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Preliminary studies on *in vitro* methods for the evaluation of anticoccidial efficacy/resistance in ruminants

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A B S T R A C T

Ovine *Eimeria* spp. infections cause increased mortality, reduced welfare and substantial economic losses, and anticocccidials are important for their control. Recent reports of anticoccidial resistance against ovine *Eimeria* spp. necessitate the development of *in vitro* methods for the detection of reduced anticoccidial efficacy, especially since the *in vivo* methods are both expensive, time consuming and requires the use of otherwise healthy animals. The aim of the present study was therefore to approach a preliminary standardization of *in vitro* assays for evaluation of the efficacy of the most commonly used anticoccidials in ruminants. For this purpose, apart from the evaluation of inhibition of oocyst sporulation, most effort was concentrated on assessment of the capacity of the different anticoccidials to inhibit both the invasion and further development (up to the first schizogony) of *E. ninakohlyakimovae* sporozoites in bovine colonic epithelial cells (BCEC). For this purpose, infected cultures were monitored 1, 8 and 15 days post infection to determine the infection rate, number of immature schizonts and number, size and appearance of mature schizonts, respectively. No clear inhibitory effect was found with any of the anticoccidial formulations tested, and we could not identify why there were no measurable effects from the different anticoccidials. Despite the lack of positive results, further investigations should be encouraged, as this could decrease the need for animal experiments and could be used in the initial assessment of anticoccidial efficacy of new drugs.

1. Introduction

Infections caused by *Eimeria* species are some of the most important parasitic diseases affecting the profitability of ruminant production systems (Keeton and Navarre, 2018). Young animals are particularly affected by clinical disease, often in the period around weaning. Infection may result in diarrhoea, reduced growth and occasional deaths (Daugschies and Najdrowski, 2005; Ruiz et al., 2006; Chartier and Paraud, 2012). The control of ruminant coccidiosis is traditionally based on the combination of good management together with prophylactic or metaphylactic treatment with anticoccidials (Daugschies and Najdrowski, 2015). However, for at least a decade no new drugs have been brought to market despite evidence of anticoccidial resistance (ACR) to current treatments occurring worldwide in poultry (McDougald et al., 1987; Peek and Landman, 2005; Lan et al., 2017). Toltrazuril resistance has been confirmed in porcine *Cystoisospora suis* (Sheresta et al., 2017), and in ovine *Eimeria* spp. (Odden et al., 2018a). Toltrazuril resistance in ovine *Eimeria* spp. in Norway is probably related to the widespread and extensive use of anticoccidial (AC) treatment in this country, as discussed in a recent publication based on a questionnaire study and aiming to identify potential risk behaviour for development of ACR (Odden et al., 2017).

Standard methods for *in vivo* evaluation of anthelminthic efficacy are not valid for the assessment of the efficacy of anticoccidial drugs, due to the substantial lifecycle differences between nematodes and coccidia. Accordingly, Odden et al. (2018b) recently published a new

Abbreviations: ACE, anticoccidial efficacy; ACR, anticoccidial resistance; BCEC, bovine colonic epithelial cell; BUVEC, bovine umbilical vein endothelial cells; CUVEC, caprine umbilical vein endothelian cells; FECRT, faecal egg count reduction test; FOCRT, faecal oocyst count reduction test; MIC, minimal inhibitory concentration; MDBK, Madin-Darby bovine kidney cells; WAAVP, Word Association for Advanced Veterinary Parasitology

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approach for field evaluation of anticoccidial efficacy (ACE) against ovine *Eimeria* spp. using a method based on the WAAVP recommended faecal egg count reduction test (FECRT) for identifying resistance to anthelmintics (Coles et al., 1992), with certain modifications. Changes from the original protocol included the use of geometric, instead of arithmetic, means and restriction to the exponential phase of oocyst excretion for a more accurate evaluation of the ACE. The usefulness of this new approach, named FOCRT (Faecal Oocyst Count Reduction Test), as a tool to evaluate ACE in the field was shown by the same authors in a controlled efficacy study in which the existence of reduced efficacy of toltrazuril in a field isolate of ovine *Eimeria* spp. was demonstrated (Odden et al., 2018a). In that study, 50% of the experimentally *Eimeria*-infected lambs were metaphylactically treated with the recommended dose of 20 mg/kg toltrazuril (Baycox® Sheep vet., Bayer Animal Health), but no difference in oocyst excretion between treated and control lambs was observed (Odden et al., 2018a). Furthermore, there were no differences in weight gain and macro-/microscopic findings at post-mortem examination.

The *in vitro* evaluation of drug efficacy or resistance against helminths has been extensively documented. As reviewed by Taylor et al. (2002), the available *in vitro* tests are diverse, and include: egg hatch assays, migration and motility assays, and larval and adult development tests, as well as biochemical and molecular techniques. Most of these *in vitro* tests have been used to detect and describe resistance against most anthelmintic groups, including benzimidazoles (Rialch et al., 2013; Ramünke et al., 2016; Milhes et al., 2017), imidazothiazoles (Martines-Valladares et al., 2013), and macrocyclic lactones (Almeida et al., 2013; Milhes et al., 2017). The same methodology has also been applied for the evaluation of the efficacy of a number of biological compounds or plant extracts in the last decades (Alawa et al., 2003; Iqbal et al., 2006; Al-Rofaai et al., 2012; Araújo et al., 2017; Jaso Díaz et al., 2017; Novobilsky et al., 2013). However, as for *in vivo* assessments, most of these *in vitro* tests are not appropriate for evaluation of ACE due to the complexity and particularities of the endogenous and exogenous life-cycle of *Eimeria* spp.. Accordingly, new *in vitro* methods have recently been developed, mostly for investigation of poultry coccidiosis. For instance, there are *in vitro* assays showing that different plant extracts inhibit the sporulation of oocysts, the viability of sporozoites, or the invasion rate of different *Eimeria* species of poultry (Molan et al., 2009; Khalafalla et al., 2011; Burt et al., 2013; Gadelhaq et al., 2018). Further evaluation of the efficacy of different anticoccidials has been accomplished by using a combination of cell culture and qPCR (Thabet et al., 2015). Minimum inhibitory concentrations (MIC) of monensin, maduramicin, salinomycin and lasalocid have been determined, based on the development to mature schizonts in Madin-Darby bovine kidney (MDBK) cells of sporozoites of a field *Eimeria tenella* strain (Thabet et al., 2015). The same authors recently proposed *in vitro E. tenella* assays as a replacement for animal experiments for ACE testing (Thabet et al., 2017). The efficacy of the same polyether ionophores described above and toltrazuril were tested (Thabet et al., 2017) using *in vitro* sporozoite inhibition and reproduction inhibition assays and further determination of the MIC.

In contrast to poultry, limited information is available on *in vitro* assays evaluating the ACE for ruminant *Eimeria* species. Interestingly, the launch of a *Guideline for Evaluating the Efficacy of Anticoccidials in Mammals* (Joachim et al., 2018), raised concerns about drug-resistance testing and alternative methods for evaluation of drug efficacy. For both *in vivo* and *in vitro* tests, the authors stress the need for defined strains of ruminant *Eimeria* species for protocol standardization. Ruiz et al. (2013a) reported the isolation and experimental infection of a defined strain of *E. ninkohlyakimovae*, initially isolated from the field in 2006 in Gran Canaria (Spain). This *Eimeria* strain has been subsequently used in a number of immunological, pathological, and immunoprophylactic studies (Ruiz et al., 2013b, 2014; Pérez et al., 2015, 2016; Matos et al., 2017a, 2017b; 2018).

Against this background, the aim of the present study was to address preliminary standardization of *in vitro* assays for evaluation of the efficacy of the most commonly used anticoccidials in ruminants. For this purpose, apart from the evaluation of inhibition of oocyst sporulation, most effort was concentrated on assessment of the capacity of the different anticoccidials to inhibit both the invasion and further development (up to the first schizogony) of *E. ninakohlyakimovae* sporozoites in bovine colonic epithelial cells (BCEC).

2. Material and methods

2.1. Parasite maintenance

The *E. ninakohlyakimovae* strain, GC, used in the present study was initially isolated from goats in the Gran Canaria Islands (Spain), and maintained by passage in goat kids for oocyst production. Oocysts were isolated according to Jackson (1964) with some modification. Briefly, faeces were mixed 1:1 with water and passed through sieves of decreasing pore diameter, down to approximately 100 μm. The faecal mix was subsequently mixed 1:1 with saturated sugar-solution and floated onto glass slides, which were washed every 2 h with distilled water, for three consecutive days. The washings were centrifuged at 2300 × *g* for 20 min, the supernatant was discarded, and the resulting sediment mixed 1:1 with distilled water in a glass flask. Oocysts collected in the flask were set to sporulate under constant aeration for 7 days at room temperature (RT). Sporulated oocysts were stored at 4 °C in culture flasks (Nunc) with access to air.

Oocyst purification and isolation of sporozoites were performed according to Fayer and Hammond (1967) and Pérez et al. (2015), with slight modifications. Sporulated oocysts were added to 5% (w/v) sodium hypochlorite and stirred on ice for 30 min using a magnetic flea, followed by centrifugation at 233 × g, 10 °C for 5 min. The supernatant was mixed 1:1 with distilled water and centrifuged at 1500 × *g*, RT for 10 min. The resulting sediment, containing oocysts, was suspended in sterile 0.5% L-cysteine ($C_2H_7NO_2S$, Merck) and 1.68% $NaHCO_3$ solution (Sigma Aldrich) and incubated at 100% CO_2 atmosphere, 37 °C for 20 h. Subsequently, oocysts were suspended in Hank's balanced salt solution (HBSS, Sigma Aldrich) containing 0.075% w/v % trypsin (Biowest), 0.15% w/v sodium taurodeoxycholate (Sigma Aldrich), and 8% sterile-filtered bovine serum from two different animals obtained from the local abattoir. The oocyst suspension was incubated and checked periodically (approximately every 30 min) by microscopy for up to 4 h (37 °C, 5% CO_2 atmosphere). Excysted sporozoites were washed three times (20 min, RT, 1500 × *g*) and suspended in RPMI-1640 (Sigma-Aldrich) medium with the appropriate anticoccidial concentration (see Table 1) and transferred to the cell cultures.

2.2. Cell culture

Bovine colonic epithelial cells (BCEC) (Ruiz et al., 2010) were cultured in RPMI-1640 medium (Sigma-Aldrich) in 12-well plates and incubated at 37 °C and a 5% CO_2 atmosphere until confluence. The

Table 1
Concentrations (μg/ml) of the different commercial anticoccidials included in the assay.

Concentration	Toltrazuril	Diclazuril	Decoquinate	Sulphonamide	Control DMSO
	T	D	Q	N	X
A	25.0	25.0		25.0	25.0
B	15.0	15.0		15.0	15.0
C	10.0	10.0	1.0	10.0	10.0
D	5.0	5.0	0.1	5.0	5.0
E	1.0	1.0	0.01	1.0	1.0
F	0.1	0.1	0.001	0.1	0.1
G	0.01	0.01	0.0001	0.01	0.01
H	0.001	0.001	0.00001	0.001	0.001

medium was supplemented with 500 U/ml penicillin (Sigma-Aldrich), 50 μg/ml streptomycin (Sigma Aldrich), 0.25 μg/ml amphotericine (Sigma Aldrich), 0.5 μg/ml PlasmocinTM (InvivoGen), and 20% faecal calf serum (Biowest).

2.3. Anticoccidials

Both commercial anticoccidial formulations (toltrazuril (Baycox® Sheep vet., Bayer Animal Health), diclazuril (Rumicox®, Steve Veteriaria), decoquinate (Deccox®, Zoetis) and sulphonamide (Cunitotal®, Ecuphar)), and the source anticoccidial toltrazuril and its metabolites, toltrazuril sulphoxide and toltrazuril sulphone (Sigma Aldrich), were used in the study. Additionally, two different negative controls, dimethyl sulphoxide (DMSO, Sigma Aldrich) for the commercial anticoccidials and dimethyl formamide (DMF, Sigma Aldrich) for the pure source/metabolite anticoccidials, were also included in the assays. The anticoccidials were diluted up to a 100 μg/ml stock concentration, either by using DMSO (commercial anticoccidials) or DMF (pure source/metabolite anticoccidials); further dilutions were made using cell culture medium. The anticoccidial concentrations applied to sporozoites and cells are shown in Table 1 (commercial anticoccidials) and Table 2 (pure source/metabolite anticoccidials). The DMSO and DMF maximum concentrations used were 0.4%. Anticoccidials were continuously included in the cell-culture medium throughout the study under the assumption that the drug concentrations remained stable. Monensine (Sigma Aldrich), at the same concentrations used for pure/derivate anticoccidials, was employed as positive control.

2.4. Infection of host cells and evaluation of invasion and development

Confluent BCEC cell layers containing anticoccidials or negative controls were infected with 100,000 freshly excysted sporozoites and incubated at 37 °C and 5% CO_2, in duplicates. The culture medium was changed 24 h after infection and subsequently three times a week.

At 24-h post infection, the invasion rate was determined by examining 15–20 photos taken at 400× magnification using a phase-contrast microscope (DMIL, Leica) and associated digital camera DFC299. Photos were taken systematically, covering the central area of the wells with the highest phase contrast, and scanning in a zig-zag motion. The scanning movement meant that a single field was not photographed more than once. For each of the 15–20 pictures taken of a single culture (condition), the number of intracellular sporozoites was enumerated using Carl Zeiss ZEN 2.3 lite software. To determine the average number of cells per picture, 600 pictures were evaluated. Invasion rate is given as the percentage of infected cells 24-h post infection.

Evaluation of development was assessed at days 8 and 15 post infection. At day 8, 15–20 pictures were taken systematically at 400× magnification as described for day 1, and immature schizonts were counted. Pictures were only taken when at least one immature schizont was present in a field, and the total number of fields examined, with or without immature schizonts, up to a maximum of 50 fields, was recorded. This early development is presented as the number of immature schizonts per mm^2.

At day 15, the mature schizonts were counted at 100× magnification over 15–20 pictures taken as referred previously for each condition. The degree of development is given as the number of schizonts per mm^2. In addition, the size and appearance of the schizonts were described.

Table 2
Concentrations (μg/ml) of the pure source/metabolite anticoccidials included in the assay.

Concentration	Pure toltrazuril	Toltrazruil sulfoxide	Toltrazuril sulfone	Control DMF
	TT	TD	TN	DMF
A	25.0	25.0	25.0	25.0
B	5.0	5.0	5.0	5.0
C	1.0	1.0	1.0	1.0
D	0.1	0.1	0.1	0.1
E	0.01	0.01	0.01	0.01

2.5. In vitro oocyst sporulation inhibition assay

The effects of the different anticoccidials on sporulation of *E. ninakohlyakimovae* oocysts *in vitro* were evaluated as follows. Briefly, 1.5 ml Eppendorf tubes were filled with a 20 μl suspension of 5000 unsporulated oocysts in sterile PBS, 30 μl distilled H_2O, 50 μl of 10% potassium dichromate, and 100 μl of different anticoccidials (A-D: 0.5, 0.1, 0.05 and 0.025 mg/ml). The incubations were performed at RT for 24 h and then the oocysts were washed in distilled water four times (2000 × *g*, 10 min). After the last washing step, the oocysts were resuspended in 1 ml 2% potassium dichromate, transferred into 24-multiwell tissue culture plates (Nunc) and incubated in the presence of oxygen at RT. As negative controls, similar concentrations of DMSO (commercial anticoccidials) or DMF (pure source/metabolite anticoccidials) to those used to dissolve the corresponding anticoccidials (from 8% to 1%) were used, and serial formaldehyde solutions (Panreac) served as positive controls (4, 2, 1 and 0.5%). Oocyst sporulation rate was determined after 72 h at RT by microscopic analysis, and for this purpose a minimum of 100 oocysts were counted and analysed. The assay was repeated twice including duplicates of all concentrations in both assays.

2.6. Statistical methods

Data were managed and analysed in Excel (2013) (Microsoft Inc.), in addition to analyses performed in R (R Core Team, 2017) and Sigmaplot 12.0. Data from the different *in vitro* analysis (invasion rate, development of immature and mature schizonts and inhibition of sporulation) were grouped and analysed independently for significant differences by using Chi-square tests. Effects of the different anticoccidial concentrations on the parasites were compared to the corresponding DMSO or DMF negative controls. Differences were regarded as significant at a level of $P < 0.05$.

3. Results

3.1. Sporozoite invasion

The average sporozoite invasion rate ranged from a minimum of 14.1% infected cells (25.0 μg/ml toltrazuril) to a maximum of 39.9% (control DMSO). The highest invasion rate for the commercial anticoccidials was seen for 0.1 μg/ml decoquinate at 39.7%, and for the pure source/metabolite anticoccidials for 5 μg/ml TN at 39.3%. The lowest invasion rate was seen for 25.0 μg/ml toltrazuril at 14.1% and for 0.1 μg/ml toltrazuril sulphoxide TD at 20.2%, for the commercial and pure source/metabolite anticoccidials, respectively. The average invasion rates for the controls, combining all concentrations, were 33.8 ± 1.1% (range: 26.6–39.9%) and 29.9 ± 1.6% (range: 27.4–33.2%) for DMSO and DMF, respectively.

The sporozoite invasion showed a significant difference ($P < 0.05$) for toltrazuril (25.0 μg/ml) and diclazuril (25.0 μg/ml), compared with the corresponding control DMSO concentration, but not for any of the other anticoccidials, or concentrations (Fig. 1). Although only the highest concentration of commercial toltrazuril was significantly different from the control, the following three concentrations showed a gradual increase in the percentage of infected cells of 22.6, 31.5, and 34.1%, respectively.

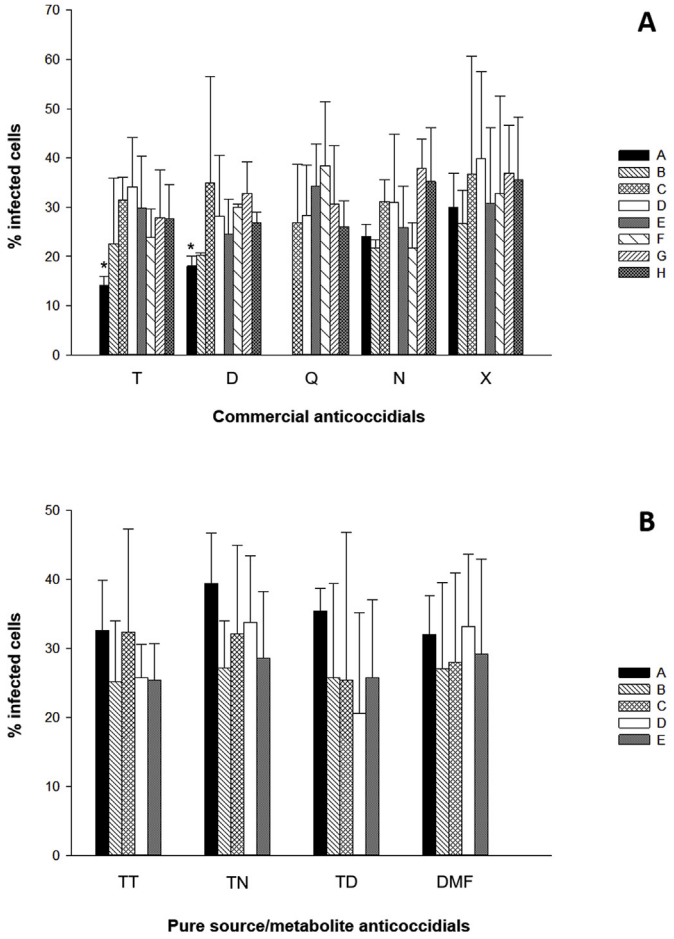

Fig. 1. Invasion rates of *Eimeria ninkohlyakimovae* sporozoites into bovine colonic epithelial cells under the influence of commercial (**A**) or pure source/metabolite (**B**) anticoccidials, calculated 24 h post infection. **A**: (T) toltrazuril, (D) diclazuril, (Q), decoquinate, (N) sulphonamide, (X) dimethyl sulphoxide – negative control. **B**: (TT) pure toltrazuril, (TD) toltrazuril sulphoxide, (TN) toltrazuril sulphone, (DMF) dimethyl formamide negative control. See Tables 1 and 2 for specific concentrations (A–H) used for each anticoccidial.

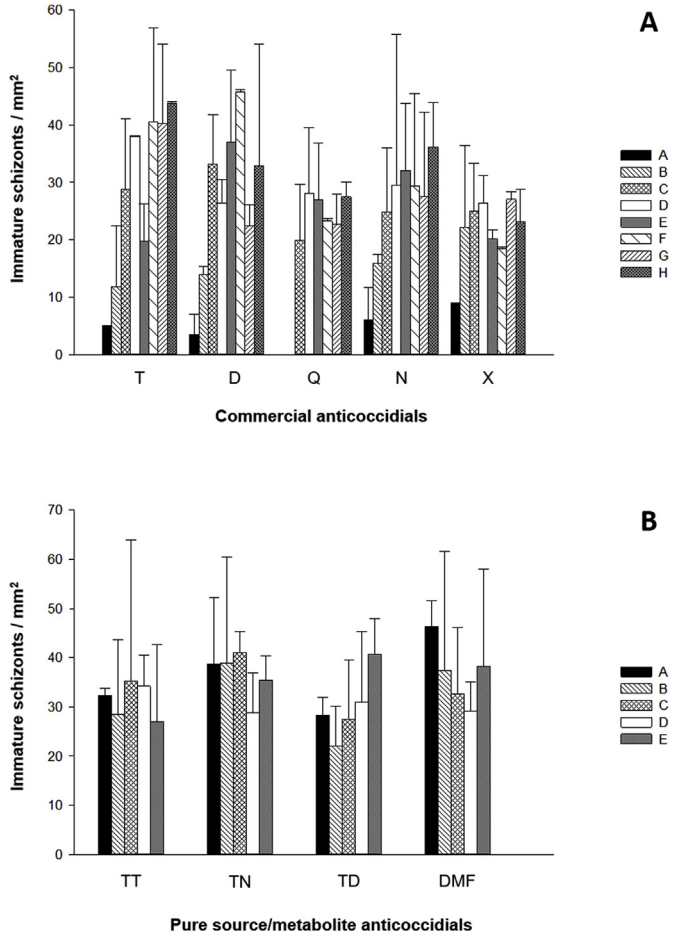

Fig. 2. Development of immature *Eimeria ninkohlyakimovae* schizonts in bovine colonic epithelial cells under the influence of commercial (**A**) or pure source/metabolite (**B**) anticoccidials evaluated 8 days post infection. **A**: (T) toltrazuril, (D) diclazuril, (Q), decoquinate, (N) sulphonamide, (X) dimethyl sulphoxide – negative control. **B**: (TT) pure toltrazuril, (TD) toltrazuril sulphoxide, (TN) toltrazuril sulphone, (DMF) dimethyl formamide negative control.

3.2. Sporozoite development

3.2.1. Immature schizonts

The average number of immature schizonts ranged from a minimum of 4.7 schizonts/mm^2 (25.0 μg/ml toltrazuril) to a maximum of 46.3 schizonts/mm^2 (5.0 μg/ml control DMF). The average number of schizonts/mm^2 was of 22.2 ± 0.8 and 36.9 ± 2.0 for DMSO and DMF, respectively. The highest number of schizonts/mm^2 recorded for the commercial anticoccidials was 125, found with two different drugs; 0.1 μg/ml toltrazuril and 1.0 μg/ml diclazuril, whereas the highest number recorded for the pure source/metabolite anticoccidials was 204.5 schizonts/mm^2 (1.0 μg/ml toltrazuril sulphone).

There was a significant difference ($P < 0.05$) in sporozoite development into immature schizonts at day 8 for 25.0 μg/ml of toltrazuril and diclazuril, compared with their corresponding concentration of the control DMSO (Fig. 2). This difference was not found in any of the other anticoccidials, at any concentrations.

3.2.2. Mature schizonts

The average number of mature schizonts/mm^2 ranged from a minimum of 3.2 ± 0.6 schizonts/mm^2 (sulphonamide 25 μg/ml) to a maximum of 21.6 ± 1.6 schizonts/mm^2 (DMSO) (Fig. 3). The lowest number of schizonts/mm^2 recorded for the commercial anticoccidials was 2.7 (Sulphonamide 25 μg/ml), whereas for the pure source/metabolite anticoccidials the lowest number recorded was 5.3 schizonts/mm^2 (toltrazuril, 5 mg/ml). For commercial anticoccidials, in particular, the schizont numbers at the highest concentration were lower than for negative controls but significant differences could not be demonstrated. The same was observed for the mean appearance of the mature schizonts at day 15 after infection, whose values ranged from 1.3 to 2.0 for all anticoccidials and corresponding controls (Fig. 4). In contrast, the size of the mature schizonts remained relatively similar, with diameters fluctuating between 46.2 ± 6.9 μm (decoquinate, 10 μg/ml) and 76.7 ± 7.1 (toltrazuril 25 μg/ml) (Fig. 5). Schizonts ≥50 μm amounted for 83.2% of all schizonts counted. However, by only evaluating large schizonts (≥50 μm), the results did not significantly change, from the evaluations of all schizonts.

3.3. Oocyst sporulation inhibition assay (OSIA)

None of the anticoccidials, commercial or pure source/metabolite, appeared to influence oocyst sporulation rate, which ranged from 81.3% (pure toltrazuril, 0.5 mg/ml) to 94.6% (diclazuril, 0.1 mg/ml). In contrast, less than 30% oocysts sporulated after incubation with the positive control (formaldehyde; data not shown).

4. Discussion

In the present study we assessed the ability of *E. ninakholyakimovae*

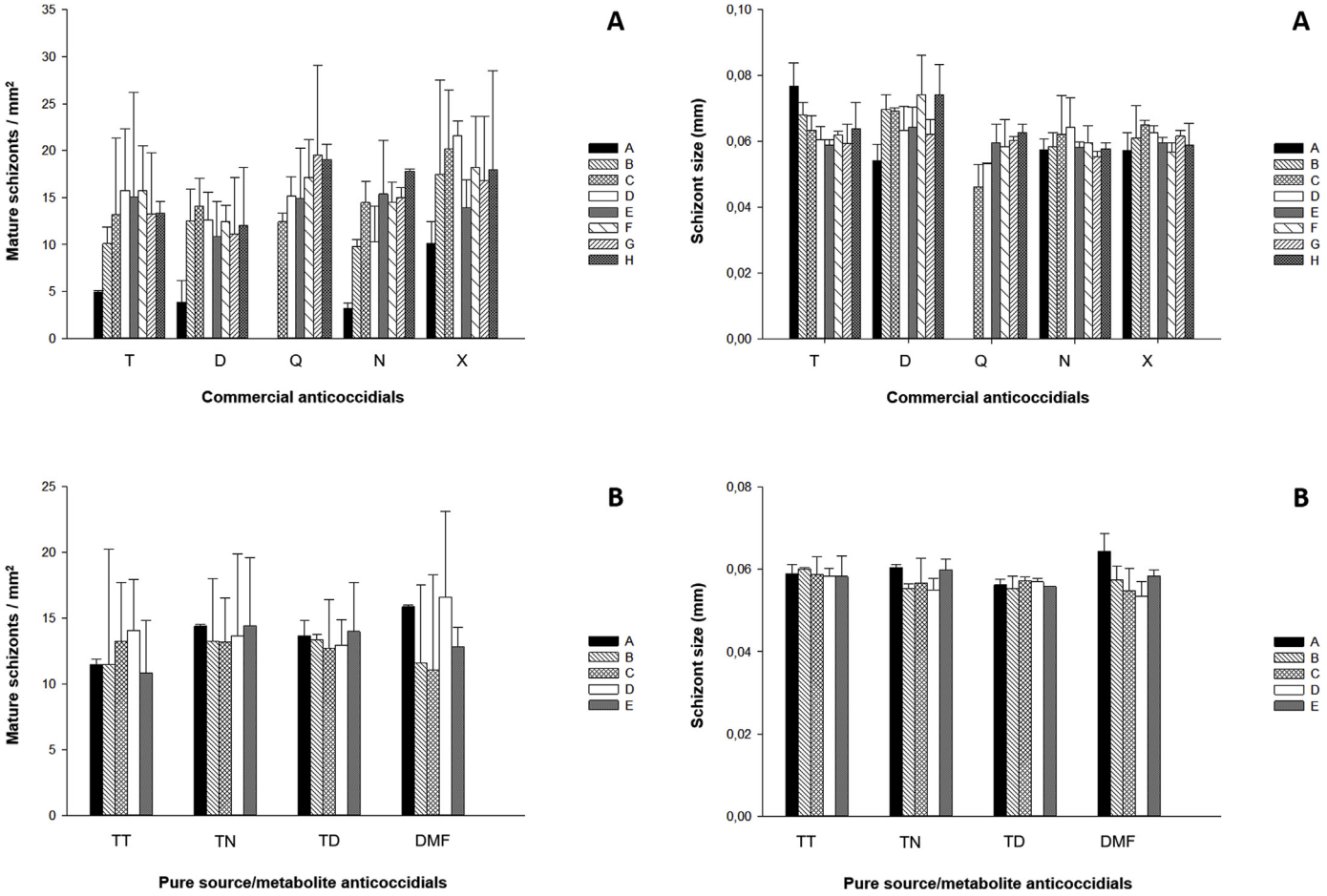

Fig. 3. Number of mature *Eimeria ninkohlyakimovae* schizonts in bovine colonic epithelial cells under the influence of commercial (**A**) or pure source/metabolite (**B**) anticoccidials evaluated 15 days post infection. **A**: (T) toltrazuril, (D) diclazuril, (Q), decoquinate, (N) sulphonamide, (X) dimethyl sulphoxide – negative control **B**: (TT) pure toltrazuril, (TN) toltrazuril sulphone, (TD) toltrazuril sulphoxide, (DMF) dimethyl formamide – negative control. The data are represented as the mean duplicates ± STD. See Tables 1 and 2 for specific concentrations (A–H) used for each anticoccidial.

Fig. 4. Size of mature *Eimeria ninkohlyakimovae* schizonts in bovine colonic epithelial cells under the influence of commercial (**A**) or pure source/metabolite (**B**) anticoccidials evaluated 15 days post infection. A: (T) toltrazuril, (D) diclazuril, (Q), decoquinate, (N) sulphonamide, (X) dimethyl sulphoxide - negative control, B: (TT) pure toltrazuril, (TN) toltrazuril sulphone, (TD) toltrazuril sulphoxide, (DMF) dimethyl formamide – neagative control. The data are represented as the mean duplicates ± STD. See Tables 1 and 2 for specific concentrations (A–H) used for each anticoccidial.

sporozoites to invade and further develop up to first schizogony in BCECs in the presence of different concentrations of anticoccidials or controls. No clear inhibitory effect was found with any of the anticoccidial formulations tested. Similar to our results, Thabet et al. (2017) did not find a significant correlation between *in vivo* data and percentage of reproduction inhibition *in vitro* for toltrazuril when evaluating the development of *Eimeria tenella* in MDBK cells. In addition, compared with different ionophores, toltrazuril showed the highest value of minimum inhibitory concentration. The authors suggested that those data probably indicated that this test system is not appropriate for assessing toltrazuril sensitivity of *E. tenella*, and the same may be the case for the non-ionophore anticoccidials evaluated here.

The commercial anticoccidials, toltrazuril (Baycox®), diclazuril (Rumicox®), and decoquinate (Deccox®), assessed in the present study have been demonstrated to be effective at reducing parasite burdens and have been associated with increased growth rates in calves (Daugschies and Najdrowski, 2005; Mundt et al., 2005; Enemark et al., 2015), lambs (Taylor et al., 2011; Taylor and Bartran, 2012; Diaferia et al., 2013), and goat kids (Foreyt et al., 1986; Ruiz et al., 2012; Iqbal et al., 2013); all three anticoccidials are registered in different EU countries for cattle and sheep. In contrast, in many countries sulphonamides are no longer authorized for anticoccidial treatment, although some derived drugs, such as sulphadimethoxine, are still commercially available for cattle coccidiosis, e.g., in the USA (Burke et al., 2013).

Despite the strong efficacy documented *in vivo* for the four commercial anticoccidials evaluated in our study, limited evidence of their activity *in vitro* could be demonstrated in the cell-culture model employed here. Indeed, only the highest concentration of toltrazuril (Baycox®) and diclazuril (Rumicox®) significantly reduced the infection rate as well as the number of immature schizonts. Low numbers of mature schizonts were also found for the two higher concentrations of both commercial anticoccidials, but the differences were not statistically significant; this probably reflects the relatively high standard deviations associated with a limited number of replicates.

Bioconversion of antiparasitic drugs in the host is not uncommon (Lanusse et al., 1995), so the apparent lack of effect of the commercial anticoccidials analysed here could be related to the inability of the current *in vitro* system to metabolize the drugs to active compounds. To assess this possibility, the anticoccidial activity *in vitro* of pure toltrazuril (without excipient) and two of its main metabolites were evaluated at different concentrations. Within the treated host, toltrazuril undergoes extensive metabolism to toltrazuril sulphoxide and then to toltrazuril sulphone (ponazuril) (Lim et al., 2010), which appears to have anticoccidial activity against *Cystoisospora suis* (Bach et al., 2003) and goat *Eimeria* infections (Gibbons et al., 2016). However, far from increasing their anticoccidial effect *in vitro* in the present

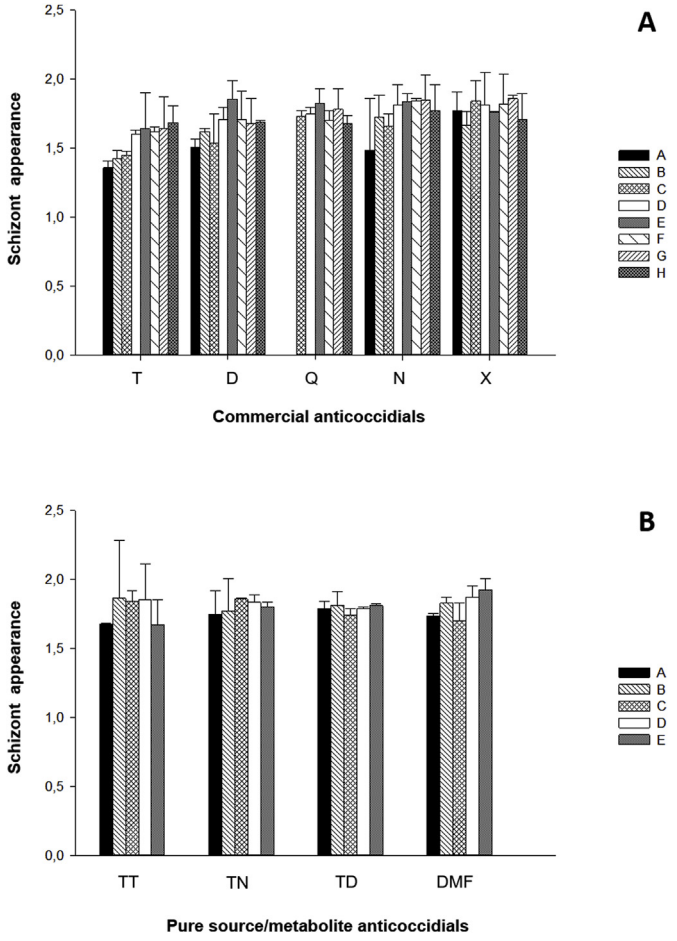

Fig. 5. Appearance of mature *Eimeria ninkohlyakimovae* schizonts in bovine colonic epithelial cells under the influence of commercial (**A**) or pure/derivate (**B**) anticoccidials evaluated 15 days post infection. The schizont condition was scored as: 1) healthy and with grey scale colour at phase contrast; 2) relatively healthy and with brownish appearance at phase contrast; 3) unhealthy and with irregular shape. A: (T) toltrazuril, (D) diclazuril, (Q), decoquinate, (N) sulphonamide, (X) dimethyl sulphoxide - negative control, B: (TT) pure toltrazuril, (TN) toltrazuril sulphone, (TD) toltrazuril sulphoxide, (DMF) dimethyl formamide – negative control. The data are represented as the mean duplicates ± STD. See Tables 1 and 2 for specific concentrations (A–H) used for each anticoccidial.

study, the metabolites of pure toltrazuril, toltrazuril sulphone and toltrazuril sulphoxide, only showed a minor inhibitory activity against *E. ninakholyakimovae* sporozoite invasion and further development.

As the *in vitro* anticoccidial effects of the drugs analysed here were mostly found at the highest concentrations, a more pronounced inhibitory response would have been expected by increasing the amount of drug available in the cultures. However, concentrations higher than those shown in Tables 1 and 2 could not be estimated in the culture system employed here, due to evidence of cell damage, either by the effect of the drug itself or the concentration of solvent used. In particular, high concentrations of decoquinate (Deccox ®) showed a strong cytotoxicity, and therefore the two highest concentrations were not evaluated for this commercial anticoccidial. Similarly, the final DMSO percentage for the highest concentration led to a significant reduction in the number of immature schizonts.

Irrespective of the drug metabolite or concentration used, the type of cells might be another important factor to be considered in an *in vitro* system for the evaluation of ACE. The cell line used here, BCEC, has previously been shown to be a suitable *in vitro* model for the development of *E. ninakholyakimovae* up to the first schizogony (Ruiz et al., 2010), which was also achieved in the present study. Example photographs of intracellular sporozoites, immature schizonts, and mature schizonts containing merozoites are provided in electronic supplementary material. These types of cells are colonic in origin and it is possible that absorption of the anticoccidials used in our study differs between intestinal segments; this is relevant because the pathogenic species mainly infect the small intestine and the caecum (Deplazes et al., 2016). It may also be that the process of creating a permanent cell line (Föllmann et al., 2000), might have influenced the cells' ability to incorporate different substances. Multi-drug resistance has been reported in colonic cancer cells, which block drug activity by efflux transporters that promote metabolism, elimination, and detoxification (Chen et al., 2012); whether the colonic cell line used in our studies share some of these features cannot be excluded and should be evaluated. The use of a different cell line from closely related hosts, possibly of small intestinal origin or primary endothelial origin, like the bovine or caprine umbilical vein endothelial cells (BUVEC and CUVEC, respectively), might thus have provided more useful results. Infections of BUVEC and CUVEC by *E. ninakohlyakimovae* sporozoites have also been shown to result in merogony and macromeront-I formation (Ruiz et al., 2010).

The development of an *in vitro* model for the study of biological processes does not necessarily take into account the complexity of circumstances occurring *in vivo*. When evaluating drug efficacy *in vitro*, the assumption is made that the mechanism of action also occurs in the test system, but this may not always be the case. Indeed, the mechanisms of action of the different anticoccidials are not always well elucidated. For example, the proposed mode of action of toltrazuril is thought to be directed against the first and second generation schizonts, microgamonts, and macrogamonts (Mehlhorn, 2008). The action is probably achieved by inhibiting mitochondrial respiration and nuclear pyrimidine synthesis in the parasite, possibly by inhibiting dihydroorotate dehydrogenase (Harder and Harberkorn, 1989). However, the distribution of this enzyme in different intestinal segments is still unknown and molecular-related reactions deserve further investigation. For diclazuril, the mechanisms of action are unknown, but it has been shown that the activity is only directed against specific endogenous stages of *Eimeria* spp. (Mehlhorn, 2008). Wood and Fildes (1940) proposed that the mechanism of action of sulphonamides is related to the ability of the drug to inhibit the synthesis of folic acid by coccidian parasites through analogous competition to PABA (p-amino-benzoic acid). Finally, decoquinate acts by arresting the development of sporozoites following their penetration of the gut epithelium (Taylor and Bartram, 2012), probably through the inhibition of mitochondrial respiration and electron transport in *Eimeria* parasites (Wang, 1975, 1976; Fry and Williams, 1984). All the anticoccidials tested here seem to act against intracellular stages of the parasites, so they should have no effect against oocysts. This assumption has not been documented in the literature as far as we know. However, as expected, no inhibitory effect on oocyst sporulation rate was shown for any of the commercial or pure/source anticcoccidials tested in the present study.

In conclusion, in this study we provide preliminary work towards the development of an *in vitro* model to evaluate ACE in ruminant hosts, using BCEC for cell culture studies and investigating different stages of development. One weakness of our study was the assumption that the anticoccidial concentrations remained stable when continuously included in the cell-culture medium throughout the study; however, we did not perform the necessary analyses to show that this was the case. We have been unable to determine the reason why, in general, we could not identify measurable effects from the different anticoccidials. Thus, further experiments, including the analysis of different cell lines, the implementation of cell permeability for non-ionophores anticoccidials, and investigation of their specific mechanisms of action are recommended. Despite these initial experiments not yielding any definitive clues, it is clear that the development of a suitable *in vitro* system for the evaluation of the ACE in ruminants would decrease the need for

animal experiments and could be used in the initial assessment of ACE of new anticoccidial drugs or bioactive substances. In our opinion, it therefore remains an important and worthy goal, and further investigations should be encouraged.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exppara.2019.04.009.

Declaration of interest

The authors have no interests to declare.

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