documented in maize, groundnut, and other crops (Nesci et al., 2005; Alaniz Zanon et al., 2013; Morteza et al., 2013; Zhao et al., 2014; Sivparsad and Laing, 2016; Siahmoshteh et al., 2017). In this study, six commercial biological products were evaluated for their ability to control *A. flavus* and aflatoxin production. Zeolite, Vacciplant®, Serenade Max®, and Mycostop® inhibited effectively the sporulation of all three *A. flavus* toxigenic strains *in vitro* by 48.2–63.1% with no statistically significant difference among the strains. In contrary, Botector® and Trianum® did not provide any significant inhibition in the sporulation of the three toxigenic strains when they were tested *in vitro*, but led to a significant reduction of AFB1 and *Aspergillus* ear rot severity in the field under a high *A. flavus* inoculum pressure per plant. Moreover, Mycostop® has the ability to inhibit the AFB1 content in maize field experiments, despite the fact that it did not suppress the ear rot severity by more than 10%. *In-vitro* tests do not resemble the natural environmental variation but they are always essential for the first screening of all plant protection products.

The two-year field experiment showed that when we applied the biological products Botector® (*A. pullulans*) and Mycostop® (*S. griseoviridis*) twice during the silk stage, they were able to reduce AF production. The most effective commercial biopesticide was Botector® that showed an inhibition of *Aspergillus* ear rot severity by 22% and a significant reduction of aflatoxin content by 46%. *Bacillus* spp. and yeasts are growing at a faster rate than *A. flavus* and as a consequence, they can demonstrate a higher biocontrol efficacy during the first steps of incubation (Siahmoshteh et al., 2017). Based on several studies, the mode of action of *Bacillus* strains is the inhibition of mycelial growth and the antibiosis (Baysal et al., 2008; Zhao et al., 2014). Other studies, by Chan et al. (2003), mention that *Bacillus* strains have the same mode of action for other fungi except *A. flavus*, such as *Fusarium* sp., *Alternaria* sp., and *Phytophthora* sp. Mannaa et al. (2017) mentioned that some *Bacillus* strains reduced significantly the aflatoxin production in rice grains produced by *A. flavus* due to their volatiles. In our study, Serenade Max® (*Bacillus subtilis*) did not reduce the AFB1 content when tested by TLC.

Liu et al. (2013) reported that yeasts, such as *A. pullulans*, grow rapidly and as a result, deplete available nutrients and physically occupy the given space. After the colonization, other modes of action can play a significant role in concert with nutrient competition and niche exclusion to disclose decay management (Droby et al., 2000, 2009; Wisniewski et al., 2007). Moreover, the study of Ponsone et al. (2011) shows that some yeasts are able to deliver promising results against the grape rot by *Aspergillus* section *Nigri*. In accordance with our study, Dimakopoulou et al., 2008 mention that an *A. pullulans* isolation offered a significant reduction on *A. carbonarius* strain in grapes. Moreover, Prasongsuk et al. (2013) found that the components that lead to a reduction in AFB1 content are the aureobasidins.

Concerning Vacciplant® that is based on laminarine, Hu et al. (2011) mention that a specific concentration of laminarine could decrease the infection of peanut seeds by *A. flavus* as well as the contamination by AFB1. In the present study, we found that laminarine inhibits conidia germination but did not provide any protection against AFB1 biosynthesis.

In our studies, Trianum® (*T. harzianum*) led to a significant reduction of conidia production *in vitro*, but in the field experiments, did not significantly reduce the *Aspergillus* ear rot severity or the aflatoxin production. The mode of action of *T. harzianum* is based on its ability to successfully colonize a wide array of ecological niches (Schuster and Schmoll, 2010). The competitive exclusion of *T. harzianum* involves the utilization of limited resources, and as a result, the pathogen is unable to grow. Alamene (2015) found that *Trichoderma* strains from a commercial biocontrol product (Tusal)® can effectively inhibit toxigenic *A. flavus* species and AFB1 concentrations *in vitro* and *in planta*, to a level below that recommended by the European Commission of 15 ppb in peanuts. Gachomo and Kotchoni (2008) mention that two strains of *T. harzianum* and two strains of *T. viride* were found to efficiently suppress the growth of peanut molds and to significantly reduce aflatoxins (AFB1 and AFB2), contents in infected peanut kernels due to their extracellular enzymatic activities and mycoparasitism. Abdel-Megeed (2013) found that a *T. harzianum* strain provided significant suppression of AFB1 content by 91.2% in *in vitro* tests and Sivparsad and Laing (2016) found that *T. harzianum* colonizes the silk of sweet corn by inhibiting the *A. flavus* infection.

Finally, our results showed that zeolite has the capacity to inhibit conidia germination *in vitro*. These data are in agreement with the study of Savi et al. (2017) who present that the ion-exchanged zeolites with Li^+ and Cu^{2+} have antifungal activity against *A. flavus*, including negative effects on conidia germination, hyphae morphological alterations, and inhibition of AFB1 production. Another study by Marković et al. (2015) indicates that zeolite can provide AFB1 adsorption. However, in our experiments, zeolite did not reduce AFB1 content neither in the field nor in *in vitro* tests. These results show that probably the application dose and application timing are crucial factors in the efficacy of zeolite *in planta*.

To date, there have been several studies demonstrating the efficacy of some microorganisms against *A. flavus* (Mannaa et al., 2017; Shakeel et al., 2018; Zeidan et al., 2018; Feng et al., 2019; Kagot et al., 2019; Mwakinyali et al., 2019; Peromingo et al., 2019). However, none of these studies have been conducted at field level and their tested microorganisms are not commercial formulations. Several factors can influence the efficacy of the biocontrol agents such as the cultivar response, the plant nutrition, the environmental variables, and the climate change. Furthermore, experiments about the right application and the appropriate number of application and dose could help to improve their efficacy against aflatoxins.

The European Commission suggests that, in southern Europe, climate change may lead to temperature increases of 4–5°C, in combination with increased drought periods (García-Cela et al., 2011; Battilani et al., 2012), conditions that will favor the production of aflatoxins in maize and other crops. An integrated approach of pre-harvest biological control, in conjunction with other post-harvest management strategies constitutes a very promising method for a long-term reduction in aflatoxin contamination in maize.

CONCLUSIONS

The findings of these studies demonstrated for the first time, the potential of commercial non-chemical products (e.g., Botector® and Mycostop®) to suppress disease ear rot severity symptoms and decrease significantly AFB1 content in maize fields. Taking everything into account, the biological control of aflatoxigenic fungi, the control of insects, and the investigation on new maize aflatoxin tolerant hybrids/varieties along with effective chemical products (Lagogianni and Tsitsigiannis, 2018), disease forecasting models and decision support systems can lead to a successful IPM system in order to eliminate the aflatoxins problem in maize and other crops.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

CL performed the experiments, analyzed the data, and wrote the manuscript. DT conceived the study, analyzed the data, and edited the manuscript.

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CORRECTION
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Corrigendum: Effective Biopesticides and Biostimulants to Reduce Aflatoxins in Maize Fields

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Keywords: ***Aspergillus*** **ear rot, aflatoxins,** ***Aspergillus flavus*****, biological control, mycotoxins**

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A Corrigendum on

Effective Biopesticides and Biostimulants to Reduce Aflatoxins in Maize Fields
by Lagogianni, C. S., and Tsitsigiannis, D. I. (2019). Front. Microbiol. 10:2645. doi: 10.3389/fmicb.2019.02645

In the original article, there was a mistake in **Table 1** as published. The company that provided the product named "Botector" with the biological agent *Aureobasidium pullulans* strains is not "Syngenta" company but "BIO-FERM." Also, the company that provided the product named "Vacciplant" with the active ingredient *Laminarin* is not "Arysta" company but "GOEMAR."

The corrected **Table 1** appears below.

In the original article, there was an error. The wrong company name was used in the **ACKNOWLEDGMENTS** section.

A correction has to be made to **ACKNOWLEDGMENTS** paragraph:

"The authors are grateful to BIO-FERM, Koppert, BASF, and GOEMAR for providing the commercial formulations used in this study."

The authors apologize for these errors and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

TABLE 1 | Commercial biopesticides and biostimulants used in the present study, active ingredients and applied doses according to manufacturer's instructions and company.

Product name	Active ingredient/ biological agent	Applied dosage[a]	Company
Botector®	*Aureobasidium pullulans* strains	1 g L^{-1}	BIO-FERM®
Trianum®	*Trichoderma harzianum*	3 g L^{-1}	Koppert®
Mycostop®	*Streptomyces griseoviridis*	0.5 g L^{-1}	Verdera®
Serenade Max®	*Bacillus subtilis* QST 713	4 g L^{-1}	BASF®
Zeolite®	Mineral	10 g L^{-1}	Olympos®
Vacciplant®	Laminarin	2 g L^{-1}	GOEMAR®

[a]*Highest recommended dosage according to manufacturer's instructions.*

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ORIGINAL RESEARCH
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Ethanol Inhibits Aflatoxin B_1 Biosynthesis in *Aspergillus flavus* by Up-Regulating Oxidative Stress-Related Genes

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As the most carcinogenic, toxic, and economically costly mycotoxins, aflatoxin B_1 (AFB_1) is primarily biosynthesized by *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin biosynthesis is related to oxidative stress and functions as a second line of defense from excessive reactive oxygen species. Here, we find that ethanol can inhibit fungal growth and AFB_1 production by *A. flavus* in a dose-dependent manner. Then, the ethanol's molecular mechanism of action on AFB_1 biosynthesis was revealed using a comparative transcriptomic analysis. RNA-Seq data indicated that all the genes except for *aflC* in the aflatoxin gene cluster were down-regulated by 3.5% ethanol. The drastic repression of aflatoxin structural genes including the complete inhibition of *aflK* and *aflLa* may be correlated with the down-regulation of the transcription regulator genes *aflR* and *aflS* in the cluster. This may be due to the repression of several global regulator genes and the subsequent overexpression of some oxidative stress-related genes. The suppression of several key aflatoxin genes including *aflR*, *aflD*, *aflM*, and *aflP* may also be associated with the decreased expression of the global regulator gene *veA*. In particular, ethanol exposure caused the decreased expression of stress response transcription factor *srrA* and the overexpression of bZIP transcription factor *ap-1*, C_2H_2 transcription factors *msnA* and *mtfA*, together with the enhanced levels of anti-oxidant enzymatic genes including *Cat*, *Cat1*, *Cat2*, *CatA*, and Cu, Zn superoxide dismutase gene *sod1*. Taken together, these RNA-Seq data strongly suggest that ethanol inhibits AFB_1 biosynthesis by *A. flavus* via enhancing fungal oxidative stress response. In conclusion, this study served to reveal the anti-aflatoxigenic mechanisms of ethanol in *A. flavus* and to provide solid evidence for its use in controlling AFB_1 contamination.

Keywords: aflatoxin B_1, *Aspergillus flavus*, transcriptome, RNA-seq, oxidative stress, ethanol

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INTRODUCTION

Aspergillus flavus is a saprophytic fungus being often found in mildewed grains, grain products, and other moldy organic matter, and causes the wastage of several important agricultural crops (Wild and Gong, 2010; Liang et al., 2015). In addition, this fungus is an opportunistic human and animal pathogen causing aspergillosis diseases (Amaike and Keller, 2010). It is more important to notice that this fungus can produce aflatoxins (AFs), the most potent natural carcinogen and

toxic compounds ever characterized (Da Rocha et al., 2014). In 1993, AFs are classified as a Class 1 carcinogen by the (International Agency for Research on Cancer [IARC], 1993, 2002), and were estimated to induce up to 28% of the total global cases of hepatocellular carcinoma (HCC) (Wu, 2014; Liu et al., 2017). AFs are mainly produced by *A. flavus* and *Aspergillus parasiticus*, and the former is the predominant aflatoxigenic species of contaminated foods and feeds in China (Xing et al., 2017). The most common AF-contaminated food and feed are aflatoxin B_1 (AFB_1), B_2, G_1, and G_2 (Bennett and Klich, 2003). Among AFs, AFB_1 is the most potent natural carcinogen and toxic compound known (Squire, 1981; Marin et al., 2013). Therefore, it is urgent to develop simple, economical, and effective ways to control *A. flavus* and subsequent AF contamination in food and feed, especially during storage and processing.

As we all know, ethanol is an inhibitor of the growth of bacteria and fungi (Ma et al., 2019). Previous studies showed that the accumulation of ethanol inhibited yeast cell growth and viability, affected the integrity of the cell membrane, and inactivated cellular enzymes, resulting in cell death during fermentation (Gibson et al., 2007; Kim et al., 2016; Ma et al., 2019). Ma et al. (2019) indicated that ethanol stress induced an obvious suppression of *Aspergillus oryzae* growth and conidia formation, and the inhibitory effect increased with ethanol concentration. As a general cell toxic substance, ethanol disturbed many cellular processes, such as irregular nuclei, the aggregation of scattered vacuoles, the increase of unsaturated fatty acid, and the overexpression of related fatty acid desaturases (Ma et al., 2019).

Transcriptional sequencing (RNA-Seq) has been widely applied to study lots of eukaryotic transcriptomes because of high sensitivity, low false-positive rates, and broad expression range coverage (Wilhelm et al., 2008; Wang et al., 2009; Lin et al., 2013; Lv et al., 2018). For *A. flavus*, this technology has been used to explore the mechanism of action of water activity (a_w) and temperature on fungal growth and AF production (Yu et al., 2011; Zhang et al., 2014; Bai et al., 2015). Moreover, it also has been used to decipher the inhibitory mechanism of 5-azacytidine (5-AC) (Lin et al., 2013), 2-phenylethanol (Chang et al., 2015), eugenol (Lv et al., 2018), gallic acid (Zhao et al., 2018), and cinnamaldehyde (Wang et al., 2019) on *A. flavus* growth and AF formation. The objective of this study was to determine transcriptomic changes in *A. flavus* treated with ethanol and untreated samples using RNA-Seq technology. In particular, ethanol's molecular mechanism of action on AF biosynthesis was elucidated. This study may pave a way for further understanding the inhibitory mechanism of action of ethanol on AF formation at the transcriptomic level.

MATERIALS AND METHODS

Chemicals, Fungal Strain, and Growth Conditions

Ethanol (100% purity) was purchased from Beijing Chemical Works (Beijing, China). Chromatographic grade methanol and acetonitrile were purchased from Thermo Fisher Scientific (Waltham, MA, United States). The AFB_1 standard was purchased from Sigma-Aldrich (Sigma-Aldrich Chemicals, St. Louis, MO, United States).

The *A. flavus* strain NRRL3357 was obtained from Dr. Wenbing Yin, Institute of Microbiology, Chinese Academy of Sciences, and was maintained in the dark on potato dextrose agar (PDA, purchased from Hopebio, Qingdao, China) at 4°C. Conidia suspension of 1×10^7 conidia/ml was prepared by surface washing PDA culture with 0.1% Tween-80 solution.

In order to investigate the effect of ethanol on *A. flavus* growth, after filtering with 0.22 μm filters, ethanol was added into the autoclaved PDA medium to obtain the final concentrations of 2, 2.5, 3, 3.5, 4, 5, and 6%. As the control group, PDA plates without ethanol were prepared. Then, 5 μl of 10^3–10^7 conidia/ml suspension was inoculated on PDA medium and incubated at 28°C for 7 days. A requisite amount of the ethanol was added to the autoclaved yeast extract sucrose (YES, purchased from Hopebio, Qingdao, China) broth to obtain the final concentrations of 1, 2, 2.5, 3, 3.5, and 4%. Then, 100 μl of 10^7 conidia/ml suspension was added to 100 ml of YES broth containing different concentrations of ethanol. The control cultures were treated similarly but without ethanol. After incubation at 28°C and 180 rpm/min in the dark for 7 days, fungal mycelia were collected. Each treatment was conducted in triplicate.

Determination of Mycelia Weights and AF Production

The dry weights of fugal mycelia were determined according to the method described by Yamazaki et al. (2007). AFB_1 levels were determined according to the method described by Liang et al. (2015). It was extracted with acetonitrile:water (84:16) mixture from 10 ml of culture broth and purified using a ToxinFast immunoaffinity column (Huaan Magnech Biotech, Beijing, China). AFB_1 was quantified using an HPLC system with a fluorescence detector (Agilent 1220 Infinity II System, Santa Clara, CA, United States) and a post-column derivation system (Huaan Magnech Biotech), and a TC-C18 column (250 mm × 4.6 mm, 5 μm particle size; Agilent, Santa Clara, CA, United States). The mean recovery of AFB_1 (1–100 ng/ml) was 95.3% ± 7.5%, and the lowest detection limit was 1 ng/ml.

Preparation of cDNA Libraries, RNA Sequencing, and Data Analysis

RNA extraction, cDNA libraries preparation, and data analysis were conducted according to the methods described by Lv et al. (2018). An Illumina® HiSeq 4000™ system (San Diego, CA, United States) was used to sequence the cDNA libraries. The RNA-seq data have been deposited in the NCBI Sequence Read Archive with accession code SRP217458.

The EST sequencing, rRNA sequencing, and assembling were performed using the programs TopHat v2.0.12 (Trapnell et al., 2009), Bowtie2 (Langmead et al., 2009), and Cufflinks, respectively. The transcription levels of genes were normalized using the FPKM values (Trapnell et al., 2010). The differential

expression of genes was analyzed using DEseq software (Anders and Huber, 2010). The significant differentially expressed genes were identified as $\log_2$Ratio ≥ 1 and $q < 0.05$ between these compared samples (Zhao et al., 2018).

Quantitative Reverse Transcription QRT-PCR Analysis of AF Biosynthesis Genes

All genes in the AF biosynthesis cluster were analyzed using QRT-PCR according to the methods described by Lv et al. (2018).

RESULTS

Inhibitory Effect of Ethanol on Fungal Growth and AFB_1 Production by *A. flavus*

As shown in **Figure 1**, some significant morphological changes of mycelial colonies were observed in *A. flavus* treated with ethanol compared with the control. The diameters of *A. flavus* colonies appeared much smaller than the control after treatment with 2–6% ethanol in a dose-dependent manner, and the mycelia growth was completely inhibited by 6% ethanol when the initial concentration was $\leq 10^4$ conidia/ml (**Figure 1A**). In YES broth, as shown in **Figure 1B**, the dry mycelia weights of *A. flavus* appeared much lower in 3.5–4.0% ethanol application compared to the control. AFB_1 production was significantly inhibited by 3.0–4.0% ethanol with the inhibition rate up to 99.8%. Interestingly, the mycelia weight was higher in 2.0–2.5% ethanol application compared to the control, but the AFB_1 level was obviously decreased. Taken together, these findings suggested that ethanol significantly inhibited fungal growth and AFB_1 production by *A. flavus*. Moreover, the suppressive effect increased with the rising levels of ethanol.

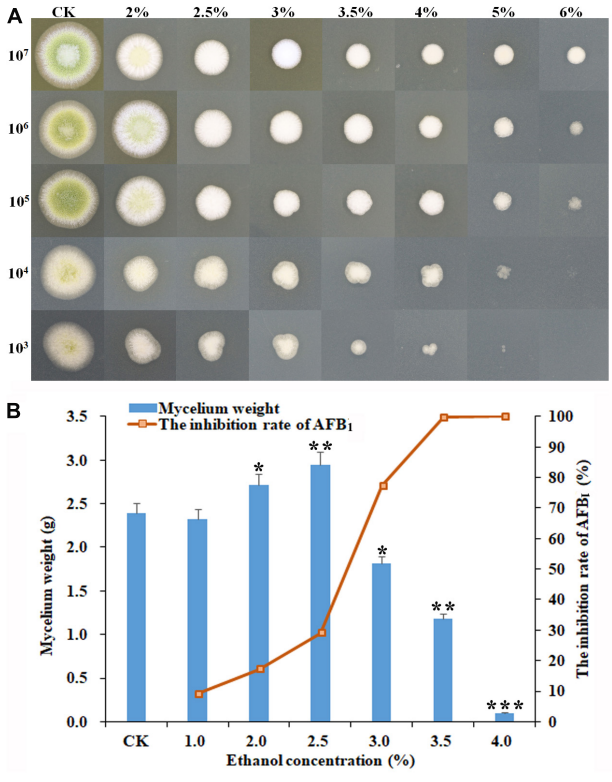

FIGURE 1 | Effect of ethanol on the mycelial growth and AFB_1 production of *A. flavus* NRRL3357. **(A)** The inhibitory effect of ethanol at different concentrations (from 0 to 6%) on mycelial colonies on PDA plates by inoculating the serial dilutions of *A. flavus* conidia (from 10^7 to 10^3) at 48 h post-treatment. **(B)** The mycelia biomass of *A. flavus* and the inhibition rate of AFB_1 in YES broth at 120 h post-treatment. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Overall Transcriptional Response Profile of *A. flavus* to Ethanol

To decipher the potential inhibitory mechanism of ethanol on *A. flavus* growth and AFB_1 biosynthesis, a transcriptome analysis was carried out. Via RNA-seq, averagely 47.81 million, 46.01 million, and 49.49 million raw reads were generated from control, 2.5 and 3.5% of ethanol treatment samples, respectively. After filtering, 46.30 million, 44.85 million, and 47.34 million clean reads were obtained, and 96.09, 93.99, and 94.32% of total clean reads from control, 2.5 and 3.5% ethanol group were aligned to reference sequences. Based on the FPKM values with FDR ≤ 0.05 and Log2Ratio ≥ 1 or ≤ -1, 2240 and 2434 differentially expression genes (DEGs) were down-regulated and up-regulated under 2.5% ethanol treatment compared with control. Under 3.5% ethanol treatment, 2636 and 3105 DEGs were down-regulated and up-regulated compared with control, respectively. Compared with 2.5% ethanol, 973 and 1547 DEGs were down-regulated and up-regulated under 3.5% ethanol treatment, respectively.

Functional and Pathway Analysis of DEGs

The DEGs between the ethanol treatment and the control provided an important clue to decipher the molecular mechanism of action of ethanol on fungal growth and AFB_1 production. The functions, metabolic pathways and interactions of these DEGs were analyzed using GO and KEGG enrichment analysis. **Figure 2A** showed the top 30 enriched functional categories of 2240 down-regulated DEGs in *A. flavus* treated with 2.5% ethanol. Therein, cellular protein metabolic process, organonitrogen compound metabolic process, organonitrogen compound biosynthetic process, etc. were obvious enrichment terms in the biological process. Adenyl nucleotide binding, adenyl ribonucleotide binding, ATP binding, etc. were the main terms in molecular function. For the up-regulated DEGs in the 2.5% ethanol group (**Figure 2B**), carbohydrate metabolic process, phosphorus metabolic process, phosphate-containing compound metabolic, etc. were the predominant terms belonging to the biological process. The significant enrichment terms in the molecular function were hydrogen ion transmembrane transporter activity, monovalent inorganic cation transmembrane transporter activity, cation transmembrane transporter activity, etc. For the down-regulated

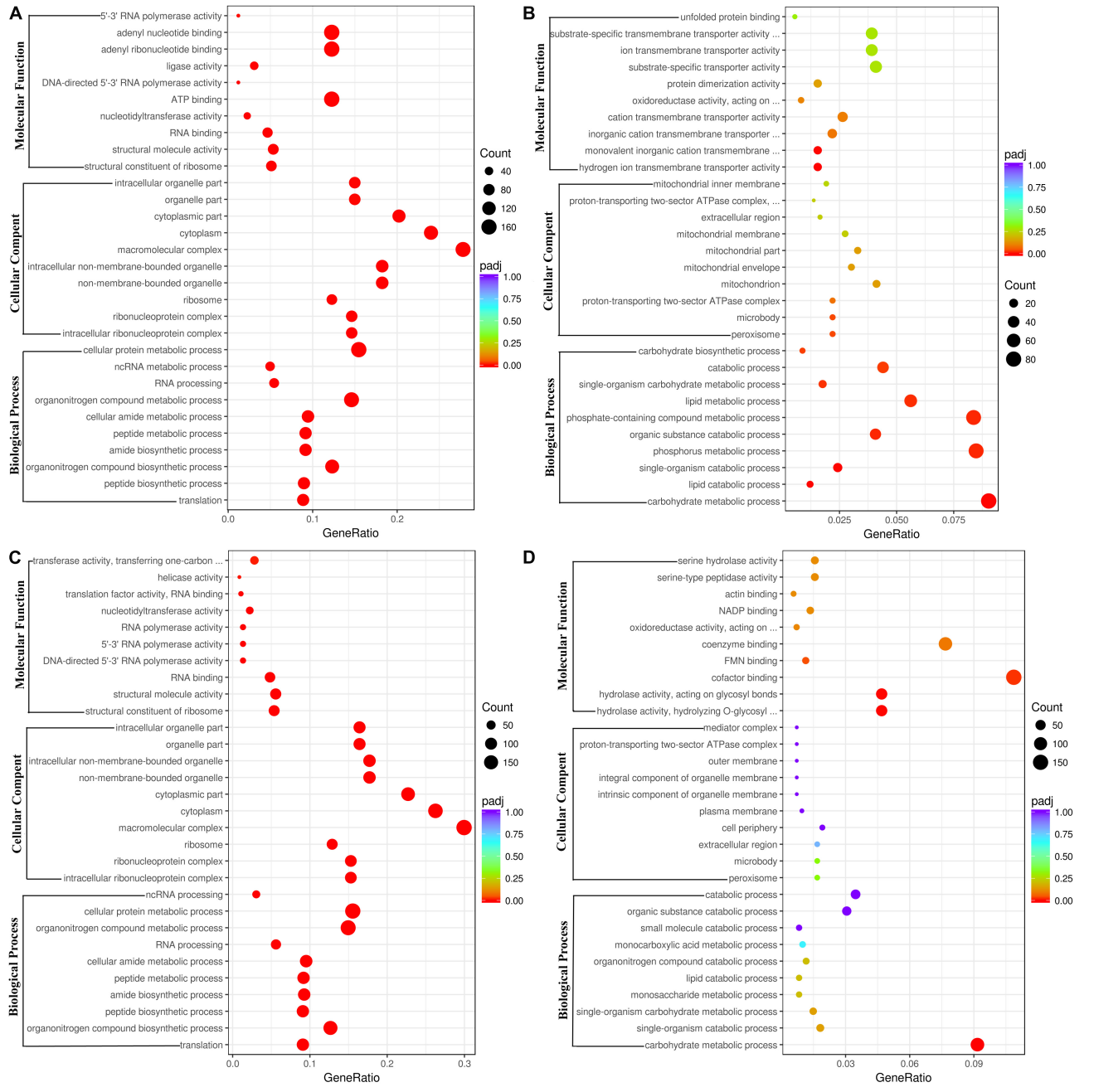

FIGURE 2 | Go functional classification of down-regulated **(A,C)** and up-regulated **(B,D)** DEGs. **(A,B)** The ordinate means with 2.5% ethanol treatment. **(C,D)** The ordinate means with 3.5% ethanol treatment. The size of the plot represents the number of DEGs in one GO term; the color of the plot close to red represents more significant enrichment.

DEGs in the 3.5% ethanol group (**Figure 2C**), cellular protein metabolic process, organonitrogen compound metabolic process, and organonitrogen biosynthetic process were the most abundant in the biological process. Structural constituent of ribosome, structural molecule activity, and RNA binding were the most abundant in the molecular function. For the up-regulated DEGs in this group (**Figure 2D**), carbohydrate metabolic process, single-organism catabolic process, and single-organism carbohydrate metabolic process were the main terms belonging to the biological process. Hydrolase activity, cofactor binding, FMN binding, etc. were the main enrichment terms in molecular function.

In *A. flavus* treated with 2.5% ethanol, the top 20 enriched KEGG pathway were shown in **Figures 3A,B**. For the down-regulated DEGs, the most abundant genes (83 DEGs) were enriched in ribosome (afv03010), and 54, 50, and 50 DEGs were

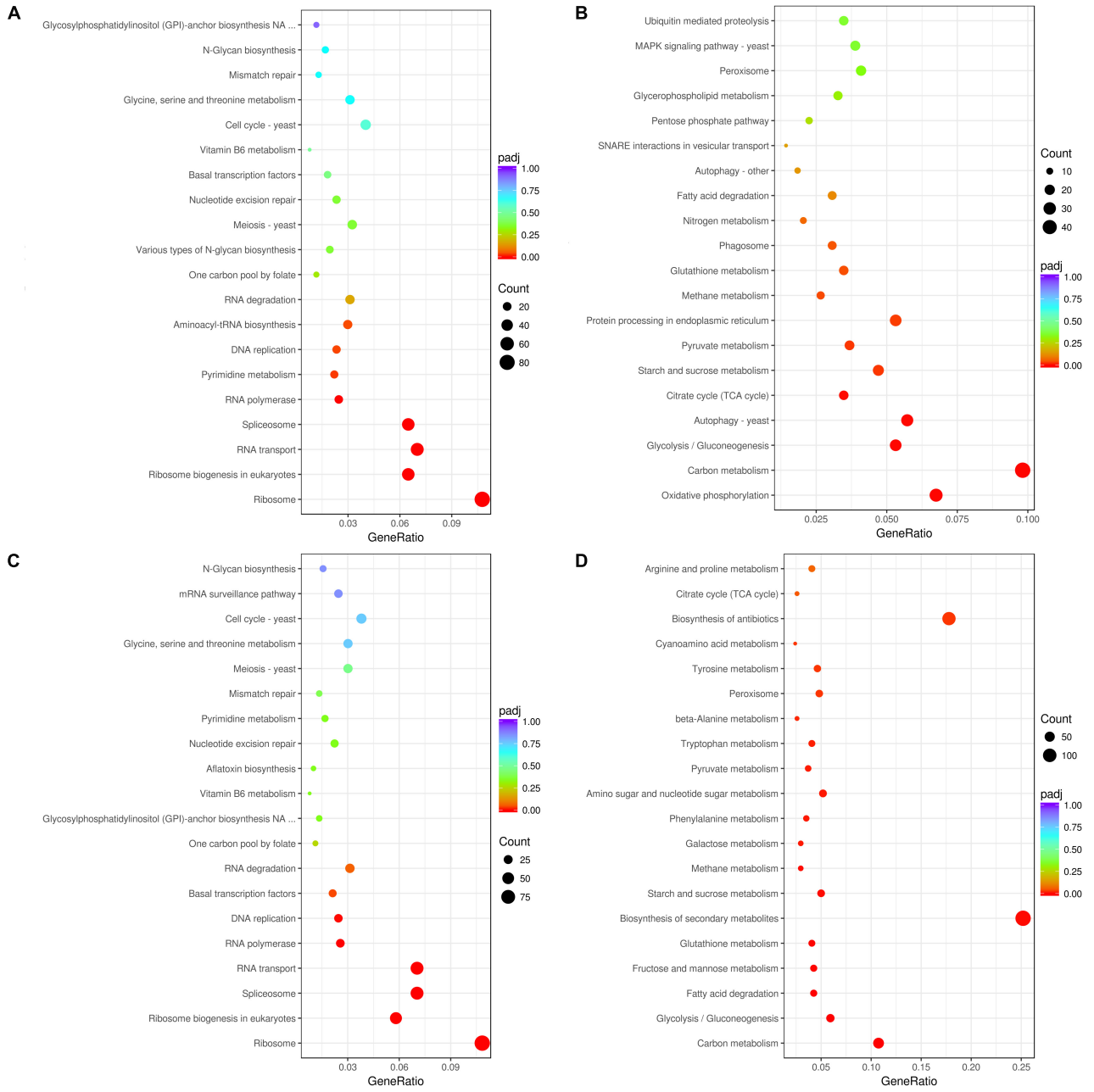

FIGURE 3 | KEGG enrichment of down-regulated **(A,C)** and up-regulated **(B,D)** DEGs. **(A,B)** The ordinate means with 2.5% ethanol treatment. **(C,D)** The ordinate means with 3.5% ethanol treatment. The size of the plot represents the number of DEGs in one GO term; the color of the plot close to red represents more significant enrichment.

enriched in RNA transport (afv03013), ribosome biogenesis (afv03008), and spliceosome (afv03040), respectively. For the up-regulated DEGs (**Figure 3B**), the most abundant genes (48 DEGs) were enriched in carbon metabolism (afv01200), and 33, 28, 26, and 26 DEGs were enriched in oxidative phosphorylation (afv00190), autophagy-yeast (afv04138), glycolysis/gluconeogenesis (afv00010), and protein processing in endoplasmic reticulum (afv04141), respectively. For the 3.5% ethanol group, the most abundant down-regulated DEGs (**Figure 3C**) were enriched in ribosome (afv03010, 97 DEGs), and 63, 63, and 52 DEGs were enriched in spliceosome (afv03008), RNA transport (afv03013), and ribosome biogenesis in eukaryotes (afv03008), respectively. The most abundant up-regulated DEGs (**Figure 3D**) were enriched in biosynthesis of secondary metabolites (afv01110, 136 DEGs), and 96, 58, and 32 DEGs were enriched in biosynthesis of antibiotics (afv01130), carbon metabolism (afv01200), and glycolysis/gluconeogenesis (afv00010), respectively.

Expression Analysis of Pigment (#10), Aflatrem (#15), Aflatoxin (#54), and Cyclopiazonic Acid (#55) Biosynthesis Genes in Response to Ethanol

As shown in **Table 1**, in pathway #10, AFLA_016120 encoding an O-methyltransferase family protein and AFLA_016130 were down-regulated by 2.5% ethanol, but all three genes in this pathway were up-regulated by 3.5% ethanol. In pathway #15, the expression levels of most genes were very low except for AFLA_045450. In pathway #55, AFLA_139470 encoding a FAD-dependent oxidoreductase, AFLA_139480 encoding a tryptophan dimethylallyl transferase, and AFLA_139480 encoding a hybrid PKS/NRPS enzyme were down-regulated by 2.5% ethanol, while AFLA_139460 coding a MFS multidrug transporter was up-regulated. Under 3.5% ethanol treatment, four genes in pathway #55 were all down-regulated. In AF pathway #54, *aflLa* (a similar hypothetical gene of *aflL*), and *aflG* were up-regulated by 2.5% ethanol, while *aflYd* and *aflYb* (*aflYa-e* are genes in sugar cluster and the last letters indicate the sequence of genes in the cluster) were down-regulated. The expression changes of other genes in pathway #54 were slight after 2.5% ethanol treatment. Interestingly, all of AF cluster genes were down-regulated by 3.5% ethanol except for *aflC*. The two key regulator genes *aflR* and *aflS* were both down-regulated by 3.5% ethanol compared to the control with $\log_2$FC values −1.31 and −1.73, respectively. For the structural genes, the expression of *aflK* and *aflLa* was completely inhibited, and *aflV*, *aflP*, *aflO*, *aflL*, and *aflM* were markedly down-regulated with $\log_2$FC values ≤ -10, and *aflY*, a*flX*, *aflW*, *aflQ*, *aflI*, *aflG*, *aflN*, *aflMa*, *aflE*, and *aflJ* were down-regulated with $\log_2$FC values ≤ -5. It is worth mentioning that *aflY(a–d)* genes belong to the sugar cluster and most of them appear to be more down-regulated when 2.5% ethanol was applied. However, the *aflYa* gene encoding NADH oxidase was significantly down-regulated by 3.5% ethanol, while the other four genes did not change significantly.

The RNA-seq results were confirmed by analyzing the expression of AF cluster genes in *A. flavus* treated with 3.5% ethanol using qRT-PCR method. As shown in **Figure 4**, the expression mode of these genes was consistent with the RNA-seq data.

Genes Involved in the Development

The transcription levels of genes involved in development are shown in **Supplementary Table S1**. From the expression profile data, we found that some genes involved in conidiophores development including *FlbA*, *FlbC*, *FlbD*, and *HymA* were down-regulated by 2.5 and 3.5% ethanol. For the velvet complex, *VeA* was up-regulated by 2.5% ethanol, but was down-regulated by 3.5% ethanol. *FluG* (AFLA_039530) and *VosA* were down-regulated by 2.5 and 3.5% ethanol. However, *LaeA* did not show a significant differential expression with ethanol treatment. *AbaA* controlling phialide differentiation, development regulator *Mod-A* (AFLA_009340), and conidial hydrophobin *RodB* were down-regulated by 2.5 and 3.5% ethanol. The *BrlA* mediating conidiophores was up-regulated by 3.5% ethanol.

Genes Involved in Fungal Oxidative Stress

The expression levels of genes involved in oxidative stress response are shown in **Supplementary Table S2**. The catalase/peroxidase/superoxide dismutase genes were all significantly modulated by ethanol. The expression of *Cat1*, *Cat2*, *CatA*, and *sod1* were up-regulated by 2.5 and 3.5% ethanol while mnSOD was down-regulated. The transcriptional levels of *Cat* were down-regulated by 2.5% ethanol, but were up-regulated by 3.5% ethanol. The bZIP transcription factor *ap-1* and two C_2H_2 transcription factors *msnA* and *mtfA* were up-regulated by 2.5 and 3.5% ethanol. However, the stress response transcription factor *srrA* was down-regulated by 2.5 and 3.5% ethanol. The MAP kinase *sakA* gene was obviously down-regulated by 2.5 and 3.5% ethanol. The transcriptional level of fatty acid oxygenase *ppoA* was down-regulated by 2.5 and 3.5% ethanol, but *ppoC* was up-regulated. Meantime, *ppoB* was expressed at a very low level. The expression of GPCRs *gprC*, *gprH*, *gprM*, *gprR*, and *gprS* was down-regulated by 2.5 and 3.5% ethanol, while that of *gprD* and *gprG* was up-regulated. The transcriptional level of *gprK* was down-regulated by 2.5% ethanol, but was up-regulated by 3.5% ethanol.

Genes Involved in Metabolism of Ethanol

The expression levels of genes involved in metabolism of ethanol are shown in **Figure 5**. After treatment with 3.5% ethanol, most of the genes involved in the metabolism of ethanol were up-regulated except for the two alcohol dehydrogenase genes, AFLA_016380 and AFLA_138950, involved in the process converting ethanol to acetaldehyde and the acetate and CoA ligase gene AFLA_027070 involved in the conversion of acetate to acetyl-CoA. The four alcohol dehydrogenase genes AFLA_085950, AFLA_048690, AFLA_073680, and AFLA_0133830 were up-regulated by 3.5% ethanol with Log_2FC values of 2.94, 1.48, 2.82, and 1.54, respectively. The two aldehyde dehydrogenase *AldA* genes were up-regulated by 3.5% ethanol with Log_2FC values of 2.33 and 1.69, respectively. The NADPH flavin oxidoreductase gene AFLA_077220 and P450 family fatty acid hydroxylase AFLA_085490 involved in the conversion of fatty acid to α-hydroxy fatty acid were up-regulated by 3.5% ethanol with Log_2FC of 1.65 and 1.86, respectively.

DISCUSSION

AF biosynthesis needs more than 23 enzymatic reactions (Cleveland et al., 2009). In *A. flavus*, the genes encoding these enzymes are located in an AF pathway gene cluster and are regulated by AFLR and AFLS (Bhatnagar et al., 2003; Cleveland et al., 2009). In our RNA-Seq data, the transcriptional level changes of the AF cluster genes were stronger in *A. flavus* treated with 3.5% ethanol compared to 2.5% ethanol. Of 30 AF cluster genes, the expression of 27 genes was significantly down-regulated by 3.5% ethanol except for *aflA*, *aflC*, and *aflU*. It is important to notice that the two key regulator genes *aflR* and *aflS* were both down-regulated by 3.5% ethanol, together with the down-regulation of the structural genes in the cluster. The

TABLE 1 | Transcriptional activity of genes in the biosynthesis of conidial pigment (#10), aflatrem (#15), aflatoxin (#54), and cyclopiazonic acid (#55).

Cluster ID	Gene ID (AFLA_x)	CK* (FPKM)	E2.5* (FPKM)	E3.5* (FPKM)	E2.5* Log	E3.5* Log	Annotated_gene_function
10	AFLA_016120	6.91	3.54	15.79	−0.95	1.15	O-methyltransferase family protein
10	AFLA_016130	4.02	1.64	8.55	−1.29	1.05	Hypothetical protein
10	AFLA_016140	25.18	29.44	98.18	0.23	1.92	Conidial pigment biosynthesis scytalone dehydratase Arp1
15	AFLA_045450	26.95	38.23	45.50	0.51	0.72	Ankyrin repeat-containing protein, putative
15	AFLA_045460	0.63	1.56	3.25	1.32	2.28	Hypothetical protein
15	AFLA_045470	0.05	0.03	0.12	−0.65	1.23	Non-sense-mediated mRNA decay protein, putative
15	AFLA_045480	0.00	0.00	0.09	/	/	Conserved hypothetical protein
15	AFLA_045490	0.09	0.02	0.26	−1.91	1.55	Dimethylallyl tryptophan synthase, putative
15	AFLA_045500	0.24	0.18	0.89	−0.35	1.88	Cytochrome P450, putative
15	AFLA_045510	0.13	0.19	0.23	0.54	0.77	integral membrane protein
15	AFLA_045520	0.06	0.00	0.09	/	/	Integral membrane protein
15	AFLA_045530	0.10	0.21	0.33	1.08	1.73	Conserved hypothetical protein
15	AFLA_045540	0.03	0.21	0.20	2.89	2.77	Cytochrome P450, putative
15	AFLA_045550	0.86	0.10	0.41	−3.18	−1.08	Hypothetical protein
15	AFLA_045560	2.67	0.48	0.85	−2.48	−1.69	Carboxylic acid transport protein
15	AFLA_045570	0.62	15.47	1.71	4.64	1.41	Acetyl xylan esterase, putative
54	AFLA_139100	1.14	0.61	1.84	−0.89	0.66	*aflYe/orf*/Ser-Thr protein phosphatase family protein
54	AFLA_139110	0.86	0.38	0.77	−1.14	−0.19	*aflYd/sugR*/sugar regulator
54	AFLA_139120	1.02	0.62	1.17	−0.72	0.16	*aflYc/glcA*/glucosidase
54	AFLA_139130	5.30	2.15	3.57	−1.30	−0.61	*aflYb/hxtA*/putative hexose transporter
54	AFLA_139140	14.65	16.49	0.33	0.18	−5.48	*aflYa/nadA*/NADH oxidase
54	AFLA_139360	80.84	81.82	33.67	0.02	−1.31	*aflR/apa-2/afl-2/*transcription activator
54	AFLA_139340	116.22	66.04	35.10	−0.81	−1.78	*aflS*/pathway regulator
54	AFLA_139150	60.40	61.41	0.74	0.03	−6.39	*aflY/hypA/hypP/*hypothetical protein
54	AFLA_139160	104.46	63.29	2.85	−0.72	−5.23	*aflX/ordB*/monooxygenase/oxidase
54	AFLA_139170	56.80	49.75	0.50	−0.19	−6.86	*aflW/moxY*/monooxygenase
54	AFLA_139180	54.39	69.01	0.04	0.35	−10.28	*aflV/cypX*/cytochrome P450 monooxygenase
54	AFLA_139190	38.21	51.44	0	0.43	Down	*aflK/vbs*/VERB synthase
54	AFLA_139200	6.99	8.62	0.01	0.31	−9.58	*aflQ/ordA/ord-1*/oxidoreductase/cytochrome P450 monooxigenase
54	AFLA_139210	25.03	38.34	0.01	0.62	−10.92	*aflP/omtA/omt-1*/O-methyltransferase A
54	AFLA_139220	52.83	43.14	0.03	−0.29	−10.83	*aflO/omtB/dmtA*/O-methyltransferase B
54	AFLA_139230	5.24	9.54	0.03	0.87	−7.17	*aflI/avfA*/cytochrome P450 monooxygenase
54	AFLA_139240	20.69	47.89	0	1.22	Down	*aflLa/hypB*/hypothetical protein
54	AFLA_139250	46.25	52.77	0.03	0.20	−10.53	*aflL/verB*/desaturase/P450 monooxygenase
54	AFLA_139260	13.18	32.24	0.07	1.29	−7.40	*aflG/avnA/ord-1*/cytochrome P450 monooxygenase
54	AFLA_139270	744.25	461.76	51.46	−0.68	−3.90	*aflNa/hypD*/hypothetical protein
54	AFLA_139280	23.45	19.62	0.24	−0.25	−6.57	*aflN/verA*/monooxygenase
54	AFLA_139290	140.71	177.05	0.35	0.34	−8.66	*aflMa/hypE*/hypothetical protein
54	AFLA_139300	479.94	507.79	0.09	0.09	−12.46	*aflM/ver-1*/dehydrogenase/ketoreductase
54	AFLA_139310	104.67	119.71	0.78	0.20	−7.10	*aflE/norA/aad/adh-2*/NOR reductase/dehydrogenase
54	AFLA_139320	169.61	176.89	4.63	0.07	−5.24	*aflJ/estA*/esterase
54	AFLA_139330	263.26	286.95	9.08	0.13	−4.90	*aflH/adhA*/short chain alcohol dehydrogenase
54	AFLA_139370	25.57	24.00	10.68	−0.09	−1.31	*aflB/fas-1*/fatty acid synthase beta subunit
54	AFLA_139380	7.60	9.94	3.98	0.39	−0.98	*aflA/fas-2/hexA*/fatty acid synthase alpha subunit
54	AFLA_139390	101.40	127.23	4.63	0.33	−4.50	*aflD/nor-1*/reductase
54	AFLA_139400	41.13	73.10	3.82	0.83	−3.47	*aflCa/hypC*/hypothetical protein
54	AFLA_139410	5.11	8.34	5.59	0.71	0.09	*aflC/pksA/pksL1*/polyketide synthase
54	AFLA_139420	82.13	98.69	41.71	0.27	−1.02	*aflT/aflT*/transmembrane protein
54	AFLA_139430	9.30	8.65	8.15	−0.10	−0.23	*aflU/cypA*/P450 monooxygenase
54	AFLA_139440	37.20	29.33	18.42	−0.34	−1.06	*aflF/norB*/dehydrogenase
55	AFLA_139460	659.14	1823.80	260.75	1.47	−1.38	MFS multidrug transporter, putative
55	AFLA_139470	30.56	18.67	7.29	−0.71	−2.11	FAD dependent oxidoreductase, putative
55	AFLA_139480	45.38	23.43	16.66	−0.95	−1.49	Dimethylallyl tryptophan synthase, putative
55	AFLA_139490	0.49	0.11	0.28	−2.17	−0.84	Hybrid PKS/NRPS enzyme, putative

**CK, Control; E2.5, 2.5% ethanol; E3.5, 3.5% ethanol.*

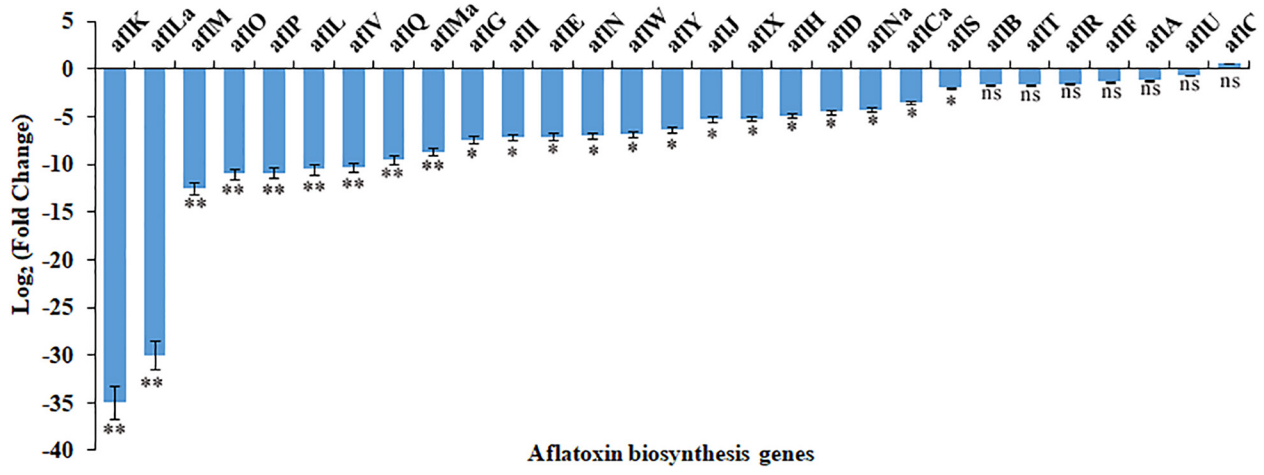

FIGURE 4 | The differential expression of genes in aflatoxin biosynthesis cluster in response to 3.5% ethanol. ns, not significant; *$p < 0.05$; **$p < 0.01$.

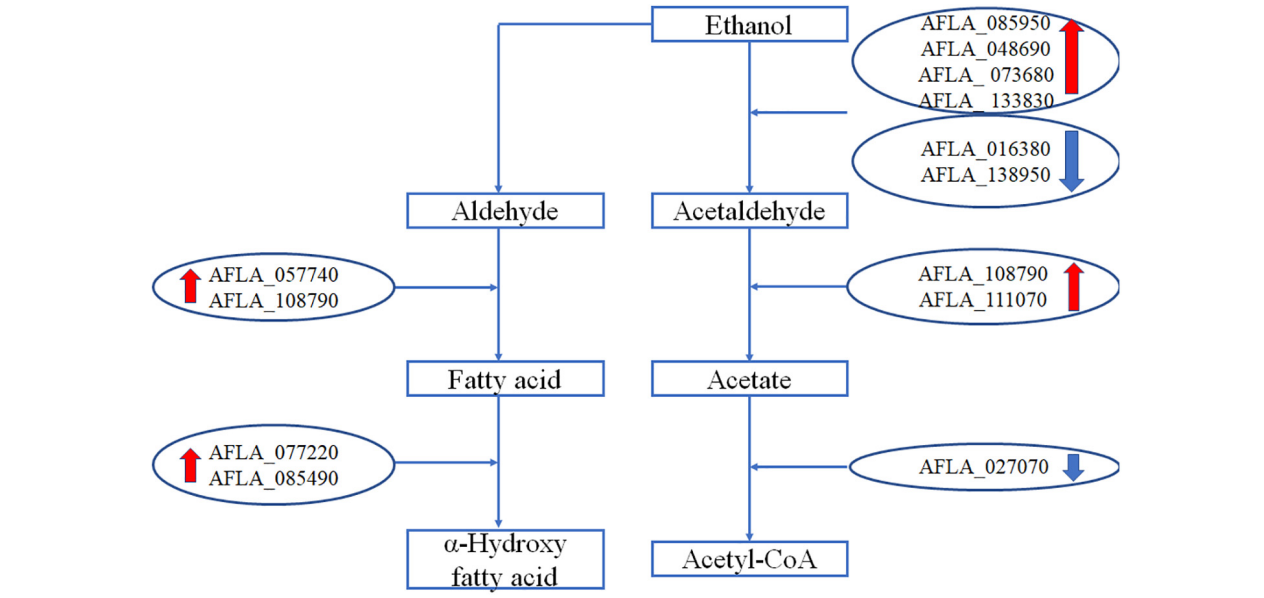

FIGURE 5 | The differential expression of genes involved in metabolism of ethanol.

gene *aflK*, encoding a versicolorin (VERB) synthase involved in conversion of versiconal (VAL) to VERB (McGuire et al., 1996; Silva and Townsend, 1996; Silva et al., 1996), was completely inhibited. This conversion is a critical step in AF biosynthesis because it closes the bifuran ring of AFs, which is a prerequisite for binding to DNA and gives AFs the mode of action as a mutagen (Yu et al., 2004). In addition, the expression of *aflLa/hypB*, a hypothetical protein gene, was also completely inhibited by 3.5% ethanol. Similarly, Lin et al. (2013) found that *aflLa/hypB* was completely inhibited by 5-azacytidine (5-AC), an inactivator of DNA methyltransferase. It was reported that *aflLa/hypB* might be involved in the second oxidation step converting O-methylsterigmatocystin (OMST) to a 7-membered ring lactone, the precursor for AFB_1 formation (Ehrlich, 2009). Our previous study indicated that *aflLa/hypB* was one of the target genes for rapid identification of atoxigenic strains (Wei et al., 2014). These findings suggested that 3.5% ethanol inhibited AF biosynthesis by down-regulating the transcriptional levels of transcriptional factor *aflR*, the cofactor *aflS*, and subsequently most of the structural genes.

As a general cell toxic substance, ethanol affects the integrity of the cell membrane, inactivates cellular enzymes, and destroys protein structure, leading to the inhibition of fungal growth, viability, and conidia formation (Ma et al., 2019). In addition, ethanol triggered internal cellular perturbations like irregular nuclei and the aggregation of scattered vacuoles in fungal cells. The abovementioned disorders of cellular functions in turn could lead to the reduction of AFs biosynthesis. Moreover,

ethanol also influenced the transcription levels of some global regulator factors. The velvet family proteins VeA, VelB, and LaeA of *A. flavus* form a heterotrimeric velvet complex to coordinate sexual development and biosynthesis of several secondary metabolites in the dark (Bayram et al., 2008; Chang et al., 2013). The coordinating and balanced interactions among the velvet family proteins together with FluG play a key role in maintaining programmed AFs biosynthesis and conidiation and sclerotial production (Chang et al., 2013). After treatment with 3.5% ethanol, the expression of *veA* and *fluG* was significantly down-regulated with Log2FC −2.97 and −4.03, respectively. The down-regulation of *veA* suppressed the expression of several key AFs genes including *aflR*, *aflD*, *aflM*, and *aflP* and resulted in the inhibition of AF biosynthesis (Duran et al., 2007).

The oxidative stress was recognized as a prerequisite for AFs formation in *A. flavus* and *A. parasiticus* (Reverberi et al., 2008; Zhang et al., 2015; Lv et al., 2018; Guan et al., 2019). In the meantime, AFs biosynthesis is thought to protect the fungus against oxidative stress (Wang et al., 2019). Several previous studies have indicated that some AFs inhibitors can regulate the stress response system of fungi (Reverberi et al., 2005; Grintzalis et al., 2014; Sun et al., 2015; Caceres et al., 2017). After treatment with 3.5% ethanol, all catalase genes including *Cat*, *Cat1*, *Cat2*, *CatA*, and Cu, Zn superoxide dismutase gene *sod1* were up-regulated, while only Mn superoxide dismutase gene *mnSOD* was down-regulated. Similarly, piperine exposure significantly induced decreased expression of *veA* together with the overexpression of several bZIP transcription factors genes like *atfA*, *atfB*, and *ap-1* and genes encoding catalase such as *catA*, *cat2*, and superoxide dismutase like *sod1* in *A. flavus* (Caceres et al., 2017). Moreover, this gene response was coupled with an obvious increase of catalase enzymatic activity (Caceres et al., 2017). Cinnamaldehyde exposure resulted in the up-regulation of several transcription factors genes like *srrA*, *msnA*, and *atfB* and genes encoding catalase like *cat*, *cat1*, *catA*, and superoxide dismutase including *sod1* and *mnSOD* (Wang et al., 2019).

The transcriptional levels of genes involved in the antioxidant system were modulated by the upstream transcription factors including *ap-1*, *atfA*, *atfB*, *msnA*, *mtfA*, and *PacC* (Hong et al., 2013). As a redox-state sensor protein, the functions of Ap-1 are highly conserved in yeast, fungi, and mammals (Toone et al., 2001; Caceres et al., 2017). In fungi, the *N*- and *C*-terminal cysteine-rich domains of Ap-1-like protein might act as a sensor target of reactive oxygen species (ROS) like H_2O_2 (Sies, 2014). In *A. parasiticus*, the deletion of *ApyapA* causes the increase of AFs biosynthesis, oxidative stress, premature conidiogenesis, and an earlier transcription of AFs cluster genes like *aflR* and *aflE* (Reverberi et al., 2008; Caceres et al., 2017). The bZIP transcription factor SrrA, an ortholog of *Saccharomyces cerevisiae* Skn7 and *Saccharomyces pombe* Prr1, mediates cellular response to environmental stimuli (Hagiwara et al., 2007; Vargas-Perez et al., 2007). In *A. parasiticus*, Hong et al. (2013) identified a recognition site of SrrA in promoters of the antioxidant genes *cat1* and *mnsod*, and AFs biosynthetic genes *aflB* (*fas-1*) and *aflM* (*ver-1*). Moreover, the adjacent binding sites of SrrA and AP-1 in the promoter suggest that they can interact and are involved in the transcriptional regulation of AFs genes (Hong et al., 2013). In the present study, an up-regulation of *ap-1* and a down-regulation of *srrA* were observed upon 3.5% ethanol addition. MsnA is a C_2H_2 zinc finger transcription factor and can respond to some cellular stress such as oxidative stress, carbon starvation, heat shock, and osmotic stress (Martinez-Pastor et al., 1996; Hong et al., 2013). In *A. flavus* and *A. parasiticus*, disruption of *msnA* led to increased AFs biosynthesis and the production of conidia, ROS, and kojic acid, although fungal growth was inhibited (Chang et al., 2011). In addition, *msnA* deletion down-regulated transcription levels of genes encoding antioxidant enzymes, which protect fungus against ROS (Hong et al., 2013). Our previous studies revealed that eugenol and cinnamaldehyde up-regulated the expression of *msnA* and inhibited AFs biosynthesis (Lv et al., 2018; Wang et al., 2019). A similar finding, the up-regulation of *msnA* in *A. flavus* treated with 3.5% ethanol, was obtained in the present study. MtfA is another C_2H_2 zinc finger transcription factor, which was originally identified in *Aspergillus nidulans* and was involved in sterigmatocystin (ST) regulation (Ramamoorthy et al., 2013). The disruption and overexpression of *mtfA* both induced the decreased production of ST (Zhuang et al., 2016). In *A. flavus*, overexpression of *mtfA* dramatically reduced AFB_1 production accompanied by a drastic reduction of *aflR* expression compared to the WT strain while deletion of *mtfA* did not significantly influenced AFB_1 production (Zhuang et al., 2016). Caceres et al. (2016) indicated that eugenol up-regulated the expression of *mtfA* and inhibited AFB_1 production. Similarly, the transcription level of *mtfA* was up-regulated by 3.5% ethanol in the present study.

It is important to point out that the transcriptional status is very fluctuating depending on transcription rate and half-life of the mRNA, which may be very short compared to the more accumulative and stable concentration of the AF produced. This means that the transcription may not be directly

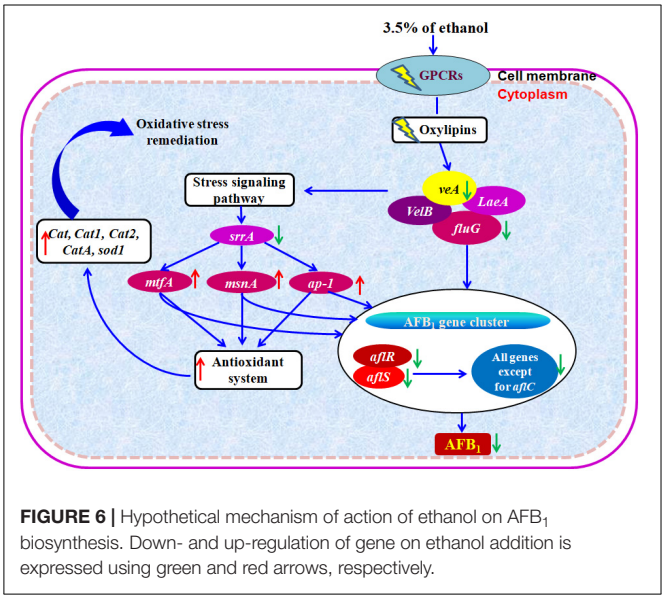

FIGURE 6 | Hypothetical mechanism of action of ethanol on AFB_1 biosynthesis. Down- and up-regulation of gene on ethanol addition is expressed using green and red arrows, respectively.

correlated with the amount of AF produced at each time point. Therefore, the following mechanism of action of ethanol on the inhibition of AFs proposed in this study is based on the RNA-seq data on the 7th day.

Based on the abovementioned results, we proposed a hypothetical mechanism of action of ethanol on the inhibition of AFs (**Figure 6**). Taken together, the enhanced transcription levels of the stress response system, such as bZIP transcription factor *ap-1*, C_2H_2 transcription factors *msnA* and *mtfA*, the down-regulation of stress response transcription factor *srrA*, and the overexpression of genes encoding for antioxidant system including catalase genes and superoxide dismutase gene in *A. flavus* treated with ethanol, significantly down-regulate the expression of AF biosynthesis genes and in turn result in the inhibition of AFs production.

CONCLUSION

In the present study, we reveal the transcription modulation mechanism behind ethanol's AFB_1-repressing action using an RNA-Seq. The RNA data indicated that (1) with ethanol treatment, AFB_1 cluster genes were dramatically down-regulated following the up-regulation of their specific regulators *aflS*/*aflR*; (2) ethanol's mechanism of action involved the down-regulation of the global regulator *veA* and *fluG*; (3) ethanol's transcription modulation mechanism involved the decreased expression of stress response transcription factor *srrA* together with overexpression of bZIP transcription factor *ap-1* and C_2H_2 transcription factors *msnA* and *mtfA*; (4) ethanol induced enhanced levels of anti-oxidant enzymatic genes including *Cat*, *Cat1*, *Cat2*, *CatA*, and Cu, Zn superoxide dismutase gene *sod1*. In conclusion, these results strongly suggest that ethanol inhibits AFB_1 biosynthesis by *A. flavus* via enhancing fungal oxidative stress response.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA558521.

AUTHOR CONTRIBUTIONS

FX and QY conceived and designed the experiments. YR, JJ, and MZ performed the experiments. YR and FX analyzed the data and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02946/full#supplementary-material

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Requirement of LaeA, VeA, and VelB on Asexual Development, Ochratoxin A Biosynthesis, and Fungal Virulence in *Aspergillus ochraceus*

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Aspergillus ochraceus is reported to be the major contributor of ochratoxin A (OTA), classified as one of the possible human carcinogen (group 2B) by the International Agency for Research on Cancer. The heterotrimeric velvet complex proteins, LaeA/VeA/VelB, have been most studied in fungi to clarify the relation between light-dependent morphology and secondary metabolism. To explore possible genetic targets to control OTA contamination, we have identified laeA, veA, and velB in *A. ochraceus*. The loss of *laeA*, *veA*, and *velB* yielded mutants with differences in vegetative growth and conidial production. Especially, ΔlaeA almost lost the ability to generate conidiaphore under dark condition. The deletion of *laeA*, *veA*, and *velB* drastically reduced the production of OTA. The wild-type *A. ochraceus* produced about 1 and 7 μg/cm^2 OTA under light and dark conditions on media, whereas the three gene deletion mutants produced less than 20 ng/cm^2 OTA, which was correlated with a down regulation of OTA biosynthetic genes. Pathogenicity studies of ΔlaeA, ΔveA, and ΔvelB showed their reduction in disease severity in pears. Furthermore, 66.1% of the backbone genes in secondary metabolite gene cluster were significantly regulated, among which 81.6% were downregulated. Taking together, these results revealed that velvet complex proteins played crucial roles in asexual development, secondary metabolism, and fungal virulence in *A. ochraceus*.

Keywords: *Aspergillus ochraceus*, ochratoxin A, LaeA, VeA, VelB, secondary metabolism, development, virulence

INTRODUCTION

Ochratoxin A (OTA) is the secondary metabolite of *Aspergillus* and *Penicillium* species (Wang et al., 2016a,b). That poses a serious health hazard according to its mycotoxic properties (Taniwaki et al., 2018). It is classified as a possible human carcinogen (group 2B) by the International Agency for Research on Cancer (IARC, 1993). OTA was first isolated from *A. ochraceus* in 1965 (van der Merwe et al., 1965). And it was reported to be the major contributor of OTA in cereal, *Zea mays*, coffee, fruits, and beverage (Mantle, 2002).

The biosynthetic pathway of OTA has been extensively studied in the past decades (William and Hamilton, 1979; Wang et al., 2015; Gallo et al., 2017; Geisen et al., 2018). Wang has identified a conserved OTA biosynthetic gene cluster by comparatively analysis of six OTA-producing fungi and clarified its biosynthetic pathway by deletion mutants of four structural genes (*otaA*, *B*, *C*, and *D*) and one regulatory gene (*otaR1*) (Wang et al., 2018a,b). Environmental factors are crucial to regulation of OTA production (Selouane et al., 2009; Abarca et al., 2019). The mechanism of OTA biosynthesis is very complex and acts at different levels. Generally, environmental signals transmit to biosynthetic cluster to activate/repress the production of OTA by global regulators and multiprotein complexes. For example, *Aoyap1*, a transcription factor related to oxidative stress, regulated OTA synthesis by controlling cell redox balance in *A. ochraceus* (Reverberi et al., 2012). The transcriptional factors *AopacC* (Wang et al., 2018a,b) and *hog* (Schmidt-Heydt et al., 2012) that are functionally performed pH signaling and osmotic stress were also involved in the regulatory mechanism of OTA biosynthesis at pH stress and osmotic stress, respectively. The heterotrimeric velvet complex, VelB/VeA/LaeA, has been most studied in fungi to clarify the relation between light-dependent morphology and secondary metabolism. In *A. nidulans*, VeA bridges VelB to LaeA, the nuclear master regulator of secondary metabolism (Bayram et al., 2008). LaeA has also been suggested as an epigenetic regulator for its methyltransferase functions toward amino acid lysine and arginine. Several structure homologous LaeA proteins have been identified in *A. fumigatus* (Bok et al., 2005), *A. oryzae* (Oda et al., 2011), *Cochliobolus heterostrophus* (Wu et al., 2012), *Fusarium oxysporum* (Lopez-Berges et al., 2014), *P. chrysogenum* (Veiga et al., 2012), and *Trichoderma reesei* (Karimi-Aghcheh et al., 2013) and demonstrated profound influence on sporulation capacity, mycelial growth, sclerotia formation, and secondary metabolite production.

Several studies have been conducted to regulate mycotoxin biosynthesis by LaeA. The deletion of *laeA* in *A. flavus* led to the loss of aflatoxin mediated by the expression loss of *aflR*, specific transcription factor in aflatoxin biosynthetic cluster. The conidial production, sclerotia formation, and host colonization were repressed in the ΔlaeA of *A. flavus* (Kale et al., 2008). Deletion of *laeA* and *veA* greatly reduced sporulation and strongly copromised the alternariol and alternariol monomethyl ether production (Estiarte et al., 2016). In *A. carbonarius*, Crespo-Sempere suggested that VeA and LaeA have an important role regulating conidiation and OTA biosynthesis (Crespo-Sempere et al., 2013). The *veA* gene was proven to act as a positive regulator of conidia production, OTA biosynthesis, and oxidative stress tolerance in *A. niger* (Zhang et al., 2018). *A. steynii*, *A. niger*, *P. nordicum*, and *P. verrucosum* were described about their ability to produce OTA response to light (Schmidt-Heydt et al., 2010, 2011). However, comprehensive study about velvet complex regulated OTA biosynthesis responding to light is needed.

There is still limited information regarding to the link of light and OTA biosynthesis and their regulatory mechanism in *A. ochraceus*, except Aziz reported white and UV light affected mycelial growth and OTA production in 1997 (Aziz and Moussa, 1997). Nothing has been reported about the function of velvet complex proteins in *A. ochraceus*. For this purpose, we have identified and deleted the members of velvet complex (laeA, veA, and velB) in *A. ochraceus* and explored their regulatory role in growth morphology, OTA biosynthesis and fungal virulence on pears. Furthermore, we demonstrated how LaeA affects secondary metabolism in *A. ochraceus* at gene expression level.

MATERIALS AND METHODS

Strains and Growth Conditions

The wild type (WT) strain *A. ochraceus* fc-1 used in this study was isolated, characterized, and genome sequenced in our laboratory (Wang et al., 2018a,b). WT and mutant strains were routinely cultured at 28°C under dark condition. For phenotype and gene expression studies, all utilized strains were cultured on potato dextrose agar (PDA, BD DifcoTM, USA) at 28°C. Each strain was cultured on four plates as technical replicates, and each experiment was repeated three times as biological replicates.

Phylogenetic Tree and Functional Analysis

LaeA, VeA, and VelB amino acid sequences from *A. nidulans* (Bayram et al., 2008), *A. flavus* (Kale et al., 2008) and *Cochliobolus heterostrophus* (Wu et al., 2012) were used as queries, and basic local alignment search tool algorithm was used to search LaeA, VeA, and VelB from the genome of *A. ochraceus*, *A. niger*, *A. welwitschiae*, *A. lacticoffeatus*, *A. sclerotioniger*, *A. steynii*, and *P. nordicum* from the National Center for Biotechnology Information resources (NCBI). The amino acid sequences of LaeA were aligned by MUSCLE, and a maximum likelihood phylogeny was constructed by treeBeST using 1,000 bootstrap replicates.

Generation of Gene Deletion Mutants

To construct *laeA*, *veA*, and *velB* mutants, previous approach reported in our group was used, and the deletion cassettes were generated by overlap PCR procedures (Wang et al., 2018a,b). Primers utilized in this study were listed in **Supplementary Table S1**. And then fusion PCR products were transformed into the protoplasts of *A. ochraceus*. Transformants were verified by Southern blotting. Briefly, approximately 20 μg genomic DNA of each sample was complete-digested and separated 1% agarose gel and transferred to a Hybond-N+ nylon membrane (GE healthcare, UK). After alkali denaturation and neutralization, hybridization was detected with digoxigenin-labeled probes using DIG high-prime DNA labeling and detection starter Kit II (Roche, Basel, Switzerland) according to the instructions of the manufacturer. Primers for probe amplification were listed in **Supplementary Table S1**.

Phenotypic Studies of Mutants

For mutant's growth assessment, PDA plates were inoculated at center with 1 μl of conidia suspension (10^6 conidia/ml) of

each strain and cultures were incubated at 28°C for 9 days under two conditions, white light (Mazda, 23 W CFT/827, 1,485 lm) and darkness. The growth rate was analyzed by measuring the colony diameter of each mutant. For phenotypic study, the hyphae and spores were observed under optical microscope and electron microscope. For further analysis, conidia were collected from six agar plugs (1 cm diameter) from equivalent zones of fungal surface of PDA. The collected samples were homogenized and diluted in 0.1% Tween-80 and counted by a hemocytometer.

Analysis of Ochratoxin A Production

For the investigation of OTA, WT, ΔlaeA, ΔveA, and ΔvelB of *A. ochraceus* were cultured on PDA for 9 days under light and dark conditions. Six agar plugs (1 cm diameter) from equivalent zones of fungal surface of PDA were collected and extracted with 6 ml methanol ultrasonically. Then, the supernatant was filtered through a 0.22 μm filter into a vial. Next, HPLC analysis was performed on an Agilent HPLC system for analyzing the concentration of OTA as previously described method (Wang et al., 2018a,b).

Pathogenicity Assay

Fresh pears (*Pyrus* × *bretschneideri*) were selected to test the pathogenicity of WT and mutant strains of *A. ochraceus in vitro*. The upper surface of pears were disinfected three times with 0.1% sodium hypochlorite (NaClO) for 10 s and rinsed with sterilized water for 30 s. Each pear was punctured by sterilized needle to approximately 2 mm depth to make a wound (2 mm diameter) for inoculation, injected 2 μl conidia suspension (10^6 conidia/ml) in wound, in contrast sterilized water was served as control and incubated at 28°C under dark condition. The diameter of scab was measured after 5 and 9 days.

DNA and RNA Isolation

The mycelium of *A. ochraceus* strains were harvested *via* filtration. Genomic DNA was isolated using a Qiagen DNeasy kit, according to the manufacturer's protocol. For RNA isolation, the *A. ochraceus* mycelium tissues were grown on PDA medium at 28°C for 9 days under light condition. RNA was extracted using TRIZOL reagent (Invitrogen, USA) following the manufacturer's protocol.

Real-Time Polymerase Chain Reaction Analysis and Quantitative Real-Time Polymerase Chain Reaction Analysis

Three biological replicates were performed for each analysis of the relative expression levels. Reverse transcription of 500 ng RNA was performed with a TIANScript II RT Kit (TIANGEN, China). The *A. ochraceus gadph* gene served as an internal standard. Primers for the RT-PCR amplification were listed in **Supplementary Table S2**. The cDNA was analyzed by qRT-PCR using SYBR Premix Ex Taq™ II (TAKARA) on a BIO-RAD CFX96 (BIO-RAD). The *gadph* gene serving as house-keeping gene was used for normalization. The relative expression values were calculated and the expression ratios were quantified using the $2^{-\Delta\Delta Ct}$ method. Primers were listed in **Supplementary Table S3**.

Statistical Analysis

All data were analyzed with IBM SPSS statistics version 20 and presented with the means and standard deviation. The statistical significances among sample groups were calculated with ANOVA and means were compared by least significant difference (LSD) and Duncan's test. The difference was regard to be statistic significant at $p < 0.05$.

RESULTS

Identification, Analysis, and Disruption of LaeA, VeA, and VelB in *A. ochraceus*

In order to identify velvet protein homologs in *A. ochraceus*, the genome sequence of *A. ochraceus* was interrogated using Blast alignment approach. BlastP searches were performed using LaeA, VeA, and VelB amino acid sequences from *A. nidulans*, *A. flavus* and *Cochliobolus heterostrophus* as the probes and the homologs AoFC_03061, AoFC_07220 and AoFC_09406 were identified. LaeA from *A. steynii* (XP_024703593.1), VeA from *A. tanneri* (THC96327.1), and VelB from *A. tanneri* (THC97134.1) were found to be most related to velvet complex proteins in *A. ochraceus*, with the identity of 95.2, 72.5 and 89.6%, respectively. A phylogenetic tree of evolutionary relationship of LaeA proteins from various species including OTA producing fungi was constructed (**Figure 1**), revealing that LaeA was conserved among the *Aspergillus* species. Inactivation of LaeA, VeA, and VelB locus was obtained by homologous replacement of the genes by encoding gene of hygromycin B phosphotransferase (*hygR*). The strategy of mutant generation was shown in **Supplementary Figure S1A**. The isolate resistant to hygromycin B was screened by PCR using primers in marker gene namely *hygR* and outside the knockout cassette (**Supplementary Figure S1B**). At least three transformants of each gene disrupted mutant were obtained from the mutant generation. Southern blot analysis also showed that *ΔlaeA* (**Figure 2A**), *ΔveA* (**Figure 2B**), and *ΔvelB* (**Figure 2C**) lack the target genes (*laeA, veA*, and *velB*).

Involvement of LaeA, VeA, and VelB in Asexual Development, Growth Rate, and Conidiation

A series of difference related to colony morphology, asexual development and conidiation were observed in ΔlaeA, ΔveA, and ΔvelB compared with the WT of *A. ochraceus* on PDA media under light and dark conditions. Under light condition as shown in **Figure 3A**, the WT colonies grew in yellow uniform layer while the laeA deletion mutant grew as a white-yellow cover. We also observed a pigment reduction for ΔlaeA, and a pigment increasing for ΔveA and ΔvelB in the back of the Petri dishes. Under dark condition, the WT *A. ochraceus* showed more pigmentation compared to the light condition. The ΔlaeA grew as a white color for the decrease of spores and pigment (**Figure 3A**). A reduction of conidiophore in ΔlaeA compared with the other strains from the colony edge under dark condition by scanning electron micrograph was observed (**Figure 3B**).

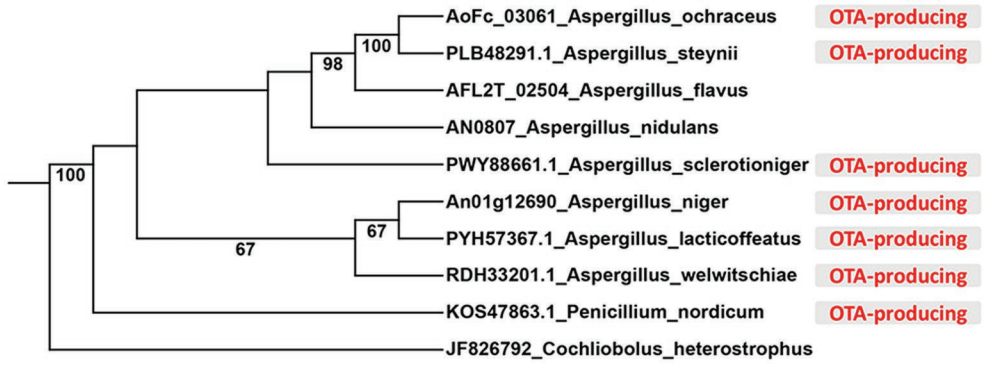

FIGURE 1 | Phylogenetic relationship of LaeA protein from different species. The OTA-producing fungi were marked in red color.

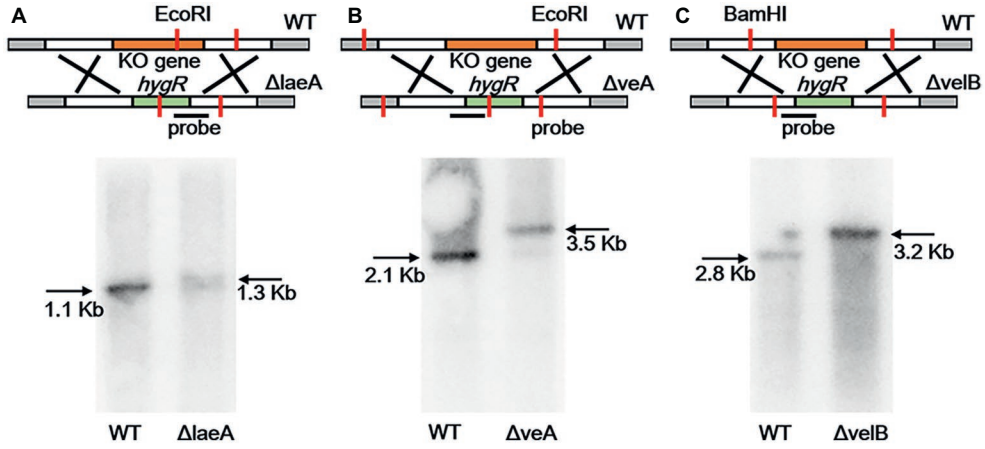

FIGURE 2 | Southern blotting verification of laeA, veA, and velB gene deletion. **(A)** The WT and ΔlaeA isolates were digested with *EcoR*I. A fragment amplified from ΔlaeA was used as the probe. **(B)** The WT and ΔveA isolates were digested with *Eco*RI. A fragment amplified from ΔveA was used as the probe. **(C)** The WT and ΔvelB isolates were digested with *BamH*I. A fragment amplified from ΔvelB was used as the probe.

Light condition had no effect on the growth rate of *A. ochraceus* strains for WT and ΔlaeA, while repressing the growth of ΔveA and ΔvelB ($p < 0.05$). The growth rate was significantly decreased in ΔlaeA, ΔveA and ΔvelB compared with the WT (**Figure 4A**). Mycotoxin-producing fungi caused extensive infestations by generating asexual spores called conidiaspore. To investigate the involvement of LaeA, VeA and VelB in conidiation, the conidiaspore number was counted for strains cultured for 9 days under light and dark conditions. We found conidial generation was increased in the light condition for the *A. ochraceus* strains, although the conidiaspore amount of ΔvelB under light and dark condition demonstrated non-significant difference at statistic level (**Figure 4B**). The deletion of laeA resulted in a drastic reduction of conidial generation, whose inactivation leading to *A. ochraceus* almost loss the ability to generate conidiaspore under dark condition (**Figure 4B**). The conidiaphore amount of ΔlaeA and ΔvelB under light condition demonstrated significant difference compared with the WT. These results indicated the velvet complex proteins (LaeA, VeA and VelB) play important roles in colony phenotype, growth rate and conidiation.

Requirement of LaeA, VeA, and VelB in Ochratoxin A Biosynthesis

In order to investigate whether LaeA, VeA, or VelB is linked to secondary metabolism related to OTA biosynthesis, the crude extractions of *A. ochraceus* of 9-day-old cultures were analyzed by HPLC. The results showed the deletion of *laeA*, *veA*, and *velB* drastically reduced the production of OTA. The WT *A. ochraceus* produced about 1and 7 μg/cm^2 OTA under light and dark condition on media, while the three gene deletion mutants produced less than 20 ng/cm^2 OTA (**Figure 5A**). We observed white light was an inhibitory factor for OTA biosynthesis. To further elucidate the function of LaeA as regulator of OTA biosynthesis, the expression level of genes in the OTA biosynthetic cluster was comparatively examined in WT and ΔlaeA in the dark condition. As shown in **Figure 5B**, the results of qRT-PCR analysis confirmed the expression level of *otaA*, *otaB*, *otaC*, *otaR1*, and *otaD* was downregulated 2–40-fold in ΔlaeA compared to those genes in WT. The upstream gene AoFC_09697 and downstream gene AoFC_09703 showed different expression profiles in WT and also *ΔlaeA* with respect

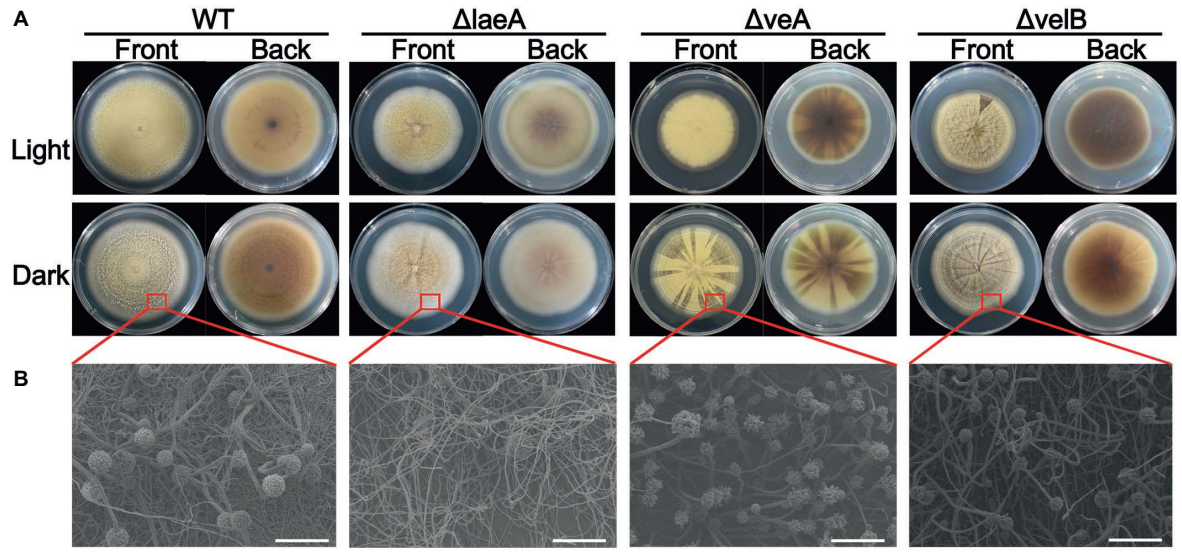

FIGURE 3 | Colony view of the WT, ΔlaeA, ΔveA, and ΔvelB strains of *A. ochraceus*. **(A)** The front and back of *A. ochraceus* colony under light and dark conditions. **(B)** Scanning electron micrograph of *A. ochraceus* strains (scale bar = 200 μm). The red box represented the part of colony for observation.

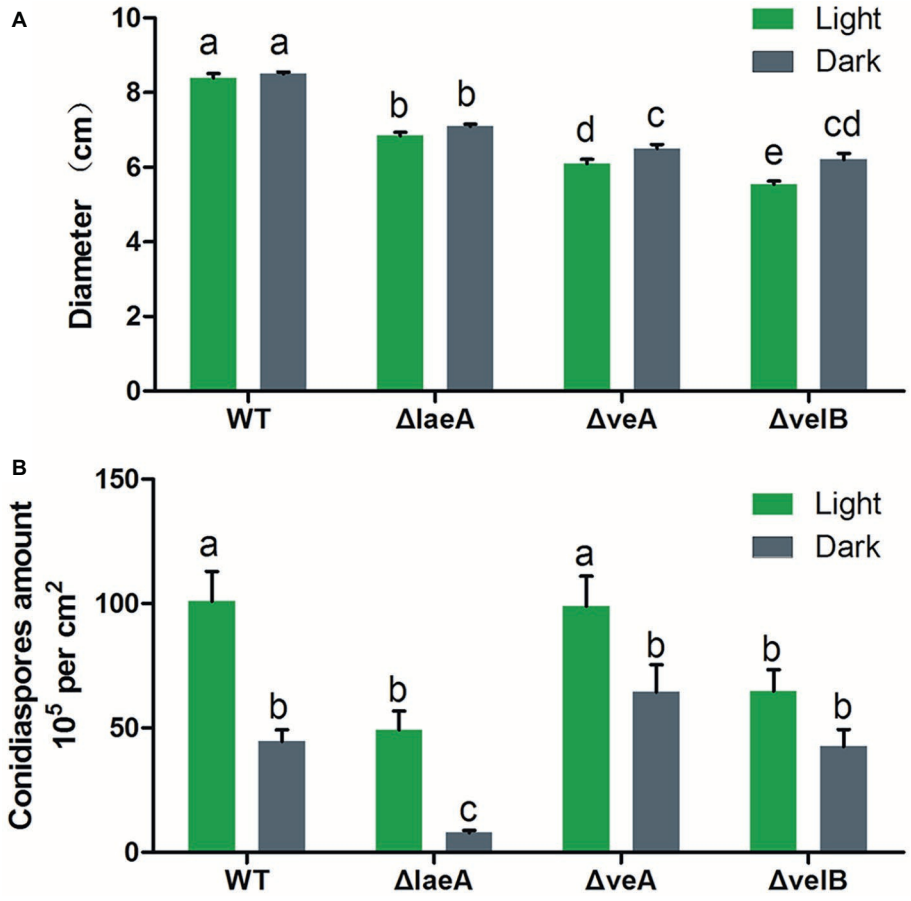

FIGURE 4 | Effect of LaeA, VeA, and VelB deletion on the colony growth and conidiation of *A. ochraceus*. **(A)** Diameter of WT, ΔlaeA, ΔveA, and ΔvelB under light and dark conditions. **(B)** Conidiaspore production of WT, ΔlaeA, ΔveA, and ΔvelB under light and dark conditions. Different letters indicate a significant difference between the corresponding values ($p < 0.05$) with three biological replicates.

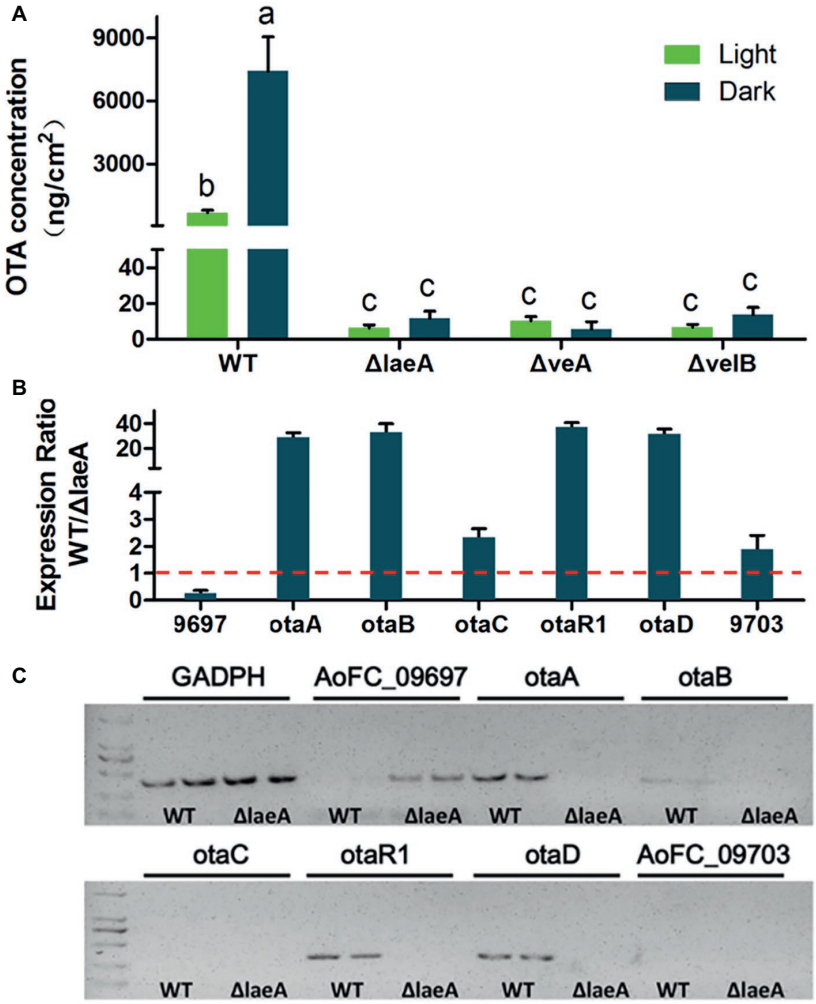

FIGURE 5 | OTA production in WT, ΔlaeA, ΔveA, and ΔvelB of *A. ochraceus*. **(A)** OTA concentration in WT, ΔlaeA, ΔveA, and ΔvelB under light and dark conditions. Different letters indicate a significant difference between the corresponding values ($p < 0.05$) with three biological replicates. **(B)** qRT-PCR was run to check the expression ratio of the genes which are involved OTA biosynthesis and as well as present inside and outside of OTA biosynthetic gene cluster in WT and compared to ΔlaeA mutant. **(C)** RT-PCR amplification of the genes in and out OTA biosynthetic gene cluster.

to the OTA biosynthetic gene. The transcripts of the four OTA biosynthetic genes (*otaA*, *otaB*, *otaD*, and *otaR1*) were detected in WT by amplification by RT-PCR but not in ΔlaeA. The otaC gene was not detected in WT because of its low level of expression (**Figure 5C**). These results were consistent with the production of OTA, which could be detected in WT and could not be detected in ΔlaeA.

Roles of LaeA, VeA, and VelB in Fungal Virulence

The influence of LaeA, VeA, and VelB on the capacity of *A. ochraceus* to infect pears was ascertained. Lesion diameters were measured at 5 and 9 days after infection. After incubation for 5 days, lesions infected by all *A. ochraceus* strains were observed. Obviously, the lesions infected by ΔlaeA, ΔveA, and ΔvelB were repressed when compared with the lesions infected by WT (**Figure 6A**). **Figure 6B** demonstrated the significant difference in statistic level. After incubation for 9 days, the lesion infected by WT obviously increased. Lesions infected by ΔveA and ΔvelB had little change compared with incubation for 5 days. This study illustrated that the loss of velvet proteins would weaken the infection ability of *A. ochraceus* on pear.

LaeA Extensively Regulated Secondary Metabolism in *A. ochraceus*

As earlier reported, the *A. ochraceus* genome contains 99 secondary metabolite biosynthetic gene clusters (Wang et al., 2018a,b). The expression level of backbone genes in secondary metabolites cluster were checked by qRT-PCR (**Figure 7**). About 66.1% of the backbone genes in the cluster were differentially expressed at $p < 0.01$, and 81.6% of the differential expression genes were down-regulated in *laeA* deletion mutant. About 58.6% of the backbone genes' expression level were regulated at least two folds, among which 81.2% were down-regulated.

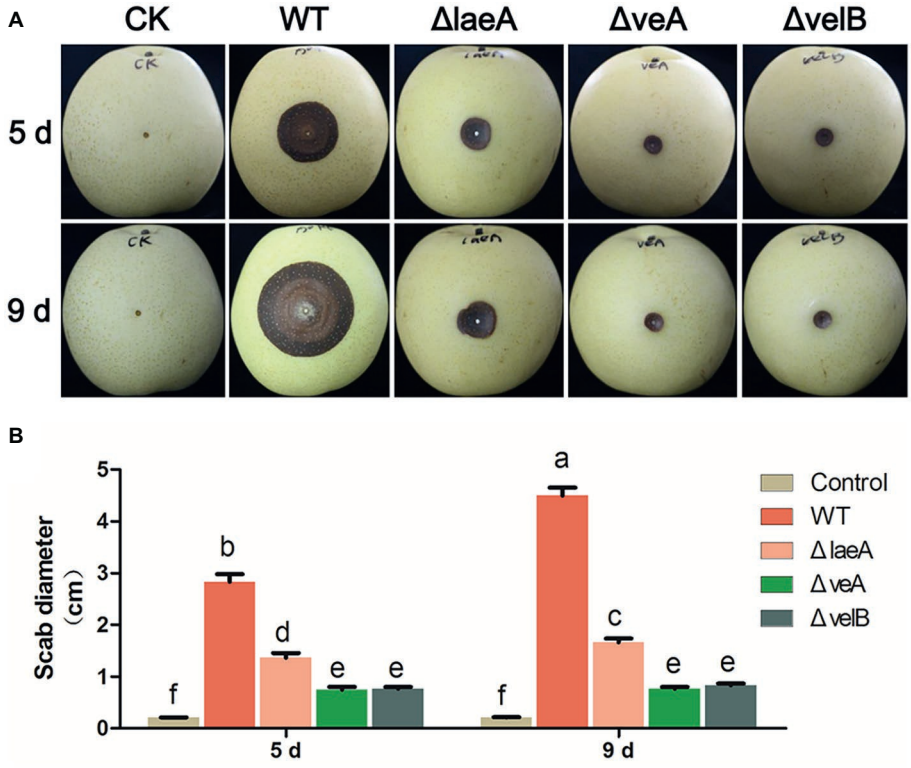

FIGURE 6 | Pathogenicity assay for WT and mutants of *A. ochraceus* on pears. **(A)** Pears infected by WT, ΔlaeA, ΔveA, and ΔvelB incubated at 28°C for 5 and 9 days under dark condition and photographed. **(B)** The scab diameters of pears measured using cross method. Different letters indicate a significant difference between the corresponding values ($p < 0.05$) with three biological replicates.

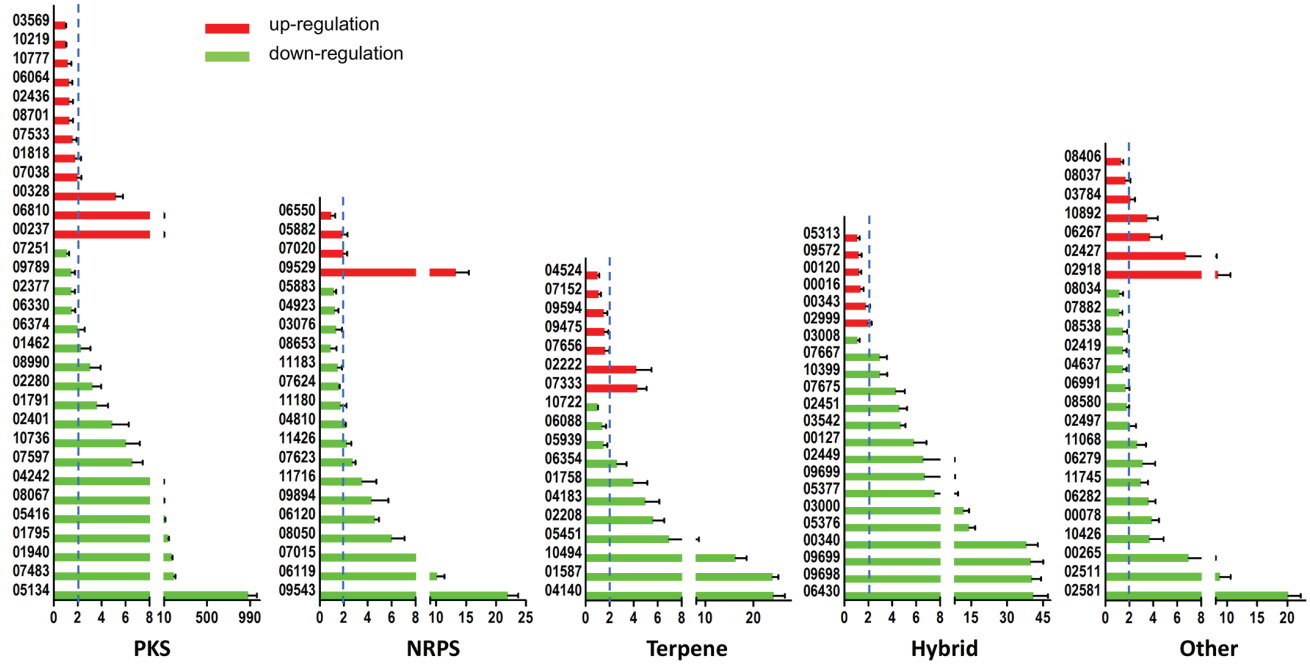

FIGURE 7 | LaeA influenced the expression level of secondary metabolite biosynthetic genes. Both WT and ΔlaeA had three biological replicates. Y axes represented the backbone genes in PKS, NRPS, Terpene, Hybrid, and other gene clusters. X axes represented the expression ratio of genes expressed in WT compared to that expressed in ΔlaeA.

These results indicated that LaeA was essential for the expression of considerable part of secondary metabolite encoding genes.

DISCUSSION

OTA contamination of food, feed, and fruits is a significant health concern worldwide. *A. ochraceus* is the major producer of OTA, with a wide range of host. Furthermore, a number of secondary metabolites, such as circumdatin G and H (Dai et al., 2001; Lopez-Gresa et al., 2005), stephacidin A and B (Jingfang Qian-Cutrone et al., 2002), Speramides A and B (Chang et al., 2016), and waspergillamide B (Frank et al., 2019), could be produced by *A. ochraceus* and researchers never give up to isolate new compounds from this fungus. However, the role of secondary metabolites except ochratoxins on health and virulence is unknown. And little is known about the genetic regulation of the lots of secondary metabolites including OTA biosynthesis process. Thus, deep inspection of the regulatory genes involved in metabolic pathways could provide a better understanding the mechanism of regulation of secondary metabolites.

In 2008, it was revealed that LaeA and two velvet families, VeA and VelB, confirmed a trimetirc complex that is essential to coordinate secondary metabolism and development in *A. nidulans* under dark condition (Bayram et al., 2008). VeA forms the light-responsive bridge that links VelB and LaeA. Three proteins were conserved in various fungi. In the WT of *A. ochraceus*, light cause a 50% increase of conidiospore and a 92% reduction of OTA. It is found that OTA biosynthesis was reduced under light condition for other ochratoxingenic fungi such as *A. carbonarius*, *A. niger*, *P. verrucosum*, and *P. nordicum* (Schmidt-Heydt et al., 2010; Crespo-Sempere et al., 2013), indicated that the development and secondary metabolism was regulated by light condition and might be explained according to the role of velvet complex. Here, we are reporting first time the function of LaeA, VeA, and VelB in *A. ochraceus*, and also providing the vision on light regulating OTA biosynthesis mechanism.

Thus, we obtain the deletion mutants of *laeA*, *veA*, and *velB* of *A. ochraceus* and compare their characteristic for development, OTA biosynthesis and fungal virulence on pears. Deletion of *laeA* led to the dramatic reduction of conidiaspore, and deletion of laeA, VeA, and VelB led to the slowing down of growth rate. The biosynthesis of OTA was strongly regulated by LaeA, VeA, and VelB, for the production of OTA was decreased by three order of magnitude in the deletion mutants. All the three proteins affected the pathogenicity of *A. ochraceus* on pears. However, we could not confirm whether pathogenicity be related to OTA biosynthesis. Some studies were reported to prove the role of mycotoxin in fungal virulence (Barad et al., 2014), whereas others not (Ballester et al., 2015). It is meaningful to in-depth study the relationship among development, OTA biosynthesis and fungal virulence of *A. ochraceus* for exploring strategies of OTA contamination.

The mechanism of LaeA playing its regulatory role is unclear until now, although a number of studies referring to various fungi focus on LaeA. Being a member of velvet complex is only one of the mechanisms. The S-adenosyl methionine-binding site contained in LaeA presumably indicates its methyltransferase activity. Additionally, it has been suggested that this protein has been linked to changes in chromatin structure because loss of LaeA leads to increased hetero-chromatin marks and its often precise regulation of secondary metabolites (Bok and Keller, 2016). In this study, we focused on the regulatory role of LaeA on secondary metabolite biosynthetic genes for its widely accepted function. About 66.7% backbone genes in NRPS cluster were significantly regulated by LaeA, among which about 85.7% of the genes were down-regulated. In addition to backbone genes in PKS, Terperne, hybrid, and other clusters, 66.1% of the genes were significantly regulated, and 81.6% of differential expression genes were downregulated (**Figure 7**). These data proved the role of LaeA in secondary metabolite biosynthesis regulation, and deletion of laeA repressed the expression of many compounds as reported previously (Bok and Keller, 2004; Perrin et al., 2007). Although the structure of compounds corresponding to each cluster was not clear, this study would gain insights to the link between compounds and biosynthetic gene clusters.

In conclusion, results from this study have provided some evidence that velvet complex proteins (LaeA, VeA, and VelB) play important roles in morphology development, OTA biosynthesis and fungal virulence in *A. ochraceus*. And we further demonstrated LaeA widely affect gene expression of *A. ochraceus* genome, with a focus on secondary metabolites. The down regulation effect of LaeA was more than up regulation effect in secondary metabolism. Given the strong effect of *laeA*, *veA*, and *velB* on OTA biosynthesis, these genes could be designed as target sites to develop new strategies for OTA control and prevention.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

YL and GW designed the experiment. GW, YW, FL, EL, JM, BY, and CZ performed the experiments. GW, LL, and HZ analyzed the data. GW wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02759/full#supplementary-material

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frontiers in Microbiology

ORIGINAL RESEARCH
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The pH-Responsive Transcription Factor PacC Governs Pathogenicity and Ochratoxin A Biosynthesis in *Aspergillus carbonarius*

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Pathogenic fungi must respond effectively to changes in environmental pH for successful host colonization, virulence and toxin production. *Aspergillus carbonarius* is a mycotoxigenic pathogen with the ability to colonize many plant hosts and secrete ochratoxin A (OTA). In this study, we characterized the functions and addressed the role of PacC-mediated pH signaling in *A. carbonarius* pathogenicity using designed *pacC* gene knockout mutant. Δ*AcpacC* mutant displayed an acidity-mimicking phenotype, which resulted in impaired fungal growth at neutral/alkaline pH, accompanied by reduced sporulation and conidial germination compared to the wild type (WT) strain. The Δ*AcpacC* mutant was unable to efficiently acidify the growth media as a direct result of diminished gluconic and citric acid production. Furthermore, loss of *AcpacC* resulted in a complete inhibition of OTA production at pH 7.0. Additionally, Δ*AcpacC* mutant exhibited attenuated virulence compared to the WT toward grapes and nectarine fruits. Reintroduction of *pacC* gene into Δ*AcpacC* mutant restored the WT phenotype. Our results demonstrate important roles of PacC of *A. carbonarius* in OTA biosynthesis and in pathogenicity by controlling transcription of genes important for fungal secondary metabolism and infection.

Keywords: ***Aspergillus carbonarius*, PacC, pH regulation, OTA biosynthesis, post-harvest disease**

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INTRODUCTION

In order to survive and proliferate under diverse conditions, fungal microorganisms must be able to sense and respond to rapidly changing environmental stresses (Prusky et al., 2013). Fungal pathogens are not only able to survive in a diverse range of environmental conditions, but also have evolved abilities to recognize, penetrate and attack their hosts, while responding to chemical and physical signals from the host. Ambient pH is one of these environmental conditions and acts as an important signal for fungal growth, development, secondary metabolism and host infection (Drori et al., 2003; Li et al., 2010; Vylkova et al., 2011; Barad et al., 2014, 2016). *Aspergillus carbonarius* is frequently responsible for post-harvest decay of various fresh fruits, including grapes, peaches, pears, citrus and nectarines (JECFA, 2001). In addition to its pathogenicity, *A. carbonarius* is also

considered as the main producer of ochratoxin A (OTA) – a potent nephrotoxin which may exhibit carcinogenic, teratogenic and immunotoxic properties in animals and possibly in humans (IARC, 1993; Pfohl-Leszkowicz and Manderville, 2007). Our recent study demonstrated that ambient pH plays an important role in *A. carbonarius* pathogenicity and OTA biosynthesis (Maor et al., 2017). Secretion of gluconic acid (GLA) by *A. carbonarius* caused direct fruit tissue acidification and induced accumulation of OTA in colonized grapes. Previous findings indicated that acidification of the apple fruit host environment through secretion of organic acids enhanced maceration and colonization of the fruit by *Penicillium expansum* (Prusky et al., 2004; Barad et al., 2012). Barad et al. (2014) pointed out the importance of the acidification process driven by GLA production in the activation of patulin biosynthesis and its contribution to the enhanced pathogenicity of *P. expansum* in apples.

Ambient pH signaling in filamentous fungi was first discovered in *Aspergillus nidulans*; it's mediated by transcription factor PacC and six Pal proteins (PalA, PalB, PalC, PalF, PalH, and PalI), which may regulate both acid- and alkaline-expressed genes in several fungal species (Caddick et al., 1986; Tilburn et al., 1995; Penalva et al., 2008; Ment et al., 2015). The external pH signal is transmitted by fungal signaling pathway from the extracellular environment to the nucleus, where it regulates the expressions of PacC dependent genes, which are involved in secondary metabolism, cell wall biosynthesis and fungal pathogenesis (Penalva and Arst, 2002; You et al., 2007; Luo et al., 2017). PacC has been previously shown to differentially regulate virulence as well as biosynthesis and secretion of secondary metabolites in several fungal species. Decreased fungal virulence as well as reduction in biosynthesis of secondary metabolites, as a result of *pacC* knockout, have been observed in several *Aspergillus* and *Penicillium* species (Suarez and Penalva, 1996; Keller et al., 1997; Bergh and Brakhage, 1998; Zhang et al., 2013; Chen et al., 2018; Wang et al., 2018). In contrast, disruption of *pacC* resulted in increased pathogenicity of *Fusarium oxysporum* (Caracuel et al., 2003) and higher trichothecene and fumonisin production in *F. graminearum* and *Fusarium verticillioides*, respectively, compared to the wild type strains (Flaherty et al., 2003; Merhej et al., 2011), suggesting that this transcription factor acts as a negative regulator of virulence and secondary metabolism in *Fusarium* spp.

It has been already reported that OTA biosynthesis is regulated by PacC in *Aspergillus ochraceus* (Wang et al., 2018). Although the growth of the Δ*AopacC* mutant and its ability to produce OTA were compromised to some degree, an increase in conidia formation has been observed compared to that of the wild type strain, suggesting that PacC in *A. ochraceus* positively regulates growth and OTA biosynthesis, but has a negative regulatory role in sporulation. In the present study we investigated the role of the pH regulatory factor PacC in virulence and OTA production in *A. carbonarius*, which is one of the most important mycotoxigenic pathogens. *AcpacC* gene deletion showed the significance of this transcription factor in germination, sporulation, mycelial growth, OTA biosynthesis and virulence in fruits. Our results suggest that AcPacC is a positive regulator of virulence in *A. carbonarius* by mediating expression of the glucose oxidase encoding gene (*gox*) and the genes encoding for cell wall degrading enzymes.

MATERIALS AND METHODS

Fungal Strain and Growth Conditions

The wild type (WT) strain *A. carbonarius* NRRL 368 was obtained from USDA Agricultural Research Service Culture Collection (Northern Regional Research Laboratory, Peoria, IL, United States). The WT strain and mutants generated in this study were grown at 28°C and maintained on Potato Dextrose Agar (PDA) plates (BD, Franklin Lakes, NJ, United States). Conidia were harvested and adjusted using a haemocytometer to the indicated concentrations.

Gene Knockout and Complementation

Construction of the *AcpacC* gene replacement plasmid was achieved by PCR-amplifying genomic flanking regions using specific primer pairs that incorporated a single 2-deoxyuridine nucleoside near the 5′ ends (primers U-f1 × U-r1 for the promoter region and primers D-f1 × D-r1 for the terminator region). Both DNA fragments and the pre-digested pRFHU2 binary vector (Frandsen et al., 2008) were mixed together and were treated with the USER enzyme (New England Biolabs, Ipswich, MA, United States) to obtain the plasmid pRFHU2-AcpacC. An aliquot of the mixture was used directly in chemical transformation of high-efficiency *Escherichia coli* DH5α cells (New England Biolabs, Ipswich, MA, United States) without prior ligation. Kanamycin resistant transformants were screened by PCR for validation of proper fusion events. Then, the plasmid was introduced into electro-competent *Agrobacterium tumefaciens* AGL-1 cells.

A single colony of *A. tumefaciens* AGL-1 carrying plasmid pRFHU2-AcpacC was used to inoculate a starter culture and incubated for 24 h. Bacterial cells were centrifuged, washed with induction medium (10 mM K_2HPO_4, 10 mM KH_2PO_4, 2.5 mM NaCl, 2 mM $MgSO_4$, 0.6 mM $CaCl_2$, 9 μM $FeSO_4$, 4 mM $(NH_4)_2SO_4$, 10 mM glucose, 40 mM MES pH 5.3, 0.5% glycerol) and diluted in the same medium amended with 200 μM acetosyringone (IMAS) to an OD600 = 0.15. Cells were grown at 28°C and 200 rpm until they reached an OD600 = 0.75. Equal volumes of IMAS induced bacterial culture and conidial suspension of *A. carbonarius* (10^6 conidia/ml) were mixed and spread onto Whatman filter papers, which were placed on agar plates containing the co-cultivation medium (same as IMAS, but containing 5 mM instead of 10 mM of glucose). After co-cultivation at 28°C for 48 h, the membranes were transferred to PDA plates containing hygromycin B (100 μg/mL) as the selection agent for fungal transformants, and cefotaxime (200 μg/mL) to inhibit growth of *A. tumefaciens* cells. Hygromycin resistant colonies appeared after 3–4 days of incubation at 28°C. Disruption of *AcpacC* was confirmed by PCR analyses of the transformants.

A restriction free cloning method (van den Ent and Lowe, 2006) was employed in order to replace the hygromycin

resistance gene *hph* of the pRFHU2 vector with the phleomycin resistance gene *ble* amplified from the pBC-Phleo plasmid (Silar, 1995) using primer pair RFC-f1 x RFC-r1. Proper substitution of the resistance genes was confirmed by DNA sequencing and the new plasmid was termed pRFPU2. For the construction of the complementation vector, two genomic fragments consisting of the entire *AcpacC* cassette (primer pair U-f1 × U-r2) and the gene's terminator region (primer pair D-f1 × D-r1), were USER cloned to the pre-digested pRFPU2 vector to generate pRFPU2-AcpacC-c as described before. Conidia of the Δ*AcpacC* knockout strain were transformed using *A. tumefaciens* AGL-1 cells carrying the plasmid pRFPU2-AcpacC-c as described before. For selecting phleomycin resistant complementation transformants, phleomycin (50 μg/mL) was enough to prevent growth of untransformed conidia. Analysis of transformants for reintroduction of the endogenous *AcpacC* cassette was done by PCR. All primers used to create and confirm the mutant and complement strains are listed in **Supplementary Table S1**.

Physiological Analysis

Radial growth and sporulation (conidial production) were assessed on solid YES media (20 g bacto yeast extract, 150 g sucrose and 15 g bacto agar per liter) adjusted with 10 M HCl or 10 M NaOH to pH 4.0, 7.0 and 8.0, while conidial germination was evaluated in YES broth media (20 g bacto yeast extract and 150 g sucrose per liter) adjusted to pH 4.0 and pH 7.0.

For radial growth assessment, 90 mm agar plates were point inoculated with 10^2 conidia of either the WT, knockout or complement strain and incubated at 28°C. Growth was monitored by diameter measurements on a daily basis for 10 days using three replicate plates per strain.

For conidial production quantification, 55 mm agar plates containing 10^5 conidia of each strain were incubated at 28°C for 7 days. To accurately count conidia, two 1 cm plugs from each plate were homogenized in 3 ml water containing 0.01% Tween 20, diluted and counted with a haemocytometer. Conidial production was quantified starting by the 3rd day post-inoculation using three replicate plates per strain.

For germination evaluation, the conidia concentration of all strains was adjusted to 10^4 conidia/ml in the medium. 0.5 ml of each conidial suspension was distributed into three replicate wells of a 24-well sterile culture plate (SPL Life Sciences, Pocheon, South Korea). Time-course microscopy was carried out over 24 h at 28°C using a Nikon Eclipse T*i* inverted microscope (Nikon, Tokyo, Japan) equipped with a ProScan motorized XY stage (Prior Scientific, Cambridge, United Kingdom) with a LAUDA ECO RE 415 temperature-controlled incubator (LAUDA-Brinkmann, Delran, NJ, United States). Images were captured at 1-h intervals, beginning 2 h post-incubation using an ANDOR zyla 5.5 MP sCMOS camera (Andor Technology, Belfast, Northern Ireland) and processed using the NIS elements AR 4.6 (64 bit) software package. Conidia were considered to have germinated when germ tubes arose from the swollen conidial base. Number of conidia germlings were counted for each strain and the percent of germinated conidia was plotted against time.

pH Measurements, Organic Acids and OTA Analysis

A 10^6 fungal conidia/ml solution (100 μl) was inoculated onto 55 mm petri dishes containing 10 ml of solid YES media adjusted to pH 4.0 or pH 7.0. The plates were incubated at 28°C in the dark for 2–13 days as needed for sample collection.

pH was measured directly in the agar cultures with a double pore slim electrode connected to a Sartorius PB-11 Basic Meter (Sartorius, Göttingen, Germany).

For assessment of organic acids production, five 1-cm diameter discs of agar were placed in 5 ml of sterilized water and crushed to homogeneity. A 1 ml aliquot of the solution was sampled in a 1.5 ml microcentrifuge tube and centrifuged for 10 min at 20,800 *g*. The supernatant was taken for GLA and citric acid analysis using test kits applying enzymatic methods for the specific measurement of total D-Gluconic acid and citric acid contents (Megazyme, Wicklow, Ireland) according to the manufacturer's instructions.

To evaluate OTA levels, five 1-cm diameter discs of agar were added to 1.7 ml of HPLC grade methanol (Bio-Lab, Jerusalem, Israel) and crushed to homogeneity. OTA was extracted by shaking for 30 min at 150 RPM on an orbital shaking platform and centrifuged for 10 min at 20,800 *g*. The supernatant was filtered through a 0.22 μm PTFE syringe filter (Agela Technologies, Tianjin, China) and kept at −20°C prior to HPLC analysis. OTA was quantitatively analyzed by injection of 20 μl into a reverse phase UHPLC system (Waters ACQUITY Arc, FTN-R, Milford, MA, United States). The mobile phase consisted of acetonitrile:water:acetic acid (99:99:2, v/v/v) at 0.5 ml/min through a Kinetex 2.6 μm XB-C18 (100 × 2.1 mm) with a security guard column C18 (4 × 2 mm) (Phenomenex, Torrance, CA, United States). The column temperature was maintained at 30°C. The OTA peak was detected with a fluorescence detector (excitation at 330 nm and emission at 450 nm) and quantified by comparing with a calibration curve of the standard mycotoxin (Fermentek, Jerusalem, Israel).

Colonization and Pathogenicity Experiments

"Zani" seedless grapes and "Sun Snow" nectarines were obtained from a local supermarket. Fruits were subjected to surface sterilization using 1% sodium hypochlorite solution for 1 min, and immediately rinsed in sterile distilled water. A 10 μl conidial suspension containing 10^6 conidia/ml of either the WT, the Δ*AcpacC* mutant strain or the *AcpacC*-c complement strain was injected directly into the sterilized fruits at 2 mm depth. Following inoculation, the fruits were incubated in covered plastic containers at 28°C for 2–9 days as needed for symptom monitoring and sample collection, and the diameters of the rotten spots were recorded daily.

The pH of nectarine tissues was measured by inserting a double pore slim electrode directly into the tested area. To analyze GLA content in inoculated nectarine fruits, 1.7 gr of the macerated necrotic area were taken, 5 ml of sterilized water were added and the tissues were homogenized. A 1 ml aliquot of the solution was sampled in a 1.5 ml microcentrifuge tube,

centrifuged for 10 min at 20,800 *g*, and the amounts of GLA produced were measured as described above.

For OTA analysis in colonized grapes and nectarines, 1.7 gr of the macerated necrotic area were taken, 1.7 ml of HPLC grade methanol (Bio-Lab, Jerusalem, Israel) were added and the tissues were homogenized. Then, OTA was quantitatively analyzed as described above.

RNA Extraction and qRT-PCR Analysis of Gene Transcription Profile

Mycelia from the *in vitro* experiments were harvested at the appropriate time, weighed, frozen in liquid nitrogen, lyophilized for 24 h and kept at −80°C until use. In colonized nectarines, mycelia containing exocarp (peel) was removed, frozen in liquid nitrogen and lyophilized prior to RNA extraction. Total RNA was extracted from 100 mg of lyophilized tissue of the selected samples using the Hybrid-R RNA isolation kit (GeneAll, Seoul, South Korea) according to the manufacturer's protocol. The DNase and reverse-transcription reactions were performed on 1 μg of total RNA with the Maxima First-Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, United States) according to the manufacturer's instructions. The cDNA samples were diluted 1:10 (v/v) with ultrapure water. The quantitative real-time PCR was performed using Fast SYBR green Master Mix (Applied Biosystems, Waltham, MA, United States) in a StepOnePlus Real-Time PCR System (Applied Biosystems, Waltham, MA, United States). The PCR conditions were as follows: 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 20 s. The samples were normalized using β-*tubulin* as endogenous control and the relative expression levels were measured using the $2^{(-\Delta\Delta Ct)}$ analysis method. Results were analyzed with StepOne software v2.3. Primer sequences used for qRT-PCR analysis are listed in **Supplementary Table S2**.

Statistical Analysis

Student's *t*-test was performed when data was normally distributed and the sample variances were equal. For multiple comparisons, one-way ANOVA was performed when the equal variance test was passed. Significance was accepted at $p < 0.05$. All experiments described here are representative of at least three independent experiments with the same pattern of results.

RESULTS AND DISCUSSION

Creation and Validation of *pacC* Deletion and Complementation Strains of *A. carbonarius*

In order to explore the functional roles of PacC in the physiology and pathogenicity of *A. carbonarius*, *AcpacC* deletion and complementation strains were generated. For *AcpacC* deletion, a targeted gene deletion strategy was employed using *A. tumefaciens* mediated transformation of *A. carbonarius* NRRL 368 WT strain (**Supplementary Figure S1**). Gene replacement plasmid, pRFHU2-AcpacC, was obtained by a USER Friendly cloning system (Frandsen et al., 2008). Co-cultivation of *A. tumefaciens* cells carrying pRFHU2-AcpacC with the conidia of *A. carbonarius* led to the appearance of hygromycin B-resistant colonies approximately 4 days after transfer to the selective PDA plates. Disruption of *AcpacC* was confirmed by several PCR analyses for the introduction of the hygromycin resistance gene coding sequence, correct genomic placement of the 5′ and 3′ flanking sequences and the absence of the *AcpacC* sequence. With respect to the *AcpacC* complement strain, twelve single transformants were initially selected on PDA medium supplemented with phleomycin (50 μg/ml). The phleomycin resistant strains were diagnosed by PCR to confirm the integration of the WT allele in the Δ*AcpacC* strain using the same set of primers that amplify the *AcpacC* ORF. As expected, the *AcpacC-c* strain revealed the expected band of 472 bp (**Supplementary Figure S2**). One of the correct *AcpacC-c* strains was used for the following experiments.

AcPacC Is Required for Fungal Growth, Conidial Formation and Germination

Physiological analysis revealed that compared to the WT strain the growth of the Δ*AcpacC* mutant was reduced on YES synthetic media under acidic condition at pH 4.0, significantly impaired at pH 7.0 and completely inhibited under alkaline condition at pH 8.0 (**Figures 1A,B**). Due to the inability of the mutant strain to grow in alkaline conditions, the following experiments were performed under acidic and neutral pH conditions. Conidia production in Δ*AcpacC* mutant strain was severely inhibited at all examined time points under both acidic and neutral pH conditions (**Figure 1C**). Our study demonstrated that pH, as an environmental factor, may affect conidial germination of *A. carbonarius*. Germination rate of *A. carbonarius* was inhibited by 29% under ambient pH 7.0 (**Figure 1D**). It has been previously reported that germination of *P. expansum* conidia was inhibited significantly under ambient pH 2.0 and 8.0, probably through impairing protein synthesis and folding (Li et al., 2010). Conidial germination in the Δ*AcpacC* mutant strain was delayed compared to the WT at both pH conditions, although 100% germination of these conidia has been observed following 17 h of incubation (**Figure 1D**). By this time point, the ability of the mutant strain to develop hyphae in liquid culture at acidic pH was normal as was the ability to maintain hyphal growth (**Figure 2A**). In contrast, at pH 7.0 hyphal formation and growth of Δ*AcpacC* strain was significantly stunted and characterized by severe hyper-branching compared to the WT (**Figure 2B**). Normal growth, sporulation, conidial germination and hyphal formation were recovered in the *AcpacC-c* strain, indicating that AcPacC is required for morphogenetic development of *A. carbonarius*, especially in alkaline conditions.

Similarly to Δ*AcpacC*, growth of the *pacC* disrupted mutants in *Sclerotinia sclerotiorum*, *F. oxysporum*, *Colletotrichum acutatum*, *F. graminearum* and *Penicillium digitatum* was slightly reduced under acidic pH but severely impaired under alkaline conditions (Caracuel et al., 2003; Rollins, 2003; You et al., 2007; Merhej et al., 2011; Zhang et al., 2013). However, deletion of *pacC* showed poor growth phenotype under both acidic and alkaline pH conditions in *P. expansum*, *Metarhizium robertsii* and

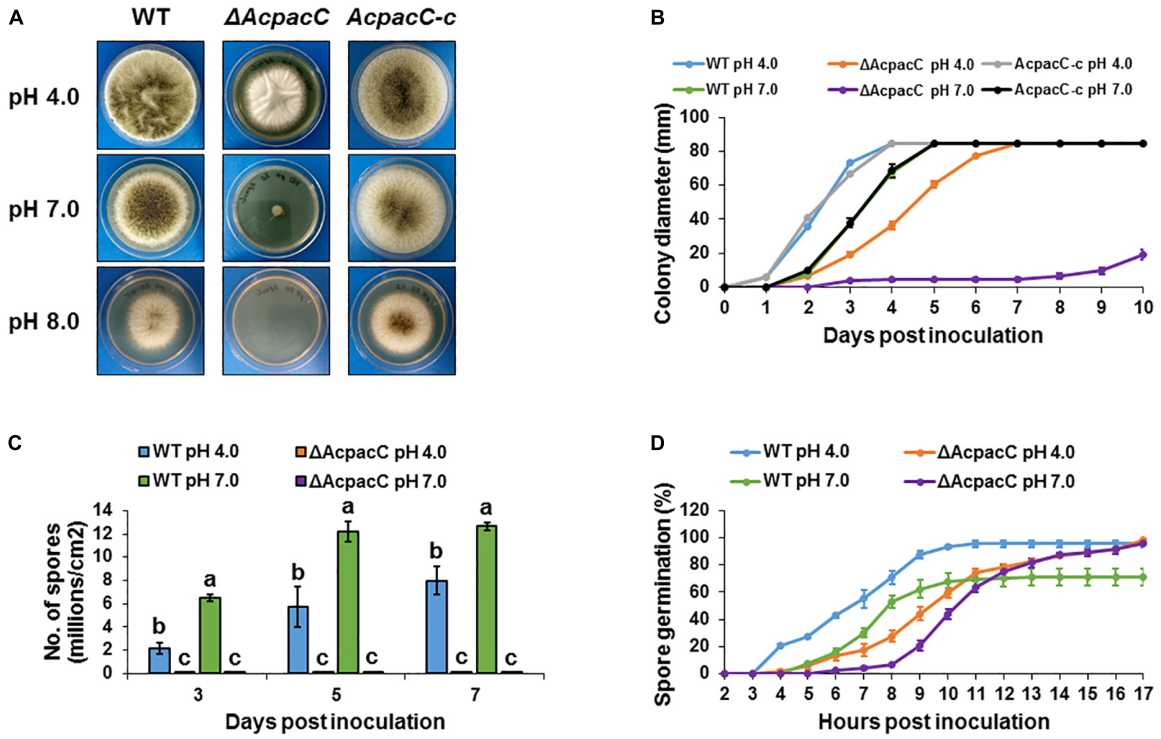

FIGURE 1 | Physiological analyses of the WT and mutant strains of *A. carbonarius* at different pH conditions. **(A)** Growth phenotype and **(B)** radial growth of the WT, Δ*AcpacC* and *AcpacC-c* strains on solid YES media at 28°C under pH 4.0, 7.0, and 8.0. **(C)** Conidiation of the WT and Δ*AcpacC* strains on solid YES media at pH 4.0 and 7.0. **(D)** Germination rates in the WT and Δ*AcpacC* strains were assessed in static YES broth media at 28°C under pH 4.0 and 7.0. Error bars represent the standard error of the mean (SEM) across three independent replicates. Different letters above the columns indicate statistically significant differences at $p < 0.05$, as determined using the Tukey's honest significant difference test.

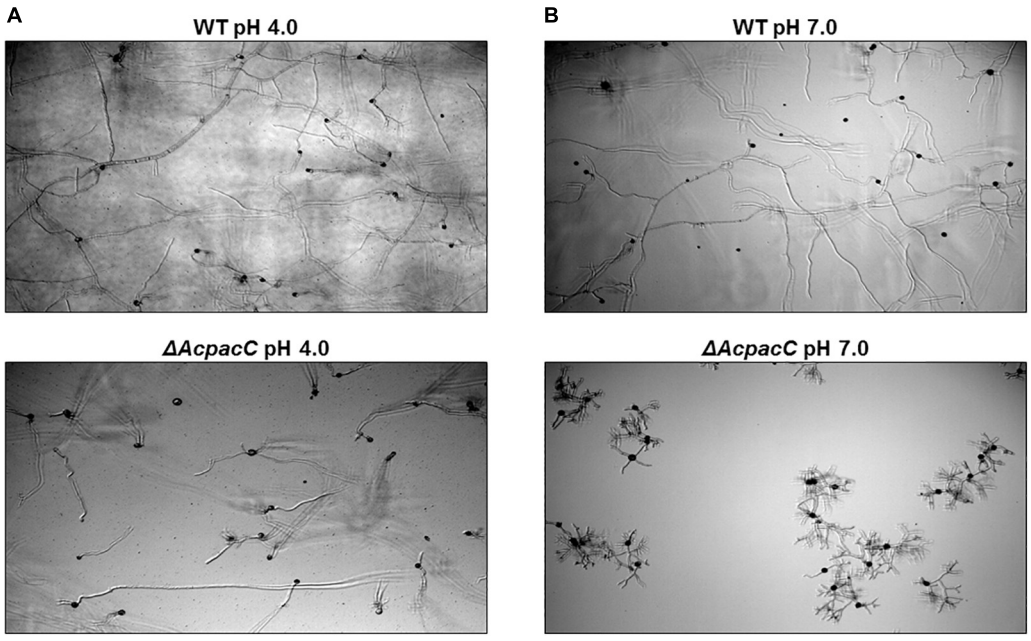

FIGURE 2 | Microscopic observation of hyphal morphology of WT and Δ*AcpacC* strains. Images of time-course microscopy were captured 17 h following incubation of WT and Δ*AcpacC* conidia suspensions in YES broth media adjusted to pH 4.0 **(A)** and pH 7.0 **(B)**.

Ganoderma lucidum (Huang et al., 2015; Wu et al., 2016; Chen et al., 2018). Interestingly, *A. ochraceus* Δ*pacC* mutant strain had slightly impaired growth under alkaline conditions, but similar growth rate to the WT in acidic pH. On the contrary, unlike Δ*AcpacC* strain, an increase in conidia formation was observed in *A. ochraceus* Δ*pacC* mutant compared to that of the WT strain under all the pH conditions (Wang et al., 2018). Therefore, it is likely that PacC plays different roles in mycelial growth and sporulation of different fungal pathogens.

AcPacC Regulates Production of Organic Acids in *A. carbonarius*

Post-harvest fungal pathogens were reported to enhance their virulence by locally modulating the host's ambient pH (Prusky et al., 2014). Previous studies have shown that *Penicillium* spp. (*P. expansum*, *P. digitatum*) and *A. carbonarius* acidify the ambient environments of deciduous fruits during decay development by secretion of significant amounts of organic acids, mainly citric and gluconic acids (Prusky et al., 2004; Barad et al., 2012, 2014; Maor et al., 2017). Gluconic acid accumulation by *P. expansum* and *A. carbonarius* is pH-dependent and is mainly regulated by glucose oxidase (GOX) that catalyzes the oxidation of glucose to gluconic acid. In the current study, a significant decrease in the formation of both citric and gluconic acids was observed in Δ*AcpacC* knockout mutant compared with the WT under acidic and neutral pH conditions at all tested time points (**Figures 3A,B**). The kinetics of the gene transcript level shows that *Acgox* gene expression was markedly down-regulated in Δ*AcpacC* in both acidic and neutral pH conditions, compared to the WT strain (**Figure 3C**), indicating that AcPacC directly regulates gluconic acid production by positive modulation of *A. carbonarius* glucose oxidase-encoding gene. In *P. expansum*, two *pacC*-RNAi mutants with downregulation of PacC (silenced by RNAi technology) resulted in a 63 and 27% reduction in gluconic acid production, respectively (Barad et al., 2014). This relatively moderate reduction could be attributed to residual PacC expressions in *pacC*-RNAi mutants. A recent study reported that GOX, which was identified by proteome analysis as an alkaline-expressed protein, is directly regulated by *P. expansum* transcription factor PacC (Chen et al., 2018). At acidic pH, the PacC protein is inactive and therefore unable to bind to the promoter sites of the target genes, however, under alkaline conditions PacC acts as an activator of alkaline-expressed genes and as a repressor of acid-expressed genes (Penalva et al., 2008).

OTA Biosynthesis in *A. carbonarius* Is Regulated by AcPacC

Among black aspergilli, *A. carbonarius* has shown a consistent ability to produce OTA and is the most probable source of this mycotoxin in a wide range of foods. Our previous study showed a clear pattern of pH modulation through secretion of organic acids by *A. carbonarius*, which acidify the ambient environment and induce OTA production in culture (Maor et al., 2017). In addition, Barad et al. (2014) demonstrated that down-regulation of *gox* gene in *P. expansum* (using RNAi technology) resulted in impaired ability to produce gluconic acid, which was accompanied by down-regulation in the relative expression of *idh* gene (encodes the isoepoxydon dehydrogenase enzyme, a key enzyme in the patulin biosynthesis pathway) and reduction in patulin accumulation. As shown in **Figure 4A**, OTA production by WT strain was significantly higher throughout the experiment under acidic condition, at pH 4.0, compared to the accumulation at pH 7.0. Deletion of *AcpacC* resulted in complete inhibition of OTA production at both pH 4.0 and 7.0 during the first 6 days after inoculation (**Figure 4A**). Nevertheless, under acidic condition a small amount of OTA was secreted by Δ*AcpacC* at day 8 of the experiment (**Figure 4A**). These results indicated that AcPacC is an important regulator in OTA biosynthesis in *A. carbonarius* under different pH conditions. Our findings suggest that not only the organic acids production could influence the accumulation of OTA, but also low pH itself might stimulate the mycotoxin biosynthesis. Moreover, we investigated the differential expression of all the five OTA biosynthetic cluster genes in Δ*AcpacC* and WT strains at ambient pH 4.0 and 7.0. As shown in **Figure 4B**, expressions of all five genes at day 4 of the experiment were down-regulated in Δ*AcpacC* at acidic pH. Under this condition, the relative expression of bZIP transcription factor, halogenase (HAL) and polyketide synthase (PKS) encoding genes in Δ*AcpacC* was similar to that of the WT strain at day 7 post-inoculation (**Figure 4B**); apparently, this is reflected in OTA production by the mutant at day 8 of the experiment. Although Δ*AcpacC* lost the ability of OTA production under neutral condition, the expression levels of several genes in the mutant strain, encoding bZIP transcription factor, PKS or HAL, were either unaffected or upregulated at pH 7.0 compared to the WT strain (**Figure 4B**), suggesting that these genes might be regulated by other transcription factors. PacC may act either as a positive or negative regulator of secondary metabolites biosynthesis (Brakhage, 2013). Similar to our findings, PacC was found to serve as a positive regulator of penicillin synthesis in *A. nidulans* and patulin biosynthesis in *P. expansum* (Bergh and Brakhage, 1998; Barad et al., 2014; Chen et al., 2018). On the contrary, PacC negatively regulates fumonisin biosynthesis in *F. verticillioides* and trichothecene biosynthesis in *F. graminearum* (Flaherty et al., 2003; Merhej et al., 2011). In *A. ochraceus*, another OTA producing pathogen, PacC played a positive role in regulating OTA biosynthesis, which was slightly impaired in *AopacC* loss-of-function mutant (Wang et al., 2018). Thus, PacC appears to function differently in regulating secondary metabolites in different fungal pathogens; yet, many unanswered questions remain on the mechanism of this regulation.

PacC Is Required for Pathogenicity in *A. carbonarius* and OTA Contamination of Deciduous Fruits

Colonization of "Sun Snow" nectarines and white "Zani" grape berries by Δ*AcpacC* strain showed a significant reduction in the rotten colonized area relative to that of the WT strain (**Figures 5A,B** and **Supplementary Figures S3A,B**). Four days

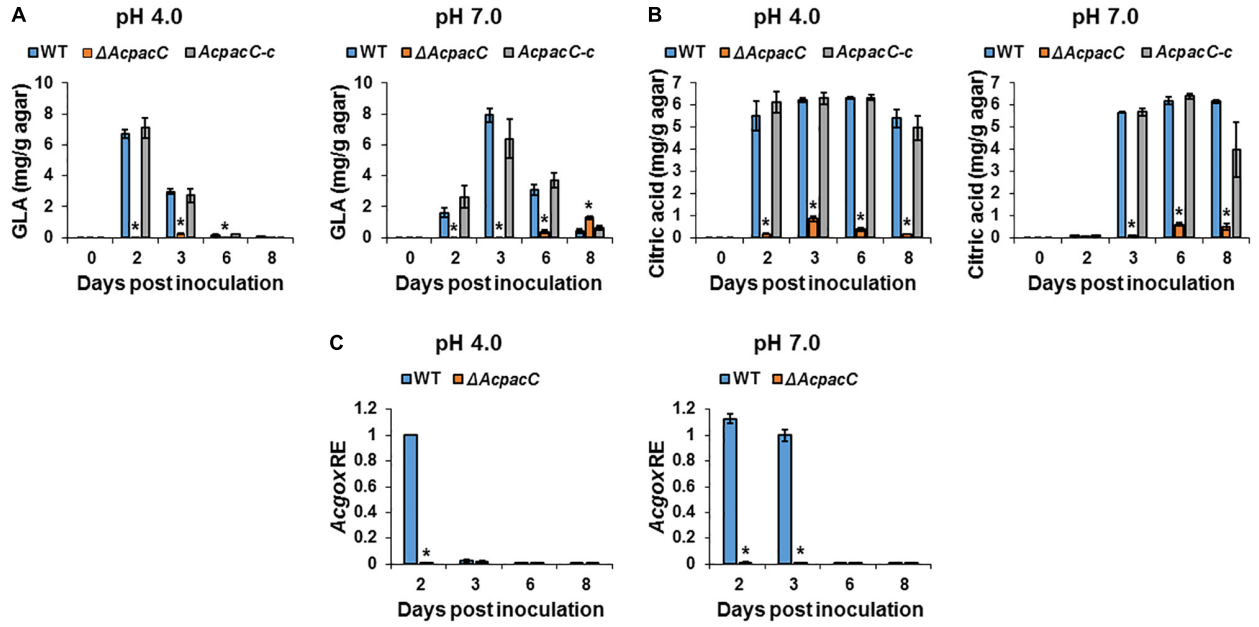

FIGURE 3 | Effects of AcPacC on organic acids production in *A. carbonarius*. Gluconic acid (GLA) **(A)** and citric acid **(B)** accumulation by the WT, Δ*AcpacC* and *AcpacC-c* strains under different pH conditions. **(C)** Differential expression of the *Acgox* gene between WT and Δ*AcpacC* at pH 4.0 and 7.0. Average values of three replicates (± standard error) are reported. Experiments were repeated three times and results of a single representative experiment are shown. Asterisks denote significant differences between strains at $p < 0.05$.

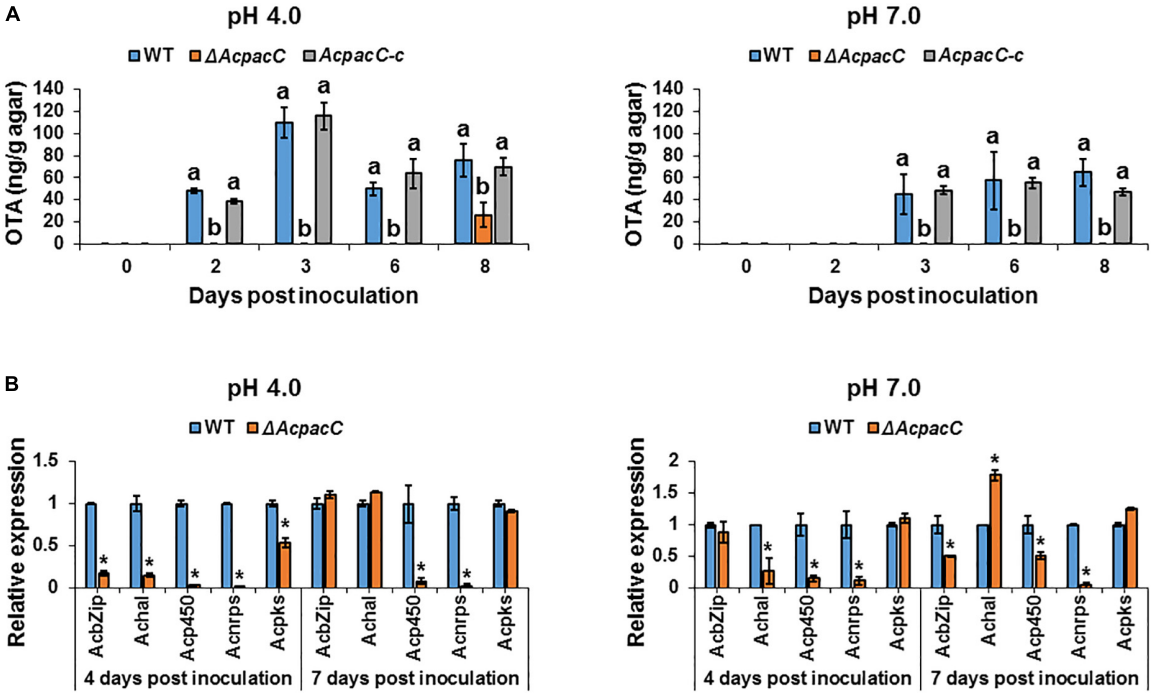

FIGURE 4 | Effects of AcPacC on OTA biosynthesis in *A. carbonarius* at different pH conditions. **(A)** OTA production by the WT, Δ*AcpacC* and *AcpacC-c* strains at pH 4.0 and pH 7.0. **(B)** Relative expression of OTA cluster genes in WT and Δ*AcpacC* at days 4 and 7 post-inoculation. Relative expression was normalized using β-*tubulin* as an internal control. Average values of three replicates (± standard error) are presented. Experiments were repeated three times and results of a single representative experiment are shown. Different letters above the columns indicate statistically significant differences at $p < 0.05$, as determined using the Tukey's honest significant difference test. Asterisks denote significant differences between strains at $p < 0.05$.

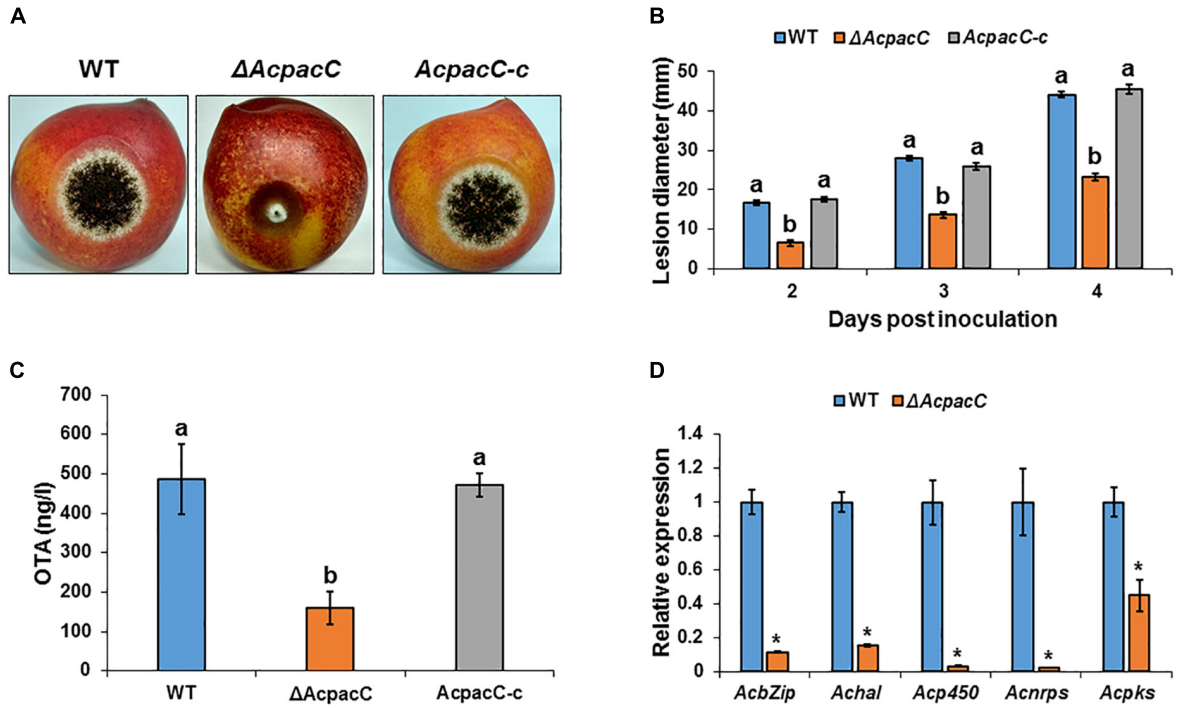

FIGURE 5 | Effects of AcPacC on pathogenicity of *A. carbonarius* and OTA production in nectarines. **(A)** Disease symptoms on nectarine fruits inoculated with conidia of WT, Δ*AcpacC* and *AcpacC-c* strains at 3 days after inoculation. **(B)** Histogram showing the diameters of the rotten spots on infected nectarines. **(C)** OTA accumulation in infected nectarines, and **(D)** relative expression of OTA cluster genes in WT and Δ*AcpacC* strains. RNA was extracted from infected nectarines at day 4 post-inoculation. Relative expression was normalized using β-*tubulin* as an internal control. Error bars represent standard error of three independent biological replicates. Different letters above the columns indicate statistically significant differences at $p < 0.05$, as determined using the Tukey's honest significant difference test. Asterisks denote significant differences between strains at $p < 0.05$.

after inoculation, Δ*AcpacC* strain showed an inhibition of the rotten area in nectarines and grape berries by up to 47 and 26%, respectively, compared with the WT strain (**Figure 5B** and **Supplementary Figure S3B**). The virulence was reverted by the complemented *AcpacC-c* strain (**Figure 5A**). The analysis of OTA accumulation in the infected nectarine tissue 5 days after inoculation revealed a three-fold reduction in OTA synthesis by the mutant strain, compared with the WT strain (**Figure 5C**); at the same time point, no OTA accumulation was detected by the Δ*AcpacC* in the inoculated grape berries (**Supplementary Figure S3C**). Nectarines infected with the Δ*AcpacC* mutant showed a 2–10 fold down-regulation of the transcript levels of all the five genes involved in OTA biosynthesis, compared with infections with the WT strain (**Figure 5D**), suggesting that AcPacC is essential for OTA production and probably directly involved in regulating transcription of the genes in OTA biosynthetic pathway. One would expect that reduction in OTA production by Δ*AcpacC* mutant may contribute to a reduction in virulence in this strain, however, in this regard, it should be noted that pathogenicity of the *A. carbonarius pks* mutant, which is unable to produce OTA, remained very similar to that of the WT (data not shown).

To gain an understanding of the potential mechanism underlying the reduced pathogenicity of the Δ*AcpacC* strain, the mutant was assessed for several physiological characteristics that have previously been associated with virulence in this pathogen. It has been proposed that one of the factors that contribute to pathogenicity of *A. carbonarius* is its ability to reduce the pH of infected grape tissue through the production of gluconic acid (Maor et al., 2017). Indeed, 4 days after inoculation, colonization of nectarine tissue by *A. carbonarius* WT strain reduced pH from 4.3 in the healthy part of the fruit to 3.5 in the decayed tissue (**Figure 6A**). This further acidification of the rotten tissue was accompanied by an accumulation of 9 mg/g of gluconic acid (**Figure 6B**). In contrast, the Δ*AcpacC* strain showed a smaller reduction in pH and resulted in the accumulation of minimum amount of the gluconic acid (0.24 mg/g; **Figures 6A,B**). Gene expression analysis in the tissue inoculated with the Δ*AcpacC* mutant showed a 10-fold down-regulation of *Acgox* expression, which may explain poor gluconic acid formation *in vivo* (**Figure 6C**). Thus, our data indicate that AcPacC is required for *A. carbonarius* virulence in fruits, most likely by the regulation of the expression of the *gox* gene.

Disruption of *pacC* resulted in reduced pathogenicity of *P. expansum* in pear and apple fruits through mediating a virulence factor glucose oxidase (Chen et al., 2018). In that study, glucose oxidase was identified as alkaline-expressed protein by proteome analysis and proved to be involved in the virulence

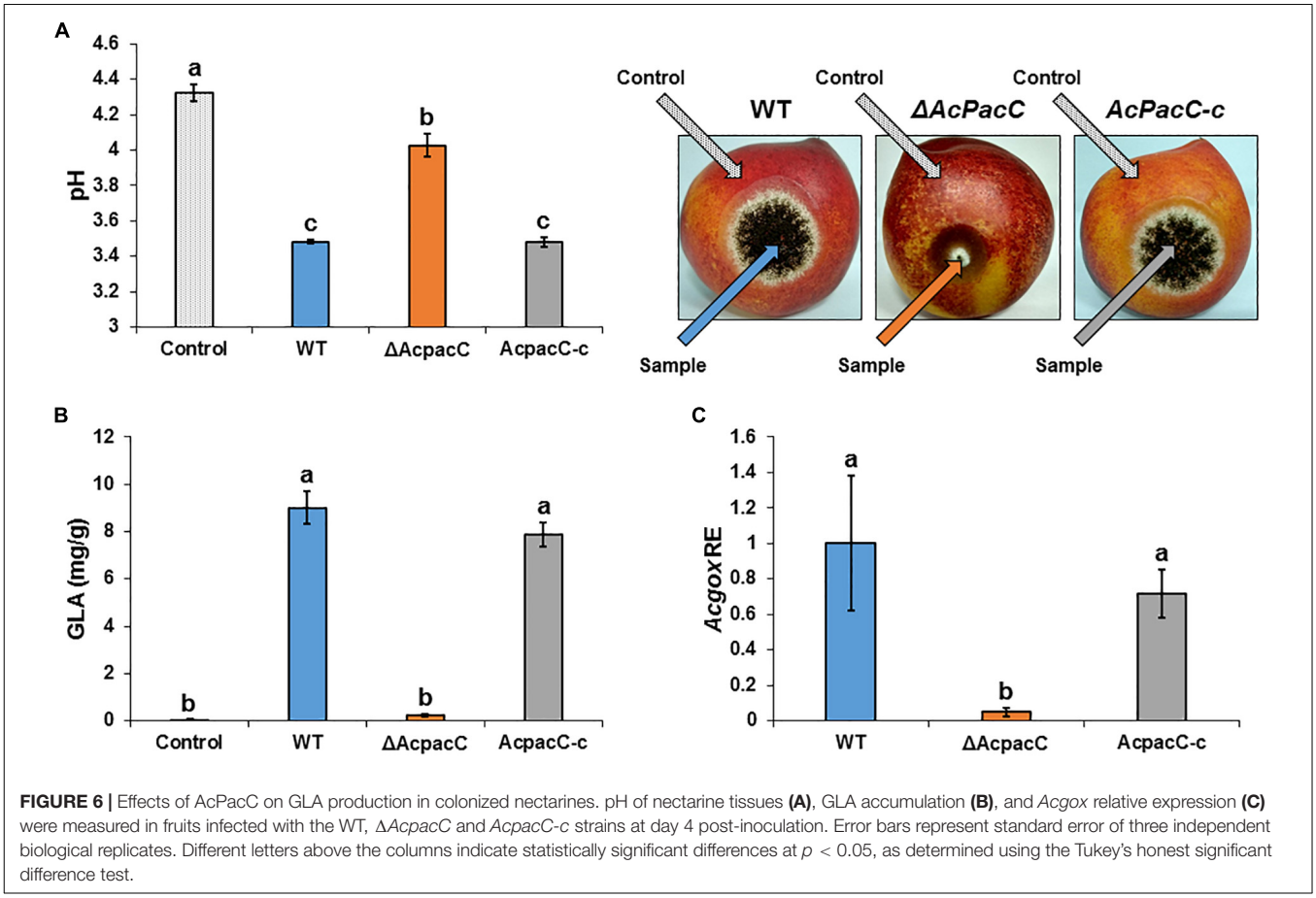

FIGURE 6 | Effects of AcPacC on GLA production in colonized nectarines. pH of nectarine tissues **(A)**, GLA accumulation **(B)**, and *Acgox* relative expression **(C)** were measured in fruits infected with the WT, Δ*AcpacC* and *AcpacC-c* strains at day 4 post-inoculation. Error bars represent standard error of three independent biological replicates. Different letters above the columns indicate statistically significant differences at $p < 0.05$, as determined using the Tukey's honest significant difference test.

of *P. expansum*. These results confirmed the findings of an earlier study of Barad et al. (2014), where *P. expansum pacC*-RNAi mutants reduced gluconic acid (which is regulated by *gox* expression) and patulin accumulation in apples and showed a 45% reduction in fungal pathogenicity, compared to the WT.

Furthermore, since *Aspergillus* enzymes are involved in degradation of plant cell wall polysaccharides, the expression levels of four genes encoding polygalacturonase, pectate lyase, cellulase and hemicellulase were also analyzed during fruit colonization, and all of them were down-regulated in the Δ*AcpacC* strain compared with the WT (**Supplementary Figure S4**). This down-regulation suggests possible involvement of AcPacC in the regulation of the cell wall-degrading enzymes during fruit colonization. Zhang et al. (2013) have reported similar findings, where PdPacC has been shown to be important for the pathogenicity of *P. digitatum* in citrus fruits via regulation of polygalacturonase and the pectin lyase genes. In *Colletotrichum gloeosporioides*, *pac1* mutants showed reduction of *pelb* gene expression level, with consequent delayed pectate lyase secretion and dramatically reduced virulence in avocado fruits (Miyara et al., 2008). Overall, our results suggest that AcPacC may contribute to the pathogenesis of *A. carbonarius* through regulating different PacC-dependent genes or pathways involved in virulence.

CONCLUSION

In this study we demonstrated that disruption of the pH signaling transcription factor PacC significantly decreased the virulence of *A. carbonarius* on deciduous fruits. This phenotype is associated with an impairment in fungal growth, decreased accumulation of gluconic acid and reduced synthesis of pectolytic enzymes. We showed that glucose oxidase-encoding gene, which is essential for gluconic acid production and acidification during fruit colonization, was significantly down-regulated in the Δ*AcpacC* mutant, suggesting that *gox* is PacC-responsive gene. Recently we have provided evidence that deletion of *gox* gene in *A. carbonarius* led to a reduction in virulence toward nectarine and grape fruits (data not shown), further indicating that GOX is a virulence factor of *A. carbonarius*, and its expression is regulated by PacC. The deletion of *AcpacC* may also affect the pathogenesis of *A. carbonarius* through the down-regulation of the cell wall-degrading enzymes such as polygalacturonase, pectate lyase, cellulase and hemicellulose. It is also clear from the present data that PacC in *A. carbonarius* is a key factor for the biosynthesis of secondary metabolites, such as OTA. Additional work is needed in order to gain a genomic perspective of the function of PacC during pathogenesis. Therefore, comparison of the transcriptomes of the WT and the Δ*AcpacC* mutant during fruit infection would contribute for the better understanding

of the molecular regulatory network in pathogenicity and OTA biosynthesis of *A. carbonarius*.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

OB, DP, YB, and ES conceived and designed the experiments. OB, UM, SS, and VZ performed the experiments. OB, SS, DP, YB, and ES analyzed the data. OB, DP, and ES wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00210/full#supplementary-material

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frontiers in Microbiology

REVIEW
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Plant Bioactive Compounds in Pre- and Postharvest Management for Aflatoxins Reduction

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Aflatoxins (AFs) are secondary metabolites produced by *Aspergillus* spp., known for their hepatotoxic, carcinogenic, and mutagenic activity in humans and animals. AF contamination of staple food commodities is a global concern due to their toxicity and the economic losses they cause. Different strategies have been applied to reduce fungal contamination and AF production. Among them, the use of natural, plant-derived compounds is emerging as a promising strategy to be applied to control both *Aspergillus* spoilage and AF contamination in food and feed commodities in an integrated pre- and postharvest management. In particular, phenols, aldehydes, and terpenes extracted from medicinal plants, spices, or fruits have been studied in depth. They can be easily extracted, they are generally recognized as safe (GRAS), and they are food-grade and act through a wide variety of mechanisms. This review investigated the main compounds with antifungal and anti-aflatoxigenic activity, also elucidating their physiological role and the different modes of action and synergies. Plant bioactive compounds are shown to be effective in modulating *Aspergillus* spp. contamination and AF production both *in vitro* and *in vivo*. Therefore, their application in pre- and postharvest management could represent an important tool to control aflatoxigenic fungi and to reduce AF contamination.

Keywords: *Aspergillus*, aflatoxins, reduction, bioactive compounds, plant extracts

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INTRODUCTION

Aflatoxins (AFs) are toxic secondary metabolites, mainly produced by *Aspergillus* spp., which are recognized as human carcinogens (AFs of the B and G series) and possible carcinogens (AFs of the M series). They represent a great health concern (Kumar et al., 2017). Toxic outcomes, also known as aflatoxicosis, may include liver cancer, hepatotoxicity, immune system depression, and impaired growth both in humans and animals (IARC, 2012). AF maximum limits are regulated in Europe; products exceeding the maximum levels cannot be placed on the market nor mixed with uncontaminated ones (European Commission, 2006). From a chemical point of view, AFs (**Figure 1**) are difuranocoumarins composed of two furan rings arranged to a coumarin moiety in a rigid and planar structure (Loi et al., 2017). The high chemical stability endows them with high resistance to heat treatments, extreme pH values, high pressures, and mild (food grade) chemical treatments. As a result, the contamination persists in processed products, including those deriving from animals. Meat, milk, and eggs may also be contaminated with

AF metabolites, mainly originating from *in vivo* hydroxylation reactions (AF of the series M, aflatoxicol, aflatoxin Q_1, and aflatoxin P_1). AF contamination is a major problem in tropical and subtropical regions, where the environmental conditions are extremely favorable to fungal growth and AF production. However, in the last years, also Mediterranean areas have suffered from severe AF contamination due to climate change, temperature rise, and recurrent droughts (Moretti et al., 2019). AF management is a complex task, requiring actions at every stage of the supply chain (**Figure 2**). The application of the Good Manufacturing Practices (GAPs), i.e., crop rotation, the use of fungicides, and resistant varieties, is the first critical practice to prevent and reduce fungal contamination. However, the GAPs alone are not sufficient to avoid AF contamination, as it may depend upon several biotic and abiotic factors, also during storage (Mahuku et al., 2019). Therefore, the postharvest management is essential to manage AF contamination throughout the whole supply chain (Leslie and Logrieco, 2014).

Aspergillus spp. contamination can be detected in samples by several approaches. A basic microbiological diagnosis with chromogenic substrates was developed for the detection of toxigenic fungi, including *Aspergillus flavus*, *Aspergillus carbonarius*, and *Aspergillus ochraceus*. The great advantage is the use of basic laboratory equipment, a relatively low cost, and time for analysis (48–72 h). However, being a very generic growth test, it can be used only as a rapid screening test (Jefremova et al., 2016). On the contrary, advanced molecular PCR-based tools can be used to tackle conserved genes in *Aspergillus* spp. and AF biosynthetic gene cluster in contaminated materials (Moretti and Susca, 2017).

Controlling humidity, temperature, and moisture are among the most effective management strategies to cope with fungal spoilage and AF production during the storage and transport of susceptible commodities (Neme and Mohammed, 2017). Physical methods, such as sorting, dehulling, cleaning, and milling, are widely used to remove highly contaminated fractions from cereals during processing. Other physical methods include the use of microwave, UV, pulsed light, electrolyzed water, cold plasma, ozone, and irradiation. Despite their potentialities, their use is still limited due to the high technology cost and the residual toxic potential (Mahato et al., 2019).

Biological methods rely on the application of microorganisms (Liuzzi et al., 2017), pure enzymes (Loi et al., 2018), or enzyme extracts (Branà et al., 2020) able to degrade and, possibly, detoxify mycotoxins. In Europe, they can be authorized as postharvest treatments in feed, as long as safety, efficacy, and non-interference with feed nutrients is proved (Commission Regulation (EU) 2015/786, 2015).

The use of chemicals to prevent fungal growth in the field, in food, and feed products is a common practice worldwide. The use of fungicides and artificial preservatives has raised concern in consumers, researchers, and stakeholders because of the possible residual toxicity, carcinogenicity, and environmental pollution. The possible development of new resistant fungal strains is also a matter of great concern. Therefore, the use of natural compounds may encounter higher consumers' and stakeholders' acceptability (Onaran and Yanar, 2016). Bioactive compounds deriving from plant metabolism belong to greatly diverse chemical groups and possess different biochemical and physiological roles. Therefore, they are considered versatile molecules. Indeed, determining the exact and univocal function of secondary metabolites in plants is a difficult task.

Nonetheless, they share common antimicrobial (Bassolé and Juliani, 2012), antifungal (Tabassum and Vidyasagar, 2013), antioxidant properties (Miguel, 2010), and the capability of improving the postharvest management of vegetable crops (Sivakumar and Bautista-Baños, 2014). Moreover, particular attention is paid to these molecules as bioactive compounds in the human diet because of their high antioxidative capacity (Pisoschi et al., 2016).

Despite their potentialities having been widely investigated in the past, their application as AFs control agents in pre- and postharvest remains still poorly explored. Bioactive compounds have been widely used to inhibit *Aspergillus* growth at different levels (mycelia growth, spore production, germ tube formation), to inhibit the secondary metabolism and AFs production. In addition, their direct use was also found to degrade AFs and, in some cases, detoxify them.

BIOACTIVE COMPOUNDS IN PLANT METABOLISM

Plants are the richest source of bioactive compounds. Bioactive metabolites are classified into primary or secondary metabolites, depending on their functional role (Sharma et al., 2019). Plants and fungi produce thousands of secondary metabolites according to the physiological stage, tissue localization (floral and non-floral leaves, fruits, or bark), environmental conditions, and other biotic or abiotic stress. These compounds may be involved in the primary physiological function of the cell; they may participate in the control of cell growth and cell development, acting as plant growth substances, i.e., plant hormones. Among them, ethylene, auxin, gibberellins, abscisic acid, cytokinins, brassinosteroids, and polyamines are the most important ones (Depuydt et al., 2016). Nevertheless, their main function is ecological, especially with regards to the plant defense against herbivores, bacteria, and fungi (Mithöfer and Maffei, 2017).

Plants cope with pathogen attacks by different types of defense mechanisms, based on either anatomical or biochemical features (passive and constitutive defense), or active changes induced by pathogens (active and inducible defense). In some cases, like for terpenes, compounds can be secreted in low basal amounts constitutively, and expression can be triggered to produce higher amounts upon tissue damage or pathogen attack. Passive or constitutive defense compounds include glucosides, saponins, antifungal proteins, inhibitors of enzymes, and antifeedants, while inducible molecules include phytoalexins, pathogen-related (PR) proteins, chitinases, and glucanases (Walters, 2011).

Metabolites involved in the defense mechanism may occur in glycosylated or conjugated forms, which allow the plant to synthetize and store them in a non-toxic form. The conjugation or their specific localization (i.e., in the vacuoles or other

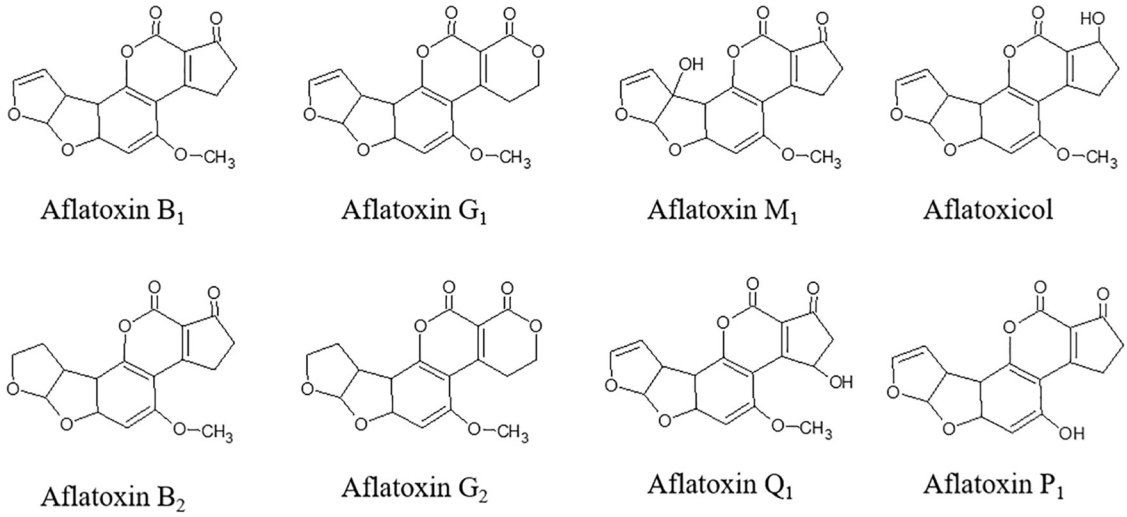

FIGURE 1 | Chemical structure of aflatoxins and their metabolites.

subcellular compartments) are strategies to avoid autoallelopathy and to produce active forms quickly and only when needed (Chaves Lobón et al., 2019).

Conversely, the *de novo* synthesis of antifungal molecules is also observed during the infection process in many plants. These substances are called phytoalexins, and they are similar to the constitutive antifungal toxins, although they show a more lipophilic character. Plants can also produce compounds with animal hormonal activity, the phytoecdysones, which can alter or cause precocious insect development. Finally, they may have a role in establishing the symbiotic processes with beneficial fungi and lichens (Ghasemzadeh and Ghasemzadeh, 2011).

Bioactive compounds can be extracted by different techniques: Soxhlet extraction, maceration, and hydrodistillation are classically used. The use of ultrasounds, microwaves, electric fields, high pressures, or supercritical fluids have been investigated to reduce the use of solvents and apply gentler extraction conditions (Azmir et al., 2013; Giacometti et al., 2018). Water traces can be removed to obtain a concentrated extract, also referred as to essential oil (EO). On the basis of the biosynthetic origin, secondary metabolites can be divided into three main groups: (i) phenolics, (ii) terpenes, and (iii) nitrogen-containing compounds. With regards to the antifungal and antiaflatoxin activity, the most important bioactive secondary compounds are reported in **Figure 3**.

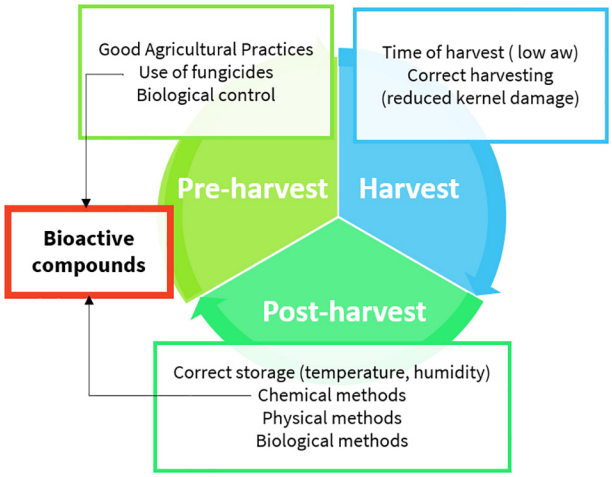

FIGURE 2 | Aflatoxin management practices (details are provided in the text).

(i) Phenolic Compounds

The term phenolic compounds generally includes compounds bearing one or more hydroxylated aromatic rings and are subgrouped into phenolic acids, stilbenes, flavonoids, lignans, and ellargic acids. The flavonoids subgroup comprises a wide variety of simple compounds like anthocyanins, flavonols, chalcones, flavanones, flavones, and isoflavones or complex ones, such as condensed tannins (Zhang and Tsao, 2016). Thanks to their hydroxyl and carboxyl moieties, polyphenols act as antioxidants. They modulate the cellular redox status by directly quenching free radicals and chelating metal ions (promotors of oxidative reactions). They also activate redox-sensitive transcription factors for the antioxidative enzymes (superoxide dismutase, catalase, and glutathione peroxidase) (Upadhyay and Dixit, 2015). Protein binding and inhibition is mediated by hydrogen bonds between hydroxyl moiety of phenols and the carboxyl and thiol groups of proteins. Conversely, the aromatic ring is able to interact with proteins through van der Waals (hydrophobic) interaction.

Structure–activity relationships of two phenol derivatives (cinnamaldehyde and eugenol) were studied on two phytopathogenic fungi, namely, *Rhizoctonia solani* and

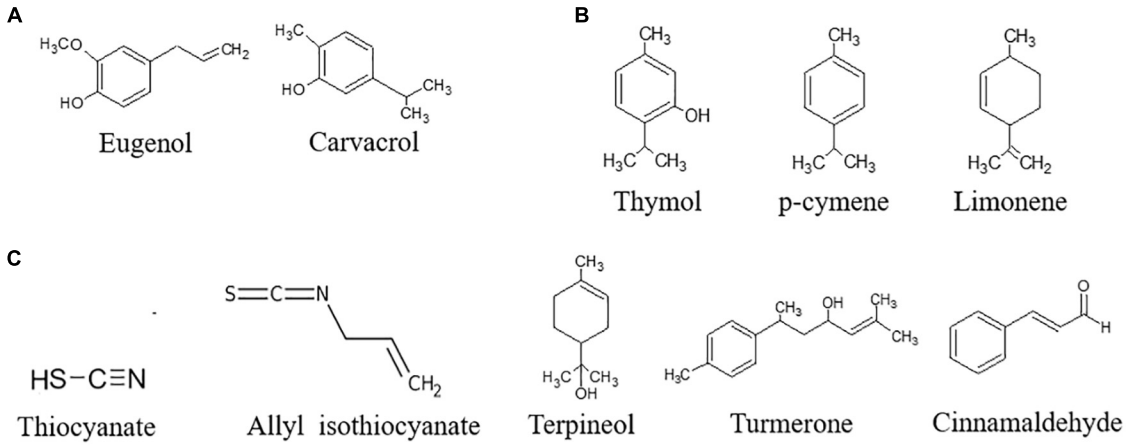

FIGURE 3 | Chemical structure of the most important bioactive secondary compounds with antifungal and antiaflatoxin activity. **(A)** Phenols, **(B)** Terpenes, **(C)** N-containing compounds.

Fusarium oxysporum. Phenol antifungal activity was shown to depend on the chemical structure. In particular, aldehydes, acid groups, conjugated double bonds, and the length of CH chain outside the ring have increased the antifungal activity (Xie et al., 2017). While aldehydes and acid groups may be more prone to react with amino acid residues of proteins through hydrogen bonds and induce conformational modification because of the proton release ability, the length of the CH chain increases hydrophobicity, a major determinant of phenol capability to enter the plasma membrane (Ben Arfa et al., 2006; Dambolena et al., 2011).

(ii) Terpenes

Terpenes are volatile compounds deriving from the condensation of two or more isoprene molecules. They represent the largest class of plant compounds, with more than 40,000 different chemical structures. They are usually synthetized and stored in trichomes or secretory glands to be secreted constitutively or released as a consequence of tissue damages. Their function in plant metabolism is extremely diverse. Terpenes act as radical scavenging molecules against UV light damage and other environmental stresses. The double bonds can absorb high-energy radiation or scavenge free radicals, functioning as a first defense mechanism. Nonetheless, not all terpenes have a defensive function. Volatile terpenes are generally released constitutively to act as attractants to pollinators and symbionts, repellents to herbivores, or as signaling molecules to other plants or plant tissues. Polyisoprene intermediates are used in the post-translational modification of prenylated proteins (Pichersky and Raguso, 2018). Limonene, carvone, carvacrol, linalool, thymol, terpineol, myrcene, linalool, and pinene are the most important ones, with regards to the antifungal activity against *Aspergillus* spp. The latter activity is mainly due to their lipophilic nature, which allows them to enter the cell and interact with the cellular and mitochondrial membranes, and cause alteration in cell permeability and electrochemical potential (Tian et al., 2012b).

(iii) Nitrogen-Containing Compounds

Nitrogen-containing compounds are a heterogeneous group, which share the presence of at least one nitrogen atom in their chemical structure: glucosinolates, alkaloids, and cyanogenic glucosides are the main classes. All of them have defensive functions, but only glucosinolates have been recently exploited as antifungal and antiaflatoxin agents (Kaur et al., 2011).

With this regard, volatile compounds from Brussels sprouts (*Brassica oleracea* L. var. gemmifera DC.), cabbage (*Brassica oleracea* L.), kale (*Brassica oleracea* var. sabellica), radish (*Raphanus sativus* L.), and broccoli (*Brassica oleracea* L. var. botrytis L.) were extensively studied. Among them, the most important one is ally-l-isothiocyanate, a β-thioglycoside formed after the hydrolysis of glucosinolates by the enzyme myrosinase (Kumar et al., 2019). Hydrolysis occurs upon tissue damage, since glucosinolates are safely stored in the vacuole. Nitriles may be also produced as secondary products of the reaction. Thiocyanates and nitriles are hydrophilic compounds with high antioxidant capacity. They participate in plant defense systems as allelochemicals, volatile repellents, in the transcriptional regulation of the heat stress response, sulfur metabolism, water transport, stomatal opening, cell growth, and apoptosis (Bones et al., 2015). The isothiocyanate group (–N=C=S) is highly nucleophilic and able to bind thiols, amino groups of amino acids, peptides, and proteins. The antifungal and antiaflatoxin properties are mainly due to the inactivation of crucial enzymes, such as reductases, acetate kinases, and oxidases (Nazareth et al., 2016).

ANTIFUNGAL ACTIVITY OF BIOACTIVE COMPOUNDS

Natural plant extracts have been widely used since ancient times for their antimicrobial activity against insects, bacteria, and fungi (Bakkali et al., 2008). Many of them are already

employed as pharmaceuticals, feed and food additives, cosmetics and perfume ingredients because of their antioxidant capacity and strong organoleptic properties. Recently, their composition and biological activity have been investigated in relation to the antifungal activity and the ability to inhibit AF production by *Aspergillus* spp.

Carvacrol (Gómez et al., 2018), cinnamaldehyde (Bang et al., 2000; Xie et al., 2004; Tian et al., 2012b; Sun et al., 2016; Khorasani et al., 2017; Gómez et al., 2018), eugenol (Khorasani et al., 2017), limonene (Sharma and Tripathi, 2008; Rammanee and Hongpattarakere, 2011), p-cymene (Pinto et al., 2013), terpineol (Tian et al., 2012b; Kohiyama et al., 2015), thymol (Marei et al., 2012; Gorran et al., 2013; Kohiyama et al., 2015; Shen et al., 2016), and turmerone (Ferreira et al., 2013) are the main active compounds of cinnamon (*Cinnamomum verum* J. Presl), clove [*Syzygium aromaticum* (L.) Merr & L.M. Perry], lemon [*Citrus* × *limon* (L.) Burm. f.], oregano (*Origanum vulgare* L.), and thyme (*Thymus vulgaris* L.) extracts. Their structure is shown in **Figure 2**, and the main effects as antifungal agents in plant extracts or as pure compounds are presented in **Tables 1A** and **1B**, respectively.

Those compounds generally act synergistically and in a dose-dependent manner. The highest effects were registered using increasing amounts of bioactive compounds and the whole EOs instead of single compounds (Tian et al., 2012a; Ferreira et al., 2013; Pinto et al., 2013).

Plant extracts are very complex mixtures, and their composition varies according to plant species and chemotype, phenological stage, tissue, and method of extraction (Figueiredo et al., 2008). Accordingly, their effect often has multiple targets (**Figure 4**) and different modes of action (**Figure 5**). They induce cytotoxicity through multiple pathways: (i) disrupting cell membrane permeability and functionality; (ii) inhibiting enzymes involved in the synthesis of cell wall components; (iii) impairing ergosterol metabolism; (iv) inducing ultrastructural alterations in cell compartments leading to swelling, vacuolations, and cation leakage; (v) inhibiting cytoplasmic and mitochondrial enzymes; and (vi) altering the osmotic and the redox balance.

Effects on Cell Wall and Cell Membrane

Fungal cell wall is a dynamic component, essential to assure cell viability. Moreover, it is involved in multiple cell functions, including morphogenesis and pathogenesis. Chitin, glucans, and pectins are the major building blocks, and they are continuously remodeled to cope with cell growth and differentiation by enzymes, such as chitin and glucan synthases, glycohydrolases, and transglycosidases (Gow et al., 2017). Therefore, these enzymes are perfect physiological targets to inhibit fungal growth.

An extensive survey on the antifungal activity of 13 different commercially available monoterpenes was performed by Marei et al. (2012). Among all tested compounds, thymol, followed by limonene, had the highest inhibitory effect on cellulase and pectin methyl esterase enzymes of *Aspergillus niger*, *F. oxysporum*, and *Penicillium digitatum*. The rate of inhibition on *A. niger* was higher for the pectin methyl esterase (IC_{50} at 1.28 mg L^{-1}) rather than for the cellulase (IC_{50} at 44.56 mg L^{-1}). Cinnamaldehyde was found to be a non-competitive inhibitor of chitin synthase (IC_{50} at 111.0 mg L^{-1}) and *b*-(1,3)-glucan synthase (IC_{50} at 190.3 mg L^{-1}) (Bang et al., 2000).

Ergosterol is the main sterol derivative of fungi, and it is essential to preserve cell membrane functionality as cholesterol does in animal cells. In addition, it is essential to ensure the activity of membrane-bound enzymes. Owing to its essential role in fungal cells, many fungicides act by inhibiting its biosynthesis or binding it in the cell membrane (Sant et al., 2016). Phenols and aldehydes possess a sufficient hydrophobicity to pass the double phospholipid bilayer, to interact with ergosterol in the cell membrane, or to enter the nucleus and act as regulators for its biosynthesis. As a consequence, alteration of fatty acid profiles along with modification of cell membrane, osmotic imbalance leading to irreversible damage of the hyphae membranes, conidiophores, and death occur (Ansari et al., 2013).

Cinnamomum spp. EO or its main component, cinnamaldehyde, were reported to impair ergosterol biosynthesis at concentrations as low as 2 mg L^{-1} (Tian et al., 2012b) and to cause irreversible deleterious morphological and ultrastructural degenerative alterations of the fungal cell membrane at 104 mg L^{-1} (Sun et al., 2016; Khorasani et al., 2017). The same effect on fungal morphology was described for *Thymus vulgaris* L. (at 2,500 mg L^{-1}) (Kohiyama et al., 2015), *Curcuma longa* L. (Ferreira et al., 2013; Hu et al., 2014, 2017), and *Anethum graveolens* L. EOs (at 2 and 100 mg L^{-1} *in vitro* and in cherry tomatoes, respectively) (Tian et al., 2011).

Ergosterol biosynthesis may be regulated at the genomic level. Downregulation of ERG7, ERG11, ERG6, ERG3, and ERG5 genes by citral, the major component of lemongrass EO, was indeed reported for *P. digitatum* (OuYang et al., 2016).

Mitochondrial Dysfunction

Mitochondrial membrane potential is maintained in healthy cells by an electrochemical gradient through the electron transport chain, which is, ultimately, the major source of ATP molecules. As ATP levels decrease, the normal metabolic functions slow down until cell death occurs. The mechanism of action is not clearly understood. Several hypotheses have been made, including a direct inhibition of ATPases (see *Enzyme Inhibition*) and disruption of the osmotic balance, mainly causing calcium and protons leaking and, consequently, of the electrochemical potential. As for polygodial, a naturally occurring sesquiterpene dialdehyde isolated from different plant species, the mechanism was studied in depth, although with mammalian mitochondrial preparations. In this case, direct inhibition of enzymes was excluded. Indeed, the mechanism was supposed to rely on the uncoupling of the mitochondrial ATPase due to the modification of the electric properties of the membrane surface (Castelli et al., 2005). In yeasts, carvacrol was also responsible for the induction of calcium stress, mediated by the activation of specific intracellular signaling pathways (Rao et al., 2010).

TABLE 1A | Antifungal activity of plant extracts on *Aspergillus* spp.

Plant/compounds	Type of extract or oils	Major components	Antifungal activity	Concentration of active compound(s) (mg L^{-1} or ml L^{-1})	References
Jojoba oil		Gadoleic acid, erucic acid	Growth inhibition of *Aspergillus parasiticus*, *A. ochraceus*, *Fusarium solani*, *Penicillium* sp.	n.p.	Badr et al. (2017)
Jojoba pomace extract	Aqueous isopropyl extract, pH 4–5	84.7% phenols, 15.3% flavonoids			
Jatropha oil		Linoleic acid, oleic acid			
Jatropha pomace extract	Aqueous isopropyl extract, pH 4–5	78.4% phenols, 21.6% flavonoids			
Mentha (*Mentha pulegium L.)*	Aqueous extract	n.p.	Growth inhibition of *A. flavus*	8,000	Omidpanah et al. (2015)
Senna (*Cassia senna* L.)				6000	
Basil (*Ocimum basilicum* L.)				8,000	
Thyme (*Thymus vulgaris* L.)				2,000	
Safflower (*Carthamus tinctorius* L.)				4,000	
Hairy cistus (*Cistus incanus* L.)	Methanolic extract	n.p.	Growth inhibition of *A. parasiticus*; up to 90% of reduction of AFB_1 production in YES medium and 86% in macadamia nuts	n.p.	Kalli et al. (2018)
Cinnamon (*Cinnamomum zeglanicum* Garcin ex Blume)	Diluted water extract (3, 5, 7, and 9% *v/v*)	88.7% cinnamaldehyde	Up to 100% growth inhibition of *Aspergillus flavus* on PDA extract (using 3% extract after 1 day); 100% reduction of AFB_1 production in pistachio nuts	n.p.	Khorasani et al. (2017)
Clove (*Caryophyllus aromaticus* L.)		71.1% eugenol			
Thyme (*Thymus daenensis* Celak)		Thymol (73.9%) and carvacrol (6.7%)	Up to 100% growth inhibition of *Aspergillus flavus* on PDA extract (using 7% extract after 1 day); up to 100% reduction of AFB_1 production in pistachio nuts		
Oregano (*Origanum vulgare* L.)	Commercially available essential oil	86% carvacrol	Growth inhibition of *A. parasiticus and A. flavus* in maize extract medium under different environmental conditions (25–37°C, aw 0.99–0.96)	152–505	Gómez et al. (2018)
Cinnamon (*Cinnamomum verum* J. Presl)		66.5% cinnamaldehyde		295–675	

(Continued)

TABLE 1A | Continued

Plant/compounds	Type of extract or oils	Major components	Antifungal activity	Concentration of active compound(s) (mg L^{-1} or ml L^{-1})	References
Cinnamon *(Cinnamomum jensenianum* Hand.-Mazz.*)*	EO obtained by hydrodistillation	17.3% eucalyptol, 12.5% α-terpineol	Growth inhibition of *A. flavus, A. oryzae*, *A. niger*; up to 100% of reduction of AFB_1 production	2 (*in vitro*)–120 (*in vivo*)	Tian et al. (2012b)
Dill (*Anethum graveolens* L.)	EO obtained by hydrodistillation	n.p.	Growth inhibition of *A. flavus* by disruption of mitochondrial membrane potential (MMP), acidification of external medium, and mitochondrial ATPase and dehydrogenase activities	25–2,000	Tian et al. (2012a)
Dill (*Anethum graveolens* L.)	EO obtained by hydrodistillation	n.p.	100% growth inhibition of *Aspergillus* spp.	2	Tian et al. (2011)
			Up to 86.1 and 94.4% growth inhibition of *Aspergillus* spp. in healthy and wounded cherry tomatoes, respectively	100	
Thyme (*Thymus vulgaris* L.)	EO obtained by hydrodistillation	40.6% borneol, 19.9% α-terpineol, 12.3% camphene	Growth inhibition of *A. flavus*, up to 100% of reduction of AFB_1 production	1,500 (for AFB_1 reduction); 2,500 (for *A. flavus* growth inhibition)	Kohiyama et al. (2015)
Thyme (*Thymus daenensis* Celak)	Hydrodistillates resuspended in ethanol	n.p.	Mycelial growth and spore production inhibition of *A. flavus*, up to 100% of reduction of AFB_1 production	350	Gorran et al. (2013)
Savory (*Satureja khuzestanica*)					
Savory (*Satureja macrosiphonia* Bornm)			Mycelial growth and spore production inhibition of *A. flavus*, up to 56.8% of reduction of AFB_1 production	500	
Turmeric (*Curcuma longa* L.)	Hydrodistillates resuspended in 5% (*v/v*) Tween-20	Oxygenated sesquiterpenes (60.7%) Sesquiterpene hydrocarbons (34.3%)	Up to: 93.41% mycelial growth inhibition; 93.41% spore germination inhibition; 74.6% activity inhibition of mitochondrial ATPase and 84.7% dehydrogenases activity inhibition	n.p.	Hu et al. (2014)
Turmeric (*Curcuma longa* L.)	EO obtained by hydrodistillation	ar-turmerone (33.2%), α-turmerone (23.5%), ß-turmerone (22.7%)	Up to 99.0% inhibition of AFB_1 production using 5% (*w/w*) of extract	n.p.	Ferreira et al. (2013)
Ferula (*Ferulago capillaris Link ex Spreng.)*	EO obtained by hydrodistillation	α-Pinene (35.8%) and limonene (30.9%)	Inhibition of *Aspergillus* spp. growth	640–1,250	Pinto et al. (2013)

n.p., not provided.

TABLE 1B | Antifungal activity of pure commercial compounds on *Aspergillus* spp.

Plant/compounds	Antifungal activity	Concentration of active compound(s) (mg L^{-1} or ml L^{-1})	References
Isothiocyanate	Up to 100% of inhibition of *A. parasiticus* growth and aflatoxin production	0.01	Nazareth et al. (2016)
Isothiocyanate	Corn kernels	≥0.00005	Tracz et al. (2017)
Allyl isothiocyanate	Inhibition of *A. flavus* growth and aflatoxin production in corn, barley, and wheat in simulated silo system	0.0005	Quiles et al. (2015)
Allyl isothiocyanate	Inhibition of *Aspergillus parasiticus* growth and aflatoxin production in Brazil nuts	0.0000025	Lopes et al. (2018)
Curcumin	Up to 96.0% inhibition of AFB_1 production using 0.5% (*w/w*) of extract	n.p.	Ferreira et al. (2013)
Cinnamaldehyde	Inhibition of radial growth, spore, and aflatoxin production of *A. flavus*	104	Sun et al. (2016)
Camphene	Mycelial growth inhibition of *F. oxysporum, A niger, P. digitatum;* inhibition of pectin methyl esterase, cellulase, and polyphenol oxidase enzymes	From 121.5 to 314.2	Marei et al. (2012)
(R)-Camphor		From 157.1 to 367.0	
(R)-Carvone		From 432.5 to 120.0	
1,8-Cineole		From 36.4 to 148.4	
Cuminaldehyde		From 79.5 to 363.5	
(S)-Fenchone		From 193.8 to 330.6	
Geraniol		From 73.9 to 357.0	
Carbendazim		From 13.6 to 37.38	
(R)-Linalool		From 266.6 to 73.7	
(1R,2S,5R)-Menthol		From 121.9 to 394.4	
Myrcene		From 95.5 to 336.9	
Thymol		From 20.1 to 50.4	
(S)-Limonene		From 26.8 to 153.2	

n.p., not provided.

Enzyme Inhibition

Mitochondrial dysfunction may also occur via ATPase inhibition. Dill (*Anethum graveolens* L.) EO was shown to affect mitochondrial and plasma membrane ATPase at 0.08–0.64 ml L^{-1} (Pinto et al., 2013), while turmeric (*C. longa* L.) EO was shown to suppress mitochondrial dehydrogenases and mitochondrial ATPase at 2–8 ml L^{-1} (Hu et al., 2014). Turmeric EO was also found to exert antifungal activity via ATPase, malate dehydrogenase, and succinate dehydrogenase inhibition at 1–8 ml L^{-1} *in vitro* and 4 ml L^{-1} in maize (Hu et al., 2017). The reactivity of phenols and aldehydes in EOs to proteins and enzymes is the major mechanism, as reported for isothiocyanates.

Isothiocyanate were successfully used to inhibit *Aspergillus parasiticus in vitro* at doses of 5 mg (Manyes et al., 2015) or even in gaseous form in foods at concentrations of 100.01 ml L^{-1} in wheat flour (Nazareth et al., 2016), at ≥0.05 ml L^{-1} in corn kernels (Tracz et al., 2017), at 0.5 ml L^{-1} in corn, barley, and wheat in simulated silo system (Quiles et al., 2019), at 0.0025 ml L^{-1} in Brazil nuts (Lopes et al., 2018), and at 46,040 and 78,250 mg/kg in the Italian "piadina" (Saladino et al., 2016).

INHIBITORY EFFECT ON AFLATOXIN B_1 PRODUCTION

Aflatoxins are polyketide-derived furanocoumarins, the production of which depends upon 25 different genes, clustered together in a 70-kb DNA sequence region. The majority of the genes encodes for enzymes involved in the synthesis and participates as transcription factors, while others do not have a clear assigned function (Yu et al., 2004).

Many physiological events in fungal cells are regulated by oxidative bursts such as differentiation, switch from conidia to germ tube development, and the onset of secondary metabolism. In particular, oxidants are able to induce AF biosynthesis (Reverberi et al., 2006). In the presence of oxidative stress, the fungal antioxidant molecules (tocopherols, ascorbic acid, carotene, reduced glutathione) and enzymes (superoxide dismutase, catalase, and glutathione peroxidase) are induced concomitantly to AF biosynthetic gene cluster (Reverberi et al., 2010). Therefore, it was also suggested that AF production may also be a way to incorporate oxygen atoms and protect cells from oxidative damage (Kim et al., 2005). The mechanism of EOs or their components may be associated with their antioxidant activity, responsible for the attenuation of the fungal oxidative stress responses, thus AF production (Kim et al., 2005; Reverberi et al., 2005).

Different compounds have been proven to inhibit the production of secondary metabolites like AFB_1, at comparable or slightly lower concentrations than those that inhibit the mycelial growth, which is consistent with their supposed mode of action. The different inhibition pattern suggests that the suppressive effect is elicited

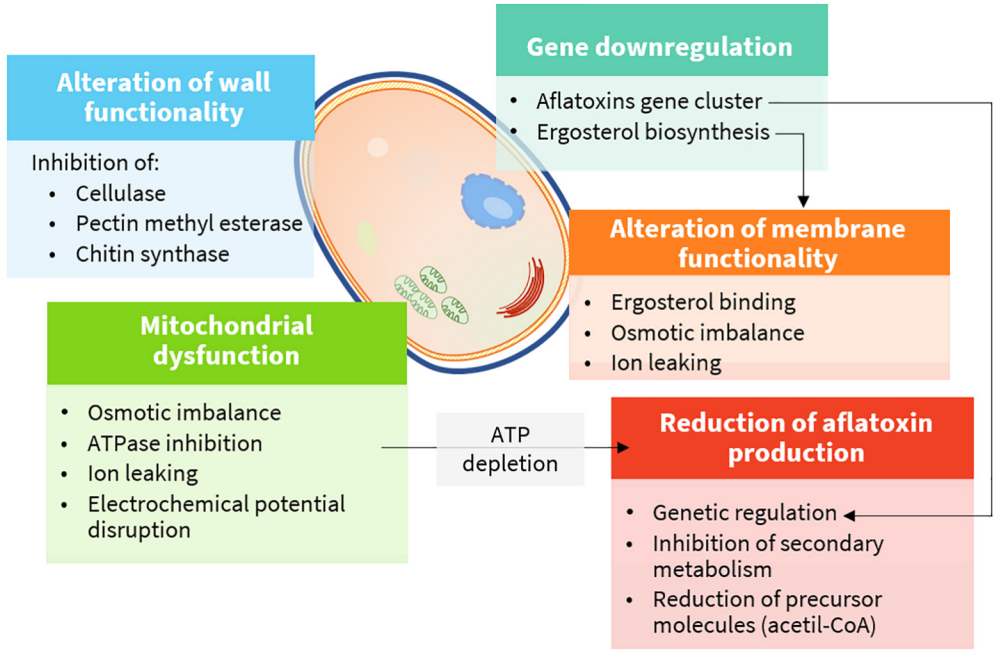

FIGURE 4 | Cellular targets and mechanisms of action of bioactive compounds (details are provided in the text).

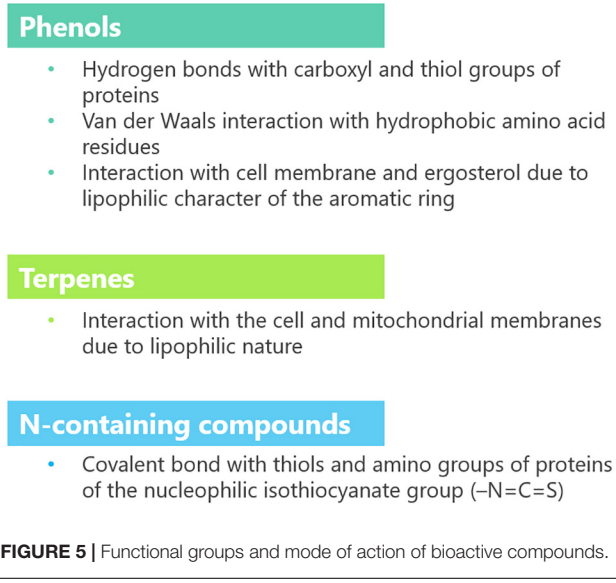

FIGURE 5 | Functional groups and mode of action of bioactive compounds.

on transcriptional regulators (AflR and AflS) as well as on structural genes (Georgianna and Payne, 2009), as reported in **Table 2**.

Eugenol was proved to be effective in downregulating aflM, aflD, aflC, aflP, aflR (Jahanshiri et al., 2015), aflP, aflM, aflD, and aflT (Liang et al., 2015; Lv et al., 2018) genes. Conversely, turmeric EO downregulated aflD, aflM, aflO, aflP, and aflQ genes (Hu et al., 2017). In a recent study by Moon et al. (2018) γ-terpinene was found to downregulate aflC, aflD, aflE, aflK, aflO, and aflQ genes, whereas citral downregulated aflD, aflE, aflK, aflL, aflO, aflQ, aflR, aflS, aflC, and aflG.

Finally, inhibition of the secondary metabolism as a consequence of the reduced fungal growth and ATP and AF precursor depletion (acetyl coenzyme A) by mitochondrial dysfunction may contribute to the general antiaflatoxigenic effect of these compounds (Tian et al., 2011).

AFLATOXIN DEGRADATION ACTIVITY

Various plant extracts were reported to degrade AFB_1 as well as other mycotoxins both *in vitro* and *in vivo*, as reported in **Table 3**.

In most of the studies, the active agents were water soluble, belonged to the flavonoids and phenol groups. Besides the activity of those low molecular weight compounds, the possible coextraction of enzymes able to degrade mycotoxins has to be taken into account. In fact, a detrimental effect on the degrading activity was observed after boiling, while no effect was registered after dialysis with 10–14 kDa cutoff membrane. This suggests that heat-sensitive, high-molecular weight compounds may play a significant role in AF degradation (Vijayanandraj et al., 2014; Ponzilacqua et al., 2019). Indeed, many enzymes, also belonging to plants, have been described for their ability to degrade AFs (Loi et al., 2017; Lyagin and Efremenko, 2019). Among them, polyphenol oxidases and laccases may also use low molecular weight compounds as redox mediators, thus enhancing their degradation capability through a synergistic or additive mechanism (Loi et al., 2018).

Although the mechanism of action is not clearly understood, some authors evaluated the outcome of the degradation

TABLE 2 | Aflatoxins genes regulated by bioactive compounds.

Gene	Function	Bioactive compound and references
aflC previously known as pksA	Polyketide synthase	Eugenol (Jahanshiri et al., 2015) γ-terpinene (Moon et al., 2018)
aflD previously known as nor-1	Reductase	Eugenol (Jahanshiri et al., 2015; Liang et al., 2015; Lv et al., 2018) Turmeric EO (Hu et al., 2017) γ-terpinene and citral (Moon et al., 2018)
aflE	Reductase	γ-Terpinene (Moon et al., 2018)
aflK	Versicolorin synthase	γ-Terpinene (Moon et al., 2018)
aflL	Desaturase	Citral (Moon et al., 2018)
aflM previously known as ver-1	Dehydrogenase/ ketoreductase	Eugenol (Jahanshiri et al., 2015; Liang et al., 2015; Lv et al., 2018) Turmeric EO (Hu et al., 2017)
aflO	Oxidoreductase/P450 monooxygenase	Turmeric EO (Hu et al., 2017) γ-Terpinene and citral (Moon et al., 2018)
aflP previously known as omtA	Methyltransferase	Eugenol (Jahanshiri et al., 2015; Liang et al., 2015; Lv et al., 2018) Turmeric EO (Hu et al., 2017)
aflQ	*O*-Methyltransferase	γ-Terpinene (Moon et al., 2018) Citral (Moon et al., 2018)
aflR	Transcriptional regulator	Eugenol (Jahanshiri et al., 2015 Citral (Moon et al., 2018)
aflS	Transcription enhancer	Citral (Moon et al., 2018)
aflT	Transmembrane protein	Eugenol (Liang et al., 2015; Lv et al., 2018)

by high-performance liquid chromatography (HPLC) and liquid chromatography tandem MS (LC-MS/MS). The chemical properties of AFs were deeply modified upon incubation with plant extracts. AFB_1 was modified in different ways, including the removal of the double bond of the furan ring and the modification of the lactone ring, resulting in a significant decrease in the cytotoxicity, evaluated on Hela cells (Velazhahan et al., 2010) and by Brine shrimps (*Artemia salina*) bioassay (Iram et al., 2015, 2016a,b). The toxic and carcinogenic potential of AFB_1 was indeed attributed to the difuran ring, which *in vivo* is quickly oxidized to 8,9-epoxy-AFB_1 and, to a lesser extent, to the lactone moiety (Loi et al., 2016).

DISCUSSION

The use of natural compounds in pre- and postharvest appears appealing, especially when compared to the use of antibiotics or fungicides from synthetic origin. Natural flavoring compounds derived from plants were listed as GRAS compounds in Europe and the United States: among others, clove, marjoram, thyme, nutmeg, basil, mustard, and cinnamon. However, despite their proven *in vitro* efficacy and their GRAS status, the use of those compounds as a pre- or postharvest treatment has different limitations: high volatility, poor stability due to oxidation reactions, and strong organoleptic features. This latter may lead to unpleasant tastes and off-flavors in food and feed or interfere with the signaling pathway mechanisms mediated by volatile compounds in the field. To overcome these limitations, different technologies have been studied to deliver bioactive components while preserving them from unwanted chemical reactions and controlling the organoleptic impact. Emulsification, spray drying, coaxial electrospray system, freeze drying, coacervation, *in situ* polymerization, extrusion, fluidized bed coating, and supercritical fluid technology are the most promising ones (Bakry et al., 2016). EOs can be also incorporated in edible coatings (Peretto et al., 2014; Alotaibi et al., 2019), films (Giteru et al., 2015), or even sprayed on food in a vapor form (Gao et al., 2014).

Among the different proposed technologies, the encapsulation of EOs has many advantages, i.e., even dispersion and release of EOs, odor masking, increased shelf life, and improved technological properties (easy dosing and pouring, increased solubility, dust-free material) (Wu et al., 2012; da Rosa et al., 2015).

The antifungal activity of encapsulated eugenol, menthol, and t-anethole (Kumar et al., 2019), *Illicium verum* Hook. f. (Dwivedy et al., 2018), *Cinnamomum zeylanicum* Garcin ex Blume (Kiran et al., 2016), and *Coriandrum sativum* L. (Das et al., 2019). EOs was investigated *in vitro* toward *A. flavus*, and was shown to reduce AFB_1 production with promising results. A recent study by Mateo et al. (2017) investigated the antiaflatoxigenic potential of a bioactive packaging based on ethylene-vinyl alcohol copolymer films incorporating EOs from *O. vulgare* L., *C. zeylanicum* Garcin ex Blume, or their major active constituents, carvacrol and cinnamaldehyde. On the contrary, the antifungal activity of allyl isothiocyanate was completely lost upon encapsulation (Janatova et al., 2015). This means that specific delivery systems have to be developed for each EO or bioactive compound.

Moreover, the effectiveness of the preharvest treatments also depends upon several biotic and abiotic factors. The treatment response may vary according to the specific plant species or cultivar, due to the activation of cultivar-specific defense pathways and different host–pathogen interaction patterns (Feliziani et al., 2015). Weather conditions and the phenological stage at the delivery may also affect the results of the treatment in the field.

Few *in vivo* trials were conducted to evaluate the efficacy of the use of natural compounds as antifungal agents, even though they focused on the reduction in the postharvest decay (Sivakumar and Bautista-Baños, 2014; Feliziani et al., 2015).

As regards the postharvest treatments, food matrix and composition, lipid content, water activity, pH, and enzymes can decrease their effectiveness as an antimicrobial or antifungal compound (Hyldgaard et al., 2012). Therefore, with respect to the *in vitro* studies, 1–3% higher amounts may be needed to achieve the same results (Firouzi et al., 2007). Nonetheless, when high amounts are used, the organoleptic properties of the food may be impaired. To overcome this issue, lower concentrations

TABLE 3 | Degradation activity of plant extract on aflatoxin B_1 (AFB_1).

Plant	Type of extract/oils	*In vitro/in matrix* AFB_1 reduction	Relative toxicity of AFB_1 degradation products	References
Araçá (*Psidium cattleianum*)	Aqueous extract	Up to 30% of AFB_1 degradation (16.67 μg/L) after 48 h of incubation in aqueous medium, pH 6.0–7.0	n.p.	Ponzilacqua et al. (2019)
Rosemary (*Rosmarinus officinalis* L.)		Up to 60% of AFB_1 degradation (16.67 μg/L) after 48 h of incubation in aqueous medium, pH 6.0–7.0	n.p.	
Oregano (*Origanum vulgare* L.)		Up to 38% of AFB_1 degradation (16.67 μg/L) after 48 h of incubation in aqueous medium, pH 6.0–7.0	n.p.	
Basil (*Ocimum basilicum* L.)	Aqueous extract	Up to 90% of AFB_1 degradation (100 μg/L) after 72 h at 60°C in aqueous extract;	70% of mortality reduction by Brine shrimps (*Artemia salina*) bioassay	Iram et al. (2016a)
		In matrix degradation (maize) up to 90.4% of degradation after 72 h of incubation at 30°C, pH 8		
Golden tree *(Cassia fistula* L.*)*		Up to 54% of AFB_1 degradation (100 μg/L) after 72 h at 60°C in aqueous extract;	n.p.	
		Up to 62.5% of AFB_1 degradation (100 μg/L, spiked) in maize after 72 h of incubation at 30°C, pH 8		
Ajowan caraway (*Trachyspermum ammi* L.) Sprague ex Turrill	Aqueous extract	Up to 92.8% of AFB_1 degradation (100 μg/L) after 72 h of incubation at 30°C, pH 8	72% of mortality reduction by Brine shrimps (*Artemia salina*) bioassay	Iram et al. (2016b)
		Up to 89.6% of AFB_1 degradation (100 μg/L, spiked) in maize after 72 h of incubation at 30°C, pH 8		
Lemon Scented Eucalyptus (*Corymbia citriodora*)	Leaf aqueous extract	Up to 95.21% of AFB_1 degradation (100 μg/L) after 72 h of incubation at 30°C, pH 8;	75% of mortality reduction by Brine shrimps (*Artemia salina*) bioassay	Iram et al. (2015)
		Up to 70.26% of AFB_1 degradation (100 μg/L, spiked) in maize after 72 h of incubation at 30°C, pH 8		
Garlic (*Allium sativum* L.)	Aqueous extracts	61.7% of AFB_1 degradation (50 μg/L) after 1 h of incubation at 37°C in PBS medium; 68.3% after 1 h of incubation at 37°C in real-contaminated sample using 50 mg/L of extract	n.p.	Negera and Washe (2019)
Lemon (*Citrus limon* L.)		56.0% of AFB_1 degradation (50 μg/g, spiked) after 1 h of incubation at 37°C in PBS medium; 60.6% after 1 h of incubation at 37°C in real-contaminated sample using 50 mg/L of extract		
Thyme (*Thymus daenensis* Celak)	Hydro-distillates	Up to 97% of AFB_1 degradation (2,000 μg/L) using 2,000 mg/L aqueous extract	n.p.	Gorran et al. (2013)
Savory (Satureja khuzestanica)		Up to 5% of AFB_1 degradation (2,000 μg/L) using 2,000 mg/L aqueous extract		
Savory (*Satureja macrosiphonia* Bornm)		Up to 13% of AFB_1 degradation (2,000 μg/L) using 2,000 mg/L aqueous extract		
Ajowan (*Trachyspermum ammi* L.) Sprague ex Turrill	Seeds aqueous extract	Up to 61% of AFB_1 degradation after incubation at 38°C for 48 h	No chromosomal aberrations induced in corn	Velazhahan et al. (2010)
Basil (*Ocimum tenuiflorum* L.)	Leaves aqueous extract	Up to 74.7% of AFB_1 degradation after incubation at 85°C for 4 h;	73.7% of cytotoxicity reduction on Hela cells	Panda and Mehta (2013)
		Up to 70.2% of AFB_1 degradation (1 μg/g, spiked) in rice after 4 h of incubation at 85°C		

n.p., not provided.

with bacteriostatic or fungistatic effects can be used, or they can be applied in combination with other antimicrobial compounds in a "multiple-hurdle approach" (Prakash et al., 2015; Sudharsan et al., 2019). Few authors evaluated the application in food to reduce AFB_1 contamination, mainly nuts like macadamia (Kalli et al., 2018) and pistachio (Khorasani et al., 2017), obtaining comparable results with respect to the *in vitro* analyses.

Feed Applications

Bioactive compounds are used in feed to enhance (i) the organoleptic characteristics of feed (as feed flavorings), (ii) feed stability (as antioxidants), and (iii) feed digestibility and gut flora stability (as zootechnical additives) [Regulation (EC) No 1831/2003, 2003].

The European Commission approved the use of linalool, thymol, eugenol, carvone, cinnamaldehyde, vanillin, carvacrol, citral, and limonene as flavorings in food products with no restriction. A stepwise approach was adopted to evaluate the safety of those compounds, including the evaluation of the structure–activity relationships, intake from current uses, toxicological threshold of concern, and available data on metabolism and toxicity [Commission Implementing Regulation (EU) No 872/2012, 2012].

Simple and substituted phenols like thymol and carvacrol, have been proposed so far as flavoring additives in feed for all animal species; thus, the demonstration of efficacy was not considered necessary for their approval by the European Food Safety Authority [EFSA], 2012. Thanks to their antioxidant capacity, these compounds enhance the stability, the quality, the palatability of animal feed, and prolong the shelf life.

The so-called "phytogenic" feed additives (PFAs) are simple or complex mixtures of compounds belonging to a wide variety of herbs, spices, EO, or non-volatile extracts, which can be used in feed for various purposes. PFAs can be applied as solid powders, granulated, or also in liquid form to premixtures or complete feeds (Steiner and Syed, 2015).

Bioactive compounds are widely used as zootechnical additives to increase animals weight gain and performance. A general positive effect was shown for feed intake, weight gain, and feed conversion rate in piglets, sows, and poultry, while inconsistent data were registered for apparent digestibility in piglets (Franz et al., 2010; Christaki et al., 2012; Zeng et al., 2015) possibly due to improved secretion of digestive enzymes and bile secretion (Hafeez et al., 2015). A positive effect on gut microbiota in monogastric animals was also reported by several authors (Tiihonen et al., 2010; Bento et al., 2013). On the contrary, there is still no evidence of the *in vivo* efficacy on ruminants, while discordant data are available from *in vitro* studies with ruminal models. EOs may improve nitrogen uptake and energy production but at the same time be toxic for the ruminal microbiota, which produces volatile fatty acid and inhibits ruminal methanogenesis (Giannenas et al., 2013).

Two feed additives made of a mixture of encapsulated EOs (carvacrol, methyl salicylate and L-menthol, thymol, D-carvone) from oregano (*O. vulgare* L.) and from caraway seed (*Carum carvi* L.) were positively evaluated by EFSA as growth enhancers for weaned piglets, chickens for fattening, chickens reared for laying, and minor avian species to the point of lay (European Food Safety Authority [EFSA], 2019a,b).

Despite the different uses in animal nutrition, the use as AF-reducing agents in feed is still unexplored. To be used as a feed additive to reduce AF contamination, EOs shall undergo a scientific assessment by EFSA to assure that several requirements are met: (i) the chemical compound is fully characterized and safe to be used; (ii) it leads to an irreversible and effective detoxification; (iii) the products of the detoxification process are not harmful or are less harmful than the contaminant itself to animals, people, or the environment; and (iv) the chemical and organoleptic characteristics of the feed are not altered (Commission Regulation (EU) 2015/786, 2015). A clear gap of knowledge for the identification of the degradation products and the evaluation of their toxicity currently limits this application.

In vivo studies often show low reliability because the EO composition is usually not fully characterized and active compounds quantified; the effects are not clearly defined because there may be differences in gastrointestinal tract anatomy and functionality also within the same species. When the studies are commercially oriented, some information may be voluntarily scarce (Stevanović et al., 2018). Eventually, limited information is available regarding the interaction between EOs and feed ingredients or other feed additives, such as fibers, probiotics, vitamins, and organic acids (Zeng et al., 2015).

CONCLUSION AND FUTURE PERSPECTIVES

Bioactive compounds from plant species are recognized for their pharmacological and nutraceutical value and are endowed with antifungal and antiaflatoxin activities.

The application of natural compounds deriving from plants to control aflatoxigenic fungi and AF production has been explored mostly *in vitro* in the last 10 years. The mechanisms of action are diverse and mainly target the cell wall, the plasmatic membrane, proteins, and the mitochondrial functionality of fungal cells. Some compounds also act as downregulators of AF biosynthetic pathway, while others have a direct degrading activity toward AF molecules. Limited studies evaluate the applicability of such compounds in food and feed to reduce *Aspergillus* spp. and AFs contamination. Nonetheless, many compounds possess the GRAS status and can be used as food and feed additives in Europe. Bioactive compounds are used as flavoring, antioxidant, and zootechnical additives to improve weight gain and digestibility of feeds in non-ruminant species. Exploring new technologies to extract and use antifungal compounds from food wastes, such as olive oil wastewater or winery by-products, or to deliver such compounds can increase sustainability and lower the cost of these compounds.

Enriching and expanding the genetic repertoire of plant secondary metabolites could help in increasing the plant defense systems. The identification of biosynthetic pathways, plant–host interactions, and varieties with higher content of bioactive compounds are crucial to allow the production of molecules of high commercial value and to improve the safety and quality of plant products. Another possible strategy to counteract AF contamination may be to increase the production of bioactive compound in susceptible commodities.

The major challenges that have to be overcome are the characterization of the active(s) compounds, the standardization of doses and biological activity, the evaluation of interactions in the field or with the food/feed matrix, the identification and the toxicological characterization of the degradation products in the case of the application to AF-contaminated commodities. Nonetheless, the potentialities of these compounds are diverse and may represent a powerful to counteract *Aspergillus* spp. contamination and AF production both in pre- and postharvest.

AUTHOR CONTRIBUTIONS

ML and CP wrote the manuscript. AL conceived the review. GM coordinated the contributions. All authors contributed to manuscript revision, read and approved the submitted version.

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Cinnamaldehyde, a Promising Natural Preservative Against *Aspergillus flavus*

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The problem of food spoilage due to *Aspergillus flavus* (*A. flavus*) needs to be resolved. In this study, we found that the minimum inhibitory concentration of cinnamaldehyde (CA) that inhibited *A. flavus* was 0.065 mg/ml and that corn can be prevented from spoiling at a concentration of 0.13 mg/cm^3. In addition to inhibiting spore germination, mycelial growth, and biomass production, CA can also reduce ergosterol synthesis and can cause cytomembrane damage. Our intention was to elucidate the antifungal mechanism of CA. Flow cytometry, fluorescence microscopy, and western blot were used to reveal that different concentrations of CA can cause a series of apoptotic events in *A. flavus*, including elevated Ca^{2+} and reactive oxygen species, decrease in mitochondrial membrane potential ($\Delta\psi_m$), the release of cytochrome c, the activation of metacaspase, phosphatidylserine (PS) externalization, and DNA damage. Moreover, CA significantly increased the expression levels of apoptosis-related genes (*Mst3*, *Stm1*, *AMID*, *Yca1*, *DAP3*, and *HtrA2*). In summary, our results indicate that CA is a promising antifungal agent for use in food preservation.

Keywords: cinnamaldehyde, *Aspergillus flavus*, antifungal, apoptosis, food preservation

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INTRODUCTION

Aspergillus flavus (*A. flavus*) is one of the most common species among the filamentous fungi. In addition, *A. flavus* is reported to be the second largest cause of aspergillosis infection in humans (Varga et al., 2011). The notorious *A. flavus* metabolite aflatoxin B_1, which has been recognized by the World Health Organization (WHO) as a primary carcinogen, is absorbed by humans and animals through contaminated agricultural crops and animal feed, such as maize, peanuts, nuts, cottonseed, and edible oil (Yang et al., 2015). Therefore, controlling the food spoilage mediated by *A. flavus* at its source is critical to limiting the health hazards of aflatoxin and to preventing substantial economic losses. Nevertheless, traditional antifungal drugs have continuously posed problems, which include the increasingly serious problem of drug resistance, the toxicity of the chemical antifungal compounds, drug interactions, and the high costs. Research into new antifungal agents needs to be carried out urgently because of the drug resistance and the toxicity of the compounds currently available (Sarkar et al., 2014; Qu et al., 2019). Consequently,

increasing numbers of scientists are exploring novel natural products from medicinal plants such as Geraniol and Citral in an attempt to solve the question of fungal drug resistance and with consideration for the natural low toxicity and high antifungal activity of these products (Atanasov et al., 2015; Tang et al., 2018).

Apoptosis is a form of cell death that plays a vital role in the normal development and maturation cycle. In routine physiological processes, the homeostatic balance between cell proliferation and cell death is critical (Fuchs and Steller, 2011). Some scholars have suggested that phenolics can damage mitochondrial function through targeting antioxidative signal transduction and thereby inhibit pathogenic *Aspergillus* (Kim et al., 2004, 2006). Our own previous studies have indicated that apoptosis-promoting compounds are a promising direction in the exploration for a novel antimicrobial drug, and many antifungal agents have been investigated through the apoptotic pathway, such as amphotericin B and anacardic acid (Tian et al., 2017; Qu et al., 2019). In addition, our recent research has shown that Nerol possesses an anti-*A. flavus* ability through apoptosis. Other researchers have indicated that cinnamaldehyde (CA) can decrease the expression of the aflatoxin biosynthetic gene and inhibit the biosynthesis of aflatoxin B1 (Liang et al., 2015; Tian et al., 2018).

Cinnamaldehyde is an α, β-unsaturated aldehyde, abundant in cinnamon and widely used as a food additive in products such as drinks, candies, ice cream, chewing gum, and condiments (Cabello et al., 2009). Furthermore, CA is a traditional Chinese medicine used for gastritis, indigestion, blood circulation disorders, and inflammation (Liao et al., 2012; Chen et al., 2019). CA has been reported to inhibit *Geotrichum citri-aurantii* in citrus fruits and *Phytophthora capsici* in peppers, both of which result in food decay (Hu et al., 2013; OuYang et al., 2019). CA is well-tolerated in humans and animals and is considered a safe natural active ingredient. The FDA and the council of Europe have accepted this concept and recommend daily intake of 1.25 mg/kg (Zhu et al., 2017). Furthermore, CA has been reported to remove natural or chemical toxicities such as ochratoxin A and to protect human health (Dorri et al., 2018; Wang et al., 2018a). The antioxidant activity and the anti-cerebral thrombosis ability of CA have been proven in mice (Zhao et al., 2015; Buglak et al., 2018). Some reports have indicated that CA can initiate the production of reactive oxygen species (ROS) and damage the mitochondrial membranes of *Penicillium expansum* (Wang et al., 2018b,c). The use of CA as a preservative in food storage and transportation is widely recognized to be beneficial. In a recent publication, CA is reported to inhibit *A. flavus* at lower concentrations, and CA has also been recognized as able to induce apoptosis in cancer cells (Roth-Walter et al., 2014; Li et al., 2015). Another report indicates that CA mediates *A. flavus* oxidative stress, but it only detected changes in antioxidant enzyme activity, and the follow-on mechanism of ROS in *A. flavus* is not clear (Sun et al., 2016). Nevertheless, the mechanism by which CA inhibits *A. flavus* is considered worth exploring. Therefore, this research investigated the apoptotic effects of CA in *A. flavus*, such as intracellular ROS, calcium concentration, mitochondrial membrane potential, cytochrome c, phosphatidylserine, metacaspase, and DNA damage.

MATERIALS AND METHODS

Materials and Strain

Cinnamaldehyde (CAS registry no. 104-55-2) was purchased from Shanghai Macklin Biochemical, Co., Ltd. (Shanghai, China) and prepared as a stock solution in 0.1% (*v/v*) Tween-80. The *A.* flavus (NRRL 3357) used in this research was purchased from the China General Microbiological Culture Collection Center (CGMCC). It was cultured in potato dextrose agar (PDA: 200 g peeled potato, 20 g dextrose, 15 g agar powder, and 1000 ml distilled water) for 4 days at 28°C and stored at 4°C.

Antifungal Susceptibility Testing

Broth microdilution methods are commonly used for *in vitro* antifungal assays (Tian et al., 2017). For our experiments, 80 μl of different concentrations of CA, 100 μl of potato dextrose broth (PDB), and 20 μl 5×10^6 spores/ml *A. flavus* were added to each of 10 wells. The 11th well was used as a blank control without the CA, and the 12th well was used as a negative control, without the fungal suspension. After incubation at 28°C for 48 h, the minimal drug concentration that inhibited the growth of the *A. flavus* was described as its minimum inhibitory concentration (MIC).

Effect of CA on *A. flavus* Pathogenicity in Corn

Fungal infection in corn was investigated using a method described previously with minor modifications (Yang et al., 2018). After the tip of each corn kernel was scratched with a knife, it was immersed in 5% sodium hypochlorite and then placed in a shaker for 10 min. The corn was washed twice with sterile water, twice with 70% ethanol, and again twice with sterile water. It was finally shaken with *A. flavus* for 30 min. Six corn kernels were placed in each Petri dish, and then sterile water and various concentrations of CA were added, and sterile filter paper was put it on the bottom of the plate. For the control, the corn was not treated with CA, and neither was it co-incubated with *A. flavus*. All samples were incubated for 5 days at 28°C after sealing.

Fungal Culture Conditions

Fungal cells were suspended in phosphate buffered saline (PBS) and adjusted to 5×10^6 spores/ml with a hemocytometer. The PDB and different concentrations of CA (0, 0.033, 0.065, 0.13, 0.26, and 0.52 mg/ml) were mixed and added to the *A. flavus* spore suspension; they were then cultured in a shaker at 28°C for 6 h. At least 200 spores were observed in each treatment group, confirming the spore germination. Nine mm agar disks were prepared on an *A. flavus* plate with a puncher and placed in the center of a PDA medium with the CA at 28°C. The colony diameters were measured after 3 days to measure mycelial growth. The cells were cultured at a constant temperature in a shaker at 28°C for 72 h, and then the hyphae were collected. The hyphae were treated in an oven at 60°C for 24 h and then weighed to determine their biomass.

Effect of CA on Biofilm of *A. flavus*

A. flavus cells were treated with various concentrations of CA for 12 h at 28°C. The morphological changes in the *A. flavus* were observed and analyzed by the forward-scattered light (FSC) and the side-scattered light (SSC) channels of flow cytometry (BD Biosciences, San Jose, CA, United States). As in the previous method, the content of ergosterol in the *A. flavus* cell membrane was analyzed by spectral scanning (Tian et al., 2012). Membrane integrity was determined by monitoring the uptake of fluorescent nuclear staining propidium iodide (PI)—a DNA-stained fluorescent probe. Cells were incubated with 5 μg/ml PI for 30 min at room temperature in the dark and detected by an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, United States). *A. flavus* cells were centrifuged, and the supernatant was taken out. After dilution, the $OD_{260\ nm}$ value was measured for soluble content release by using an ultraviolet-visible spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States).

ROS, $\Delta\psi_m$, and Ca^{2+} Measurement

The *A. flavus* cells treated with CA were analyzed by flow cytometry with DCFH-DA (Sigma-Aldrich, St. Louis, MO, United States) to detect the production and accumulation of ROS. JC-1 (Sigma-Aldrich, St. Louis, MO, United States) staining was used to measure $\Delta\psi_m$. Fluo-3/AM (Sigma-Aldrich, St. Louis, MO, United States) and Rhod-2/AM (Sigma-Aldrich, St. Louis, MO, United States) are commonly used to detect cytoplasmic and mitochondrial Ca^{2+} levels (Yun and Lee, 2016). The cells treated with different concentrations of CA (0, 0.033, 0.065, 0.13, 0.26, and 0.52 mg/ml) at 28°C for 12 h were harvested by centrifugation at 5000 × *g* for 5 min, washed twice, and then resuspended in PBS. Cells were then incubated with 10 μM DCFH-DA, 10 μg/ml JC-1, Fluo-3/AM and Rhod-2/AM at 28°C for 30 min in the dark. Finally, the cells were washed in PBS and then analyzed using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, United States).

Analysis of Cytochrome c Release

The *A. flavus* cells were treated with various concentrations of CA for 12 h at 28°C for the detection of cytochrome c. The cells were then harvested, and mitochondrial and cytosolic fractions were prepared with an ultrasonic cell disruptor. Mitochondrial fractions were collected by using a filamentous fungus mitochondrial protein extraction kit (BestiBio, Shanghai, China), and cytoplasmic proteins were collected with a filamentous fungal cytoplasmic protein extraction kit (BestiBio, Shanghai, China). The protein concentration was tested using a microplate reader and a bicinchoninic acid (BCA) Protein Assay Kit (Solarbio, Beijing, China). Sixty micrograms of total cellular proteins were separated by 15% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and transferred to the polyvinylidene difluoride (PVDF) membrane (Merck Millipore, Billerica, MA, United States). The PVDF membrane was blocked with 5% non-fat milk (m/v) for 1 h and then washed with 0.1% Tween-20 in Tris saline buffer. It was then incubated with rabbit anti cytochrome c (Proteinsimple, Silicon Valley, CA, United States) and mouse anti-GAPDH (Bioss, Beijing, China) for 12 h at 4°C. The membrane was investigated with western blot chemiluminescence reagents (GE Healthcare, Little Chalfont, United Kingdom), and the reactive density was measured using ImageJ software 1.48 V.

Detection of Metacaspase Activity

Activated metacaspases in *A. flavus* cells were measured with the CaspACE FITC-VAD-FMK *in situ* marker (Promega, Madison, WI, United States). *A. flavus* cells were treated with various concentrations of CA for 12 h at 28°C. The cells were then harvested by centrifugation at 5000 × *g* for 5 min, washed twice, and then resuspended in PBS. The cells were stained with 10 μM of CaspACE FITC-VAD-FMK for 30 min at room temperature in the dark. Finally, the samples were analyzed by using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, United States).

Detection of PS Externalization

PS externalization was detected by fluorescence microscopy using Annexin V-FITC and PI. The method used to prepare the protoplasts has been described in a previous study (Tian et al., 2018). Subsequently, the protoplasts were treated with CA for 12 h at 28°C. Next, the CA-treated protoplasts were stained with 5 μl/ml of PI and FITC-labeled Annexin V, and then analyzed using the Annexin V-FITC apoptosis detection kit (BD Biosciences, San Jose, CA, United States). Finally, the test protoplasts were analyzed by using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, United States).

Analysis of DNA and Nuclear Damage

The TUNEL assay and DAPI staining were used to confirm the diagnostic markers of yeast apoptosis, including DNA and nuclear fragmentation. *A. flavus* cells were treated with various concentrations of CA for 12 h at 28°C. For the DAPI staining, the CA-treated cells were permeabilized and fixed with 70% absolute ethanol at 4°C for 30 min and then treated with 5 μg/ml DAPI (Sigma-Aldrich, St. Louis, MO, United States) for 10 min in the dark. Cells were then harvested and examined under fluorescence microscopy (Leica, Wetzlar, Germany). For the TUNEL assay, 20 μl of the *A. flavus* suspension was added to the adhering slide, and then 100 μl of 4% paraformaldehyde was added, dropwise. Subsequently, 100 μl of 0.2% Triton X-100 and 50 μl of the reaction system were added according to the instructions with the TUNEL kit (Solarbio, Beijing, China). The DNA breaks were observed under fluorescence microscopy (Leica, Wetzlar, Germany).

Quantitative Real-Time PCR

Total RNA was extracted using the TRI reagent method. RNA was extracted from the mycelium after growth in a liquid culture to ensure that a high yield was obtained with purity of RNA (Canciani et al., 2017). The *A. flavus* mycelia were treated with 0, 0.065, 0.13, and 0.26 mg/ml CA for 12 h, then collected, washed, and resuspended in sterile PBS. The collected mycelia were then fragmented with liquid nitrogen and transferred to a TRI reagent solution. We used a Micro UV spectrophotometer

(Thermo Fisher Scientific, Waltham, MA, United States) to test at 260 and 280 nm, and then the RNA was sequentially reverse transcribed to the first strand of cDNA by using the RevertAid First Strand cDNA Synthesis Kit and following the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, United States). The obtained cDNA was used in the analysis of real-time PCR (RT-PCR). The primers used in this study are presented in **Table 1**. SYBR Green was used (Takara, Tokyo, Japan), and the procedures for Q-PCR were performed on AB (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, United States) and consisted of denaturation at 95°C for 30 s and 40 cycles of 95°C for 5 s and 60°C for 40 s. The expression level of the target genes relative to the reference was determined by using $2^{-\Delta\Delta Ct}$ (Jahanshiri et al., 2012).

Statistical Analysis

All experiments were performed in triplicate (n = 3). One-way ANOVAs and Tukey tests were used and data were assessed with GraphPad Prism software, version 8.0.0. The p-values were considered significant at <0.05, <0.01, and <0.001.

RESULTS

CA Reduced Fungal Viability of *A. flavus*

The spoilage by *A. flavus* of seed crops and foodstuffs, together with the contamination by aflatoxins produced by this fungus, have caused significant concern to farmers and the food industry. To resolve the problem of food spoilage by *A. flavus*, CA, an α, β-unsaturated aldehyde that is widely used in food additives, was introduced to treat this fungus. We found that *A. flavus* treated with CA showed MIC at 0.065 mg/ml when the viability of the CA treatment was assessed after 5 days according to visual observation (**Figure 1A**).

We then examined the ability of *A. flavus* to invade maize kernels treated with CA. As shown in **Figure 1B**, the maize kernels in the control were unspoiled by fungus. In the treatment samples, maize kernels with little CA treatment were seriously invaded by the *A. flavus*, which produced a large amount of green spore. The spoilage of the maize kernels by *A. flavus* was significantly inhibited by treatment with greater concentrations of CA (0.13, 0.26, and 0.52 mg/cm^3). *A. flavus* failed to colonize the maize kernels after they were treated with a concentration of 0.13 mg/cm^3 CA (**Figure 1B**). At concentrations of 0.26 and 0.52 mg/cm^3, CA prevented all fungal spoilage. Our results indicated that CA is a promising agent for preventing the infection of seed crops by *A. flavus*.

TABLE 1 | Names and nucleotide sequences of primers used for RT-PCR in this study.

Primer name	Sequence (5′–3′)
Mst3-Forward	TGTGCATCTGGCTTGGCTTA
Mst3-Reverse	ATGGTGGGTGCTTTGACTGT
Stm1-Forward	ATTGCCTGCAACAGCGAATC
Stm1-Reverse	CTTCCTGAGTTGCGCCCTAT
AMID-Forward	TTGCGAACCGAGGCTGAATA
AMID-Reverse	ATTGGGACTCGCAGGTTCTC
Yca1-Forward	GTATTCTTGGGGAGCGCCTT
Yca1-Reverse	CTGCGCAATAGCCTACCAGA
DAP3-Forward	GGAAGACTAGAAGGAGACGCA
DAP3-Reverse	TGGTGTCAGAGGGTCAGGAA
HtrA2-Forward	GGCATGAAGCTGATTGCGTT
HtrA2-Reverse	ATGCCGTCCTTGTGTTTGGA
β-Tubulin-Forward	GCTGGAGCGTATGAACGTCT
β-Tubulin-Reverse	GGCACGAGGGACATACTTGT

CA Inhibited the Sporulation and Fungal Development of *A. flavus*

To better understand how it is that CA restricts the growth of *A. flavus* in seed crops, the effects of CA on the sporulation and development of *A. flavus* were assayed. As shown in **Figures 2A,B**, the spore germination of this fungus was significantly inhibited by CA when the concentration was 0.033 mg/ml or more. The results also show that CA had a positive inhibitory effect on the spore germination of *A. flavus*, indicating that the effect of CA on inhibiting sporulation is dose-dependent. In the assay exploring the effect of CA on *A. flavus* development, we found that increasing concentrations of CA significantly inhibited the growth of *A. flavus* (**Figures 2C,D**). Direct contact with CA significantly inhibited the growth of *A. flavus* hyphae, and this inhibition was positively correlated with the treatment concentration, such that, when the concentration was at 0.52 mg/ml, the mycelial growth of *A. flavus* was completely inhibited. The effect of CA on the biomass of *A. flavus* was also investigated, and this showed that, after treatment with different concentrations of CA, the biomass of *A. flavus* was significantly decreased (**Figures 2E,F**). The decrease in biomass was positively correlated with the concentration of CA. These results demonstrate that CA has the potential to significantly inhibit the sporulation and fungal development of *A. flavus*.

CA Destroyed the Biofilm of *A. flavus*

As the sporulation of *A. flavus* was dramatically inhibited by CA, we wondered whether the integrity of the cell membrane was impaired by the CA treatment. We then examined the cell morphology of *A. flavus* by using flow cytometry. The FSC (X-axis) indicated the size of the cells, and the SSC (Y-axis) indicated the granularity of the cells. As shown in **Figures 3A,B**, the morphological characteristics of *A. flavus* cells were changed following treatment with CA, and the extent of the cell changes differed according to the different concentrations of CA. The results showed that CA significantly changed the cell morphology of *A. flavus*.

Ergosterol is the principal sterol in filamentous fungi and it is required for fungal cell membrane growth and normal function (de Lira Mota et al., 2012). We detected the synthesis of ergosterol in *A. flavus*, and the results showed that different concentrations of CA could inhibit the synthesis of ergosterol in the *A. flavus* cell membrane (**Figures 3C,D**). Compared with the control group, the ergosterol content of *A. flavus* cells

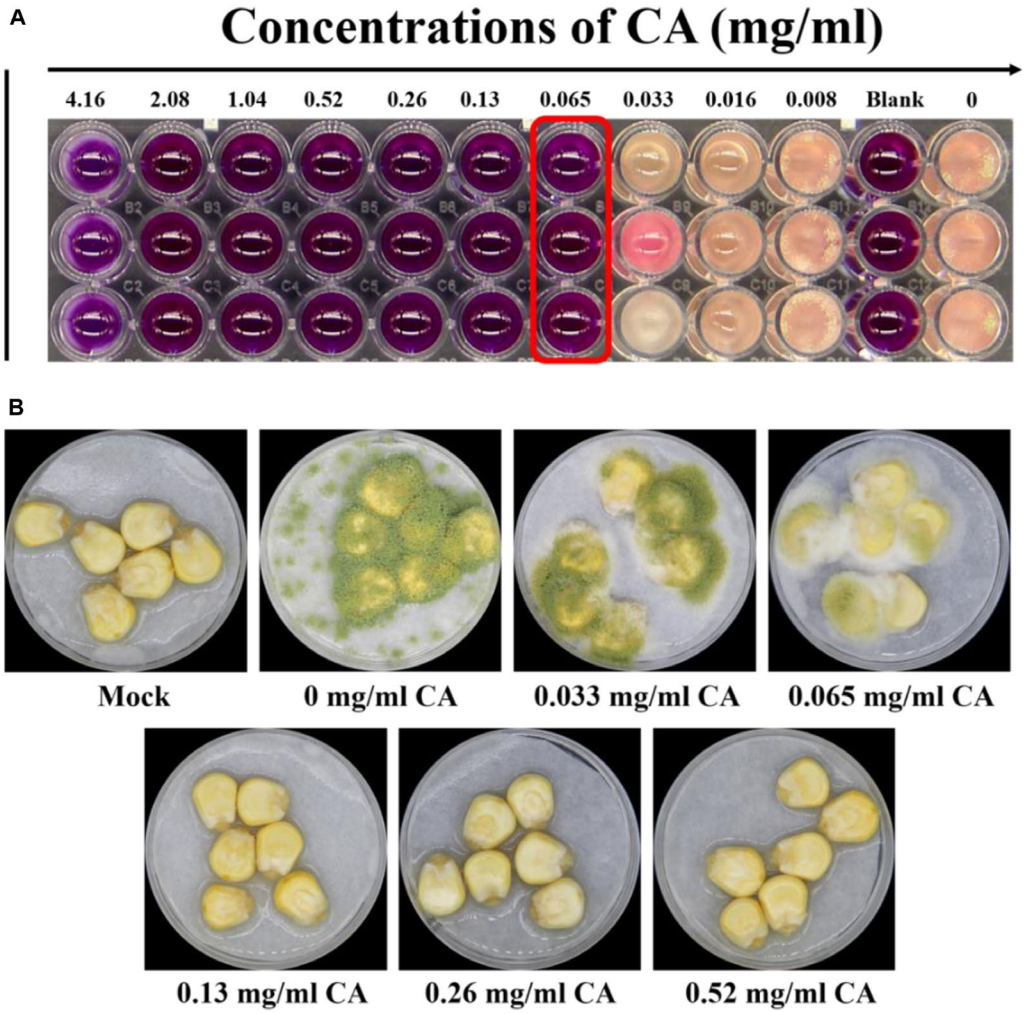

FIGURE 1 | Effects of CA on *Aspergillus flavus* viability and fungal virulence. **(A)** The MIC for *A. flavus* treated with CA was detected by the microdilution method, and the endpoint was observed by using resazurin. **(B)** The antifungal effect of CA on corn: 5×10^6 spores/ml suspension of spore in 0.01% Tween 20 was inoculated into corn, which was treated with CA volatilization, and the Petri dish was kept in a moist incubator at 28°C with 12 h cycles of light/dark for 5 days. The mock control was treated with sterile water, and spore were not inoculated into the corn.

decreased after treatment with different concentrations of CA, which indicated that CA inhibition of the synthesis of ergosterol in the *A. flavus* cell membrane was dose-dependent. The cell membrane is an important organelle in cells and plays a key role in material transport and signal transmission. This experiment used PI—a fluorescent dye that can stain nucleic acids—to detect *A. flavus* cell membrane damage after CA treatment. The *A. flavus* cells showed a more pronounced fluorescence as the concentration of CA increased. The optical density (OD) value for detecting the release of the content of *A. flavus* was recorded at a wavelength of 260 nm by an ultraviolet spectrophotometer. As the concentration of CA increased, the OD value obtained from the corresponding experimental group also increased (**Figure 3G**). The results show that, as the concentration of CA increased, the release of contents also increased. The release of contents after treatment with CA indicates that the cell membrane was destroyed.

The Accumulation of Intracellular ROS Increased With CA

In various physiological and pathological processes, ROS plays a vital role in autophagy and in cell death (Xu et al., 2017). We used the sensitive fluorescent dye DCFH-DA to investigate the production of intracellular ROS by flow cytometer. As shown in **Figure 4**, the generation of intracellular ROS increased significantly after *A. flavus* cells were treated with increasing concentrations of CA. The data indicate that CA may be conducive to an accumulation of intracellular ROS.

Effect of CA on $\Delta\psi_m$ of *A. flavus* Cells

$\Delta\psi_m$ is known to promote cell death and to act as a protease in the extracellular matrix (Kimura-Ohba and Yang, 2016). In this study, we used fluorescence microscopy and flow cytometry

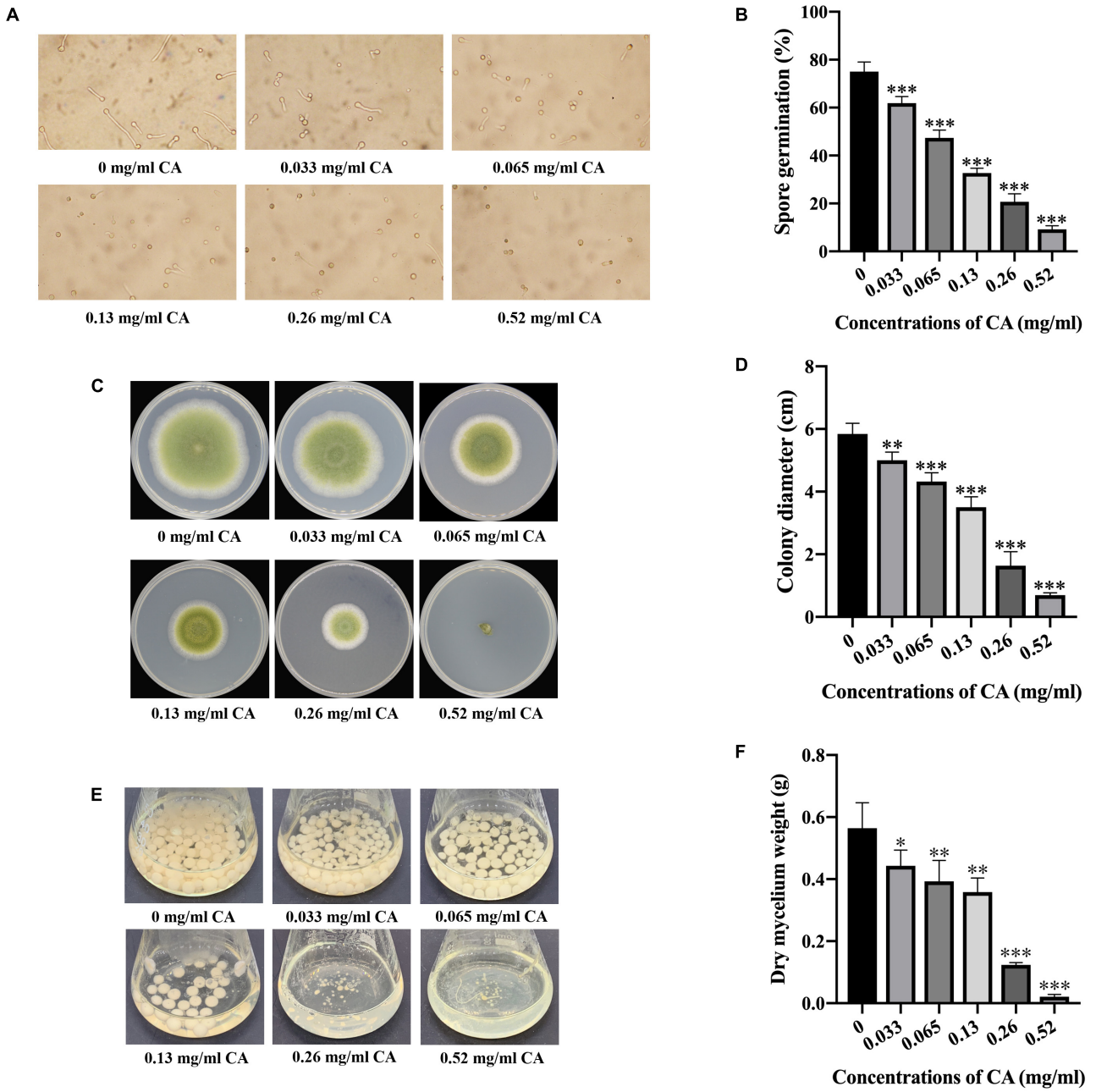

FIGURE 2 | Effects of CA on spore germination and fungal development in *A. flavus*. **(A)** Observation by microscope of spore germination of *A. flavus* treated with different concentrations of CA. **(B)** Statistical analysis of spore germination percentages. **(C)** Mycelial growth of *A. flavus* under various concentrations of CA. **(D)** Statistical analysis of colony diameters. **(E)** The biomass production of *A. flavus* under different concentrations of CA. **(F)** Statistical analysis of dry mycelium weight. In all statistical analysis, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ when compared with the 0 mg/ml CA.

with JC-1 staining to measure the effect of CA on $\Delta\psi_m$ in *A. flavus* cells: a decrease in $\Delta\psi_m$ was evident in *A. flavus* cells with increasing concentrations of CA after 12 h of treatment (**Figure 4C**). As shown in **Figure 4**, a typical fluorescence distribution of JC-1 was displayed in the non-treated group, and the J-aggregates were red. We found that the cells stained with JC-1 changed to a cytoplasmic formation of J-monomeric (green) forms with increased concentrations of CA. These results indicate that CA may decrease $\Delta\psi_m$ in *A. flavus* cells in a concentration-dependent manner.

CA Increased Cytoplasmic and Mitochondrial Ca^{2+} Levels

Ca^{2+} in the mitochondria plays an important role in the regulation of cell survival, apoptosis, and autophagy

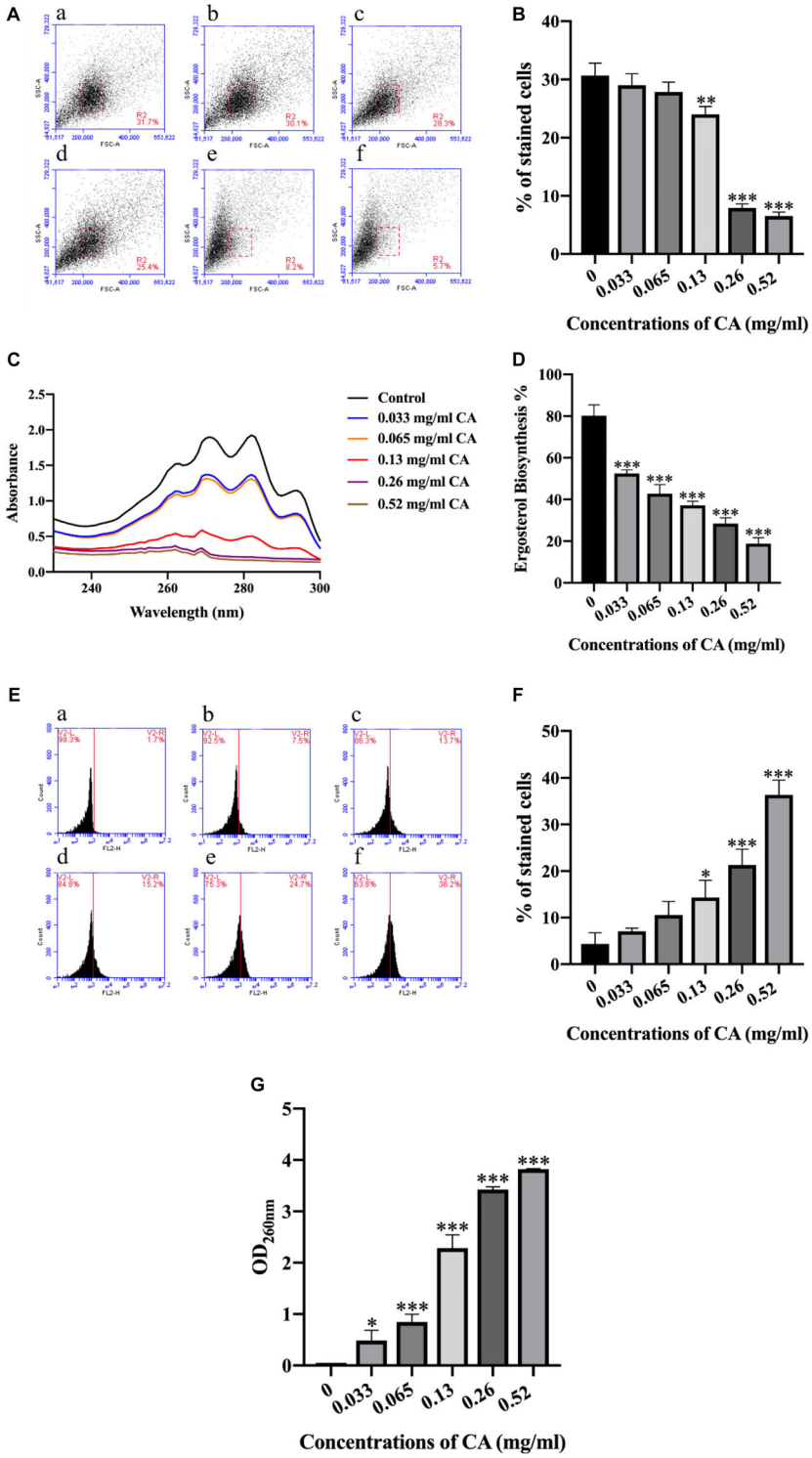

FIGURE 3 | CA destroyed the biofilm of *A. flavus*. **(A)** CA destruction of *A. flavus* cells' morphology detected by flow cytometry and a histogram analysis of the destruction of cell properties. **(a)** Fluorescence of cells without CA treatment; **(b–f)** *A. flavus* cells exposed to 0.033, 0.065, 0.13, 0.26, and 0.52 mg/ml CA. **(B)** Statistical analysis of percentage of stained cells. **(C,D)** CA inhibited the synthesis of ergosterol, which has been considered a classical antifungal target in *A. flavus* cell membranes. **(E)** PI staining was used to detect the biofilm damage level after being treated with CA and a statistical analysis of the damage to cells. (**a**) Fluorescence of cells without CA treatment; **(b–f)** *A. flavus* cells as treated with 0.033, 0.065, 0.13, 0.26, and 0.52 mg/ml CA. **(F)** Statistical analysis of percentage of stained cells. **(G)** The analysis of *A. flavus* cellular content after being treated with various concentrations of CA. In all statistical analyses $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ when compared with the 0 mg/ml CA.

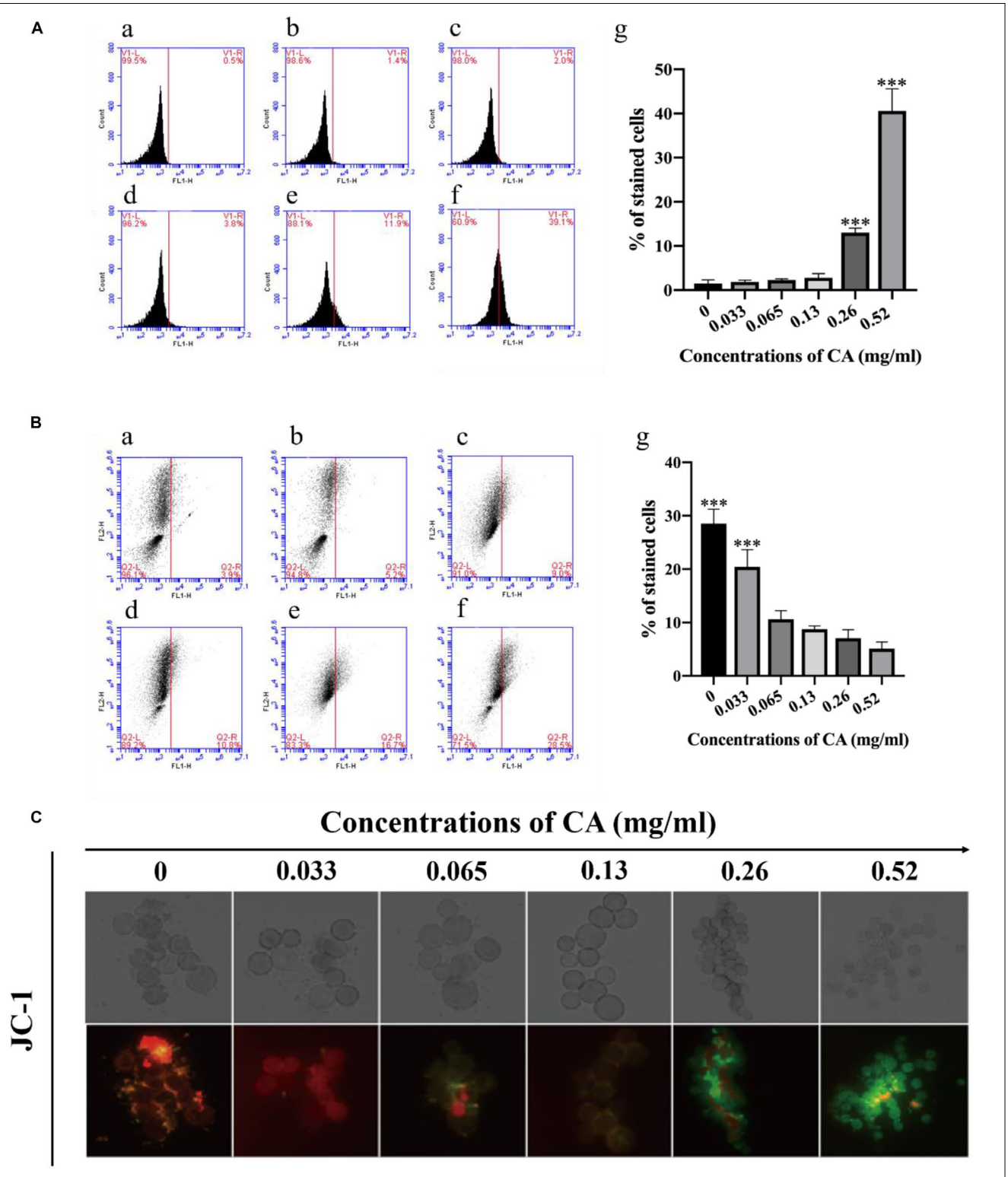

FIGURE 4 | Flow cytometry analysis of ROS content and $\Delta\psi_m$ in CA-treated *A. flavus*. **A (a)** Fluorescence of cells without CA treatment; **(b–f)** *A. flavus* cells exposed to 0.033, 0.065, 0.13, 0.26, and 0.52 mg/ml CA. **(B)** Statistical analysis of percentage of stained cells. **(C)** CA decreased the extent of mitochondrial damage to cells as detected by flow cytometry and the statistical analysis of the stained cells; **(a)** Fluorescence of cells without CA treatment; **(b–f)** *A. flavus* cells exposed to 0.033, 0.065, 0.13, 0.26, and 0.52 mg/ml CA. **A (g)** and **B (g)** Statistical analysis of percentage of stained cells. **(C)** Fluorescence microscopy analysis of the degree of mitochondrial depolarization. JC-1 generates red fluorescence when the mitochondrial membrane potential is high, and green fluorescence when the mitochondrial membrane potential is low. In all statistical analyses $^{***}p < 0.001$ when compared with the 0 mg/ml CA.

(Chemaly et al., 2018), and the Ca^{2+} level in the cytoplasm is always elevated during the process of cell apoptosis (Zhu et al., 2018). In this study, we selected the Fluo-3/AM and Rhod-2/AM stains to detect the levels of Ca^{2+} in the cytoplasm and mitochondria. Compared with the non-treated cells, the Ca^{2+} was increased in the mitochondria at different concentrations of CA (**Figures 5A,B**). Furthermore, the Ca^{2+} level in the cytoplasm was also elevated with the increasing concentrations of CA (**Figures 5C,D**). These results suggest that CA may induce an increase in cytoplasmic and mitochondrial Ca^{2+} levels.

CA Induced the Release of Cytochrome c

Cytochrome c plays an important role in initiating apoptosis, and its release from the mitochondria is a crucial event in the mammalian cell (Liu et al., 2012). The levels of cytochrome c in the mitochondria and cytoplasm were detected by western blot after *A. flavus* cells were co-incubated with

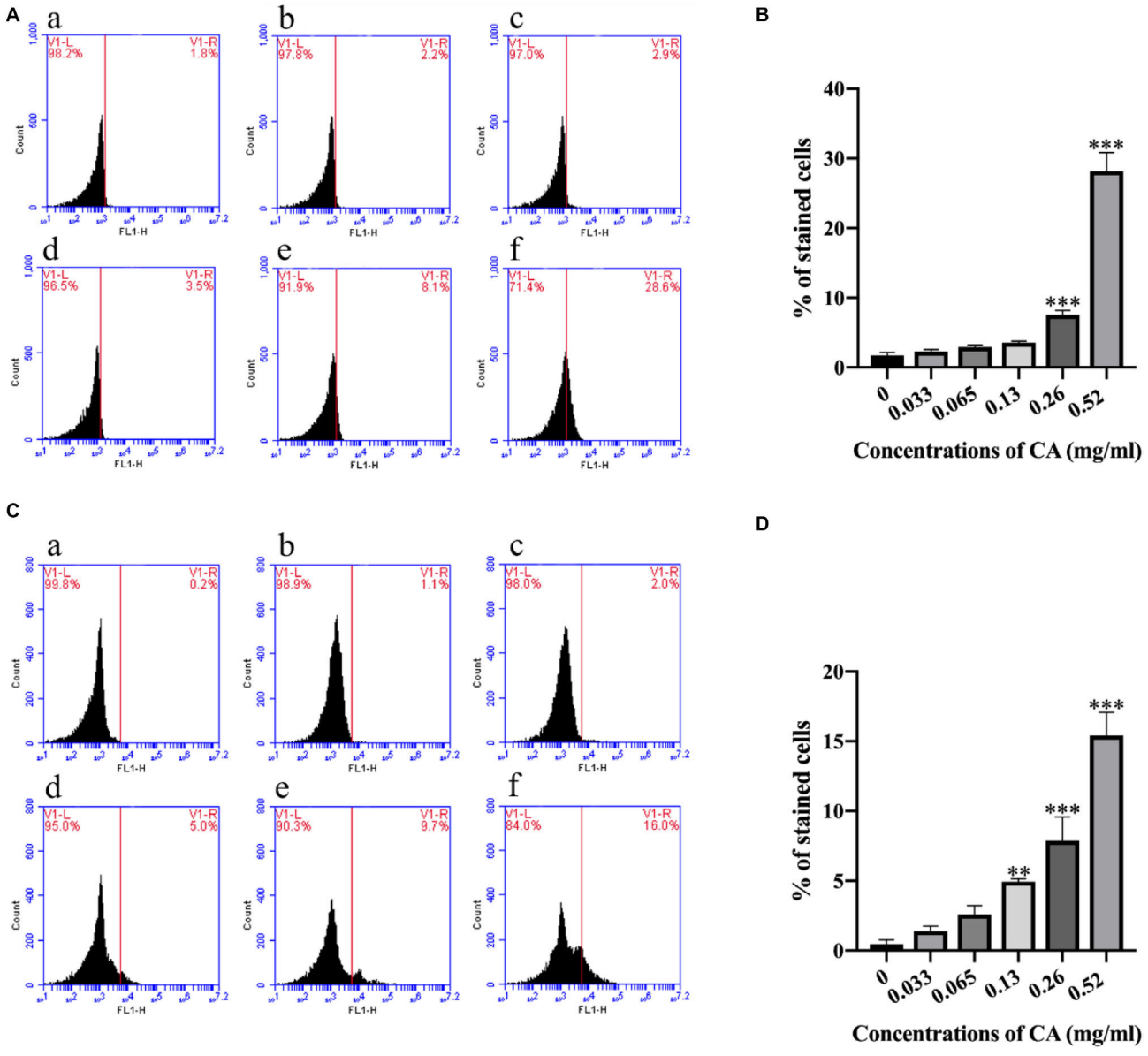

FIGURE 5 | Cinnamaldehyde promoted the Ca^{2+} accumulation in both the cytoplasm and the mitochondria. **(A)** Rhod-2/AM fluorescence probe was used to detect the content of calcium ion in the mitochondria after being treated with different concentrations of CA; **(a)** Fluorescence of cells without CA treatment; **(b–f)** *A. flavus* cells exposed to 0.033, 0.065, 0.13, 0.26, and 0.52 mg/ml CA. **(B)** Statistical analysis of percentage of stained cells. **(C)** Fluo-3/AM fluorescence probe was used to test the concentrations of Ca^{2+} in the cytoplasm after co-incubation with CA; **(a)** Fluorescence of cells without CA treatment; **(b–f)** *A. flavus* cells were treated with 0.033, 0.065, 0.13, 0.26, and 0.52 mg/ml CA. **(D)** Statistical analysis of percentage of stained cells. In all statistical analyses, $^{**}p < 0.01$ and $^{***}p < 0.001$ when compared with the 0 mg/ml CA.

various concentrations of CA. Compared with the non-treated cells, the level of cytochrome c in the mitochondria significantly decreased, while the level in the cytosol increased noticeably (**Figures 6A–C**). The results demonstrate that CA induced the release of cytochrome c from the mitochondria in *A. flavus* cells.

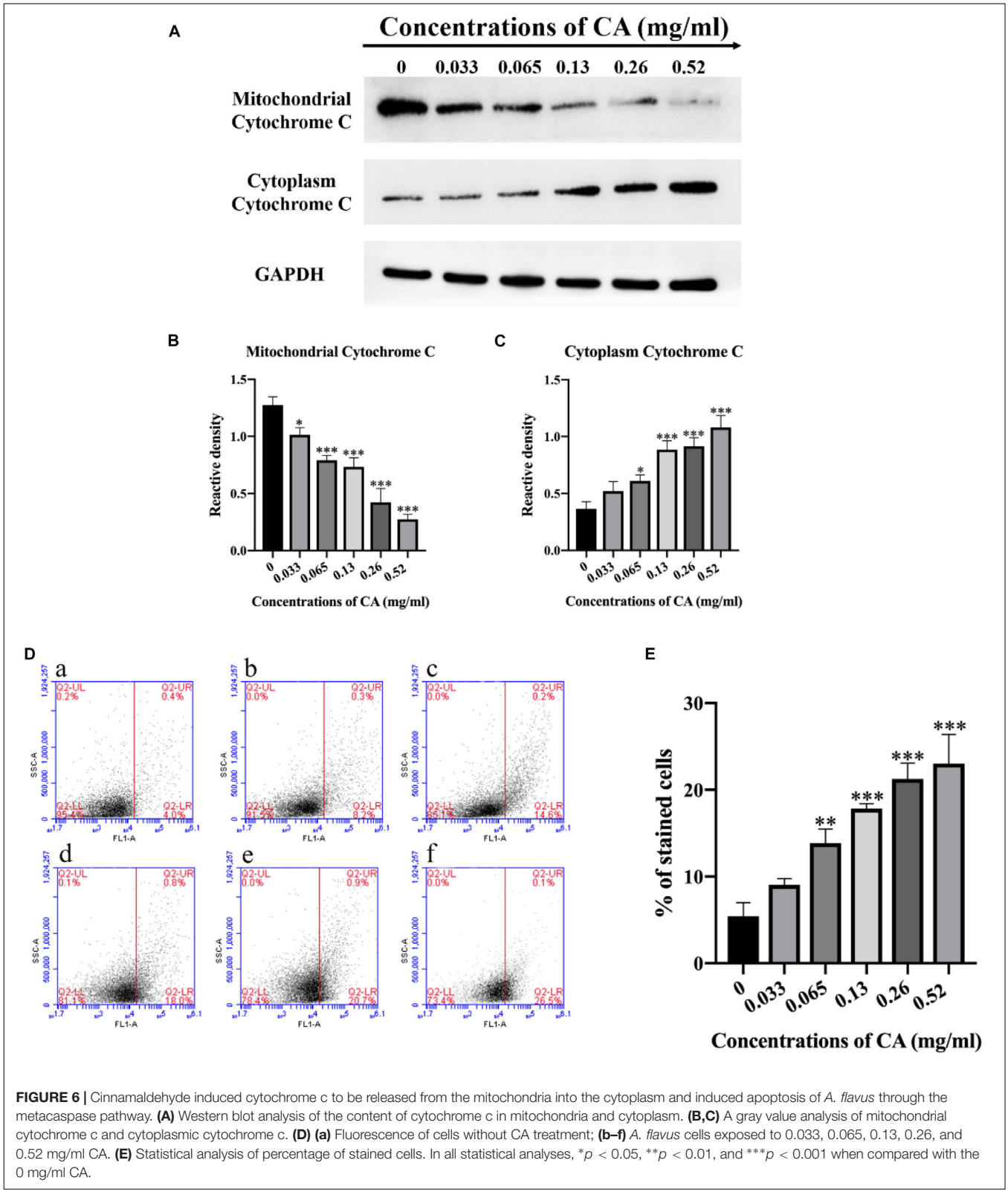

FIGURE 6 | Cinnamaldehyde induced cytochrome c to be released from the mitochondria into the cytoplasm and induced apoptosis of *A. flavus* through the metacaspase pathway. **(A)** Western blot analysis of the content of cytochrome c in mitochondria and cytoplasm. **(B,C)** A gray value analysis of mitochondrial cytochrome c and cytoplasmic cytochrome c. **(D) (a)** Fluorescence of cells without CA treatment; **(b–f)** *A. flavus* cells exposed to 0.033, 0.065, 0.13, 0.26, and 0.52 mg/ml CA. **(E)** Statistical analysis of percentage of stained cells. In all statistical analyses, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ when compared with the 0 mg/ml CA.

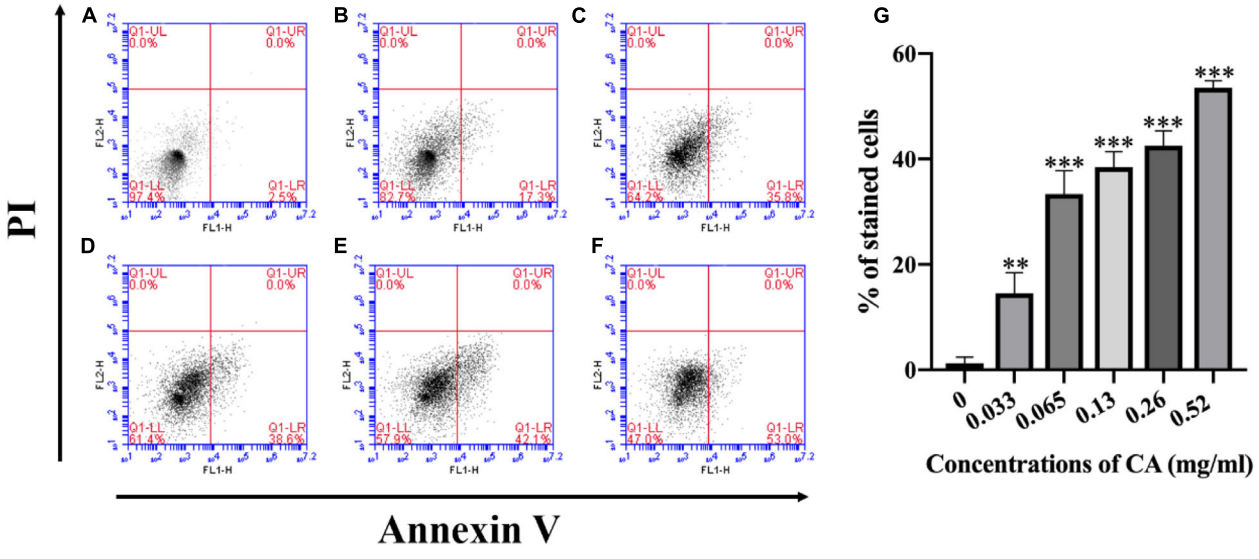

FIGURE 7 | Flow cytometry measures PS externalization in *A. flavus* after being treated with CA. **(A)** Fluorescence of cells without CA treatment. **(B–F)** *A. flavus* cells treated with 0.033, 0.065, 0.13, 0.26, and 0.52 mg/ml CA. **(G)** Statistical analysis of percentage of stained cells. In all statistical analyses, $^{**}p < 0.01$ and $^{***}p < 0.001$ when compared with the 0 mg/ml CA.

CA Caused Activation of Metacaspase

In fungi, plants, and in some bacteria, the metacaspases have been implicated in programmed cell death (PCD) (Asplund-Samuelsson et al., 2012). We stained the cells with CaspACE FITC-VAD-FMK, and the cells were incubated with CA. As shown in **Figure 6D**, the percentage of *A. flavus* cells that were significantly stained increased in a dose-dependent manner. This result indicates that CA induced the activation of the metacaspases to initiate apoptosis in *A. flavus*.

CA Caused PS Externalization

Phosphatidylserine (PS) is expressed in the outer layers of the cell membrane and is "flipped out" from the inner layers in early apoptosis (Chowdhury et al., 2014). In this assay, *A. flavus* cells were double-stained with Annexin V-FITC and PI at various concentrations of CA treatment, and apoptotic cells were identified by flow cytometry. **Figure 7** depicts the percentages of early apoptotic cells (Annexin V-positive and PI-negative) in the lower right quadrant and this increases in a time-concentration-dependent manner. The results conclusively indicate that CA can lead to apoptosis through the externalization of PS.

Effect of CA on DNA Damage and Nuclear Fragmentation

The degree of DNA damage is related to the degree of apoptosis, with DNA damage preceding apoptosis, which is consistent with the time of execution of apoptosis (Rai et al., 2015). We used DAPI and TUNEL staining to detect DNA damage and nuclear fragmentation, which are hallmarks of late apoptosis. In the microscopic analysis, the cells treated for 12 h with various concentrations of CA showed an increasing fluorescence intensity, which indicated CA induced DNA damage (**Figure 8B**). Similarly, we found that when *A. flavus* cells were exposed to CA they had a DAPI-positive phenotype and showed chromatin condensation, which suggested that the CA induced nuclear fragmentation (**Figure 8A**). Our results show that CA caused DNA damage and nuclear fragmentation in *A. flavus* cells. In recent years, the literature has demonstrated that *Mst3*, *Stm1*, *AMID*, *Yca1*, *DAP3,* and *HtrA2* are all genes associated with apoptosis (Fedorova et al., 2005). As **Figure 8C** shows, after 12 h of treatment with CA, the expression levels of *Mst3*, *Stm1*, *AMID*, *Yca1*, *DAP3,* and *HtrA2* were significantly increased. β-tubulin was selected as the reference gene as it displayed the same expression level in different samples. We found that the relative expression levels of the apoptotic genes were significantly changed after *A. flavus* cells were treated with 0.26 mg/ml CA. The results showed that CA can affect the expression of *Mst3*, *Stm1*, *AMID*, *Yca1*, *DAP3*, and *HtrA2,* which then activate related pathways to induce apoptosis in *A. flavus* cells.

DISCUSSION

Aromatic and medicinal plants have been used as pharmaceutical and food preservatives for decades. Many plants, such as cloves, thyme, and cinnamon, have been used to treat infectious diseases and to protect foods because they have been shown to have antimicrobial activity against spoilage by fungi and bacteria (Liu et al., 2017). Aromatic and medicinal plants produce essential oils in the form of secondary metabolites (Pandey et al., 2016). Essential oils have been reported to have a wide range of antifungal activities (Tian et al., 2011). CA is the main component of the cinnamon essential oil, and this has been developed as a food antimicrobial agent due to its activity against bacteria, yeast, and filamentous mold (Hu et al., 2013).

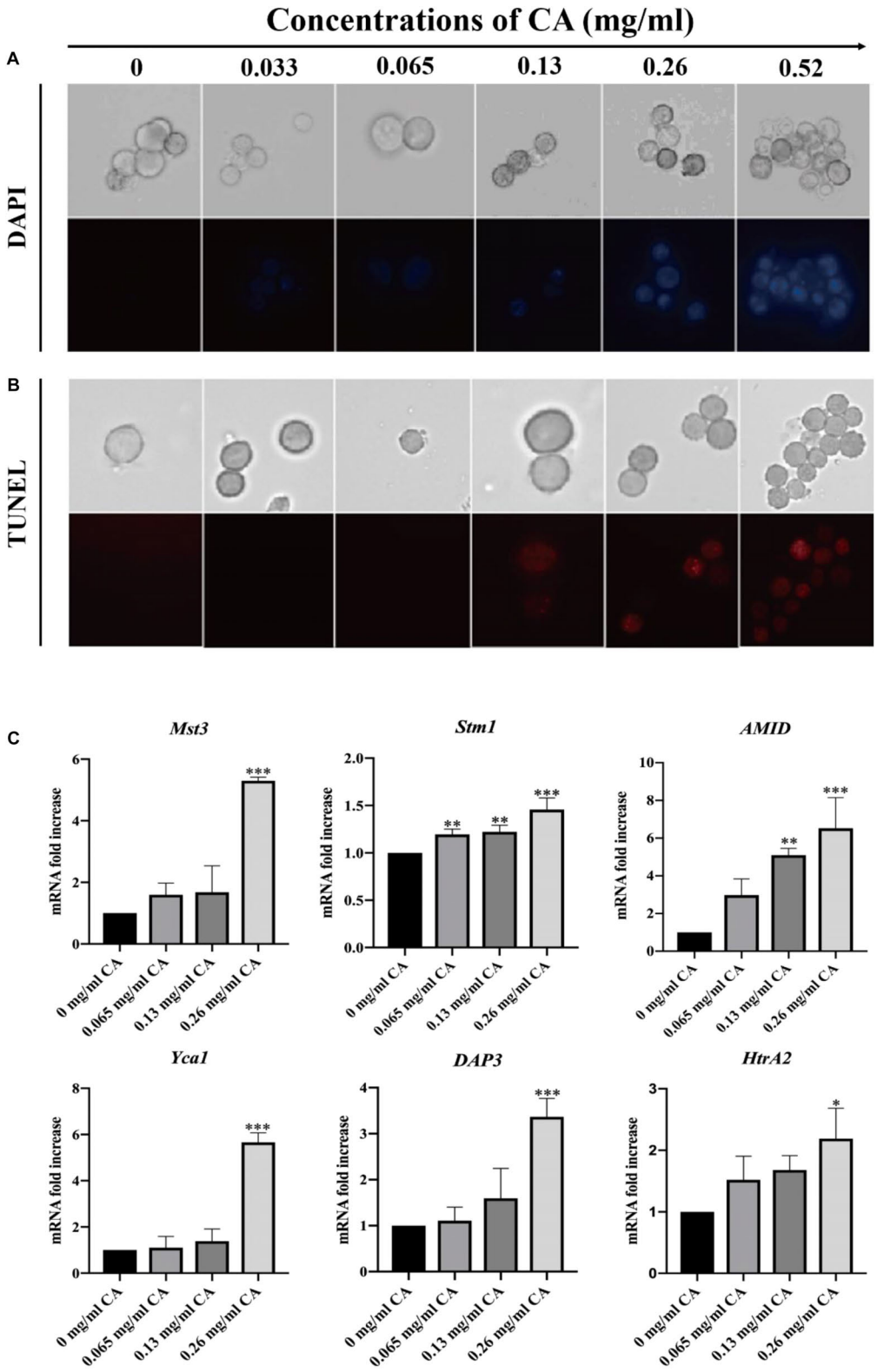

FIGURE 8 | DNA damage and nuclear fragmentation by CA were visualized with fluorescence microscopy using TUNEL and DAPI staining. **(A)** Blue fluorescence indicates a nuclear signal after staining by DAPI. **(B)** Red fluorescence means a positive signal in TUNEL staining. **(C)** The expression levels of apoptosis-related genes (*Mst3*, *Stm1*, *AMID*, *Yca1*, *DAP3*, and *HtrA2*) at various concentrations of CA (0, 0.065, 0.13, and 0.26 mg/ml) were examined by Real-Time PCR. In all statistical analyses, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ when compared with the 0 mg/ml CA.

According to the report, during corn storage, a complex essential oil rich in CA reduced the content of aflatoxin B1, zearalenone, and deoxycaprolol. Furthermore, this complex essential oil also showed the capability to reduce the contamination by *Fusarium*, *Wallemia*, *Sarocladium*, and *Penicillium* in the process of maize kernels storage (Wang et al., 2019). CA was reported to be highly safe, 20 times of effective dose (20 mg/kg) of this compound does not cause abnormal behavioral signs and serum chemical damage throughout the study (Subash Babu et al., 2007; Anand et al., 2010). Therefore, the development of CA into a new type of natural preservative has a certain safety and theoretical basis.

This study showed that CA has potential antifungal activity against *A. flavus* and may be a source of natural antifungal compounds that negatively affect the growth of *A. flavus*. The MIC of CA against *A. flavus* is 0.065 mg/ml (**Figure 1A**). When we applied CA to corn preservation we found that CA inhibited corn spoilage at a concentration of 0.13 mg/cm^3 (**Figure 1B**). CA inhibited spore germination and mycelial growth and reduced biomass production (**Figure 2**). In addition, the morphology of *A. flavus* cells changed after CA treatment (**Figures 3A,B**). These results are consistent with previous research that reported that *A. flavus* seemed to be shriveled and wrinkled after treatment by CA as shown by scanning electron microscopy (Sun et al., 2016). The ability of CA to inhibit spore germination, mycelium growth, and biomass production in *A. flavus* is consistent with the results of previous studies (Tian et al., 2015; Sun et al., 2016).

Ergosterol is a unique component in the fungal cell membrane and plays a vital role in the activity of fungal cells, where it serves to stabilize the membrane structure, regulate membrane fluidity, and ensure material transportation (de Lira Mota et al., 2012). Most antifungal drugs used clinically target ergosterol or its biosynthesis (Shapiro et al., 2011). When ergosterol synthesis is reduced, the physiological activity of the cell membrane is affected, which is likely to cause fungal cell membrane damage and cell breakage (Georgopapadakou and Walsh, 1994). Some researchers have shown that the lipophilic nature of essential oils allows them to pass easily through the cell membrane to induce biological responses (Tian et al., 2015). We examined the synthesis of ergosterol and detected cell membrane damage by monitoring the uptake of the fluorescent nuclear stain PI. The results showed that when *A. flavus* was treated with different concentrations of CA (0, 0.033, 0.065, 0.13, 0.26, and 0.52 mg/ml) it reduced the synthesis of ergosterol and caused cell membrane damage (**Figures 3C–F**). This result is consistent with previously reported results that tested products such as citral, octanal, and alpha-terpineol, which can all damage cell membranes with consequent bacteriostatic action (Zhou et al., 2014). We also detected a release of the contents of *A. flavus* and found that CA can cause this release of contents. This result indicated that the cell membrane was damaged (**Figure 3G**), verifying the previous results.

Apoptosis is a unique form of PCD in which cells activate an intrinsic suicide program for self-destruction (Perez-Garijo et al., 2013). Currently, clinically used antifungal drugs such as peptaibols, anacardic acid, and amphotericin B, which are cytotoxic to pathogenic fungi through activation of an apoptotic pathway (Muzaffar et al., 2016) are considered to offer a promising approach to the prevention of fungi and food contamination. In the light of previous results, we determined that CA can effectively inhibit *A. flavus*. We therefore focused on its mechanism of apoptosis.

The redox state of cells plays a crucial role in cell fate. A slight imbalance between the rate of production and the breakdown of reactive oxygen and nitrogen species (ROS and RNS) may lead to activation of the cell death pathway (Hirpara et al., 2001). It is worth noting that mitochondria are the primary intracellular source of ROS, mainly superoxide (O_2^+) and hydrogen peroxide (H_2O_2), as electrons are promoted leading to oxygen leakage through high-throughput electron transport chains (ETCs) (Finkel and Holbrook, 2000). The accumulation of ROS is considered to be one of the earliest changes associated with PCD (Tian et al., 2016). With this in mind, a DCFH-DA assay was applied to detect changes in the levels of ROS in CA-treated *A. flavus* cells. Compared with the non-CA-treated *A. flavus* cells, our results indicated that intracellular ROS levels increased significantly in CA-treated *A. flavus* (**Figures 4A,B**). The emergence of high levels of ROS can lead to mitochondrial damage, cell membrane damage, and even DNA breaks (Phaniendra et al., 2015). It has also been reported that an increase in $\Delta\psi_m$ is associated with high intracellular ROS accumulation (Sukumar et al., 2016).

Mitochondria are essential regulators of cellular bioenergetics, and mitochondria that are damaged by ROS tend to produce more ROS, thereby activating mitochondria-mediated apoptosis or necrosis pathways. The opening of mitochondrial permeability transition pores (MPT pores) leads to a loss of the mitochondrial inner membrane integrity. MPT pores allow flux of small molecules, <1500 Da, and protons, leading to mitochondrial swelling, loss of $\Delta\psi$m, rupture of the outer membrane, and death through apoptosis or necrosis. The formation of these pores can occur in response to several stimuli, including Ca^{2+} overload and oxidative stress (Handy and Loscalzo, 2012). Therefore, we measured changes in $\Delta\psi$m in CA-treated *A. flavus* cells by using a JC-1 probe. As shown in **Figure 4C**, $\Delta\psi$m significantly decreased after incubation with different concentrations of CA (0, 0.033, 0.065, 0.13, 0.26, and 0.52 mg/ml), and this result is consistent with the results of previous research (Yun et al., 2017). We speculated that the mitochondrial homeostasis is disrupted, causing its dysfunction and leading to changes in membrane potential.

The role of ROS and Ca^{2+} channels may potentially modulate mitochondrial dysfunction, form MPT pores, and induce apoptosis. Ca^{2+} overload can lead to cell death. Calcium, as a major second messenger in cells, is well-known for its important role in mediating PCD (Handy and Loscalzo, 2012). Increased intracellular calcium is a sign of early apoptosis in cells. When the balance of intracellular calcium is disrupted, it leads to the release of cytochrome c and other pro-apoptotic factors (Garcia-Prieto et al., 2013). The results of this study illustrated a concentration-dependent increase in cytoplasmic Ca^{2+} in CA-treated *A. flavus* as well as significant increases in mitochondrial Ca^{2+} levels (**Figure 5**), which echo the

results of previous reports. Overloading of mitochondrial Ca^{2+} disrupts mitochondrial function and depolarization of $\Delta\psi_m$. This result confirms the pathological events leading to apoptosis (Yun and Lee, 2016).

Given the above-mentioned disruption of the intracellular calcium balance leading to the release of cytochrome c, the collapse of $\Delta\psi_m$ is closely related to a series of events, including the release of cytochrome c, the activation of metacaspase, DNA damage, and nuclear fragmentation. Cytochrome c is a pro-apoptotic protein, and the opening of the MPT pores causes the mitochondrial membrane rupture to release cytochrome c (Mallick et al., 2015). In our study, we measured the release of cytochrome c from the mitochondria into the cytoplasm in CA-treated *A. flavus* cells by western blot (**Figures 6A–C**). Release of cytochrome c from mitochondria is a key event initiating apoptosis. It induces the assembly of apoptotic bodies and activates downstream caspase. Further, apoptosis and caspase were initially thought to be crucial markers of apoptosis (Yuan et al., 2016).

In yeast apoptosis, there are caspase-dependent and caspase-independent cell death pathways. The yeast caspase-like protease, known as metacaspase, is encoded by *YCA1*. ROS is a major factor in inducing apoptosis in yeast cells, and it regulates cell death pathways by activating yeast metacaspase (Kim et al., 2016). Therefore, we examined the metacaspase activity of *A. flavus* cells following CA treatment and found that the activity of metacaspase increased in tandem with increased CA concentration, indicating that CA activated metacaspase (**Figures 6D,E**).

Changes in the phospholipid bilayer in the cell membrane usually occur in the early stages of apoptosis. When apoptosis occurs, the PS component of the phospholipid bilayer will move from the inner membrane to the outer membrane (Tian et al., 2016). We examined the apoptotic characteristics of CA-treated cells, and the results showed that CA caused the externalization of PS on the outer surface of the plasma membrane (**Figure 7**). DNA damage and nuclear fragmentation are typical morphological features of apoptotic cells in the late

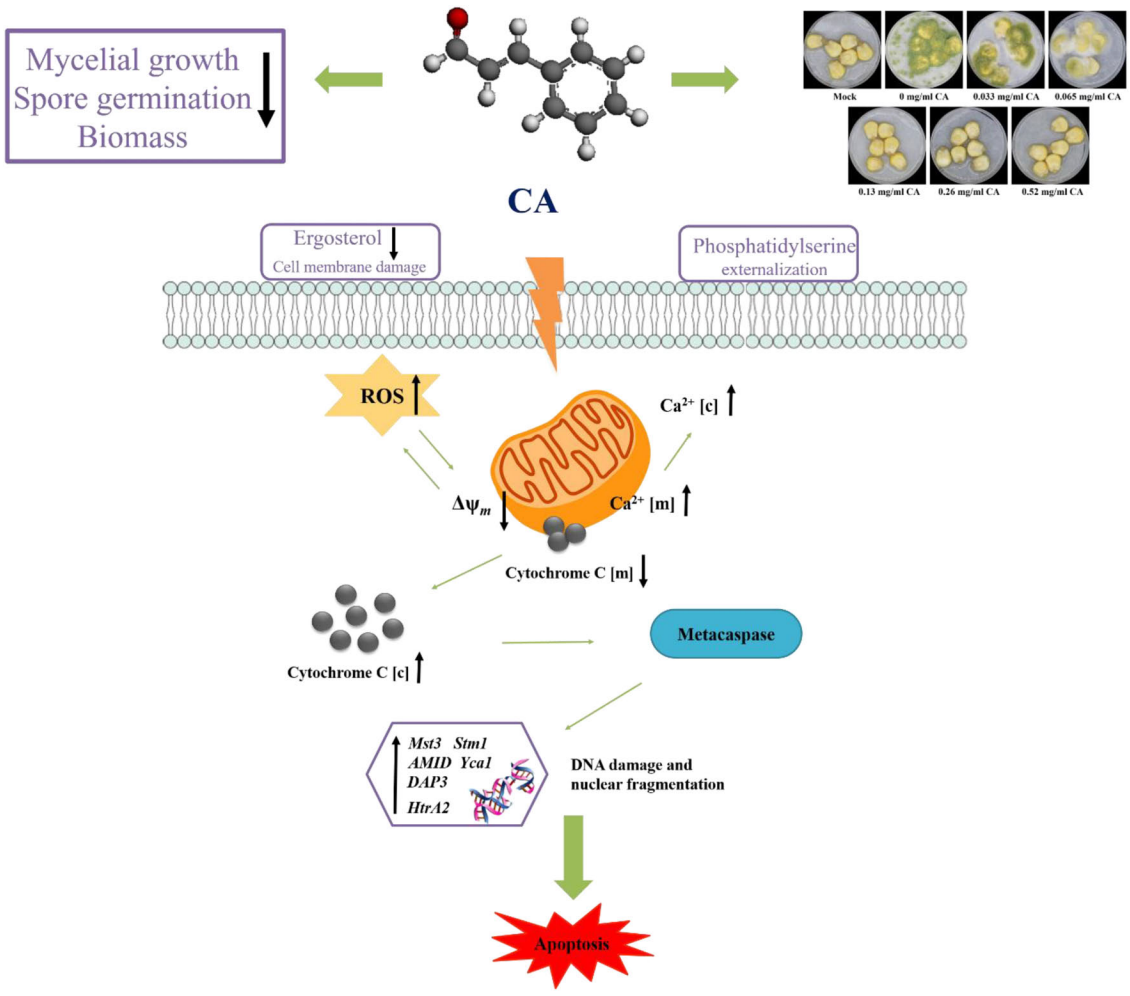

FIGURE 9 | A schematic illustration of the potential inhibition mechanism on *A. flavus* by CA.

stage of apoptosis (Tian et al., 2017). It is well-known that DAPI fluorescent probes are used to detect chromatin condensation, and TUNEL staining is one of the most reliable strategies for identifying the amount of DNA fragmentation visible. Our fluorescence results indicated that CA can significantly affect DNA damage and chromatin condensation in *A. flavus* (**Figures 8A,B**).

In summary, our study demonstrates that Ca^{2+} and ROS-mediated apoptosis can occur in *A. flavus* treated with CA. We propose a model of apoptosis mechanism, as shown in **Figure 9**. CA causes an increase in Ca^{2+} and ROS. Ca^{2+} overload and oxidative stress disrupt mitochondrial function and cause the loss of $\Delta\psi_m$, which in turn promote the release of cytochrome c from the mitochondria into the cytoplasm. PS externalization can be observed in the early stages of apoptosis, and the increase in ROS activates metacaspase, which further induces apoptosis. Finally, typical morphological features of late apoptosis, DNA fragmentation, and chromatin condensation can be observed.

To further explore the molecular mechanism of CA-induced apoptosis in *A. flavus*, we examined apoptosis-related genes by RT-PCR. As shown in **Figure 8C**, the expression levels of *Mst3*, *Stm1*, *AMID*, and *Yca1* increased in tandem with an increase in CA concentration. Previous studies have shown that these genes are all coding for the caspase family in fungi (Fedorova et al., 2005), which is consistent with our finding that CA activates metacaspase. Over-expression of these genes may be a potential mechanism by which CA activates metacaspase to induce apoptosis. The expression levels of *DAP3* and *HtrA2* exhibited the same trend, showing concentration-dependence. These two genes have been reported to be involved in mitochondrial homeostasis and injury (Fedorova et al., 2005). Over-expression of these two genes may cause CA to disrupt mitochondrial homeostasis, lead to mitochondrial damage, promote the release of apoptotic factors, and ultimately result in apoptosis.

CONCLUSION

Cinnamaldehyde can inhibit mycelial growth, buccal germination, and biomass production. It can alter cell morphology, cause cell membrane damage, and cause mitochondrial dysfunction through the interaction between Ca^{2+} and ROS, leading to apoptosis of *A. flavus*. CA is also effective in preventing corn spoilage. The results of this study indicate that CA is a potential candidate for use as a antifungal agent in food preservation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

JT, KY, and YGL designed the experiments. SQ, LC, QG, and XH performed the experiments. KY, ML, and YXL analyzed the data. SQ and LC drafted the manuscript. All authors read and approved the final manuscript.

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Synergistic Inhibition of Mycotoxigenic Fungi and Mycotoxin Production by Combination of Pomegranate Peel Extract and Azole Fungicide

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Fungal plant pathogens cause considerable losses in yield and quality of field crops worldwide. In addition, under specific environmental conditions, many fungi, including such as some *Fusarium* and *Aspergillus* spp., are further able to produce mycotoxins while colonizing their host, which accumulate in human and animal tissues, posing a serious threat to consumer health. Extensive use of azole fungicides in crop protection stimulated the emergence of acquired azole resistance in some plant and human fungal pathogens. Combination treatments, which become popular in clinical practice, offer an alternative strategy for managing potentially resistant toxigenic fungi and reducing the required dosage of specific drugs. In the current study we tested the effect of pomegranate peel extract (PPE) on the growth and toxin production of the mycotoxigenic fungi *Aspergillus flavus* and *Fusarium proliferatum,* both alone and in combination with the azole fungicide prochloraz (PRZ). Using time-lapse microscopy and quantitative image analysis we demonstrate significant delay of conidial germination and hyphal elongation rate in both fungi following PPE treatment in combination with PRZ. Moreover, PPE treatment reduced aflatoxin production by *A. flavus* up to 97%, while a combined treatment with sub-inhibitory doses of PPE and PRZ resulted in complete inhibition of toxin production over a 72 h treatment. These findings were supported by qRT-PCR analysis, showing down-regulation of key genes involved in the aflatoxin biosynthetic pathway under combined PPE/PRZ treatment al low concentrations. Our results provide first evidence for synergistic effects between the commercial drug PRZ and natural compound PPE. Future application of these findings may allow to reduce the required dosage of PRZ, and possibly additional azole drugs, to inhibit mycotoxigenic fungi, ultimately reducing potential concerns over exposure to high doses of these potentially harmful fungicides.

Keywords: pomegranate peel extract, prochloraz, mycotoxigenic fungi, combination treatment, aflatoxin B1, synergistic interaction

INTRODUCTION

Many fungal plant pathogens that belong to the genera *Aspergillus* and *Fusarium* produce important mycotoxins of concern in relation to animal and human health (Tsitsigiannis et al., 2012). These fungi represent serious phytopathological and mycotoxicological risks at pre- and post-harvest stages, as well as in processed food products (Castoria et al., 2008). Mycotoxins, which are secondary metabolites produced by these fungi, have a significant economic impact worldwide as they pose a significant threat to food and feed safety, as well as in medical settings. Indeed, among natural food and feed contaminants, mycotoxins represent one of the major concerns regarding chronic toxicity, and pose critical challenges in food toxicology (Dellafiora and Dall'Asta, 2017). Although much progress has been made toward developing different agents to control mycotoxigenic pathogens at pre- or post-harvest stages, the number of efficacious antifungal drugs that can be used in food-production setting remains limited. Of these, azole-based fungicides are the most used antifungals in agriculture, due to their high efficiency and broad spectrum activity (Price et al., 2015). Thousands of tons of azoles are sold annually to control fungal infections in crops. According to the instructions of manufacturers, about 10 mg of azoles should be applied per 1 m^2 of the field (Hof, 2001). Excessive and long-term use of azole fungicides in agriculture has led to the emergence of acquired azole resistance in some plant pathogenic fungi (Serfling et al., 2007). Moreover, several recent studies demonstrated that exposure of *Aspergillus* species, especially.

Aspergillus fumigatus, to azole compounds in the environment can induce cross-resistance to medical azole drugs (Chowdhary et al., 2013; Ren et al., 2016; Verweij et al., 2016). The development of drug resistance in many fungal pathogens, as well as growing public concerns over the health and environmental impacts of fungicides, has led to a significant interest in the development of alternative, environmentally friendly methods of disease control. Plant extracts are generally considered environmentally safer (i.e., biodegradable with low toxicity to the environment) and thus preferable alternatives to synthetic compounds. Plants produce a wide diversity of secondary metabolites which serve them as defense compounds for their own protection against other plants, pests and microbes. Several plant extracts were reported to exhibit a direct antifungal activity in treated plant hosts (Tripathi and Dubey, 2004; Palou et al., 2016). These secondary metabolites exhibit a wide range of biological and pharmacological properties, leading to the use of several products isolated from plants in the treatment of microbial infections in a number of host-pathogen combinations (Wink, 2015). Combining different antifungal compounds with different modes of action could reduce the required dose of each drug while minimizing the potential for the development of drug-resistance, still allowing for effective combating of fungal infections. While a number of recent studies explored the interactions between natural products and antifungal drugs (Shin, 2003; Shin and Kang, 2003; Shin and Lim, 2004; Karioti et al., 2011), the use of such combined antifungal treatments in agricultural setting remains limited, particularly when compared to clinical applications. Pomegranate by-products, such as peel and seeds, are considered a rich source of bioactive compounds such as flavonoids, phenolic acids, and tannins which have free radical scavenging activity and antioxidant capacity (Panichayupakaranant et al., 2010; Sorrenti et al., 2019). Several studies have been reported on the effectiveness of pomegranate peel extracts (PPEs) against human and plant fungal pathogens (Dikmen et al., 2011; Glazer et al., 2012; Foss et al., 2014; Pangallo et al., 2017; Rosas-Burgos et al., 2017). However, harmful fungi often have greater tolerance to such natural compounds when compared to commercially available antifungals. Several additional factors, such as low curative effect, reduced and inconsistent efficacy, and limited range of antifungal activities, represent major barriers to the commercial acceptance of plant extracts and other natural products for controlling agriculturally relevant fungal pathogens (Campbell et al., 2012; Bautista-Baños et al., 2013). The development of treatments combining natural compounds with commercial antifungal drugs is a promising approach toward harnessing the power of naturally occurring compounds. Considering the limited number of antifungal agents available, and that most of them have similar modes of activity (Loeffler and Stevens, 2003), their combination with natural antifungals, possibly with different modes of activity, has the potential for synergistic interaction. In the present study we evaluated an antifungal activity of PPE, and its potential for synergistic combination with an agricultural azole drug prochloraz (PRZ). We show that this combination is highly effective at inhibiting growth of the most prevalent mycotoxigenic fungal pathogens and mycotoxin production, suggesting a potential for such an approach in improving food and feed safety.

MATERIALS AND METHODS

Preparation of Pomegranate Peel Extract

Pomegranate fruits (*Punica granatum* L.) from the "Wonderful" variety were purchased from local markets. Fruits were washed, and the arils were manually removed. The fruit peels were cut, frozen at −80°C, lyophilized and milled into a fine powder using an electric blender. The dried powder (100 g) was extracted with 500 ml of 80% methanol for 72 h at room temperature in the dark. The extract PPE was filtered through Whatman No. 1 filter paper, concentrated using a rotary evaporator (Buchi R-100, Switzerland) at 45°C, freeze-dried and kept at −20°C until use. PPE was dissolved in dimethyl sulfoxide (DMSO) at 100 mg/ml and kept at −20°C.

Fungal Strains, Media, Growth Conditions, and Chemicals

Aspergillus flavus (NRRL3518) and *F. proliferatum* (NRRL31866) were used throughout the study. In some susceptibility tests also *A. parasiticus* (NRRL6111), *A. fumigatus* (NRRL62427), and *F. verticillioides* (NRRL25457) were used. The isolates were obtained from USDA Agricultural Research Service Culture Collection (Northern Regional Research Laboratory, Peoria, IL, United States). Strains were refreshed from −80°C by sub

culturing on solid potato dextrose agar (PDA; 0.4% potato starch, 2% dextrose, and 2% agar) or broth (PDB) and maintained on PDA plates at 28°C before each experiment. Conidia were collected in sterile saline and the conidial suspension was adjusted to the required concentration by counting in a hemocytometer. The inoculum of the test strains was verified by plating on PDA plates for determination of colony forming units (CFU) counts. PRZ (Sigma) was prepared in DMSO at 25 mg/ml; stock solution was kept at −20°C. RPMI 1640 medium (Sigma) buffered with 0.165M MOPS (morpholinepropanesulfonic acid; pH 7) was used for antifungal microdilution susceptibility testing.

Antifungal Susceptibility Testing

The *in vitro* activities of the antifungal compounds against mycotoxigenic fungi were determined using the standardized CLSI M38-A2 broth microdilution method (CLSI, 2008), with slight modification. Briefly, antifungal agents were dispensed in 96-well microtiter plates with two-fold serial dilutions of compound. The final compound concentration was prepared from stock solution in RPMI 1640 medium. The concentration of PPE and PRZ in the wells ranged from 9.76 to 5000 μg/ml and 0.0078 to 4 μg/ml, respectively. The stock conidial suspension (10^6 spores/ml) was diluted to a final inoculum concentration of 0.4×10^4 to 5×10^4 spores/ml and dispensed into the microdilution wells. Minimal inhibitory concentrations (MIC) of the compounds were determined after 48 h incubation at 28°C. The MIC value was considered as the lowest compound concentration with no visible growth. Interactions between PPE and azole drug were assessed by checkerboard assays to determine the fractional inhibitory concentrations (FIC) of the combination of PPE and PRZ (Meletiadis et al., 2003). The first compound of the combination PPE was serially diluted along the abscissa (horizontal, *x*-axis), while the second drug PRZ was diluted along the ordinate (vertical, *y*-axis). Each microtiter well was inoculated with 100 μl of a fungal inoculum (0.4×10^4 to 5×10^4 conidia/ml), and the plates were incubated at 28°C for 48 h. The resulting checkerboard contains each combination of two compounds, with wells that contain the highest concentration of each compound at opposite corners. The FIC of each compound was calculated by using both MIC endpoints as described previously (CLSI), namely, the ratio of the concentration of the drug in combination that achieves the MIC endpoint to the MIC of the drug alone by using that endpoint. The FIC index (FICI) value was calculated by adding the FIC of PPE to the FIC of PRZ. Drug interactions were classified as follows: FICI $\leq$ 0.5, synergistic; 0.5 $<$ FICI $\leq$ 1, additive; 1 $<$ FICI $\leq$ 4, indifferent; FICI $>$ 4, antagonistic.

Live Imaging Microscopy

Aspergillus flavus and *F. proliferatum* were treated with PPE and PRZ alone, and in combination (checkerboard method), and examined under live imaging microscope. PPE and PRZ were serially diluted in 24-well microtiter plate at the concentration ranges of 156.25 to 2500 μg/ml and 0.0625 to 0.25 μg/ml, respectively. Each well containing each drug alone and combination of two compounds was inoculated with 200 μl of a fungal inoculum of 0.4×10^5 to 5×10^5 conidia/ml. The plate was placed on a motorized stage and conidial germination and hyphal growth were monitored for 24 h at 28°C under a live imaging microscope. Different parameters, such as time to germination, inhibition of conidial germination, mean hyphal elongation rate and maximum hyphal length, were determined for assessment and calculation of fungal growth inhibition. The elongation rate was calculated by averaging the changes during sequential time periods of the fungal growth. All experiments were conducted three times; a minimum of 10 conidia were examined under each treatment. Microscopic imaging was performed using a NIKON eclipse T*i* microscope (Nikon, Japan) equipped with a ProScan motorized XY stage (Prior Scientific, MA, United States) with a temperature-controlled incubator (LAUDA ECO RE 415, Korea). Bright field illumination was provided by a cool LED pE-100A (Cool LED, United Kingdom). The system is also equipped with an HF110A system, enabling rapid switching of emission wavelengths. Imaging was performed using a long working distance 10× objective (NA 0.6). Images were captured at 30 min intervals using an ANDOR zyla 5.5 MP ScMOS camera (China) and processed using the NIS elements AR 4.6 (64 bit) software package.

Sterol Analysis

Sterol profiles of *A. flavus* (NRRL 3518) were analyzed as described previously with some modifications (Sionov et al., 2009). The samples of the fungal strain, which were grown in PDB (10^7 conidia/ml) for 24 h at 28°C, included: (1) no drug control; (2) supplemented with 1250 μg/ml PPE; and (3) supplemented with 0.5 μg/ml PRZ. Another control sample included only PPE (1250 μg/ml) with no fungus (due to the adsorption of PPE into the mycelium that was detected following a change in the mycelium color). Three independent experiments were performed; each experiment included three biological replicates (n = 3) of each treatment as well as the untreated controls, with each biological replicate being one independent extraction. Mycelia were harvested by centrifugation, washed once with sterile distilled water, frozen in liquid nitrogen and lyophilized. Twenty mg of lyophilized mycelium of each sample were resuspended in 9 ml methanol; 4.5 ml 60% (wt/vol) KOH was added together with 2.5 μg cholesterol (used as an internal recovery standard). Mycelial suspension was heated to 85°C in a water bath for 2 h to complete the saponification, and the resulting mixture was cooled to room temperature. The sterols were then extracted twice with 2 ml hexane by vigorous vortex for 2 min. The upper hexane layers containing the sterols were removed, washed twice with water, and evaporated under a stream of gaseous nitrogen. Before derivatization, water residues in the sample were completely evaporated by lyophilization. Subsequently, 50 μl of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was added, the sample was shaken vigorously, and the mixture was transferred to a 2 ml auto sampler glass vial with a 100 μl conical glass insert and analyzed by GC-MS. The GC-MS system comprised an Agilent 7890A gas chromatograph equipped with split/splitless injector, and LECO Pegasus HT Time-of-Flight Mass Spectrometer (TOFMS). GC was performed on a 30 m × 0.25 mm × 0.25 μm Rxi-5Sil MS column (Restek). Samples were analyzed in both

split and splitless modes; injector and transfer line temperatures were set at 280°C. Analytes by 1 μl injected were separated using the following chromatographic conditions: Helium was used as carrier gas at a flow rate of 1.0 ml/min. The thermal gradient started at 170°C, was held at this temperature for 2 min, ramped to 280°C at 37°C/min and then ramped to 300°C at 1.5°C/min and held at 300°C for 5.0 min. Eluents were fragmented in the electron impact mode with an ionization voltage of 70 eV. The MS mass range was 50–750 m/z with an acquisition rate of 20 spectra per second. The ion source chamber was set to 230°C and the detector voltage was 1650 V. The reconstructed ion chromatograms and mass spectra were evaluated using Xcalibur software version 2.2 (Thermo Fisher Scientific). Cholesterol internal standard was identified by comparing the retention time and mass spectrometry spectra of trimethylsilyated (TMS) authentic cholesterol standard analyzed on the same instrument. The metabolites of interest were identifies by comparison with the NIST 05 Mass spectral library using NIST MS Search program v 2.3. Specifically, ergosterol (Purkait et al., 2012; Keller et al., 2015) lanosterol (Paik et al., 2008) and sitosterol (Wretensjö and Karlberg, 2002) were putatively identified based on their trimethylsilyated spectra compared with previously published spectra and relative retention times. The fragment ions at m/z 458 (M +), m/z 468 (M +), m/z 498 (M +), and m/z 486 (M +) are indicative of cholesterol, ergosterol, lanosterol and beta-sitosterol, respectively. The relative amount of each sterol was obtained by comparing the area under the curve for each sterol with that for the cholesterol internal standard in the chromatogram.

Mycotoxin and qRT-PCR Analyses

For the evaluation of AFB1 production 100 μl of *A. flavus* inoculum (10^6 conidia/ml) was inoculated in 25 ml PDB with PPE or PRZ alone, and in combination, and incubated at 28°C with shaking at 200 rpm up to 72 h. The samples included: (1) no drug control; (2) supplemented with 625 and 1250 μg/ml PPE; (3) supplemented with 0.0156 and 0.0312 μg/ml PRZ; and (4) supplemented with both PPE and PRZ. Another control sample included DMSO (because the tested compounds were dissolved in this solvent). After 48 and 72 h of incubation the mycelial biomass was collected by centrifugation, freeze-dried, weighed and stored at −80°C for RNA isolation. For the mycotoxin extraction procedure, the supernatant collected at the same time points was mixed with an equal volume of chloroform and vortexed for 15 min. The lower chloroform phase was dried at 50°C under a stream of gaseous nitrogen. The samples were redissolved in 300 μl of methanol and derivatized with 300 μl of trifluoroacetic acid solution (70% water, 20% trifluoroacetic acid and 10% acetic acid) for 20 min at 65°C. After 20 min 580 μl of water was added to the reacted samples. The samples were vortexed, filtered using 0.2 μm PTFE membrane filter, and quantitatively analyzed by injection of 20 μl into reverse phase UHPLC system (Agilent technologies) with a gradient elution of 0.1% acetic acid in water (59%), methanol (27%), and acetonitrile (14%) at 0.4 ml/min through a Kinetex 2.6 μm XB-C_{18} (100 × 2.1 mm) with a security guard column C18 (4 × 2 mm; Phenomenex, United States). AFB1 peaks were detected with fluorescence detector (excitation at 365 nm and emission at 455 nm) and quantified by comparing with calibration curves of the standard mycotoxin.

The total RNA was extracted from 100 mg of lyophilized mycelia of the selected samples using the Hybrid-R RNA isolation kit (GeneAll, Seoul, South Korea) according to the manufacturer's protocol. The DNase and reverse-transcription reactions were performed on 300 ng of total RNA with the Maxima First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, United States) according to the manufacturer's instructions. The cDNA samples were diluted 1:5 (v/v) with ultrapure water. The quantitative real time PCR was performed using Fast SYBR green Master Mix (Applied Biosystems, Waltham, MA, United States) in a StepOnePlus Real-Time PCR System (Applied Biosystems, Waltham, MA, United States). The primer pairs for the specific 3 genes involved in aflatoxin biosynthesis (*aflR*, *aflC,* and *aflD*) were synthesized based on previous studies (Zhao et al., 2018; Lan et al., 2019). The PCR conditions were as follows: 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 20 s. The sample was normalized using β-actin and the relative expression levels were measured using the $2^{(-\Delta\ \Delta\ Ct)}$ analysis method. Results were analyzed with StepOne software v2.3.

Statistical Analysis

The data were presented as the means ± SE (standard error) of three experiments by measuring three independent replicates. An unpaired *t*-test was used to compare differences in gene expression level between *A. flavus* isolate treated with the compounds and the untreated (no drug) control. A *p*-value of less than 0.05 was considered to be significant ($^*p < 0.05$, $^{**}p < 0.01$).

RESULTS AND DISCUSSION

Synergistic Antifungal Activity of PPE With Azole Drugs

In vitro susceptibility of several important mycotoxigenic species, including *A. flavus*, *A. parasiticus*, *A. fumigatus*, *F. proliferatum* and *F. verticillioides*, to a PPE extract and azole antifungal agent was examined. The pomegranate extract was found to be active against the fungal isolates at relatively high concentrations, with MIC values between 1.25 and 5 mg/ml (**Table 1**). These values are in good agreement with those previously reported for PPE on its *in vitro* and *in vivo* antifungal effect against major fungal post-harvest pathogens (Li Destri Nicosia et al., 2016; Pangallo et al., 2017). Agricultural antifungal azole drug, PRZ, had considerably lower MIC values in the range of 0.25 to 1 μg/ml. Following determination of the individual MIC values, the efficacy of PPE in combination with the azole compound was examined by checkerboard assays against the fungal isolates. Despite its apparent low antifungal activity when used alone, PPE demonstrated synergistic inhibitory effects when combined with PRZ against *A. flavus*, *A. fumigatus* and *F. proliferatum,* with FICI values of 0.25 to 0.5 (**Table 1**); additive effects were observed against *A. parasiticus* and *F. verticillioides* (FICI 0.75–1). It is noteworthy that in the presence of PPE the MIC values of PRZ for *A. flavus* and *F. proliferatum* were reduced

TABLE 1 | *In vitro* susceptibility of mycotoxigenic fungi to PPE and PRZ and in combination.

Fungal strains	MICs of compounds (μg/ml)				FICIs	Interpretation
	Alone		In combination			
	PPE	PRZ	PPE	PRZ		
A. flavus (NRRL 3518)	2500	0.25–0.5	625	0.0625	0.37	Synergy
A. parasiticus (NRRL 6111)	2500	0.5–1	1250	0.25	0.75	Additive
A. fumigatus (NRRL 62427)	2500	0.25–0.5	312.5	0.0625	0.5	Synergy
F. verticillioides (NRRL 25457)	5000	0.25–0.5	2500	0.125	1	Additive
F. proliferatum (NRRL 31866)	5000	0.5–1	625	0.0625	0.25	Synergy

PPE, pomegranate peel extract; PRZ, prochloraz; MIC, minimum inhibitory concentration; FICI, fractional inhibitory concentration index.

by 8- and 16-fold, respectively. Moreover, PRZ lowered the MIC values of PPE by four- to eight-fold against same isolates. These results indicate that the combined approach may decrease the required concentrations of the compounds to effectively inhibit mycotoxigenic fungi. Such combination may allow lower doses of the agents to be used in any application while reducing potential concerns over dosage levels and/or non-specific toxicity of the single compound.

Live-Imaging Based Investigation of the Inhibitory Effects of PPE/PRZ on Mycotoxigenic Fungi

Using time-lapse microscopy we could clearly demonstrate the effect of each compound alone, as well as their combination, on the kinetics of conidial germination, hyphal growth, and branch initiation in *A. flavus* and *F. proliferatum*. One of the most striking features of the time-lapse sequences obtained from our experiments is the apparent delay in germination following treatment with PPE at two-fold lower concentration than its MIC, with both fungi showing markedly reduced growth at 12 h compared to untreated control (**Figures 1**, **2** and **Supplementary Videos S1**, **S2**). However, after 24 h of incubation the growth of PPE-treated *A. flavus* (1250 μg/ml) was not clearly distinguishable from the untreated control. Treatment of *A. flavus* with the azole drug at sub-MIC concentration (0.125 μg/ml PRZ) proved highly effective, resulting in marked inhibition of both germination and growth over 24 h of incubation. Interestingly, a combined treatment (1250 μg/ml PPE + 0.125 μg/ml PRZ) resulted in further reduction in both parameters (**Figure 1** and **Supplementary Video S1**). A different image emerged for *F. proliferatum,* where PPE effect at 2500 μg/ml resulted in significant delay of branch initiation and reduction of lateral branches formation compared to untreated control over the course of the experiment (**Figure 2** and **Supplementary Video S2**). Surprisingly, over the 1st 12 h of incubation, PPE treatment appeared to be more effective than treatment with 0.125 μg/ml PRZ. Following 24 h of incubation, however, PRZ treatment resulted in stunted growth of *F. proliferatum* characterized by severe hyper-branching. Similar to *A. flavus,* the combined treatment here (2500 μg/ml PPE + 0.125 μg/ml PRZ) appeared to be the most effective, resulting in the least observed growth while also eliminating the hyper-branching observed for PRZ alone, suggesting the two treatments to have different modes of action and possibly synergistic effects. Interestingly, while reduced growth rate is many times accompanied by hyper-branching, as in the case of PRZ effect on *F. proliferatum,* hyphal polar extension and hyphal branching are two distinct morphological processes which are independently regulated (Seiler and Plamann, 2003; Ziv et al., 2009). This is further supported by the different changes induced in the growth and structure of hyphae, which indicate a different effect for both PPE and PRZ.

Quantification of Antifungal Effects of Sub-MIC Concentrations of PPE and PRZ by Image Analysis

A major advantage of live-imaging microscopy over more traditional approaches for measuring fungal growth is the ability to observe and quantify the growth parameters of individual spores. The usefulness of this approach has been demonstrated in a number of studies, and was applied for monitoring different dynamic processes in bacteria and fungi including sporulation, mycelial growth, and host-pathogen interactions (Löfman et al., 1997; Richter et al., 2007; Jyothikumar et al., 2008; Held et al., 2010, 2011; Grünberger et al., 2017; Marshall et al., 2017). Here we utilize the power of this approach to quantitatively compare the effect of conventional and natural antifungal agents on several growth parameters of *A. flavus* and *F. proliferatum*. Conidia or sexual spores are critical in the fungal life cycle. Fungal development starts from conidial germination under favorable growth conditions. Therefore, the dynamic analysis of early germination under treatments with antifungal compounds might improve our understanding of the physiological response of fungi and thus may help for the development a new combination strategy to fight fungal infections. An antifungal treatment may reduce fungal biomass by delaying spore germination or by reducing the overall rate of germination (i.e., percent of spores that develop into hyphae). Treatment with PPE alone did not affect germination rate in *A. flavus* regardless of concentration. The effect of PRZ treatment was dose dependent, with approximately 9% reduction in germination rate for 0.0625 μg/ml compared to 36% inhibition for 0.125 μg/ml (**Figure 3A** and **Supplementary Figure S1A**). Moreover, the combinations of PPE with PRZ at 0.125 μg/ml

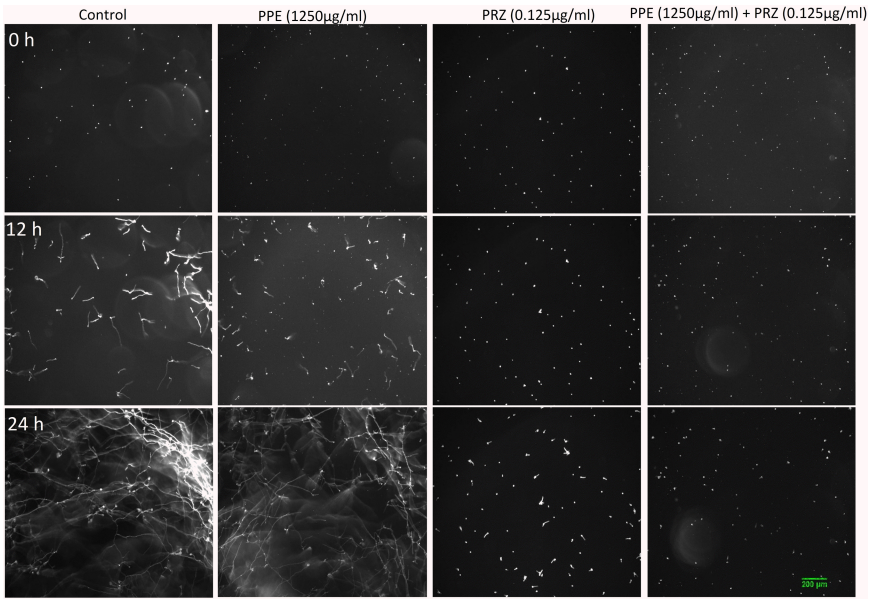

FIGURE 1 | Time-lapse microscopic images of *A. flavus* treated with antifungal compounds. Three different time points as indicated are given to visualize the images of *A. flavus* treated with PPE/PRZ alone and in combination (original magnification: ×10). Experiments were repeated three times and results of a single representative experiment are shown.

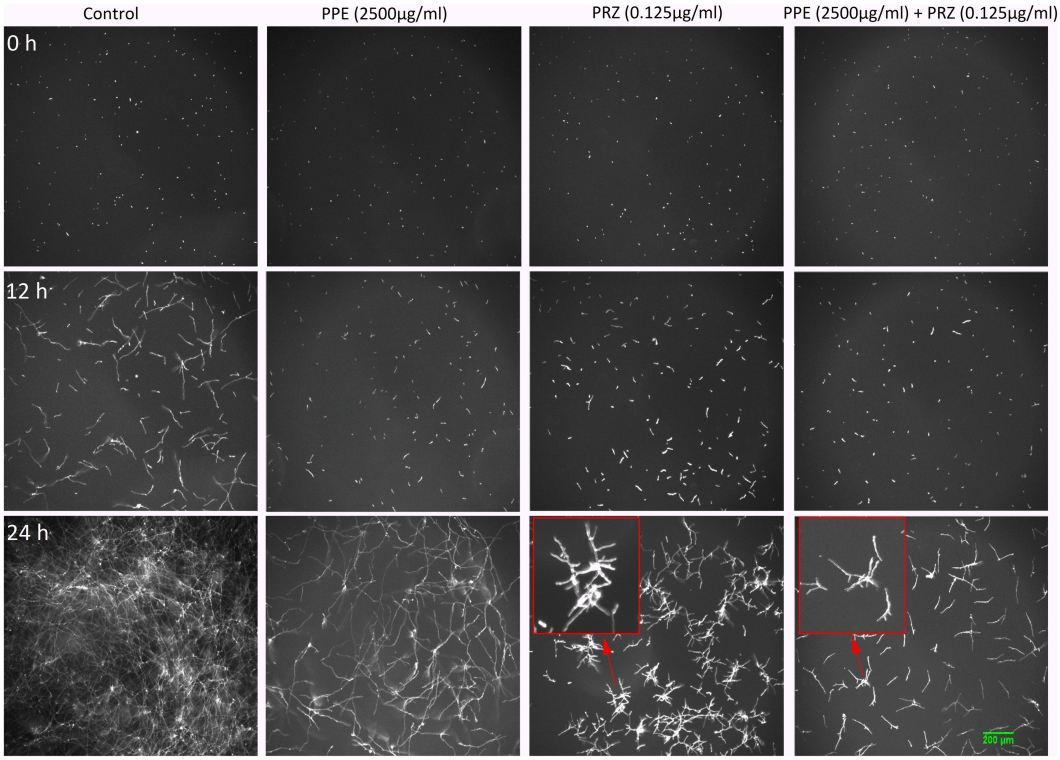

FIGURE 2 | Time-lapse microscopic images of *F. proliferatum* treated with antifungal compounds. Three different time points as indicated are given to visualize the images of *F. proliferatum* treated with PPE/PRZ alone and in combination (original magnification: ×10). Enlarged regions of the original images are marked with red box. Experiments were repeated three times and results of a single representative experiment are shown.

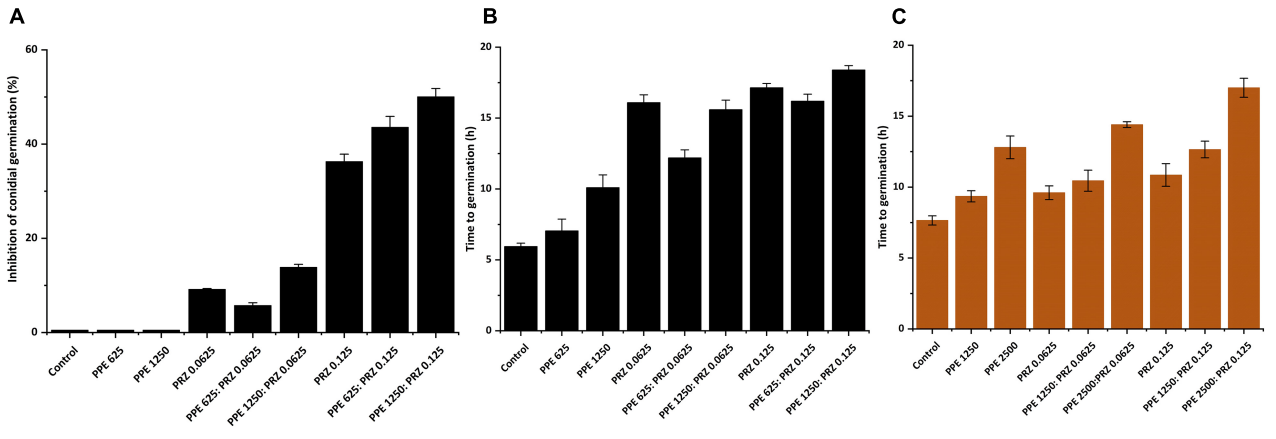

FIGURE 3 | Effect of antifungal compounds on conidial germination. **(A)** Percentages of inhibition of *A. flavus* conidial germination following treatment with different concentrations of PPE and PRZ both alone and in combination. **(B)** Effect of PPE/PRZ both alone or in combination on time required for germination of *A. flavus* conidia, and **(C)** *F. proliferatum* conidia. Results are expressed as the means ± SE of three experiments.

resulted in higher rates of inhibition, with germination rate dropping to 50%. Different from *A. flavus, F. proliferatum* conidia showed 100% germination for all treatments, pointing out that any treatment only delayed conidial germination but did not alter the overall germination rate (**Supplementary Figure S1B**). The mean time to germination, determined as the time point at which the germ tube exceeded spore diameter, was found to be affected by all tested treatments. Mean time to germination for the untreated *A. flavus* control was 5.95 h, compared to 10.1 h following treatment with 1250 μg/ml PPE and 17.15 h following treatment with 0.125 μg/ml PRZ (**Figure 3B**). Similarly, the untreated *F. proliferatum* control had a mean time to germination of 7.65 h, while treatment with 2500 μg/ml PPE extended the mean germination time to 12.8 h (**Figure 3C**). Interestingly, treatment with 0.125 μg/ml PRZ resulted in a less pronounced delay, with a mean time to germination of just 10.85 h. The effect of combined treatments on the mean time to germination in *A. flavus* varied with concentration. No synergy between the two treatments was found when 625 μg/ml PPE where added to either concentration of PRZ (**Figure 3B**). Addition of 1250 μg/ml PPE had no effect on germination time when combined with 0.0625 μg/ml PRZ, compared to the azole drug alone, but had a slight positive effect when combined with 0.125 μg/ml PRZ, extending the mean time to germination from 17.15 h for PRZ alone to 18.4 h for the combined treatment. A better synergistic inhibitory effects were found for combined treatments against *F. proliferatum*, especially when either PRZ concentration was combined with 2500 μg/ml PPE, extending the time to germination up to 17 h (**Figure 3C**). Hyphal elongation rate is another criterion used to determine the effect of the compounds on fungal growth while using live imaging microscopy techniques. PPE treatment at a concentration of 1250 μg/ml slowed the mean elongation rates of both *A. flavus* and *F. proliferatum* hyphae up to 26.34 and 30.9 μm/h, respectively, compared to those of the untreated controls (43.04 and 37.06 μm/h, respectively) (**Figures 4A,B**). When *A. flavus* was treated with the azole drug at sub-MIC concentrations of either 0.0625 or 0.125 μg/ml, the mean elongation rate of the filaments was nearly 10-fold slower (2.85–4.26 μm/h) compared to that of the untreated control. It is noteworthy that the mean hyphal elongation rate for *A. flavus* almost unchanged under combination treatment compared to the azole drug alone (**Figure 4A**). The pomegranate extract at two-fold higher concentration (2500 μg/ml) significantly inhibited *F. proliferatum* growth and led to reduction in the mean of hyphal elongation rate up to 15.05 μm/h. Under PRZ treatment *F. proliferatum* hyphal mean elongation rate considerably reduced up to 75% (9.47 μm/h) in comparison to the control (**Figure 4B**). However, the combined treatment of PPE with PRZ, when tested at higher sub-MIC concentrations, yielded synergistic inhibitory effect with further reduction of *F. proliferatum* hyphal extension rate up to 6.36 μm/h compared to each compound alone (**Figure 4B**). Following germination, the elongation rate of the hypha increases exponentially toward a maximum linear rate (Robson, 1999), which is an additional measurement standard that has been calculated in order to evaluate antifungal efficacy of the compounds and their combinations. Interestingly, unlike the mean elongation rate, the maximum rate of hyphal extension for both fungi was lower under combination treatment at higher sub-MIC concentrations of the compounds compared to the azole drug alone (**Supplementary Figures S2A,B**). These data are reflected in video-microscopy images where synergistic effect of the PPE compound combined with the azole drug PRZ was clearly observed (**Figures 1**, **2**). As was expected, hyphal elongation rate is closely correlated with the time required for conidial germination: the longer it takes to germinate due to the treatment, the lower the rate of the hyphal extension.

Effect of PPE on Sterol Composition in *A. flavus*

Ergosterol is a lipid responsible for fungal cell membrane fluidity and permeability and plays a crucial role in its viability. Several antifungals, such as azole drugs, primarily

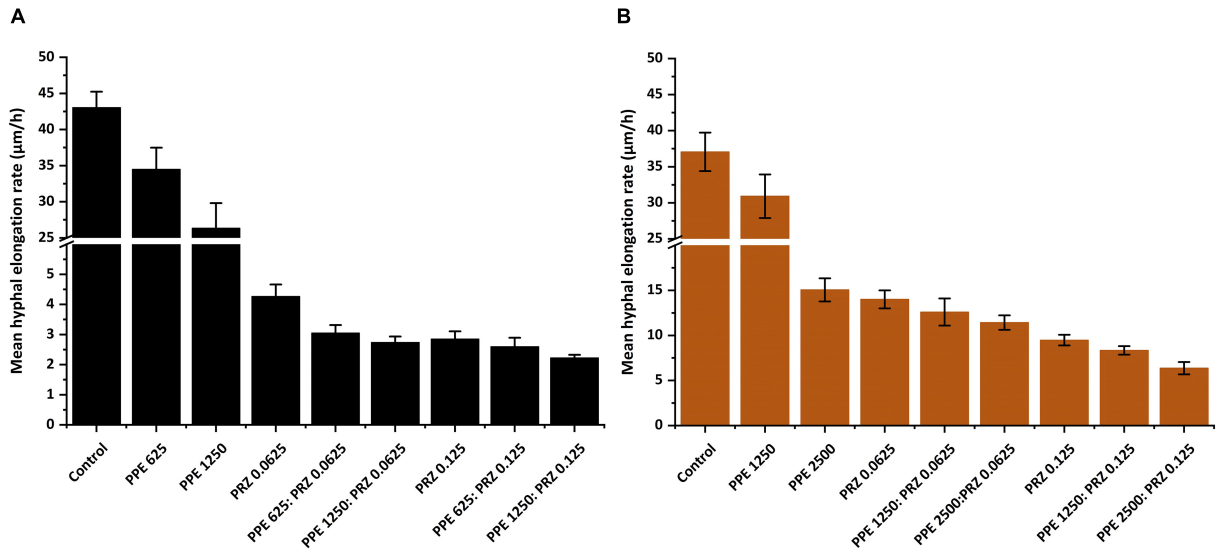

FIGURE 4 | Graphical representation of the fungal growth under treatment with antifungal compounds. Mean hyphal elongation rates of **(A)** *A. flavus,* and **(B)** *F. proliferatum* treated with different concentrations of PPE and PRZ both alone and in combination. Results are expressed as the means ± SE of three experiments.

target ergosterol biosynthesis. Azole antifungals affect ergosterol biosynthesis via inhibition of 14-α demethylase (Cyp51/Erg11), a fungal cytochrome P-450 enzyme, which mediates the conversion of lanosterol to ergosterol. Application of PRZ treatments to *Sclerotinia sclerotiorum* and *Botrytis cinerea* have indicated that sterol-inhibiting fungicides do not inhibit spore germination or initial cell growth but result in aberrant hyphal morphology, namely swollen hyphae and/or hyper-branching (Pappas and Fisher, 1979; Zhang et al., 2018) similar to our observation in *F. proliferatum* in the current work. This is also in line with the phenotypic consequence of mutating *erg11* (or *cyp51,* the suspected target of PRZ). A conditional mutant of *Neurospora crassa* demonstrate that Erg11 is required for hyphal elongation but not germination (Hu et al., 2018). However, the pronounced effect of PRZ on *A. flavus* germination and the synergistic effect with PPE may suggest a more complex effect of these drugs on the fungal physiology. We hypothesized that PPE, similarly to PRZ, may interfere with ergosterol function in the fungal cell membrane. To test this hypothesis, the sterol profile of *A. flavus* was compared with that of *A. flavus* treated with PPE and/or azole antifungal PRZ. Without any drug treatment, ergosterol was the major fraction of the total sterol content (**Figure 5** and **Supplementary Figure S3**). Following PRZ treatment, the ergosterol content was dramatically reduced in *A. flavus* in parallel to an increase of lanosterol (**Supplementary Figure S3**), consistent with the classic pattern of *Aspergillus* sterol 14α-demethylase activity following treatment with azoles (Parker et al., 2014). Interestingly, PPE treatment of the isolate also resulted in a significant decrease of ergosterol content in comparison to the untreated control (**Figure 5**). No lanosterol was detected in the PPE treatment, however, this could be due to the wide peak of β-sitosterol which co-eluted with lanosterol and was highly abundant in the PPE itself, as indicated by the PPE control samples (blank, without fungus) (**Supplementary Figure S3**). The results suggest that PPE compound may act upstream or downstream of the azole target Erg11 and might interfere with sterol function through potential inhibition of certain enzymatic steps in the ergosterol biosynthesis pathway.

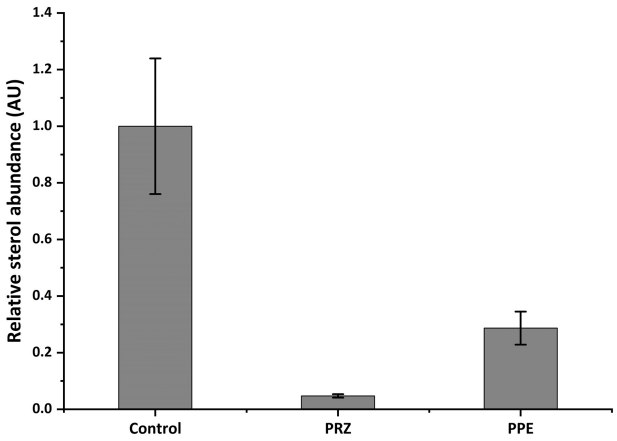

FIGURE 5 | Relative abundance of ergosterol in *A. flavus* as detected by GC-MS. Data presented is peak area of ergosterol relative to cholesterol standard, normalized to control. Results are expressed as the average ± SE of three independent experiments, each with three biological replicates; AU, arbitrary units.

Anti-mycotoxigenic Activity of PPE/PRZ

Pomegranate peel extract treatments appear to have the capability of inhibiting aflatoxin production by *A. flavus* (**Table 2**). In

TABLE 2 | Effect of antifungal compounds and their combination on aflatoxin B1 production by *A. flavus*.

Compounds[a]	AFB1 (ng/ml)	
	48 h incubation	72 h incubation
Control 1 (no treatment)	65.33 ± 2.1[b]	147.79 ± 6.18
Control 2 (DMSO)	73.87 ± 3.42	157.37 ± 5.94
PPE 625 μg/ml	nd	188.84 ± 6.63
PPE 1250 μg/ml	nd	48.97 ± 4.22
PRZ 0.0156 μg/ml	nd	217.57 ± 11.7
PRZ 0.0312 μg/ml	nd	nd
PPE 625 + PRZ 0.0156	nd	nd
PPE 625 + PRZ 0.0312	nd	nd

[a]*No reduction in fungal biomass was observed under treatment with each compound alone and in combination at the concentrations indicated in the table;* [b]*Average values of mycotoxin concentration ± (SE) standard error (the average of three independent experiments, each with three biological replicates) "nd" not detected.*

particular, after 72 h of incubation the compound at the concentration of 1250 μg/ml inhibited AFB1 production by 67% without affecting the fungal growth. Reduction in fungal biomass by at least 50% was observed under PPE treatment at the higher concentration of 2500 μg/ml with subsequent inhibition of AFB1 production by 97%. These findings suggest that PPE inhibitory activity of fungal growth and mycotoxin formation are not directly related, and the inhibition of aflatoxin production by the extract could involve an inhibition of specific enzymes in the pathway of aflatoxin biosynthesis. Compared to the untreated controls, there was an increase in AFB1 production by the fungus when treated with an azole agent PRZ or pomegranate extract at low concentrations of 0.0156 and 625 μg/ml, respectively (**Table 2**). Among different environmental factors, such as temperature, oxidative stress, water activity and pH, application of low fungicide concentrations might be an additional stress factor stimulating mycotoxin biosynthesis by fungi as a defense response. Several studies reported that sub-lethal concentrations of synthetic or natural fungicides stimulated mycotoxin production. For example, increased production of deoxynivalenol (DON) and 3-acetyl deoxynivalenol (3-ADON) has been observed when low doses of azole fungicides were used against *F. culmorum* cultures (D'Mello et al., 1998; Magan et al., 2002). A four-fold increase in aflatoxin synthesis by *A. parasiticus* on different substrates occurred in the presence of sub-inhibitory level of miconazole (Buchanan et al., 1987). A number of studies reported that several plant essential oils at sub-lethal concentrations could reduce the growth of mycotoxigenic *Fusarium* and *Aspergillus* species, but stimulated their toxins production (Hope et al., 2005; Nerilo et al., 2016; Morcia et al., 2017). However, in the current study, combination of two compounds at suboptimal concentrations completely inhibited AFB1 synthesis by *A. flavus*, compared to increased mycotoxin production by the fungus when treated with each compound alone (**Table 2**). Furthermore, the effect of PPE, PRZ and their combination at suboptimal concentrations on the expression level of key genes in the aflatoxin biosynthesis cluster, *aflR* (aflatoxin transcription factor), *aflC* (polyketide synthase) and *aflD* (nor-1/reductase), was analyzed by qRT-PCR. The results indicated that the expression levels of these genes were down-regulated under combined treatment of PPE with PRZ at low doses, directly causing depression of aflatoxin production (**Figure 6**). These findings are consistent with a recent study of Wang et al. (2018) where combination of cinnamaldehyde and citral (the major components of *Cinnamon bark* essential oil) at sub-MIC concentrations resulted in a significant decrease of patulin biosynthesis by *Penicillium expansum*. According to the RNA sequencing results in that study, the expressions of all the 15 genes involved in patulin biosynthetic pathway were down-regulated under cinnamaldehyde and citral combined treatment at sub-MIC concentrations (Wang et al., 2018). Taking these results together, we suggest that the complete elimination of the mycotoxin can be achieved by an azole fungicide application at

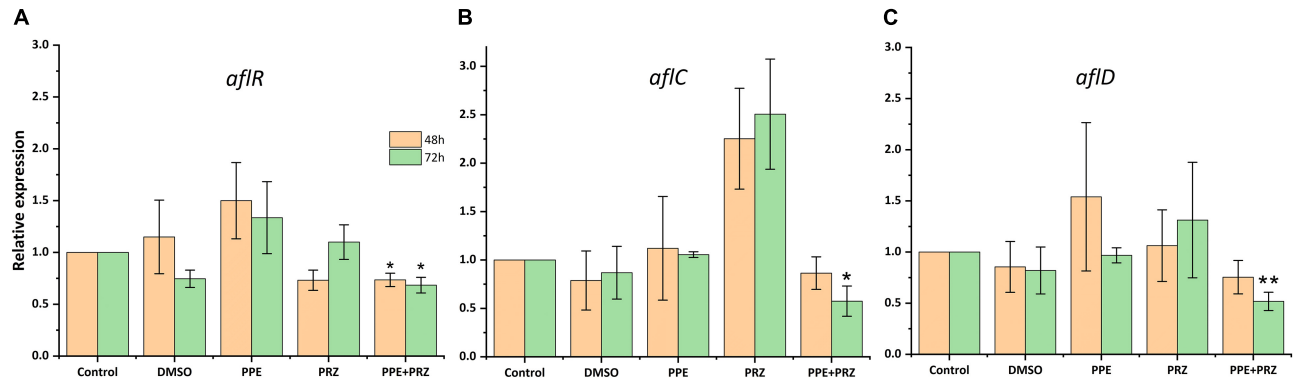

FIGURE 6 | Effect of antifungal compounds on the expression of key aflatoxin biosynthetic pathway genes in *A. flavus*. Expression ratios of **(A)** *aflR* (aflatoxin transcription factor), **(B)** *aflC* (polyketide synthase), and **(C)** *aflD* (nor-1/reductase) in *A. flavus* treated with PPE (625 μg/ml) and PRZ (0.0156 μg/ml) both alone and in combination compared to untreated (no drug) control; DMSO control was also included. The expression of each gene in the untreated (no drug) control samples was normalized as 1.0. Results are expressed as the average ± SE of three independent experiments, each with three biological replicates, $^{*}p < 0.05$, $^{**}p < 0.01$. SE in normalized control (no drug) samples is 0 due to the $2^{(-\Delta\Delta Ct)}$ analysis method.

very low concentrations with less toxicity to the environment when combined with PPE, through modulating the expression of key aflatoxin biosynthetic pathway genes.

CONCLUSION

In summary, our study demonstrated that the combination of natural antifungal compound with conventional synthetic fungicide is highly effective at inhibiting growth of certain major mycotoxigenic and food-spoilage fungi. This combination was particularly effective while producing a synergistic suppression effect at considerably lower doses on aflatoxin biosynthesis by *A. flavus*. Moreover, the results may provide flexibility to determine the dose range of the compounds that can be used in combination for practical applications. Therefore, it is proposed that this combination approach can offer an effective strategy for controlling fungal growth and mycotoxin production in agricultural commodities.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

SS, OS, and ES conceived and designed the experiments. SS, CZ, OB, and VZ performed the experiments. SS, OS, CZ, OB, and ES analyzed the data. SS, OS, CZ, and ES wrote the manuscript. All authors read and approved the final manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01919/full#supplementary-material

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Aflatoxin B_1-Adsorbing Capability of *Pleurotus eryngii* Mycelium: Efficiency and Modeling of the Process

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Aflatoxin B_1 (AfB_1) is a carcinogenic mycotoxin that contaminates food and feed worldwide. We determined the AfB_1-adsorption capability of non-viable *Pleurotus eryngii* mycelium, an edible fungus, as a potential means for removal of AfB_1 from contaminated solutions. Lyophilized mycelium was produced and made enzymatically inert by sterilization at high temperatures. The material thus obtained was characterized by scanning electron microscopy with regard to the morpho-structural properties of the mycotoxin-adsorbing surfaces. The active surfaces appeared rough and sponge-like. The AfB_1-mycelium system reached equilibrium at 37°C, 30 min, and pH 5–7, conditions that are compatible with the gastro-intestinal system of animals. The system remained stable for 48 h at room temperature, at pH 3, pH 7, and pH 7.4. A thermodynamic study of the process showed that this is a spontaneous and physical adsorption process, with a maximum of 85 ± 13% of removal efficiency of AfB_1 by *P. eryngii* mycelium. These results suggest that biosorbent materials obtained from the mycelium of the mushroom *P. eryngii* could be used as a low-cost and effective feed additive for AfB_1 detoxification.

Keywords: biosorption, aflatoxin, *Pleurotus eryngii*, feed additive, king oyster mushroom

INTRODUCTION

The contamination of food with mycotoxins is a worldwide problem with impact on the health of humans and animals and on the economy of many countries, especially in sub-tropical and temperate areas. The problem is caused by the spoilage of agricultural products by microscopic filamentous fungi, mostly belonging to species and strains in the genera *Aspergillus*, *Fusarium,* and *Penicillium*, which in favorable environmental conditions are able to produce toxic secondary metabolites that accumulate into food and feedstuffs. Aflatoxin B_1 (AfB_1) is the most toxic mycotoxin and is classified in the "group 1" substances (carcinogenic to humans) by the International Agency for Research on Cancer (World Health Organization and International Agency for Research on Cancer, 1993). AFB_1 has potent hepatotoxic, carcinogenic, and mutagenic effects on humans and animals and is produced mainly by isolates of the species *Aspergillus flavus* and *A. parasiticus*. AFB_1 occurrence is a major problem in a number of crops, including

cereals, groundnuts, legumes, and cotton seeds, which can be contaminated at any stage from field to storage. Human exposure to AFB_1 can result from ingestion of contaminated food or from consumption of meat, eggs, and dairy products from animals that have been fed with contaminated feed (Rushing and Selim, 2019).

Much attention is devoted to measures of prevention and monitoring that aim at the reduction of contamination levels in commodities. Nevertheless, often the contamination occurs despite careful application of prevention means, and it is necessary to put into action decontamination measures to avoid the complete loss of the produce and mitigate the risk of mycotoxins in food and feed. Different chemical and physical methods for decontamination and detoxification have been developed, but their use is often limited by high cost, lack of information on nature and toxicity of degradation products and, above all, loss of nutritional, organoleptic, and visual qualities (Boudergue et al., 2009).

For protection of animals from aflatoxicosis, the use of adsorbent materials which are able to bind with high efficiency the mycotoxins in feeds is being receiving growing interest (Williams et al., 2004). The adsorbents reduce the bioavailability of mycotoxins in the gastro-intestinal tract and thus their diffusion into the bloodstream and transport to the target organs (Kabak et al., 2006; Kolosova and Stroka, 2011). Aluminosilicates are the most used adsorbents, followed by activated carbon and special polymers (Huwig et al., 2001; Vila-Donat et al., 2018). The EU has approved the use of various adsorbent materials as food additives, for example the use of bentonite as a feed additive for all animal species is regulated by the Commission Implementing Regulation (EU) No. 1060/2013. The efficiency of these mycotoxin ligands differs considerably, depending on the chemical structures of both the adsorbent and the toxin (Fu and Viraraghavan, 2001; Crini, 2006). Also, the use of these materials may have a negative impact on the quality of decontaminated feeds. For this reason, scientific interest is partly shifting toward the use of less expensive, though effective, and environmentally friendly materials such as microbial biomasses (Low et al., 2008).

Some studies have demonstrated the ability of some strains of lactic and bifidus bacteria to efficiently bind AfB_1 (El-Nezami et al., 1998; Peltonen et al., 2001), through a chemical-physical phenomenon related to the features of structural elements of the bacterial wall such as peptiglycans and polysaccharides (Kabak et al., 2006). However, few materials have been studied for this purpose and to our knowledge there are no studies concerned with the use of non-viable fungal mycelium as mycotoxin adsorbent. The mycelium of fungi has noteworthy adsorbing properties, mostly due to the ability of the polysaccharides constituting the cell wall to form hydrogen, ionic, or hydrophobic interactions with organic and inorganic molecules (Huwig et al., 2001). These properties are the subject of research with practical applications in different contexts, including bioremediation of soils and wastewater from heavy metals and organic pollutants (Gavrilescu, 2004; Gadd, 2009). Hence, the use of fungal mycelium also as biosorbent for mycotoxins appears conceivable.

The present work is focused on a new adsorbent material made from mushroom mycelium. This novel biosorbent is different from those already on the market, since it is palatable and has nutritional value. Particularly, we report on the characterization of the biosorbent properties of the mycelium of *Pleurotus eriyngii* (DC.) Quél. (king oyster mushroom). This fungus combines several advantageous features. Species of *Pleurotus* can be grown easily and are cultivated worldwide. They can be grown on a variety of lignocellulosic materials, including wastes which are produced through agricultural, forest, and food-processing activities. They grow faster than other cultivable mushrooms and cultivation has no particular technical hurdles (Sánchez, 2010); therefore, large biomasses of *Pleurotus* spp. can be obtained at sensible cost. Besides being easily cultivable and edible, *Pleurotus* spp. exhibit some properties of biotechnological interest. These fungi are high producers of extracellular ligninolytic enzymes, namely phenol oxidases (mainly laccases) and peroxidases (lignin peroxidase and Mn peroxidase) (Sánchez, 2010). Because of the low substrate specificity of these ligninolytic enzymes, applications of *Pleurotus* have been investigated for bioremediation purposes, e.g., decontamination of wastewater and water sediments from phenolic endocrine disruptors (Loffredo et al., 2013), degradation of dyes (Kalmiş et al., 2008) and mycotoxins (Alberts et al., 2009; Branà et al., 2017) and of other recalcitrant environmental pollutants (Rigas et al., 2009; Purnomo et al., 2010). In addition, mycelia of *Pleurotus* spp. have been reported to have binding and sequestering capabilities for heavy metals (recently reviewed by Kapahi and Sachdeva, 2017).

Fungal cell walls have already received attention as biosorbents for bioremediation of polluted soils and wastewaters (Wang and Chen, 2009). We here propose a novel application intended for feed industry. In particular, we have investigated the capability of non-viable mycelium of *P. eryngii* to bind AfB_1; in addition, the effects of physical and chemical conditions on the binding efficiency were studied through a Design of Experiment (DOE) methodology. In most of the works concerned with the adsorption process, an approach that takes into account one factor at a time is used, while there are few studies that use a factorial design model to evaluate the relative importance and the interaction of different operative factors on the biosorption process (Manal, 2007). The design determines which factors have significant effects on the response, as well as the cases in which the effect of a factor varies with the level of another (Brasil et al., 2006), using the least possible number of experiments. The determination of interactions between factors is the key for optimization of complex processes. In the absence of such a study, important interactions might remain undetermined and the optimization becomes difficult to achieve (Brasil et al., 2006). For this reason, our study firstly evaluated the effects of different factors on the adsorption process and then we proceeded with the assessment of the stability of the mycelium-mycotoxin system and the identification of the experimental conditions that achieve

Abbreviations: ΔG^0, Gibbs free energy; ΔH^0, Standard enthalpy; ΔS^0, Standard entropy; ABTS, 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonate; Ads%, Percentage of adsorption; AfB_1, Aflatoxin B_1; FLD, Fluorescence detector; LOQ, Quantification limit of the method; MEA, Malt extract agar; MEB, Malt extract broth; PBS, Phosphate buffer saline; SE, Secondary electrons; SEM, Scanning electron microscope.

the highest efficiency in mycotoxin binding of AfB1 and its removal from a solution. Our results show that in the optimized process, non-viable mycelium of the fungus *P. eryngii* is able to absorb up to 85% of AfB_1 at temperature (37°C) and pH (5 and 7) conditions that are compatible with animal physiology, and a possible development of fungal mycelium-based biosorbent as feed additive can be conceived.

MATERIALS AND METHODS

Reagents and Standards

The standard solution of AfB_1 at 1 mg/ml was prepared by dissolving the solid commercial mycotoxin (SigmaAldrich, Milan, Italy) in toluene/acetonitrile (9,1, v/v). The stock solution was diluted, at a concentration of 10 μg/ml and quantified according to AOAC Official Method 971.22 (AOAC, 2000). The stock solution was evaporated at 50°C in an air stream and dissolved in appropriate buffers (pH 5 or 7) at a concentration of 500 ng/ml. The calibration solutions were obtained by diluting at 0.6, 1.2, 2.4, 5.7, 11.0, 23.0, 57.0 ng/ml. The solutions were stored at −20°C and warmed to room temperature before use. All solvents (grade HPLC) were purchased by VWR. International S.r.l (Milan, Italy), water was of Milli-Q quality (Millipore, Bedford, MA, USA). Regenerated cellulose membrane filters (RC 0.2 μm) were obtained from Phenomenex (Bologna, Italy). The filter paper used was Whatman # 4 (Whatman, Maidstone, UK). The 0.1 mol/L phosphate buffer (PBS) was prepared by dissolving the tablets (SigmaAldrich, Milan, Italy) in water and adjusted to pH 7 or to pH 7.4 with sodium hydroxide. The 0.01 mol/L acetate buffer (pH 5) was prepared by dissolving tri-hydrate sodium acetate (SigmaAldrich, Milan, Italy) in water adjusted to pH 5 with acetic acid. The 1 mmol/L citrate buffer (pH 3) was prepared by dissolving tri-sodium citrate 2-hydrate in water and adjusted to pH 3 with citric acid.

Preparation of the *Pleurotus eryngii* Mycelium

The isolate *P. eryngii* ITEM 13681 that was used in this study was obtained from the collection of Institute of Sciences of Food Production (ITEM Collection, http://www.ispa.cnr.it/Collection/, Bari, Italy). The culture was grown in purity on malt extract agar (MEA, Oxoid, Basingstoke, UK) slants, for 30 days at 28°C, which were used as sources of inoculum for subsequent cultures in malt extract broth (MEB). Five mycelial plugs (8 mm diameter) were transferred onto Roux flasks filled with 200 ml of MEB and incubated under static conditions for 20 days at 28°C. After incubation, the mycelium was separated from the culture broth by filtration through filter paper by applying vacuum and then washed four times with 25 ml of sterile distilled water. The biomass collected was then inactivated by autoclaving at 121°C for 20 min, lyophilized for 3 days, varying the temperature from −20 to 20°C and maintaining the pressure of 0.030 mbar, and finally ground with a mortar and then sieved to collect a fine powder (particle size ≤500 μm).

Dosage of Laccase Activity

A 100 mmol/L sodium malonate buffer (pH 4.5) was prepared by dissolving sodium malonate hydrate in distilled water. The solution was adjusted to pH 4.5 with 100 mmol/L malonic acid. An amount of 0.1 gram of ground autoclaved mycelium was extracted with 5 ml of 100 mmol/L phosphate buffer (PBS) at pH 7.3 and incubated for 60 min, at 25°C in a rotary shaker at 150 rpm. The extract obtained was filtered and used for the enzymatic assay. The laccase activity was determined spectrophotometrically by oxidation of 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonate (ABTS) at 37°C (Li et al., 2008). The reaction mixture (1.5 ml) contained 0.75 ml of sodium malonate buffer (100 mM, pH 4.5), 0.075 ml of ABTS (2 mM in water solution), 0.655 ml of H_2O, and 0.02 ml of enzyme extract. The oxidation of the ABTS was evaluated spectrophotometrically (Varian Cary 50) by the increase of absorbance at 420 nm. One laccase unit was defined as the quantity of enzyme able to oxidize 1 μmol of ABTS in 1 min, given a molar extinction coefficient ε_{420} = 36,000 M^{-1} cm^{-1}.

Analysis of Aflatoxin B_1

The chromatographic analysis of the AfB_1 was performed by high-performance liquid chromatography (HPLC, Agilent Technology Series 1,260) associated to a fluorescence detector (FLD). Before the injection, the mycotoxin was derivatized by a photochemical post-column derivatization reaction (UVE™ LCTech GmbH, Obertaufkirchen, Germany). A Synergi 4U MAX-RP 80A reverse phase column (150 mm × 4.6 mm, 4.0 μm) (Phenomenex, Torrance, California, USA) was used, preceded by a pre-column (MAX-RP, 4 mm × 3.0 mm, Phenomenex) thermostatically controlled at 40°C. The mobile phase consisted of water-acetonitrile, 60:40, with a flow rate set at 1 ml/min. The fluorometric detector was set at the wavelengths of 365 nm (excitation) and 435 nm (emission). Under these analytical conditions, the retention time of the AfB_1 was about 6 min. AfB_1 was quantified by measuring the peak area and comparing it with the calibration curve obtained with standard solutions. The quantification limit of the method (LOQ) was 0.6 ng/ml, based on a 10:1 signal-to-noise ratio.

Determination of Major Variables Affecting Adsorption and Optimization of the Process

A factorial design was employed to reduce the total number of experiments needed to achieve the optimization of the system. The design adopted determined which factors have significant effects on the response and how the effect of one factor varies with the levels of the other factors (interactions). A full factorial design 2^4 was adopted. All the experiments were done in duplicate, the experiments were arranged in random blocks to avoid systematic errors, and the experiments were performed in two different working days. The variables studied were pH of solution (5 and 7), time of interaction t (30 and 120 min), mass of adsorbent (50 and 500 mg), and concentration of AfB_1 (50 and 500 ng/ml). In order to evaluate both the stability of AfB_1 in the buffer solutions under the

experimental conditions and any nonspecific interaction of the toxin with the buffer components or the test tube surface, we prepared blank controls. A blank control consisted of a standard working solution of AfB_1 in the absence of adsorbent material, which was treated in the same way as the experimental treatments. In addition, negative controls (solution containing the adsorbent material in the absence of AfB_1) were set up during each test to assess the absence of potential matrix constituents that could interfere with the chromatographic analysis. Reduction of AfB_1 in the treatments was compared to the blank control. The values of p from the analysis of variance (ANOVA) were used to check the significance ($p < 0.05$) of the effect of different parameters and of the interactions between variables (Kavak, 2009). Optimization of the process was carried out, considering the two most significant parameters obtained from the previous analysis: mass of adsorbent (m) and concentration of AfB_1. A completely randomized factorial experimental design 3^2 was used for optimization, in which the mass of adsorbent (400, 700, 1,000 mg) and AfB1 concentration (50, 525, 1,000 ng/ml) were investigated at three levels and five center points. Blank controls and negative controls were set up for this experiment as described above.

Aflatoxin Adsorption Experiments

Different amounts of powdered mycelium were transferred into 15-ml test tubes and 8 ml of AfB_1 solution at different concentrations at pH 5 or 7 were added. The suspensions were mixed on a vortex to ensure homogeneity and placed in an orbital shaker at 250 rpm, in the dark, at different temperatures and for different periods of time. Subsequently, the samples were centrifuged for 10 min at 10397 × *g* at 25°C, the supernatant was recovered, and the pellet was washed twice with the same buffer used for suspension. The supernatant and the washing solutions were collected and analyzed by HPLC/FLD. For each experiment, a control was prepared using AfB_1 standard solution in buffer without adsorbent material, in order to evaluate the stability of mycotoxins in the buffer solution under the experimental conditions or the occurrence of any nonspecific toxin interaction with the surface of the tubes. A negative control consisting of the buffer solution without the adsorbent material and AfB_1 was also analyzed to evaluate the absence of potential matrix constituents able to interfere with the chromatographic analysis of the toxin. The experiments were carried out in triplicate.

The percentage of adsorption (Ads%) was calculated using the following equation:

$$Ads\% = \frac{(C_0 - C_e)}{C_0} \times 100$$

where C_0 was the initial concentration of AfB_1 in solution and C_e was the mycotoxin concentration measured in the supernatant and the washing solutions after the adsorption.

Desorption

Aliquots of *P. eryngii* powdered mycelium were weighed and subjected to the treatment to assess the adsorption of AfB_1. After recovery of the supernatant and the washing solutions, the remaining pellet was treated with 8 ml of either citrate buffer (pH 3) or phosphate-buffered saline buffer (PBS, pH 7.4). The tubes were kept at room temperature in the dark for 48 h and subsequently centrifuged for 10 min at 9500 × *g* at 25°C. The supernatant was recovered and analyzed by HPLC/FLD. The experiments were carried out in triplicate.

The percentage of desorption was determined by comparing the quantity of mycotoxin released (q_{des}) and that adsorbed (q_a) on mycelium, according to the following equation:

$$\%D = \frac{q_{des}}{q_a} \cdot 100$$

The mycotoxin released (q_{des}) per gram of biomass was calculated from the concentration of mycotoxin after desorption (C_{des}):

$$q_{des} = C_{des} \frac{V}{m}$$

where V was the volume of the solution and m was the weight of the biosorbent.

To test the biosorbent for re-usability, the AfB_1 adsorbed was then extracted with methanol. The tubes were kept at 40°C in the dark for 1 h and subsequently centrifuged for 10 min at 8422 × *g* at 25°C. The supernatant was recovered and analyzed by HPLC/FLD.

Adsorption Isotherms

The adsorption isotherms were determined to study the effect of the amount of adsorbent (isotherm I) and of the AfB_1 concentration (isotherm II) on the mycotoxin binding. Equilibrium experiments were set up according to the result of a preliminary screening, using 30 min of contact time at pH 7. For isotherm I, the concentration of AfB_1 was 200 ng/ml and the amount of mycelium varied from 600 to 1,200 mg. For isotherm II, the amount of mycelium was 250 mg and the concentration of AfB_1 varied from 200 to 2000 ng/ml.

The amount of adsorbed mycotoxin (q_a) ng of mycotoxin absorbed per milligram of fungal mycelium (ng/mg) was calculated as the difference between the concentration of mycotoxin in the test solution (C_0) and the concentration of mycotoxin recovered from the supernatant of (C_e), according to the following equation:

$$q_a = \left(\frac{C_0 - C_e}{m} \right) \cdot V$$

where V was the volume of solution (ml) and m was the mass of fungal mycelium (mg).

The adsorption isotherms were obtained by plotting the values of the amount of mycotoxin adsorbed in mg/g at equilibrium (q_a) as a function of the amount of residual mycotoxin in solution in ng/ml at equilibrium (C_e), and reporting the percentage of adsorption as a function of the dosage of the adsorbent in mg/ml. The data were fitted by the Langmuir and Freundlich isotherm models (Freundlich, 1906; Langmuir, 1916).

A dimensionless constant known as the separation factor (K_R) derived from the Langmuir (K_L is the Langmuir constant) equation was used to assess the favorability of adsorption:

$$K_R = \frac{1}{1 + K_L C_0}$$

The Gibbs free energy change (ΔG^0, kJ/mol), the standard enthalpy (ΔH^0, kJ/mol), and the standard entropy (ΔS^0, kJ/mol·K) were calculated according to Kavak, 2009.

SEM Characterization

For scanning electron microscope (SEM) investigations, the samples were previously fixed on an aluminum stub with a carbon-based, electrically conductive, double-sided adhesive disc and then sputtered with a 30-nm-thick carbon film using an Edwards Auto 306 thermal evaporator.

Images of the samples were taken with a secondary electrons (SE) detector mounted on a SEM of LEO, model EVO50XVP. Operating conditions of the SEM were: 7.5 kV accelerating potential, 500 pA probe current, and 9 mm working distance.

Statistics

Adsorption/desorption experiments were performed in triplicate. The results obtained were subjected to one-way ANOVA with a significance level of $p < 0.05$. The data-processing software used were Excel 2016 (Microsoft Corporation, Redmond, Washington, USA) and OriginPro 2017 (OriginLab Corporation, Northampton, Massachusetts, USA). The statistical software used was STATGRAPHICS® centurion XVII (Statpoint Technologies, Inc. The Plains, Virginia, USA).

RESULTS

Laccase Activity in the Autoclaved Mycelium

In order to rule out the occurrence of enzymatic degradation in the reduction of AfB1 concentration in the solutions exposed to the adsorbent, ground mycelium of *P. eryngii* was autoclaved to obtain denaturation of the proteins and subsequently extracted with PBS (pH 7.3); the extract was then analyzed for laccase activity. No laccase activity was found in the *P. eryngii* mycelium subjected to the heat treatment. This allowed to clarify that the removal of AfB_1 in the working solutions treated with autoclaved *P. eryngii* mycelium was not due to enzymatic degradation.

Identification of Major Variables Affecting Adsorption

The ANOVA was employed to analyze the role of different variables (pH, time, mass of the adsorbent, and concentration of AfB1) on the adsorption process. The main factors and interaction effects are shown in **Table 1**. Only two factors, that is, mass of adsorbent and concentration of AfB_1, were significantly different from 0 at the 95.0% confidence level ($p < 0.05$). Time, pH, and interaction between factors were not statistically significant. The Pareto chart of standardized effects at $p = 0.05$ is presented in **Figure 1**. The same two factors (mass of adsorbent and concentration of AfB1) showed a statistically significant effect ($p = 0.05$), with absolute values higher than 2.3.

Optimization of Adsorption

Mass of adsorbent (m) and concentration of AfB_1 were identified as effective factors of adsorption and their effect was optimized by a factorial experiment in which the two variables were investigated at three levels. The 3^2 factorial design matrix and the results of the experiments are shown in **Table 2**.

The model expressed by Eq. (1), where the variables are expressed in their original units, represents the removal efficiency of AfB_1 (Ads %) as a function of m and AfB_1.

$$\begin{aligned} Ads\% = {} & 70.1995 + 0.0154816m - 0.0640237AfB_1 \\ & + 0.00000784314m^2 - 0.00000701754mAfB_1 \\ & + 0.0000385856AfB_1{}^2 \end{aligned} \quad (1)$$

TABLE 1 | Effect of pH, mass of mycelium (m), AfB1 concentration (AfB1), and time (t), and interactions thereof on AfB_1 adsorption by *P. eryngii* mycelium.

Source	Sum of squares	Df	Mean square	F-ratio	*p*
pH	69.0313	1	69.0313	0.20	0.6613
m	3260.28	1	3260.28	9.34	0.0062
AfB_1	16607.5	1	16607.5	47.57	0.0000
t	247.531	1	247.531	0.71	0.4097
pH × m	57.7813	1	57.7813	0.17	0.6885
pH × AfB_1	11.2813	1	11.2813	0.03	0.8591
pH × t	13.7813	1	13.7813	0.04	0.8445
m × AfB_1	205.031	1	205.031	0.59	0.4524
m × t	344.531	1	344.531	0.99	0.3324
AfB_1 × t	0.03125	1	0.03125	0.00	0.9925
blocks	38.2813	1	38.2813	0.11	0.7440
Total error	6982.13	20	349.106		
Total (corr.)	27837.2	31			

The effects are statistically significant if p < 0.05 (95% confidence level).

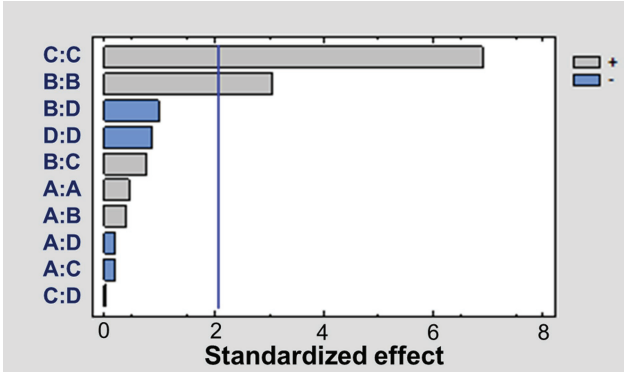

FIGURE 1 | Pareto chart of the standardized effect for AfB_1 adsorption. A is the pH, B is the adsorbent mass, C is the mycotoxin concentration, and D is the time. The effect of one factor is statistically significant ($p < 0.05$) if its absolute value is higher than 2.3 (sector of the chart at the right of the vertical line).

The model equation is useful in indicating the direction in which the variables should be changed in order to optimize the AfB_1-removal efficiency of the adsorbent. The results of ANOVA are presented in **Table 3**. The statistical significance of each coefficient was determined by values of *p*: the smaller the values of *p*, the more significant is the coefficient. This implies that the first-order main effects of mass of adsorbent and mycotoxin concentration are more significant than their quadratic main effect. However, the quadratic main effect of AfB_1 concentration is more significant than other second main effect.

The fit of the model was checked by the determination of the coefficient (R^2). In this case, the value of the determination coefficient (R^2 = 0.8873) indicated that the 11.27% of the total variable was not explained by the model.

Figure 2 shows the effect of the initial concentration of AfB_1 and the quantity of the mycelium on mycotoxin removal efficiency.

The working conditions at the optimum point for removal efficiency of AfB_1 were determined as follows:

$$m = 1000\ \text{mg}$$
$$AfB_1 = 50\ \text{ng/ml}$$

Application of the optimum parameter *m* = 1,000 mg and AfB_1 = 50 ng/ml to our model resulted in a theoretical optimum removal efficiency of AfB_1 by *Pleurotus* mycelium of 90.07%. The experimentally determined removal efficiency for the same levels of "*m*" and "AfB_1" was 85 ± 13% showing a satisfactory goodness-to-fit of the model.

TABLE 2 | The coded values for experimental design and the results.

Run	Mycelium (mg)	AfB1 (ng/ml)	Absorption (%)
1	1,000	525	62
2	700	525	58
3	700	1,000	59
4	700	525	59
5	1,000	50	96
6	700	525	61
7	700	525	61
8	400	1,000	46
9	700	50	72
10	400	50	78
11	700	525	62
12	400	525	53
13	1,000	1,000	60
14	700	525	60

TABLE 3 | Statistical significance of coefficients assessed by ANOVA.

Source	Sum of squares	Df	Mean square	F-ratio	*p*
m	280.167	1	280.167	10.65	0.0115
AfB_1	1093.5	1	1093.5	41.57	0.0002
m^2	1.41176	1	1.41176	0.05	0.8226
$mAfB_1$	4.0	1	4.0	0.15	0.7067
AfB_1^2	214.745	1	214.745	8.16	0.0212
Total error	210.422	10	26.3027		
Total (corr.)	1867.21	13			

SEM Analysis

A SEM micrograph of the *P. eryngii* mycelium is shown in **Figure 3**. The surface appears rough and sponge-like. The approximate pore size of 5–15 μm was measured from SEM analysis.

Adsorption Isotherms

Several adsorption isotherm models have been used to describe experimental adsorption data. The Langmuir and Freundlich models are the most frequently employed models. In this work, both models were used to describe the effect of mycotoxin concentration (**Figure 4**) and the effect of adsorbent dosage (**Figure 5**).

The linear regression analysis was applied to assess the goodness of the fits and to calculate the parameters involved in the adsorption mechanism (**Table 4**). The results obtained by comparing R^2 and SS_{res} showed that, for both the effect of adsorbent quantity and the effect of AfB_1 concentration, the isotherm that fits the experimental data is the Langmuir

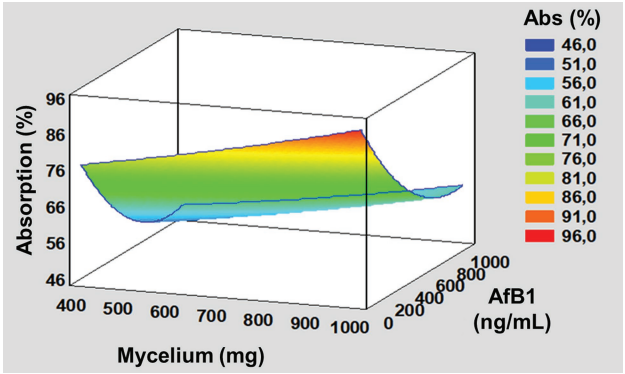

FIGURE 2 | Estimate response surface plot for the effect of mass of adsorbent and mycotoxin concentration on the AfB_1 removal.

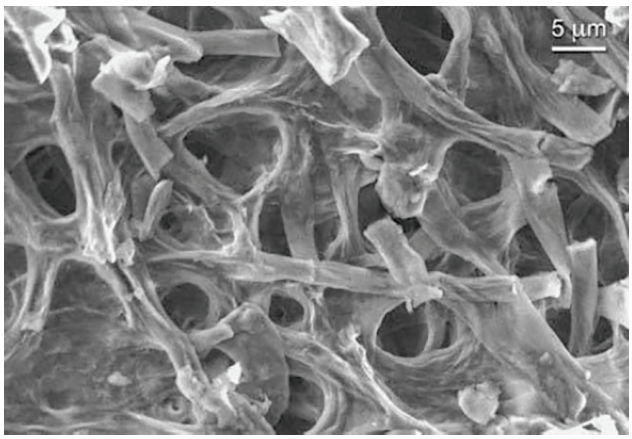

FIGURE 3 | SEM secondary electron micrograph of *P. eryngii* mycelium.

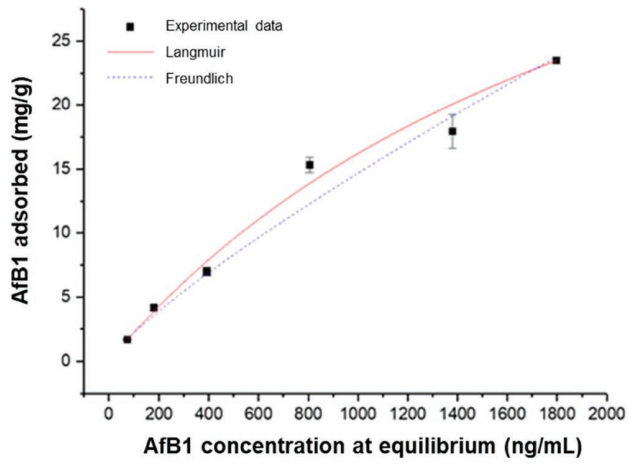

FIGURE 4 | Effect of mycotoxin concentration on AfB_1 adsorption by mycelium. Equilibrium adsorption isotherms were obtained at constant temperature (37°C) and pH (7) by testing a fixed amount of mycelium with increasing mycotoxin concentration.

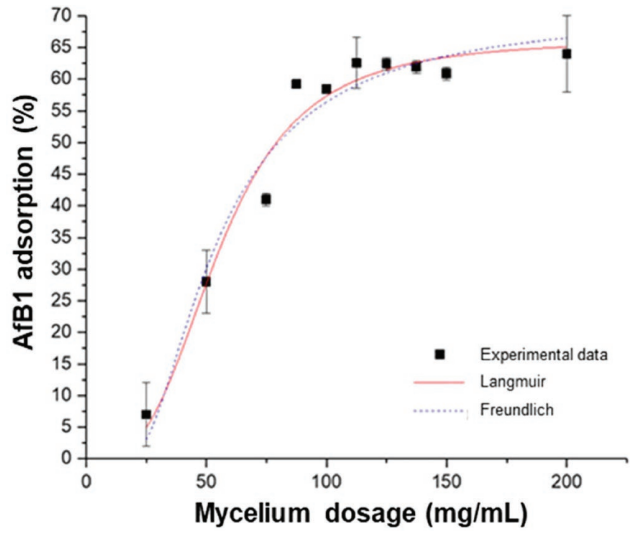

FIGURE 5 | Effect of adsorbent dosage on AfB_1 adsorption by mycelium. Equilibrium adsorption isotherms were obtained at constant temperature (37°C) and pH (7) by testing a fixed amount of mycotoxin with increasing adsorbent dosage.

isotherm. This suggests that the AfB_1 adsorption mechanism is monolayer and that occurs at a finite (fixed) number of definite equivalent sites. The model describes a homogeneous adsorption in which each molecule has enthalpy and activation energy of the constant process and is graphically characterized by a plateau, such as a saturation point where each molecule occupies a site and there can be no further adsorption.

The Langmuir model can be used to predict whether the adsorption system is favorable or unfavorable by calculating the dimensionless constant K_R (Weber and Chakravorti, 1974). For favorable adsorption, the K_R value should fall in the range 0–1. The adsorption is considered unfavorable when $K_R > 1$, the isotherm is linear when $K_R = 1$, and the adsorption is irreversible when $K_R = 0$. In this study, the values of K_R for AfB_1 adsorption on *P. eryngii* mycelium are comprised between 0 and 1, which suggests a favorable process for the system.

TABLE 4 | Isotherm model parameters for the adsorption of AfB_1 by *P. eryngii* mycelium.

Model	Parameter	Effect of AfB_1 concentration	Effect of adsorbent dosage
Langmuir	K_L (±SE)	$(4.3 \pm 0.6)\cdot10^{-4}$	$(3 \pm 7)\cdot10^{-6}$
	q_m (±SE)	53 ± 19	66 ± 3
	R^2	0.9976	0.9698
	SS_{res}	16.47	100.70
Freundlich	K_F (±SE)	$(50 \pm 8)\cdot10^{-3}$	70 ± 6
	1/n (±SE)	0.82	2.2 ± 0.5
	R^2	0.9942	0.9580
	SS_{res}	39.03	140.20

TABLE 5 | Thermodynamic parameters for the AfB_1 adsorption.

Temperature (°C)	$\ln K_0$	ΔG^0 (kJ/mol)	ΔH^0 (kJ/mol)	ΔS^0 (kJ/mol K)
22	0.65	−1.59	30.62	0.11
37	0.71	−1,83		
50	1.77	−4.76		

Thermodynamic Parameters

The effect of temperature on the adsorption of AfB_1 by *P. eryngii* mycelium was investigated. The uptake of AfB_1 was found to increase when temperature increased: 66 ± 3% at 22°C, 67 ± 0% at 37°C, and 85 ± 3% at 50°C The increase of adsorption at increasing temperature indicates an endothermic nature of the adsorption process, as confirmed also by a positive ΔH^0.

Molar free energy change of the adsorption process (ΔG^0), standard enthalpy change (ΔH^0), and standard entropy change (ΔS^0) are shown in **Table 5**.

The negative ΔG^0 values are indicative of a spontaneous adsorption process. The ΔG^0 values decreased as the temperature was raised, which is an indication of a physical adsorption nature of the process. Generally the free energy variation for the physical adsorption is between −20 and 0 kJ/mol, while in chemisorption, the range is −80 to 400 kJ/mol (Kavak, 2009). Besides the physical nature of the process, the experimental data show that the adsorption process needs to be activated by a moderately high temperature. This implies that the process is reversible and that the material can be regenerated by an appropriate treatment.

Desorption Experiments

To verify the stability of the system over time, the percentage of desorption of the mycotoxin adsorbed on *P. eryngii* mycelium was assessed at room temperature and at the pH values of 3 and 7.4.

Desorption studies showed a very low desorption after 48 h at 25°C, at all the pH values tested. The percentage of desorption was 10 ± 4% at pH 3 and 7 ± 4% at pH 7.4. These results indicate a good stability of the system.

However, treatment with methanol resulted in a complete desorption of AfB_1 from *P. eryngii* mycelium (recovery percentage 108 ± 6%). This result supports a possible re-utilization of the adsorbent after use, by regeneration of the adsorbing properties with an appropriate chemical treatment.

DISCUSSION

AfB_1 is one of the most important mycotoxins. It is produced by different species of *Aspergillus*, mainly *A. flavus* and *A. parasiticus*, in a number of agricultural products, including cereals, wine, spices, flavor products, peanuts, and soy. In this research work, we studied a method for the removal of AfB_1 from a solution by absorption, a promising detoxification technology that is growing in industrial interest and economic prospect. In particular, we investigated the AfB_1-adsorbing capability of the fungal mycelium of *P. eryngii*, an edible mushroom. The mycelium was produced and then processed, making it enzymatically inert by sterilization at high temperatures and subsequent lyophilization. The material thus obtained was morphologically characterized by SEM and subjected to various batch tests to assess its performance as biosorbent.

The adsorbents for mycotoxins are high-molecular weight compounds that are able to bind mycotoxins in contaminated feeds without releasing them into the gastro-intestinal tract of the animal. In this way, the toxin-adsorbent complex passes through the animals' intestine and is eliminated with the feces. This prevents or minimizes the exposure of the animal to mycotoxins (Kabak et al., 2006). The temperature and pH conditions during animal digestion vary according to the class they belong to. In particular for ruminants, which are polygastric animals (cattle, sheep), the bolus temperature is 38–40°C and the pH is 6.2–6.5. In the case of monogastrics, such as pigs, poultry, dogs, and cats, the pH varies during digestion from 4 to 6 and the temperature is between 38 and 40°C. For horses, the pH during digestion is 7.4–7.6 and the temperature is 37.5 –38.5°C. The biosorbent capability of *P. eryngii* mycelium was studied at the temperature of 37°C and at the pH values of 5 and 7, which are compatible with the temperature and pH of the gastro-intestinal apparatus of most farm animals (Cunningham and Klein, 2007). In contrast with the approach used in most of the studies on adsorbents, which is based on variation of one factor at a time, we applied a factorial design model (DOE) to evaluate the influence of the different operative factors on the biosorption process (Manal, 2007). Concentration of mycotoxin present in the solution and the quantity of adsorbent material were identified as determinants of the process. The pH of the solution was irrelevant in a range from 5 to 7 (range compatible with the pH of application). Zavala-Franco et al. (2018) studied the adsorbing capability of different biosorbents by an *in vitro* poultry digestive model. In that study, the same variables as in our work, that is mass of adsorbent and AfB_1 concentration, were assumed to be the main variables affecting the system. Also, Phillips et al. (1988) and Diaz et al. (2002) reported that pH had no influence on AfB1-binding by inorganic adsorbents. The DOE method, that we adopted herein, is intended to describe the variation of outcomes under conditions that are hypothesized to reflect the variation. This mathematical approach was developed to extrapolate the information needed through the least number of independent experiments. The fact that the results of our study are consistent with those obtained by more traditional approaches corroborates the validity of the DOE approach.

The system works in the same way over a range of time that goes from 30 to 120 min. This allowed to obtain a system that reaches equilibrium in a very short time (30 min) and that was found to remain stable for 48 h at room temperature, at pH 3 and pH 7.4, giving a desorption of 10 ± 4% and 7 ± 4% respectively. In optimal conditions, the mycelium of *P. eryngii* reaches 85 ± 13% of Afb_1 removal efficiency, values slightly lower than those achieved by other adsorbent materials, such as aluminosilicates (Phillips et al., 1988) and bentonites (Diaz et al., 2002), both of which can remove up to 95% of Afb_1. However, the latter have the disadvantage of showing high inclusion rates for vitamins and minerals, while mycelium of *P. eryngii* can be used as an alternative adsorbent material that is effective without causing nutritional losses. In a recently reported study, the adsorbing capability of different biosorbents, i.e., banana peel, *Pyracantha* leaves, and *Aloe* powder, were compared to that of zeolite in a laboratory model that simulated the conditions of the poultry gastro-intestinal tract (Zavala-Franco et al., 2018). The adsorption values assessed were 70, 69, 46, and 28% for zeolite, *Aloe* powder, *Pyracantha* leaves, and banana peel, respectively. Although determined in a different experimental system and therefore hardly comparable, these values appear significantly lower than adsorption achieved with *Pleurotus* mycelium.

The value of ΔH^0 of mycelium sorption is positive, indicating that the reaction is endothermic. The magnitude of ΔH^0 gives an indication of the type of adsorption, which can be either physical or chemical (Della Gatta, 1985). In the first case, the energy requirement is small (<40 kJ/mol) allowing the equilibrium to be attained rapidly and the process to be easily reversible (Ringot et al., 2005). On the contrary, chemical adsorption involves higher enthalpy changes (>40 kJ/mol). In this study, the value of enthalpy is less than 40 kJ/mol, indicating a physical adsorption phenomenon. The positive and small value of ΔS^0 reflects the little increasing randomness at the solid/liquid interface during the adsorption of AfB_1 on *P. eryngii* mycelium. The reaction was reversible and optimization of the process resulted in 85 ± 13% of AfB_1 removal.

The effectiveness of adsorption processes depends on the chemical structures of the adsorbent and the mycotoxin involved. The most important feature for adsorption is the physical structure of the adsorbent, that is, its total charge, the charge distribution, the pore size, and the accessible surface area. The properties of the adsorbed mycotoxins, such as polarity, solubility, shape, and charge distribution, also play a significant role (Huwig et al., 2001). To the best of our knowledge, there is no previously published study on the mycotoxin-binding capability of fungal mycelium, though the adsorbing capability

of fungal biomass has been shown for several organic and mineral (heavy metals) pollutants (Ahmaruzzaman, 2008; Wang and Chen, 2009). To date, the biosorption mechanism of organic compounds and metal ions by fungal biomass has been studied largely in relation to chitin and its deacetylated derivative, chitosan. The carboxylate and/or phosphate ligands along with the hydroxy and amide functional groups on the fungal cell wall components, which form relatively weak bonds with adsorbed molecules, have been proposed to be involved. Our SEM observations showed that the cell walls of *P. eryngii* mycelium are highly porous, with a pore size of 5–15 μm, which significantly increases the exposure of the cell wall active surfaces and of the sites of binding, thus making the process more efficient.

CONCLUSIONS

Our results show that non-viable mycelium of the fungus *P. eryngii* is able to efficiently adsorb AfB_1 in conditions (temperature and pH) compatible with the physiology of animals' digestion. A study was conducted to identify the major factors involved in the process. The concentration of mycotoxin in the solution and the quantity of adsorbent material were identified as determinants of the process. The pH of the solution was irrelevant in a range from 5 to 7 (range compatible with the pH of possible application). In addition, the system worked with no significant variation in the time lapse 30–120 min. of exposure. This allowed to obtain a system that reached equilibrium in a short time (30 min) and that remained stable in both acidic and slightly alkaline conditions that are compatible with pH values of the gastro-intestinal trait of farm animals. The thermodynamic study of the process showed that it is a spontaneous process with $\Delta G^0 = -2.73$ kJ/mol (average of ΔG^0 at three temperatures 22, 37 and 50°C), endothermic ($\Delta H^0 = 30.62$ kJ/mol and $\Delta S^0 = 0.11$ kJ/mol·K) and that it is a physical adsorption, regulated by weak and reversible interactions, whereby the material can be regenerated with an appropriate treatment such as quantitative extraction with methanol. Optimization of biosorption resulted in 85 ± 13% of removal efficiency by *P. eryngii* mycelium.

The mycelium of *P. eryngii* is a biological and edible material and this characterizes this adsorbent as completely different from the materials currently used in the industry. The ongoing proof of concept and validation studies *in vitro* rumen models and *in vivo* might open the path for practical use of new, efficient though low-cost fungal mycelium-based feed additives for mycotoxin-biosorbtion and mitigation of mycotoxin risk.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

CA and MH conceived the research. CA, MH, and AL wrote the manuscript. MB carried out the microbiological work. EC carried out the DOE study. PA performed the scanning electron microscope (SEM) study. All authors designed the experiments, analyzed the data, contributed to manuscript revision, read and approved the submitted version.

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