

Alma Mater Studiorum Università di Bologna
Archivio istituzionale della ricerca

An effective self-control strategy for the reduction of aflatoxin M1 content in milk and to decrease the exposure of consumers

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:

An effective self-control strategy for the reduction of aflatoxin M1 content in milk and to decrease the exposure of consumers / Kerekes, Kata; Bonilauri, Paolo; Serraino, Andrea; Giacometti, Federica; Piva, Silvia; Zambrini, Vittorio; Canever, Alessandra; Farkas, Zsuzsa; Ambrus, Árpád. - In: FOOD ADDITIVES & CONTAMINANTS. PART A. CHEMISTRY, ANALYSIS, CONTROL, EXPOSURE & RISK ASSESSMENT. - ISSN 1944-0049. - ELETTRONICO. - 33:12(2016), pp. 1840-1849. [10.1080/19440049.2016.1241895]

Availability:

This version is available at: <https://hdl.handle.net/11585/571399> since: 2020-10-21

Published:

DOI: <http://doi.org/10.1080/19440049.2016.1241895>

Terms of use:

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>).
When citing, please refer to the published version.

(Article begins on next page)

This is the final peer-reviewed accepted manuscript of:

Kata Kerekes, Paolo Bonilauri, Andrea Serraino, Federica Giacometti, Silvia Piva, Vittorio Zambrini, Alessandra Canever, Zsuzsa Farkas & Árpád Ambrus (2016): *An effective self-control strategy for the reduction of aflatoxin M1 content in milk and to decrease the exposure of consumers*, Food Additives & Contaminants: Part A, Volume 33, Issue 12, 1 December 2016, Pages 1840-1849.

The final published version is available online at:

<http://dx.doi.org/10.1080/19440049.2016.1241895>

Rights / License:

The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

An effective self-control strategy for the reduction of aflatoxin M1 content in milk and to decrease the exposure of consumers

Kerekes, Kata^{a,*}; Bonilauri, Paolo^b; Serraino, Andrea^c; Giacometti, Federica^c; Piva, Silvia^c; Zambrini, Vittorio^d; Canever, Alessandra^d; Farkas, Zsuzsa^a; Ambrus, Árpád^a

a. *Directorate for Food Safety Risk Assessment, National Food-Chain Safety Office*; b. *Institute for Zooprophyllaxis in Lombardy and Emilia-Romagna*; c. *Department of Veterinary Medical Sciences, Alma Mater Studiorum, University of Bologna*; d. *Granarolo S.p.A.*

Contact of authors:

Kata Kerekes (corresponding author)

Postal address: National Food Chain Safety Office,
Directorate for Food Safety Risk Assessment, Kitaibel
Pál u. 4., 1024 Budapest, Hungary
Phone: +36 20 23 5 6231
Email: kerekes.kata@gmail.com

Bonilauri Paolo

Postal address: Institute for Zooprophyllaxis in
Lombardy and Emilia-Romagna, Via Pitagora 2,
42100 Reggio Emilia, Italy
Phone: +39 052 26 4 0613
Email: paolo.bonilauri@izsler.it

Andrea Serraino:

Postal address: Dept. Scienze Mediche Veterinarie,
Universita degli Studi di Bologna, via Tolara di Sopra
50, 40064 Bologna, Italy
Phone: +39 051 20 9 7323
Email: andrea.serraino@unibo.it

Federica Giacometti

Postal address: Dept. Scienze Mediche Veterinarie,
Universita degli Studi di Bologna, via Tolara di Sopra
50, 40064 Bologna, Italy
Phone: +39 051 20 9 7320
Email: federica.giacometti@unibo.it

Silvia Piva

Postal address: Dept. Scienze Mediche Veterinarie,
Universita degli Studi di Bologna, via Tolara di Sopra
50, 40064 Bologna, Italy
Phone: +39 051 20 9 7064
Email: silvia.piva@unibo.it

Angelo Vittorio Zambrini

Postal address: Granarolo S.p.A., Via Cadriano 27/2 -
40127 Bologna, Italy
Phone: +39 051 41 6 2311
Email: vittorio.zambrini@granarolo.it

Alessandra Canever

Postal address: Granarolo S.p.A., Via Cadriano 27/2 -
40127 Bologna, Italy
Phone: +39 051 41 6 2311
Email: alessandra.canever@granarolo.it

Zsuzsa Farkas

Postal address: National Food Chain Safety Office,
Directorate for Food Safety Risk Assessment, Kitaibel
Pál u. 4., 1024 Budapest, Hungary
Phone: +36 30 77 7 9725
Email: farkas.zsuzsa13@gmail.com

*Árpád Ambrus (Retired scientific adviser, previously
at National Food-Chain Safety Office, Directorate for
Food Safety Risk Assessment)*

Postal address: Hómező u. 41., 1221 Budapest,
Hungary
Phone: +36 30 93 5 2432
Email: ambrusadr@yahoo.co.uk

*Corresponding author. E-mail: kerekes.kata@gmail.com; Phone: + 36 20 235 6231; Postal address: National Food Chain Safety Office, Directorate for Food Safety Risk Assessment, Kitaibel Pál u. 4., 1024 Budapest, Hungary

Abstract

The study reports on the results of testing the sensitivity of an early warning sampling plan in detecting milk batches with high AFM₁ concentration. The effectiveness of the method was investigated by the analysis of 9017 milk samples collected in Italian milk processing plants that applied control plans with different action limits (AL). In the case of milk processing plants, where 30 ng kg⁻¹ AL has been applied, the AFM₁ contamination was significantly lower at or above the 95th percentile of the milk samples compared to plants that used 40 ng kg⁻¹ AL. The results show that the control plan can be used effectively for early warning of occurrence of high AFM₁ contamination of milk and to carry out pro-active measures to limit the level of contamination. Estimation of dietary exposure was also carried out, based on the aflatoxin M₁ content of the milk samples and on Italian food consumption data. Estimated Daily Intakes (EDI) and Hazard Indices (HI) were calculated for different age groups of the population. HIs show that in case of adult population no adverse effects are expected, but in case of children under age three, the approximate HI values were considerably higher, which underlines the importance of the careful monitoring and control of aflatoxin M₁ in milk and dairy products.

Keywords: Aflatoxins; Milk; Statistical analysis; Exposure assessment

Introduction

Aflatoxins are produced by *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* under favourable growing and storage conditions (WHO 1997; Giorni et al. 2007). They may contaminate feed, especially in tropical and subtropical climatic environment, but they have also been detected in maize grown in other areas such as Italy (Prandini et al. 2009) and Spain (Cano-Sancho et al. 2013). According to the 2013 RASFF Annual Report, most of the notifications related to mycotoxins in feed (21 out of 37) concerned maize products, mostly from south-eastern Europe (EC 2014; RASFF 2013 annual report). As an impact of climate change, the infected areas may further increase (EFSA 2012a). Aflatoxin M₁ and M₂, the metabolites of aflatoxins B₁ and B₂ can be found in milk of animals fed with feed contaminated with aflatoxins, and AFM₁ contamination in milk was reported from several European countries (EFSA 2004; Cano-Sancho et al. 2013; Duarte et al. 2013; Tsakiris et al. 2013; Trevisani et al. 2014). According to Prandini and his co-workers, if contaminated feed has been used, AFM₁ appears in the milk after two or three days, and the depuration interval is about the same after the animals are again fed with AFB₁-free feed. Once milk becomes contaminated with AFM₁ neither pasteurization nor sterilization makes a significant change in the concentration of AFM₁ in it. Moreover, since AFM₁ associates with the casein fraction of milk, processes like cheese-making carries over AFM₁ to the final products (Prandini et al. 2009). Considering these facts and also that there is no procedure at the moment for complete elimination of AFM₁ from milk (Ismail et al. 2015), preventing or limiting contamination of milk with aflatoxins should have priority among the reduction strategies.

Milk is a basic food item for all age groups and gender of the human population, especially for children, due to its nutritional value. Milk contains high-quality protein, which includes all essential amino acids and has high antioxidant capacity - especially its casein fraction (Zeluta et al. 2009). Milk contributes significantly to the intake of calcium, magnesium, selenium, riboflavin, vitamin B₁₂ and pantothenic acid. Milk lipids serve as a carrier for fat-soluble vitamins, and the complex fat content of milk contains around 400 types of fatty acids (Weaver et al. 2013). According to EFSA's Comprehensive Food Consumption Database, the mean daily consumption of cow milk is in the range of 42.3 to 292.9 g/day among adults in Europe. The large portion size represented by the 95th percentile average consumption is 592 g/day for toddlers and 483 g/day for the adult population. Aflatoxins are carcinogenic, mutagenic and genotoxic substances. AFB₁ becomes activated during the metabolism and its epoxide form binds strongly to the DNA, causing damages in the helix. The metabolites of AFB₁ - such as AFM₁ - are poorer substrates for epoxidation and therefore, are less toxic (Wild & Turner 2002). Although the carcinogenicity of AFM₁ is considered 10 times lower than that of AFB₁. It has been classified as "possibly carcinogenic to humans" by the International Agency for Research on Cancer (WHO and IARC, 1993), therefore their level in milk should be kept as low as reasonably achievable (WHO 1997; EFSA 2007). In order to reach this goal, the European Commission introduced the principle of establishment of early warning system and taking pro-active

measures for reducing the contamination (Directive 2002/32/EC of the EP and the Council). The maximum level for AFM₁ in raw milk, heat-treated milk and milk for the manufacture of milk-based products set by EC No 1881/2006 is 50 ng kg⁻¹. Consequently, the Italian Ministry of Health defined an 'attention limit' of 40 ng kg⁻¹ (Ministero della Salute 2013), which accommodates the uncertainty of analytical results.

Farkas and co-workers elaborated an early warning sampling plan, based on random sampling and analysis of commingled bulk milk collected by the transport tankers typically from 1-6 dairy farms (Farkas et al. 2014). If the AFM₁ concentration exceeded the action limits ranging from 16.7 to 40 ng kg⁻¹ - depending on the number of dairy farms -, the milk samples taken from individual farms were consequently analysed and the necessary pro-active actions were recommended to reduce the chance of mixing the contaminated milk with those complying with the legal limit of 50 ng kg⁻¹. The random sampling plan elaborated is applicable only if the number of milk collecting zones remains constant during a given period of time.

The objective of the present study was to develop and test a generally applicable control plan, which can be used effectively for the early warning of occurrence of AFM₁ contamination in milk and making timely pro-active measures to limit the level of contamination. Additionally, the study aimed to use the results of the great number of analyses carried out to estimate the level of exposure of different population groups by the consumption of cow milk.

Materials and methods

AFM₁ data collection

Altogether 9017 milk samples were collected and analysed for AFM₁ between April 2013 and May 2014. 3303 samples were taken during the implementation of a self-control plan of a milk processing plant (called Plant A hereunder) located in Southern Italy, which collects about 40 million litres of milk per year. The milk was collected from 85 farms by eight tankers. In addition, 5714 results of AFM₁ analyses carried out by four dairy plants (indicated as NP hereunder), in the Northern, Central and Southern regions were also collected and used for assessing the efficiency of the control plan developed within this study. Two types of milk were collected separately in both cases: high quality milk (HQM) and normal quality milk (NQM), which are differentiated by Italian law, on the basis of their content in fat, protein, somatic cell count, and total bacterial count. The collection route, including 1-15 farms for one tanker was optimized depending on the production and accessibility of the farms. At each occasion, the quantity and type (HQM or NQM) of milk loaded from a farm were recorded, and a sample of milk was collected and labelled for later investigations if needed.

Analysis of samples for AFM₁

AFM₁ concentration was measured using the ELISA Immunoscreen AFM₁ kit (Tecnas.r.l., Trieste, Italy), which was validated within the range of 5-100 ng kg⁻¹ (Rosi et al. 2007). An HPLC method was used for quantitative confirmation of values above 50 ng kg⁻¹ (legal limit) (Dragacci & Grosso 2001).

Pro-active measures for reducing AFM₁ contamination of commingled milk

In order to avoid the contamination of huge milk quantities with aflatoxin M₁, dairy plants applied a self-control plan for the monitoring of the AFM₁ content of the consignments. Prior to unloading, samples had been taken from the compartments of each truck, in which milks of different qualities were collected. Depending on the detected AFM₁ levels, the following actions had been carried out:

- i) AFM₁ content of a consignment $\leq 30 \text{ ng kg}^{-1}$: milk was unloaded and processed without further actions.
- ii) AFM₁ content of a consignment $> 30 \text{ ng kg}^{-1}$ but $\leq 40 \text{ ng kg}^{-1}$: the milk was unloaded and processed, but each of the samples taken at the dairy farms when the truck had been loaded was also analysed separately.
 - a. If the sample collected at a farm contained AFM₁ above 40 ng kg^{-1} but less than 50 ng kg^{-1} , the veterinarian visited the farm on the same day and corrective action was started by changing the feed ration of the cows;
 - b. If the sample of a farm contained AFM₁ above 50 ng kg^{-1} (legal limit), the collection of milk from the farm was suspended. Moreover, not only the corrective action had been applied in these cases, but samples were also taken and analysed every three days until the AFM₁ concentration declined below 40 ng kg^{-1} .
- iii) AFM₁ content of a consignment $> 40 \text{ ng kg}^{-1}$ but $\leq 50 \text{ ng kg}^{-1}$, the milk was processed, and the respective authority was notified according to the regulation of the Ministry of Health. Corrective actions at farms providing milk with AFM₁ content above 40 or 50 ng kg^{-1} were applied as reported under points ii)a. and ii)b.
- iv) AFM₁ content of a consignment $> 50 \text{ ng kg}^{-1}$: the same actions took place as reported under point iii). In addition, the milk consignment was discarded.

In case of NP, points iii) and iv) of the above described action plan were applied.

Statistical evaluation of the distribution of AFM₁ concentrations

The P percentiles of the AFM₁ concentrations were calculated by the NIST procedure (NIST/SEMATECH 2003). The rank numbers of their standard deviations were calculated based on normal approximation (Diem & Seldrup 1982):

$$s = \sqrt{N \times P \times q} \quad \text{Equation (1)}$$

where N is the number of AFM₁ concentration values in the dataset, P is the selected percentile and $q = 1 - P$.

The 95% confidence intervals are calculated as:

$$Rp_{lower}^{upper} = R_p \pm 1.96s \quad \text{Equation (2)}$$

where R_p is the rank number of selected percentile.

The AFM₁ concentrations corresponding to the calculated rank numbers of the ordered dataset encompass the 95% concentration range of the AFM₁ concentration corresponding to the R_p. The confidence intervals for the mean AFM₁ concentration values were calculated as:

$$CI_{0.95} = \bar{C}_{AFM1} \pm 1.96 \times \frac{SD}{\sqrt{N}} \quad \text{Equation (3)}$$

where SD is the standard deviation of AFM₁ concentrations in N samples and \bar{C}_{AFM1} is the average concentration. No correction was needed for the calculation of SD due to the large number of concentration values.

Dietary risk assessment and risk characterization

Food consumption data were obtained from the EFSA Comprehensive European Food Consumption Database. The original data were reported by Italy based on the results of the Italian National Food Consumption Survey (INFCS) conducted from October 2005 to December 2006. It involved 3322 consumers from 1329 households located in the four main geographical areas of Italy (North-West, North-East, Centre and South and Islands) (Leclercq et al. 2009; EFSA Comprehensive Database). The average with standard deviation, median, 95th and 99th percentile consumption data of cow milk were reported for each age class: infants (≤ 11 months); toddlers (from 12 to 35 months); children (36 months to 9 years); adolescents (10-17 years); adults (18-64 years); elderly (65-74 years); very elderly (75 years and older). The consumption data of dairy products and milk based beverages were not included in the exposure assessment.

Left censored data (AFM₁ concentrations < LOD) were calculated with 0.5 LOD as recommended for cases where the non-quantified results were < 60% (EFSA 2010).

As 80th percentile milk consumption values were not available, therefore the 80th percentile AFM₁ concentration was selected as the basis for worst-case scenario calculation as a conservative estimate of long-term intake. For the EDI calculation, the weighted mean AFM₁ concentrations unloaded from each tanker and the average consumption were used. The weighted monthly mean AFM₁ values for each month were computed by multiplying the quantities of milk and their AFM₁ concentrations (including left censored data) in the sampled tanker and the sum of these values were divided by the sum of total milk quantities of the given month. For the conservative intake estimation, the same method was used, but the weighted 80th percentile AFM₁ concentrations were inserted into the equation instead of the weighted means.

The estimated daily intakes (EDI: ng kg⁻¹ bw day⁻¹) for the population groups were calculated with a deterministic method:

$$EDI = \frac{\sum C_{AFM1} * AMC}{bw * 365} \quad \text{Equation (4)}$$

where C_{AFM1} is the aflatoxin M₁ concentration (ng kg⁻¹), AMC is the average milk consumption (kg/year) and bw is the average body weight (kg) of the given population group.

In view of the facts that aflatoxins are genotoxic and carcinogenic, it is not possible to identify an intake without risk (EFSA, 2004). Kuiper-Goodmann (1990) proposed to derive a safe dose of 0.2 ng kg⁻¹ for AFM₁ from the dose causing 50% of the animals developing tumour (TD₅₀) divided by a safety factor of 50 000. The Hazard Index (HI) was calculated by dividing the estimated daily intake with 0.2. The same approach was also used in other studies (Shundo et al. 2009, Duarte et al. 2013; Tsakiris et al. 2013;).

Following the EFSA advice, to carry out risk assessment of substances, which are both genotoxic, and carcinogenic, the Margin of Exposure (MoE) was calculated as follows:

$$\text{MoE} = \frac{\text{BMDL}_{10}}{\text{EDI}} \quad \text{Equation (5)}$$

where BMDL₁₀ is the benchmark dose for a 10% increase in Hepatocellular carcinoma (HCC) incidence compared to the control group. In the case of AFB₁ (used as a conservative value) BMDL₁₀ is 870 ng kg⁻¹ bw day⁻¹ (EFSA, 2007), which was divided by the mean and 80th percentile EDI values for each age category of consumers. The calculation was carried out for each month from April 2013 to May 2014.

Risk Potency (RP) was calculated assuming that 2% of the population is HBV⁺ (considering that the prevalence of carriers of hepatitis B (HBV) in the Italian population is between 1.2% and 2%).

Because the carcinogenic potency of aflatoxin is higher in carriers of hepatitis B virus surface antigen than in individuals not being carriers, the potency values suggested by JECFA: 0.3 and 0.01 in HBV⁺ and HBV⁻ population respectively (JECFA 2001) were used as follows (Cano-Sancho et al. 2013):

$$RP = (0.01 \times 0.98) + (0.3 \times 0.02) = 0.016 \text{ HCC per year per } 100,000 \text{ person}$$

$$\text{Equation (6)}$$

The Risk Potency value was then used to calculate the fraction of incidence of liver cancer (LCI) in the Italian population attributable to the intake of milk contaminated with AFM₁ in the following way:

$$LCI = \frac{RP \times \text{BMDL}_{10}}{\text{MoE}} = \frac{0.016 \times 870}{\frac{870}{\text{EDI}}} = 0.016 \times \text{EDI} \quad \text{Equation (7)}$$

The fraction of incidence of HCC attributable to intake of AFM₁ was calculated on the basis of MoE considering the mean and the 80th percentile of exposure estimation (MoE_{mean} and MoE_{P0.8}).

Results and discussion

Distribution of AFM₁ concentrations and comparison of milk contaminations in plants A and NP

Distributions of AFM₁ concentrations in commingled milk discharged by the tankers are illustrated in Table 1. In Plant A, where the more stringent control plan was applied, even the highest values were below the legal limit, unlike in the case of NP, where some values were twice or three times higher.

The selected percentile values of AFM₁ concentrations with their 95% confidence limits are given in Table 2. The results show that there was no significant difference between the AFM₁ concentration of high and normal quality milks, either in Plant A or in NP. The applied stricter sampling plan in Plant A mainly influenced the frequency of occurrence of high concentration values in the 95th-99th percentile range.

The number of samples taken from Plant A and from NP is presented in Table 3. The table shows the percentages of samples in each plant that resulted in 30 ng kg⁻¹ AFM₁ concentration or above. The measured values are aggregated in the table in order to show the distribution of the high values in both plants. The table shows that in 2013 as well as in 2014 less samples resulted in AFM₁ concentration values at or above 30 ng kg⁻¹ in the case of Plant A, where the action limit was set at 30 ng kg⁻¹ compared to NP, which applied the higher (40 ng kg⁻¹) action limit. In the NP 31 milk samples (0.54%) resulted in AFM₁ concentration values at or above the legal limit (50 ng kg⁻¹), but in Plant A, none of the samples resulted in AFM₁ concentration above 50 ng kg⁻¹ during the examined period. Figure 2 shows the monthly distribution of AFM₁ contamination of the milk sampled in plants A and NP. Figure 1 as well as Tables 2 and 3 demonstrate the advantage of the action plan used by Plant A, where the stricter (30 ng kg⁻¹) action limit was applied compared to 40 ng kg⁻¹ used in NP. Between the 50th and 90th percentile, there was no significant difference between the AFM₁ concentrations of milk processed in the different plants. On the contrary, the AFM₁ contamination in NP was higher at or above 95th percentile of the milk samples (Table 2). This indicates that the difference observed above the 90th percentile is attributable to the different control plans applied.

As it was demonstrated by Trevisani et al. (2014), the AFM₁ contamination in milk has a substantial seasonal variation due to the change of the AFB₁ contamination of feed. The action limit of 30 ng kg⁻¹ enables the early detection of the beginning of the increase of the AFM₁ contamination of milk. Table 4 gives some examples of the results of the control plans applied in Plant A. The milk collected from various farms by the tanker was sampled at the time of discharge at the processing plants and the samples taken at individual farms were analysed for AFM₁, if the discharged commingled milk contained AFM₁ above 30 ng kg⁻¹. The example shows that in case the aflatoxin levels of feed are not controlled properly, it is very important to screen the individual batches of milk at farm level. This way it is possible to prevent the contamination of milk with high levels of AFM₁, which could otherwise result in the disposal of entire commingled milk causing great economic losses for the producers.

According to one of the latest reviews on this subject (Flores-Flores et al. 2015), studies conducted in Italy and in other countries show the occurrence of AFM₁ levels in milk within the same order of magnitude as it was during our study period. Nonetheless the results cannot be directly used as a basis for the evaluation of the control plan, because the aflatoxin concentrations vary within the year and depend on the geographical location and climatic conditions.

The AFM₁ concentration values of milk samples show the effectiveness of the applied self-control plans. The continuous monitoring of consignments makes possible the early detection of elevated AFM₁ levels in milk and the localization of farms, where cows are being fed by contaminated feed. The results show that the 30 ng kg⁻¹ action limit applied in the self-control plans is effective in keeping the AFM₁ concentration below the legal limit.

Risk characterization and exposure assessment

Because of the carcinogenic potential of aflatoxins, JECFA (2001) did not specify a numerical tolerable daily intake (TDI) for aflatoxins and concluded that daily exposure, even <1 ng kg⁻¹ bw, contributed to the risk of liver cancer.

Figure 2 shows the seasonal variation of estimated daily intakes (EDI) for NQM during the study period (from April 2013 till May 2014) in the populations of adults and toddlers. The aflatoxin concentrations of the two different milk qualities were similar, therefore the HQM values are not shown in the figures. Two different EDI values were calculated using equation (4) in the case of both milk qualities. EDI_{mean} values were computed by multiplying the weighted mean AFM₁ concentration of the given month. The EDI_{P0.8} values were calculated in a similar way but using the 80th percentile of AFM₁ concentration instead of the weighted mean. For the adult population, EDI values were calculated with a mean body weight of 70 kg, and mean milk consumption of 0.102 kg day⁻¹. For infants, a mean body weight of 5 kg and the mean milk consumption of 0.086 kg day⁻¹; for toddlers, a mean body weight of 12 kg and the mean milk consumption of 0.246 kg day⁻¹ were used (EFSA Comprehensive Database, EFSA 2012b).

Figure 2. clearly shows that the estimated intakes (ng kg⁻¹ bw day⁻¹) were significantly higher in the case of toddlers.

The seasonal variations of EDI values are in line with the variation of aflatoxin levels in feed reported by other studies (Farkas et al. 2014; Bilandžić et al. 2015).

Figure 3 shows the average EDI values of the different population groups in case of normal quality milk consumption. The lowest values were observed in the group of adults, while the highest values occurred in the groups of infants and toddlers. The intakes of “high consumers” were approximated by the calculation of EDI_{P0.8} values. According to the results, the estimated daily intake of large portion size consumers is about 40-50% higher than the average consumers.

The Hazard Indices (HI) were calculated by dividing the estimated daily intake values with 0.2. In the case of adult and elderly population (Figures 4 and 5), the HIs were less than 0.5, which means a level at which no adverse effects are expected. The average HI values of all age groups were below one, except for infants and toddlers, where the HIs were between 1 and 2.5 in most months of the studied period indicating that the youngest population groups might be exposed to AFM₁ above the safe level. It is important to note that according to the Italian food consumption survey, only 25% of the infant population is being fed with cow milk, but among toddlers, the ratio of cow milk consumers is 91.7%.

Taking into account that the daily consumption values included not all kinds of milk and dairy products, only cow milk, the overall HI values of AFM₁ for this food category might be even higher. The liver cancer incidence attributable to AFM₁ was calculated using the cancer potency of 0.016 hepatocellular carcinoma/year/100,000 persons. In the study period, the incidence estimated with the mean MoE for the adult population ranged between 0.0001 and 0.0006 cancer cases/year/100,000 persons in the case of HQM and from 0.0002 to 0.0007 cancer cases/year/100,000 persons for NQM. The hepatocellular carcinoma (HCC) incidence estimated with the mean MoE for the population of toddlers ranged between 0.0018 and 0.0067 cancer cases/year/100,000 persons calculated with the HQM results and from 0.0018 to 0.0079 in the case of NQM.

The cancer incidence estimated with the 80th percentile MoE for the adult population ranged between 0.0001 and 0.0006 cancer cases/year/100,000 persons in the case of HQM and from 0.0001 to 0.0007 cancer cases/year/100,000 persons in the case of NQM. The HCC incidence estimated with the 80th percentile MoE for the population of toddlers ranged between 0.0008 and 0.0089 cancer cases/year/100,000 persons in the case of HQM and from 0.0008 to 0.0105 cancer cases/year/100,000 persons in the case of NQM. The average liver cancer incidence (LCI) values with standard deviations are indicated in Figure 6 for each population groups. The average LCI values are the highest in the groups of infants and toddlers.

According to our results, the most exposed Italian population groups are the infants (≤ 11 months) and toddlers (from 12 to 35 months), but especially toddlers, because, while only 25% of the infants consume cow milk, this ratio among toddlers is over 90%.

In order to reduce the exposure of children, especially of the ones less than 3 years old, the most sensitive population group - specifically selected milk batches should be used for producing milk and milk products for young children. For each population group, an average EDI less than 0.2 ng kg⁻¹ bw day⁻¹ is necessary to reach in order to get a HI below 1, a level at which no adverse effects are expected. Considering the average body weights and consumption levels in the Italian population groups, the AFM₁ concentration below 11.7 ng kg⁻¹ for infants and a concentration below 9.7 ng kg⁻¹ in the case of toddlers would result an EDI less than 0.2 ng kg⁻¹ bw day⁻¹ and consequently a HI below 1. Therefore, milk containing AFM₁ ≤ 10 ng kg⁻¹ should be used for producing milk and milk-based products specifically for young children. Considering the uncertainty of analytical measurements (~18%), it means that those commingled milk batches discharged from the tankers which contain ≤ 8 ng kg⁻¹ AFM₁ should be stored and processed separately for the youngest population groups.

The results underline the importance of regular control of produced milk, applying appropriate action limit in combination with immediate corrective actions at farm level. Our study has shown that the applied monitoring plan is sensitive enough to reveal milk batches with high AFM₁ concentration and keep the AFM₁ concentration below 50 ng kg⁻¹ in commingled milk for general use and selection of batches with low (≤ 8 ng kg⁻¹) AFM₁ contamination for preparing milk and milk products for infants and toddlers.

Abbreviations used

| | |
|---------------------|---|
| AFB ₁ | Aflatoxin B ₁ |
| AFM ₁ | Aflatoxin M ₁ |
| AMC | Average milk consumption |
| BMDL ₁₀ | Benchmark dose lower confidence limit for a 10% response |
| BW | Body weight |
| CL | Confidence level |
| CI _{0.95} | 95% confidence intervals |
| EDI | Estimated Daily Intake |
| EFSA | European Food Safety Authority |
| ELISA | Enzyme-linked immunosorbent assay |
| 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 |
| IARC | International Agency for Research on Cancer |
| INFCS | Italian National Food Consumption Survey |
| JECFA | Joint FAO/WHO Expert Committee on Food Additives |
| LCI | Liver cancer incidence |
| LCL | Lower confidence limit |
| LOD | Limit of detection |
| MoE | Margin of Exposure |
| MoE _{mean} | Margin of Exposure considering the mean of exposure estimation |
| MoE _{P0.8} | Margin of Exposure considering the 80 th percentile of exposure estimation |
| NIST | National Institute of Standards and Technology |
| NP | Dairy plants in the Northern, Central and Southern regions of Italy |
| NQM | Normal quality milk |
| SD | Standard deviation |
| TD ₅₀ | Dose causing 50% of the animals developing tumour |
| TDI | Tolerable daily intake |
| UCL | Upper confidence limit |
| WHO | World Health Organization |

References

- Bilandžić N, Varenina I, Kolanović BS, Božić Đ, Đokić M, Sedak M, Tanković S, Potočnjak D & Cvetnić Ž. 2015. Monitoring of aflatoxin M1 in raw milk during four seasons in Croatia. *Food Control*. doi: 10.1016/j.foodcont.2015.02.015.
- Cano-Sancho G, Sanchis V, Marín S, Ramos AJ. 2013. Occurrence and exposure assessment of aflatoxins in Catalonia (Spain). *Food Chem. Toxicol.* 51:188-193.
- Diem K, Seldrup J. 1982. Lenter C. *Statistical Tables Mathematical Formulae: Introduction to statistics*. Ciba-Geigy Basel (Switzerland); p. 219.
- Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed, *Official Journal of the European Union*. L140, 30:10-21.
- Dragacci S, Grosso F. 2001. Immunoaffinity Column Cleanup with Liquid Chromatography for Determination of Aflatoxin M1 in Liquid Milk: Collaborative Study. *J. AOAC Int.* 84:437-443.
- Duarte SC, Almeida AM, Teixeira AS, Pereira AL, Falcão AC, Pena A, Lino CM. 2013. Aflatoxin M1 in marketed milk in Portugal: Assessment of human and animal exposure. *Food Control*. 30:411-417.
- European Commission. 2014. Rapid Alert System for Food and Feed (RASFF) annual report 2013. EU Commission - Working Document. p.19. ISBN 978-92-79-38196-6 (PDF). doi:10.2772/33031 (PDF).
- European Food Safety Authority. 2004. Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the Commission related to Aflatoxin B1 as undesirable substance in animal feed. *EFSA Journal*. 39:1-27. Available from: <http://www.efsa.europa.eu/en/efsajournal/pub/39> (accessed Mar 26, 2016)
- European Food Safety Authority. 2007. Opinion of the scientific panel on contaminants in the Food Chain on a request from the commission related to the potential increase of consumer health risk by a possible increase of the existing maximum levels for aflatoxins in almonds, hazelnuts and pistachios and derived products. *EFSA Journal*. 446: 1-127.
- European Food Safety Authority. 2010. Management of left-censored data in dietary exposure assessment of chemical substances. *EFSA Journal*. 8:1557-1853.
- European Food Safety Authority 2012a. Scientific report submitted to EFSA: Modelling, predicting and mapping the emergence of aflatoxins in cereals in the EU due to climate change. Question No

EFSA-Q-2009-00812. Available from: <http://www.efsa.europa.eu/en/supporting/doc/223e.pdf> (accessed Mar 26, 2016)

European Food Safety Authority 2012b. Scientific Committee; Guidance on selected default values to be used by the EFSA Scientific Committee, Scientific Panels and Units in the absence of actual measured data. EFSA Journal. 10:2579. [32 pp.] doi:10.2903/j.efsa.2012.2579.

European Food Safety Authority Comprehensive European Food Consumption Database [date unknown] [Internet]. Available from: <http://www.efsa.europa.eu/en/food-consumption/comprehensive-database> (accessed Mar 26, 2016)

Farkas Zs, Trevisani M, Horváth Zs, Serraino A, Szabó IJ, Kerekes K, Szeitzné Szabó M, Ambrus Á. 2014. Analysis of industry generated data Part II. Risk based sampling plan for efficient self-control of aflatoxin M1 contamination in raw milk. Food Addit. Contam. A. 31:1257-1273.

Flores-Flores ME, Lizarraga E, Lopez de Cerain A, Gonzalez-Penas E. 2015. Presence of mycotoxins in animal milk: A review. Food Control. 53:163-176.

Giorni P, Magan N, Pietri A, Bertuzzi T, Battilani P. 2007. Studies on *Aspergillus flavi* isolated from maize in northern Italy. Int. J. Food Microbiol. 113:330-338.

Ismail A, Akhtar S, Levin ER, Ismail T, Riaz M, Amir M. 2015. Aflatoxin M1: Prevalence and decontamination strategies in milk and milk products. Crit. Rev. Microbiol. 8:1-10.

Joint FAO/WHO Expert Committee on Food Additives (JECFA). 2001. Safety evaluation of certain mycotoxins in food, WHO/FAO, Geneva, Food Additive Series No. 47.

Kuiper-Goodmann T. 1990. Uncertainties in the risk assessment of three mycotoxins: aflatoxin, ochratoxin, and zearalenone. Can. J. Physiol. Pharm. 68:1017-1024.

Leclercq C, Arcella D, Piccinelli R, Sette S, Le Donne C, Turrini A. 2009. The Italian National Food Consumption Survey INRAN-SCAI 2005–06: main results in terms of food consumption, Public Health Nutr. 12:2504–2532

Ministero della Salute. 2013. Nota del Ministero della Salute prot. n. 855 del 16/1/2013. Procedure operative straordinarie per la prevenzione e la gestione del rischio contaminazione da aflatossine nella filiera lattiero-casearia e nella produzione del mais destinato all' alimentazione umana e animale, a seguito di condizioni climatiche estreme. [Extraordinary operating procedures for the prevention and management of the contamination risk by aflatoxins in the dairy supply chain and in maize production intended for 'human and animal nutrition, as a result of extreme weather conditions.]

- National Institute of Standards and Technology. [date unknown] E-Handbook of Statistical Methods. [Internet] Available from: <http://www.itl.nist.gov/div898/handbook> (accessed Jan 17, 2016)
- Prandini A, Tansini G, Sigolo S, Filippi L, Laporta M, Piva G. 2009. On the occurrence of aflatoxin M1 in milk and dairy products. *Food Chem. Toxicol.* 47:984-991.
- Rosi P, Borsari A, Lasi G, Lodi S, Galanti A, Fava A, Girotti S, Ferri E. 2007. Aflatoxin M1 in milk: Reliability of the immunoenzymatic assay. *Int. Dairy J.* 17:429-435.
- Shundo L, Navas SA, Conceição L, Lamardo A, Ruvieri V, Sabino M. 2009. Estimate of aflatoxin M1 exposure in milk and occurrence in Brazil. *Food Control.* 20:655-657.
- Trevisani M, Farkas Zs, Serraino A, Zambrini VA, Pizzomiglio V, Giacometti F, Ambrus Á. 2014. Analysis of industry generated data Part I. A baseline for the development of a tool to assist milk industry in the design of sampling plans for controlling aflatoxin M₁ in milk Part I. *Food Addit. Contam. A.* 31:1246-1256.
- Tsakiris IN, Tzatzarakis MN, Athanasios K, Alegakis AK, Vlachou MI, Renieri EA, Aristidis M, Tsatsakis AM. 2013. Risk assessment scenarios of children's exposure to aflatoxin M1 residues in different milk types from the Greek market. *Food Chem. Toxicol.* 56:261-265.
- Weaver C, Wijesinha-Bettoni R, McMahon D, Spence L. 2013. Milk and Dairy Products in Human Nutrition. Muehlhoff E, Bennett A, McMahon D. Chapter 4, Milk and dairy products as part of the diet. p. 106-111. Food and Agriculture Organization of the United Nations (FAO): Rome, Italy, E-ISBN: 978-92-5-107864-8
- Wild CP, Turner PC. 2002. The toxicology of aflatoxins as a basis for public health decisions. *Mutagenesis.* 17:471-481.
- World Health Organization. 2012. [date unknown] [Internet] Template for the calculation of the IESTI. Available from: http://www.who.int/entity/foodsafety/chem/IESTI_calculation_13c.xlt (accessed Mar 26, 2016)
- World Health Organization. 1997. Evaluation of Certain Food Additives and Contaminants, Forty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives, WHO Tech. Rep. Ser. 884:69-77.
- World Health Organization, International Agency for Research on Cancer. 1993. [Internet] Monographs on the Evaluation of Carcinogenic Risks to Humans, Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins, vol. 56.

Available from: <http://monographs.iarc.fr/ENG/Monographs/vol56/mono56-14.pdf> (accessed Aug 18, 2016)

Zeluta A, Maurizi A, Frigola A, Esteve MJ, Coli R, Burini G. 2009. Antioxidant capacity of cow milk, whey and deproteinized milk. *Int. Dairy J.* 19:380–385.

Figure Captions

Figure 1: AFM₁ contamination [ng kg⁻¹] found in milk samples from the collection tankers in Plants NP and A in a monthly distribution. Note: AFM₁ concentration values below 30 ng kg⁻¹ and ranging from 60 to 150 ng kg⁻¹ are not shown in the figure.

Figure 2: Monthly variation of the mean and 80th percentile Estimated Daily Intake (EDI) values of the adult population (Adults) and the children between 1 and 3 years of age (Toddlers) from normal quality milk (NQM). Note: the months are indicated with their first character e.g. A13 corresponds to April 2013.

Figure 3: Average Estimated Daily Intake of AFM₁ attributable to normal quality milk (NQM) consumption in the different population groups of Italy.

Figure 4: Monthly variation of the Hazard Indices (HI) in case of consumption of normal quality milk (NQM) calculated from the AFM₁ contamination measured in Plant A and the mean and 80th percentile consumption of the adult population and the group of toddlers.

Figure 5: Average Hazard Index values attributable to AFM₁ contamination normal quality milk (NQM) consumption in the different population groups of Italy.

Figure 6: Comparison of the average estimated liver cancer incidences (LCI) in the different population groups of Italy attributable to AFM₁ in case of average and high contamination of normal quality milk.

Word count: 6643

Note: The authors declare no competing financial interest.