In vitro anticoccidial activity of thymol, carvacrol, and saponins

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ABSTRACT The anticoccidial activity of thymol, carvacrol, and saponins was assessed in an in vitro model of coccidiosis. *Eimeria* spp. sporozoites were collected from field samples, characterized, and used for 2 different invasion assays on Madin-Darby Bovine Kidney cells (**MDBK**). The cells were challenged with 5×10^4 sporozoites without (control) or with various treatments: saponins (10 ppm), thymol, and carvacrol (7 ppm each) or a combination of saponins, thymol, and carvacrol at 2 doses; MIX 1 (saponins 5 ppm, thymol 3.5 ppm, and carvacrol 3.5 ppm) and MIX 2 (saponins 10 ppm, thymol 7 ppm, and carvacrol 7 ppm). The treated cells were incubated at 37°C for 24 h (invasion assay 1) and for 2, 24, and 48 h (invasion assay 2). The efficiency of invasion was determined by counting the sporozoites left in the supernatant that were not able to invade the cells, whereas intracellular *Eimeria* DNA was detected by qPCR to confirm the data. Data were analyzed with ANOVA, and differences were considered significant when P value was ≤ 0.05. Data from invasion assay 1 showed that the thymol and carvacrol-containing blends significantly reduced invasion, especially in combination with saponins at the highest dose. Saponins alone did not have a strong inhibiting activity but acted synergistically with the other molecules. Interestingly, in invasion assay 2, it was found that the effect of the highest dose of the blend of saponins, thymol, and carvacrol was already visible at 2 h postinfection, whereas the other treatments were significantly successful at 24 h postinfection. The invasion assay protocol was designed to screen molecules in vitro starting from field fecal samples, and it can represent a potential tool in *Eimeria* research. Moreover, this study shows that invasion in MDBK cells by *Eimeria* sporozoites is inhibited in presence of thymol, carvacrol, and saponins, thus highlighting the anticoccidial potential of these compounds.

Key words: *Eimeria*, in vitro, botanical, MDBK

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INTRODUCTION

Eimeria is an important avian parasite which causes severe enteritis, leading to relevant economic losses in poultry industry, estimated to be more than 3 billion US dollars per year (Cobaxin-Cardenas, 2016). Five are the species of *Eimeria* mainly involved in the disease onset: *Eimeria acervulina*, *Eimeria maxima*, *Eimeria brunetti*, *Eimeria necatrix*, and *Eimeria tenella*, with the latter 3 associated with the highest mortality rate and majority of symptoms, whereas the others lead to subclinical signs, which are often hard to recognize (Quiroz-Castañeda and Dantán-González, 2015). In addition, coccidia can contribute to the outbreak of secondary infections, such as clostridiosis, responsible for severe necrotic enteritis (Moore, 2016). Methods for controlling the disease include the use of ionophores and synthetic anticoccidial drugs applied with rotation programs or vaccination with live *Eimeria* oocysts. However, vaccines can trigger undesired reactions that affect the birds' performance, and recently, many cases of resistance to anticoccidial drugs have been documented (Abbas et al., 2012). As a consequence, research is now focusing on finding new cost-effective alternatives to control these pathogens (Peek and Landman, 2011). Botanicals and nature identical compounds are well renowned for their antimicrobial and antiparasitic activity, so they can represent a valuable tool against *Eimeria* (Cobaxin-Cardenas, 2016). The mechanisms of action of these molecules include degradation of cell wall, cytoplasm damaging, ion loss with reduction of proton motive force, and also induction of oxidative stress, that lead to inhibition of invasion as well as impairment

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of *Eimeria* spp. development (Abbas et al., 2012; Nazzaro et al., 2013). These compounds are often tested in vivo, but ethical concerns for animal welfare and high cost are pushing toward the assessment of standardized in vitro methods to screen new molecules (Singh et al., 2016). Among botanicals, thymol, carvacrol, and saponins are promising molecules because they can interfere with the membrane permeability of pathogens, causing a cascade of reactions that involve the entire cell and eventually leads to its death (Nazzaro et al., 2013). These compounds are naturally found in plants: thymol and carvacrol are major constituents of oregano, thyme, and basil (Sakkas and Papadopoulou, 2017), whereas *Quillaja* spp. and *Yucca* spp. are common sources of saponins, amphipathic glycosides used as defense mechanisms (Francis et al., 2002). The aim of this study was to evaluate the antiparasitic effect of different blends of thymol, carvacrol, and saponins on the invasion efficiency of *Eimeria* sporozoites in vitro. The protocol was designed to test alleged anticoccidial compounds starting from field samples, thus respecting animal welfare and without animal sacrifice, in agreement to the "3 Rs" guidelines (Russel and Burch, 1959).

MATERIALS AND METHODS

Eimeria *Sporozoites Recovery From Field Samples*

Eimeria spp. oocysts were collected from fecal samples of nonvaccinated animals showing coccidiosis symptoms. The samples were processed as indicated in *Guidelines on techniques in coccidiosis research* (Shirley, 1995) with some changes. Oocysts were resuspended in potassium dichromate 2% (Cat.#P5271, Sigma-Aldrich, St. Louis, MO) to allow sporulation. The oocyst samples were cleaned with Dulbecco's modified phosphate buffered saline (**DPBS**, Cat.#D8537, Sigma-Aldrich) from potassium dichromate, and then, they were resuspended in lysis buffer T1 (Cat.# 740952.240 C, MACHEREY-NAGEL Inc., Bethlehem, PA) and stored at −80°C until qPCR analysis was performed as described below. After sporulation, the oocysts were washed and resuspended in sodium hypochlorite for sterilization, and then, they were washed and lysed with glass beads (0.5 mm) for 1 min with Disruptor Genie (Cat.# SI-D258, Scientific Industries, Bohemia, NY) to obtain sporocysts. Those were washed and resuspended in excystation medium, containing 2.5 g/L trypsin (Cat.#T4049, Sigma-Aldrich), 5 g/L bile salts (Cat.#B3301, Sigma-Aldrich), 2 g/L pancreatin (Cat.#P1750, Sigma-Aldrich), and 2 g/L $MgCl_2$ (Cat.# 459337, Carlo Erba Reagents, Milan, Italy). The suspension was incubated for 90 min at 39°C. Afterward, the obtained sporozoites were washed and resuspended in cell medium to initiate the invasion assay.

Cell Culture

Madin-Darby Bovine Kidney (**MDBK**, Cat.# CCL-22, ATCC, Manassas, VA) cells were seeded (1×10^5 cells/well) on 24-well plates (Cat.#353047, Corning Incorporated, Corning, NY) and grown until confluency for 48 h in basal medium containing Dulbecco's Modified Eagle's Medium (Cat.#D1145, Sigma-Aldrich), 10% fetal bovine serum (Cat.#F7524, Sigma-Aldrich), 1x Penicillin-Streptomycin (Cat.#P4333, Sigma-Aldrich), and 10 mM L-glutamine (Cat.#G7513, Sigma-Aldrich). Cells were incubated at 37°C and 5% CO_2.

Invasion Assay 1

Confluent cells were infected with 5×10^4 sporozoites per well and treated with one of the treatments based on thymol (Cat.#T0501, Sigma-Aldrich), carvacrol (Cat.#W224511, Sigma-Aldrich), and saponins (Vetagro S.p.A., Reggio Emilia, Italy). The treatment groups were negative control (no sporozoite and no treatment), infected control (**C+**), saponins 10 ppm (**SAP**), thymol and carvacrol (7 ppm each) (**THY:CAR**), saponins 5 ppm + thymol 3.5 ppm + carvacrol 3.5 ppm (**MIX1**), or saponins 10 ppm + thymol 7 ppm + carvacrol 7 ppm (**MIX2**). The efficacy of the treatments was studied at 24 h postinfection (**hpi**). After the invasion assay, cells were stained with Giemsa to observe the actual internalization of the processed sporozoites. Moreover, the cells were accurately washed with DPBS until most of the residual debris was removed. Then cells were detached with trypsin 0.25%, washed with DPBS, resuspended in lysis buffer T1, and stored at −80°C until analysis. To measure the efficiency of invasion, the noninvading sporozoites found in the supernatant of 18 wells per group (n = 18) were counted on 4 squares of a Burker chamber under inverted microscope (Nikon Eclipse TS100, Nikon corporation, Tokyo, Japan), and the resulting number was used to estimate invasion efficiency with the following formula:

$$100 - \left[\left(\frac{number\ of\ sporozoites\ in\ the\ supernatant\ after\ treatment}{number\ of\ starting\ sporozoites}\right) \times 100\right]$$

Invasion Assay 2

Confluent cells were infected with 5×10^4 sporozoites per well and treated with one of the treatments described before. The efficiency of invasion was estimated at time points (2 hpi, 24 hpi, and 48 hpi) with sporozoites counts of 12 wells per treatment (n = 12), as defined above.

DNA Extraction and qPCR

DNA extraction and qPCR was performed to characterize the oocyst samples and to detect intracellular *Eimeria* DNA in the cells that were harvested after the assays. NucleoSpin DNA extraction kit (Cat.# 740952.240 C, MACHEREY-NAGEL Inc.) was used according to the manufacture instructions. DNA concentration was measured using Denovix DS-11 Series Spectrophotometer/Fluorometer (Microvolume Mode with Smart Path Technology–Cat.# DS11, Denovix, Hanby Building, Wilmington, NC) at 260 nm, and quality was verified by 260/280 ratio. The PCR reaction was prepared in a final volume of 10 µL, including 5 µL of iTaq Universal SYBR Green Supermix (Cat.# 1725120, Bio-Rad Laboratories, Hercules, CA), 500 nM of forward and reverse primers, and 2 µL of DNA, and the instrument used was the CFX96 TouchTM Real-Time PCR Detection System (Cat.# 1855195, Bio-Rad Laboratories). The primers used to detect *Eimeria* spp. were supplied by Sigma-Aldrich and are listed in Table 1. Cycling reaction was carried out under the following conditions: 3 min at 95°C, followed by 40 cycles of 10 s at 95°C and 30 s at 60°C. Nonspecific product formation was monitored with a melting curve analysis. The melting cycle included of 0.5°C increments from 55°C to 95°C for 5 s. A relative quantification method ($2^{-\Delta\Delta Ct}$) was used to verify the efficiency of invasion, using as reference the gene of Bovine cytochrome B (Livak and Schmittgen, 2001).

Statistical Analysis

GraphPad Prism 8.1.1 was used to perform statistical analysis. Descriptive analysis of data was done, and normality was checked with Shapiro-Wilk test. Normally distributed data were analyzed with a parametric one-way ANOVA test, whereas non-normal data were analyzed with Kruskal-Wallis tests. For the timepoint invasion assay, the comparison was done with two-way ANOVA. Post-hoc multiple comparison was done with Tukey's test, and differences were considered significant when P value was ≤ 0.05.

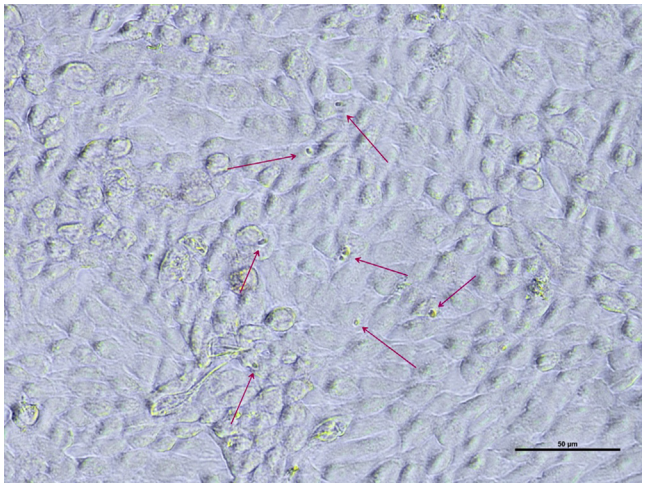

Figure 1. Eimeria sporozoites inside MDBK cells, Giemsa stained. The arrows indicate intracellular sporozoites after 24 h of invasion.

RESULTS

Sample Characterization

Different samples were characterized with qPCR to detect the species of *Eimeria* inside the processed samples. *E. tenella*, *E. brunetti*, and *E. acervulina* were detected. Then, the actual invading capability of the processed sporozoites was visualized by Giemsa staining (Figure 1).

Invasion Assay 1

The results of the first invasion assay are reported in Figure 2. The counts highlighted significant decreases of the invasion efficiencies among all the thymol and carvacrol-based treatments ($P \leq 0.0002$) compared with C+, especially THY:CAR and MIX2, whereas saponins effect was not significant ($P = 0.409$). The qPCR results confirmed the counts, as the same inhibition trend was visible (data are shown in Figure 3). THY:CAR and MIX2 significantly reduced *E. tenella* DNA quantity inside the cells compared with C+ (P values are respectively 0.022 and <0.0001), whereas

Table 1. Primers used to detect the Eimeria spp.

Target		Primer sequence (5′→3′)	Product size (bp)	Accession number	Reference
E. brunetti	F	TTGCGTAAATAGAGCCCT	148	AF026383	(Kawahara et al., 2008)
	R	CATGCAGAAAACTCCAAAAG			
E. maxima	F	GTTGCGTAAATAGAGCCCTCT	152	AF065094	(You, 2014)
	R	ACCAATGCAGAACGCTCCAG			
E. necatrix	F	GCAGTCGTTCTTGGGTGT	148	AF026385	(Kawahara et al., 2008)
	R	TGCTCACGCCCATACTAC			
E. tenella	F	TGGAGGGGATTATGAGAGGA	147	AF026388	(Kawahara et al., 2008)
	R	CAAGCAGCATGTAACGGAGA			
E. acervulina	F	GCAGTCCGATGAAAGGTATTTG	103	Ac-AD18-953	(Siddiki et al., 2014)
	R	GAAGCGAAATGTTAGGCCATCT			
Bovine Cytochrome B	F	CGGAGTAATCCTTCTGCTCACAGT	116	D34635	(Dooley et al., 2004)
	R	GGATTGCTGATAAGAGGTTGGTG			

DNA in the samples and inside the cells after the invasion assays. Forward primer (F), reverse primer (R), base pair (bp).

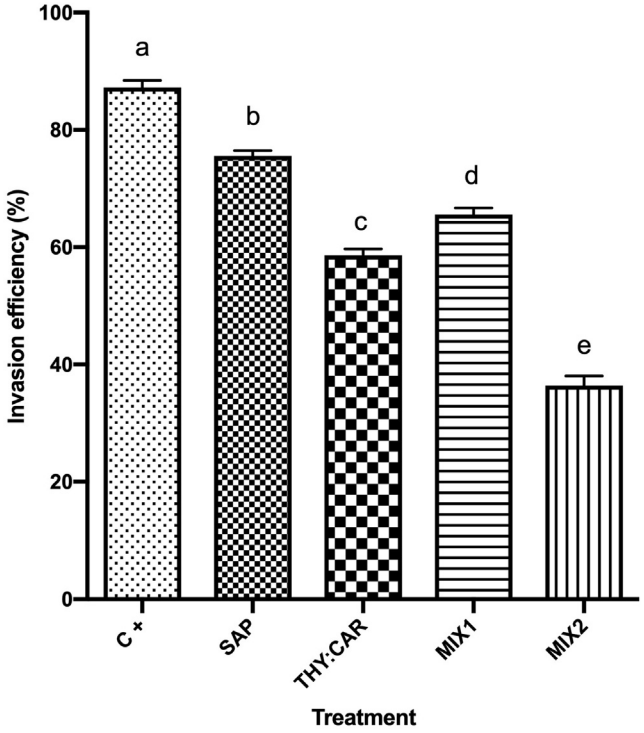

Figure 2. Invasion efficiency determined by sporozoites count. Percentual values are presented as means, and SEM is symbolized with a bar; n = 18. Statistical analysis was performed with one-way ANOVA, letters above the columns represent significant differences among treatments ($P \leq 0.05$). The treatments were infected control (C+), saponins 10 ppm (SAP), thymol 7 ppm + carvacrol 7 ppm (THY:CAR), saponins 5 ppm + thymol 3.5 ppm + carvacrol 3.5 ppm (MIX1), and saponins 10 ppm + thymol 7 ppm + carvacrol 7 ppm (MIX2).

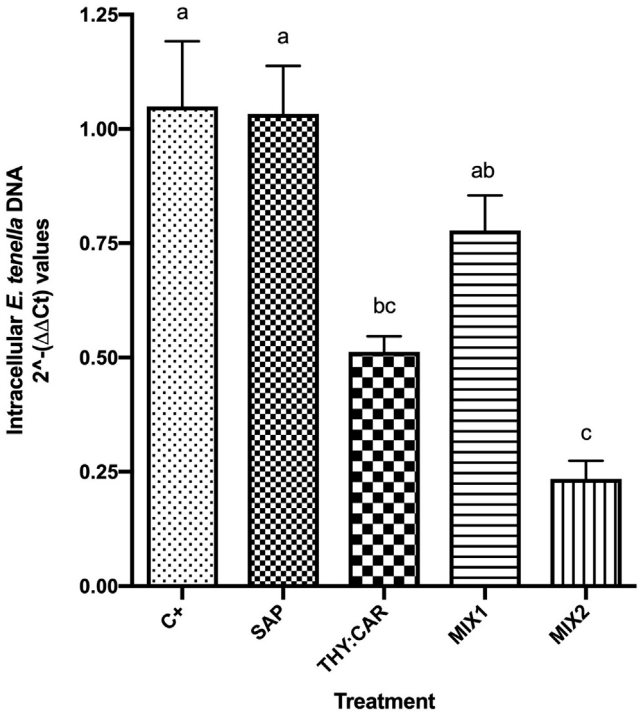

Figure 3. Intracellular *E. tenella* DNA relative quantity. Mean $2^{-(\Delta\Delta Ct)}$ values are represented in the graph, and SEM is symbolized with a bar; n = 6. Statistical analysis was performed with one-way ANOVA, letters above the columns represent significant differences among treatments ($P \leq 0.05$). The treatments were infected control (C+), saponins 10 ppm (SAP), thymol 7 ppm + carvacrol 7 ppm (THY:CAR), saponins 5 ppm + thymol 3.5 ppm + carvacrol 3.5 ppm (MIX1), and saponins 10 ppm + thymol 7 ppm + carvacrol 7 ppm (MIX2).

SAP and MIX1 did not differ from it. The DNA of other *Eimeria* spp. was not detected inside the cells.

Invasion Assay 2 (Timepoints)

In the second invasion assay, supernatant counts were carried out at timepoints. The inhibition trend was visible at 2 hpi: SAP and MIX1 did not differ from C+, whereas THY:CAR and MIX2 were significantly reduced compared with C+ ($P = 0.003$ and 0.001, respectively). At 24 hpi, THY:CAR and MIX2 invasion efficiency was significantly lower than C+ ($P < 0.0001$); at 48 hpi, all of the treatments reduced invasion efficiency compared with C+ ($P < 0.05$) (Figure 4).

In this assay, changes in invasion efficiency over time were also compared within each treatment (Figure 5). Over time, MIX2 was the only treatment able to inhibit invasion within 2 hpi, whereas all the other treatments reached a plateau after 24 hpi.

DISCUSSION

Recently documented cases of resistance to the classic anticoccidial treatments and restrictions to the use of antibiotics for livestock have increased the need of screening new compounds to control *Eimeria* (Giannenas et al., 2003). In vivo anticoccidial efficacy tests represent a routine tool but problematic issues, like high costs and ethical concerns are pushing toward the assessment of new methods to screen substances in vitro (Thabet et al., 2017). The aim of this study was to replicate an in vitro invasion model for *Eimeria* with a suitable method to estimate invasion efficiency on MDBK and to use it to test botanical molecules.

Eimeria sporozoites were extracted from multispecies field fecal samples to use them in an in vitro assay. Field samples are a valid source for in vitro experiments because they are more representative of the disease; indeed, field cases of coccidiosis are usually characterized by multiple strains of *Eimeria*. Despite this, only *E. tenella* DNA was detected inside the cells, suggesting that there might be a specific mechanism for internalization; in fact, there is little evidence that other *Eimeria* spp. sporozoites can be internalized by MDBK, and this aspect should be further investigated (Burt et al., 2013; Alnassan et al., 2015; Jitviriyanon et al., 2016). The natural target of avian *Eimeria* is the intestinal epithelia, but a chicken intestinal epithelial cell model is not available yet. Other cell lines are used to study *Eimeria*, but some are more permissive than others (Augustine, 2001a). Tierney and Mulcahy (2003) demonstrated that *E. tenella* sporozoites can be internalized by different cell models, especially baby hamster kidney cells, MDBK, and rabbit kidney cells, with different efficiencies (Tierney and Mulcahy, 2003). In another study, Augustine (2001) demonstrated that

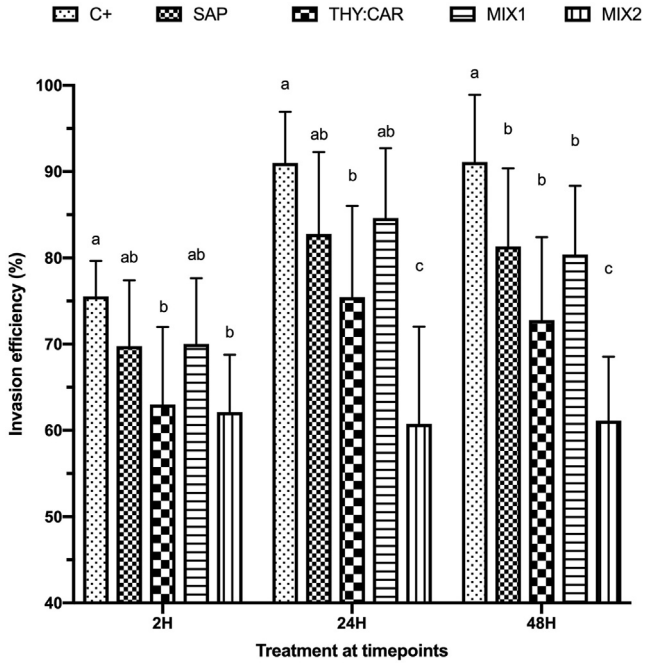

Figure 4. Invasion efficiency determined by sporozoites count. Percentual values are presented as means, and SEM is symbolized with a bar; n = 12. Statistical analysis was performed with two-way ANOVA, letters above the columns represent significant differences among treatments within the same timepoint ($P \leq 0.05$). The treatments were infected control (C+), saponins 10 ppm (SAP), thymol 7 ppm + carvacrol 7 ppm (THY:CAR), saponins 5 ppm + thymol 3.5 ppm + carvacrol 3.5 ppm (MIX1), and saponins 10 ppm + thymol 7 ppm + carvacrol 7 ppm (MIX2).

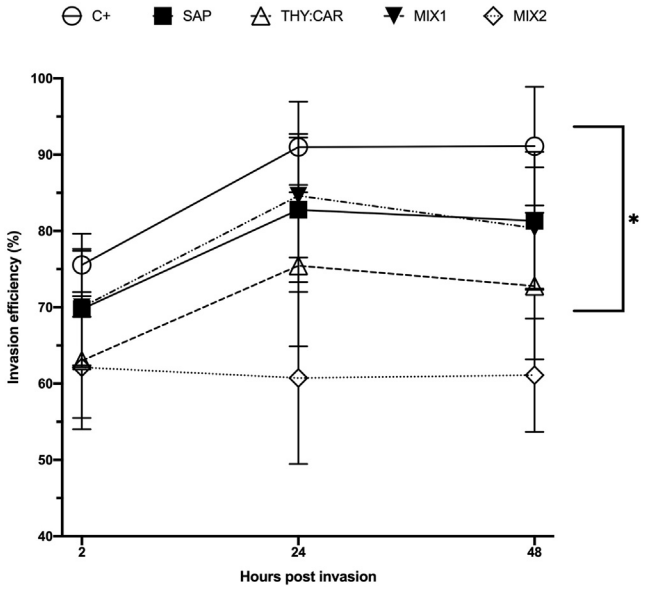

Figure 5. Overtime changes in invasion efficiency determined by sporozoites count. Percentual values are presented as means and SEM is symbolized with a bar; n = 12. Statistical analysis was performed with two-way ANOVA. The asterisk means that 24hpi and 48hpi values are significantly different from the 2 hpi values for C+, SAP, THY:CAR, MIX1 ($P \leq 0.05$). The treatments were: infected control (C+), saponins 10 ppm (SAP), thymol 7 ppm + carvacrol 7 ppm (THY:CAR), saponins 5 ppm + thymol 3.5 ppm + carvacrol 3.5 ppm (MIX1), and saponins 10 ppm + thymol 7 ppm + carvacrol 7 ppm (MIX2). Abbreviation: hpi, hours postinfection.

different species of *Eimeria* diverged in their ability to invade cells because of both sporozoital and cellular factors (Augustine, 2001b). Actually, invasion seems to occur by recognition of receptive molecules; in this study, the conditions might have allowed only *E. tenella* to successfully invade the cells.

To estimate invasion efficiency, supernatant sporozoites counts and qPCR were applied. Both are cheap, fast, and reliable. Former studies applied different quantification methods, but most of these rely on the use of expensive items and dangerous reagents such as antibodies or radioactive compounds. qPCR was also previously used by Alnassan et al. (2015) and Thabet et al. (2017) to quantify *E. tenella* in MDBK, but they applied an absolute quantification method instead, using internal transcribed spacer 1 gene (*ITS-1*) of *E. tenella* from a pSCA-amp/kan plasmid (Alnassan et al., 2015; Thabet et al., 2017).

In the present study, bovine cytochrome B was used as a housekeeping gene to detect the quantity of *Eimeria* DNA in the invaded cells, thus applying a relative quantification method. Supernatant sporozoites count was a newly assessed method of quantification, and we are not aware of other studies where it was used. It was a robust and precise tool, and it allowed fast estimation of invasion. However, by comparison of the results obtained with qPCR and sporozoites count, some divergences came out; even though the trend on inhibition is maintained for all treatments, the values were quite different between the 2 methods. This might be explained by the fact that for sporozoites counts, the percentage of inhibition is calculated in relation to all the free sporozoites, which are of various species, whereas in the case of qPCR, it is calculated on the DNA of one specific *Eimeria* spp. (in this case it was *E. tenella* only). This is probably why the 2 methods were not fully comparable, so sporozoites counts were chosen as the best marker for this study.

Among the tested treatments, thymol and carvacrol–based blends were the most effective. Also other studies have demonstrated that thymol and carvacrol exert an antiparasitic activity on *Eimeria* spp. and their mode of action is linked to the destruction of sporozoites' membrane and consequent loss of calcium ions from the parasite, essential for invasion in *E. tenella* (Sárközi et al., 2007; Bozkurt et al., 2013). Studies on saponins reveal that these compounds may interact with cholesterol on the sporozoites' membrane, thus hindering *Eimeria* lifecycle (Bozkurt et al., 2013). In the present study, the results suggest that thymol and carvacrol exert the main inhibiting effect on sporozoites and saponins act as adjuvants, but they do not have a strong inhibiting action by themselves. Saponins might instead facilitate the activity of thymol and carvacrol on the sporozoites. In fact, data from the second invasion assay highlight that sporozoites take 24 h to complete the invasion process. The action of thymol, carvacrol, and saponins was visible at 2 hpi already in THY:CAR and MIX2 groups. However, only MIX2 managed to stop invasion at 2 hpi, whereas in all the other treatments, the process went on

for longer. This suggests that saponins might increase the anticoccidial power of thymol and carvacrol allowing a very rapid action of these compounds.

The activity of thymol and carvacrol against *Eimeria* was previously discussed by Giannenas et al. (2003) and Küçükyilmaz et al. (2012) in in vivo trials. Both found that oregano essential oils, rich in thymol and carvacrol, contribute to improve animal's health during a coccidia challenge and reduce the number of oocysts shed in feces. Giannenas et al. detected an increase in body weight gain similar to the uninfected group in chickens treated oregano EO. Küçükyilmaz et al. also found an improvement in immunity linked to oregano EO. Burt et al. analyzed the effect of carvacrol containing blends on MDBK in vitro and found that carvacrol significantly inhibited MDBK invasion by *E. tenella*. In these studies, the composition of the mixtures was variable, and other methods of detections were used, so it is difficult to compare the results (Giannenas et al., 2003; Küçükyilmaz et al., 2012; Burt et al., 2013). However, the anticoccidial efficacy of these molecules has been confirmed again by the outcomes of this study.

Scientific literature lacks studies on *Eimeria* spp., and those available are very different and hard to compare to one another. The protocol we applied can be used to screen fast and successfully other compounds at various doses to replace and reduce animals sacrifice. A fine and universal method for *Eimeria* research that respects the "3 Rs" guidelines should be assessed, and this study is one of the first to use field samples in a successful way to screen substances in vitro. We also found that thymol and carvacrol blends are interesting compounds to treat coccidiosis; the actual modes of action need to be elucidated in future, by investigating sporozoites' metabolic pathway and life cycle.

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Conflict of Interest Statement: Andrea Piva serves as a professor at the University of Bologna and is a member of the board of directors of Vetagro S.p.A. (Reggio Emilia, Italy). Ester Grilli serves as an advisor of Vetagro S.p.A.

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