Aus dem Institut für Parasitologie, Justus-Liebig-Universität Giessen, Deutschland
und der Universität von Antioquia, Medellín, Kolumbien

Eimeriosis in ruminants: large-scale epidemiological survey, isolation of a new *Eimeria zuernii* strain and novel data on *Eimeria* spp.-host cell interactions

INAUGURAL DISSERTATION

zur Erlangung des Grades eines

Dr. med. vet.

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Submitted by

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López-Osorio S, Silva LMR, Velazquez ZD, Taubert A, Hermosilla C (2019) Modulation of lipid uptake during *Eimeria arloingi* macromeront formation and impact on parasite intracellelar development *(manuscript in preparation).*

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Lopez-Osorio S, Villar D, Piedrahita D, Failing K, Taubert A, Hermosilla C, Chaparro-Gutierrez JJ (2019) Epidemiological survey and associated risk factor analysis on *Eimeria* infections in Colombian calves. German Veterinary Medicine Society (DVG): 17-19/09/2019, Leipzig, Germany. Oral presentation.

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Lopez-Osorio S, Velasquez ZD, Conejeros I, Hermosilla C, Taubert A (2019) 3D-holotomographic analysis of aerobic *Eimeria bovis* oocyst sporogony. German Veterinary Medicine Society (DVG): 17-19/09/2019, Leipzig, Germany. Poster.

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Abbreviations

BFGC: Bovine fetal gastrointestinal cells

BSLEC: Bovine spleen lymphatic endothelial cells

BUVEC: Bovine umbilical vein endothelial cells

CHO: Cholesterol

Ck. Creatine kinase

CMI: Internal membrane complex

CNS: central nervous system

CO_2: carbon dioxyde

d.p.i: Days *post infectionem*

d: days

DC: Dendritic cells

ER: Endoplasmic reticulum

HUVEC: Human umbilical vein endothelial cells

IFN- γ: Interferon γ

LDL: Low density lipoproteins

MDBK: Madin-Darby bovine kidney

MPO: Myeloperoxidase

NE: Neutrophile elastase

NETs: Neutrophile extracellular traps

NO: Nitrogen oxide

ND: Not described

O.P.G: Oocyst per gram

OR: Oocyst residual body

p.i: post infectionem

PMN: Polymorphonuclear neutrophils

PP: prepatency period

PUVEC: Porcine umbilical vein endothelial cells

RT: Room temperature

SEM: Scanning electron microscope

SOCE: Store-operated calcium entry

SQLE: Squalene epoxidase

SR: Sporocyst residual body

VP: Parasitophorus vacuole

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Preface

This doctoral thesis focuses on various aspects of the apicomplexan parasite genus *Eimeria*, which is considered as an important protozoan genus of domestic ruminant species (i. e. goats, sheep and cattle) worldwide. Important aspects such as parasite host cell invasion, obligate intracellular development, epidemiology, parasite metabolism, and host innate immune reactions against *Eimeria* spp. will be covered throughout the thesis.

This manuscript is divided by chapters and the first chapter covers a literature review on most relevant ruminant *Eimeria* spp. which summarizes the epidemiology, biology, metabolism and diagnosis of these enteric parasites.

The second chapter composes published data entitled: "*Concomitant* in vitro *development of* Eimeria zuernii- *and* Eimeria bovis-*macromeronts in primary host endothelial cells*", and summarizing methodology used for first isolation and replication of a Colombian bovine *E. zuernii* strain, and further the establishment of a suitable *in vitro* culture system for investigations on host cell-parasite interactions and for comparative studies to other highly pathogenic ruminant *Eimeria* species.

The third chapter has the basis of another publication entitled: "*Epidemiological survey and risk factor analysis on bovine* Eimeria *infections in Colombia*". This study includes analysis of 1333 stool samples from calves under one year of age distributed across the Colombian territories, in addition to some risk factor analyses associated with the presence of different *Eimeria* species.

Chapter four includes another manuscript currently under revision entitled: "*Optimized excystation protocol for ruminant* Eimeria *spp. sporulated oocysts (Apicomplexa, Coccidia)*". Here, an improved protocol for the *in vitro* release of the viable sporozoites is described and adapted for two highly pathogenic ruminant *Eimeria* species: *E. bovis* (bovine) and *E. arloingi* (caprine).

Chapter five has also become another submitted manuscript entitled: "*3D-holotomographic live cell microscopy analysis of aerobic* Eimeria bovis *oocyst*

sporogony". The aim of this study was to investigate in depth for the first time the aerobic-dependent sporogony of *E. bovis* oocysts with a novel live cell imaging technique such as 3D-holotomographic microscopy (Nanolive®) to simulate the *in vivo* situation in high resolution imagines of external and internal changes attained while *E. bovis* sporogony process took place.

Chapter six is based on the manuscript entitled: "*Modulation of cholesterol during* Eimeria arloingi *macromeront formation and impact on parasite intracellular development".* With this work, we explored some metabolic requirements for the formation of the the first generation of merozoites I *in vitro*.

Chapter seven includes another manuscript "*Metabolic requirement for NET formation and simultaneous autophagy in* Eimeria bovis *sporozoite-exposed bovine polymorphonuclear neutrophils".* In this chapter we determined the relevance of distinct PMN-derived metabolic pathways via pharmacological inhibition experiments and assessed final merozoite I production.

Finally, a discussion and conclusion section are presented at the end of the thesis, with a general view on future investigations of some here developed experimental techniques to gain access to sporozoites (excystation protocol), detailed host innate immune reactions and further work on metabolic requirements of the genus *Eimeria*.

1. Chapter: Literature review

1.1 Introduction

Coccidiosis is the term used to describe a disease caused by infection with one or more species of *Eimeria* (Oluwadare, 2004) which have high economic impact on cattle and goat industry worldwide (Oluwadare, 2004; Hermosilla *et al.*, 2002a; Silva *et al.*, 2015). This intestinal disease is caused mainly by pathogenic *Eimeria* species which belong to the phylum Apicomplexa (cattle: *E. zuernii, E. bovis* and *E. alabamensis;* goats: *E. ninakohlyakimovae, E. arloingi, E. alijevi*) (Matjila and Penzhorn, 2002; Chartier and Paraud, 2012). These are obligate intracellular protozoan parasites infecting mainly epithelial and endothelial cells of the intestinal tract of susceptible host (Oluwadare, 2004; Hermosilla, Ruiz and Taubert, 2012). *Eimeria* spp. are distributed worldwide and infection practically occurrs in all kinds of vertebrates such as cattle, sheep, goats, fowls, cat, dogs, rabbits and horses (Deplazes *et al.*, 2016). Mostly all cattle and goats are infected with coccidians at some point during their life, but only few of them develop clinical manifested coccidiosis. The clinical symptoms occur mainly in young animals, but occasionally affect animals over 6 months of age or even adult animals (Davies, Joyner and Kendall, 1972; Chartier and Paraud, 2012). Challenge with low levels of *Eimeria* can stimulate the protective host immune response and this is the basis of vaccination strategies for avoidance of coccidiosis outbreaks (Catchpole, Norton and Gregory, 1993).

This parasite exhibits a high degree of host and site specificity. One single animal species can be host to several *Eimeria* spp., each with its distinct location in the intestine. Every single *Eimeria* spp. produces different host parasite interactions explaining why there are many degrees of coccidiosis (Deplazes *et al.*, 2016). The disease occurs if the animal is exposed to a high infective dose or its immunity is rather low (Chapman, 2014). Due to the self-limiting nature of the life cycle and enhanced resistance to reinfection, coccidiosis is rarely a problem in extensively raised livestock systems, but it becomes important in closely confined

and highly intensively reared animal systems (Daugschies and Najdrowski, 2005).

Therapy options for many of the diseases caused by apicomplexan parasites include: culling of severly infected livestock, prevention of infection by vaccination or chemotherapy. Thus, knowledge on suitable targets for intervention must be generated and candidate compounds must be characterised with regard to their mechanisms of action. For such drug testing studies, *in vitro* culture systems for *Eimeria* species have increasingly been applied thereby paving the way to exploit the basic biology as well as metabolic necessities of these organisms, and had a major impact on the development of tools for diagnostic purposes. With adequate *in vitro* culture systems, studies on complex host cell–parasite interactions, on factors involving innate and adaptive resistance, stage conversion, differentiation, genetics and transfection technology, vaccine candidates and drug effectiveness could be carried out (Müller and Hemphill, 2013a). Because the pathogenesis occurs through intracellular interactions, it seems necessary to use *in vitro* systems based on primary specific host cells which allow investigation of these detailed molecular mechanisms. Several authors agree that an *in vitro* culture system is essential for the study of obligate intracellular parasites, since it is the way to obtain direct information from the infected host cells, and compare them with non-infected ones. In addition, it is the ideal tool for molecular studies in their early stages of parasite replication and to identify compounds of interest for treatment such as parasite-induced anti-apoptotic factors (Lang *et al.*, 2009; Müller and Hemphill, 2013b).

1.2 Systematic of *Eimeria* spp.

Infrakingdom:	Alveolata
Phylum:	Apicomplexa
Class:	Conoidasida
Order:	Eucoccidiorida
Family:	Eimeriidae
Genus:	*Eimeria* (Schneider, 1875)

Phylum: Apicomplexa

Apicomplexa constitute an eukaryotic infraphylum (Levine 1970) that is part of the superphylum Alveolata (Cavalier-Smith 1991) under the protozoan kingdom Chromista (Levine, 1973; Adl *et al.*, 2005; Sato, 2011). Until now, approximately 6,000 apicomplexan species have been described, but sequencing data of environmental samples suggest there may be million more species belonging to this phylum (Adl *et al.*, 2007). All apicomplexans are obligate parasites, and some cause either human or animal diseases such as coccidiosis (caused by *Eimeria* spp.), toxoplasmosis (*Toxoplasma gondii*), cystoisosporosis (*Cystoisospora* spp.) babesiosis (*Babesia* spp.), theileriosis (*Theileria* spp.), cryptosporidiosis (*Cryptosporidium* spp.), sarcocytiosis (*Sarcocystis* spp.) and malaria (*Plasmodium* spp.). However, many apicomplexans are not pathogenic for their host (Sato, 2011). Because of their clinical and economical importance in livestock, disease-related apicomplexan genera have extensively been investigated in the past decades. The taxonomic classification of the phylum Apicomplexa with relevance for veterinary medicine is represented in Fig. 1.

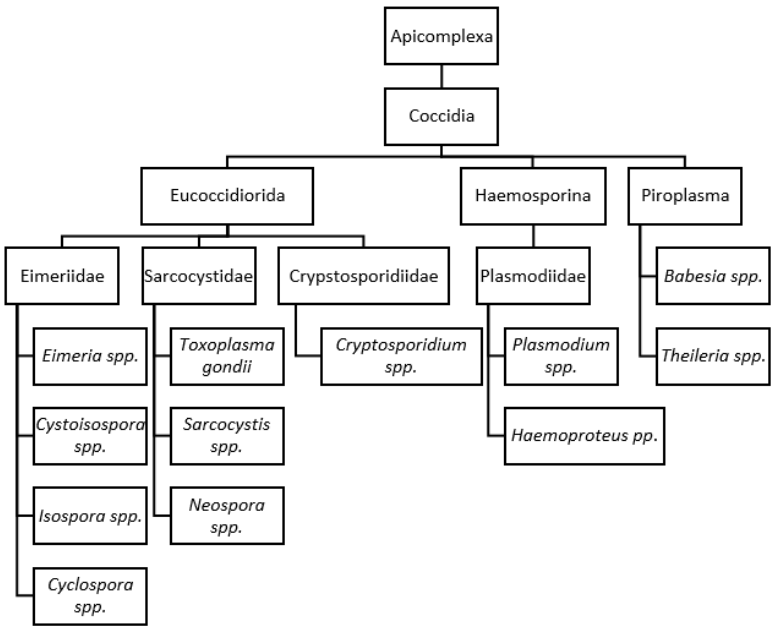


Figure 1. Taxonomic classification of the phylum Apicomplexa of veterinary medicine importance. Modified from Roberts, Janovy, and Gerald 2009.

Family: Eimeriidae

There are 16 genera and approximately 1,340 species in this family but the most important genera in veterinary medicine are *Eimeria* and *Cystoisospora.* Infections with these two genera are usually refered as coccidiosis (M. Taylor, Coop and Wall, 2007).

Genus *Eimeria*

This genus is composed of approximately 1,700 species, affecting mainly domestic mammals and birds. All *Eimeria* spp. are species-specific and known as monoxenous parasites (Müller and Hemphill, 2013a). The genus *Eimeria* contains the species of most economic impact for ruminants. To distinguish this genus from several others, the internal structure of infective sporulated oocysts is shown in Fig 2. In general, all freshly shed oocysts consist of a thickened outer wall and rounded mass with nucleated zygotus (Fig. 2), but after sporulation the distinguishing characteristics of each species become more apparent. For *Eimeria* genus four sporocysts develop within the circumplasm of the oocyst, each containing two banana-shaped sporozoites (Silva *et al.*, 2017) (Fig. 3). In contrast, the other genus *Cystoisospora* contains two sporocysts each containing four sporozoites (Fig. 2). This genus infects principaly species from wild birds, dogs, cats and pigs.

The initial infective unit of all *Eimeria* spp. is the sporozoite stage, which is a banana-shaped motile cell. Detailed sporozoite morphology is shown in Fig. 4. The sporozoite is the beginning and the end of the life cycle of coccidian (Bowmann, 2014). Sporozoites are the infective forms found in sporulated oocysts and are the result of protoplasm segmentation (Bowman, 2014). The protoplasm (sporont) is surrounded by a resistant oocyst wall and is eliminated in the stool (non-sporulated oocyst, Fig. 2).

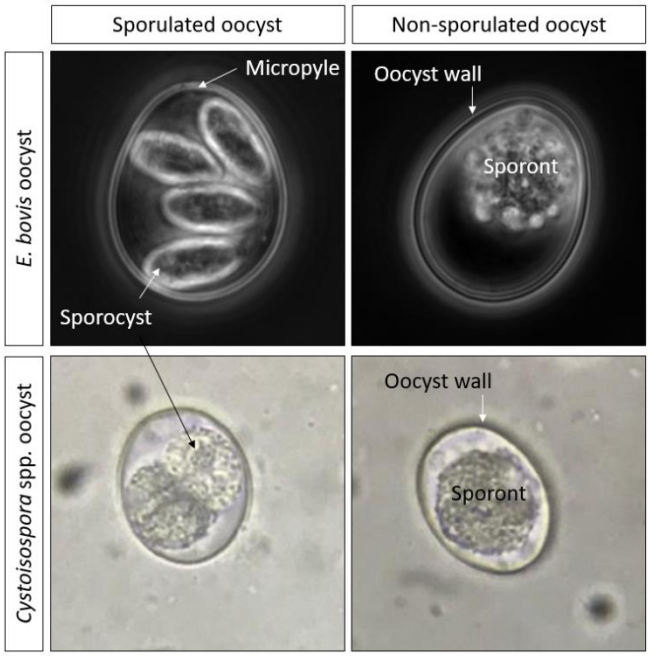
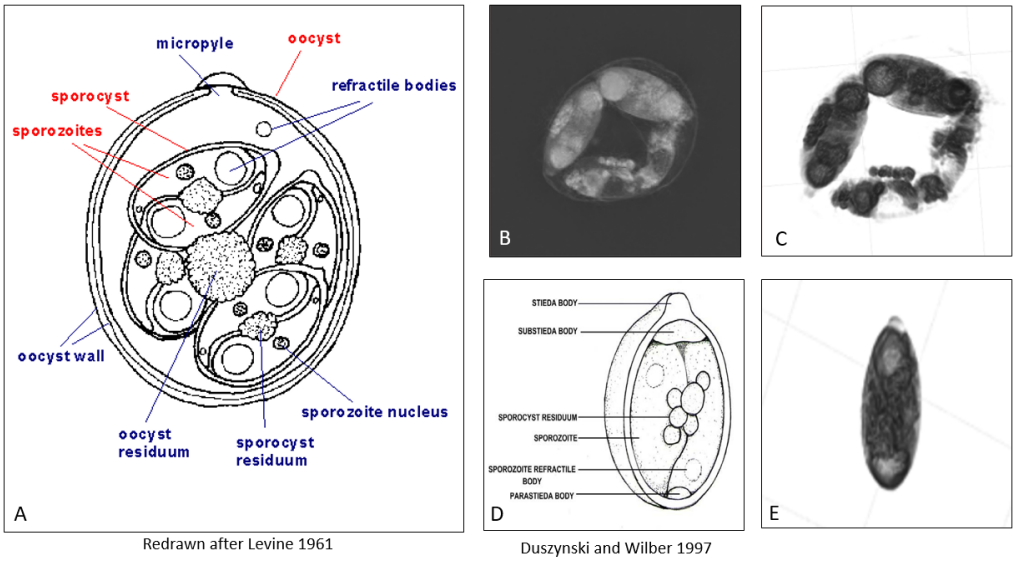


Figure 2. Sporulated and non-sporulated oocyst from *Eimeria* and *Cystoisospora* spp. *Eimeria bovis* oocyst (Strain H). Picture taken by: Sara López, 2018. Olympus inverted microscope (IX81, Olympus®). Magnification 600X. *Cystoisospora* spp. oocyst. Olympus microscope (Olympus®). Magnification 1000X. Dog faeces sample. Picture taken by: Sara López, 2018.



Redrawn after Levine 1961

Duszynski and Wilber 1997

Figure 3 Schematic representation of *Eimeria* spp. oocyst and its parts (A) (D.N. Levine, n.d.). Detailed structures are shown in an *E. bovis* sporulated oocyst by 3D-holotomography microscopy (B) and rendering format (C). Sporocyst scheme (D). Sporocyst rendering format by 3D-holotomography analysis (E). Picture taken by: Lopez-Osorio et al., 2019

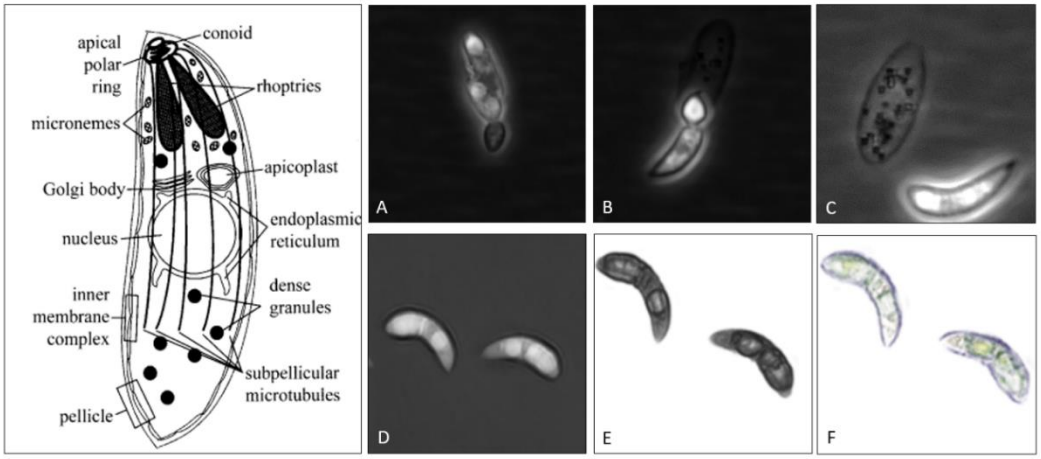
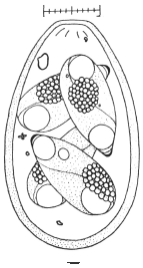
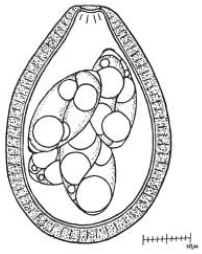
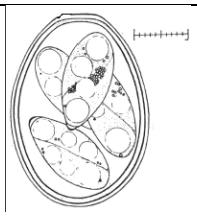
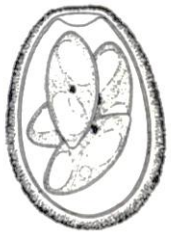


Figure 4 Schematic representation of *Eimeria* spp. sporozoite (left panel) (Morrissette and Sibley 2002). Pictures A-C shows the egress of *E. bovis* sporozoites from the sporocyst. *Eimeria bovis* free sporozoites were obtained using STEVE® software (Nanolive) to generate a refractory index-based z-stack (D) (3D-holotomography), the rendering format (E) and the digital staining (F) based on the refractory index. Lopez-Osorio et al., 2019.

More than 20 morphologically different *Eimeria* species have been reported in cattle (Daugschies and Najdrowski, 2005; Kokusawa, Ichikagua-Seki and Itagaki, 2013), but only 13 are currently considered as valid bovine species (Faber *et al.*, 2002a). Among these, two are regarded as highly pathogenic: *E. bovis* and *E. zuernii*, which are responsible for severe clinical symptoms, generating profuse bloody diarrhoea, and sometimes even death (Mundt *et al.*, 2005a). In addition, it has been reported that *E. alabamensis* can also cause diarrhoea in elderly animals, after feeding on highly contaminated pastures with sporulated oocysts (Measures, 1956; Hooshmand-Rad, Svensson and Uggla, 1994; Svensson, Uggla and Pehrson, 1994). Natural occurring *Eimeria* spp. infections are typically involving a combination of several species, and sometimes adquiring clinical relevance in the face of other predisposing factors (e. g. coinfection with other parasites, primary infection, low immunity, malnutrition, etc). Table 1 summarizes the characteristics of the most important *Eimeria* species affecting cattle industry worldwide.

***Eimeria* spp.**	**Shape of oocyst**	**Size (µm)**		**Micropyle**	**Sporulation time (days) RT**	**Main localization and disease**
		Oocyst	Sporocyst			
E. alabamensis	 Oocyst ovoid, colourless, without OR[2] and SR.	13-24x11-16 (18.9x13.4)[3]	10-12x4-6	No	5-8	Jejunum and especially ileum (in nuclei of epithelial cells): Meronts I-II and gamonts; rarely gamonts in colon and caecum. **Catarrhailic enteritis** PP:6-8 d ++[1]
E. auburnensis	 Oocyst elongated oval, yellowish-brown, wall smooth or finely granulated surface without OR, SR.	32-46x20-25 (38.4x23.1)	16-23x7-11	Yes	2-3	Jejunum, ileum (epithelium): large meront I (up to 240 µm) and meronts II; gamont in lamina propria. **Catarrhailic enteritis** PP:>16 d ++
E. bovis	 Oocyst ovoid or subspherical, orange-brown, micropyle indistinct, with OR and SR.	25-34x17-23 (27.7x20.3)	13-18x5-8	Yes	2-3.	Ileum (endothelial cells of central lymph capillaries of villi): large meront I (>200 µm); epithelial cells of caecum and colon: small meronts II and gamonts. **Haemorrhagic typhlitis and colitis** PP: 18-21 d +++

E. brasiliensis	 Oocyst ellipsoid, yellow-brown, micropyle with a clear plug, without SR, OR.	33-43x24-30 (37x27)	17-21x8-10	Yes	12-14.	Endogenous development unknown.
E. bukidnonensis	 Oocyst pear-shaped or oval, one pole tapered, thick, radially striated, yellowish-brown wall.	47-50x33-38 (48.6x35.4)	20-10.	Yes	6-7.	Endogenous development unknown.
E. canadensis	 Oocyst ovoid or subspherical, orange-brown, micropyle indistinct, with OR and SR.	28-37x20-27 (32.5x23.4)	15-22x6-9	Yes	3-4.	Endogenous development unknown.
E. cylindrica	 Oocyst oblonged, colourless, without OR, SR.	16-27x12-15 (23.3x12.3)	12-16x4-6	No	2.	Endogenous development unknown.

E. ellipsoidalis	 Oocyst ellipsoidal, colourless, without OR, SR.	20-26x13-17 (23.4-15.9)	11-16x5-6	No	2-3	Jejununm, especially ileum (epithelium): meronts I-II and gamonts **Catarrhalic enteritis** PP: 8-10 d +
E. illinoisensis	 Oocyst ellipsoidal or ovoid	24-29x19-22	13-16x6-7	No	Unknown	Endogenous development unknown.
E. pellita	 Oocyst oval, thick brown wall with uniformly distributed protuberances, without OR, SR	34-41x26-30 (40x28)	14-20x6-8	Yes	10-12.	Endogenous development unknown.
E. subspherica	 Oocyst round or subspherical, colourless, without OR and SR	9-14x8-13 (11x10.4)	7-10x3-5	No	4-5.	Endogenous development unknown.

E. wyomingensis	 Oocyst ovoid, thick-walled, yellow brown, without OR, SR	37-45x26-31 (40.3x28.1)	18x9	Yes	5-7.	Distal small intestine. PP: >13 d -
E. zuernii	 Oocyst subspherical, colourless, without OR but with SR	15-22x13-18 (17.8x15.6)	7-3x4-7	No	2-3.	Ileum (lamina propria): large meronts I (> 200 µm); epithellial cells of caecum and colon: small meronts II and gamonts. **Haemorrhagic typhlitis and colitis**. PP: 15-17 d +++

Table 1 Key scheme for the identification of sporulated *Eimeria* oocysts of cattle.

[1]Degree of pathogenicity: +++ = high; ++ = moderate; + = Low; - = none. (Deplazes *et al.*, 2016). [2]OR= Oocyst residual body; SR: Sporocyst residual body; PP: prepatency period; d: days. [3]Average size. Adapted from: (Davies, Joyner and Kendall, 1972; D Norman Levine, 1973; Arguello and Campillo, 1999; Daugschies and Najdrowski, 2005; Almeida *et al.*, 2011; Deplazes *et al.*, 2016; Florião *et al.*, 2016)

In the case of goats, at least nine *Eimeria* species are reported so far, including *E. ninakohlyakimovae*, *E. arloingi, E. alijevi, E. caprina, E. christenseni, E. caprovina* and *E. hirci* (Fig. 5). The three first listed here are regarded as pathogenic species and inducing diarrhoea in affected goat kids. For a long time, the species of *Eimeria* in sheep and goats have been considered to be identical on the basis of morphology, nevertheless there is no cross infection due to their strict host specificity (monoxenous parasites).

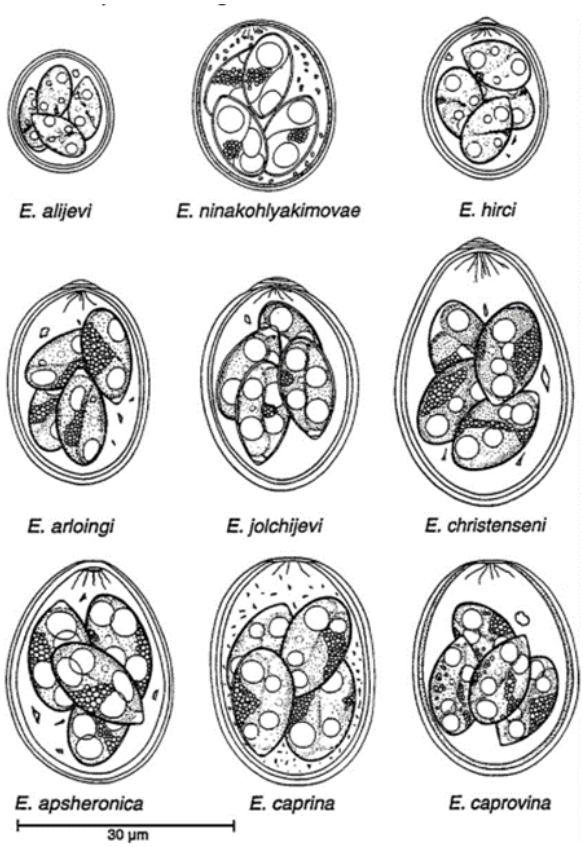


Figure 5 Sporulated oocysts of the principal species of *Eimeria* in goats (Eckert et al., 1995).

1.3 Economic impact

The great majority of *Eimeria* spp. are monoxenous enteropathogens of vertebrates and cause mild pathology and non-clinical infection. Nonetheless, certain species such as *E. bovis, E. zuernii, E. alabamensis* (cattle), *E. ninakohlyakimovae* and *E. arloingi* (goat) are considered highly pathogenic, and can cause severe intestinal lesions (Silva *et al.*, 2017). Coccidiosis is particularly relevant to ruminant and poultry production worldwide (Daugschies and Najdrowski, 2005; Chapman, 2014). In both industries, the economic impact is high and was recently estimated in 6–9% reduction in gross margin for ruminants, and to exceed US$3 billion for poultry (Lassen and Ostergaard, 2012; Blake and Tomley, 2014). The main factors influencing economic losses are costs for coccidiosis prevention and treatments, combined with morbidity and mortality of heavily infected individuals (Silva *et al.*, 2017).

1.4 Life cycle

After the exogenous phase of sporogony, sporulated oocysts can initiate replication once they become orally ingested by a susceptible host. In this host, the parasite reaches the small intestine, where sporozoites are released from oocysts by action of digestive enzymes among others. For sporozoite egress, two separated stimuli must be present: First, stress by carbon dioxide (CO_2) causes rupture of the micropyle and leads to a change in oocyst permeability. Consequently, the oocyst content collapses in a hypertonic salt solution (intestinal content) (Ryley, 1972). The optimal concentration of CO_2 and time of incubation differs according to the species. In *E. bovis* for example, it has to be 100% CO_2 atmosphere for at least 24 h (Lang *et al.*, 2009). The temperature is also essential for the liberation of infective sporozoites (i. e. body temperature) (Pyziel and Demiaszkiewicz, 2015).

The second stimulus consists in the action of compounds such as trypsin and bile. Both compounds activate the sporozoites inside the sporocyst and additionally digest the Stieda body generating a hole in the sporocyst membrane. Bile can either facilitate entrance of digestive enzymes through altered micropyle into oocyst, or can alter lipoproteins of the Stieda body of *Eimeria* oocysts. Although bile is not strictly necessary for activation of sporozoites, it has been demonstrated that lack of bile for many *Eimeria* spp. results in a slower release and mobility of egressed sporozoites. Trypsin digests the sporocyst wall, alongside with parasite-specific enzymes secreted by activated sporozoites. Thanks to the continuous movement of sporozoites, the Stieda body swells and then disappears, leaving a small hole through which the sporozoites escape. This process is very fast and involves a strong constriction of the sporozoite to go through the hole generated by the rapid movement and its pressure (Andrews, 1930; Nyberg and Hammond, 1964; Hibbert, Hammond and Simmons, 1968; Woodmansee, 1986). Up to this moment of egress, no damage is still induced to the host [for more details on *E. bovis*- and *E. arloingi-in vitro* excystation please refer to Chapter 4] (Fig.6).

During the excystation and invasion of the host cell, the sporozoite uses its stored amylopectin to cover its energy requirements. Vetterling and Doran (1969) observed that during the 30 min period of excystation at 42.9°C, carbohydrate reserve levels decreased 2/3 in activated sporozoites. This is also correlated with the consumption of oxygen and other lipid compounds (Levine, 1973).

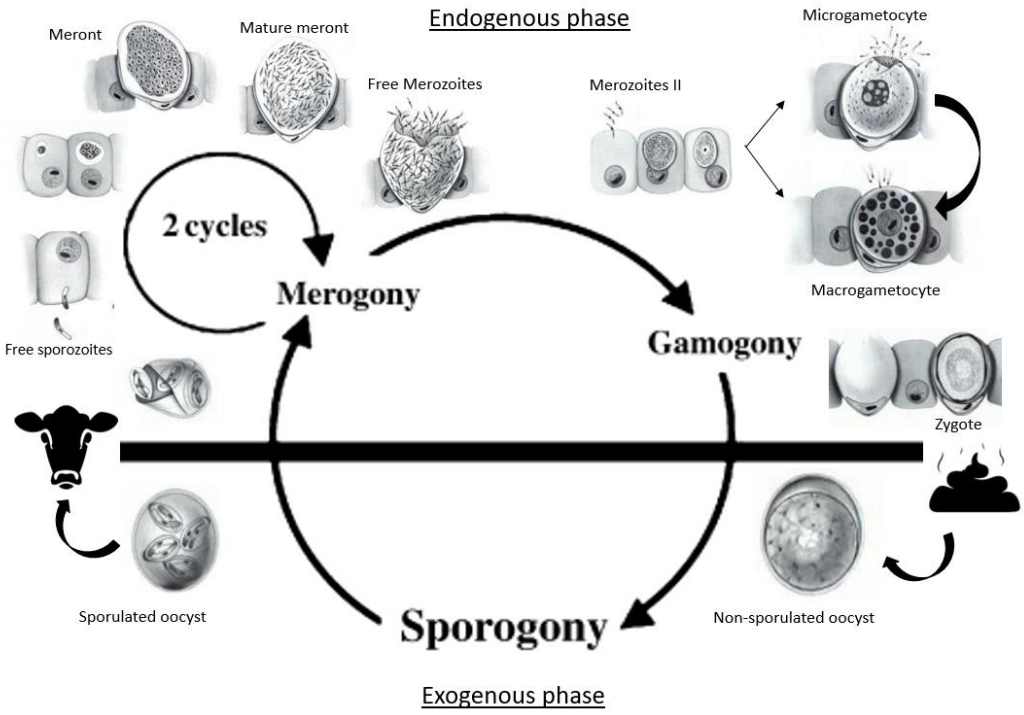


Figure 6 Eimeria spp. life cycle. Modified from Daugschies and Najdrowski 2005

Free sporozoites infect intestinal cells of jejunum or ileum, and develop inside a parasitophorus vacuole (PV) into a rounded and growing organism called the trophozoite, which becomes a meront during the first merogony generation (Hermosilla *et al.*, 2002a, 2008; Daugschies and Najdrowski, 2005; Deplazes *et al.*, 2016). In case of *E. bovis* and *E. zuernii*, sporozoites must cross the epithelium of the small intestine to invade the host endothelial cells of the lymphatic capillaries of the ileum, where the first merogony occurs. It is believed that lectin-like receptors mediate the invasion of the host cell (Hammond and Long, 1973). As the sporozoite grows, the endothelial cell gets hypertrophic and its nucleus undergoes alterations, becoming larger and with an enlarged

nucleolus with scattered chromatin; its cytoplasm is organized in two concentric zones and shows no vacuolated appearance (Hammond, Ernst and Goldman, 1965). At first the host cell nucleus has a random distribution, but 8 d.p.i it migrates to the periphery in order to give space to the macromeront development (Hammond, Ernst and Goldman, 1965; Fayer and Hammond, 1967; Bedrnik, 1969).

The merogony starts with multiple nucleus division of the *Eimeria* trophozoite without the division of the cytoplasm, resulting in the formation of ellipsoidal structures called blastophores with a peripheral layer of nuclei. The merozoite forms around each nucleus and grows radially. At the end of the phase, the division of the cytoplasm starts resulting in the formation of mononuclear spindle-shaped, motile daughter cells, known as merozoites (Deplazes *et al.*, 2016). Mature merozoites I are separated by the residual body, a remnant of the blastophore (Hammond, Davis and Bowman, 1944; Hammond *et al.*, 1946; Hammond, Ernst and Goldman, 1965; Fayer and Hammond, 1967; Hammond and Long, 1973). The first generation of macromeronts produce up to 170,000 merozoites I which invade new host epithelial cells resulting in severe destruction of the gut mucosa (Hermosilla *et al.*, 2002b; Daugschies and Najdrowski, 2005). [For detailed information on first merogony of *E. bovis* and *E. zuernii* please refer to Chapter 2]. Once the macromeront is mature, the merozoites I rupture the cell and escape into the lumen of the small intestine and are most probably transported by intestinal stream to the large intestine, where merozoites I enter the crypts of Lieberkühn (*E. bovis*). Merozoites I are 11-16 µm long x 1-3 µm wide. These stages have a polar ring containing a conoid with fibers grouped in a narrow helix. Two rhoptries extend from the cone to the back of the parasite with a parallel bar in its neck. The anterior region contains abundant micronemes, 22 subpelicular tubes, of which three have granules of glycogen, many ribosomes, one or two mitochondria, a micropore and endoplasmic reticulum (Hammond, Ernst and Goldman, 1965; Fayer and Hammond, 1967). These merozoites I enter into epithelial cells of the crypt and develop into second meront stages which mature in 1-2 days measuring 10 µm in diameter and releasing only

30 to 36 merozoites II. For each sporulated oocyst that is ingested by a calf, approximately 24 million second generation merozoites are formed (i. e. 8 sporozoites x 100,000 merozoites I x 30 merozoites II) involving the massive destruction of approximately 24 billion intestinal cells (Hammond and Long, 1973; Arguello and Campillo, 1999).

After the maturation of second meront stages, released merozoites II invade adjacent epithelial cells undergoing sexual gamogony. During the gamogony, most merozoites II develop into a single, large, mononuclear, spheroid cell, the female macrogamete. The macrogametes have characteristic eosinophilic granules [outer granule layer containing glycoproteins and an inner granule layer containing protein-rich molecules; both commonly known as 'wall forming bodies' (WFB1, WFB2)]. Few merozoites II develop into large, polynucleated cells (male microgamonts) which form many spindle-shaped cells with two flagella, the microgametes.

The gamonts quickly generate alterations in the host cell, which distorts and loses its columnar structure (Hammond, Andersen and Miner, 1963). For *E. bovis* this phase occurs in epithelial cells of the intestinal glands of caecum/colon and appears at 17 d.p.i. For *E. zuernii* it is known that the first gamonts are seen 12 d.p.i in epithelial cells of the glands, jejunum, large intestine, caecum/colon and rectum. The pathological changes and the clinical signs associated with *Eimeria* are generated mainly by the gamonts (Levine, 1973), since they generate destruction of the mucous membrane of the jejunum, caecum and colon, causing imbalances in the absorption (especially water and electrolytes) and resulting in diarrhoea.

Free-released microgametes fertilise thereafter surrounding macrogametes and forming the zygotes. The eosinophilic granules converge and form a resistant oocyst wall surrounding the zygote which decreases in size and becomes a sporont. The oocysts are finally released from ruptured epithelial cells and excreted with the faeces into the environment (Daugschies and Najdrowski, 2005; Chartier and Paraud, 2012; Deplazes *et al.*, 2016). The un-sporulated

Eimeria spp. oocyst excreted from the host contains a diploid sporont stage which develops further by meiosis. In a first step, four haploid sporoblasts are generated, and enclosed by a shell thereby becoming sporocyst. In each sporocyst, two sporozoites are newly formed. The sporont, after meiosis generates also a refractile polar body. The haploid number of chromosomes is two (Walton, 1959). This exogenous sporulation process (also known as sporogony) requires optimal enviromental conditions, including sufficient oxygen, moisture and adequate temperature [for detailed description of *E. bovis* sporogony please refer to Chapter 5]. Sporulation seems to be a strictly aerobic process. Dur and Pellerdy in 1969 found that for sporulation approximately 390 µL of O_2 is required for every 10^6 sporocysts. Once the sporulation ends, the metabolism and respiration of the oocyst is reduced, however, it uses its reserves of polysaccharides, and after a while it becomes non-infective because the parasite runs out of energy to carry out the process of final endogenous excystation in the gut lumen (Ryley, 1972).

It has been described that merogony and gamogony always take place within specific host cells and sites of the intestinal mucosa (Silva *et al.*, 2017). Most ruminant apathogenic *Eimeria* species replicate in intestinal epithelial host cells, nevertheless, other species [e. g. *E. bovis, E. zuernii* (cattle), E*. arloingi, E. ninakohlyakimovae, E. christenseni* (goats)] replicate in host endothelial cells of the lymph capillaries of the lacteals of the small intestine. Consistently, phylogenetic tree demonstrates that most pathogenic species which replicate in highly immunocompetent host endothelial cells and forming huge first-generation macromeronts (up to 400 µm), share a common evolutionary history (please see Fig. 7).

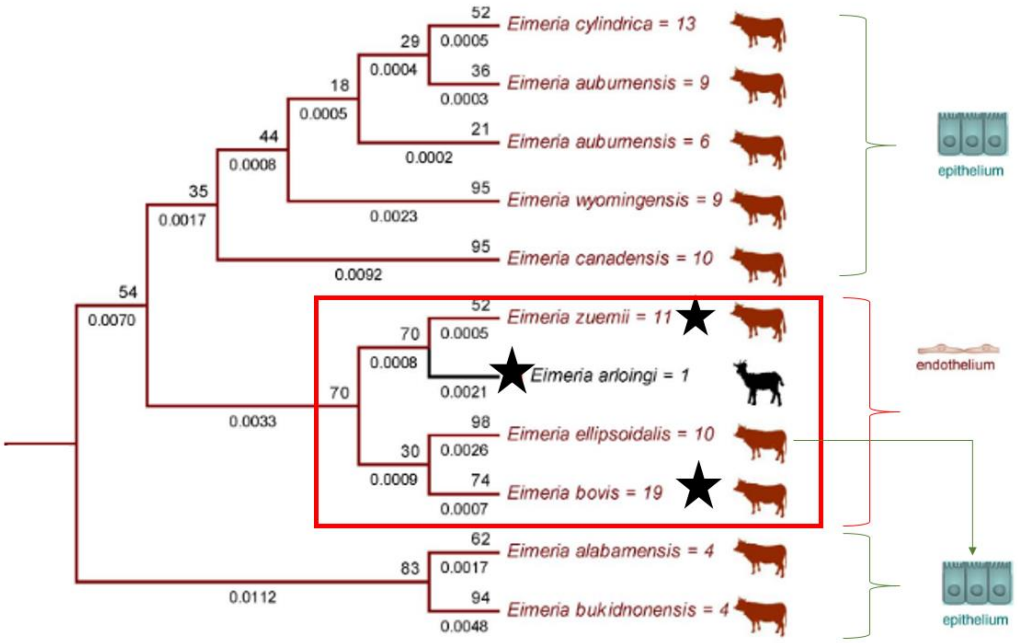


Figure 7 Neighbor-Joining phylogenetic tree with partial 18S-ITS1-5.8S- partial ITS2 sequences from different ruminants *Eimeria* sequences available in GenBank. Adapted from (Liliana M.R. Silva et al. 2017). The consensus of 1000 bootstrap replicates is shown. The sequences of interest are marked with a redsquare and a black star. The species marked with green infect epithelial cells, while the ones indicated with red infect host endothelial cells. The number at the end of each node indicates how many sequences constitute each of the collapsed branches. The host species of each parasite is shown: bovine (cow drawing); caprine (goat drawing).

1.5 Mechanism of invasion

To generate disease, apicomplexan parasites first need to invade susceptible host cells. To achieve this, the process of recognition and initiation of the infection are key points that might be used as targeting factors for potential treatment. Currently, there are abundant studies on this process for parasites such as *Toxoplasma gondii* and *Plasmodium* spp. (Augustin, 2001; Kim and Weiss, 2004; Friedrich, Matthews and Soldati-Favre, 2010; Kemp, Yamamoto and Soldati-Favre, 2013; Foquet *et al.*, 2014). However, information on *Eimeria* spp. invasion is poor, and there are still gaps in the understanding of how infection occurs (Augustine, 2001).

Sequentially, we can divide the process of invasion of target cell into 5 essential steps (Fig. 8), which allows the better understanding of this event.

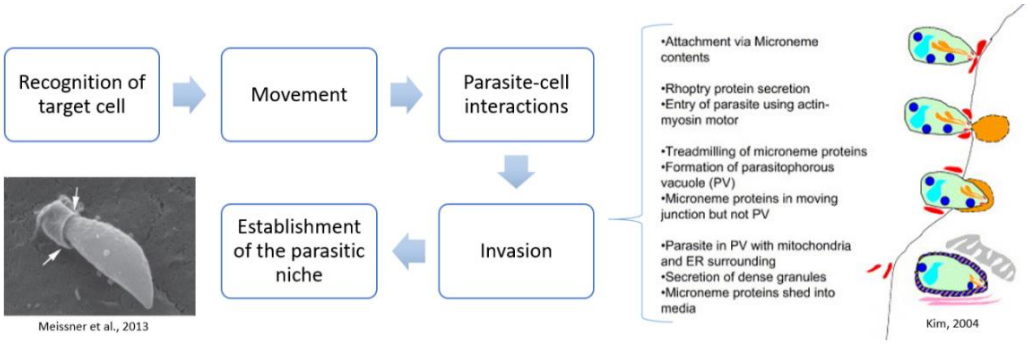


Figure 8 Sequential process of host-cell *Eimeria* invasion. In detail, the steps of sporozoite invasion described by Kim 2004. Sequential secretion of micronemes (red), rhoptries (orange) and dense granules (blue). The parasite enters the host cell using its actin–myosin motor. Microneme proteins are found at the moving junction, a constriction in the parasite that is seen as it enters host cells. After successful entry the parasite lives within a vacuole surrounded by mitochondria and ER from the host. Dense granule secretion occurs after entry is complete (Kim 2004). Picture from (Meissner et al 2013). Formation of a collar-like structure during host cell invasion. Scanning electron micrographs of tachyzoites of *Toxoplasma gondii* invading cultured human umbilical vein endothelial cells (HUVEC).

Recognition of the target cell

The invasion requires recognition and interaction of the sporozoite with the host cell. It is considered that only in specific host, *Eimeria* spp. is able to complete its life cycle and to produce oocysts shed with the faeces; however, for this specific process the parasite requires a series of stimuli (Andrews, 1930; Hammond, Clark and Miner, 1961; Marquerdt, 1966). Between 1929 and 1954 a large number of studies were carried out trying to infect different host species with several types of *Eimeria*, however, most of the experiments were unsuccessful, and only experimental infections of chickens with the turkey species (*E. gallopavonis* and *E. meleagridis*) were successfully achieved (Hammond and Long, 1973). These observations suggested that some recognition molecules were probably necessary for sporozoites in order to enter specific host cells.

Although host cells do not have an active role in the physical process promoting entry of the parasite inside the cell, they provide appropriate surface molecules and receptors, or secret metabolites which are believed to initiate attraction or activation of apicomplexan parasites and thereafter to initiate their recognition. *In vivo*, *Eimeria* spp. show a high degree of specificity on host cells for their development. *Eimeria* spp. usually infect a limited number of host cells, and

specifically a portion of an organ or system (Joyner, 1982; Augustine, 1986). This specificity may be associated with unique conditions of the intestinal lumen, such as pH, enzymes, mucous, metabolites, concentration of nutrients, microbes etc. (Bumstead and Tomley, 1997).

The motility, structure and secretions of sporozoites allow them to penetrate the cell, however, there is evidence that the host cell also provides characteristics which are key to host cell infection (Nichols, Chiappino and O'Conner, 1983; Dolbrowolski and Sibley, 1996). Among these, there are some molecules of the surface of cells of the intestinal epithelium that act as receptors or recognition sites for the sporozoites. The last was demonstrated by *in vitro* studies in which the invasion of the parasite was inhibited with a variety of compounds that altered the host cell membrane. Some examples of these compounds are cationic molecules, enzymes and protease inhibitors (Fayer, 1971; Augustine, 1985b, 1985a, 1986; Augustine and Danforth, 1986, 1987; Fuller and McDougald, 1990; Crane and McGaley, 1991).

More specific evidence has shown the presence of parasite antigens that bind to molecules present on host cell surface. Antigens of 22, 31 and 37 kDa, membrane glycoconjugates, epitopes of host cell and sporozoites, which could act as receptors and ligands have been identified. However, their inhibition does not completely affect the invasion process of the parasites, so there must be more mechanisms to be involved (Augustine, 2001). Some studies showed that *E. adenoeides* sporozoite antigens bind to specific components of host cell. Augustine (1989) developed a monoclonal antibody directed to a 40 kDa antigen of the sporozoite, which markedly decreased cell invasion, thus testing the hypothesis of at least one specific receptor for invasion (Augustine, 1989).

To date, it has been considered that the mechanisms of invasion are similar for all apicomplexan parasites, however ligands/receptors may change between different species. The recognition of glycosylated groups, such as heparan sulfate and chondroitin sulfate on host cells, seem to be the rule, and may be

responsible for differences in target cell specificity (Carruthers *et al.*, 2000; Naguleswaran, Muller and Hemphill, 2003; Vonlaufen *et al.*, 2004). Apicomplexan parasites can invade several host cells *in vitro* (Hermosilla et al., 2002), suggesting that receptors used are widely distributed in host cells. However, these can vary according to the stage of the cell cycle (Grimwood et al., 1996), thus limiting the entry of the parasite. For *T. gondii*, adhesion to different host cell types increases if the cell goes from G1, to half of the S phase, decreasing its entry into cells in G2 phase. Other characteristics, such as membrane and cytoskeletal fluidity might also play a fundamental role in host cell invasion, so not only the appropriate receptor is needed, but a set of characteristics of the cell membrane for successful apicomplexan invasion process (Grimwood et al., 1996).

Consistently, some membrane glycoproteins have also been identified as potential cellular receptors for invasion. For several protozoa it has been proposed that adhesion is mediated by binding to lectin receptors, since it is observed that the distribution of carbohydrate residues on the luminal surface of the intestine is different according to the region (Suprasert and Fujioka, 1988; Alroy *et al.*, 1989). For *E. bovis, E. zuernii* and *E. arloingi*, the specific receptors involved in recognition and invasion of the host endothelial cells have not been yet described. Concerning the selection of host cells and the invasion of cells *in vitro* however, there is neither host- nor cell type-specificity, since many cell types can be infected by sporozoites of *E. bovis* and *E. arloingi*. This behaviour in principle had been reported earlier by Fayer and Hammond (1967) and Hammond and Fayer (1968). More recently, it has been demostrated that *E. arloingi* sporozoites were able to infect primary BUVEC (bovine), permanent MDBK (bovine) and permantent MARC (non-bovine) cell lines and developing first macromeronts but with clear different efficiency rates (Silva *et al.*, 2015). Nevertheless, BUVEC were the most suitable cells tested by showing higher infection rates and allowing further development of *E. arloingi* macromeronts. Additionally, Hermosilla et al., 2002 demostrated that *E. bovis* was able to infect several cell lines (BFGC, BUVEC, BSLEC, MDBK, VERO, HUVEC and PUVEC),

which suggest that the receptors for this species are expressed in more than one cell type. The speed of sporozoites' invasion did not depend on cell type source, as sporozoites invaded HUVEC and PUVEC faster than cattle-derived MDBK cells. Possibly, such differences are related to the abundance of target molecules on the host cell surface, which are recognized by the parasite for adherence or penetration (Augustine, 2001). These molecules are not yet known in the case of *E. bovis, E. zuernii* and *E arloingi* (Hermosilla *et al.*, 2002b)

Movement

The invasive stages of apicomplexan parasites are characterized by having a complex of specialized structures (e. g. conoid, polar ring, apicoplast) and organelles attached to their membranes. This complex is located at the anterior end of the parasite and the excreted substances are essential for the recognition, adhesion and invasion of the host cell. Previously it was believed that the internalization of parasites occurred by passive phagocytosis, however, an active participation of the parasite in the process has been demonstrated (Russell and Sinden, 1981; Nichols, Chiappino and O'Conner, 1983; Dolbrowolski and Sibley, 1996).

Although the sporozoites can move by gliding, flexing and rotating, they do not have visible organs of locomotion, such as cilia, flagella or pseudopods. The function of the rhoptries, micropores, micronemes and conoid, are associated with penetration into the host cell and the creation of an intracellular environment suitable for the growth of the parasite (Nichols, Chiappino and O'Conner, 1983).

The apical complex is composed of unique secretory elements (micronemes and rhoptries) and structural elements (polar rings and conoid). During the active process of cell invasion, the content of the secretory organelles is released forming a mobile union that allows the formation of the parasitophorous vacuole. The conoid is surrounded by polar rings composed of microtubules and is believed to be the mechanical support of host cell invasion (Nichols and O'Connor, 1981; Nichols and Chiappino, 1987; Carruthers and Sibley, 1997;

Mordue *et al.*, 1999; Morrissette and Sibley, 2002). In addition, the content of the rhoptries together with the dense granules reprogram cellular functions, such as cellular immune response (Melo, Jensen and Saeij, 2011).

The functionality of the parasite is dependent on the cytoskeletal network that supports the membranous trilaminar film, which consists of a plasma membrane and several elongated vesicles (alveolar membranes). The latter, together with cytoskeleton, constitutes the internal membrane complex (IMC) (Heintzelman, 2015). The structure of the IMC is intimately supported by filaments (subpelicular network), which has a family of proteins known as alveolins (Mann and Beckers, 2001; Gould *et al.*, 2008; Kudryashev *et al.*, 2010). This complex is essential for the gliding motility process (Preston and King, 1992).

The sporozoites recognize, contact and enter the cell through a circular sliding movement (gliding). This movement is essential for invasion both *in vivo* and *in vitro*. In the *in vivo* situation, the sporozoites excyst from the oocyst in the intestine of the host, and subsequently migrate to the intestinal lumen where they make contact to host epithelial cells where the sporozoite invasion occurs, or travel through the mucosa to the lymph vessels, where endothelial host cells will be infected (e. g. *E. bovis*). Once this first contact is made the sporozoite penetrates the cell thanks to its apical complex machinery. In the *in vitro* model gravity helps sporozoites to achieve contact to host cells, since they usually grow as monolayers, nevertheless it is known that also gliding motility is essential for the invasion (Augustin, 2001). Parasites can adjust their gliding motility motor to activate migration through different tissues, to force the invasion of cells, and under certain circumstances, to activelyegress of infected host cell. This movement is regulated by internal and external factors, with the cascade of calcium signaling playing a central role in the process (Lavine and Arrizabalaga, 2008; Tardieux and Menard, 2008; Sibley, 2010; Blackman and Carruthers, 2013; Harker, Ueno and Lodoen, 2015; Lourido and Moreno, 2015). Detailed studies of the gliding motility show that both actin and myosin are involved in this process (the entire complex of proteins is known as glideosome) (Schwartzman and Pfefferkorn, 1983; Pinder *et al.*, 1998; Opitz and Soldati, 2002; Foth, Goedecke

and Soldati, 2006). The primary components of this apparatus have been characterized using biochemical and molecular methods, together with immunohistochemistry and ultrastructural tests (Boucher and Bosch, 2015).

Parasite-host cell binding

The ultimate objective of gliding motility of the parasite is to establish temporary adhesion to the cells in order to generate enough traction to propel itself inside the host cell. This initial contact is mediated by adhesion molecules that are released from the micronemes towards the membrane (pellicula) of the parasite. Of these proteins, the most characterized is AMA1 and members of the anonymous proteins related to thrombospondin, which bind directly to the motor complex of the adhesion site (Carruthers and Tomley, 2008; Morahan, Wang and Coppel, 2009; Tyler, Treeck and Boothroyd, 2011).

Invasion of the host cell

Once the sporozoite is adhered to the cell, an invagination of the cell membrane occurs in front of the advancing parasite, which produces changes in the cell membrane. There is evidence that *Eimeria* spp. secrete materials that favor the invagination of the membrane (Sheetz, Painter and Singer, 1976). Studies in *T. gondii* reveal that the invasion is an orchestrated process accompanied by a sequential release of micronemas, roptrias and dense granules (Opitz and Soldati, 2002; Sibley, 2003).

Microneme proteins are rich in adhesive domains, similar to those found in mammals, although there is little homology between the proteins. Secreted microneme adhesins, such as TgMIC2, are translocated from the surface of the parasite by an actin-myosin motor during their entry into the cell (Opitz and Soldati, 2002; Sibley, 2003). The content of the roptrias is secreted during the invasion and promotes the formation of the parasitophorous vacuole. For *T. gondii*, it is suggested that the content of the roptrias is responsible for prevent that PV fuses with the lysosomes. Also this proteins recruit the mitochondria and

endoplasmic reticulum from the host cell (Sinai, Webster and Joiner, 1997; Hakansson, Charron and Sibley, 2001; Sinai and Joiner, 2001).

Establishment of an intracellular niche

It was demonstrated that apicomplexans such as *T. gondii* have an active role in the construction of this intracellular niche that depends to a large extent on the cytoskeleton.Its closure is similar to that performed when receptor-mediated phagocytosis occurs (Boyle and Radke, 2009). *In vitro* studies showed that the PV membrane functions as a molecular sieve, being permeable to molecules between 1300 and 1900 Da. It also has transmembrane proteins which are derived from infected host cells. Farther, PV does not bind to lysosomes and is rapidly associated with organelles and cellular components. It has been shown that microtubules and intermediate filaments of vimetin surround the PV within few minutes after invasion, and that some organelles are attached to it. The latter is essential to prevent PV-lysosome union (Jones and Hirsch, 1972; Sinai, Webster and Joiner, 1997; Melo, Carvalho and De Souza, 2001). For *T. gondii,* it has been described 2 ways to access content of cellular organelles: firstly, intimate association with organelles maintained by parasite-derived proteins of the PV and secondly, manipulation of the cytoskeleton to recruit vesicles to the PV. At 4 h p. i. from 20 to 50% of the PV is covered by host cell mitochondria and host cell ER (de Melo, de Carvalho and de Souza, 1992; Coppens *et al.*, 2006).

In addition, it has been proposed that mitochondria are bound to the PV due to rhoptry-derived proteins such as ROP2. ROP2 is anchored to the PV membrane by hydrophobic interactions and ionic interactions with the N-terminus of the protein (Labesse *et al.*, 2009). ROP2 contains two domains that target the mitochondrial matrix and ER domain exposed in the cytosol. The intimate contact between the organelles and the PV facilitates the transfer of some products to the parasites through channels located in the membrane. Nevertheless, this channels are not yet characterized (Coppens, 2014).

After the previously described interactions, the parasite begins massive modulation of the host transcriptome. The genes modulated in response to *T. gondii* for example, can be divided into 3 classes: genes involved in the defense of the host, genes beneficial to the parasite replication, and genes that are affected as a result of the last two functional pathways (Blader and Koshy, 2014).

The transcription of genes that have impact on host defense and apicomplexan parasites' development are regulated by the activation of transcription factors. Within these factors is the NF-ƙB family, which comprises p50 (NF-ƙB1), p52 (NF-ƙB2), and subunits RelA, RelB and c-Rel. This factor is usually associated with its inhibitor (IκBs) molecule and thereby being efficiently inactived. Due to external stimuli, the phosphorylation of serine residues of IƙBs is generated leading to the degradation by proteasome and generating the activation of this transcription factor. The active heterodimer is translocated to the nucleus, where transcription of genes involved in cell growth, apoptosis and immune response begins (Caamano and Hunter, 2002). NF-ƙB is activated during host cell infection by various pathogens, and its activation benefits obligate intracellular apicomplexans. For *T. gondii* the activation of this factor in fibroblasts increased its survival strategy. The anti-apoptotic machinery of NF-ƙB has also been reported in *C. parvum*. However, the parasite can also block the translocation of the factor to the nucleus, thus diminishing the inflammatory response (Caamano, 2000; Butcher *et al.*, 2001; Chen, 2001; Molestina *et al.*, 2003). On the other hand, for *P. falciparum* the factor is also activated in the endothelial cells, generating an increase of ICAM-I expression, which is associated with the sequestration of red blood cells on the endothelium to escape phagocytosis of the spleen (Tripathi, Sullivan and Stins, 2006). Additionally to NF-ƙB, proteins of the STAT family (STAT1-4, STAT5a, STAT5b, and STAT6) are activated in response to apicomplexans and leading to cytokine production. This family regulates the transcription of genes related to cell differentiation, growth and survival, together with immune response. The phosphorylation of STAT proteins is mediated by cytosine-activated Janus kinase, which produces nuclear dimerization and translocation. The activity of STAT1 is important for cellular

defense mechanisms while STAT3/6 promotes the intracellular development of *T.gondii* (Fig. 9.) (Ihle, 2001; Gavrilescu *et al.*, 2004; Lieberman *et al.*, 2004; Phelps, Sweeney and Blader, 2008; Ong, Reese and Boothroyd, 2010).

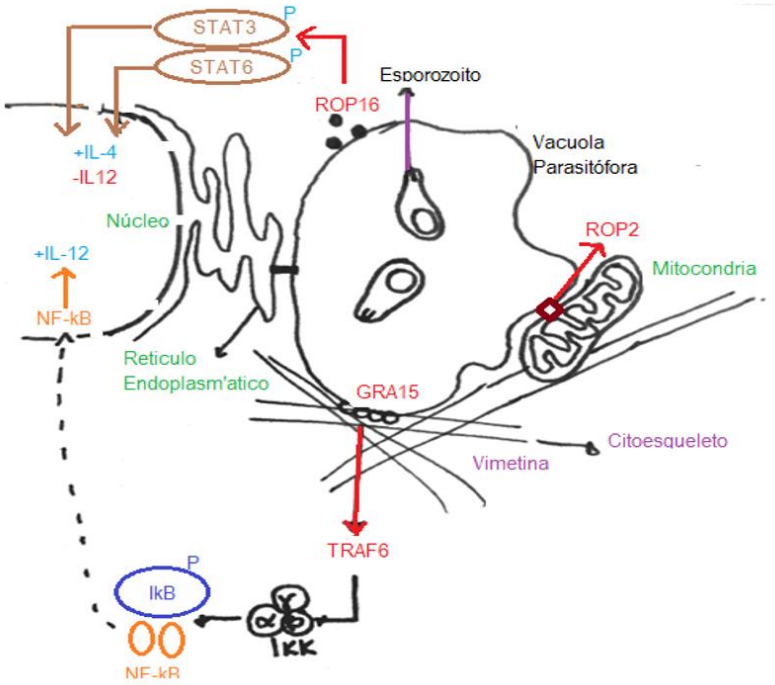


Figure 9 Interaction of *T. gondii* parasitophore membrane with host cell organelles. The triggering of the phosphorylation of STAT 3/6 by the ROP16 protein of *T. gondii* and the NF-kB factor by the GRA15 protein is illustrated. After parasite activation of these transcription factors translocation into the host cell nucleus occurs and transcription of genes related to the immune response is initiated.

1.6 Pathogenesis

E. bovis

E.bovis, described by Fiebiger in 1912, is one of the most pathogenic species in cattle coccidiosis. The prepatent period has been reported between 18 to 21 days p. i. and the patency period with oocyst shedding is 5-7 days long. The asexual merogony consists of two generations of meronts which are responsible for deforming and destroying the villi when the merozoites are released (Behrendt *et al.*, 2004). The first generation of macromeronts take place in host endothelial cells of the central lymphatic vessels within ileum villi. *E. bovis* macromeronts can measure up to 400 µm, containing more than 120,000 merozoites I, which are

released at 14-18 days p. i. (Sheffield and Hammond, 1967). The second generation of meronts occurs in host epithelial cells of caecum and colon; the meront II stages measure 10 µm and contain 30 to 36 merozoites II, which mature within 2 days after the 1st generation. The gamogony is located in the ileum (in severe infections), caecum and colon generating the greatest intestinal damage when the oocysts are released (Arguello and Campillo, 1999). The gametocytes mature in epithelial cells of intestinal glands after 3 days. Depending on the intensity of *E. bovis* infection either focal or vast extended ephitelial cell rupture areas with necrosis are seen. Dilation of blood and lymph vessels, cellular infiltration and oedema can develop in affected tissues. Due to these mucosal lesions, fluids, minerals, protein and blood are lost into the intestinal lumen. As such, severe *E. bovis* infections generate hypoalbuminaemia, hypoproteinaemia and hypo-γ-globulinaemia, while the α and β globulins might be increased (D Norman Levine, 1973). The uptake of 125,000 oocysts can generate marked clinical signs, which include diarrhoea, anorexia, weight loss and fever. Mucosa of the caecum and colon becomes congested, presents oedema, petechiae and haemorrhages. The contents of the lumen liquefy, and the watery faeces contain blood, fibrin and mucosal shreds. If the calf survives, the mucosa of the intestine can regenerate completely (Hermosilla et al., 2012).

E. zuernii

E. zuernii, described by Martin, 1909, is the most pathogenic *Eimeria* of cattle. The prepatent period has been reported between 15 to 17 days and elimination of oocysts can be up to 11 days. Bangoura et al. (2007) showed the presence of oocysts in faeces from 16 d.p.i. in calves experimentally infected with *E. zuernii* with a maximum of 101,550 OPG at day 23, which slowly decreased until day 28 p. i. As seen for *E. bovis*, *E. zuernii* has also two merogony stages, one in the ileum (endothelium) and another in the caecum and colon (in host cells of the lamina propria). First merogony I occurs until day 15 p.i, when the macromeront diameter is >200 µm in diameter, containing >100,000 merozoites I. This stage takes place in endothelial cells of the lymphatic vessels within ileum villi. In the second merogony, meronts II measure up to 13.2 µm in diameter and contain

between 24 and 36 merozoites II. Gamogony occurs in the ileum, caecum and colon (Lieberkühn crypts) from day 12 to 17 onwards. In contrast to *E. bovis*, it can cause chronic infections due to repeated reinfections, generating pasty bloody and mucous diarrhoea, with low oocyst shedding. The acute *E. zuernii* infection generates severe bloody diarrhoea, anaemia, weakness, dysentery and secondary infections. This clinical phase lasts 3 to 4 days. It can also generate catarrhal enteritis, ulcers in the mucosa and diffuse haemorrhages (D Norman Levine, 1973; Bangoura and Daugschies, 2007). Bangoura et al. (2007), found that *E. zuernii*-infected calves had diarrhoea (for one to 11 days), starting at 28 d.p.i. However, Mundt et al., 2005, found diarrhoea from day 16 p.i. onwards, with a maximum of 53,200 OPG counts. The faeces are watery and bloody, and contain fragments of intestinal mucosa and fibrin. Infection with *E. zuernii* also causes alterations in water balance, protein catabolism and lipolysis. Furthermore, it produces changes in bilirubin and cholesterol concentration in serum, in addition to an alteration of CK activity. At histopathological level, slight inflammatory changes are observed at 16 d.p.i. (late pre-patent period), with an increase of detritus (containing merozoites) in the crypts and glands of the jejunum and colon. Minor to moderate irregularities of infected epithelium of the ileum can also be observed (Mundt *et al.*, 2005b; Bangoura, Daugschies and Fuerll, 2007). After 21 days, acute typhlitis and partial necrosis can be observed together with granulocytic infiltration in the mucosa and abundant detritus. By transmission electron microscopy, focal damage of the villus structures and the presence of some bacteria in jejunum can be observed. Loss of epithelial layer occurs in the caecum and colon. At 26 d.p.i., the villi from jejunum diminish in size and reepithelialization and hyperplasia of crypt epithelium are observed with an increase of goblet cells within caecum and colon mucosa. At this stage, there is hardly any evidence of parasitic structures and only seldomly in deeper layer of the mucosa (Mundt *et al.*, 2005a; Bangoura and Daugschies, 2007).

E. arloingi

The endogenous development of *E. arloingi* involves the asexual replication, with two generations of meronts, and the sexual replication (gamogony) (Silva *et al.*,

2015). The first generation of macromeronts can grow up to 240 µm in host endothelial cells (ECs) of the lacteals of the villi of duodenum, jejunum and ileum and producing >120,000 merozoites I within 9–12 days p. i. (M. A. Taylor, Coop and Wall, 2007; Hashemnia *et al.*, 2012). The second generation of meronts is smaller and producing only 8 to 24 merozoites II (Sayin, Dincer and Milli, 1980; Hashemnia *et al.*, 2012) within 12 days p.i. in epithelial cells of the villi and the crypts of jejunum (M. A. Taylor, Coop and Wall, 2007).

The excretion of oocysts occurs 15–18 days after inoculation and can continue for 14–15 days. In goat kids 9 days p.i. marked inflammation and diffusely scattered pale-yellow plaques are evident in mucosa of the small intestine. Inflammatory reactions including oedematous swelling, epithelial necrosis, leucocyte infiltration and hyperplasia of varying severity are present in the small intestine.

1.7 Clinical signs

Bovine

In most cases, *Eimeria* infections in calves remain asymptomatic. Those affected by disease are usually animals at an age of 6 to 18 months, rarely younger or older. Changes in the housing conditions and rearrangement of young animals' groups are often followed by an increasing intensity and duration of oocyst excretion. When the infection dose is low, the animals excrete pasty or liquid faeces. These animals usually recover quickly. In contrast, severely diseased calves are febrile and suffer considerable pain (tenesmus, rectal prolapse), excrete liquid, bloody faeces with mucus and mucosal shreds. Sometimes, CNS symptoms may be present in *E. bovis* infections. In these cases, mortality can be high (Hermosilla et al., 2002). The surviving calves and heifers recover slowly (after weeks) but they never catch up with the status of the non-infected animals (Mundt *et al.*, 2005b; Deplazes *et al.*, 2016).

Goats

Clinical coccidiosis in goat kids is usually diagnosed at 2– 4 weeks after weaning. The main symptom is diarrhoea and weight loss. In contrast to bovine coccidiosis, caprine *Eimeria*-derived diarrhoea is less frequently haemorrhagic. The faeces are watery with clumps of mucus and colour changes from brown to yellow or dark tarry (B Koudela and Boková, 1998). In addition, there is decreased appetite and dehydration during severe caprine coccidiosis. In certain conditions, caprine coccidiosis can be characterised by sudden mortality without preceding digestive signs, in particular amongst young animals between 2 and 4 months of age (Chartier and Paraud, 2012).

1.8 Diagnosis

Diagnosis of coccidiosis is based on clinical signs and coprology (detection of large number of oocysts i. e. >10.000 OPG). The involved *Eimeria* spp. should be determined by oocyst morphology (Florião *et al.*, 2016) (Table 1, Fig. 5) or molecular tools (PCR) (Kawahara *et al.*, 2010; Kokusawa, Ichikagua-Seki and Itagaki, 2013). Nevertheless, oocyst shedding may be very low at the late phase of patency. In severe infections, gamonts can be demonstrated in rectaly taken mucosal samples. Animals kept on pasture frequently show mixed infections with gastrointestinal nematodes. In cases of bloody diarrhoea, differential diagnosis should include *Salmonella* spp., *Escherichia* spp. and *Clostridium* spp. infections (M., Joe and M., 2002; Radostits *et al.*, 2008; Deplazes *et al.*, 2016).

1.9 Epidemiology

Bovine

This parasite has a worldwide distribution and affects mainly calves between 3 and 6 months, while in adults asymptomatic infection is the rule (Faber *et al.*, 2002b; Radostits *et al.*, 2008). Clinical disease depends on the *Eimeria* species, pressure of infection and immune status of the host (Faber *et al.*, 2002b). Other factors such as stress (temperature, transport, humidity, change of diet), nutritional deficiencies, coinfections with viruses, bacteria (*E. coli*) and parasites

(*Giardia duodenalis, Cryptosporidium parvum, Trichostrongylus columbiformis, Cooperia punctata*), can contribute to trigger the clinical disease (Arguello and Campillo, 1999). In places highly contaminated with oocysts and with a high density of animals, the disease is common with prevalences of 46% in calves, 43% in animals of one year and up to 16% in adult cows (Radostits *et al.*, 2008).

Animals develop the infection after ingesting sporulated oocysts in food or water, which depending on the dose may cause disease, even in adults. If the dose is low or of a low pathogenic species, no clinical signs will be generated and may result in induced protective immunity against homologous *Eimeria* species. Although the cycle of *Eimeria* is self-limiting (Measures, 1956), reinfections and incomplete immunity can give rise to asymptomatic carriers. Those animals are responsible for a distribution of the infection throughout the herd (D Norman Levine, 1973).

As mentioned above, infections by single *Eimeria* species are rare under natural conditions, so the mixed infections are the ones that predominate worldwide (D Norman Levine, 1973). Exogenous oocysts are quite resistant to cold temperatures (it can resist -19 to -35 ºC for months) but sporulated oocysts are more sensitive to heat (they are inactivated at 40 ºC within 4 days, or faster at higher temperatures). In addition, disinfectants such as sodium hypochlorite (1.25%), phenol (5%) and formaldehyde can partially destroy oocysts or inhibit sporulation (Davies, Joyner and Kendall, 1972; Arguello and Campillo, 1999).

Since the disease has a cosmopolitan distribution, in all types of cattle production (Daugschies and Najdrowski, 2005), abundant studies on the prevalence of fecal oocysts in calves and cattle around the world are available. Table 2 shows consolidated result of some of them, with their prevalences to single *Eimeria* species. These studies were mainly performed on coprological analyses (McMaster and flotation) and further identification of species by morphology of the sporulated oocyst. Some of them presented the dynamics of excretion by ages (Sanchez, Romero and Founroge, 2008; Lucas *et al.*, 2014), concluding

that the highest prevalence and number of oocysts were registered in the rainy season.

In the case of Colombia, the current status of *Eimeria* spp. infections in calves and young animals (<1-year-old) was determined as part of this work (see chapter 3: Epidemiological survey and risk factor analysis on bovine *Eimeria* infections in Colombia). Some other small local studies have been also published (Chaparro *et al.*, 2016).

Goats

More than 15 *Eimeria* species have been identified in goats but four of them including *E. ninakohlyakimovae, E. arloingi, E. christenseni* and *E. caprina* are considered to be the most pathogenic species (Hashemnia *et al.*, 2012). The species *E. ninakohlyakimovae* and *E. arloingi* are the predominant species in The Netherlands (Borgsteede and Dercksen, 1996), Czech Republic (B Koudela and Boková, 1998), Malaysia (Jalila *et al.*, 1998), Poland (Balicka-Ramisz *et al.*, 2012), South Africa (Harper and Penzhorn, 1999), Sri Lanka (Faizal and Rajapakse, 2001), Iraq (Al-Amery and Hasso, 2002), Jordan (Abo-Shehada and Abo-Farieha, 2003), Turkey (Gul, 2007), Iran (Razavi and Hassanvand, 2007), Kenya (Githigia, Munyua and Kanyari, 1992), Zimbabwe (Chhabra and Pandey, 1991) and Tanzania (Kimbita *et al.*, 2009). In temperate areas like western Europe, the most prevalent *Eimeria* species are also *Eimeria ninakohlyakimovae* and *Eimeria arloingi* (Yvoré, Esnault and Guillimin, 1981). In semi-arid zones of Gran Canaria (Spain), the most frequent species are *E. ninakohlyakimovae, E. arloingi* and *Eimeria alijevi* (Ruiz *et al.*, 2006). In mid-western states of USA, the most frequent species are *E. arloingi* (98.8%), *E. christenseni* (58.2%), *E. ninakohlyakimovae* and *E. parva* (33.3%) (Lima, 1980).

Heavy excretion of oocysts by goat kids aged between 2 and 4 months (>10,000 OPG), and a rapid decrease of the excretion of oocysts with age have been reported (Chartier and Paraud, 2012). In older goats (over 7 years), a slight increase in the excretion of oocysts can be noticed. Ruiz et al. (2006) recorded

higher oocyst counts in adult goats during the hot season on the island of Gran Canaria.

Table 2 Summary of bovine *Eimeria* spp. prevalence in different countries.

(Das et al. 2015; Tomczuk et al. 2015; Lucas et al. 2014; Eidari et al. 2014; Dong et al. 2012; Koutny et al. 2012; Almeida et al. 2011; Bruhn et al. 2012; Rehman et al. 2012; Bruhn et al. 2011; Berit Bangoura et al. 2012; Sanchez, Romero, and Founroge 2008; YOMAR and ESPINOSA 2007; von Samson-Himmelstjerna et al. 2006; Oluwadare 2004; Kasim and Al-Shawa 1985)

Country	Year	Autor	Number of samples	Age	Prevalence	# especies	*E. bovis*	*E. zuernii*	*E. alabamensis*	*E. ellipsoidalis*	*E.cylindrica*	*E. auburnensis*	*E. canadensis*	*E. subspherica*	*E. bukidnonensi*	*E. wyomingensis*	*E. pellita*	*E. brasiliensis*	*E. illinoisensis*
India	2015	*M.Das et al.*	535	< 1 year	33.2	7	7%	2.35	0.21	0.13	0	0.86	0	0.68	0.94	0	0	0	0
Poland	2015	*Tomczuk et al.,*	356	various	52.8		37%	19.9	<1	<1		3.9	12.1	0	5.9	<1	0	0	0
USA	2014	*Aaro S. Lucas et al.,*	414	< 1 year		12	100%	91	90	91	91	89	64	36	44	29	73	27	0
Iran	2014	*Heidari et al.,*	470	< 1 year	9.36	9	23%	18.2	11.3	11.4	4.6	6.8	13.6	0	2.3	0	9.1	0	0
Dinamarc	2013	*Enemark HL et al.,*	100	3- 4 weeks	96,2%	12	28%	32%	7%	37%	23%	23%	10%	8%	3%	1%	0,2%	0,2%	
China*	2012	*Dong H et al.*	324	< 1 year	53.30%	14	23%	54.9	7.1	14.1	16.8	4.4	33.6	17%	2.70%	8	35.4	13.3	5.30%
Austria	2012	*Koutny H. et al.,*	868	<1 year	83.67	11	28%	13.86	11.56	14.38	7	13.41	5.74	5.05	0.59	0.16	0.5	0	0
Brazil	2012	*Pascoti et al.,*	356	<1 year		10	38%	17.9	4.5	17.3	2.3	9.5	4.9	3.1	1.5	1.9	0	0	0
Pakistan	2011	*Rehman et al.,*	234	<1 year	60.68	6	52%	48.27	24.14	29.31	8.62	0	34.83	0	0	0	0	0	0
Holand	2011	*Pascoti et al.,*	37	3-7 months		11	23%	22.6	2.4	20.3	14.1	2.4	3.5	5.4	1.9	0	2.1	2	0
Brazil	2011	*das Anjos Almeida et al.,*	117	various	33.33	10	25%	6.83	0.85	5.99	3.42	3.42	8.55	0.85	1.71	0	0	2.56	0
Germany	2011	*Bangoura et al.*	633	various	59.4	2	29%	29.1	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Argentina	2008	*Sanchez et al.,*	862	<50 days	48	13	24%	11	<1	38	<1	19	4	3	<1	<1	NE	<1	<1
Colombia	2007	*Yomar et al.*	64	<1 year	67	2	58%	11	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Germany	2006	*G. von Samson et al.,*	164	5 a 15 months	100%	3	58.90 %	3.10%	83.30%	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Nigeria	2004	*TITILAYO et al.,*	2500	various	62.68	12	37%	41.72	8.8	28.8	22.24	32.24	9.44	11.8	20.2	17.8	11.6		
Venezuela	1998	*Tamasaukas R*	636	various	54%	6	x	x	x	0	x	x	0	0	0	x	0	0	0
Kenya	1990		620	various	67.40%	8	79%	60.20 %	10.30%	26.10 %	13.40 %	28.40 %	0	5%	0	6.10%	0	0	0
Arabia Saudi	1985	*AA. Kasim et al.*	205	various	34.10%	7	x	x	0	x	x	x	0	x	0	x	0	0	0

*CHINA: Yacks. *USA: high average in calves 0: Not found NR: No report

1.10 Transmission and predisposing factors of clinical coccidiosis

Clinical coccidiosis is a self-limiting disease. Nevertheless, two conditions can lead to clinical coccidiosis: firstly, massive ingestion of sporulated oocysts due to a highly contaminated environment; secondly, a significant asexual multiplication in the host, in relation to a lowered resistance of the animal. These conditions are normally found under natural conditions. For example, housing or pasturing of animals in modern industry (overcrowding, muddy zones) predispose to massive contamination of the environment and high infection pressure (Cai and Bai, 2009; Chartier and Paraud, 2012). In addition, physiological stress (cold/heat stress or feeding: weaning and early weaning, under-feeding), management practices and transportation are likely to perturb the immune system. In Northern Europe, cold and wet weather in spring occurring in heavily stocked lowland farms predispose to clinical coccidiosis (Taylor, 2009). For more details of predisposing factors please refer to Chapter 3.

1.11 *Immune response against* Eimeria *spp.*

Adaptive protective immunity to coccidia is fairly acquired but differs for each *Eimeria* species, even within the same host species, and depends on the way of immunization (e. g. age, size, and intervals between the immunizing inocula). Partial protective immunity results from a single immunizing *Eimeria* spp. infection, but complete immunity (no oocyst production) may require more than two immunizing infections, depending on *Eimeria* spp. Protective immunity is generally regarded as being species-specific as no cross immunity between *Eimeria* species develops. Nevertheless, there have been some reports of cross-immunity between closely related species, but there is conflicting evidence and mainly restricted to murine *Eimeria* species (Rose, 1972). In the case of chicken immunity against *Eimeria*, there are many studies on innate and acquired immune response (Yun, Lillehoj and Lillehoj, 2000; Chapman, 2014), however, in the case of bovine and caprine species there are only few studies so far (Hermosilla, Zahner and Taubert, 2006; Behrendt *et al.*, 2008, 2010; L. M. R. Silva *et al.*, 2014; Muñoz-Caro *et al.*, 2015)

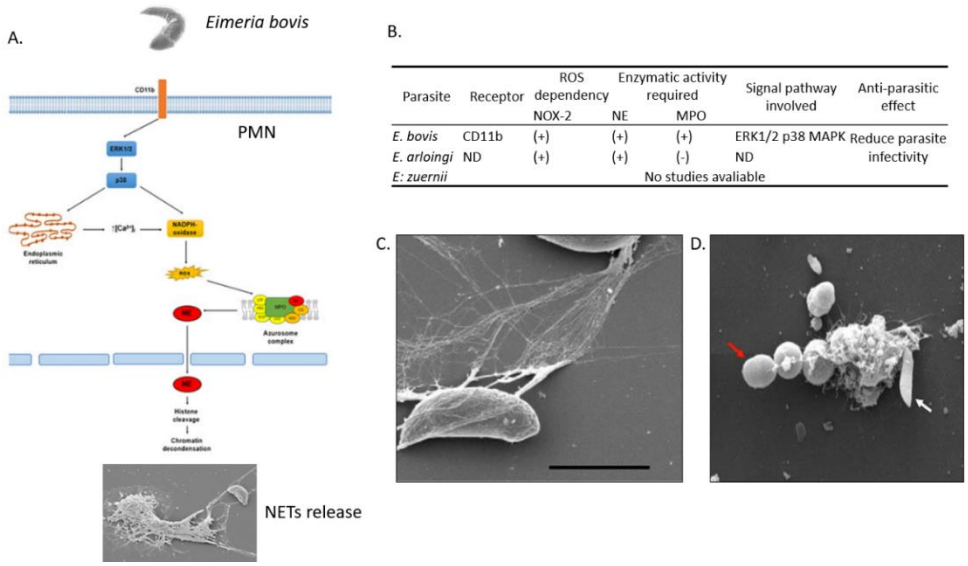
In general, the first line of defense against invading pathogens is represented by professional phagocytes, such as macrophages, dendritic cells and polymorphonuclear neutrophils (PMN). PMN respond to pathogens with diverse mechanism, which include phagocytosis of foreign material, the release of oxidative radicals as a result of oxidative burst activities and production of immunomodulatory molecules, such as cytokines and/or chemokines, contributing to initiate acquired immune responses. As additional effector mechanism, PMN-mediated killing of pathogens by forming neutrophil extracellular traps has recently been described (Behrendt *et al.*, 2010). PMN-mediated reactions play an important role in early host innate immune effector mechanisms against *E. bovis* infections in calves (Behrendt *et al.*, 2004, 2010). After exposure to *E. bovis* sporozoites, PMN-mediated reactions include enhanced transcription of IL-6, MCP-1, GROa, TNF-a, and iNOS genes. Stimulation with merozoite I-antigen, in addition, upregulated IL-8, IL-10 and IL-12 gene transcription. Furthermore, enhanced *in vitro* oxidative burst and phagocytic activities were observed after contact of PMN with viable sporozoites of *E. bovis* (Behrendt *et al.*, 2010).

E. bovis sporozoites have to cross the mucosal layer of the ileum to reach the lymphatic capillary for infection of the adequate host cells (lymphatic endothelial cells). Furthermore, these stages are able to egress from once-invaded cells (Hermosilla et al., 2002; Behrendt et al., 2004), a phenomenon that is frequently observed in cell culture systems. For that reason, sporozoites are exposed to the interstitial fluid and to the lymph *in vivo* becoming potential targets for circulating and recruited PMN to the site of infection (Muñoz-Caro et al., 2016).

In vitro and *ex vivo* assays demonstrated that PMN are involved in early innate immune reactions against *E. bovis* sporozoites and that they utilize some of their effector mechanisms, such as oxidative burst or phagocytosis to attack these parasitic stages (Behrendt *et al.*, 2008). Additionally, the sporozoite stage of *E. bovis* also induces NET formation as additional effector mechanism of PMN

(Behrendt *et al.*, 2008, 2010). PMN-derived NET structures firmly attached to *E. bovis* sporozoites, nevertheless, no lethal effects of NETs on sporozoites was detected (Behrendt et al., 2010; Muñoz-Caro et al., 2015). On the contrary, SEM analyses suggested immobilization of these parasite stages which may have a preventive effect on host cell invasion *in vivo* (Behrendt *et al.*, 2010 Muñoz-Caro et al., 2015, 2016).

The *Eimeria*-triggered NETosis is dependent on ROS generated by NADPH oxidase (classical NETosis pathway). Furthermore, sporozoites co-cultured with PMN which undergo NETosis, show reduced infectivity to BUVEC monolayers *in vitro*. Munoz-Caro et al. (2015) described that CD11b receptor of PMN (an integrin component of complement receptor 3) was implicated in this *Eimeria*-triggered NETosis process. In addition, NETosis is also dependent on calcium mobilization from store-operated calcium entry (SOCE), as well as on NE and MPO activities (Muñoz-Caro *et al.*, 2015). Merozoites I and oocyst stages of *E. bovis* also trigger NETosis, indicating that this process is not stage-specific. The NETosis process is also regulated by ERK1/2 and p38 MAPK pathways (Fig. 10) (Díaz-Godínez and Carrero, 2019).



Parasite	Receptor	ROS dependency	Enzymatic activity required		Signal pathway involved	Anti-parasitic effect
		NOX-2	NE	MPO		
E. bovis	CD11b	(+)	(+)	(+)	ERK1/2 p38 MAPK	Reduce parasite infectivity
E. arloingi	ND	(+)	(+)	(-)	ND	
E: zuernii	No studies avaliable					

Figure 10 NETosis mechanism in *Eimeria*. A. Schematic representation of mechanism described for NETosis triggered by *E. bovis*. Adapted from (Díaz-Godínez and Carrero 2019). B. Summary of mechanism of NETosis in *Eimeria* spp. SEM for *E. bovis* (C) and *E. arloingi* (D) triggered NETosis. (Behrendt et al. 2010; Silva et al. 2014). ND: No described.

For *E. arloingi* there is also evidence of *in vitro* NETosis induction in caprine exposed PMN (Silva *et al.*, 2014). *E. arloingi* sporozoites induce a rapid NETosis process which depends on ROS via NADPH oxidase activation; also, NETs entrap and reduce the infectivity of *E. arloingi* parasites *in vitro* (Silva *et al.*, 2014). Nevertheless, extruded DNA does not affect the viability of sporozoites. Similar to *E. bovis*, *E. arloingi*-triggered NETosis was not a stage-specific mechanism since oocysts were also able to induce NET release (Silva *et al.*, 2014).

Eimeria-induced NETosis *in vivo* has been also documented. In cross-sections from bovine and caprine intestinal samples infected with *E. bovis* and *E. arloingi*, the presence of leukocytic infiltration mainly composed of PMN. The immune cells were observed in close contact to oocysts, macrogamonts, and macromeronts. Presence of extracellular DNA co-localizing with histones and NE was also detected in this samples, indicating the occurrence of NETosis in infected tissues (Muñoz-Caro *et al.*, 2016).

In the case of acquired immune response, colostral antibodies, especially isotypes IgG1 but also IgG2 and IgM, are passively transferred to calves. IgG2 is the main fraction in the humoral response to infection and this has been attributed to a type 2 response to *E. bovis*. This response stimulates IgG2 synthesis via natural killer (NK) cells releasing interferon γ (IFN- γ). Although antibodies reflect exposure to coccidia they do not confer protection (Fiege *et al.*, 1992). Protective immunity is mainly of a cellular type, species-specific and can break under high infection pressure (Daugschies and Najdrowski, 2005). CD4+ T cells and other lymphoid cells are particularly important in this response and may be transferred via colostrum to the calf (Fiege *et al.*, 1992; Faber *et al.*, 2002a). Prolonged reactivity of the T-cell population to a specific antigen stimulus results after *E. bovis* infection. Activated T cells are not capable of abrogating the parasite life cycle in primary infections, nevertheless, T-cell response may interact with duration and intensity of oocyst excretion (Hermosilla et al., 1999) and may also be related to immunological control of further infections.

Hermosilla et al. (1999) also suggested that lymphoid CD4+ cells may reflect a TH1 response to infection that, by IFN-γ triggered NO release, could participate in the termination of a primary infection. In addition, CD8+ cells may play a role in the suppression of inflammation during bovine eimeriosis, nevertheless, these theories derived from other models (i. e. mouse and chicken), which should not be uncritically transferred to bovine or caprine coccidiosis.

1.12 Metabolic pathways and signaling during infection with Eimeria *spp.*

Since intracellular *Eimeria* species require a large amount of neoformation of membranes for the new progeny, it seems necessary to scavenge for essential nutrients from infected host cells (Taubert et al., 2010; Lutz et al., 2011; Hermosilla et al., 2012; Hamid *et al.*, 2015). This, together with the stress that the cell suffers due to the enlargement of the meronts, leads to the release of some inducers of cellular immunity and, of course, apoptosis (Taubert *et al.*, 2010). Endothelial cells are highly reactive, producing a series of adhesion molecules, cytokines and proinflammatory substances after their activation, generating a recruitment of leucocytes to the affected site (Locati *et al.*, 2002). In order to fulfill *E. bovis* macromeront development the parasite relies on some regulatory and evasion mechanisms of the cellular and host immune response, and additionally scavenge nutrients from infected host cells within cytoplasmic PV.

After successful intracellular development of *E. bovis* sporozoites, changes in the morphology of the endothelial cell are detected *in vivo* due to the growth of macromeronts. *In vitro*, Hermosilla et al. (2002) demonstrated that infected host cells grow up to 40 times their original sizes and produce accumulation and rearrangement of actin filaments, microtubules and spectrin around the parasitophorous vacuole; also, dense filaments cross the entire cytoplasm (Hermosilla et al., 2012). The distribution of the organelles in the cytosol depends on the distribution of the microtubule networks, and some authors have described that the mitochondria, ER and other organelles migrate near the PV (Hermosilla *et al.*, 2002b; Ramakrishnan, S. *et al.*, 2012; Jacot *et al.*, 2016). Currently there

is few information about the molecular mechanisms of modulation of the cytoskeleton by *E. bovis*, however several genes that code for binding proteins responsible for the assembly of microtubules and actin, are regulated positively in the *in vitro* system (Taubert *et al.*, 2010). Examples of these are the transcription of genes encoding tubulin (TUBB, TUBB4 and TUBB6), actin (Vinculin, azrin, CAPG, CNN2, TAGLN and PALLD) and others involved in the organization of microtubules (TPPP, DOCK7, CKAP4, DCT3) (Hermosilla *et al.*, 2008; Taubert *et al.*, 2010).

On the other hand, at the end of macromeron formation, proteins related to the elongation and depolymerization of actin are regulated negatively (α-actin-1, gelsolin, actin-like protein-2, gelsolin-like capping protein, tropomodulin-3, and transgelin). Also with myosin (myosin-10, β-tropomyosin, myosin regulatory light chain 2) and tubulin (β-tubulin 5 and 6) (Lutz *et al.*, 2011). It has been demonstrated that different mechanisms act during the formation of macromeront, initiating with a positive regulation, and at the end of the phase, a negative regulation when the merozoites I are released (Taubert *et al.,* 2010; Lutz *et al.,* 2011).

The formation of macromeront is also accompanied by a change in nuclear morphology in the *in vivo* and *in vitro* model (Fig. 11 and 12). When the sporozoite enters the host endothelial cell, it is located near the nucleus, thus implying a direct influence of this organelle during the early phase of development (4 to 7 d.p.i). The nucleus of an infected cell shows a stained content, which corresponds to a large amount of inactive heterochromatin (Taubert et al., 2010). On day 8 p. i., the proliferation of the parasite starts and the nucleus increases the amount of clear euchromatin, and large nucleoli is then observed, suggesting an enhancement of protein synthesis and gene transcription. The reorganization and increase in cell size during the late phase of macromeront formation, causes considerable cellular stress, reflected in the positive regulation of heat shock proteins (HSP90, HSP70, HSP27, HSPB6) and other stress-related molecules (SERP1 and STIP1). These molecules coordinate the functions of HSP90 and

70, which are involved in the internal pathway of apoptosis. Also, some studies suggest that *E. bovis* protects the cell from apoptosis by increasing the expression of c-AIP1 and c-FLIP (Lang *et al.*, 2009), which are involved in apoptosis through receptors (Lang *et al.*, 2009).

The positive regulation of NF-ƙB is also observed in cells infected with *E. bovis*. This type of regulation has been evidenced in infection by *Eimeria* in chickens, where antiapoptotic molecules NF-ƙB and Bcl-xL are overexpressed (Cacho *et al.*, 2004; Alcala-Canto and Ibarra-Velarde, 2008). Additionally, whole proteome analysis of *E. bovis*-infected host cells reveals a marked reduction of caspase 8, although it is not known if this is due to the action of the IAP / C-FLIP, or via another cellular mechanism (Lutz *et al.*, 2011). Recent data suggest that some candidate molecules may be involved in this regulation through activation of DDIT4 and BCL2A1 (Taubert *et al.*, 2010).

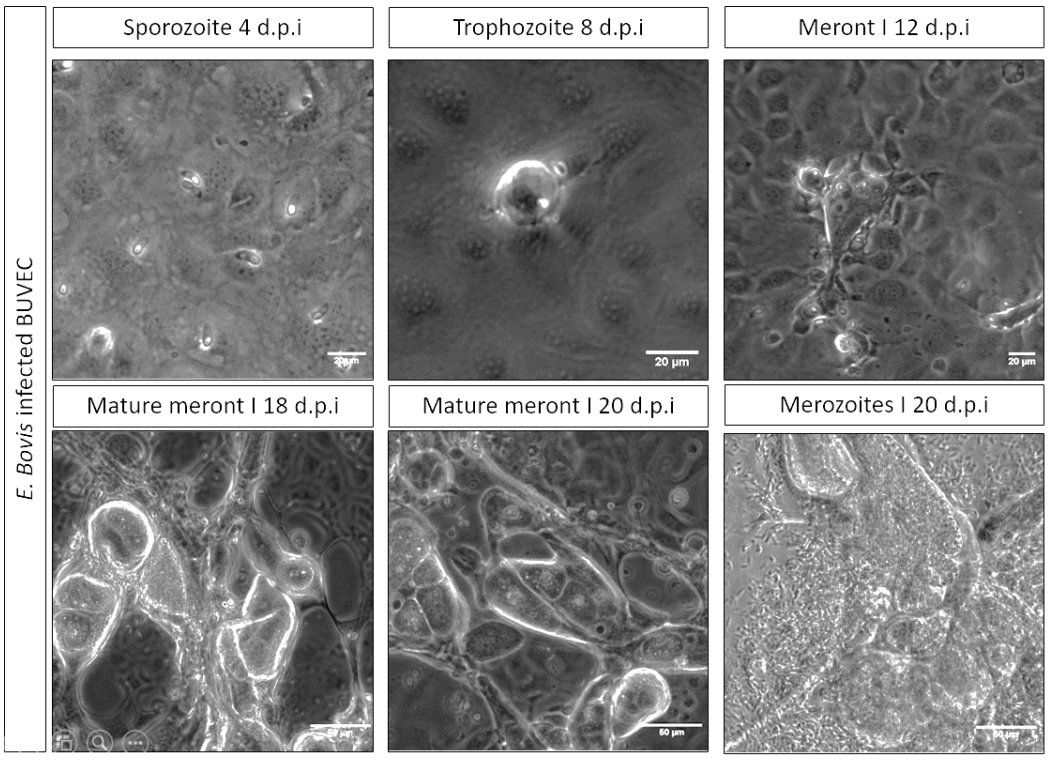


Figure 11 Eimeria bovis in vitro first merogony in BUVEC. Different stages of the first merogony are presented above. Trophozoites were seen at 4 dpi. and macromeronts at 12 dpi. Free merozoites I were visible after 18 dpi. Sporozoites 14x2 µm (*n* = 60), Trophozoites 13x12 µm (*n* = 20), Merozoites I 10.1x1.2 µm (*n* = 20).

E. bovis, like *T. gondii*, takes nutrients from its host cell, modulating molecules involved in glycolysis, citric acid cycle and degradation of lipids and alcohols (Lutz *et al.*, 2011). The upstream regulation of squalene epoxidase (SQLE) is one of the limiting factors for the mevalonate pathway route. However, while *T. gondii* can increase the expression of LDL receptors and therefore cholesterol processing, or induce de *novo* synthesis via the mevalonate route (Blader, Manger and Boothroyd, 2001; Okomo-Adhiambo, Beatti and Rink, 2006), for *E. bovis* the range of molecules associated with cholesterol suggests the use of both mechanisms (Taubert et al., 2010). In addition, the regulation of two other molecules, INSIG1 and SCAP, suggest that additional mechanisms are regulated in *E. bovis* that have not yet been described in another apicomplexan parasites (Taubert *et al.*, 2010)

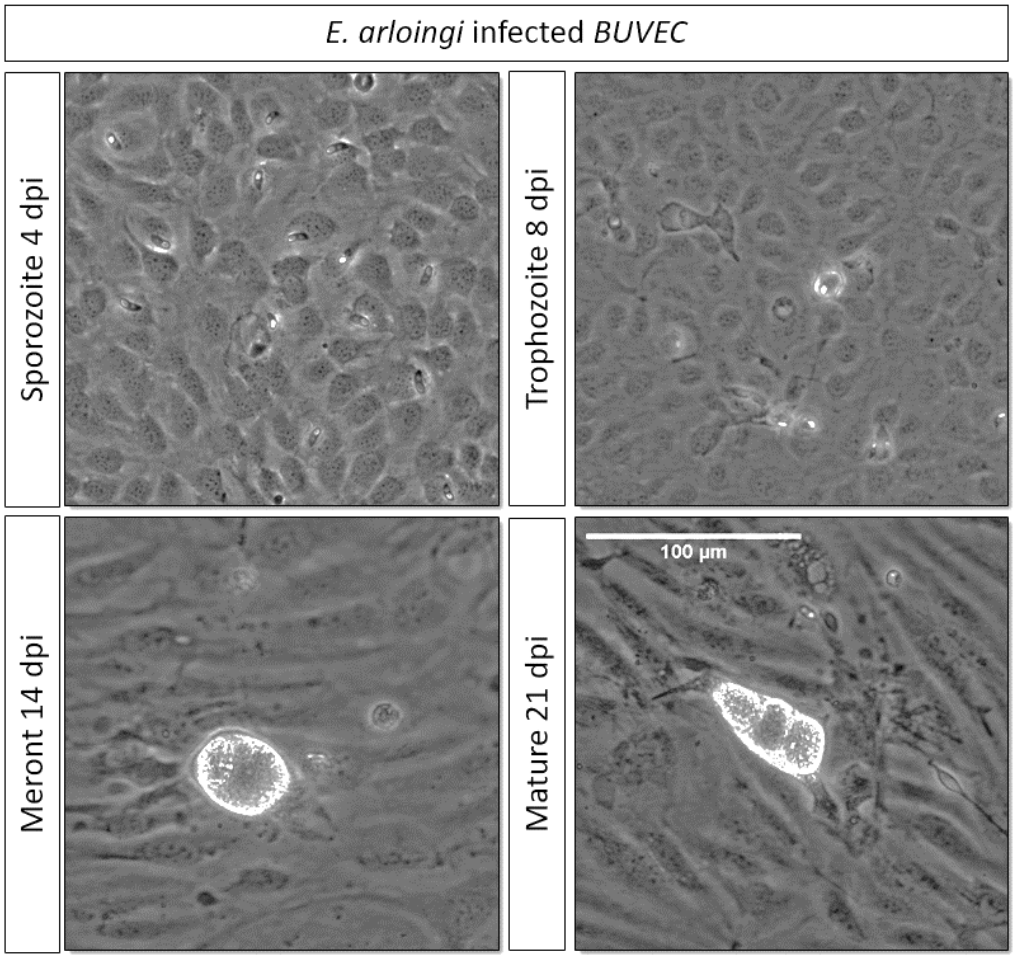


Figure 12 Eimeria arloingi in vitro first merogony in BUVEC. Different stages of the first merogony are illustrated above. Trophozoites were seen after 7 d.p.i. and macromeronts at 15 d.p.i. Free merozoites I were visible after 21 d.p.i. Sporozoites 8.3 µm (*n* = 60), Trophozoites 18x15.69 µm (*n* = 20), Merozoites I 10 µm (*n* =20).

2. Chapter: Concomitant *in vitro* development of *Eimeria zuernii*- and *Eimeria bovis* macromeronts in primary host endothelial cells

This chapter is based on the following published paper:

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Concomitant *in vitro* development of *Eimeria zuernii*- and *Eimeria bovis*-macromeronts in primary host endothelial cells

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ABSTRACT

Eimeria zuernii and *E. bovis* are host-specific apicomplexan parasites of cattle causing haemorrhagic typhlocolitis in young animals worldwide. During first merogony, both *Eimeria* species form giant macromeronts (> 300 μm) in host endothelial cells containing > 120,000 merozoites I *in vivo*. During the massive intracellular replication of macromeronts, large amounts of cholesterol and fatty acids are indispensable for enormous merozoite I-derived membrane production. From a metabolic perspective, host endothelial cells might be of advantage to the parasite, as transcription of several genes involved in both, cholesterol *de novo* biosynthesis and low density lipoprotein (LDL)-mediated uptake, are up-regulated in *Eimeria* macromeront-carrying host endothelial cells. In order to analyse further influence of *E. zuernii*/*E. bovis* infections on the metabolism of cholesterol, fatty acids, and glycolysis of the host endothelial cells, suitable *in vitro* cell culture systems are necessary. So far, *in vitro* cell culture systems based on primary bovine umbilical vein endothelial cells (BUVEC) are available for *E. bovis*-macromeront I formation, but have not been evaluated for *E. zuernii*. A novel *E. zuernii* (strain A), initially isolated from naturally infected calves in Antioquia, Colombia, was used for sporozoite isolation. Primary BUVEC monolayers were concomitantly infected with *E. zuernii*- and *E. bovis*-sporozoites, resulting in large sized macromeronts whose morphological/morphometric characteristics were compared. BUVEC carrying *E. zuernii*-macromeronts resulted in the release of viable and highly motile merozoites I. Overall, *E. zuernii*-merozoites I differed morphologically from those of *E. bovis*. The new *E. zuernii* (strain A) will allow detailed *in vitro* investigations not only on the modulation of cellular cholesterol processing (i. e. cholesterol-25-hydroxylase and sterol O-acyltransferase) but also on the surface expression of LDL receptors during macromeront formation.

1. Introduction

At least thirteen *Eimeria* species have so far been reported to infect domestic cattle and buffalo worldwide [1, 2]. Among these species, *E. zuernii* and *E. bovis* are considered as the most pathogenic ones causing severe typhlocolitis with clinical manifestations such as haemorrhagic diarrhoea, dehydration, weight loss and poor growth rates, mainly in calves [3–5].

In contrast to other bovine *Eimeria* species, *E. zuernii*- and *E. bovis*-sporozoites must invade host endothelial cells of central lymph capillaries of ileum villi *in vivo* [6–8] where first generation macromeronts are formed. As these macromeronts develop and become mature, dramatic morphological changes have been observed in infected host endothelial cells *in vivo* [6, 7] and *in vitro* [9, 10]. As such, host cell nucleus of *Eimeria* macromeront-carrying host endothelial cells changes from having heterochromatine with a dark spotted-content to a 'fried-egg'-shape containing euchromatine and a nucleolus coalescing to form single or multiple nucleoli [11, 12]. Similar host cell nuclear morphological alterations are reported to occur in host cells infected with pathogenic caprine and ovine *Eimeria* species, i. e. *E. ninakohlyakimovae* [13, 14], *E. arloingi* [15] and *E. ovinoidalis* [16].

In agreement with these common replication features, recently published phylogenetic analysis on pathogenic ruminant *Eimeria* suggests shared evolutionary history for ruminant *Eimeria* replicating in highly immunocompetent endothelium and by forming large-sized macromeronts [17]. It is hypothesized that sporozoites of a common ancestor species were able to migrate deeper in order to infect lymph endothelium, thereby colonizing a new niche in small intestine of ruminants *in vivo* [17], probably to fulfil specific nutritional requirements as recently demonstrated for *E. bovis* [11, 18, 19]. Accordingly, *E. bovis*

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strictly depends on the host endothelial cell supply of energy and on pivotal building molecules, such as cholesterol, for its massive intracellular replication [11, 18, 19], and thereby differing from other fast replicating apicomplexan parasites (i. e. *Toxoplasma gondii*, *Cryptosporidium parvum*) on metabolic requirements [20, 21].

All cattle kept under conventional husbandry conditions are unavoidably exposed to concomitant *Eimeria* spp. infections worldwide [22, 23] and infection-induced impaired animal performance, mortality and anticoccidial therapy costs generally result in considerable economic losses [19, 23, 24]. *E. zuernii/E. bovis* are strictly obligate intracellular protozoan parasites residing within a parasitophorous vacuole (PV). Both require cholesterol for PV establishment and host cell membrane enlargement but also for massive offspring production, i. e. > 120,000 merozoites I [9].

Nevertheless, usage of calves for detail research on *Eimeria*-infected host cells cholesterol- or glycolysis-metabolism is becoming more difficult to justify as requirements for housing conditions and principles of animal welfare have become more restrictive worldwide. Thus, we here provide a new *in vitro* system for *E. zuernii* as an alternative for experimental animal studies and useful for further investigations on complex parasite-host endothelial cell interactions, indispensable for better understanding of pathogenesis but also for identification of potential targets for anticoccidial drug development. This new *in vitro* culture system for *E. zuernii* is in line with 3R principles (i. e. replace, refine, reduce) of animal use, and could be used in many laboratories with not to high specific requirements as described elsewhere [25].

Thus, main aims of this study were first, to provide a suitable *in vitro* culture system based on primary bovine host endothelial cells for *E. zuernii* macromeront- and merozoites I-production being as close as possible to *in vivo* situation, and second, to generate new data on morphological, morphometric and molecular characteristics of this new *E. zuernii* field strain.

2. Materials and methods

2.1. Parasites

Eimeria zuernii oocysts used in this study were initially isolated from naturally infected calves in Antioquia, Colombia, which contained 95% *E. zuernii*- and 5% *E. bovis*-oocysts, respectively. This new *E. zuernii* (strain A), was thereafter propagated in a parasite-free male Holstein-Friesian calf according to Hermosilla et al. [26]. All animal procedures were performed following the guidelines of the Ethic Committee for Animal Experimentation, approved by the Institutional Committee for Care and Use of Animals of the University of Antioquia (Act No.102, 2016) in accordance to current Colombian Animal Protection Laws.

Briefly, for oocysts production, an 8-week-old calf (without previous *Eimeria* oocyst exposure) was infected orally with 3×10^5 sporulated *E. zuernii* (strain A) oocysts. Then, oocysts were isolated from faeces beginning at 15 days p. i. according to Jackson [27]. Oocysts were incubated in 2% (*w/v*) potassium dichromate (Merck) solution at room temperature (RT, 25 °C) and constant mixing until complete sporulation. Sporulated oocysts were stored in 2% (*w/v*) potassium dichromate solution at 4 °C until further use. Oocysts of *E. zuernii* (strain A) were identified based on morphological/morphometric characteristics of sporulated oocysts as was previously described [28–30].

2.2. Excystation

A total of 2.5×10^6 sporulated oocysts of *E. zuernii* (strain A) were excysted following oocyst excystation protocols of Hermosilla et al. [9] with some slight modifications [15]. Briefly, sporulated oocysts were added into a 4% (*v/v*) sodium hypochlorite solution and thereafter magnetically stirred on ice for 20 min. Then, oocysts were mixed by vortexing for 15 s and thereafter centrifuged (300 ×*g*, 5 min). Supernatant was collected and mixed with distilled water (1:1) and pelleted (600 ×g, 20 min). Oocysts pellet was then layered in 60% Percoll™ (GE Healthcare, UK) gradient and centrifuged for 20 min at 400 ×*g* to remove remaining faecal debris. After centrifugation, oocysts bands were suspended in sterile 0.02 M L-cysteine HCl/0.2 M NaHCO3 (Merk) solution and incubated for 20 h at 37 °C in a 100% CO_2 atmosphere. Thereafter, the oocysts were suspended in Hank's balanced salt solution (HBSS, Gibco) containing 0.4% (*w/v*) trypsin (Sigma-Aldrich) and 8% (*v/v*) sterile filtered bovine bile obtained from a local butchery, up to 4 h at 37 °C in a 5% CO_2 atmosphere. Every hour, sporulated oocysts and released sporocysts were counted using an inverted microscope (IX81, Olympus®) to estimate the number of free-released sporozoites. Free sporozoites were then washed twice with modified endothelial cell growth medium [ECGM (PromoCell) diluted in M199 medium (Gibco) (3:7), 1% penicillin-streptomycin (both Sigma-Aldrich) supplemented with 10% foetal calf serum (FCS, Biochrome)], and thereafter counted in a Neubauer counting chamber (1:10 and 1:100 dilution).

2.3. Host cells

Primary bovine umbilical vein endothelial cells (BUVEC) used in this study were isolated using the methodology previously described in detail by Taubert et al. [11]. Three different BUVEC isolates were used for host cell infection experiments. BUVEC isolates ($n = 3$) were seeded in two 25 cm^2 cell tissue culture plastic flasks (Greiner) and maintained in modified ECGM supplemented with 10% FCS. The culture medium was changed every 48–72 h after infection.

2.4. Host cell infection, development of *E. zuernii*/*E. bovis*-macromeronts and merozoite I production

BUVEC monolayers were infected with 2.5×10^5 freshly isolated sporozoites of *E. zuernii* (strain A; composed of 95% *E. zuernii* and 5% *E. bovis* oocysts) on cell monolayers with 80–90% confluency. Culture medium was changed 24 h after sporozoite infection and thereafter every two days. Using microscopy and photography, the *Eimeria*-infected host cells were evaluated daily with the aim to follow parasite development and to register measurement of different parasitic stages (i. e. intracellular sporozoites, trophozoites, immature meronts, mature macromeronts), in addition to monitor morphological changes of *Eimeria*-infected host endothelial cells.

The number of *E. zuernii*- and *E. bovis*-infected host endothelial cells was calculated by counting at least 3 different 400-fold magnification power vision fields at 24 h p. i. with their corresponding parasitic stage measurements. The total number of immature- and mature-*E. zuernii*, as well as *E. bovis*-macromeronts present per BUVEC monolayer was also determined and expressed as percentage of numbers of sporozoites initially applied to the BUVEC culture.

In addition, randomly infected cells ($n = 15$) were measured every day p. i. by using Cellsens Dimention® software (Olympus) according to Silva et al. [15]. When *E. zuernii*- and *E. bovis*-merozoites I were found free in the cell culture medium, these stages were carefully harvested daily by aspiration of the supernatant, counted, and thereafter frozen for further PCR analyses as described elsewhere [19].

2.5. DNA extraction

DNA from *E. zuernii*- and *E. bovis*-oocysts, -sporozoites, -macromeronts, and -merozoites I was extracted according to Hamid et al. [19]. After excystation, approximately 2×10^4 sporozoites of each species were used for DNA extraction. Infected BUVEC were harvested at 21 and 25 days p. i. for macromeront-derived DNA extraction. Furthermore, merozoites I were collected from infected cell cultures from 16 to 23 days p. i. onwards [19]. DNA was extracted using the DNeasy Blood and Tissue Kit® (QIAGEN), following the manufacturer's instructions for cultured cells. DNA from oocysts was extracted using the commercial kit for DNA purification NucleoSpin® Soil (Macherey-

Table 1
Eimeria zuernii- and *E. bovis*-species specific oligonucleotides used for ITS-1 PCR.

Gene	Name	Oligonucleotide	Product lenght	Description	reference
ITS-1 Genus	*ITS-1 F*	5′gcaaaagtcgtaacacggtttccg-3′	380 bp *E. bovis*	PCR. Identification of species	Kawahara F., et al., 2010
	ITS-1 R	5′ctgcaatttcacaatgcgtatcgc 3′	404 bp *E. zuernii*		
ITS-1 E. zuernii	*ITS-1 zf*	5′-aacatgtttctacccactac-3′	334 bp		
	ITS-1 zr	5′-cgataaggaggaggacaac-3′			
ITS-1 E. Bovis	*ITS-1 bf*	5′-tcataaaacatcacctccaa-3′	238 bp		
	ITS-1 br	5′-ataattgcgataagggagaca-3′			
18S rDNA	*TK2*	5′-ggt tga tcc tgc cag tag tc-3′	1800 bp	Nested PCR.	Kokuzawa et al., 2013
	ets2	5′-aat ccc aat gaa cgc gac tca-3′			
	TK1	5′-agt agt cat atg ctt gtc tc-3′			
	18S-14	5′-acg gaa acc gtg tta cga ct-3′			
18S rDNA	*18S-9*	5′-aca att gga ggg caa gtc t-3′		Sequencing primers	
	18S-13	5′-atg cct acc ttg gct tct cc-3′			
	Eb1-R	5′-agc ctg ctt gaa aca ctc t-3′			
	18S-12	5′-gaa ccg tag ttc ccg atc ct-3′			

Bp: Base pair.

Nagel GmbH & Co.), following the manufacturer's instructions with SL2 buffer plus enhancer (Sigma-Aldrich). Sample lysis was performed with the usage of Bead Ruptor 24® (OMNI), using 2 cycles of 2 and 30 min at 4 m/s with the use of 0.2 mm diameter ceramic beads.

2.6. PCR- and nested PCR-analyses

To determine if DNA extracted from samples were positive for genus *Eimeria*, we amplified the whole ITS-1 region, which is located between the 3′ 18S and 5′ 5.8S rRNA gene. The genus-specific primers set used were the following: forward 5′GCA AAA GTC GTA ACA CGG TTT CCG 3′, and reverse 5′CTG CAA TTC ACA ATG CGT ATC GC 3′ [31]. The total of 25 μL volume was used, containing 0.4 mM dNTPs mix, 1 μL of DNA (7,95 ng), 2 mM MgCl, 0,4 μM each primer, 2.5 U Taq polymerase and 1 × reaction buffer (Thermo Scientific™). An initial denaturing step was performed at 94 °C for 30 s, followed by 35 cycles at 94 °C for 10 s, 55 °C for 30 s, 72 °C for 30 s with a final extension at 72 °C for 2 min. For the specific species of *Eimeria*, primers used are described in Table 1. ITS-1 regions were composed of 380 bp fragments for *E. bovis* and of 403–404 bp fragments for *E. zuernii*, respectively.

Additionally, two primer sets for nested PCR were used to amplify an approximately 1800 bp fragment of 18S rDNA: TK2 and ets2 for the first PCR and TK1 and 18S-14 for the second PCR (see Table 1). The first PCR reaction was performed in a 25 μL reaction volume containing 6.65 μL of DNA template, 0.2 mM of each dNTP, 0.1 mM of each primer, 0.25 U polymerase, and 1 × reaction buffer. Thermocycler program conditions used were 94 °C for 10 min, 20 cycles of 98 °C for 10 s, 67 °C for 3 min and 72 °C for 10 min. The second PCR reaction was performed using 1 μL of the first PCR reactions as template DNA. The thermal conditions were 94 °C for 2 min, 30 cycles of 98 °C for 10 s, 56 °C for 10 s and 72 °C for 90 s, and 72 °C for 7 min according to Kokuzawa et al. [32].

2.7. Sequencing and Eimeria zuernii (strain A) molecular characterization

ITS-1 gene PCR products were purified directly from gel using the QIAquick Gel Extraction Kit® (QIAGEN) following manufacturer's recommendations. Purified products were stored at 4 °C until required for further sequencing. Sequence data were assembled and edited using the SeqMan® program (DNA Star Laser gene software package, WI). Nucleotide BLAST (Basic Local Alignment Search Tool) program was used to explore sequence similarity of *E. zuernii* (strain A) to some available sequences of *E. zuernii* ITS-1 gene in NCBI nucleotide databases.

2.8. Statistical analysis

The data were analysed using Microsoft Excel® 2016. Values of mean and standard deviation of length and width were calculated for each stage of the parasite, and the *t*-test: paired two samples for means was performed to determine if there were statistical differences between the size of different species. Differences were considered as significant at the level of $P < 0.05$.

3. Results

3.1. E. zuernii (strain A), subsequent sporogony and sporozoite excystation

A new bovine *E. zuernii* (strain A) was successfully isolated from a field case, and successfully replicated through experimental calf infections under parasite-free conditions in Antioquia, Colombia, as previously demonstrated for other available ruminant *Eimeria* strains [14–16].

Purified Colombian *E. zuernii* (strain A) was composed of *E. zuernii* (95%) and *E. bovis* (5%) unsporulated oocysts, respectively. Oocysts of both species were different by their morphology and morphometry as well as inner circumplasm-located structures after fulfilled exogenous sporulation. *E. zuernii*-sporulated oocysts presented a subspherical shape with no micropyle as described elsewhere [33]. The outer and inner oocyst walls of *E. zuernii* were smooth and colourless. The average size of oocysts was 16.18 ± 1.0 × 15.77 ± 1.13 μm (Fig. 1). There was no visible oocyst residual body. Conversely, *E. bovis*-sporulated oocysts presented an ovoid shape with a single micropyle and oocyst residual body within circumplasm. The average size of *E. bovis* oocysts was 32.6 ± 1.5 × 23.4 ± 2.6 μm (Fig. 1). The sporogony at RT (18–25 °C) and permanent oxigenation varied from 3 to 10 days for both species. *E. zuernii*-sporocysts were ovoid in morphology and presented an average length of 8.92 ± 1.2 μm and an average width of 5.35 ± 0.4 μm. In contrast, *E. bovis*-sporocysts were 14.26 ± 2.1 × 7.85 ± 0.6 μm in size [7, 29].

After 3 h of incubation in excystation medium, fully viable and extremely motile sporozoites were obtained from both *Eimeria* species. *E. bovis*-oocysts showed a thinner wall than *E. zuernii*-oocysts, and resulting *E. bovis*-sporozoite release from oocysts was faster than for *E. zuernii*. For instance, at 2 h of excystation process, approximately 70% of *E. bovis* oocysts were excysted, whilst only 30% of *E. zuernii*-oocysts were excysted and releasing sporozoites. In case of *E. bovis* excystation, the oocyst wall was completely disrupted and sporozoites hatched from free-released sporocysts into medium, while in *E. zuernii* sporozoites left sporocysts into oocyst circumplasm and then finally egressed from *E. zuernii* oocyst. Freshly released sporozoites of both species showed typical movements of gliding motility and contractility (see

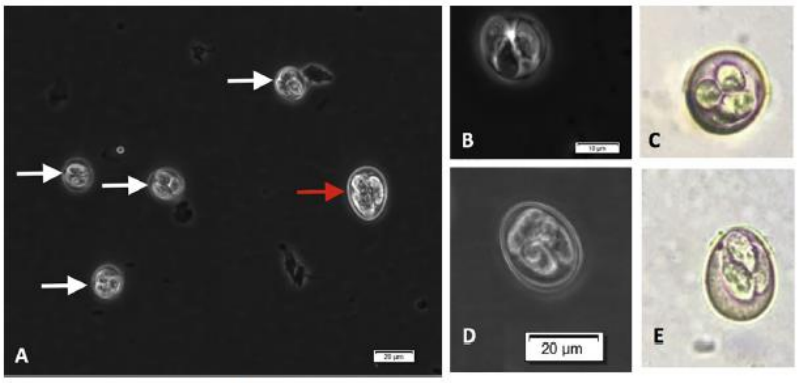

Fig. 1. A: Oocysts of *E. bovis* (red) and *E. zuernii* (white). B and C: *E. zuernii* oocyst. Size: 15.31 × 16 µm (sporocyst 10.16 × 6.14 µm). D and E: *E. bovis* oocyst. Size: 26 × 34 µm (sporocyst 18.72 × 7.8 µm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

supplementary data video 1).

3.2. Sporozoite-motiliy, –host cell invasion and macromeront development of E. zuernii and E. bovis

Viable sporozoites exposed to endothelial cells showed typical movements of sporozoite-gliding motility, -erection and -circular twisting on surface of host endothelial cells. Morphology of each extracellular *Eimeria* sporozoite species was clearly different (see Table 3, Fig. 2A) and easily recognized by their different sizes. As such, *E. bovis*-sporozoites (14.05 ± 1 × 2 ± 0.2 µm) were longer and wider than E. *zuernii*-sporozoites (10.09 ± 1.7 × 1.5 ± 0.2 µm) (Table 3, Fig. 2). Furthermore, *E. zuernii*-sporozoites had only one large visible refractile body (± 2.45 µm diameter) in posterior part whereas *E. bovis*-sporozoites showed two to three refractile bodies (the largest one with ± 3.18 µm diameter) in posterior part.

At 24 h p. i. an average infection rate of 5.9% (2.34% - 9.73%) was determined (Table 2). Active sporozoite host cell invasion was similar for both *Eimeria* species using mainly their gliding motility to get in first close contact to host cell membrane, and thereafter attaching to surface of host cell membrane in order to penetrate into cytoplasm (for more details see video 1). Sporozoites of *E. zuernii* and *E. bovis* extended their anterior parts (i. e. apical complex) and performed a small breach into host cell membrane, then sporozoites constricted themselves, became pear-shaped and underwent amoeboid deformation before penetration (Fig. 2B). The constriction observed while sporozoite invasion is a sort of tight junction between sporozoite pellicula (outer sporozoite membrane) and host cell membrane moved along sporozoites bodies as sporozoites moved into host cell cytoplasm. When active cell invasion ended, intracellular sporozoites became round at their anterior end and assumed characteristic intracellular forms (Fig. 2.D) as described elsewhere [9, 11]. The complete invasion time for *E. zuernii*-sporozoites was 29 s and is comparable to ones observed for *E. bovis*-sporozoites *in vitro* [9]. Intracellularly *E. zuernii/E. bovis*-sporozoites were most of the time found in close proximity to host cell nucleus (Fig. 2D).

3.3. Early trophozoite development

Between first and fifth day p. i. sporozoites became shorter and in case of multiple sporozoite host cell infections, all sporozoites oriented into one direction. Furthermore, some host endothelial cells were co-infected with both sporozoite species (Fig. 2C). Certain intracellular sporozoites of both species, also actively egressed from previously infected host cells. This egress behaviour was seen from day 1 until 20 days p. i. At 4–6 days p. i., trophozoites were firstly detected in infected BUVEC. Differences in trophozoite size of two *Eimeria* species were constantly observed and trophozoites of *E. zuernii* measured 7.63 ± 1.03 × 7.91 ± 1.1 µm in average size, whilst *E. bovis* were 11.95 ± 0.8 × 12.51 ± 0.96 µm in size. Both trophozoite stages presented one large refractile body in addition to some smaller refractile granules. At 7 days p. i., some of *E. bovis*- as well as *E. zuernii*-infected endothelial host cells presented drastic morphological changes in size and shape due to the intracellular development of immature macromeronts. The nucleus of infected host cells was condensed and thereby showing a 'fried-egg'-shape [11] containing 2 to 4 nucleoli. *E. zuernii*-immature macromeronts were less structured but more granulated when compared to *E. bovis*, which had several nucleoli and additional refractile granular material (see Fig. 3). Development of macromeronts was not a synchronized event for both *Eimeria* species. Some sporozoites and trophozoites persisted without changes until the end of the study, while others started further development few days later *in vitro*.

3.4. Mature macromeront formation

Further development of trophozoites into meront stages occurred from day 7 p. i. onwards, but as stated previously not all trophozoites developed into immature/mature macromeronts. In only 4.05% (602/14,458) of initially infected host endothelial cells, mature macromeronts containing fully developed merozoites I were observed. The resulting number of *E. bovis*-macromeronts was higher than for *E. zuernii* (62% *vs* 38%) (Table 2) and this in spite of the fact that only 5% *E. bovis* oocysts were available in the initial inoculum.

From day 8 to 16 p. i., macromeront growth was slow but constant. Average size of *E. zuernii*-mature macromeront was 89 × 20 µm (27–168 × 33–141 µm) and for *E. bovis* 107 × 28 µm (50–150 × 33–200 µm). In former case, merozoites I were seen inside the PV within infected host cells (Fig. 3E–F). Moreover, and in contrast to *E. zuernii, E. bovis*-mature macromeronts were multi-chambered and often elongated or had a rather 'bubble'-like form, with merozoites I easily distinguishable inside these chambers (17 days p. i.) (Fig. 3F). Conversely, morphology of *E. zuernii*-macromeronts showed asymmetrical multi-chambered structures, with 'cauliflower'-like forms and containing several refractile material and particles (15 days p. i.) (Fig. 3).

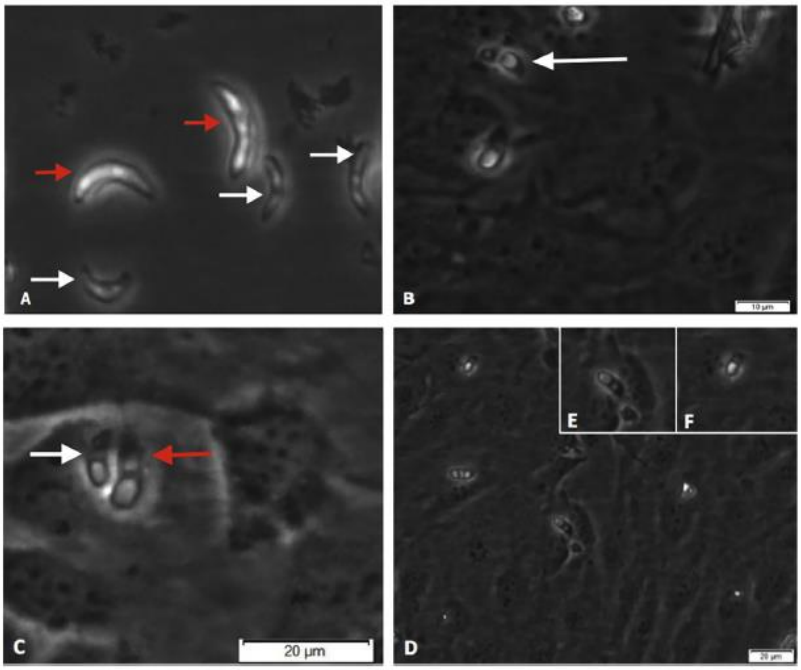

Fig. 2. A. Free *Eimeria* sporozoites. *E. bovis* (red) *versus E. zuernii* (white) sporozoites. *E. bovis* sporozoite was 14.05 μm length. *E. zuernii* sporozoite was 10.09 μm length. B. *Eimeria zuernii* cell penetration. *Eimeria* sporozoite invading host cell. Constriction of refractile body while entering host cell. C–F. BUVEC cell line infected with bovine *Eimeria* 6 dpi. C. *Eimeria zuernii* and *E. bovis* infecting same host cell. Notice the size difference between both *Eimeria* species. Average size: *E. zuernii* 9.0 × 2.1 μm *E. bovis* 13.0 × 3.1 μm D. Host cell infected with single *Eimeria* 7 dpi. In case of *E. bovis* (E) two refractile bodies were seen. *E. zuernii* (F) only one refractile body was seen. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. *E. zuernii*-merozoite I release

At 15 days p. i., first free *E. zuernii*-merozoites I were observed in the supernatant of cell culture medium (Fig. 3). At 17 days p. i., free and highly motile merozoites I of both species were found. Average size of *E. zuernii*-merozoites I was 6 ± 0.88 × 1.6 ± 0.31 μm and average size of *E. bovis*-merozoites I was 10.1 ± 1.26 × 1.5 ± 0.34 μm. Mature macromeronts continued growing until 25 days p. i. and maximum merozoite I production was observed at 20–22 days p. i. for both species.

3.6. Specific PCR of *E. zuernii* (strain A)

To confirm *in vitro* development of *E. zuernii* in BUVEC, each parasitic stage (i. e. sporulated oocysts, sporozoites, trophozoites, macromeronts and merozoites I) was tested with specific *E. zuernii* ITS-1 PCR with positive results (Fig. 4), proving suitability of this *in vitro* system for this bovine *Eimeria* species.

Sequencing analysis also confirmed presence of both species in our *in vitro* BUVEC culture system infected with *E. zuernii* (strain A). Sequence from the band expected for *E. zuernii* had 98% of identity to *E. zuernii* gene ITS-1 (AB769657.1, LC171333.1 and AB769660.1) while band expected for *E. bovis* had 98% of identity to *E. bovis* gene ITS-1 (AB769575.1, AB769572.1 and KU351711.1).

4. Discussion

The two most pathogenic bovine coccidian species are *E. bovis* and *E. zuernii* [4, 34, 35], and both species have been described as the most prevalent ones in stabled calves worldwide [3, 4, 35]. In contrast to other cattle *Eimeria* infecting gut epithelial host cells, *E. zuernii* and *E. bovis* replicate within host endothelial cells of central lymph capillaries of lacteals *in vivo*, and corresponding to a molecular phylogenic cluster of macromeront-forming ruminant *Eimeria* species [17].

Despite the fact that some data are available on *E. zuernii* infections *in vivo* [8, 36, 37], very little is known in literature on appropriate *in*

Table 2
Percentage of infection and development of *Eimeria zuernii*/*E. bovis* in BUVEC layers.

BUVEC line	Number of intracellular sporozoites (5 days)	Number of mature schizonts (20 days)	Percentage of maduration	Number of *E. zuernii* schizont	%	Number of *E. bovis* Schizont	%
284	13,977	202	1.45%	52	26%	150	74%
285	24,661	1375	5.58%	530	39%	845	61%
286	5937	230	3.87%	100	43%	130	57%
Average	14,858	602	4.05%	227	38%	375	62%

Table 3
Summary of different sizes and parasitic stages of the two species of *Eimeria* zuernii/*E. bovis* in infected BUVEC layers.

Stage	Species	Length µm		Width µm		Reference	
		Average	SD	Average	SD	Length	Width
Free sporozoites $n = 50$	*E. bovis*	14.05[a]	1	2	0.2	14	2
	E. zuernii	10.09[a]	1.7	1.5	0.2		
Sporozoites $n = 100$	*E. bovis*	11.15[a]	0.84	3.61	0.28		
	E. zuernii	8.53[a]	1.27	3.55	0.31	8.3	2.4
Trophozoites n = 15	*E. bovis*	11.95[a]	0.8	12.51[a]	0.96	11.5	8.7
	E. zuernii	7.63[a]	1.03	7.91[a]	1.1	9.5	8.2
Merozoite I n = 100	*E. bovis*	10.10[a]	1.26	1.59	0.34	13	10
	E. zuernii	6.03[a]	0.88	1.60	0.31	6.5	1.5
Meronts $n = 35$	*E. bovis*	107.80[a]	24.72	69.93	28.71	87.4	42.9
	E. zuernii	89.93[a]	35.60	62.26	20.99	61	53

Reference sizes: Fayer et al., [30]; Speer et al., [31]; Bagoura, [32]; and Hermosilla et al., [11].
[a] Significant statistical difference.

vitro culture systems for this species. So far, the only available *in vitro* culture report of *E. zuernii* [29], described development of first merogony in different permanent host cells types but not including primary host endothelial cells. In contrast to *E. zuernii*, vast amount of data exist on suitable *in vitro* culture systems for closely related ruminant *Eimeria* species [*E. bovis* (cattle) [9, 18, 38], *E. ninakohlyakimovae, E. arloingi* (goats) [14, 15] and *E. ovinoidalis* (sheep) [16]] in primary ruminant host endothelial cells, allowing investigations on early molecular host cell-parasite interactions, such as transcriptomics and proteomics [11, 12].

The reduction and refinement of animal experiments have been the aim of investigations in recent studies, but final goal should be replacement of animal testing, if possible, in the future [39]. Consistently, we here provide a suitable *in vitro* host cell-parasite interaction system for the new isolated *E. zuernii*. This *in vitro* culture method will hopefully allow performing different experiments to better understand in detail nutritional requirements (i. e. cholesterol metabolism) during massive intracellular replication, which could result in development of new drug targets without the use of live animals.

During *in vitro* excystation protocol, *E. zuernii*-sporozoites egressed with ease from oocysts after being released into oocyst circumplasm, as already reported to occur for *E. arloingi* and *E. ovinoidalis*, respectively [15, 16, 27]. However, due to lack of micropyles in *E. zuernii*-oocysts, a specific area of sporozoite egression was not determined. *E. zuernii* excystation contrasted to what occurs during *E. bovis*- or *E. ninakohlyakimovae*-excystation process [13] since firstly oocyst wall was disrupted releasing sporocysts and at last sporozoites egressed from sporocysts. Excystation of *E. zuernii* resulted in the release of viable and highly motile sporozoites, which were able to infect new host endothelial cells and to continue further intracellular macromeront development culminating in merozoite I production. This accomplishment is of importance as it will allow to gain access to different *E. zuernii*-parasitic stages, i. e. trophozoites, macromeronts and merozoites I. This BUVEC-based *in vitro* system for *E. zuernii* will permit investigations on parasite-triggered modulation of apoptosis [38], cytoskeleton [40], transcription, cell cycle, endothelial cell-derived immune reactions [11], NETosis [41], modulation of metabolic pathways [18, 19], as well as for testing novel anticoccidial compounds and drugs.

In contrast to *E. zuernii*, *E. bovis* sporozoites egressed faster from oocysts and thereafter infected rapidly BUVEC monolayers. However, development of *E. zuernii*-trophozoites and -macromeronts was faster when compared to *E. bovis*, which might reflect existing differences in prepatent periods observed with *in vivo* infections [3, 4, 7].

E. zuernii-sporozoite egression from infected host endothelial cells was also observed until the end of the experimental period and corresponding to previous reports of ruminant *Eimeria* species [42, 43]. These *E. zuernii*-sporozoites might have been able to re-invade more suitable host endothelial cells as suggested for other ruminant *Eimeria* sporozoites [15, 16, 42]. The migration capacity of certain apicomplexan sporozoites through host cells by breaching host cell plasma membranes without forming a PV, have been reported so far only for *Plasmodium falciparum* [44] and *E. bovis* [42, 43]. This alternative sporozoite host cell invasion strategy has been explained in necessity of sporozoites to transmigrate through tissues in order to reach deeper lying specific host cells, as is the case for *P. falciparum*-sporozoites hepatocytes [44] and in case of *E. bovis*-sporozoites lymphatic endothelial cells of lacteals of ileum [42, 43].

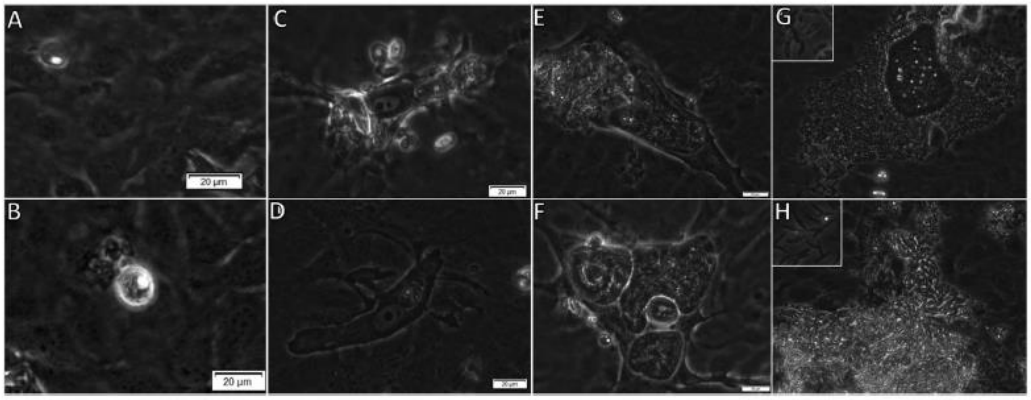

Fig. 3. Trophozoites of *E. zuernii* (A) and *E. bovis* (B) in BUVEC cell lines infected with bovine *Eimeria* 7 dpi. Average size: *E. zuernii* 7.63 × 7.91 µm *E. bovis* 11.9 × 12.5 µm. Early meront of *E. zuernii* (C) and *E. bovis* (D) in BUVEC cell lines infected with bovine *E. zuernii* (strain A). At this time point, meronts were similar in size, but the organization was different. The *E. zuernii* showed a more granular form at 14 dpi. *E. bovis* 115.5 × 24.2 µm. Mature meront of *E. zuernii* (E) and *E. bovis* (F) in BUVEC cell lines infected with bovine *E. zuernii* (strain A). In average, *E. bovis* mature macromeronts were larger in size than *E. zuernii* (107 × 69 µm *vs* 89 × 62 µm). *E. bovis* macromeronts showed more organization and multi-chambered development. 21 dpi. Merozoite I of *E. zuernii* (G) and *E. bovis* (H) in BUVEC cell lines infected with bovine *E. zuernii* (strain A). *E. zuernii* merozoites I were 4–6 × 1.6 µm in average size while *E. bovis* merozoites I were 10–12 × 1.6 µm in average size).

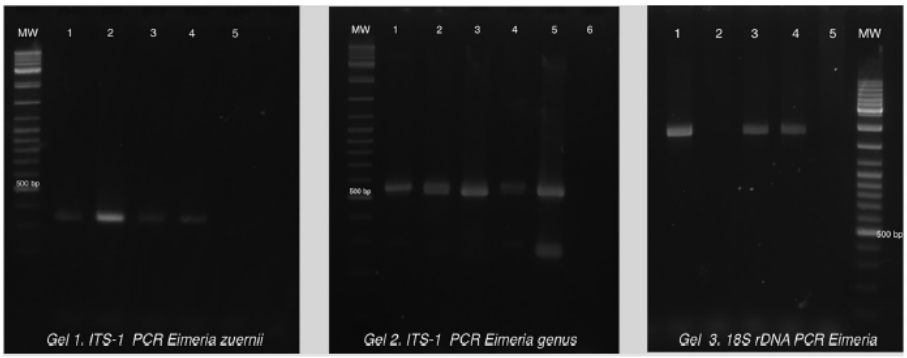

Fig. 4. Electrophoresis in agarose gel 1.5% of the *ITS-1* PCR product and 18S rDNA. MW: Molecular weight 100 bp. Gel 1. *ITS-1* PCR for *E. zuernii*: Samples: 1: Sporozoites, 2: Macromeront 21 dpi, 3: Merozoites 18 dpi. 4: Oocyst, 5: Negative control. Expected band for *E. zuernii*: 344 bp. Gel 2. *ITS-1* PCR for *Eimeria* genus. Expected band 348–546 bp. Samples: 1: Sporozoites, 2: Macromeront 21 dpi, 3: Merozoites 18 dpi. 4: Oocyst, 5: Positive control (*E. bovis* oocyst), 6: Negative control. Gel 3. 18S rDNA PCR. Expected band 1800 bp. Samples: 1: Sporozoites, 2: Macromeront 21 dpi, 3: Merozoites 18 dpi. 4: Oocyst, 5: Negative control.

Not all *E. zuernii*-sporozoites continued their intracellular development synchronically. Some sporozoites remained in 'hynozoite/dormozoite'-like stages for an indefinite period of time as demonstrated for *E. bovis in vivo* [45] and *in vitro* [9]. Correspondingly, we observed some sporozoite- as well as trophozoite-stages of both species remaining as such until end of *in vitro* study [15, 16].

In vitro development of *E. zuernii* was similar to only existing report published by Speer et al. [29]. As such, sizes of intracellular *E. zuernii*-sporozoites were found to be identical, but former authors indicated that sporozoites had none or up to 6 small refractile bodies randomly located in cytoplasm. Nevertheless, in our study only one large refractile body was detected. Likewise, first *E. zuernii*-trophozoites were found as early as 6 days p. i., while in past study trophozoites were identified at 9 days p. i. [29]. Former report described *E. zuernii*-macromeront development from day 9 p. i. onwards and at 18 days p. i. the release of fully developed merozoites I, while in our case macromeront formation occurred from day 7 p. i. onwards. Rupture of fully mature *E. zuernii*-macromeronts, with subsequent merozoites I extrusion, started at 15 days p. i. Developmental discrepancies to previous report might result from different cell types and cell lines used in each case, and by the fact that primary BUVEC are much closer to *in vivo* parasitized host

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cells, namely lymphatic endothelial cells. Conversely, it was previously described that *E. zuernii*-first merogony also occurred in lamina propia of ileum *in vivo* [29] reflecting certain flexibility on its host cell specificity. Similar findings were reported for closely related *E. ninakohlyakimovae*, where sporozoites can also infect epithelial cells of bile ducts in goats [13, 46].

Interestingly, *E. zuernii*-infected host cells presented the typical 'fried-egg'-morphology of nucleus as described for other ruminant *Eimeria* with macromeront development [14–16]. In accordance to these *in vitro* observations, Pasternak et al. [47] described the same nuclear morphological features for *E. zuernii*-infected intestinal epithelial host cell nuclei at 6–8 days p. i. *in vivo* and showing that nuclear hypertrophy was dissociated from DNA replication. At most, 20% of *E. zuernii* first-generation macromeronts developed within host cells which had proliferative potential [47], suggesting less host cell specificity when compared to *E. bovis*.

The existence of *E. zuernii*-macromeronts (200–300 μm) *in vivo* were firstly described by Marotel (1907) [48], which is close to the largest macromeront size found in our current *in vitro* study. Speer et al. [29] described similar *E. zuernii*-macromeront sizes by using permanent bovine kidney epithelial cells (MDBK) *in vitro*. *E. zuernii*-macromeronts were reported to contain 500 to 1000 rod-shaped 6.5 μm in length measuring merozoites I, with a centrally located nucleus, and lacking refractile globules [29]. *E. zuernii*-merozoites I isolated from our primary host endothelial cell culture were similar in size than previously described [29], but merozoite production was much higher. Notwithstanding, in previous *E. zuernii in vitro* report [29], no morphological differences to *E. bovis*-macromeronts were detected. Conversely, we clearly observed different morphological/morphometric characteristics for these two *Eimeria* species. Since *E. bovis*-macromeronts have been described in detail by others [9, 19, 38], this comparative study was able to better characterize morphological differences, nonetheless based exclusively on *in vitro*-derived observations.

E. bovis forms large macromeronts of up to 400 μm in size and containing 120,000–170,000 merozoites I within a well-defined PV *in vivo* [7, 49]; however, in this concomitant *Eimeria* infection, *E. bovis*-macromeronts reached sizes of up to 200 μm, which might be related to possible antagonist and/or synergistic interactions between both *Eimeria* species. Correspondingly, in rat coccidiosis existence of synergistic interactions of *Eimeria* species *in vivo* have been reported by Duszynsk [50]. Synergistic interactions were found in concomitant *Eimeria*-rat infections (i. e. *E. separata*/*E. nieschulzi*) resulting in increased discharge of oocysts of *E. separata* when compared to single *Eimeria*-

discharge of oocysts of *E. separata* when compared to single *Eimeria*-

infected controls [50]. A plausible reason for this synergism was that multiple *Eimeria* species infections might contribute to overall debilitation of animals or that one *Eimeria* species inhibits growth of others (e. g. through crowding effects) [50].

On the contrary, it could be an evolutionary strategy of certain ruminant *Eimeria* to achieve its massive replication by infecting specifically endothelium. Natural cattle coccidiosis usually occurs with two or more species. Klockiewicz et al. [51] found that almost 70% of calves infected with *Eimeria* had at least 2 species to be involved, with a maximum of 7 species.

In our study, the total number of macromeronts was higher in *E. bovis* than *E. zuernii* (with more production of merozoites I) and this although strain A contained only 5% of this species, which might suggest that this species has a higher proliferation capacity when compared to *E. zuernii*. More detailed investigations on early endothelial cell-mediated innate immune reactions, host cell-parasite interactions and parasite-parasite interactions are therefore needed to understand how these *Eimeria* species complete their massive intracellular replication within a hostile and highly immune-reactive endothelium.

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Conflict of interest

Authors declare that research was conducted in absence of any commercial or financial relationships that could be a potential conflict of interest.

Authors and contributors

SL and LS carried out *in vitro* experiments in Germany; SL, and JC isolated the new *E. zuernii* strain A in Colombia; SL, LS, and CH drafted and edited the manuscript. CH and AT designed, planed and coordinated the *E. zuernii* project. All authors have read and approved the manuscript as submitted.

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3. Chapter: Epidemiological survey and risk factor analysis on bovine *Eimeria* infections in Colombia

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Epidemiological survey and risk factor analysis on *Eimeria* infections in calves and young cattle up to 1 year old in Colombia

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Abstract

A large scale cross-sectional epidemiological study was conducted to evaluate prevalence, species diversity and associated risk factors of *Eimeria* infections in 55 cattle farms across seven States of Colombia, including subtropical and tropical regions. In total, 1333 faecal samples from young animals (<1 year of age) were examined at a single sampling date from August 2016 to December 2016. Flotation and McMaster techniques were conducted for parasitological investigation. Excreted *Eimeria* oocysts were allowed to sporulate *in vitro* and thereafter identified to species level based on morphological and morphometric characteristics. The overall *Eimeria* prevalence was 75.5% (1006/1333), with no difference observed between age categories. In total, 13 different *Eimeria* species were identified. The most prevalent species was *E. bovis* (33.5%), followed by *E. auburnensis* (12.5%) and *E. zuernii* (11.9%). Analysis of extrinsic associated risk factors revealed the floor type, feeding system, watering system and herd size as significant ($p < 0.05$) risk factors for *Eimeria* spp. infections. Based on these data, it can be assumed that bovine coccidiosis infections occur ubiquitously in the country and might play an important role especially in its subclinical form by affecting production parameters in conventional cattle management systems.

Key words: *Eimeria*, risk factors, coccidiosis, Colombia, calves.

1. Introduction

Eimeria species are common gastrointestinal parasites and the etiological cause of bovine coccidiosis which mainly affects young animals worldwide. Bovine coccidiosis is known as a limiting factor of cattle production causing economic losses mainly by subclinical infections (Fox, 1985; Lassen and Østergaard, 2012). Typically, when few calves show clinical signs (e. g. diarrhoea, straining, dehydration), the majority of calves in the same environment are likely to be subclinically infected and undiagnosed, and hence, associated costs are easily overlooked. Thus, all calves/young animals in the group should be treated to reduce losses due to reduced growth rates and the following production parameters: time to weaning, finishing, delayed onset of puberty, suboptimal weight at first calving and increased feeding costs (Daugschies and Najdrowski, 2005). In this respect, metaphylactic approaches applying toltrazuril or diclazuril have now become common in both dairy and beef industries (Enemark et al., 2013; Philippe et al., 2014) and showed to ameliorate negative effects of subclinical coccidiosis on growth performance (Daugschies et al., 2007; Veronesi et al., 2011).

A recent study on cattle industry from the Colombian Cattle Producers Association (Fedegan, 2017) cited a population of approximately 22.5 million heads accounting for 950,000 tons of meat and 3,200 million liters of milk produced annually. The annual consumption of meat and milk for an average Colombian person is 19 kg and 140 liters, respectively. With 33% of the Colombian population currently estimated to live on < 3 US$ per day, the capacity of cattle industry to reduce poverty may depend on the ability of poor households to participate in this economy sector. By number of animals per farm, Colombian farms present as following: 81% (< 50 heads), 10% (50-100 heads), 8% (101-500 heads) and 1% (> 500 heads).

Despite the fact that bovine *Eimeria* infections occur globally, little is still known on infection dynamics and associated risk factors influencing the outcome of subclinical and/or clinical coccidiosis in subtropical/tropical regions. In order to improve prevention and control of cattle coccidiosis (eimeriosis), large scale epidemiological studies reporting on prevalence, risk factors and infection pressure of pathogenic *Eimeria* species are urgently needed. So far, there are no reports available on the prevalence and species diversity of *Eimeria* with respect to Colombian cattle. For cattle, more than 20 *Eimeria* species are described, so far (Daugschies and Najdrowski, 2005), which differ in terms of pathogenicity and endogenous development. Amongst these, *E. bovis*, *E. zuernii* and *E. alabamensis* are the most pathogenic ones. Due to the development of *E. bovis* and *E. zuernii* macromeronts in host endothelial cells of lymph capillaries of the ileum (> 120.000

66 merozoites I per meront stage (Taubert et al., 2010); massive infections of caecum/colon host epithelial cells
67 resulting in second generation meronts and gamonts will lead to severe haemorrhagic typhlocolitis (Hermosilla
68 et al., 2012). In the past, identification of *Eimeria* species was based on clinical features (i. e. haemorrhagic vs.
69 catarrhalic diarrhoea) and on morphological criteria of sporulated oocysts. More recently, molecular (PCR)
70 techniques have been developed since the former morphological parameters are not fully accurate due to
71 overlapping characteristics (Kawaharaa et al., 2010).

72 Several studies identified management practices and other risk factors enhancing the likelihood of clinical
73 eimeriosis for non-tropical cattle rearing systems (Lassen et al., 2009a and 2009b; Bangoura et al., 2011;
74 Rehman et al., 2011). In general, such conditions which allow faecal contamination of feed and water are
75 considered as high risk, thereby rendering overcrowding and poor sanitation as optimal conditions for infection
76 transmission (Daugschies and Najdrowski, 2005). Consequently, the detailed identification of risk factors for
77 clinical and subclinical coccidiosis in calves of different tropical/subtropical management systems is essential
78 to establish preventive and control measures.

79 This study was conducted in seven regions across the subtropical and tropical Colombian territory from August
80 to December of 2016. It was performed on 1333 faecal samples from calves and young animals with the
81 objective to analyze the prevalence, distribution and risk factors associated with *Eimeria* infections in animals
82 reared with conventional management systems and different production systems, i. e. dairy, meat, or dual
83 purpose.

2. Materials and methods

2.1 Sample collection, study design, questionnaire and animals

In total, 1333 rectally obtained faecal samples from young animals (<1 year of age) were collected at a single time point between August and December 2016. Overall, 55 cattle farms allocated in seven States (i. e. Antioquia, Córdoba, Meta, Eje Cafertero, Arauca, Boyacá, Cundinamarca) of Colombia were sampled (Fig. 1). The distribution of the current samples by type of production system was as follows: 14.5% beef (8/55), 20% dual-purpose (11/55) and 65.5% dairy (36/55).

Calves and young animals were assigned to five different age groups as follows: ≤1, 1-3, 3-6, 6-9 and 9-12 months of age. At least 10 g of faecal samples were processed within 24 h by flotation with saturated sodium chloride solution and examined with a modified McMaster chamber for quantitative determination of *Eimeria* spp. oocysts (sensitivity of 16 oocysts/g). The oocyst number per gram of faeces (OPG) were counted and arbitrarily classified as low (16-1000), medium (1000-5000) and high (> 5000). For species identification, oocysts from each individual sample were allowed to sporulate in 2.5% potassium dichromate under constant oxygenation (Hermosilla et al., 2002). *Eimeria* species were identified based on morphological/morphometric parameters of sporulated oocysts using the taxonomic key described by Floriao et al. (2016).

Additionally, a questionnaire with closed (dichotomous and multiple choice) questions was developed for risk factor analysis. Therefore, information on animals, herd size and herd management practices were collected to determine single risk factors which are associated with the presence of distinct *Eimeria* species as published elsewhere (Thrusfield, 2008, Carrau et al., 2018). In detail, the questionnaire collected data on animal-related factors (e. g. age, breed, body weight, body condition and consistence of faeces), on herd management practices related to coccidiosis, such as general information on the farm (e. g. farm size, access to veterinary services, production type, cattle breed population), information on the occurrence of coccidiosis (e. g. actual cases of symptomatic animals and of clinical coccidiosis observed during the last two years), management factors affecting transmission between herds (e. g. cattle purchase, own animals grazing on foreign pastures, foreign animals grazing on own pastures), and factors regarding housing and hygiene conditions (i. e. existence of a calving area, type of calf housing before weaning, feeding of calves before weaning, spreading of manure on pastures).

112 The choice of farms depended on the willingness of cooperation of the farmers. The current sample size was
113 estimated to represent an entire population of 1.614.906 calves (ICA, 2016), with an expected *Eimeria*
114 prevalence of 70.5%, a 3% standard error and 95% confidence level. Tab. 1 shows the number of farms and
115 animals, and the type of production system for each Colombian State.
116 All animal procedures were performed according to the guidelines of the Ethic Committee for Animal
117 Experimentation, approved by the Institutional Committee for Care and Use of Animals of the University of
118 Antioquia (Act No.105; 2016) in accordance to current Colombian Animal Protection Laws.

119 *2.2 Statistical analysis*

120 Differences on *Eimeria* prevalence in different age groups were evaluated using a Chi square test (SPSS 12.0
121 for Windows, SPSS Inc. Chicago, IL) and $p < 0.05$ was considered as significant. A Kruskall-Wallis one-way
122 analysis of variance was used to compare OPG counts between different age groups and variation is presented
123 as standard error of means. Descriptive statistics were provided for other variables of interest: overall prevalence
124 of infection, prevalence of different *Eimeria* species, coinfection rates, OPG and demographic data.
125 Furthermore, multiple logistic regression models (MLRM) were calculated using generalized mixed model
126 analysis for hierarchical designs (animals were nested within the farm, within the strata as random factors) with
127 the statistical program package R (Free Software Foundation's GNU project, 2016). In the first step of the
128 analysis for each of the independent variables two-way frequency tables were built to describe rough
129 relationships between these variables and different *Eimeria* prevalences. Quantitative independent variables
130 were transformed by common logarithm (lg). In that case, descriptive statistics was given by geometric means
131 and dispersion factors. In the second step, regression analyses were performed to identify risk factors of
132 extensity and intensity of *Eimeria* oocyst excretion. Due to the high number of independent variables a stepwise
133 procedure was applied. Rough association of the variables to *Eimeria* spp. prevalence was analyzed for each
134 variable separately in order to filter the most conspicuous factors. Then, a multifactorial analysis was performed
135 using these variables in a common model. For oocyst excretion intensity, the number of different *Eimeria*
136 species found per sample and the log of total OPG number (without differentiating between species) were here
137 considered. For both variables, a multiple linear regression model was adjusted, again using generalized mixed
138 model analysis for hierarchical designs. Outcomes of statistical tests (Wald tests) were considered to indicate
139 statistical significant effects when $p \leq 0.05$.

3. Results

Sampled farms were located in different climate regions, including subtropical and tropical regions, as illustrated in Fig. 1. The elevation in meters above sea level (masl) of these 55 cattle farms was as follows: 100-500 masl (44%), 1001-2000 masl (6%), > 2000 masl (45%). The management practices within single farms differed according to their type of production system. For beef cattle, management practice was predominated by 'low input-low output systems' in which calves were allowed to graze freely with their dams on premises or were stocked together and dams brought in for lactation twice a day. Specialized dairy cattle farms occurred in highland tropics (≥ 2000 masl) in which calves were either individually raised or stocked together on pasture and exclusively given milk replacers and supplemental feed for 1-2 months. Dual-purpose farm systems occurred in the low tropics (≤ 1200 masl) and in these farms calves were allowed to suck residual milk and grazed with their dams until midday.

Overall, diarrhoea-related calf deaths were reported in 63% of all farms. In line, only 7% of farm owners reported on never observing clinical coccidiosis, while 62% of the farmers recognized clinical coccidiosis outbreaks in last two years and 31% had observed some sporadic cases of clinical coccidiosis, recently. Regarding housing conditions, hutches were used in 85% of the farms with the ground being composed of grass (49%), cement (15%), soil (6%), straw (4%) or combinations of these forms (26%). In 70% of the farms, the drinking water supply for calves originated from own properties thereby lacking any water treatments (non-potable).

3.1 Prevalence of Eimeria spp. *infections*

Eimeria oocysts were detected in 75.5% (1006/1333) of faecal samples, with at least one positive animal in each cattle farm. Taxonomic identification of bovine *Eimeria* species revealed the presence of 13 species in farms from two States Antioquia and Arauca, of 12 species in Córdoba, Cundinamarca, Boyacá and Eje Cafetero, and of 11 species in Meta (Fig. 2). The most prevalent species was *E. bovis* (33.5%), followed by *E. auburnensis* (12.5%) and *E. zuernii* (11.9%). Less prevalent species were *E. pellita* (7.4%), *E. ellipsoidalis* (5.4%), *E. canadensis* (5.3%), *E. wyomingensis* (5.3%), *E. bukidnonensis* (5.3%), *E. brasiliensis* (3.4%),

167 *E. alabamensis* (3.1%), *E. subspherica* (2.9%), *E. cylindrica* (1.6%), and *E. illinoiensis* (0.8%) (Fig. 2).
168 Exemplary images on sporulated *Eimeria* spp. oocysts found in this survey are depicted in Fig. 3.

169 The Colombian state-related distribution of different *Eimeria* species is shown in Fig. 2A, the overall diversity
170 in all samples in Fig. 2B. Infections with a single *Eimeria* species were most frequently detected (26.9%,
171 358/1333), followed by mixed infections with two (15.6%, 208/1333) or three species (6.5%, 86/1333) (Tab.
172 2). When related to age groups, a significant relationship between age and infection rates could be stated with
173 lower infection rates in the neonate group [≤1 month, χ^2 (4 df, n =1333) = 70.2, $p < 0.01$] (Tab. 3). However,
174 when *Eimeria* prevalence was related to age categories of three-month intervals > 1 month of age, no significant
175 differences were observed. Given that the overall prevalence of *Eimeria* spp. infections was quite similar in
176 cattle farms of the same Colombian State, data were pooled by State for ease of data presentation and are shown
177 in Tab. 4 and Fig. 3A.

178 Analyses on oocyst shedding showed a wide range from 16-360,000 OPG in each farm, with an overall median
179 value of 100-200 OPG (Tab. 4). The distribution of low-, medium- and high OPG levels for animals assigned
180 by age classes is presented in Tab. 5. Overall, in all age groups oocyst shedding mainly occurred in the range
181 of 16-1000 OPG. Nevertheless, Chi-square analysis showed that there were significant differences of OPG
182 levels among different age groups [χ^2 (9 df, n =1333) = 98.1, $p < 0.01$] since higher OPG were observed in
183 younger animals (<6 months) when compared to older ones (>6 months). For animals aged ≥ 6 months, only 1-
184 2% showed high OPG levels, few (4-6%) demonstrated medium OPG values, and most (70%) showed low
185 OPG values. In total, 23% of animals ≥ 6 months of age were *Eimeria* spp. negative. However, when overall
186 OPG counts were compared between age groups using Kruskall-Wallis analysis of variance, no significant
187 differences were observed in spite of the apparent large differences in means (± S. E.) (Tab. 5).

188 *3.2 Identification of risk factors associated with* Eimeria *infections*
189 Logistic regression analysis on the presence of *Eimeria* spp., excretion extensity and qualitative risk factors was
190 also performed by MLRM analysis. Respective data on the level of *Eimeria* species are summarized in Tab. 6.
191 Overall, the presence of *Eimeria* stages and corresponding OPG counts showed significant association to the
192 herd size in terms of animal numbers (total number of cows = OR: 0.7; C.I: 0.517 to 1.014), which indicates

193 that a reduced number of animals per farm was considered as protective factor for *Eimeria* infections. For
194 pathogenic *Eimeria* species, we found that *E. bovis*-related OPG counts were significantly associated with the
195 type of housing ground of calves (grass = OR: 1.81; 95% confidence interval (C.I.) 1.138 - 2.88; cement and
196 straw = OR: 2.648 C.I.: 1.382 - 5.075) and the mode of drinking water supply (i. e. potable and non-potable =
197 OR: 2.816; C.I.: 1.338 - 5.923). In the case of *E. zuernii,* the factors associated with its presence were the size
198 of pasture premises (OR: 3.038; C.I.: 1.344 – 6.867) and the presence of floodable zones on pastures (OR:
199 2.226; C.I.: 1. 087 to 4,558). This suggests that the environment was the main factor influencing the presence
200 of bovine coccidiosis for both pathogenic *Eimeria* species. For non-pathogenic *Eimeria* species, some of the
201 factors influencing the occurrence of coccidiosis were the type of food used (i. e. grass, cut grass, concentrated
202 feed) and presence of a veterinarian in the farm.

204 **Discussion**

205 Most studies on bovine coccidiosis emphasize the key role of *E. bovis, E. zuernii* and *E. alabamensis* not only
206 as dominant but also as most pathogenic species. Nonetheless, several other *Eimeria* species frequently occur
207 and thus should not be ignored since they contribute to subclinical coccidiosis. The biodiversity of bovine
208 *Eimeria* species affecting cattle often differs between geographic regions of a country (Daugschies and
209 Najdrowki, 2005, Tomczuk et al. 2015) as also described for small ruminant coccidian infections (Catchpole et
210 al., 1976; Carrau et al., 2018). So far, no epidemiological data are available on bovine coccidiosis in tropical
211 and subtropical regions of Colombia. To our best knowledge, this is the first large-scale epidemiological survey
212 on bovine *Eimeria* spp. infections and related risk factors.

213 Mean prevalence data confirmed *Eimeria* spp. infections as a frequent intestinal disease of cattle in
214 subtropical/tropical regions of seven States of Colombia, since an overall herd-prevalence of 75.5% was
215 estimated. This is in line with other epidemiological studies on bovine coccidiosis worldwide which also stated
216 an *Eimeria* prevalence of > 70% thereby emphasizing the relevance of coccidiosis in dairy and beef cattle
217 industry (Faber et al., 2002; Daugschies and Najdrowki, 2005; von Samson-Himmelstjerna et al., 2006; Koutny
218 et al., 2012, Tomczuk et al. 2015). However, some studies from other tropical/subtropical regions showed lower
219 prevalences, such as 33.2% in India (Das et al., 2015) and 60.7% in Pakistan (Rehman et al., 2011). Since faecal

samples were taken from a randomly selected young animal population (n = 1333) of 55 farms in seven Colombian States, it can be assumed that *Eimeria* infections are widely spread in the country and may play important role as underestimated subclinical or clinical disease affecting growth rate performance. We found 26.9% (n = 358) samples with single *Eimeria* species infections and 26.45% (n = 352) with mixed infections. Surprisingly, the actual impact of *Eimeria* spp. co-infections on calf performance remains uncertain since diarrhoea was only found associated with either total OPG counts or single *E. zuernii* or *E. bovis* infections (Hermosilla et al., 1999; Bangoura and Daugschies, 2007; Bangoura et al., 2011; Enemark et al., 2013). Thus, experimental infections with *E. zuernii* consistently caused diarrhoea accompanied by reduced body weight gains and haemoconcentration (Bangoura and Daugschies, 2007). Although oocyst shedding does not correlate well to the degree of clinical disease, a correlation between diarrhoea and OPG ≥ 500 was demonstrated in case of *E. bovis* and *E. zuernii* (Bangoura et al., 2011), which are the two most commonly reported species worldwide (Faber et al., 2002; Dong et al., 2012; Rehman et al., 2011; Kennedy and Kralka, 1987; Lucas et al., 2014, Tomczuk et al. 2015). Consequently, the differentiation between pathogenic and non-pathogenic *Eimeria* species is necessary better pre-estimate the outcome of infection (Daugschies and Najdrowki, 2005). In total 16.9% of all examined animals were infected with one or both of these species and indeed excreted ≥ 500 OPG values and thereby most likely suffering from production losses due to clinical or subclinical coccidiosis as postulated elsewhere (Hermosilla et al., 2002; Faber et al., 2002; Daugschies and Najdrowki, 2005; Taubert et al., 2010).

The number of different *Eimeria* species present in mixed infections ranged from two to eight. This finding correlates to data from Ethiopia (Abebe et al., 2008), USA (Ernst et al., 1987), The Netherlands (Cornelissen et al., 1995) and in Turkey (Arslan and Tuzer, 1998). In line to data from the Czech Republic (Chroust, 2000), the current study also showed that *E. bovis* (33.5%) was the most prevalent species followed by *E. auburnensis* (12.5%) and *E. zuernii* (11.9%) in Colombian animals. *E. bovis* and *E. zuernii* are the most frequently reported species during outbreaks of clinical coccidiosis (Deplazes et al., 2016; Waruiru et al., 2000; Faber et al., 2002; Speer, 1999; Lopez-Osorio et al., 2018) and previously been reported in South America (Reboucas et al., 1994). In line to current findings, >30% and 7-23% of Brazilian cattle herds were found infected with *E. bovis* and *E. zuernii*, respectively (Reboucas et al., 1994; Almeida et al., 2011; Bruhn et al., 2011, 2012; Tosi Cardim et al., 2018).

It is well known that young animals are more susceptible to *Eimeria* infections than adults based on the lack of protective immunity at young age (Hermosilla et al., 1999, 2002, 2012; Taubert et al., 2008, 2009, 2010). Especially calves exposed to low doses of oocysts are reported to develop protective adaptive immunity against homologous *Eimeria* species (Hermosilla et al., 1999; Sühwold et al., 2008; Taubert et al., 2010) resulting in reduced oocyst shedding and clinical manifestations (Sanchez et al., 2008; Rind et al., 2007). In contrast to other reports (Dong et al., 2012; Daugschies and Najdrowki, 2005), the large variation in OPG counts present in all age categories prevented any age-related statistical differences of OPG counts in this study. In general, current OPG counts were within the range recorded for naturally but subclinical infected animals (Lucas et al., 2014; Rehman et al., 2011; Klockiewicz et al., 2007). The majority of other surveys have shown age-related differences in *Eimeria* prevalence (Gorsich et al., 2014; Rehman et al., 2011), a finding which we could only confirm for the comparison of neonate (< 1 month) vs. animals older than 2 months.

The identification of risk factors related to the occurrence of cattle coccidiosis is important to prevent clinical infections. In this study we identified factors related to management and husbandry practices that influence the occurrence of infections, such as non-potable water (for *E. bovis, E. canadensis, E. subspherica*), floor type (for *E. bovis*), herd size (for *E. subspherica*), type of food (for *E. alabamensis, E. canadensis, E. cylindrica*), source of colostrum (for *E. alabamensis*) and floodable zones and size of the pastures (for *E. zuernii*). More importantly, the herd size represented a key factor for *Eimeria* spp. infections in calves, meaning that small herds (< 50 animals) are less affected by these parasites than large herds. Consistently, Klockiewicz et al. (2007) reported that highly pathogenic *Eimeria* species occurred more frequently in large than in small cattle farms. Additionally, Kusiluka et al. (1998) concluded that small herd sizes show lower environmental contamination than larger ones. Furthermore, McKellar (2008) stated that clinical coccidiosis is more prevalent under poor sanitation conditions, bad nutrition and overcrowding. In the current study the type of flooring had a marked influence on the presence of *E. bovis* which is in agreement to former findings (Gräfner et al., 1978, 1985). Thus, keeping calves on cement floors with embedded straw revealed critical for coccidiosis outbreaks (Gräfner et al., 1978, 1985). Generally higher oocyst contamination of shed floors compared to smooth floors are most likely due to suboptimal cleaning options and optimal humidity and temperature conditions for oocyst sporulation and survival in strawbeds. In line, Gulliksen et al. (2009) determined a lower incidence of diarrhoea in calves kept on slatted floors and Rehman et al. (2011) recorded higher *Eimeria* prevalence in animals kept

276 on non-cemented floors. Ernst et al. (1987) concluded that clinical coccidiosis bovine is more common in
277 housed animals than in those on pastures. However, in the case of *E. zuernii*, Colombian animals kept on
278 pastures with floodable zones had a higher probability for coccidiosis, which may be due to higher oocyst
279 contamination at point sources, such as around limited food and water sources, leading to host and oocyst
280 concentration at restricted areas (Rehman et al., 2011). Identified risk factors associated with clinical/subclinical
281 coccidiosis outbreaks include higher risk of infection when calves feed at ground level and/or drink from pond
282 water (Rehman et al., 2011). Apart from husbandry practices mentioned above, other factors were reported to
283 increase *Eimeria* prevalence, such as season (wet > dry season; Waruiru et al., 2000), temperature (warm >
284 cold; Makau et al., 2017), size of herds (larger > smaller; Klockiewiez et al.,2007; Chibunda et al., 1997) and
285 stocking density (Sanchez et al., 2008).

286 In conclusion, this study revealed that *Eimeria* infections frequently occur in Colombian calves/young animals
287 regardless of the type of production system. Given that clinical and subclinical *Eimeria* infections are well-
288 known to dampen bovine production parameters, regular monitoring, including diagnosis of species biodiversity
289 and metaphylactic treatments could help to prevent in future *Eimeria*-induced economic losses in Colombian
290 cattle rearing.

292 **Conflict of interest**

293 Authors declare that research was conducted in absence of any commercial or financial relationships that could
294 be a potential conflict of interest.

295

296 **Authors and contributors**

297 SL, JC and DV designed, planed and coordinated study. SL carried out the sampling and processing of the
298 samples; SL, DV, JC, CH and AT drafted and edited the manuscript. FK carried out the statistical analysis of
299 the data. All authors have read and approved the manuscript.

300

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Tab. 1. Number of calves registered in Colombian states and number of sampled farms and animals.

Department	total No. of animals *	No. of farms tested	No. of animals tested	Production type		
				Dairy	Beef	Dairy + Beef
Antioquia	461,883	18	338	78%	5%	17%
Córdoba	345,829	9	322	11%	33%	56%
Meta	139,862	8	190	75%	-	25%
Eje cafetero	82,517	3	28	100%	-	-
Arauca	174,307	6	193	-	50%	50%
Boyacá	133,824	4	136	100%	-	-
Cundinamarca	276,684	7	126	100%	-	-
TOTAL	**1,614,906**	**55**	**1333**			

*< 1 year of age (ICA 2016)

Tab. 2. Number of *Eimeria* species present in individual fecal samples.

# *Eimeria* species	0	1	2	3	4	5	6	7	8	UND*	Total
# calves	327	358	208	86	28	20	7	1	2	296	**1333**
Percentage (%)	24.5	26.9	15.6	6.5	2.1	1.5	0.5	0.1	0.2	22.2	**100.0**

*UND: Undetermined since oocyst failed to sporulate

Tab. 3. *Eimeria* spp. infections in different age groups of naturally infected cattle

Age (months)	% positive animals	*Eimeria*-infected animals		No. of animals
		+	-	
0-1	50.6[a]	86	84	170
2-3	75.7	240	77	317
4-6	82.5	349	74	423
7-9	79.3	191	50	241
10-12	76.9	140	42	182
				1333

[a] Column data marked with the different superscript show significant differences ($P < 0.05$)

Tab. 4. *Eimeria* spp. prevalence in calves from each state of Colombia.

State	Prevalence (%)	# *Eimeria* species	Median	OPG	
				min	max
Antioquia	78.0 (262/336)	13	80	16	230400
Córdoba	72.8 (235/323)	12	64	16	78864
Cundinamarca	71.4 (90/126)	12	192	16	362880
Meta	74.7 (142/190)	11	64	16	194400
Arauca	81.4 (157/193)	13	144	16	153216
Boyacá	69.1 (94/136)	12	48	16	124992
Eje cafetero	92.9 (26/28)	12	152	16	4208

Tab. 5. Age group-related OPGs in Colombian calves

Range of OPG	Age (0-3 months)		(> 3-6 months)		(> 6-9 months)		(> 9-12 months)		Total (n = 1333)	
	#	%	#	%	#	%	#	%	#	%
Negative	161	33.1	74	17.5	50	20.7	42	23.1	327	24.5
16-1000	224	46.0	292	69.0	177	73.4	125	68.7	818	61.4
1001-5000	56	11.5	44	10.4	11	4.6	11	6.0	122	9.2
> 5000	46	3.1	13	3.1	3	1.2	4	2.2	66	5.0
mean OPG	4,742 ± 1,123		1,325 ± 410		399 ± 107		414 ± 110		2281 ± 434	

Tab. 6. Multiple Logistic Regression Model for the *Eimeria* spp. presence in Colombia

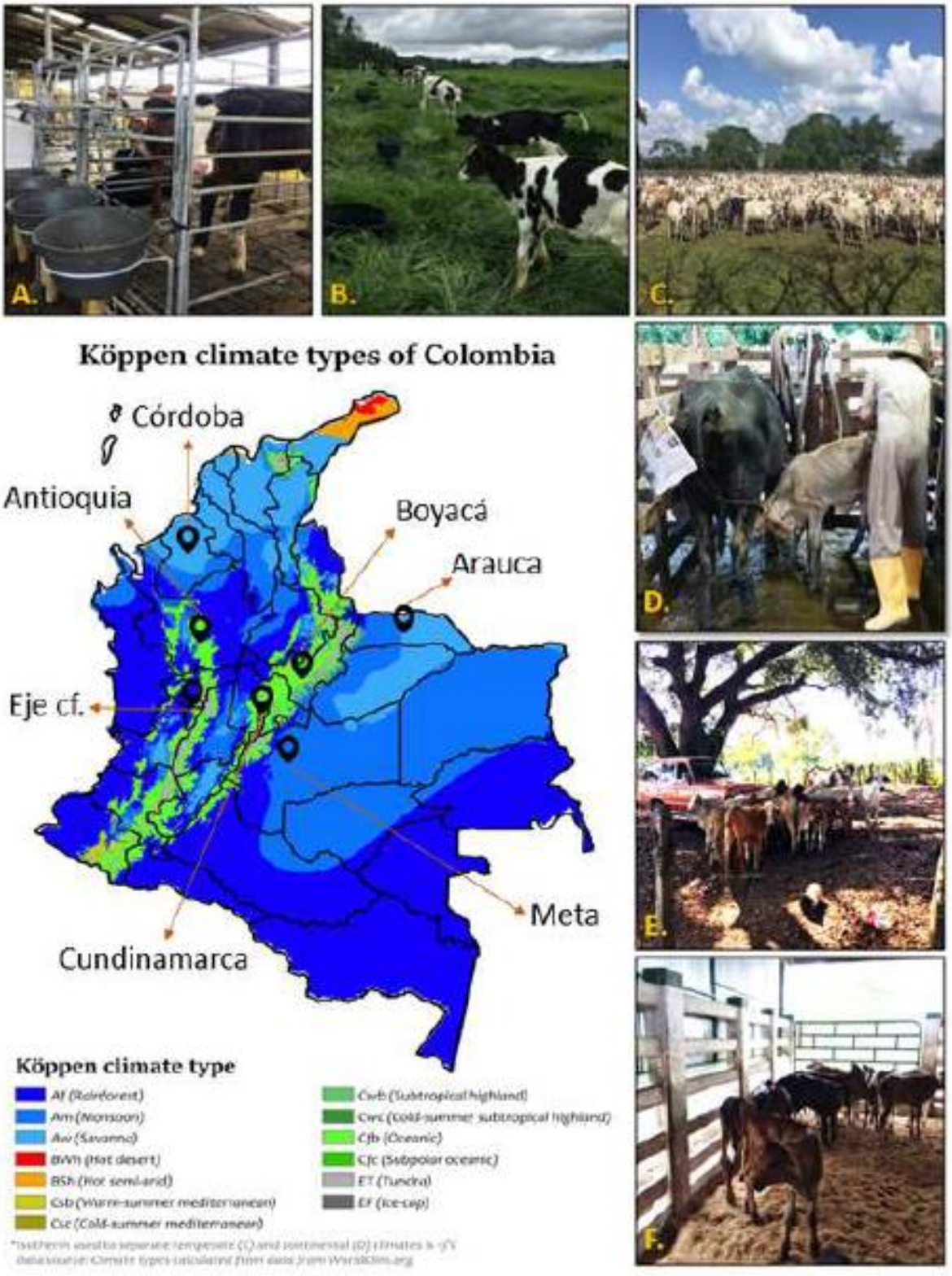
Independent variable	Variable	Std. Error	z value	Pr(>|z|)	OR	95 % CI of the OR lower limit	upper limit
Eimeria spp	total cow number/farm	0.172	-1.880	0.050	0.724	0.517	1.014
Eimeria OPG	weight of calves	0.001	-2.608	0.009	0.999	0.998	1.000
E. alabamensis	colostrum: Bank	1.033	-2.881	0.004	0.051	0.007	0.386
	food: Grass, cutted grass, concentrated	0.614	-2.770	0.006	0.183	0.055	0.608
E. bovis	floor: grass only	0.237	2.504	0.012	1.810	1.138	2.880
	floor: cement and straw	0.332	2.935	0.003	2.648	1.382	5.075
	water: non potable and potable	0.379	2.728	0.006	2.816	1.338	5.923
E. canadensis	water: non potable	0.482	2.410	0.016	3.197	1.242	8.226
	water: non potable and potable	0.708	2.146	0.032	4.565	1.141	18.272
	food: Grass, cutted grass, concentrated	0.531	-2.852	0.004	0.220	0.078	0.623
E. cylindrica	food: Grass, cutted grass, concentrated	1.533	-1.880	0.060	0.056	0.003	1.130
E. illinoinensis	no veterinary	0.909	-2.444	0.015	0.108	0.018	0.644
E. subspherica	total cow number/farm	0.518	2.116	0.034	2.995	1.084	8.272
	water: non potable and potable	1.100	1.902	0.057	8.113	0.939	70.117
E. zuernii	floodable zones	0.366	2.188	0.029	2.226	1.087	4.558
	size of pastures	0.416	2.670	0.008	3.038	1.344	6.867

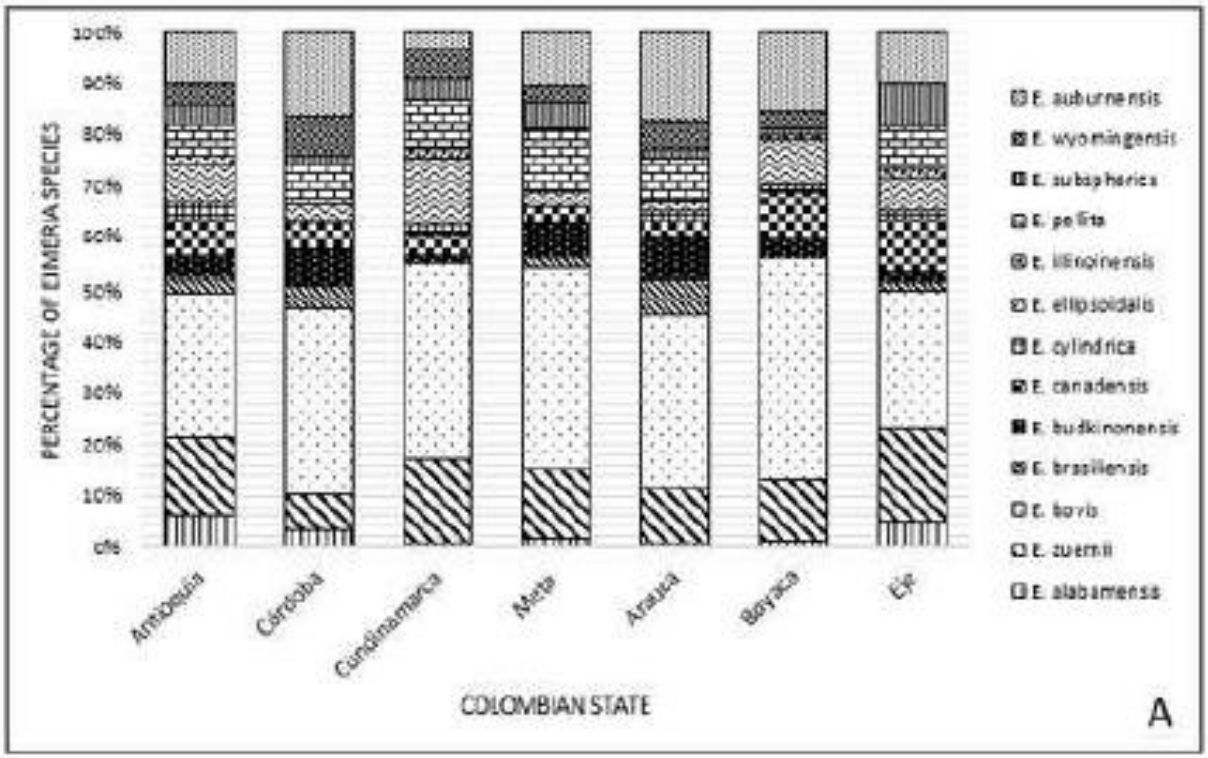
Figure legends

Fig. 1. Exemplary illustration of typical Colombian cattle management systems (A-F) and illustration of sampling areas (G). (A) Milk and beef production farm in Cordoba. (B) Milk production farm in Antioquia. (C) Milk production farm in Boyacá. (D) Beef production farm in Arauca. (E) Milk and beef production farm in Arauca. (F) Milk and beef production farm in Meta. Map modified from Koppen climate Map (Beck et al., 2018)

Fig. 2. Regional (A) and mean (B) diversity of bovine *Eimeria* species in Colombian cattle.

Fig. 3. Exemplary illustration of *Eimeria* spp. oocyst morphology (×1000 magnification). Scale bar 10 µm.


A.
B.
C.
D.
E.
F.
Köppen climate types of Colombia
Córdoba
Antioquia
Boyacá
Arauca
Eje cf.
Meta
Cundinamarca
Köppen climate type
Af (Rainforest)
Am (Monsoon)
Aw (Savanna)
BWh (Hot desert)
BSh (Hot semi-arid)
Csb (Warm-summer mediterranean)
Csc (Cold-summer mediterranean)
Cwb (Subtropical highland)
Cwc (Cold-summer subtropical highland)
Cfb (Oceanic)
Cfc (Subpolar oceanic)
ET (Tundra)
Ef (Ice-cap)


PERCENTAGE OF EIMERIA SPECIES
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Antioquia
Córdoba
Cundinamarca
Meta
Arauca
Boyaca
Cal
COLOMBIAN STATE
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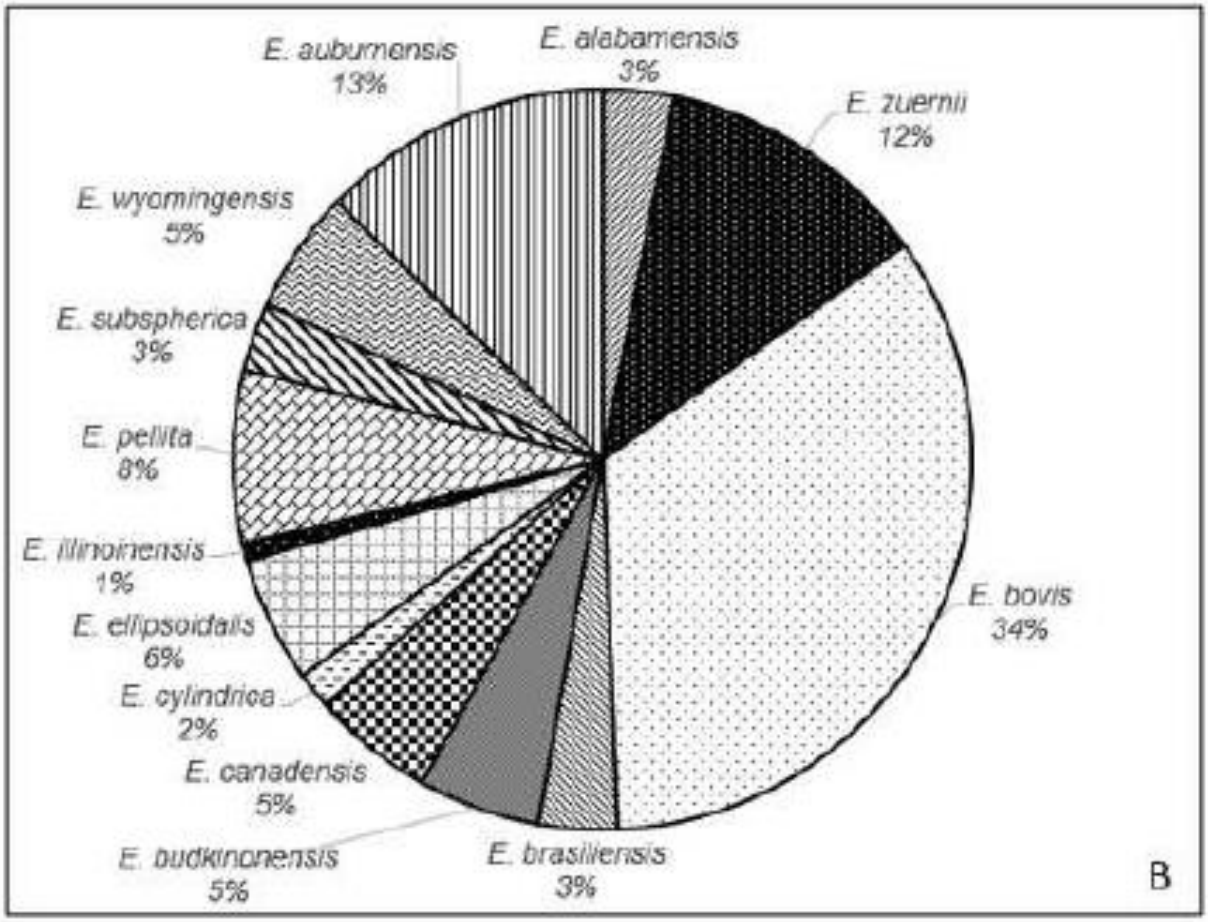
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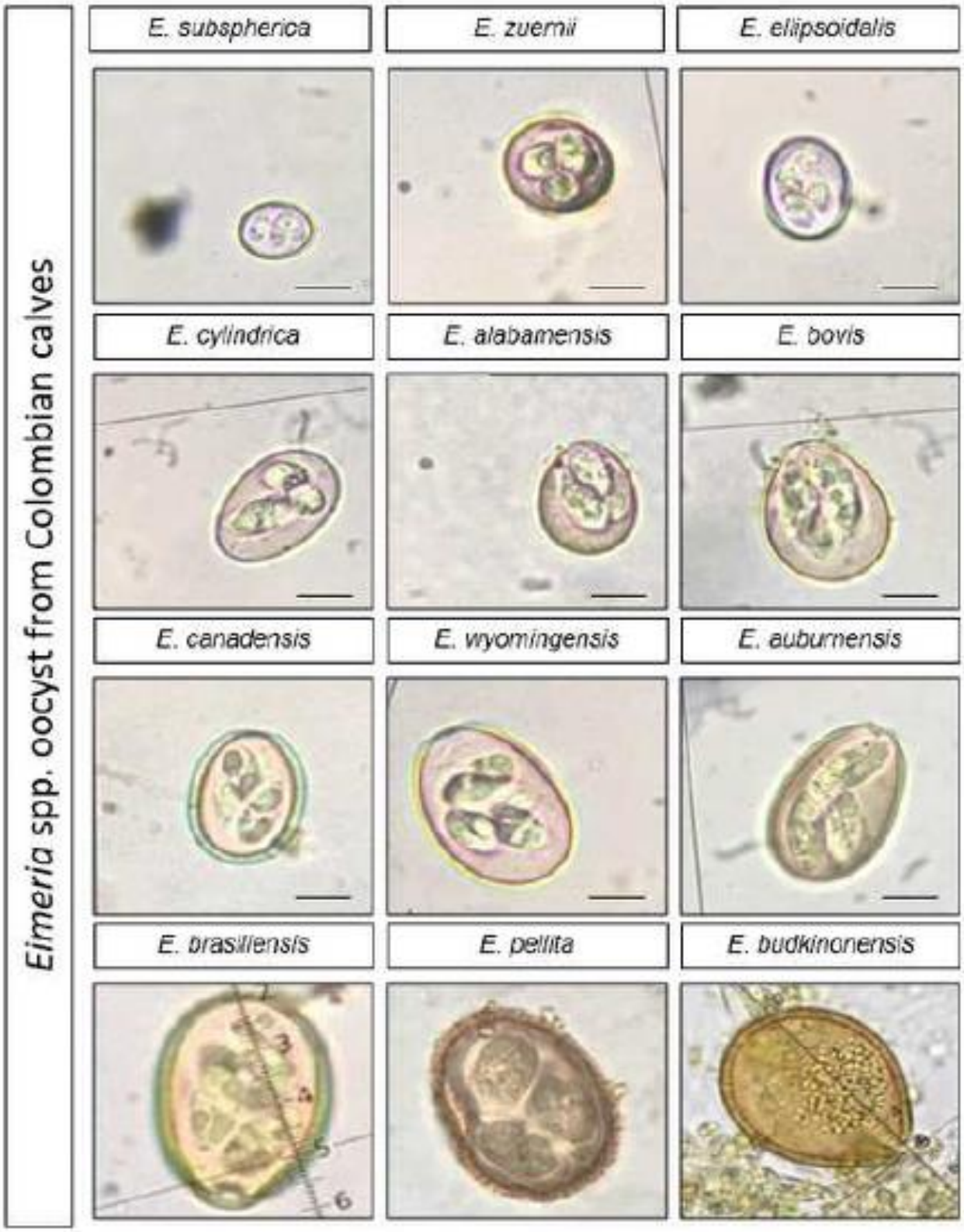
Eimeria spp. oocyst from Colombian calves
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E. wyomingensis
E. auburnensis
E. brasiliensis
E. pellita
E. budkinonensis

4. Chapter: Optimized excystation protocol for ruminant *Eimeria* spp. sporulated oocysts (Apicomplexa, Coccidia)

This chapter is based on the following summited paper:

López-Osorio S, Silva LMR, Velazquez ZD, Taubert A, Hermosilla C (2019) Optimized excystation protocol for ruminant *Eimeria* spp. sporulated oocysts (Apicomplexa, Coccidia) *(manuscript summited)*

Own contribution in the publication
Initiative: essential
Project planning plan: essential
Carrying out the experiment: essential
Evaluation de experiment: as far as possible
Creation of the publication: as far as possible

Optimized excystation protocol for ruminant *Eimeria bovis*- and *Eimeria arloingi*-sporulated oocysts and first 3D holotomographic microscopy analysis of differing sporozoite egress

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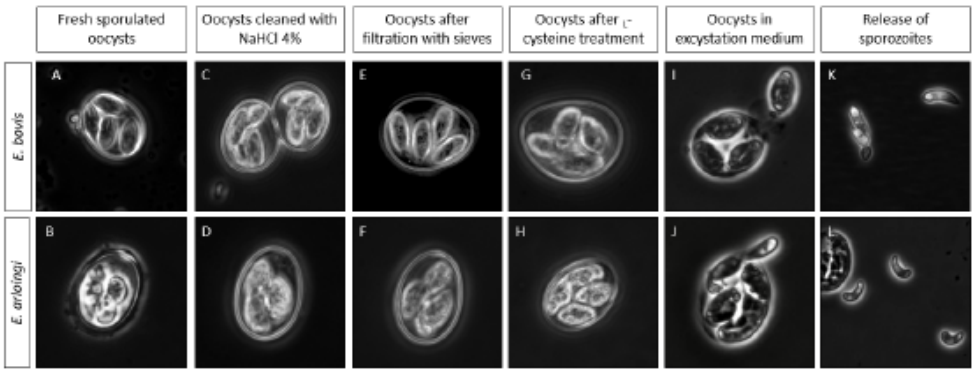
Graphical abstract



Highlights

- Present study provides an improved excystation protocol to obtain viable sporozoites from ruminants *Eimeria* spp.
- The improved method is cheaper, faster, and accessible for all labs with minimum equipment, without the requirement of expensive reagents or instruments.
- By using the novel sieve-based protocol, we achieved a higher excystation rate, which reduced the initial number of oocysts per sporozoites needed.

Abstract

Successful excystation of sporulated *Eimeria* spp. oocysts is an important step to acquire large numbers of viable sporozoites for molecular, biochemical, immunological and *in vitro* experiments for detailed studies on complex host cell-parasite interactions. An improved method for excystation of sporulated oocysts and collection of infective *E. bovis*- and *E. arloingi*-sporozoites is here described. *Eimeria* spp. oocysts were treated for at least 20 h with sterile 0.02M $_{L}$-cysteine HCl/0.2M $NaHCO_3$ solution at 37 °C in 100% CO_2 atmosphere. The last oocyst treatment was performed with a 0.4% trypsin/8% sterile bovine bile excystation solution, which disrupted oocyst walls with consequent activation of sporozoites within oocyst circumplasm, thereby releasing up to 90% of sporozoites in approximately 2 h of incubation (37 °C) with a 1:3 (oocysts:sporozoites) ratio. Free-released sporozoites were filtered in order to remove rests of oocysts, sporocysts and non-sporulated oocysts. Furthermore, live cell imaging 3D holotomographic microscopy (Nanolive®) analysis allowed visualization of differing sporozoite egress strategies.Sporozoites of both species were up to 99% viable, highly motile, capable of active host cell invasion and further development into trophozoite- as well as macroment-development in primary bovine umbilical vein endothelial cells (BUVEC). Sporozoites obtained by this new excystation protocol were cleaner at the time point of exposure of BUVEC monolayers and thus benefiting from the non-activation status of these highly immunocompetent cells through debris. Alongside, this protocol improved former described methods by being is less expensive, faster, accessible for all labs with minimum equipment, and without requirement of neither expensive buffer solutions nor sophisticated instruments such as ultracentrifuges.

Keywords: *Eimeria bovis*, *Eimeria arloingi*, sporulated oocysts, excystation, sporozoites, endothelial cells

1. Introduction

Eimeria is an apicomplexan parasite genus with a monoxenous life cycle, which requires the natural specific host animal for its propagation. In the case of ruminant *Eimeria* spp. (e. g. cattle, goats, sheep, cervids) it is still difficult and expensive to experimentally infect high animal numbers for replication of these monoxenous parasite species, which increases the need of an effective excystation protocol resulting in high excystation rates of sporozoites with reduced oocyst numbers [1] . Therefore, a considerable amount of viable sporozoites [2] can be isolated and applied for detailed and complex host-parasite as well as host cell-parasite interactions studies *in vitro* [3-4].

In contrast to avian *Eimeria* species, the excystation process for *Eimeria* spp. infecting mammalian species usually dispense from a mechanical step to release adequate sporocysts and sporozoites from oocysts [5]. According to the host species, different enzymes are necessary to disrupt the oocyst wall and liberate the sporocysts and sporozoites [2]. Hence, this physiological fact should be taken into account in order to achieve a successful *in vitro* excystation rate of viable and infective sporozoites. Previous reports on *Eimeria* excystation protocols intended to mimic intestinal environment *in vivo* of in order to achieve sporozoite release. As such, the stomach environment of rats (*Rattus norvegicus*) was mimic to successfully excyst *Eimeria separata* oocysts with ease [2], while susceptibility of proteases was considered in case of rodent *E. falciformis* and rabbit *E. stiedae* [6].

In case of ruminant *Eimeria* species, first description of a successful *in vitro* excystation protocol was published by Nyberc and Hammond (1964) [7], which used a combination of mechanical (beads) and enzymatic (trypsin, steapsin and bovine bile) oocyst disruption. Later on, Hermosilla et al. (2002) [8] improved this protocol for bovine *E. bovis* by avoiding the usage of mechanical

disruption, by using discontinuous Percoll gradients and thus consequent impairment of recovered sporozoites. In this former method, a 60% Percoll gradient was used to purify oocysts and eliminate a vast amount of faecal debris resultant from oocyst isolation process [3, 9]. This enzymatic excystation protocol has successfully been extended to other ruminant *Eimeria* species: bovine *E. zuernii* [10], caprine *E. ninakohlyakimovae* and *E. arloingi* [11-14] and ovine *E. ovinoidalis* [15].

Although this enzymatic protocol simulating intestinal *in vivo* conditions allows recovery of viable sporozoites, many oocysts are lost during the purification step with Percoll gradients, leading to an increase of the initial amount of oocysts required for each experiment, and which in turn requires more experimental animals for parasite propagation, generating an increase in the costs of *Eimeria*-related investigations.

Highly enteropathogenic ruminant *Eimeria* species often resulting in haemorrhagic coccidiosis, i. e. *E. bovis* and *E. zuernii* (cattle), *E. arloingi* and *E. ninakohlyakimovae* (goat), and *E. ovinoidalis* (sheep), have the peculiarity of replicating in host endothelial cells of lymphatic vessels during their first merogony *in vivo* [8, 10, 11, 13, 15], and thereby differing form most non-pathogenic species proliferating in intestinal epithelial cells [3, 4]. Host endothelial cells are highly immunoreactive cells [16] which might become activated with ease in presence of debris or oocyst-/sporocyst-wall detritus hampering proper *in vitro* sporozoite development as previously discussed [17].

Here, an improved excystation protocol based on the previous enzymatic method firstly described by Hermosilla et al. (2002) [8] is reported for excystation of ruminant *E. bovis* and *E. arloingi*, which are the most prevalent and pathogenic species of *Eimeria* in bovine and caprine, respectively. Furthermore, live cell imaging 3D holotomographic microscopy (Nanolive®)

analysis allowed visualization of differing sporozoite egress strategies, i. e. sporozoite egress from sporocysts within oocyst circumplasm and thereafter through oocyst micropyle (*E. arloingi*) or via rupture of internal oocyst wall, subsequent sporocyst liberation and external sporozoite egress from sporocyst (*E. bovis*). Particularly in case of ovine, caprine and camelid coccidiosis there is still an urgent need not only for suitable *in vitro* culture systems but also for proper oocysts excystation protocols.

2. Materials and methods

2.1. Parasites

For oocyst production, two 8-weeks-old calves, kept in isolation conditions without *Eimeria* spp. exposure, were infected orally with 3×10^4 sporulated *E. bovis* oocysts (strain H). The same procedure was performed for *E. arloingi* replication with 8-weeks-old goat kids, with an infection dose of 1×10^4 sporulated *E. arloingi* (strain A) oocysts. Then, oocysts were isolated from faeces beginning at 18-20 days p. i. (*E. bovis*) and 22 d. p. i (*E. arloingi*), according to Jackson, 1964 [9]. The faeces were washed through a set of three metal sieves (pore sizes 850, 250 and 80 µm; Retsch ®) with tap water. The final suspension was let to sediment overnight. The sediment was then mixed 1:1 with saturated sucrose solution ($\rho = 1.3$ g/mL) to a final density of 1.15 g/mL. The suspension was transferred into plastic trays (30 × 20 × 5 cm) horizontally adjusted. The trays were filled to the top and were covered with clean glass plates, allowing complete contact of the suspension with the surface of the glass following Jackson (1964) [9]. Every 4 h the glasses were carefully removed and adherent oocysts were washed off with tap

122 water into a container. The remaining suspension in the plastic trays was stirred up and the
123 process was repeated up to six times or until few oocysts were left (microscopic examination,
124 less than 5 oocysts per power vision field at 20 x magnification). The oocysts collected were
125 diluted with tap water (1:1) and then centrifuged at 600 × *g*, 12 min. The pellet was resuspended
126 in potassium dichromate (Merck) solution (final concentration 2%, *w*/*v*), at room temperature
127 (RT), with constant aeration until the oocysts were sporulated. After 90% of the oocysts have
128 completely become sporulated, suspension was centrifuged (600 × *g*, 12 min) and sediment
129 containing oocysts was suspended in fresh 2% (*w*/*v*) potassium dichromate (Merck) solution and
130 stored at 4 °C until further use [2, 8].
131
132 **2.2 Excystation processes**
133 The previous described gradient-based excystation protocol [8] was compared with a novel
134 filtering excystation process (sieve-based protocol) as following:
135
136 a) Cleaning of oocysts suspension
137 A total of 6.25 ×10⁶ sporulated oocysts of *E. bovis* or *E. arloingi* were centrifuged at 600 × *g* for
138 12 min to remove the potassium dichromate. Then, the pellet containing oocysts was
139 resuspended in a 4% (*v*/*v*) sodium hypochlorite solution (1:9) and magnetically stirred on ice for
140 20 min, in order to eliminate all debris attached to oocyst walls. Then, oocysts were mixed by
141 vortex for 15 s and, thereafter, centrifuged again (300 × *g*, 5 min) to remove debris. The
142 supernatant containing oocysts was collected and mixed with distilled water (1:1) and pelleted
143 (600 × *g*, 15 min).
144

b) Purification of oocysts

Oocysts pellet was diluted in distilled water and then passed through a 40 µm sieve (PluriSelect), to eliminate debris larger than the oocysts. Oocysts were then filtered with a 10 µm sieve (*E. bovis*) or 5 µm sieve (*E. arloingi*) (both PluriSelect), and thereafter washed with 50 mL bidestilled water to remove all the small particles (the oocysts being retained by this sieve). The sieve was inverted and oocysts were collected after several washes of the filter mesh with distilled water. The oocysts were centrifuged at (600 × *g*, 15 min).

c) Degradation of oocyst wall and enzymatic digestion of oocyst wall

Oocysts were suspended in fresh sterile filtered (0.2 µm filter, Sarstedt) 0.02M L-cysteine HCl-H_2O/0.2M $NaHCO_3$ (Merk) solution and incubated for 20 h at 37 °C in a 100% CO_2 atmosphere in a T75 cell culture flask (closed lid, Sarstedt). After the incubation, oocysts were collected and centrifuged at 600 × *g*, 15 min. Thereafter, oocysts were suspended in sterile filtered (0.2 µm filter, Sarstedt) excystation solution [Hank's balanced salt solution (HBSS, Gibco) supplemented with 0.4% (*w*/*v*) trypsin (Sigma-Aldrich) and 8% (*v*/*v*) bovine bile (obtained fresh from a local slaughter every 3-4 weeks and kept at -20 °C)] up to 2 h at 37 °C in a 5% CO_2 atmosphere. Every hour, excystation process was controlled using an inverted microscope (IX81, Olympus®) and the number of free sporozoites was determined to estimate the excystation rate. Additionally, live cells imaging 3D holotomographic microscopy (Nanolive®) analysis was conducted in order to gain novel insight data into sporozoite egress process of these ruminant *Eimeria* species.

e) Collection and preparation of the sporozoites

Free sporozoites were collected (600 × *g*, 15 min) and filtered with a 5 µm sieve (PluriSelect) to remove remaining oocysts and sporocysts. Then, sporozoites were washed twice with modified endothelial cell growth medium [ECGM (PromoCell) diluted in M199 medium (Gibco) (3:7), supplemented with 1% penicillin-streptomycin (both Sigma-Aldrich) and 5% foetal calf serum (FCS, Biochrome)] to remove any traces of excystation medium, and thereafter counted in a Neubauer chamber. Viability of the sporozoites was calculated using trypan blue 0.04%. Viable sporozoites of *E. bovis* and *E. arloingi* were exposed to host cells *in vitro* for testing of further intracellular development.

2.3 Host cells

Primary bovine umbilical vein endothelial cells (BUVEC) used in this study were isolated followed the methodology described in detail previously by Taubert et al. (2010) [3]. Three different BUVEC isolates were used for host endothelial cell infection experiments. BUVEC isolates (n = 3) were seeded in two 25 cm^2 cell tissue culture plastic flasks (Greiner) and maintained in modified ECGM (PromoCell) supplemented with 5% FCS (*E. bovis*), or ECGM (PromoCell) 1% penicillin-streptomycin (both Sigma-Aldrich) supplemented with 10 mM glucose (*E. arloingi*).

2.3 Host cell infection

BUVEC monolayers with 90% confluency were infected with 5×10^5 freshly isolated sporozoites of *E. bovis* or *E. arloingi*. Culture medium was changed 24 h after sporozoite infection and thereafter every two days. Using inverted microscope (IX81, Olympus®), *Eimeria*-infected host cells were evaluated daily for follow-up of parasite development. The number of *Eimeria*-

infected host endothelial cells was calculated by counting at least 3 different 400-fold magnification power vision fields at 24 h p. i. and at 4 d p. i.

2.4 Live cell imaging 3D holotomographic microscopy analysis

Oocyst from different stages of excystation protocol were collected and seeded in a 35 mm imaging dish (IBIDI ®) inside IBIDI® Stage Top Incubation System at 5% CO_2. Oocyst were followed using Live 3D Cell Explorer® (Nanolive®) to explore instantly live excystation in 3D without any labeling or chemical marker (60x magnification and depth of field 30 µm). Images were analyzed using STEVE® software (Nanolive®) to obtain a refractive index-based z-stack. Images were also digitally stained based on the cell physical refractive index using STEVE® Software (Nanolive®). Image processing was done with Fiji ImageJ® using Z-projection being restricted to overall adjustment of brightness and contrast. Additionally, before and after every step, oocysts were visually controlled with an inverted microscope (IX81, Olympus®) in order to observe changes in oocyst/sporocyst morphology during sporozoite egress.

3. Results

Recovery of viable and infective sporozoites of *E. bovis* and *E. arloingi* was achieved with both excystation protocols, i. e. with gradient and sieves, respectively. At the beginning of the procedure sporulated oocysts presented debris attached to their walls (Fig. 1A-B). After incubation in 4% (v/v) sodium hypochlorite solution, oocysts became cleaner (Fig. 1C-D) and without visible alterations of oocyst wall. Subsequently, oocysts were purified by using either gradient- [8] or sieve-protocol. After the passages through sieves, the oocyst walls were intact without any detectable changes (Fig. 1E-F). For control purposes, debris were collected and

controlled for oocyst presence. Following oocyst incubation in sterile L-cysteine medium, oocyst walls became thinner and deformed (refer to Fig. 1G-H). Sporozoites egression (Fig. 1K-L; Video S1) took place during incubation in excystation medium containing trypsin and bovine bile, both from free sporocysts (Fig. 1I, *E. bovis*) or sporocysts still within circumplasm (Fig. 1J, *E. arloingi)*. Before egression, sporozoites became very active and presented typical gliding movements (Video S2). Additionally, in some cases sporocysts residual bodies were observed as small and spherical granules inside either sporocysts or oocysts (Video S2). The first sporozoites were free after 30 min of incubation, and the maximum amount of free-released sporozoites was observed after 120 min of incubation. It is noteworthy that release of sporozoites from oocysts of both *Eimeria* species was different. In the case of *E. bovis* oocysts (Fig. 1I;1K), sporocysts were firstly released from the oocysts and then sporozoites egressed from sporocysts thereafter. On the contrary, *E. arloingi* (Fig. 1J, L) sporozoites were released from the sporocyst inside oocyst circumplasm and moving afterwards towards micropyle to escape through this structure into exogenous space (please refer to Fig. 1J; Video S2).

Sporozoites of both species were viable and motile (Video S1, Video S2). Sporozoites exhibited smooth gliding movement, twirling, twisting, and typical flexion of their banana-shaped bodies. The gradient protocol resulted in a lower recovery rate of sporozoites when compared to the sieves protocol (Table 1). Using this improved protocol (sieves protocol) more than 19 million *E. bovis*- and 5.9 million *E. arloingi*-sporozoites were collected from oocysts with a 90% sporulation rate. The ratio sporozoite:oocyst (number of sporozoites obtained from each oocyst) was 3:1 for *E. bovis* and 2.6:1 for *E. arloingi*. Almost all sporozoites (99%) were vital and capable of *in vitro* BUVEC monolayer infection. The infection rate in BUVEC were 40% and 21% for *E. bovis* and *E. arloingi*, respectively (Table 1). Achieved infection rates in BUVEC

were similar for both excystation methods (see Fig. 2). Both sporozoite species were in perfect conditions for active host cell infection and further intracellular macromeront maturation with final production of viable first generation merozoites I occurred. Nevertheless, presence of non-sporulated oocysts, sporocysts, debris of broken oocysts and/or residua were present in BUVEC cultures infected with sporozoites derived from gradient protocol. This debris contamination was present throughout the development *in vitro* in spite of constant medium exchange as it attached firmly to endothelium monolayers. Debris and oocysts remains altered the morphology and architecture of BUVEC monolayers, leading to not only to suboptimal intracellular development but also to a delay growth and consequently affecting final size and assessed degree of macromeront maturation (Fig. 2).

4. Discussion

E. arloingi and *E. bovis* are strictly obligate intracellular protozoan parasites in which sporozoites have to traverse gut epithelium to reach final host endothelial cells of the lacteals of ileum [18]. For the *in vitro* study of these monoxenous parasites, large number of oocysts exclusively produced through experimental or natural infections of respective specific hosts are necessary. For all ruminant *Eimeria* species it is especially difficult and expensive to keep animals in large metabolic cages under parasite free conditions in large animal stable facilities with restricted laminar flow entrance for parasite replication [3, 8, 11, 13, and 17]. The usage of calves or goat kids for detail research on host immunity or *Eimeria*-infected host endothelial cell metabolism is becoming more difficult as requirements for animal welfare have become more restrictive worldwide [10]. This forces science to reduce, refine, replace (3R principles) to

optimize alternative methods to achieve more efficiently same amount of sporozoites with fewer oocysts for *Eimeria*-related investigations [1, 10].

The excystation process of *Eimeria* spp. oocysts is an extremely laborious technique, which includes a two-day working protocol with several steps and requiring sophisticated equipment (e. g. high speed centrifuges). Therefore, there is an urge for an improvement of the cleaning steps where usually most of *Eimeria* oocysts are lost. Here, we described a sieve-based protocol, which increased by triple the number of recovered sporozoites, due to the increment of sporozoite:oocyst ratio from 1:1 to 3:1 (*E. bovis*), from the same amount of initial sporulated oocysts. Most excystation protocols use a gradient-based method to purify oocysts [8, 10, 11, 13, 15, and 19]. These gradient-based protocols developed from protocols used previously for avian *Eimeria* species such as *E. tenella* [20].However, recuperation of oocysts after purification was not always the best and in 2008, Kurth and Entzeroth [1] reported in *E. nieschulzi* (rats) an excystation rate of 12–13%, and a ratio oocyst:sporozoite of 1:1, similar to what we found with the gradient protocol described by Hermosilla et al. (2002) [8]. In former study, purification of single *E. bovis* sporozoites was achieved by using a DEAE column according to purification protocols described for *E. tenella* sporozoites by Schmatz et al. (1984) [20] and Shirley (1995) [21], which used DE-52 anion exchange chromatography. Dulski and Turner (1988) [19] reported a purification protocol for sporocysts and sporozoites from *E. tenella* oocysts using Percoll density gradients resulting in 87% of oocyst recovery. Using a similar protocol for *E. bovis* and *E. arloingi,* the oocyst recovery percentage was suboptimal as gradients used for *E. tenella* included glass-bead grinding [20] leaving to a specific gravity of altered oocysts differing from ones with intact oocyst membranes [19]. These authors reported that the sporozoites were 99% pure with a final recovery of approximately three sporozoites per oocyst, comparable to what we achieved with our

281 improved sieve-based method. It is worth noting that for the gradient preparation described by
282 Hermosilla et al (2002) [8], an ultracentrifuge with a minimum velocity of 15,000 rpm is necessary,
283 which is a disadvantage for many parasitology labs, equipped with rather basic instruments in
284 underdeveloped countries. In fact, the most critical point of this protocol is the Percoll gradient.
285 Firstly, it is expensive, requires special equipment (ultracentrifuge), it is long lasting and somehow
286 inefficient by losing oocysts and by not achieving complete debris elimination.
287 The method described here was adapted from the one first reported by Jackson (1962) (sheep
288 *Eimeria* spp.) [22] and Nyberg and Hammond (1964) (bovine *Eimeria* spp.) [7]. This method
289 required two different stimuli for sporozoite excystation from ruminant oocysts: *i)* oocyst
290 activation with L-cysteine hydrochloride solution under a 100% CO_2 atmosphere, and *ii)* a
291 digestion with enzymes (trypsin and other enzymes present in bovine bile), without any
292 mechanical disruption of oocysts. These conditions mimic the natural intra-gastrointestinal
293 environment of ruminants, in which there is no mechanical pre-digestion (as in the case of birds).
294 Coudert et al. (1995) [23] described an additional mechanical disruption step after L-cysteine
295 hydrochloride treatment of rabbit *Eimeria* oocysts using a Potter homogenizer and/or combining
296 enzymatic treatment of oocyst walls before mechanical disruption with glass beads for rats [1].
297 Nevertheless, for ruminant *Eimeria*, these methods appeared unnecessary if incubation with L-
298 cysteine hydrochloride was conducted in a >50% of CO_2 atmosphere for at least 14 h [7].
299 In relation to ruminant sporozoite egress from oocysts, Silva et al. (2015) [13] reported for *E.*
300 *arloingi* an initial sporozoite release from sporocysts to oocyst circumplasm, and then liberation
301 of sporozoites through oocyst micropyle. Nevertheless, we also observed this behavior with
302 some *E. bovis* oocyst. This egress pattern was also observed before by Nyberg and Hammond
303 (1964) [7], which reported that oocysts which had intact membranes before enzymatic digestion

used this mechanism for liberation of sporocysts. Apparently, this alternative egress mechanism did not affect viability of free-released sporozoites [7].

Both ruminant *Eimeria* species here reported replicate within host endothelial cells during their first merogony with the formation of >170,000 merozoites within macromeronts [8, 10, 11, 13, 15]. Host endothelial cells are highly immunoreactive cells being able to produce a broad range of adhesion molecules, proinflammatory chemokines/cytokines upon activation, thereby initiating leukocyte trafficking, e. g. by recruiting polymorphonuclear neutrophils (PMN), NK cells, T lymphocytes and monocytes to the site of infection (for reviews see [24-26]). Thus, it appears likely that these host endothelial cells will actively defend themselves from intracellular parasitism as demonstrated for *E. bovis* [3, 16, 17]. Therefore, debris, sporocysts and oocyst rests, commonly present in freshly isolated sporozoite suspensions, could eventually activate host endothelial cells leading to a hampered sporozoite intracellular development. The usage of a smaller size sieve after the excystation resulted in a purified and homogenous sporozoite population ideal for *in vitro* experiments.

By using the novel sieve-based protocol, we achieved a higher excystation rate [43.5% (*E. bovis*) and 32.77% (*E. arloingi*)], and avoiding usage of ultracentrifugation steps, which reduced not only initial number of oocysts per sporozoites needed, but also the costs of specific gradients and time spent performing protocol. The improved method is cheaper, faster, and accessible for all labs with minimum equipment, without the requirement of expensive reagents or instruments.

Authors and contributors

SL, LS and ZV carried out *in vitro* experiments. SL, LS, and CH drafted and edited the manuscript. SL, LS, JC, CH and AT designed, planed and coordinated the project. All authors have read and approved the manuscript as submitted.

Conflict of interest

Authors declare that research was conducted in absence of any commercial or financial relationships that could be a potential conflict of interest.

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345

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425
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427

428 **Table 1.** Comparative results of the excystation methods: gradients vs sieves. Initial number of sporulated oocysts;
429 method of purification; number of recovered sporozoites at the end of excystation process; recuperation rate (%)
430 given by number of recovered sporozoites divided by total number of sporozoites in oocysts (8 sporozoites per
431 oocyst); ratio recovered sporozoites:initial sporolulated oocysts; infection rate (%) given by number of infected
432 BUVEC in total monolayer.
433
434
435

Eimeria species	sporulated oocysts	method	recovered sporozoites	recuperation rate (%)	ratio sporozoites:oocysts	infection rate (%)
E. bovis	5.625×10^6	gradients	5×10^6	11.11	0.8:1.0	40
		sieves	19.6×10^6	43.55	3.0:1.0	41
E. arloingi	2.250×10^6	gradients	6.75×10^5	3.75	1.0:3.7	19
		sieves	5.9×10^6	32.77	2.6:1.0	21

436 **Legends to figures**

437

438 **Fig. 1. *In vitro* excystation of *Eimeria bovis*- and *Eimeria arloingi*-oocysts.**

439 Sporulated oocysts of *E. bovis* (35 × 28 µm; strain H) with debris attached to the outer wall (A)
440 and sporulated oocysts of *E. arloingi* (29.5 × 22 µm; strain A) (B). Oocysts after sodium
441 hypochlorite 4% incubation (C-D). Oocysts after filtration through sieves (E-F). Neither changes
442 in oocyst morphology nor shape were detected after this step, which removed debris from medium.
443 Oocysts after L-cysteine incubation under 100% CO_2 atmosphere became thinner (G-H). The
444 sporozoites inside sporocysts became activated thereby leading to deformation of oocyst
445 membrane (*E. bovis*) or sporocyst membrane (*E. arloingi*). During enzymatic digestion, *E. bovis*
446 oocyst walls degraded and sporocysts were observed free in medium (I). Later, sporozoites egress
447 from the sporocysts (K). For *E. arloingi*, sporozoites excysted from sporocysts while inside oocyst
448 circumplasm (J), and leaving through micropyle afterwards (L).

449

450 **Fig. 2. Compared *in vitro* development of first merogony of *Eimeria bovis* and *Eimeria***
451 ***arloingi* in BUVEC.**

452 Sporozoites obtained with both protocols (gradient-based and sieve-based protocols) were used
453 for BUVEC infection and further comparative *in vitro* development. Both protocols resulted in
454 viable and motile free sporozoites capable of infecting BUVEC. Nevertheless, with the gradient
455 protocol some un-sporulated oocysts, sporocyst and rest of oocysts were present together with
456 sporozoites and altered BUVEC morphology during *in vitro* development. Intracellular
457 sporozoites are indicated with orange arrow (24 h p. i.). For *E. bovis*, trophozoites were seen after
458 4 days p. i. and immature meronts were first observed at 12 days p.i. Free merozoites I were visible

after 18 days p. i. proving successful of parasite replication after excystation with both protocols. Different stages of *E. bovis* presented the following measurements: sporozoites (14 × 2 µm; *n* = 60), trophozoites (13 × 12 µm; *n* = 20), merozoites (10.1 × 1.2 µm; *n* = 20). For *E. arloingi*, trophozoites were seen after 7 days p. i. and immature meronts at 15 days p. i. Free-released merozoites I were visible after 21 days p. i. Different stages of *E. arloingi* presented the following measurements: sporozoites (8.3 × 1.9 µm; *n* = 60), trophozoites (18 × 15.69 µm; *n* = 20), merozoites I (10 × 2,26 µm; *n* = 20).

Fig. 3. Live cell imaging 3D holotomgraphic microscopy analysis of *Eimeria bovis* and *Eimeria arloingi* during *in vitro* excystation.

Comparative egress of sporozoites during *in vitro* excystation of *Eimeria bovis* and *Eimeria arloingi*. Oocyst were followed using Live 3D Cell Explorer® (Nanolive®) during incubation with excystation medium to explore instantly live egress of sporozoites in 60x magnification. Images were digitally stained using STEVE® Software (Nanolive®).

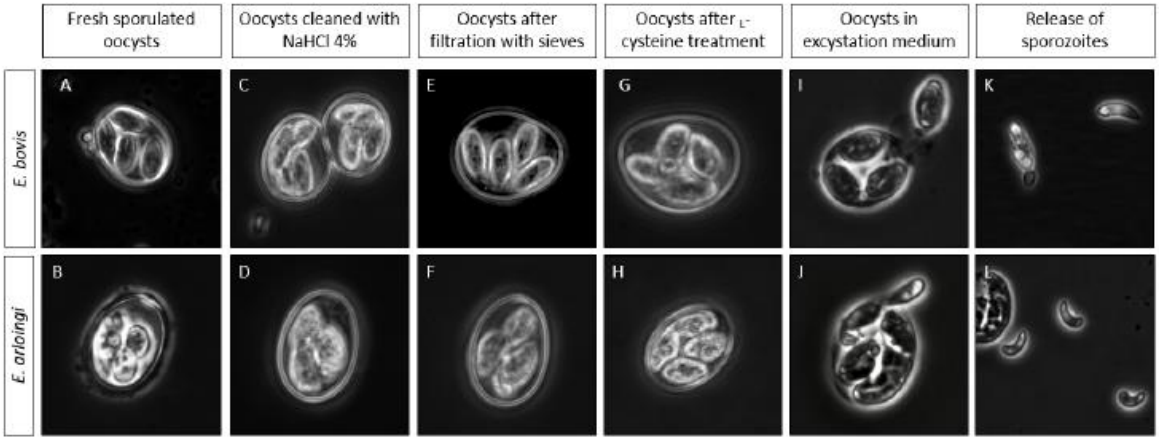
Video 1. Free *Eimeria bovis* sporozoites.

Sporozoites exhibited typical smooth gliding movement, twirling, twisting, and flexion of the banana-shaped body. Additionally, drifting (passive float in the medium) either vertical or horizontal was registered. Finally, some sporozoites remained immobile.

Video 2. Egress of *Eimeria arloingi* sporozoites

During incubation in the excystation medium, sporozoites became actived within sporocysts allocated in oocyst, and started moving actively until braking the Stieda body. Afterwards, the

sporocyst wall as opened and sporozoites left one by one and entered into circumplasm of oocyst. Then, the parasite left the oocyst through the micropyle. In this case, sporocyst residua were observed leaving ocyst prior to active sporozoite egress or following its release.


Fresh sporulated oocysts
Oocysts cleaned with NaHCl 4%
Oocysts after filtration with sieves
Oocysts after L-cysteine treatment
Oocysts in excystation medium
Release of sporozoites
E. bovis
E. arloingi
A
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K
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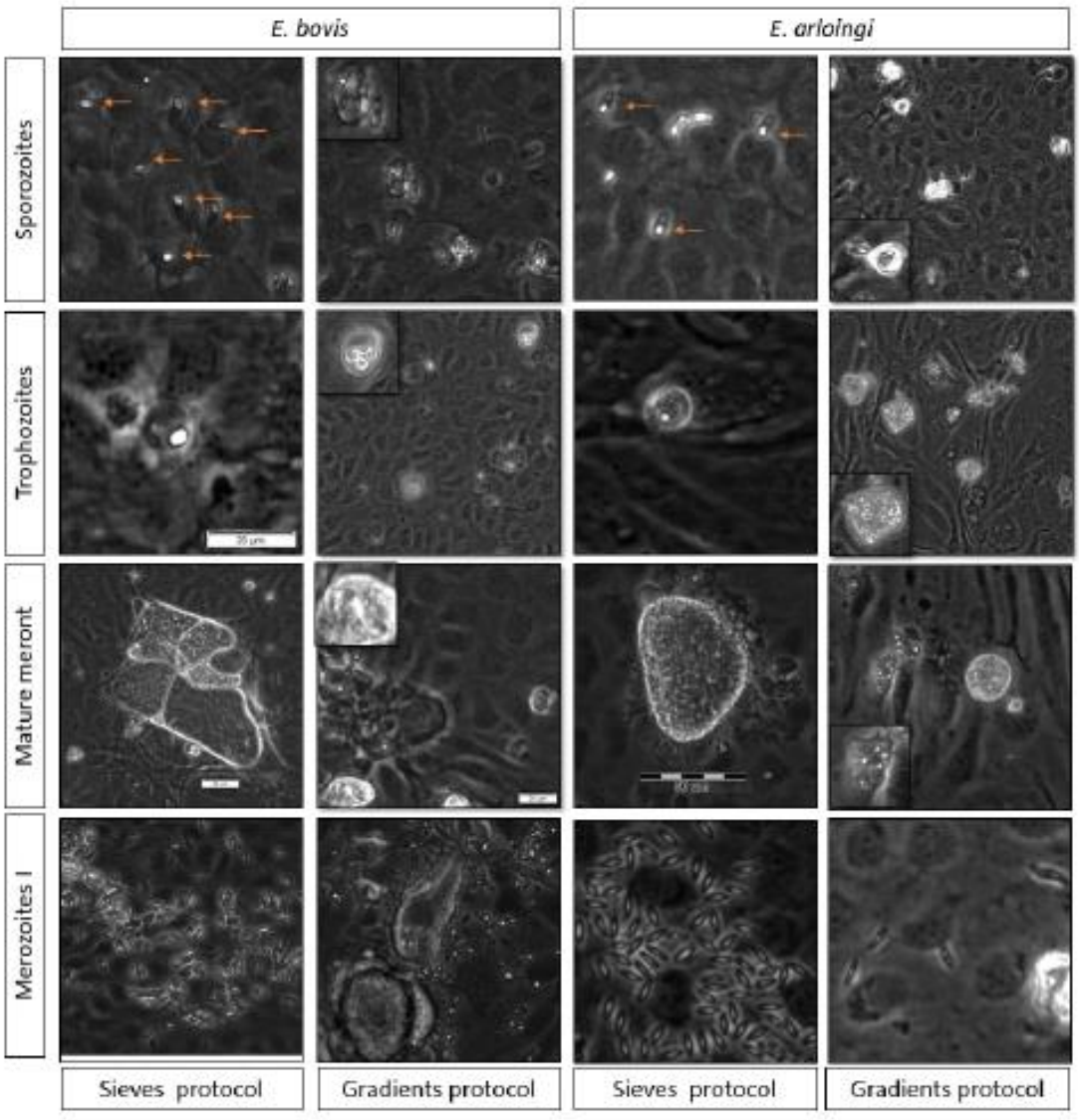
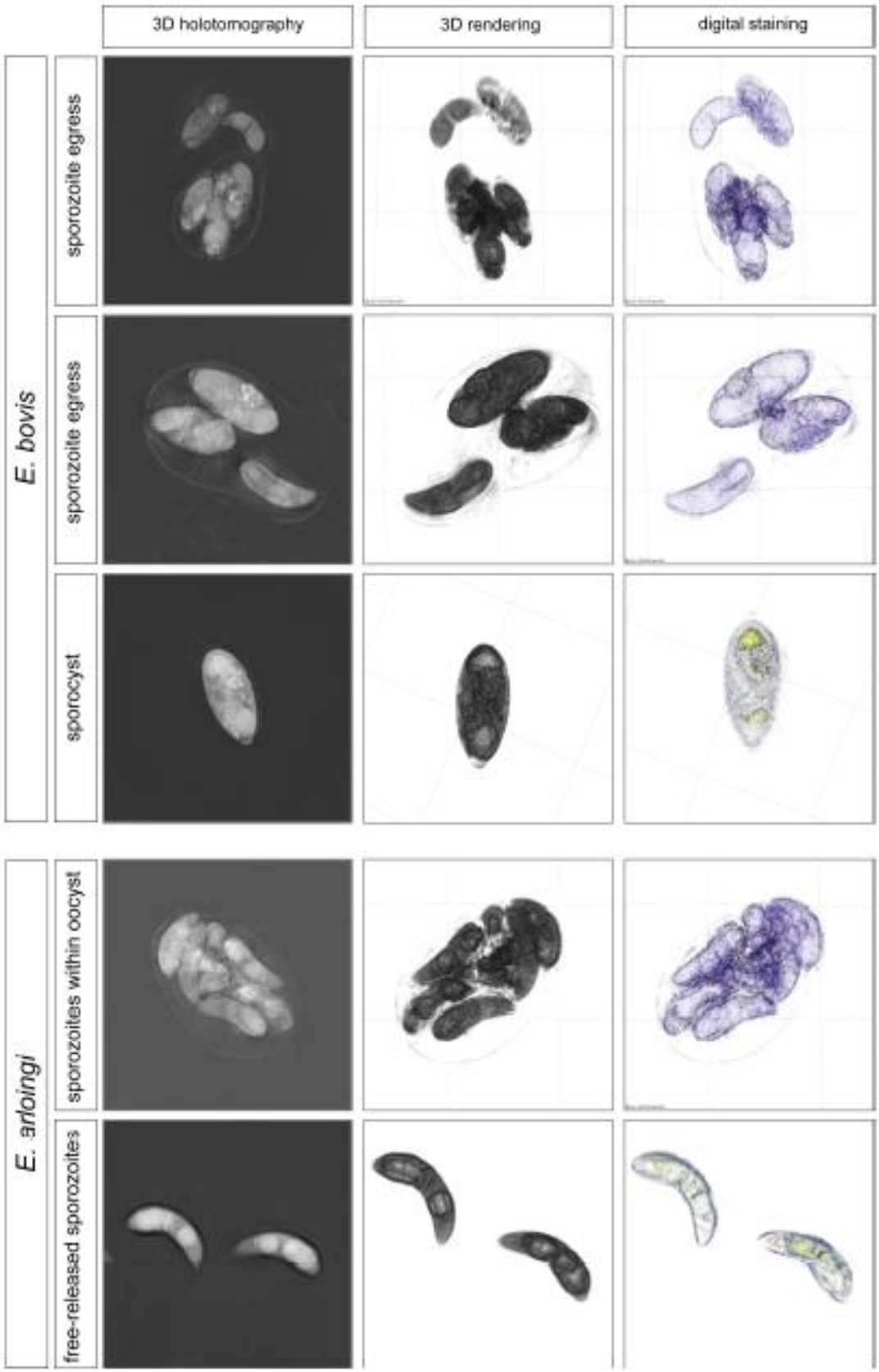

E. bovis
E. arloingi
Sporozoites
Trophozoites
Mature meront
Merozoites I
Sieves protocol
Gradients protocol
Sieves protocol
Gradients protocol


3D holotomography
3D rendering
digital staining
E. bovis
sporozoite egress
sporozoite egress
sporocyst
E. arloingi
sporozoites within oocyst
free-released sporozoites

5. Chapter: 3D-holotomographic live cell microscopy analysis of aerobic *Eimeria bovis* oocyst sporogony

This chapter is based on the following paper:

Lopez-Osorio S, Velasquez ZD, Conejeros I, Taubert A, Hermosilla C (2019) 3D-holotomographic live cell microscopy analysis of aerobic *Eimeria bovis* oocyst sporogony *(manuscript in preparation)*

Own contribution in the publication
Initiative: essential
Project planning plan: essential
Carrying out the experiment: essential
Evaluation de experiment: as far as possible
Creation of the publication: essential

First 3D-holotomographic live cell microscopy analysis of aerobic *Eimeria bovis* sporogony

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Abstract

Purpose: Monoxenous *Eimeria* species are widespread enteropathogenic apicomplexan protozoa with high economic impact in livestock. In cattle, tenacious oocysts shed by *E. bovis*-infected animals are ubiquitously found in cattle industry and making an infection of new calves almost inevitable. To become infectious oocysts, exogenous oxygen-dependent sporogony must occur leading to formation of sporulated oocysts containing four sporocysts each harbouring eight sporozoites. Investigations on sporogony by live imaging techniques of ruminant *Eimeria* species are still absent in literature as commonly used fluorescent dyes do not penetrate resistant oocyst bilayered wall. *Methods:* Sporogonial oocysts were daily analysed by a 3D Cell Explorer®/ STIS®-unit to explore ongoing aerobic sporogony as close as possible to *in vivo* situation. Subsequently, 3D-holotomographic images of sporulating *E. bovis* oocysts were digitally stained based on refractive indices of oocyst bilayered wall and sub-compartments of circumplasm using STEVE® software (Nanolive). *Results:* Overall, three different *E. bovis* sporogony phases each of them divided in two sub-stages were documented: *i)* sporoblast/sporont transformation into sporogonial stages, *ii)* cytokinesis followed by nuclear division, and *iii)* formation

of fully developed four sporocysts each with two developed sporozoites. *Conclusion:* Approximately, 60% of sporulating *E. bovis*-oocysts accomplished aerobic sporogony in a synchronized manner. *E. bovis* sporogony was delayed (i. e. 6 days) when compared to *in vivo* situation were namely 2-3 days are required but under optimal conditions.

Keyword: *Eimeria bovis*, oocyst, sporogony, 3D-holotomographic microscopy, live cell imaging.

Introduction

The genus *Eimeria* contain apicomplexan enteropathogenic protozoa with high economic impact in livestock worldwide. Prevalences of *Eimeria* infections in cattle are generally high and might reach 100% in calves [1, 2]. Of the more than dozen bovine species described so far, *Eimeria bovis* is one of the most pathogenic species causing severe typhlocolitis characterized by haemorrhagic diarrhoea with sometime fatal outcome [3, 4]. Monoxenous life cycle of all *Eimeria* species contains two phases, an exogenous- and an endogenous phase. During exogenous phase, non-sporulated oocysts found ubiquitously in contaminated cattle environments must undergo aerobic sporogony to become infective as sporulated oocysts [5]. Sporulated *E. bovis*-oocysts contain four sporocysts each harbouring two sporozoites as seen for all other *Eimeria* species [6]. The endogenous phase includes various asexual merogonies (i. e. *E. bovis* two) in specific host cells and certain sites of the intestine, followed by a sexual gamogony leading to formation of non-sporulated oocysts and to be shed with faeces to the environment [7, 8].

E. bovis oocysts are highly resistant under favourable conditions, such as moisture, temperature of -5 to -8 ° C and aeration. Furthermore, the oocyst form *E. bovis* maintain their infectivity for several months and even survive with ease Scandinavian winters [9, 10]. High tenacity of sporulated *E. bovis* oocysts was also confirmed by oocysts kept in 2% potassium bichromate solution at 4 °C for 4 ½-years of storage and still capable to induce patent infections of calves (Hermosilla, personal communication). Calves at an age of 4 weeks to 6 month

are susceptible to clinical coccidiosis [11] and becoming infected by ingesting these sporulated oocysts contaminating stable/barn floors, food, bedding or drinking water [12]. More importantly, exogenous sporogony for some bovine *Eimeria* species seems a key mechanism to overcome adversed enviromental conditions. As such, *E. zuernii*- and *E. alabamensis*-oocysts survived much better sub-zero temperatures as unsporulated oocysts while *E. bovis* resisted very low temperatures as fully sporulated oocysts [9]. Conversely, *E. alabamensis, E. zuernii,* and *E. ellipsoidalis* but not *E. bovis*, were able to undergo asexual sporogony after a month at -18 °C [13].

Thus, in cases of continuing bovine coccidiosis oubreaks the management of the herd should be critically assessed [4], particulary with respect of hygiene, feeding, animal density and floor types to achieve reduction of environmental oocyst pressure. As already stated, exogenous oocysts are capable to persist prolonged periods of time and this is attributed in part to bilayered oocyst wall [14]. Oocyst wall of many ruminant *Eimeria* is comprised of two distinct layers: the outer layer (500-200 nm), inner zone (40 nm) which is the space between the outer and inner layer, and the inner layer (40 nm) [15]. In both layers, proteins are the main composition of the wall (more than 90%), even though their relative electron densities are different [14]. Bilayered oocyst wall prevents mechanical and chemical damage of sporozoites [13]. This is one of the reason why breaking *Eimeria* oocysts in laboratory conditions requires specials protocols including use of mechanical disrupture proceedings (e. g. high speed vortexing with glass beads) or enzymatic digestion [16-19]. In fact, oocysts can be cleaned with bleach and stored in harsh oxidant (i. e. potassium dichromate), with no alterations of their infectivity [20-23]. The wall is also impermeable to many water-soluble disinfectants and detergents [24, 25]. Given that commonly used fluorescent dyes do not penetrate highly resistant *Eimeria* spp. oocyst wall a detailed and live cell imaging investigation of exogenous aerobic sporogony is still hard to achieve. Furthermore, it is extremely difficult to prepare oocysts for transmission electron microscopy (TEM) analysis by conventional techniques, which explain why all

ultrastructural aspects of *Eimeria* species life cycles have been well-documented with the exception of exogenous sporogony.

Until now, there are only few studies on ruminant coccidian sporogony [26, 27] and morphological changes during sporogony have been described for few *Eimeria* species [28-35]. For rabbit *Eimeria stiedai* 7 sporogony phases and for chicken *E. maxima* 6 sporogony phases have been described, respectively [30, 31, 35]. Nevertheless, most of these studies have been performed with either fixed oocysts for special staining techniques or simply visualized under microscopes equipped with phase optics [28, 30]. Thus, aim of this study was to investigate for the first time aerobic-dependent sporogony of *E. bovis* oocysts as close as possible to *in vivo* situation by using novel live cell imaging technique and 3D-holotomographic microscopy without any bleaching, fixation or phototoxicity**.** Generated 3D-high resolution imagines of external as well as internal changes while *E. bovis* sporogony process were completed. High specificity analysis of multiple cellular organelles, including granules and extracellular vesicles, based on different refractive indices were additionally generated. These new data will contribute as a baseline study on exogenous sporogony and allowing better understanding of different cytokinetic- and metabolic pathways to be involved in *E. bovis* sporogony.

Material and methods

Ethics

All animal procedures were performed according to the Justus Liebig University Giessen Animal Care Committee guidelines, approved by the Ethic Commission for Experimental Animal Studies of the State of Hesse (Regierungspräsidium Giessen, GI 18/ 10 No A37/2011, JLU Giessen-No. 494) and in accordance to current German Animal Protection Laws.

Parasites

For parasite propagation, two 8-weeks-old calves kept in autoclaved metabolic cages within a large animal facility unit of the Institute of Parasitology (JLU

Giessen), equipped with a laminar flow entrance and restricted access to avoid any bovine *Eimeria* spp. exposure. After deemed parasite-free, calves were orally infected with 3 × 10^4 sporulated *E. bovis* oocysts (strain H) suspended in tap water according to Hermosilla *et al.,* [16]. During patency, non-sporulated oocysts were isolated from faeces beginning at 18 days p. i. according to Jackson [36]. Faeces containing oocysts were washed through a set of three sieves (pore sizes 850, 250 and 80 µm, respectively, Retsch ®) with tap water. Final suspension was let to sediment overnight and supernatant was discarded. The sediment was mixed 1:1 with saturated sucrose solution (1.3 g/ml) to a final density of 1.15 g/ml. The suspension was transferred into plastic trays (30 x 20 x 5 cm) horizontally adjusted. Plastic trays were filled to the top, and thereafter covered with clean glass plates allowing complete contact of suspension with glass surfaces. Every 4 h the glasses were carefully removed and adherent oocysts were washed off with tap water into a container. The remaining suspension in plastic trays was stirred up and the process was repeated up to six times or until few oocysts were isolated (microscopic examination, less than 5 oocysts per power vision field, 20 x magnification). Collected *E. bovis* oocysts were diluted with tap water (1:1) and then centrifuged at 600 x *g* for 10 min.

Exogenous Eimeria bovis *sporogony*

Isolated *E. bovis* unsporulated oocysts were re-suspended in 2% potassium bichromate ($K_2Cr_2O_7$; Merck) solution at room temperature (RT) with constant oxygenation as reported elsewhere [16, 23]. Every day, oocysts were controlled with an inverted microscope (IX81, Olympus®) to observe changes of zygote morphology within circumplasm. In total, fifty oocysts per day were examined in more detail under oil immersion (x 1000 magnification) for ongoing sporogony. Finally, the proportion of sporulating oocysts in each specific sporogonic stages was expressed as a percentage.

Live cell imaging of Eimeria bovis-*oocysts by 3D-holotomographic microscopy*

E. bovis oocysts at different sporogonic stages were collected using a micropipette and washed twice with distilled water. Then, isolated oocysts were

seeded into a 35 mm imaging dish plate (Ibidi) with sterile phosphate buffered saline [(PBS) 1x, pH 7.4] inside a Stage Top Incubation System® [(STIS), Ibidi] at CO_2 5% and 37 °C. Oocyst were followed using Live 3D Cell Explorer® (Nanolive) to explore instantly live sporogony in 3D without any labeling or chemical marker (60x magnification and 30 µm depth per vision field). Images were analyzed using STEVE® software (Nanolive) to obtain a refractive index-based z-stack according to Silva *et al*., [37]. 3D-images were additionally digital stained based on cell organelle physical refractive index using STEVE® software (Nanolive). Thereafter, image processing was performed with Fiji ImageJ® using z-projection being restricted to overall adjustment of brightness and contrast as described elsewhere [37].

Excystation of E. bovis

The following modified excystation protocol was used (Silva et al., 2015). Sporulated *E. bovis* oocysts stock solution was added to 4 % (v/v) sodium hypochlorite solution and magnetically stirred on ice for 20 min. After mixing in the vortex for 20 s, oocyst solution was centrifuged (300×g, 5 min) and supernatant was mixed with bi-distilled water (1:1). Washed oocyst solution was then filtered through 40 and 5 µm sieves to remove remaining debris. Then, oocyst were centrifugated 15 min at 600×g, and the pellet was resuspended in 0.02 M L-cysteine/0.2 M NaHCO3 (Merk) solution and incubated in a 100 % CO2 atmosphere (37 °C, 20 h). Afterwards, oocysts were re-suspended in the following excystation medium: Hank's balanced salt solution (HBSS, Gibco) containing 0.4 % (w/v) trypsin (Sigma) and 8 % (v/v) sterile filtered bovine bile (obtained from the local slaughterhouse, due to the lack of caprine bile availability). Afterwards, oocysts were incubated up to 4 h (37 °C, 5 % CO2 atmosphere). Every hour, excystation progress was checked under an inverted microscope (IX81, Olympus®) to estimate the number of free released sporozoites. Freshly released sporozoites of E. arloingi were filtrated through 5 µm and then washed two times (600×g, 15 min) with cell culture medium 199 (M199, Gibco) supplemented with 2 % (v/v) fetal calf serum (FCS, Gibco) and 1 % penicillin (v/v, 500 U/ml; Sigma-Aldrich) and streptomycin (v/v, 500 µg/ml;

PS; Sigma-Aldrich) and finally suspended in culture medium (2×106 sporozoites/ml).

Results

Up to 90 % of collected *E. bovis* oocysts completed sporogony within 6 days and allowing further sporozoite excystation for final *in vitro* experimental settings such as primary host endothelial cell invasion assays, macromeront development and production of viable merozoites I (data not shown).

Detailed sequences of exogenous *E. bovis* sporogonic stages are summarized in Fig. 1, 2 and 3. Approximately 60% of sporulating oocyst went through each stage in a synchronized manner. However, some oocyst completed sporogony later on (i. e. 8-9 days), and 10% remained without further development. Detailed live cell 3D-holotomographic microscopy illustrations of sporulated oocysts demonstrated the presence of granules and /or extracellular vesicles contained in circumplasm. These extracellular granules/vesicles were released from ruptured oocysts in a sorted manner during sporozoite excystation (video 1).

Freshly isolated *E. bovis* oocysts from faeces had a large zygote in close contact with the oocyst bilayered wall and containing several cytoplasmic granules (Fig. 1: early contraction phase). The first sporogonial stage began with the late cytoplasmic contraction phase (Fig. 1). The late cytoplasmic contraction phase occurred after oocysts have been suspended in 2% potassium dichromate at permanent RT. Zygotes became irregular and reduced their volumes. The zygote nucleus was located centrally, as a clear spot inside the cytoplasmic mass. Then, zygotes started to form a more regular shaped structure (see Fig.1) and some cytoplasmic vesicles migrated to the zygote surface. Vesicle migration occurred between 24-48 h after incubation in 2% $K_2Cr_2O_7$ under constant aeration. At this time point, fully-formed spherical sporonts of approximately 17.32 ± 0.59 µm in diameter were detected within *E. bovis* oocysts.

The second phase of *E. bovis* sporogony began with the nuclear division at 48-72 h (please refer to Fig. 2). The nucleus divided in two nuclei and migrated thereafter to opposite sides of zygotes. Thereafter, the nuclei divided again and

cytokinesis occurred leading to formation of sporoblasts. In this process, sporont stages of 21 x 18.9 µm of mean size were gradually transformed into 4 sporoblasts (9.95 x 7.88 µm) by progressive constriction of their bodies until cleavage was completed (Fig. 3). After this cleavage, newly formed sporoblasts became rounded forming a 'clover-like shape' structure and beginning to elongate forming the 'cigar-shaped' sporoblasts (12.78 ± 1.3 x 6.19 ± 0.72 µm). In this stage, refractive bodies (RF) for the first time became visible (see Fig. 3). After RF formation, the Stiedae body as well as previously formed RF were visible in newly formed sporozoites within four sporocysts. Sporocysts were considered as fully mature at 96-120 h of incubation. At the end of sporogony (final sporogonial phase), residual material inside sporocysts (14.7 ± 2.22 x 6.5 ± 0.58 µm) was clearly visible as a cumulus of extracellular vesicles. The same holds true for the oocyst circumplasm containing as well residual material collected in multiple granules or vesicles of different sizes. During *E. bovis* sporozoite excystation, circumplasm granules were released in a fast and sorted way and often surrounding free-released and highly motile sporozoites (Fig. 4)

Discussion

Complete life-cycle of *E. bovis* has been well documented in the past [5-8], except for the exogenous sporogony generation. Asexual *E. bovis* oocyst sporogony is tighly regulated, complex and representing a necessary step for becoming infective stages, namely a sporulated oocyst. Although these facts, *E. bovis* sporogony as well as for other ruminant species has been infrequently explored in the past [19, 26, 27]. To our best knowledge this work represents first documentation of whole exogenous *E. bovis* sporogony using novel live cell imaging 3D-holotomographic microscopy analysis *in vivo* without fixation or interference through staining procedures.

Exogenous sporogony is an oxygen-dependent metabolic dividing process and consequently Sengerl [27] demonstrated that at least an oxygen tension of 15 mm of Hg was necessary for completing this parasitic process. Additionally, *Eimeria*-oocysts also require suitable temperatures and relative humidity for

fulfilling exogenous sporogony. For instance, *E. bovis* sporogony naturally occurs within 2-3 days under optimal temperature, oxygen and humidity conditions [26]. The sporogony time for *E. bovis* oocysts in our study was longer than the one reported previously [26] namely of 3 days. In present study, 90% of oocyst sporulation rates were achieved after 6 days at constant RT (24-25 °C), whereas former study *E. bovis* oocysts were kept at constant 28˚C [26]. These results are consistent with observations of many authors with respect to influence of temperature on *Eimeria* spp. sporogony. Just by one degree lower, namely 23 °C under natural conditions *E. bovis* sporogony was delayed by up to 25 days [28], and at much lower temperatures (3-5 ˚C) sporogony was prolonged for up to100 days [26]. Most authors agreed that the use of anti-bacterial/fungal agents (i. e. potassium dichromate) as oocyst solution help to achieve higher sporogony rates under laboratory conditions [16, 32, 38, and 39].

Three min stages can be differentiated in *E. bovis* sporogony: *i)* early sporogony stage (subdivided in unsporulated oocyst stage, less concentrated sporoblasts and concentrated sporoblasts), *ii)* mid sporogony stage (subdivided in nuclear division and cytokinesis), and *iii)* late sporogony stage (four rounded-sporoblasts and mature sporoblasts). These sporogonial stages have also been reported for other *Eimeria* species, such as chicken *E. maxima* [31] and *E. tenella* [28]. Nonetheless, for these two avian species, the pyramidal shaped sporoblasts were documented after 24 h of sporogony, which in case of *E. bovis* oocysts was not seen. Instead, a ‘clover-like shape’ sporoblast was detected in *E. bovis* sporogony. Morphological differences of eimerian sporoblasts might result from the fact that *E. bovis* oocysts were kept in constant suspension conditions while performing asexual cellular division. Conversely, other sporogongy-related studies used oocysts added to glass slides and covered with coverslips as performed in avian *Eimeria* studies, which might have physically distorted shape of newly formed sporoblasts. Interesingly, for *Cystoisospora* (former *Isospora*) *felis* sporogony*,* Shah [40] described two spherical sporoblasts each of them with distinct nuclei either with central or peripheral localization and similar to our findings of rather spherical sporoblasts.

After being excreted the zygote cytoplasms starts to shrink within bilayered *E. bovis* oocysts and becoming a spherical structure. It has been postulated, that zygote shrinkage is associated with oocyst dehydration. After oocyst dehydration, remaining fluid between the zygote and the oocyst bilayered wall, known as circumplasm, starts to contain small refracting bodies and bacteria [41]. Process of oocyst dehydration has been reported for majority of coccidian parasites, with exception of the genera *Sarcocystis* and *Cryptosporidum*, which undergo endogenous sporogony within the host intestine under anaerobic conditions. In addition, some fish *Eimeria* species, i. e. *E. carpelli* and *E. subepithelialis*, are eliminated from infected host as sporulated oocysts [41].

The molecular content and precise function of granules in oocyst circumplasm are still unknown. During initial sporogony process of *E. bovis*, we detected via 3D-analysis tiny structures and granules with different refractive indices closely related to the oocyst wall- and to the zygote-components during. These structures/granules then disappeared when zygote became a sporoblast. Structures with same refractive indices were found later on in free-released sporozoites surrounding their refractile bodies. More importantly, small granules/vesicles were released while sporozoite egress from oocysts in s clearly sorted manner and to be found later on surrounding free-released sporozoites. These circumplasm structures might represent functional extracellular vesicles, microsomes or exosomes. Extracellular vesicles and exosomes are nowadays considered as novel mode of intercellular communication [42]. These divergent vesicles/exosomes are cell-derived membranous structures and to be present in various biological fluids and being involved in multiple physiological processes [42]. Farther, extracellular vesicles/exosomes have different membrane composition and containing specific lipids, mRNA, ncRNA, microRNA, cytokines, chemkines, peptides or even proteins with diverse cellular functions [42]. Nevertheless, the hypothesis of the existance of *Eimeria*-derived exosomes has to be proved and future investiagation will be needed to unveil their contents and resulting functional activities not only on free-released sporozoites but also on intestinal epithelial host cells. Here applied 3-D

holotomography combined with S unit might help to better understand not only formation of extracellular vesicles/granules but also their modulating on sporozoite-infected host cells *in vitro.*

Metabolic signature of *Eimeria* sporogony has not yet been deeply investigated [27]. Nonetheless, the presence of cytochrome oxidase or other cytochrome system as well as a phosphorilation system of sporulating oocysts have been described [27]. The comprehension of *E. bovis* coccidiosis has significantly increased in the past decades worldwide resulting in enhanced awareness of bovine coccidiosis among farmers and veterinary surgeons and raising further questions related to the control of resistant oocysts and problem solving strategies. Thus, we call for additional sporogony-related studies in order to gain new data on ruminant *Eimeria* spp. aerobic-dependent metabolic pathways leading to sufficient energy, blocking units and nutrients while exogenous sporozoite development.

Authors and contributors

SLO and ZDV carried out the documentation and analysis of the images; SLO, ZDV, IC and CH drafted and edited the manuscript. CH and AT coordinated the project. All authors have read and approved the manuscript as submitted.

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Figures description

Figure 1. Early sporogony stage of *E. bovis.*

First sporogony stage in *E.bovis* oocyst began with a diffuse zigote closelly related with the inner oocyst wall (early contraction). Magnification of oocyst wall in contact with the zygote is shown in the 3D rendering image. The digital staining show homogeneus distribution of the granules across the circumplasm (purple). After the cytoplasmic contraction, the zygote becomes rounded and placed in the center of the oocyst (regular form). The nucleus can be seen as a pale spot in the center of the mass. Closely related with the inner wall and the zygote, some vesicles were seen with a different refractive index (yellow). Images were analyzed using STEVE® software (Nanolive) to obtain a refractive index-based z-stack (3D holotomography), the rendering format and the digital staining based on the refractory index.

Figure 2. Second sporogony stage of *E. bovis*

The nuclear division took place in this stage. The pale spot is no longer in center of the mass. The nucleus is divided twice, to form 4 nuclei, which can be located in the perifery of the mass. Closely related with the inner wall and the zygote, some vesicles were seen with a different refractive index (yellow). Cytokinesis begin with the early formation of the four sporoblast (early citokinesis). Four protrusions, located in perpendicular direccions can be seen in the surface of the zygote (late cytokinesis). Images were analyzed using STEVE® software (Nanolive) to obtain a refractive index-based z-stack (3D holotomography), the rendering format and the digital staining based on the refractory index

Figure 3. Late sporogony stage of *E. bovis*

The protrusions from the late citokinesis become more prominent, increase in size and become spherical, forming a clever like-shape structure, until they separate from each other forming the four sporoblast. The sporoblast become to elongate and form a cigar-like shape. Each sporoblast contains granular mass and vacuoles, which develop in to the residual body of the sporocyst. The

sporoblast continued to become in to oval-shaped sporocyst. Here, a single division of the nuclei ocurred, and then a division of the citoplasm, which results in the formation of two sporozoites, each one with a visible big refractile body, and the residual body (light refracting granules). Stieda bodies can be seen as a white tiny lid in one side of the sporocyst (Mature oocyst). After the stieda body and refractile body of the sporozoites are visible, the oocyst is considered mature. Images were analyzed using STEVE® software (Nanolive) to obtain a refractive index-based z-stack (3D holotomography), the rendering format and the digital staining based on the refractory index

Figure 4. *E. bovis* oocyst

Non-sporulated.(A) and sporulated oocyst (B). The four sporocyst of *E. bovis* sporulated oocyst are ovoid and contains two sporozoites. The average lenght was 14.7µm±2.22 and width 6.5±0.58. Normally, the length and width of the sporocyst are one-fourth and one to half of the oocyst (Kheysin, 1972). Each sporozoite showed a big refractile body in the base opposite to their partner. Residual material inside the sporocyst was clearly visible as a cumulus of extracellular vesicles (C) during the excystation. The stieda body were located in the narrowed end of the sporocyst. These are plugs which are covering the sporocysts micropile.

Video 1. *Eimeria bovis* during excystation. Free sporocyst contain active sporozoites moving inside and some vesicles which are released together with the sporozoites.

Suplementary video. *E. bovis* sporulated oocyst 3D view. The video was generated using Live 3D Cell Explorer® (Nanolive) (60x magnification and 30 µm depth per vision field). Images were analyzed using STEVE® software (Nanolive) to obtain a refractive index-based z-stack according to Silva *et al*., [37].

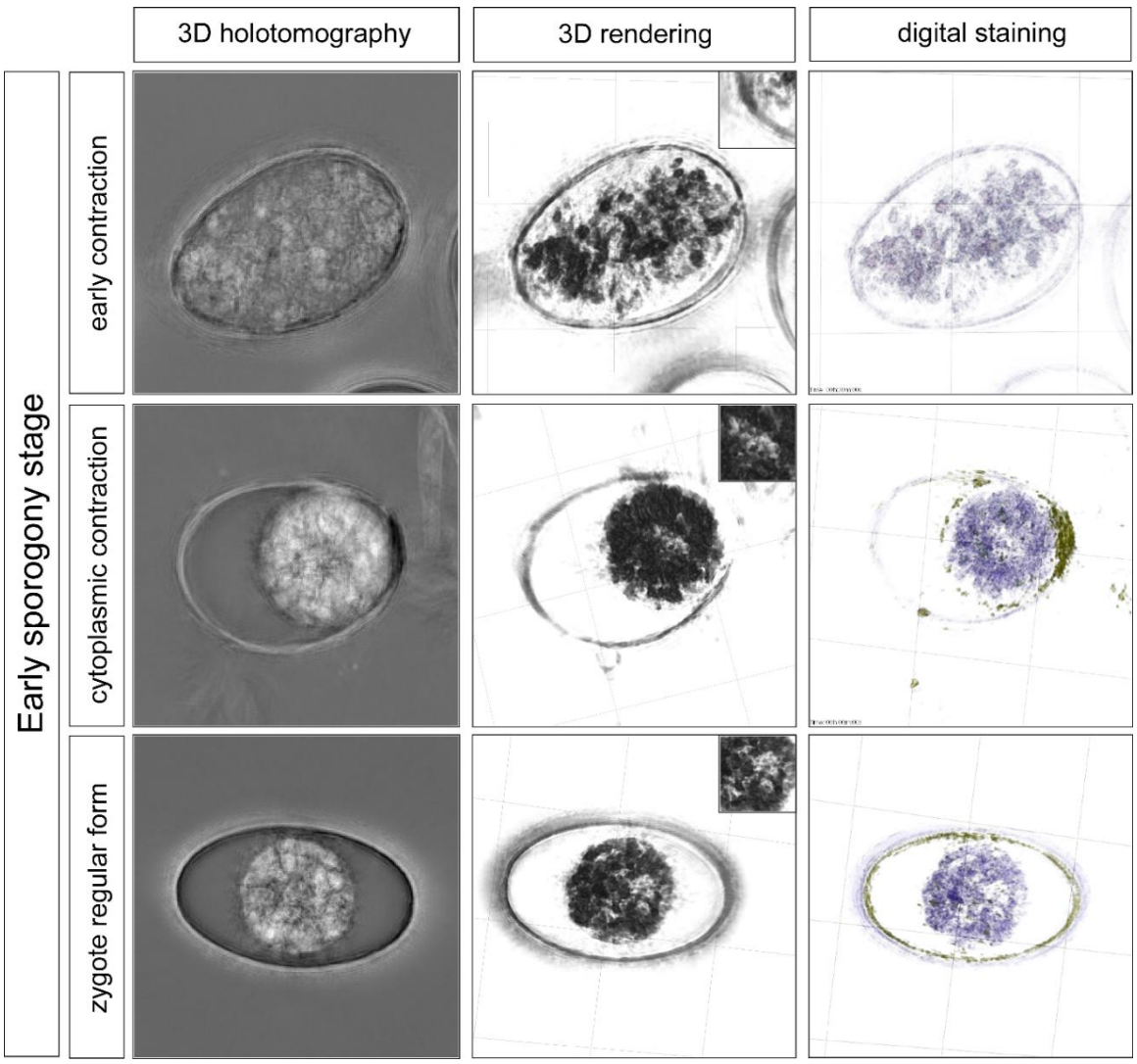


Fig. 1 Early sporogony stage of *E. bovis*.

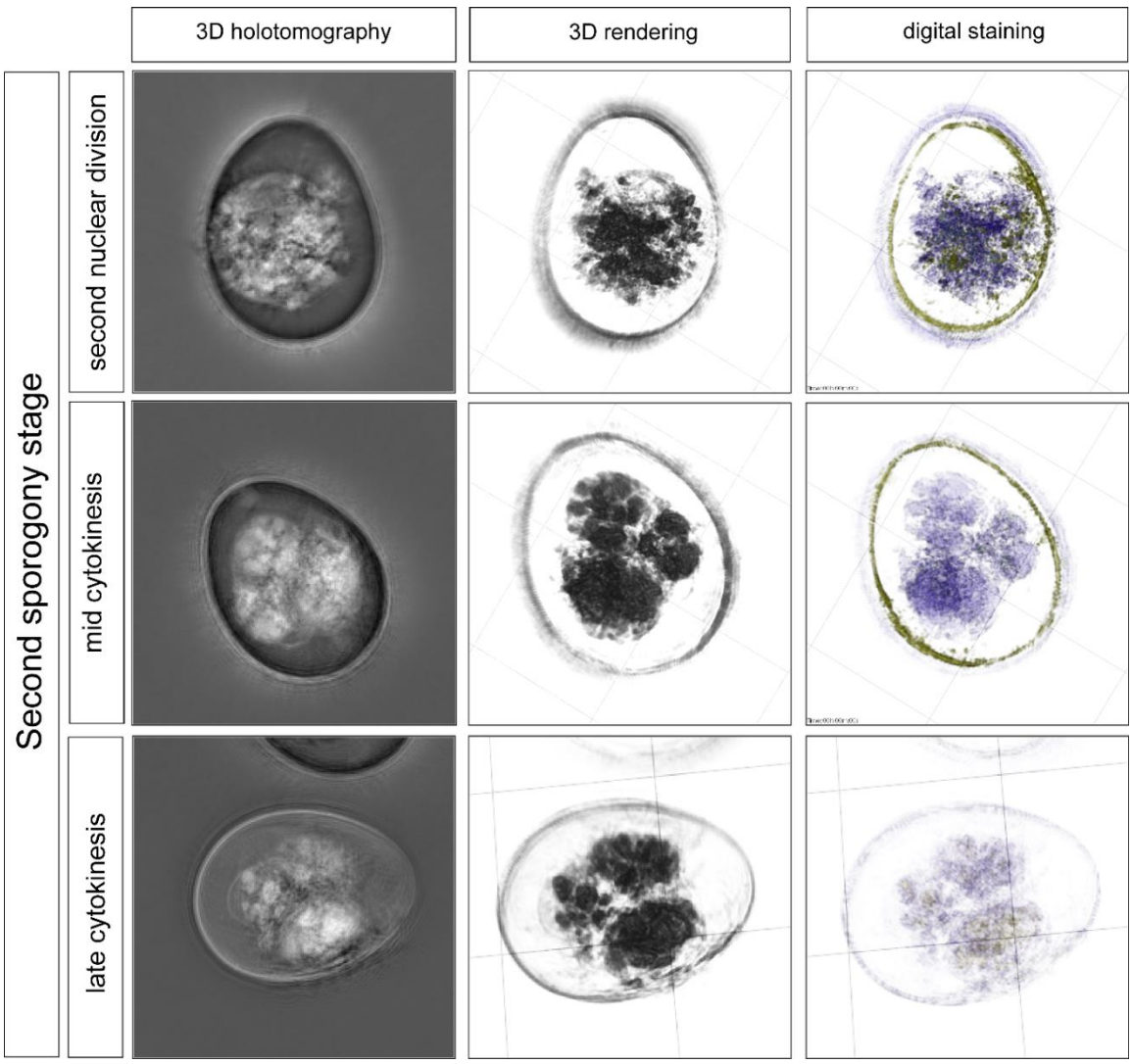


Fig. 2 Second sporogony stage of *E. bovis*

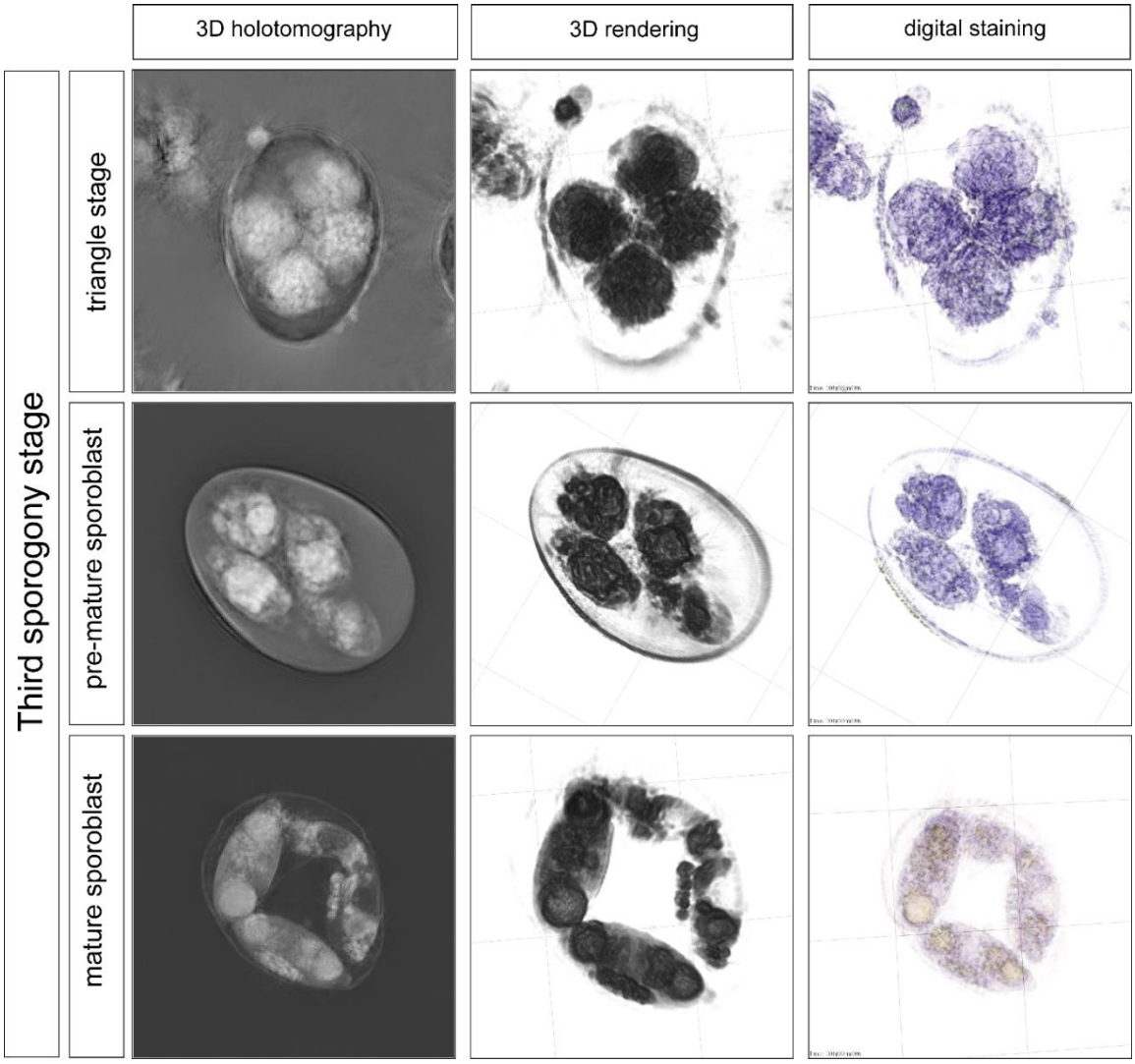


Fig. 3 Late sporogony stage of *E. bovis*

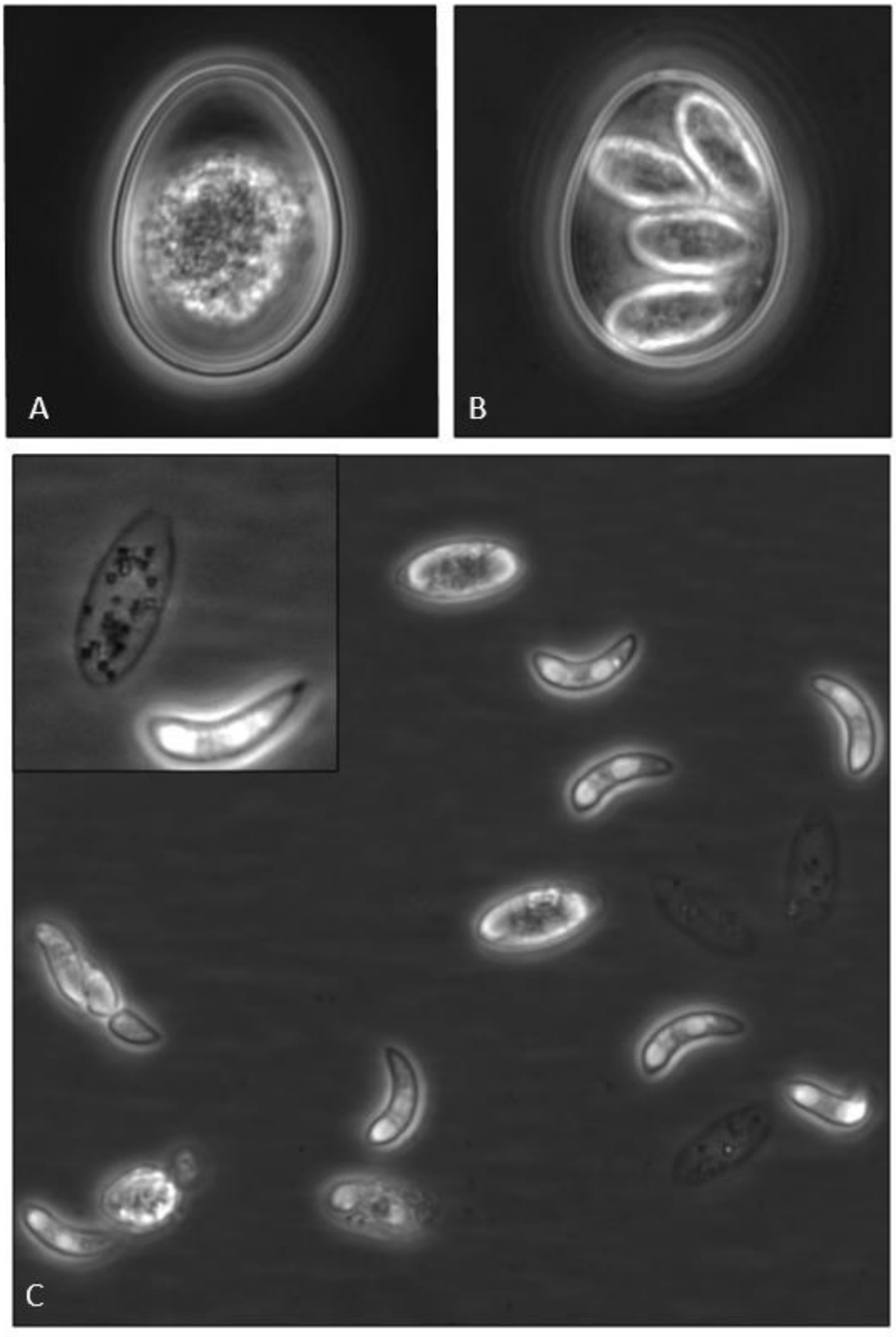
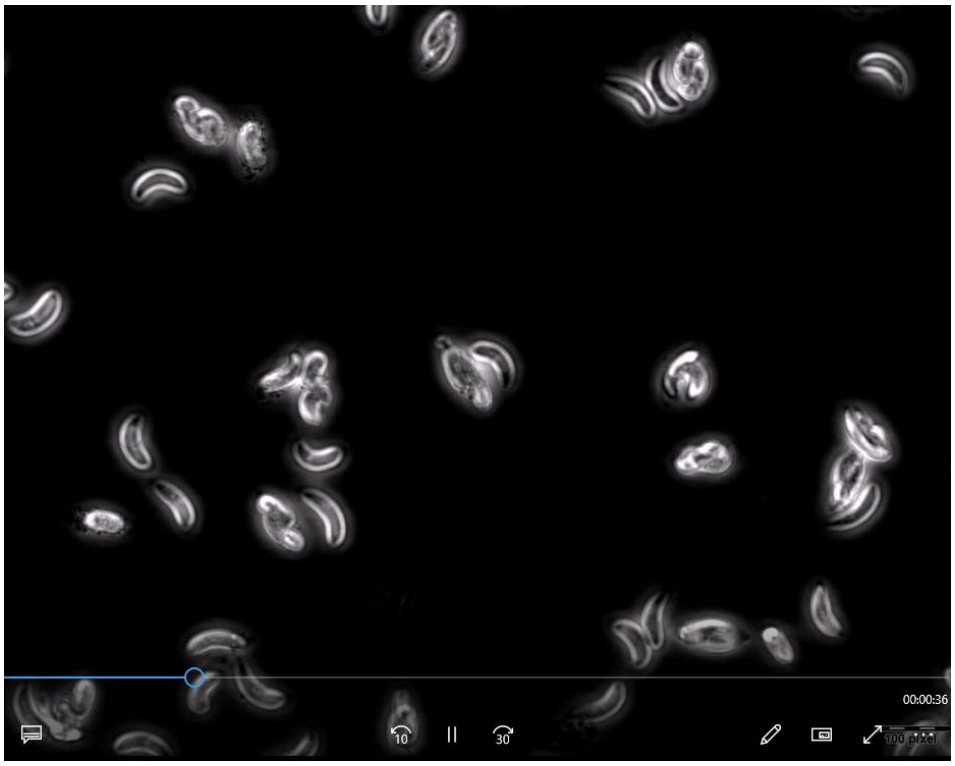
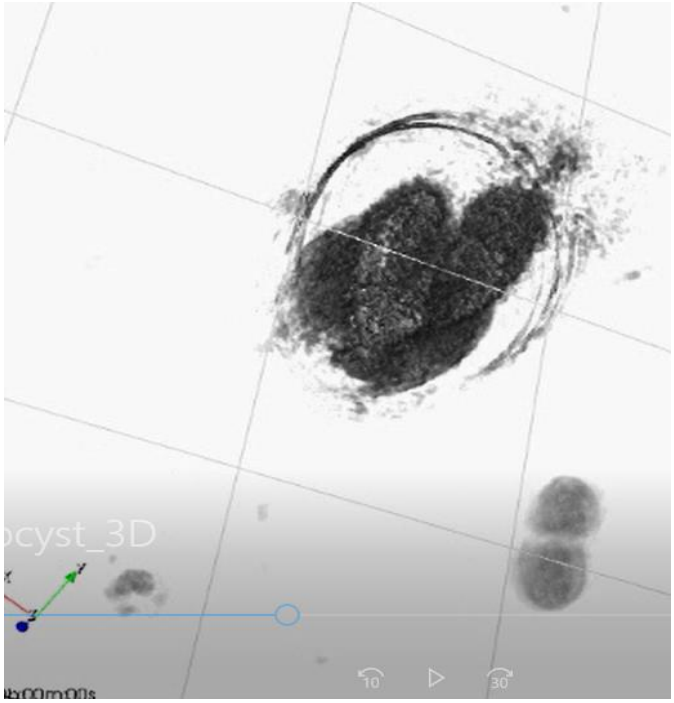


Fig. 4 E. bovis oocyst



Video 1. *Eimeria bovis* during excystation



Supplementary video*: E. bovis* oocyst 3D view.

6. Chapter: Modulation of cholesterol during *Eimeria arloingi* macromeront formation and impact on parasite intracellular development

This chapter is based on the following manuscript:

Lopez-Osorio S, Silva LMR , Velasquez ZD, Taubert A, Hermosilla C. Modulation of cholesterol during *Eimeria arloingi* macromeront formation and impact on parasite intracellular development *(manuscript in preparation)*

Own contribution in the publication
Initiative: essential
Project planning plan: essential
Carrying out the experiment: essential
Evaluation de experiment: as far as possible
Creation of the publication: as far as possible

Modulation of lipid uptake during *Eimeria arloingi* macromeront formation and impact on parasite intracellular development.

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Abstract

Eimeria arloingi is an obligate intracellular apicomplexan parasite that affects goat wordwide. The infection can lead to high economic losses in goat industry worldwide due to the diarrhea and hemorrhagic enteritis in young goat kids. As an obligate intracellular parasite, is considered inefficient in cholesterol biosynthesis and scavenge cholesterol from their host cell in a parasite-specific manner. In contrast to fast replicating coccidias, like *Toxoplasma gondii*, which produces low number of merozoites per cell, *E. arloingi* produces high number of merozoites inside a single macromeront. This stage has been proved to need large amounts of cholesterol for the offspring in other *Eimeria* spp. For example, *Eimeria bovis* drives to changes in the host cellular sterol profile, suggesting a huge demand for cholesterol during macromeront formation and its versatility in the acquisition of cholesterol sources. In order to determine the cholesterol modulation carried out by *E. arloingi* in BUVEC during the first merogony we analyzed the effect of Block Lipid Transport-1 (BLT-1) in infected cells, and compared the development and production of merozoites between treated and non-treated infected cells. The complete inhibition of merozoite I production in the treated cells suggest that the uptake of lipids via SR-B1 is necessary for the parasite´s replication in the host cell.

Keywords:

Goat eimeriosis, metabolism of cholesterol, Block lipid transport-1, Scavenger receptor class B type I.

Introduction

E. arloingi is considered one of the most pathogenic species in caprine coccidiosis. It can produce severe hemorrhagic enteritis, diarrhea, weight loss, dehydration and poor growth and in severe cases, even death (B Koudela and Boková 1998; Ruiz et al. 2010; Balicka-Ramisz et al. 2012; Rakhshandehroo et al. 2013; Liliana Machado Ribeiro da Silva et al. 2014). This apicomplexan monoxenus intracellular parasite has been associated with impact on animal health and significant economic losses in goat industry worldwide. It can affect up to 100 % of young goat kids, depending on the type of management and geographic area (Mehlhorn 2001; Carrau et al. 2015; Ruiz et al. 2006). The development of *E. arloingi* inside the host involves the asexual replication, with two generations of meronts, and the sexual replication (gametogony) (Liliana M R Silva et al. 2015). The first generation of macromeronts can grow up to 240 µm in host endothelial cells (ECs) of the lacteals of the villi of duodenum, jejunum and ileum and produces >120,000 merozoites I within 9–12 days post-infection (p.i.) (Taylor, Coop, and Wall 2007; Hashemnia et al. 2012). The second generation of meronts is smaller and produces just 8 to 24 merozoites II (Sayin, Dincer, and Milli 1980; Hashemnia et al. 2012) within 12 days p.i. in epithelial cells of the villi and the crypts of lower jejunum (Taylor, Coop, and Wall 2007)

For their intracellular replication, apicomplexan parasites need energy, building blocks, and high level of cholesterol for offspring development. Nevertheless, these parasites are generally considered as defective for *de novo* cholesterol synthesis and need to scavenge this molecule from their host cells as has been shown previously for *T. gondii*, *Neospora caninum*, *Cryptosporidium parvum*, *E. bovis* and *Plasmodium* spp. (Hamid et al. 2015; Coppens, Sinai, and Joiner 2000; Ehrenman et al. 2013; Labaied et al. 2011; Nolan et al. 2015; Grellier et al. 1994).

That is why, the host cell can enhance ist endogenous *de novo* synthesis or upregulate LDL-mediated cholesterol uptake from extracellular sources to provide *E. arloingi* with sufficient cholesterol. Hamid et al (2015), demonstrated an increase in total cholesterol contents for *E. bovis*-infected endothelial host cells (Hamid et al. 2015). On the contrary, *T. gondii* scavenges cholesterol via enhanced LDL-uptake but not via induction of *de novo* synthesis in CHO cells (Coppens, Sinai, and Joiner 2000) *N. caninum and C. parvum* also mainly relies on lipoprotein uptake (Coppens, Sinai, and Joiner 2000; Ehrenman et al. 2013).

Similarly to *E. bovis*, *E. arloingi* has high replicative capacity during first merogony and live for almost a month within its endothelial host cell for development *in vitro*. In *E. bovis*, it has been demonstrated the use of both cellular pathways of cholesterol acquisition, since it showed upregulation on a transcriptional level in infected endothelial host cells of molecules associated with *de novo* biosynthesis pathway and of LDL mediated cholesterol uptake (Taubert et al. 2010; Hamid et al. 2014). Despite that, recently was reported that BUVEC cells infected with *Besnoitia besnoiti* are not capable of upregulate LDL receptor (Liliana M.R. Silva et al. 2019). In that context LDL cholesterol acquisition could rely in other mechanism, like the uptake by Scavenger receptor, class B, type I (SR-BI). SRBI is a 82 kDa membrane protein of the CD36 family (Shen, Azhar, and Kraemer 2018) which works as a cell surface receptor that mediates the selective uptake of lipids from lipoproteins to cells (Stangl, Hyatt, and Hobbs 1999). SRBI has been described as the main receptor multiple ligands like high (HDL), low (LDL), and very low density lipoproteins, oxidized LDL, acetylated LDL, and anionic phospholipids (Stangl, Hyatt, and Hobbs 1999).

In order to determine the cholesterol modulation carried out by *E. arloingi* in BUVEC during the first merogony we analyzed the effect of Block Lipid Transport-1 (BLT-1) in infected cells, and compared the development and production of merozoites between treated and non-treated infected cells.

Methodology

Parasites

E. arloingi (strain A) was initially isolated in 2012 from naturally infected goat kids, in the province of Alentejo, Portugal. Fecal samples collected directly from the rectum of kids and dairy goats were examined with a modified McMaster technique (Whitlock 1948). For the oocysts production, two 12-week-old goats (without previous *Eimeria* oocyst exposure) were infected orally with 3×10^5 sporulated *E. arloingi* oocysts. Then, oocysts were isolated from feces beginning at 18 days p. i. according to Jackson, 1964. The feces were washed through a set of three sieves (pore sizes 850, 250 and 80 µm) with tap water. The final suspension was sediment overnight. After that, the sediment was mixed with saturated sucrose solution (specific gravity 3) relation 1:1 (final concentration: 1.5 SG). The suspension was transferred into at plastic trays (30x20x5 cm) horizontally adjusted. The trays were filled to the top and were covered with clean glass sheets, allowing complete contact of the suspension with the surface. Every 2 h the glass were removed and the oocysts adhering were washed off with water into a container. The remaining suspension in the plastic tray was stirred up and the process was repeated up to six times or until few oocysts were left (microscopic examination). The oocyst collected were diluted with water (1:1) and then centrifuge at 2000 rpm for 10 min. The pellet was re-suspend in potassium bichromate (Merck) solution (2%, w/v), at room temperature (RT, 25 °C), with constant oxygenation until the oocyst were sporulated. After 90% of the oocyst were complete sporulated, suspension was centrifuged (1700 g, 10 min) and the sediment containing the oocysts was suspended in fresh 2% (w/v) potassium bichromate solution and stored at 4°C (Kowalik S, Zahner H, 1999).

Excystation of E. arloingi

For the isolation of viable *E. arloingi* sporozoites, the following modified excystation protocol was used (Silva et al., 2015). Sporulated *E. arloingi* oocysts stock solution was added to 4 % (v/v) sodium hypochlorite solution and magnetically stirred on ice for 20 min. After mixing in the vortex for 20 s, oocyst solution was centrifuged (300×g, 5 min) and supernatant was mixed with bi-distilled water (1:1). Washed oocyst solution was then filtered through 40 and 5 µm sieves to remove remaining debris. Then, oocyst were centrifugated 15 min

at 600×g, and the pellet was resuspended in 0.02 M L-cysteine/0.2 M NaHCO3 (Merk) solution and incubated in a 100 % CO2 atmosphere (37 °C, 20 h). Afterwards, oocysts were re-suspended in the following excystation medium: Hank's balanced salt solution (HBSS, Gibco) containing 0.4 % (w/v) trypsin (Sigma) and 8 % (v/v) sterile filtered bovine bile (obtained from the local slaughterhouse, due to the lack of caprine bile availability). Afterwards, oocysts were incubated up to 4 h (37 °C, 5 % CO2 atmosphere). Every hour, excystation progress was checked under an inverted microscope (IX81, Olympus®) to estimate the number of free released sporozoites. Freshly released sporozoites of *E. arloingi* were filtrated through 5 µm and then washed two times (600×g, 15 min) with cell culture medium 199 (M199, Gibco) supplemented with 2 % (v/v) fetal calf serum (FCS, Gibco) and 1 % penicillin (v/v, 500 U/ml; Sigma-Aldrich) and streptomycin (v/v, 500 µg/ml; PS; Sigma-Aldrich) and finally suspended in culture medium (2×10^6 sporozoites/ml).

Host cells

Primary bovine umbilical vein endothelial cells (BUVEC) used in this study were isolated according to Jaffe et al. 1973. Umbilical cords from 3 animals were collected under aseptic conditions by *sectio caesaria* and kept at 4 °C in 0.9% HBSS-HEPES buffer (pH 7.4, Gibco) supplemented with 1% penicillin (500 U/mL, Sigma-Aldrich) and streptomycin (50 µg/mL, Sigma-Aldrich). Then, 0.025% collagenase type II (Worthington Biochemical Corporation) suspended in Pucks solution (Gibco) was infused into the lumen of the ligated umbilical vein and incubated for 20 min at 37 °C in 5% CO_2 atmosphere. After massaging the vein, the cell suspension was collected and supplemented with 1 mL fetal calf serum (FCS, Gibco). After two washings (400 × *g*, 10 min, 4 °C), cells were re-suspended in complete endothelial cell growth medium (ECGM, PromoCell), plated in 25 cm^2 tissue plastic culture flasks (Greiner) and kept at 37 °C in 5% CO_2atmosphere. BUVEC cell layers were used for infection after 1–2 passages *in vitro*. BUVEC were cultured in modified ECGM medium (EGCM, PromoCell, diluted 0.3× in M199 medium, Sigma-Aldrich) with medium changes every 2–3 days.

Host cell infection

Three different BUVEC isolates were used for host cell infection experiments. BUVEC isolates (n=3) were seeded in two 25cm2 cell tissue culture plastic flasks (Greiner) and maintained in ECGM supplemented with 10 mM Glucose and 1% PS. BUVEC monolayers were infected with 2.5×10^5 freshly isolated sporozoites on cell monolayers with 90% confluency. Culture medium was changed 24h after sporozoite infection and thereafter every two days. Using microscopy and photography, the *Eimeria*-infected host cells were evaluated daily with the aim to follow parasite development. The number of *Eimeria*-infected host endothelial cells was calculated by counting at least 3 different 400-fold magnification power vision fields at 4 d.p.i.

Cholesterol inhibition assay

Once sporozoites began their development inside the cell (early meront, 15 dpi), BLT-1 was used as Cholesterol inhibitor at a concentration of 2 uM. Block lipid transport-1 (BLT-1) is a specific inhibitor of the SR-BI (Scavenger receptor, class B, type I) mediated lipid transfer. The compound inhibits both cellular selective lipid uptake of HDL cholesteryl ether and efflux of cellular cholesterol to HDL.

First merogony was closely followed and arbitrarily selected meronts (n = 40/BUVEC) were measured at 17, 19, 21, 24, and 26 days p.i. using the software CellSens® Dimension 1.7® (Olympus). The area of the meronts were calculated using the software IMAGE J software (Fiji version 1.7, nih). From 21 days p.i., merozoites I were harvested and saved at -80°C for molecular quantification by qPCR.

Furthermore, *E. arloingi* sporozoites were incubated with BLT-1 (2 uM) for two hours prior to the infection and infection rate was calculated after 4 d.p.i. In addition, pre-treated cells (BLT-1 2 uM 24 hours before infection) were exposed to non-treated *E. arloingi* sporozoites and infection rate was also calculated 4 d.p.i.

Results

Eimeria arloingi *macromeront formation* in vitro

Non-treated *E. arloingi* macromeront development *in vitro* in this study was achieved as previously described (Liliana M R Silva et al. 2015). The initial infection rate with the freshly excysted sporozoites was 7.74 ± 0.9%. Nevertheless, just small proportion of these intracellular stages undergo successful macromeront development. Just 11.4% ± 0.6 % of infected-host cells achieved fully developed macromeronts at 21 days p.i. The sizes of meronts increased from day 15 to day 21, reaching a maximum value of 165,33 x 395,22 µm diameter/meront (Fig. 1) and releasing the merozoites I from day 21 to 26 p.i. (Fig. 1).

Inhibition of SRB1 blocks the proliferative capacity of E. arloingi

To estimate the effect of the BLT-1 during the first merogony, the area and number of meronts was calculated from day 15 to day 26 p.i. (Fig. 2). The development in both groups (treated and non-treated *E. arloingi* infected-BUVEC) was similar until 17 days p. i., during these days, the morphology and size of the early meronts were variable, but showed a normal distribution. Nevertheless, from 19 d.p.i to 21 d. p. i. the non-treated meronts were bigger and showed the normal morphometry of a mature and healthy meront, which produced viable merozoite I at 21 days p. i.. At day 24 p. i the distribution changed, showing bigger meronts in the treated ones than in the control, continuing with this behavior until day 26 p.i. Nevertheless, the BLT-1 treated group did not produce any merozoite I. The meronts form this group continued growing but never development merozoites inside (Fig. 1). The non-treated meronts that were seen at 24 – 26 days p. i. (smaller than the treated ones) probably are the ones that began their development late, showing the normal asynchronous development of the parasite. The infected cells were monitored daily until 35 days p. i, but no production of merozoites I were seen in the BLT-1 treated, instead a degeneration of the meronts and death of the surrounding cells were observed.

Importantly, infection rates were not affected by pre-incubation of *E. arloingi* sporozoites with BLT-1 for 2 hr prior to addition to cells, showing that BLT-1 has

no effect on sporozoite viability (data not shown). The infection rate was 8.12%, similar to the non-treated ones (8.21%). Nevertheless, pretreatment of the cells with BLT-1 generated a slight decrease in the infection rate (6.5%).

Discussion

Due to its massive replication capacity within the first merogony in host ECs, *Eimeria arloingi* is considered as one of the most pathogenic species in caprine coccidiosis, causing severe hemorrhagic enteritis (Ruiz et al. 2006; Liliana Machado Ribeiro da Silva et al. 2014). In this work, we evaluated the *in vitro* response of *E. arloingi* to the BLT-1 inhibitor, to evaluate its dependence in cholesterol uptake via SR-B1.

Many pathogens are auxotrophic for sterols and need to obtain these lipids from their hosts to maintain the structural and functional integrity of their organelles and membranes, and to produce viable progeny. To this end, we first confirmed that inhibition of SR-BI leads to a reduction in *E. arloingi* development in primary bovine endothelial cells. Because of the complete inhibition of merozoite I production in the treated cells, we suggest that the uptake of lipids via SR-B1 is necessary for the parasite´s replication in the host cell.

Pretreatment of the cells or sporozoites with BLT-1 didn't change significantly the infection rate, suggesting that this route may not be important in the early stage of the merogony (before 8 d.p.i). This was also notice by hamid et al., 2015, which found that transcription in genes that encode lipid metabolism have a significant change in times of macro- meront formation (Hamid et al. 2015) but not earlier.

Given that cellular cholesterol synthesis is tightly regulated by a complex network of cellular mechanisms, more research is needed to understand how *E. arloingi* regulates this pathways.

Authors and contributors

CH, AT, LS and ZV designed the project and experiments. SL carried the experiments. SL, LS, ZV, and CH drafted and edited the manuscript. CH, LS

and AT coordinated the project. All authors have read and approved the manuscript as submitted.

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Conflict of Interest Statement

All other authors declare no competing interests.

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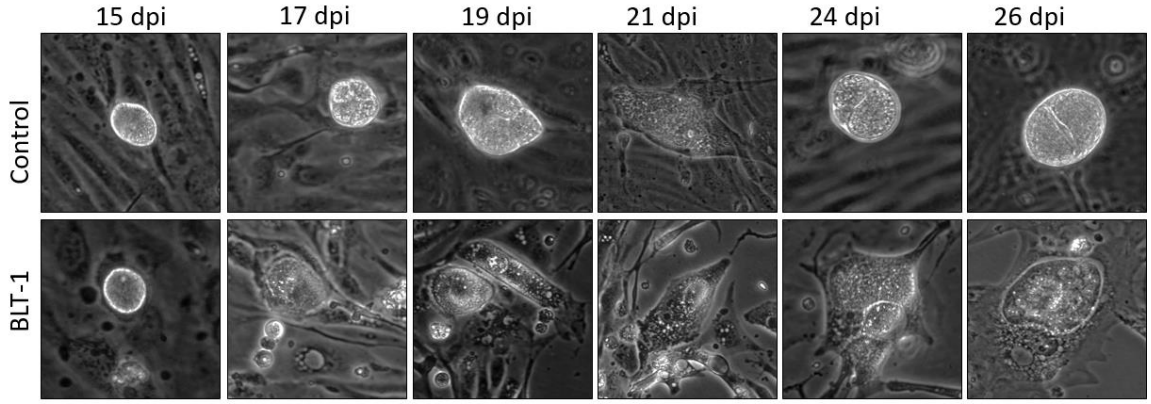
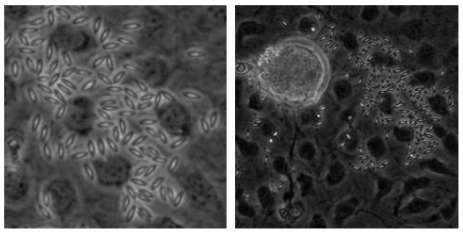
Figures

Fig. 1. *In vitro* development of *E. arloingi* in BUVEC. The panel shows pictures of *E. arloingi* in during the merogony, in both BLT-1 treated and non-treated cells. The development of the meront was similar in both groups, nevertheless, the production of merozoites was achieved only in non-treated infected cells. The sizes of different stages of *E. arloingi* are shown in the table.

Fig. 2. Effect of BLT-1 in the area (µm) of the meront during the first merogony in *E. arloingi* infected BUVEC. Left side shows the distribution of the sizes of the meronts treated (discontinuous line) and non-treated (black line). The right side shows the average size µm of the meronts in both groups in different days during the first merogony.

Fig.1

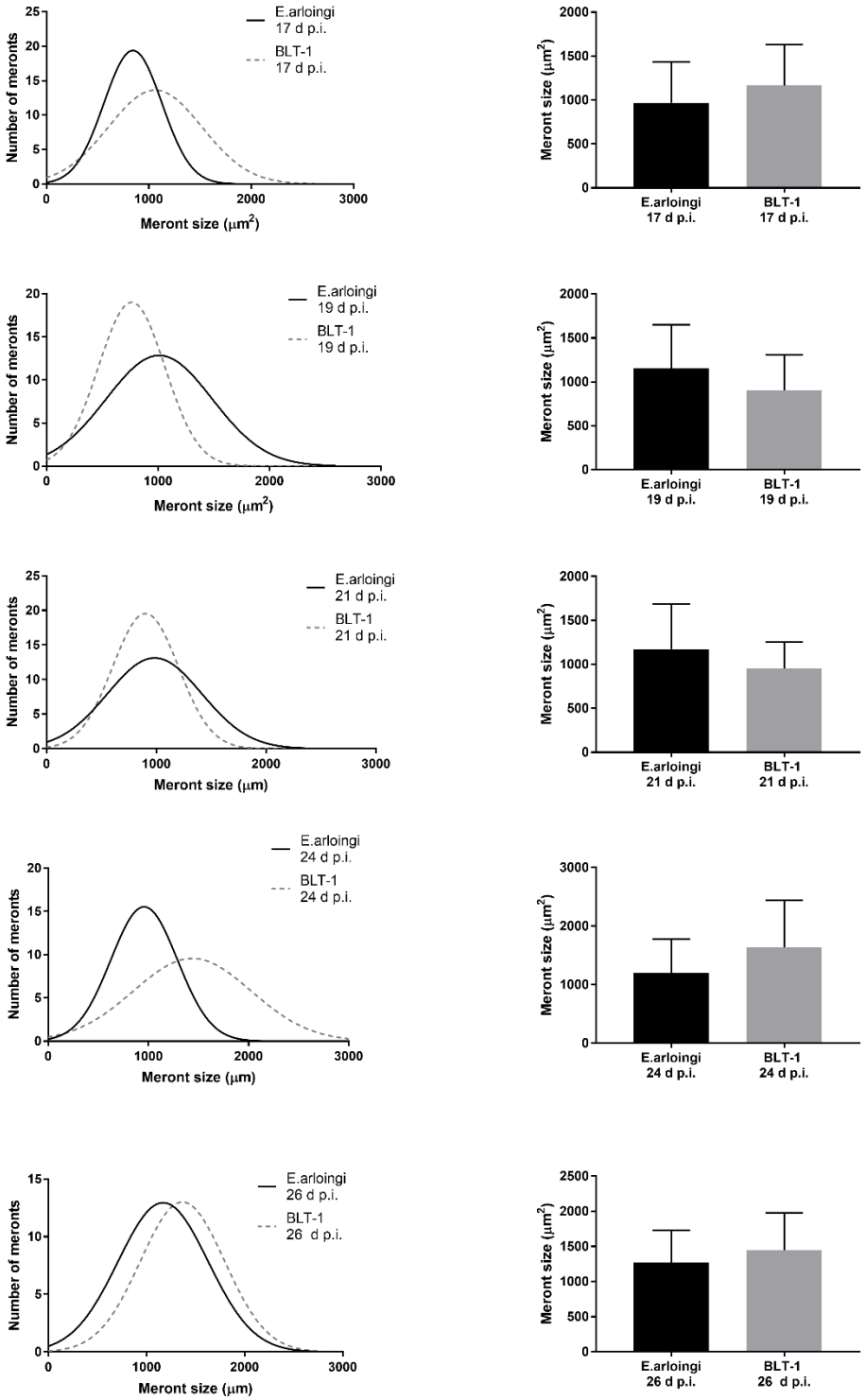




Merozoites: Control

	Size µm
Sporozoites n=67	8,3x 1,7
Trophozoite n=50	17,88 x 15.69
Early schizont n=50	35,12 x 40,22
Mature schizont n=50	165,33 x 387
Oocyst n=50	29,47 x 22,07

Fig.2


E.arloingi 17 d p.i.
BLT-1 17 d p.i.
Number of meronts
Meront size (μm²)
Meront size (μm²)
E.arloingi 17 d p.i.
BLT-1 17 d p.i.
E.arloingi 19 d p.i.
BLT-1 19 d p.i.
Number of meronts
Meront size (μm²)
Meront size (μm²)
E.arloingi 19 d p.i.
BLT-1 19 d p.i.
E.arloingi 21 d p.i.
BLT-1 21 d p.i.
Number of meronts
Meront size (μm)
Meront size (μm²)
E.arloingi 21 d p.i.
BLT-1 21 d p.i.
E.arloingi 24 d p.i.
BLT-1 24 d p.i.
Number of meronts
Meront size (μm)
Meront size (μm²)
E.arloingi 24 d p.i.
BLT-1 24 d p.i.
E.arloingi 26 d p.i.
BLT-1 26 d p.i.
Number of meronts
Meront size (μm)
Meront size (μm²)
E.arloingi 26 d p.i.
BLT-1 26 d p.i.

7. Chapter: Metabolic requirements of bovine polymorphonuclear neutrophils casting NETs formation against vital Eimeria bovis sporozoite stages

This chapter is based on the following manuscript:

Lopez-Osorio S, Conejeros I, Zhou E, Taubert A, Hermosilla C (2019). Metabolic requirements of bovine polymorphonuclear neutrophils casting NETs formation against vital Eimeria bovis sporozoite stages *(manuscript in preparation)*.

Own contribution in the publication
Initiative: as far as possible
Project planning plan: as far as possible
Carrying out the experiment: as far as possible
Evaluation de experiment: as far as possible
Creation of the publication: essential

Metabolic requirements of bovine polymorphonuclear neutrophils casting NETs formation against vital *Eimeria bovis* sporozoite stages

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Abstract

Eimeria bovis infection in cattle is a protozoan parasitosis, known as coccidiosis, causing severe clinical typhlocolitis in calves. In contrast to most other bovine *Eimeria* species, *E. bovis* sporozoites must traverse through intestinal epithelium in order to reach their final specific host endothelial cells of lymphatic vessels, and thereby becoming potential targets of polymorphonuclear neutrophils (PMN) circulating in the lymph *in vivo*. Neutrophil extracellular traps (NETs) released by bovine PMN exposed to *E. bovis* sporozoite- and merozoite I-stages have previously been reported. Nevertheless, there is no current data on metabolic requirements of *E. bovis* sporozoite-triggered NETosis. Therefore, we determined relevance of distinct PMN-derived metabolic pathways via pharmacological inhibition experiments of *E. bovis* sporozoite-triggered NETs. Furthermore, we documented in real time mitochondrial activation in bovine PMN exposed to vital *E. bovis* sporozoites by quantifiying oxygen consumption rates (OCR) and extracellular acidification rates (ECAR). Isolated *E.bovis* sporozoites-induced two phenotypes of NETs, i. e. cell free- and anchored-NETs, and both being significantly diminished via PMN-pretreatments with ATP-synthase inhibitor (oligomycin 5µM), lactate dehydrogenase inhibitor (OXA 50mM), MCT-lactate

transporter inhibitors (AR-C141900, AR-C151858, 1 µM), and P2Y1 purinergic signaling inhibitors (theobromine and NF449, 100 µM), thereby indicating a key role of ATP, pyruvate- and lactate-mediated metabolic pathways for proper sporozoite-mediated NETosis. Furthermore, anchored-NETs were increased by enhanced pH conditions. Mitochondrial OCR in PMN stimulated with sporozoites were significantly higher than control PMN, indicating activation after 30 min of exposure to *E. bovis*, whereas ECAR did not show significant differences. In summary, current data provide first evidence on carbohydrate-related metabolic pathways and purinergic energy supply of bovine PMN while casting NETs against vital *E. bovis* sporozoites.

Keywords: *Eimeria bovis*, PMN, metabolic requirements, NET formation, cattle

Introduction

Eimeria bovis is an obligate intracellular apicomplexan parasite of cattle, which causes severe hemorrhagic typhlocolitis in calves and thereby producing high economic losses worldwide in cattle industry (Daugschies and Najdrowski 2005). Despite the fact that early host innate defense reactions should be critical for the outcome of *E. bovis* coccidiosis (Behrendt et al. 2008), only few studies have been performed on early host innate immune reactions during *E. bovis* infections *in vivo* (Fiege et al. 1992; Behrendt et al. 2004, 2008; Hermosilla et al., 1999) and *in vitro* (Hermosilla et al., 2006; Muñoz-Caro et al. 2015). So far, it has been demonstrated that polymorphonuclear neutrophils (PMN) play an important role against different endogenous stages of *E. bovis,* such as sporozoites and merozoites I (Muñoz-Caro, Huertas, et al. 2015). PMN interact with these *E. bovis* stages, resulting in parasite killing through phagocytosis or production of pro-inflammatory cytokines (i. e. IFN*y,* IL-12), chemokines (CXCL8, CXCL1) and iNOS (Behrendt et al. 2008). Furthermore, PMN phagocytic and oxidative burst activities are enhanced in response to *E. bovis* sporozoites *in vitro, ex vivo* and *in vivo* during *E. bovis* infections (Behrendt et al., 2008; Muñoz-Caro et al. 2015, 2016). In addition, *E. bovis* acts as potent parasite inducer of neutrophil

extracellular traps (NETs) *in vitro* (Behrendt et al. 2010; Muñoz-Caro, Huertas, et al. 2015) and *in vivo* (Muñoz-Caro et al. 2016). Intestinal NETs formation was not only detected surrounding *Eimeria* meronts stages but also to gamonts as well as luminal oocyst stages (Muñoz-Caro et al. 2016). Interestingly, NETs can firmly attach to *E. bovis* sporozoites surface thereby entrapping them and efficiently inhibiting host cell invasion *in vitro* (Behrendt et al. 2010). Concerning signaling pathways, *E. bovis*-triggered NETosis has been known as ROS-, store-operated calcium entry (SOCE)-, NE-, MPO- as well as CD11b-dependent process (Muñoz-Caro et al. 2015). Additionally, for other closely related apicomplexans, such as *Toxoplasma gondii, Neospora caninum, Besnoitia besnoiti* and *Cryptosporidium parvum,* NETosis release is also regulated by ERK1/2 and p38 MAPK signaling pathways (Muñoz-Caro et al. 2014, 2016; Díaz-Godínez and Carrero 2019). However, studies on metabolic and mitochondrial requirements of bovine PMN during *E. bovis*-triggered NETosis are still not available in literature although PMN undergoing this process are in enormous energy need (Chacko et al. 2013; Traba et al. 2016; Conejeros et al., 2019). In fact, it has been described that PMN can use different metabolic pathways to obtain energy for displaying their effector mechanisms. Furthermore, PMN can effectively shift between a resting and/or an activated status by switching energy producing pathways on and off (Pearce et al. 2013; Traba et al. 2016) or by via autophagy (Zhou et al. 2019). One way to address the states of energy producing pathways in PMN is the simultaneous measurement of oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) in real time, as indicators of oxidative phosphorylation and glycolysis (Pelletier et al. 2014; Mookerjee and Brand 2015; Chacko et al. 2013).

In this context, we analyzed the activation of isolated bovine PMN being exposed to *E. bovis* sporozoites using an extracellular flux analyzer, the Seahorse Xfp® (Agilent), which can compare changes in OCR and ECAR in real time with different stimulation conditions. Therefore, we explored the metabolic pathways as well as the role of the purinergic receptors (P2X1 and P1A1) and the monocarboxylate transporter (MCT1 and MCT2) whilst *E. bovis*-triggered NETs formation. Furthermore, key role in glycolysis and lactate pathways was analyzed

via functional inhibition assays. Additionally, we studied the role of pH in parasite-induced NETosis via pH-adjusted media and pharmacological inhibition of MCT (lactate transporter).

Materials and methods

Ethics statement

All experiments were conducted in accordance to the Justus Liebig University Giessen Animal Care Committee Guidelines. Protocols and farther approved by the Ethic Commission for Experimental Animal Studies of the Federal State of Hesse (Regierungspräsidium Giessen; A9/2012; JLU-No.521_AZ), and in accordance to current European Animal Welfare Legislation: ART13TFEU.

Parasites

For *Eimeria bovis* oocyst production, two 8-weeks-old calves, kept in isolation conditions without *Eimeria* spp. or parasite exposure, were infected orally with 3×10^4 sporulated *E. bovis* (strain H) oocysts as reported elsewhere (C. Hermosilla et al. 2002). Then, oocysts were isolated from faeces beginning at 18-20 days *post infectionem* (p. i.) according to Jackson 1964 (Jackson AR 1964). The faeces were washed through a set of three metal sieves (pore sizes 850, 250 and 80 µm, respectively) with cold tap water. Final washing suspension was let to sediment overnight. The sediment was mixed 1:1 with saturated sucrose solution (ρ = 1.3 g/mL) to a final density of 1.15 g/mL. The suspension was transferred into plastic trays (30 × 20 × 5 cm) horizontally adjusted. Plastic trays were filled to the top and were thereafter carefully covered with clean glass plates, allowing complete contact of oocyst suspension to the glass plate surface. Every 4 h the glass plates were carefully removed and adherent oocysts were washed off with tap water into a plastic container. The remaining suspension in the plastic trays was stirred up and the process was repeated up to six times or until few oocysts were detected [microscopic examination, less than 5 oocysts per power vision field (20x magnification)]. Collected oocysts were diluted with water (1:1) and then centrifuged at 600 × *g* for 12 min. The pellet was

resuspended in potassium dichromate (Merck) solution [final concentration 2% (*w/v*), at room temperature (RT)], with constant aeration until oocysts achieved complte exogenous sporogony. When more than 90% of oocysts were completely sporulated, suspension was centrifuged (600 × *g*, 12 min, RT) and sediment containing sporulated oocysts was re-suspended again in fresh 2% (*w/v*) potassium dichromate solution and stored at 4 °C until further experimental use (Kowalik and Zahner 1999; Hermosilla et al. 2002).

For obtention of viable *E. bovis* sporozoites, the following modified excystation protocol was here applied (Silva et al. 2015). Sporulated *E. bovis* oocysts stock solution was added to 4 % (*v/v*) sodium hypochlorite solution and magnetically stirred on ice for 20 min. After mixing with vortex for 20 s, oocyst solution was centrifuged (300 × *g*, 5 min) and supernatant was mixed with bi-distilled water (1:1). Washed oocyst solution was then filtered through 40 and 10 µm sieves (PluriSelect) to remove remaining debris. Then, oocysts were centrifuged at 600 × *g* for 15 min and the pellet was re-suspended in 0.02 M L-cysteine/0.2 M NaHCO3 (Merk) solution and incubated in a 100% CO_2 atmosphere (37 °C, 20 h). Afterwards, oocysts were re-suspended in the following excystation medium: Hank's balanced salt solution (HBSS, Gibco) containing 0.4% (*w/v*) trypsin (Sigma-Aldrich) and 8% (*v/v*) sterile filtered bovine bile (obtained from a local slaughterhouse). Afterwards, sporulated *E. bovis* oocysts were incubated for upto 3-4 h at 37 °C and 5 % CO_2 atmosphere (C. Hermosilla et al. 2002). Every hour, excystation progress was controlled under an inverted microscope (IX81, Olympus®) in order to estimate the number of free-released sporozoites. Freshly released *E. bovis* sporozoites of were filtered througth 5 µm pore-sized sieves (PluriSelect) and then washed two times (600 × *g*, 15 min) with cell culture medium 199 (M199, Gibco) supplemented with 2 % (*v/v*) *Mycoplasma* spp.-free foetal calf serum (FCS, Gibco), 1 % penicillin (*v/v*, 500 U/ml; Sigma-Aldrich) and streptomycin (*v/v*, 500 µg/ml; Sigma-Aldrich), and finally suspended in RPMI 1640 cell culture medium (Sigma-Aldrich) with a final concentration of 2×10^6 sporozoites/ml.

Isolation of bovine PMN

Healthy adult dairy cows (*n* = 9) served as blood donors. Animals were bled by puncture of jugular vein and 30 ml peripheral blood was collected in 10 ml heparinized sterile plastic tubes (Kabe Labortechnik). 20 ml of heparinized blood were diluted in 20 ml sterile PBS with 0.02% EDTA (Sigma-Aldrich), layered on top of 12 ml Biocoll separating solution (density = 1.077 g/l; Biochrom AG) and centrifuged (800 × *g*, 45 min) according to Muñoz-Caro et al. (2014) (Muñoz Caro et al. 2014). After removal of plasma and peripheral blood mononuclear cells (PBMC), remaining cell pellet was suspended in 27 ml bi-distilled water and gently mixed during 30 s to lyse erythrocytes. Osmolarity was rapidly re-stored by adding 3 ml of 10 × HBSS (Biochrom AG). For complete erythrocyte lysis, this step was repeated twice and PMN were later suspended in sterile RPMI 1640 cell culture medium (Sigma-Aldrich). PMN counts were analyzed in a Neubauer haemocytometer. Finally, freshly isolated bovine PMN were allowed to rest at 37 °C and at 5% CO_2 atmosphere for 30 min until further use.

For live cell-parasite interaction experiments (i. e. metabolic assay with Agilent Seahorse Xfp® (Agilent), the protocol for erytrocyte lysis was modified. In brief, after the Biocoll gradient, plasma and buffy coat were aspirated. The remaining erythrocytes and PMN pellet were suspended in Hank's balanced salt solution (HBSS). The erythrocytes were removed by flash hypotonic lysis using a cold phosphate-buffered water solution (5.5 mM NaH2PO4, 8.4 mM HK2PO4, pH 7.2). After 1 min of incubation, a hypertonic phosphate-buffered solution (5.5 mM NaH2PO4, 8.4 mM HK2PO4, 0.46 M NaCl, pH 7.2) was used to return the isotonicity. Then, cells were centrifuged at 600 x *g* for 10 min. The lysis step was repeated twice (or until the complete lysis of erythrocytes was achieved) (Conejeros et al. 2012). The remaining PMN pellet was then washed with HBSS. PMN counts were analyzed in a Neubauer haemocytometer. Finally, freshly isolated bovine PMN were allowed to rest at 37 °C and 5% CO_2 atmosphere for 30 min until further use.

Metabolic assays

To assess if *E. bovis* sporozoites generate bovine PMN activation, quantification of oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were additionally here performed. A total of 1 x 10^5 PMN from three different animals were seeded in poly-$_L$-lysine (0.001%) coated XF96 plate in XF media (non-buffered DMEM containing 10 mM glucose, 4 mM L-glutamine, and 2 mM sodium pyruvate). Cellular bioenergetics of PMN were determined using the extracellular flux analyzer Seahorse Xfp® (Agilent), which measures O_2 and proton fluxes. This system allows non-invasive and real time measurements of OCR, the rates of total extracellular acidification, and proton efflux rates (PER), [PER (pmol H+/min) = ECAR (mpH/min) × buffer factor (mmol/L/pH) × geometric volume (µL) × Kvol], which can be correlated to PMN mitochondrial function/oxidative burst and glycolysis (Dranka et al. 2011). Changes in OCR and ECAR were measured in response to activation by *E. bovis* sporozoites (1:1) in the presence of mitochondrial inhibitors rotenone and antimycin A (Rot/AA, 14 µM). The addition of mitochondrial inhibitors in these metabolic assays before PMN activation ensures that any oxygen consumption through mitochondrial respiration can be excluded.

Extracellular DNA-based quantification of NETs

Bovine PMN were suspended in cell culture medium RPMI 1640 (Sigma-Aldrich) lacking phenol red and serum according to Muñoz-Caro et al. (2014), confronted with vital *E. bovis* sporozoites [96-well plates, duplicates; Greiner)] at a final PMN:*E. bovis* sporozoites ratio of 1:4 (2 × 10^5 PMN : 8 × 10^5 *E. bovis* sporozoites). Samples were incubated at 37 °C and 5% CO_2. For negative controls, PMN in normal serum-free cell culture medium RPMI 1640 without phenol red were employed. Zymosan (1 mg/ml; Sigma-Aldrich) stimulated PMN served as positive controls according to Muñoz-Caro et al. (2014). NETs were divided in two distinct forms (phenotypes) according to Tanaka et al. (2014) (Tanaka et al. 2014): *i)* NETs being released without further contact to PMN after extrusion, known as cell free-NETs, and *ii)* those NETs still anchored to PMN after extrusion, i. e. anchored-NETs. The 96-well plate was directly centrifuged at 300 × *g* for 5 min after incubation. The supernatants were transferred into a new

96-well plate to measure cell free-NETs and pellets were used for anchored-NETs estimation. For both sampling methods, a 1:200 dilution of Pico Green R (Invitrogen) in 10 mM Tris base buffered with 1 mM EDTA (Sigma-Aldrich) was added to each well (50 µl), and then extracellular DNA was detected and quantified by Pico Green R-derived fluorescence intensities using an automated multiplate reader (Varioskan Flash®, Thermo Scientific) at 484 nm excitation/520 nm emission (Villagra-Blanco et al. 2017; Muñoz Caro et al. 2014).

Inhibition assays and pH-related experiments

For inhibition assays, bovine PMN were pre-treated with inhibitors for 30 min and then co-cultured with *E. bovis* sporozoites (1:4 PMN:sporozoites ratio, 2 h, 37 °C, 5% CO_2). The following inhibitors were here used: 2-fluor-2-deoxy-D-glucose (FDG, 2 mM, Sigma-Aldrich; glucose analogue, inhibitor of glycolysis), sodium dichloroacetate (DCA, 8 mM, Sigma-Aldrich; inhibitor of pyruvate dehydrogenase kinase), oxythiamine (OT, 50 µM, Sigma-Aldrich; inhibitor of pyruvate dehydrogenase, α-ketoglutarate dehydrogenase and transketolase), sodium oxamate (OXA, 50 mM, Sigma-Aldrich; structural analog of pyruvate, inhibitor of lactate dehydrogenase), oligomycin A (5 µM, Sigma-Aldrich; inhibitor of ATP-synthase in mitochondrial respiration), 6-diazo-5-oxo-l-norleucin (DON; 4 µM inhibitor of glutaminolysis) theobromine (100 µM, Sigma-Aldrich; inhibitor of P1A1-mediated purinergic signaling), NF449 (100 µM, Tocris; purinergic receptor antagonist with high specificitiy for $P2X_1$), AR-C 141990 (1 µM, Tocris; MCT1 inhibitor) and AR-C 155858 (1 µM, Tocris; inhibitor of MCT1 and MCT2). All inhibitor concentrations were selected based on previous studies (Aronsen et al. 2014; Rodríguez-Espinosa et al. 2015; Seliger et al. 2013; Taubert et al. 2016; Villagra-Blanco et al. 2017; Wang et al. 2013).

For pH-related experiments, RPMI 1640 medium was adjusted to different pH values of 6.6, 7.0, 7.4 and 7.8, by HCL or NaOH (both Merck, Darmstadt, Germany) supplementation as previously described (Naffah de Souza et al. 2017). Bovine PMN were suspended in RPMI 1640 medium at diverse pH values and exposed to sporozoites. Experiments were performed as follows: 2×10^5

PMN were seeded in duplicates into 96-well plates (Greiner) and co-cultured with 8×10^5 *E. bovis* sporozoites or incubated in plain pH-adjusted medium (controls) for 2 h (37 °C, 5% CO_2 atmosphere).

NET production was quantified according to the fluorescence intensities obtained in the spectrofluorometric analysis (484 nm excitation wavelength and 520 nm emission wavelength) performed by an automated plate monochrome reader (Varioskan Flash®, Thermo Scientific) (Villagra-Blanco et al. 2017).

Statistical analysis

Statistical significance was defined by a p value <0.05. The p values were determined by applying non-parametric analyses: One-way analysis of variance was performed followed by Dunnett's multiple comparison test (DMCT). All graphs (mean ± SD) and statistical analyses were generated by the use of Graph Pad® software (v. 7.03).

Results

E. bovis *sporozoites activate bovine PMN after 30 min of exposure*

Figure 1 shows the increase in OCR observed when PMN are stimulated with *E. bovis* sporozoites, begining at 30 min post stimulation, and rising slowly over the time. OCR values for *E. bovis* exposed PMN were significantly higher than the ones of controls (p = 0.0006). This PMN activation assay shows that oxygen consumption in *E. bovis*-stimulated cells is an early measurement of PMN activation which increases slowly in time with no peaks. In the other hand, PER was higher in *E. bovis*-stimulated PMN but did not show any statisticaly differences when compared to non-stimulated PMN (controls; Fig. 1). This result indicates that extracellular acidification may not be correlated with glycolysis in sporozoite-activated bovine PMN (Dranka et al. 2011).

E. bovis-*induced 'cell free'- and ´anchored´-NETs were blocked via inhibition of lactate- and ATP- pathways*

The relevance of PMN metabolism during *E. bovis*-mediated NETosis was studied using selected metabolic inhibitors which interfere with glycolysis, ATP, lactate and purinergic pathways. Quantification of 'anchored'- and 'cell free'-NETs confirmed that vital sporozoites triggered both phenotypes in stimulated bovine PMN. Functional inhibition experiments showed that this process was independent of glucose consumption since FDG did not influenced NETs formation. In contrast, a significant decrease of 'cell free'- and ´anchored´-NET formation were observed in case of oligomycin A treatments (treated PMN + sporozoites *vs* non-treated PMN + sporozoites, oligomycin A: $p < 0.005$) suggesting that efficient *E. bovis* sporozoite-induced NETs formation seems dependent on mitochondrial ATP synthase activities. Additionally, lactate pathway appears to be pivotal for *E. bovis*-induced NETosis since OXA (inhibitor of lactate dehydrogenase), AR-C 141990 and AR-C 155858 (inhibitors of MCT1 and MCT2) significantly reduced both 'cell free'- and ´anchored´-NETs (treated PMN + sporozoites *vs* non-treated PMN + sporozoites: $p < 0.005$) (Fig. 2).

E. bovis-*induced NETosis seems dependent on P2X1-mediated ATP binding and P1A1-mediated purinergic signaling.*

To elucidate the relevance of purinergic signaling pathways in *E. bovis* sporozoite-induced NETosis, PMN were pre-treated with two specific inhibitors: theobromine (inhibits P1A1-mediated purinergic signaling) and NF449 (blocks P2X1-mediated purinergic signaling). Both PMN pre-treatments disminished parasite-triggered NET formation when compared to non-treated controls. These results suggest that sporozoite-induced NETosis depends on both P2X1-mediated ATP binding and P1A1-mediated purinergic signaling (Fig. 2).

E. bovis *sporozoite-induced NETosis was affected by pH values*

Here we investigated wether different extracellular pH conditions (6.6, 7.0, 7.4 and 7.8) would influence PMN while extruding NETs against *E. bovis* sporozoites. Production of 'anchored'-NETs in PMN stimulated with *E. bovis* sporozoites were significantly higher than in controls in all pH conditions. Additionally, the basic pH (7.8) resulted in higher amounts of 'anchored'-NETs when compared to acidic pH

(6.6) conditions, indicating a dependence of NET formation on alkaline pH (Fig. 3) and also that NET formation might be impaired in acidic conditions of inflammation. This PMN reduced activity at acidic pH has been demonstrated before (Cao et al. 2015). Nevertheless, no significant changes were seen on “cell free” NETs production after the exposure to different pH conditions, except for the basic pH (7.8) (Fig. 3).

Discusion

Hereby we investigate the relevance of selective metabolic pathways in bovine PMN for *E. bovis* sporozoite-induced NETosis. Inhibition of glycolysis with 2-FDG did not result in a significant reduction of parasite-triggered NETosis. This result is in contrast to Rodríguez-Espinosa et al. (2015) who showed that inhibition of glycolisis led to significant reduction of PMA-induced NETosis (Rodríguez-Espinosa et al. 2015). Nevertheless, this difference could be due to the differences on NET stimulation, the parasite stage or host-derived differences in response to PMA (i. e. human PMN versus bovine PMN) (Brown and Roth 1991). The process of glycolysis results in acidification of cell culture medium, which is measured here as proton efflux rates. An increase of OCR after PMN activation has been associated with a simultaneous increase of PER which is indicative for the dependence of PMN on glycolysis during activation (Mookerjee and Brand 2015; Plitzko and Loesgen 2018). Herein, we found an increase of OCR, indicating PMN activation, but not being associated with glicolisis (i. e. PER), once again suggesting that glycolisis is not determinant for the process of NETosis against *Eimeria* sporozoites.

On the contrary, the role of ATP regeneration during NETosis appears to be important. Treatment with oligomycin (inhibitor of mitochondrial ATP synthase) significantly reduced NET formation, which is in line to findings on oligomycin treatments in PMA-induced NETosis (Rodríguez-Espinosa et al. 2015). ATP is produced either by glycolysis or by mitochondrial respiration. However, PMN only have few mitochondria (Fossati et al. 2003; Maianski et al. 2004), and in contrast to current findings, older reports suggested that these organelles do not play a

key role in PMN-related energy metabolism (Borregaard and Herlin 1982). Nevertheless, blockage of mitochondrial ATP synthesis with oligomycin showed inhibitory effects on *E. bovis* sporozoite-triggered NET formation, confirming the relevance of mitochondrial ATP production for proper NETosis. Consistently, Fossati et al. (2003) demonstrated that oligomycin treatments also impaired chemotaxis and respiratory burst in human PMN (Fossati et al. 2003). In the other hand, extracellular ATP acts as second messenger molecule promoting communication between adjacent cells. Via purinergic receptors ATP drives purinergic signaling-dependent mechanisms in activated PMN. Chen et al., 2010, demonstrated that hydrolysis of extracellular ATP inhibited the process of PMN migration, and inhibition of purinergic signaling blocked PMN activation and impaired innate host responses to bacterial infection (Chen et al. 2010a). The purinergic receptors (e. g. P2X1, P1A2) are involved in PMN chemotaxis, phagocytosis, oxidative burst, apoptosis and degranulation (Tweedy et al. 2016; X. Wang et al. 2017; Chen et al. 2010b; Vaughan et al. 2007). In this study, we found that P2X1 receptors play a crucial role in *E. bovis* sporozoite-induced NETosis since inhibition of this receptor by NF449 and theobromine treatments significatively reduced ‘anchored’- and ‘cellfree’-NETs upon parasite exposure. This is in agreement with PMN data generated by Villagra-Blanco et al. (2017) who described receptors of the P2 family as important players in NET formation against *Neospora* spp. (Villagra-Blanco et al. 2017).

Interference with the lactate pathway confirmed the importance of lactate during NETosis since treatments with oxamate, AR-C 141990 and AR-C 155858 efficiently blocked *E. bovis*-induced formation of ‘cell free’- and ‘anchored’-NETs. Oxamate inhibits lactate dehydrogenase thereby reducing lactate release and regeneration of NAD+ (Ratter et al. 2018) which both may have an impact on NETosis. AR-C 141990 and AR-C 155858 are potent MCT inhibitors. MCT catalyse the bidirectional proton-linked transport of short-chain monocarboxylates such as L-lactate and pyruvate across the plasma membrane of mammalian cells (Halestrap 2012).

Since *E. bovis* sporozoite-triggered NETosis was associated with MCT1 and MCT2, we farther studied the effect of extracellular pH on *E. bovis* sporozoite-stimulated NETosis. It has been described that extracellular pH modulates the functions of immune cells (Kellum, Song, and Li 2004), including PMN (Geffner et al. 1999; Muñoz-Caro, Rubio R, et al. 2015). Recently, it was reported that extracellular acidification inhibited ROS-dependent NET formation (Behnen et al. 2017). Our data indicated that extracellular alkalization led to increase *E. bovis* sporozoite-induced NETosis, which is similar to NET-related reports in humans (Maueröder et al. 2016; Naffah de Souza et al. 2017). This enhancement of triggered NETosis by alkalinization through extracelular pH may be based on altered calcium fluxes, which leads to a PAD4-mediated citrullination of histones (H1, H2A,H2B, H3,H4) and results in significant *Neospora* spp.-triggered NETosis (Villagra-Blanco et al. 2017). Thus, acidification commonly found in conditions of inflammation may have influence on NETosis in the *in vivo* situation as postulated elsewhere (Khan et al. 2018).

In summary, this new NET-related study provides a better understanding on the relevance of metabolic pathways, purinergic signaling pathways (P2X1, P1A1), lactate transport (MCT1, MCT2), pH conditions and activation involved in *E. bovis* sporozoite-induced NETosis in exposed bovine PMN.

Authors contribution

CH, AT, and IC: designed the project and experiments. SLO: carried out most of PMN and NETosis experiments and analized data. EZ: carried out some PMN experiments ZV: performed LC3B confocal microscopy. SL: prepared the manuscript. SL and IC: prepared the figures. All authors reviewed the manuscript.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figures

Fig 1. Bovine PMN activation assay with *E. bovis* sporozoites. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) expressed as proton efflux rate (PER) for bovine PMN stimulated with *E. bovis* sporozoites were measured with extracellular flux analyzer (Seahorse XFp; Agilent). 100.000 PMN were plated in 8-well XFp Agilent plates, and rotenon/actynomicin and *E. bovis* sporozoites were injected sequentially. OCR and PER were measured during 240 min and the area under the curve of each registry was calculated for each experimental condition in order to quantify the activation of bovine PMN. Statistical significance was assessed by unpaired t-student test.

Fig 2. Inhibition of glycolysis, glutaminolysis, purinergic signaling (P2X1, P1A1) and monocarboxylate tranporters (MCTs) on *E. bovis* induced bovine NETs. Bovine PMN (n=3) were pre-treated for 30 min with FDG (2mM), DCA (8mM), OT (50µM), OXA (50mM), and Oligomycin (5µM) [glycolysis inhibitors], DON (4 µM) [glutaminolysis inhibitor], NF449 (100 µM), theobromine (100 µM) [purinergic signaling inhibitors], AR-C141990 (1 µM) and AR-C155858 (1 µM) [monocarboxylate tranporter inhibitors], followed by the exposure to *E. bovis* (ratio 1:4) at 37°C, 5% CO_2. For negative controls, PMN in normal serum-free medium RPMI 1640 without phenol red were employed. Zymosan (1 mg/ml; Sigma-Aldrich) stimulated PMN served as positive controls. After 2h incubation, samples were directly centrifuged at 300 × *g*, 5 min. The pellets were used for ‘anchored’-NETs estimation (A) and the supernatants were collected for ‘cell free’-NETs measurements (B). Extracellular DNA was detected and quantified by

PicoGreen®-derived fluorescence intensities using an automated multiplate reader (Varioskan®, Thermo Scientific). All data were performed and analyzed by one-way ANOVA followed by Dunnett's Multiple Comparison Test in GraphPad® software. Values are presented as mean ± SEM in the graphs and *p* values of <0.05 were considered statistically significant. Schematic representation of metabolic pathways and inhibitors used are presented in (C). FDG = fluoro 2-deoxy-D glucose, DCA = dichloroacetate, OT = oxythiamine, OXA = oxamate, DON= 6-Diazo-5-oxo-L-norleucine, oligomycin, NF449 (inhibitor of P2X1 receptor), theobromine (inhibitor of P1A1 receptor), AR-C141990 (MCT1 inhibitor) and AR-C155858 (inhibitor of MCT1 and MCT2).

Fig. 3 The pH dependency of *E. bovis* sporozoites induced bovine NETs. Bovine PMN (*n* = 3) were suspended in RPMI 1640 cell culture medium with different pH values (6.6, 7.0, 7.4 and 7.8), and then exposed to vital sporozoites (ratio 1 : 4) at 37 °C and 5% CO_2. After 2h of incubation, samples were directly centrifuged at 300 × *g*, 5 min. The supernatants were collected for 'cell free'-NETs measurement and the pellets were used for 'anchored'-NETs estimation. Extracellular DNA was detected and quantified by PicoGreen®-derived fluorescence intensities using an automated multiplate reader (Varioskan®, Thermo Scientific). All data were performed and analyzed by one-way ANOVA followed by Dunnett's Multiple Comparison Test in GraphPad® software. Values are presented as mean ± SEM in the graphs and p values of <0.05 were considered statistically significant.

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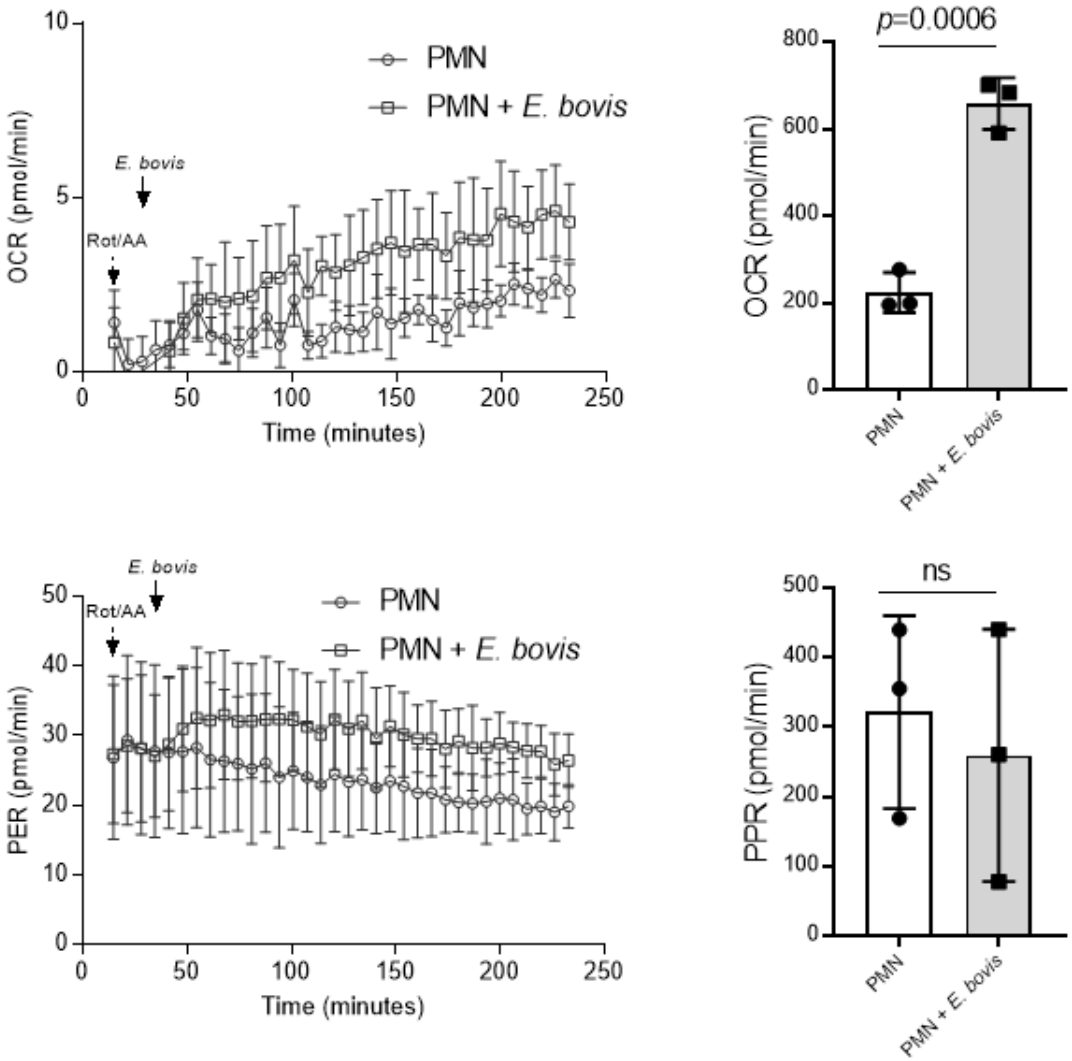
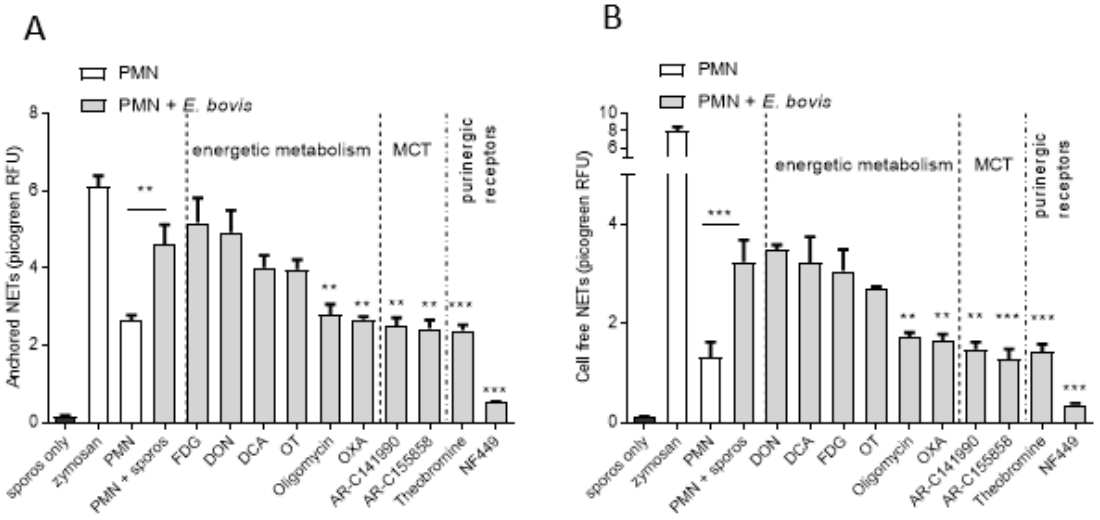


Fig 1. Bovine PMN activation assay with *E. bovis* sporozoites.


A
PMN
PMN + E. bovis
Anchored NETs (picogreen RFU)
energetic metabolism
MCT
purinergic receptors
sporos only
zymosan
PMN
PMN + sporos
FDG
DON
DCA
OT
Oligomycin
OXA
AR-C141990
AR-C155858
Theobromine
NF449
B
PMN
PMN + E. bovis
Cell free NETs (picogreen RFU)
energetic metabolism
MCT
purinergic receptors
sporos only
zymosan
PMN
PMN + sporos
DON
DCA
FDG
OT
Oligomycin
OXA
AR-C141990
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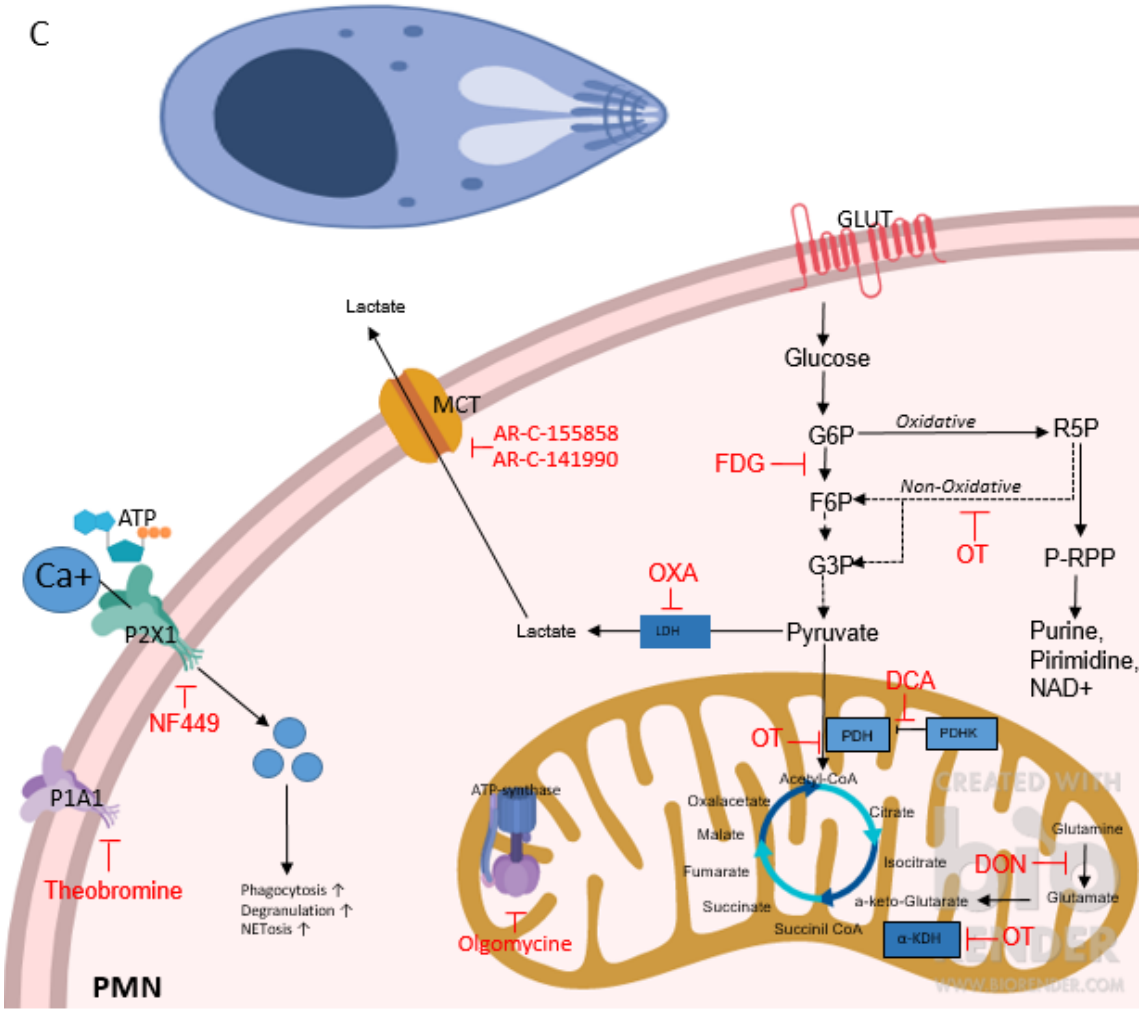
C
GLUT
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AR-C-155858
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Glucose
G6P
Oxidative
R5P
FDG
F6P
Non-Oxidative
OT
G3P
P-RPP
OXA
Lactate
LDH
Pyruvate
Purine, Pirimidine, NAD+
DCA
OT
PDH
PDHK
ATP
Ca+
P2X1
NF449
P1A1
Theobromine
Phagocytosis ↑
Degranulation ↑
NETosis ↑
ATP-synthase
Oligomycine
Acetyl-CoA
Oxalacetate
Citrate
Malate
Fumarate
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Succinate
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α-KDH
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Glutamate
OT
PMN

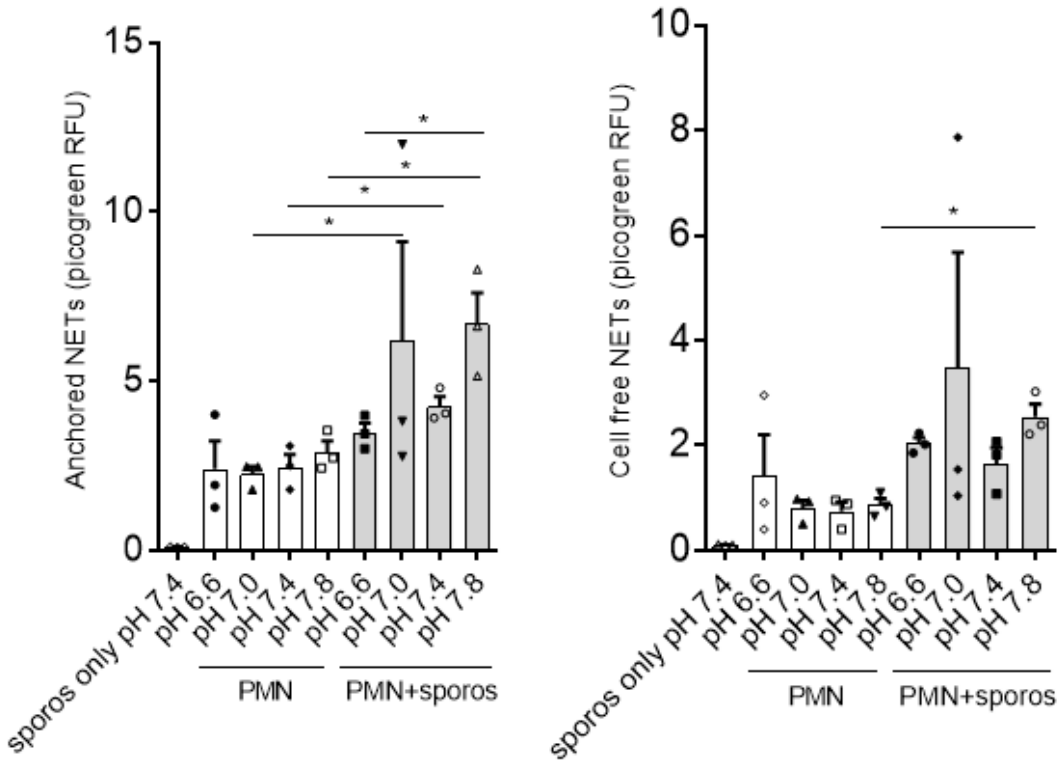

Fig. 3 pH dependency of *E. bovis* sporozoites induced bovine NETs.

8. Chapter: Co- ocurrence of authophagy and vital NETosis in *Eimeria bovis* sporozoite-exposed bovine Polymorphonuclear Neutrophils

This chapter is based on the following manuscript:

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Own contribution in the publication
Initiative: as far as possible
Project planning plan: as far as possible
Carrying out the experiment: as far as possible
Evaluation de experiment: essential
Creation of the publication: essential

Co- ocurrence of authophagy and vital NETosis in *Eimeria bovis* sporozoite-exposed bovine Polymorphonuclear Neutrophils

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Abstract

Eimeria bovis sporozoites infect bovine host endothelial cells of the central lymph capillaries of the ileum villi *in vivo*, making them potential targets for professional phagocytes when they are traveling from the intestinal lumen to reach for adequate host cells. *E. bovis* sporozoites can efficiently be trapped by neutrophil extracellular traps (NETs) released by bovine polymorph nuclear neutrophils (PMN). So far, the potential role of autophagy in parasite-triggered NET formation is unclear. Here, we aimed to analyzed autophagosome formation in potentially NET-forming PMN exposed with *E. bovis* sporozoites. Furthermore we documented in real time the formation of vital NETosis in PMN stimulated with *E. bovis* sporozoites. Blood was collected from healthy adult dairy cows, and bovine PMN were isolated via density gradient centrifugation. E*. bovis* induced LC3B-related autophagosome formation in parallel to NET formation in bovine PMN. Interesting, both rapamycin- and wortmannin-treatments failed to influence NET formation and autophagosome formation. In summary, the current data provide first evidence on autophagy simultaneously occurring in *E. bovis* sporozoites-induced NETosis.

Keywords: *E. bovis*, autophagosome, vital NETosis, cattle PMN

Introduction

Coccidiosis is the term used to describe a disease caused by infection with one or more species of *Eimeria* (Oluwadare 2004), which has high economic impact on the worldwide cattle industry (C. Hermosilla et al. 2002). Mostly all cattle are infected with coccidia at some point in their life, but only few of them develop coccidiosis. The clinical symptoms occurs mainly in young animals, but occasionally it affects cattle over 6 months of age (Davies, Joyner, and Kendall 1972). This intestinal disease is caused by *Eimeria* species that belong to the Phylum Apicomplexa (Matjila and Penzhorn 2002; Chartier and Paraud 2012). *Eimeria* spp. are distributed worldwide and the infection practically occurs in all kinds of vertebrates (Deplazes et al. 2016). More than 12 *Eimeria* spp. are described to infect bovine, nevertheless just three species are reported as pathogenic (*E. bovis, E. zuernii* and *E. alabamensis*), being the most common and severe infections generated by *E. bovis* (Carlos Hermosilla, Ruiz, and Taubert 2012; Lutz et al. 2011; Taubert et al. 2010). The last one infect endothelial cells in the intestinal tract of susceptible bovine (Carlos Hermosilla, Ruiz, and Taubert 2012). In there, sporozoites of *E. bovis* develop into huge macromeronts up to 300 µm in size within 14–18 days and produce up to 120,000 merozoites I per meront I (C. Hermosilla et al. 2002; Carlos Hermosilla, Ruiz, and Taubert 2012).

Although the intracellular stages of the parasite are protected from the immune system, there is one short extracellular phase when they are target for the immune response. This phase occurs when the host ingest the sporulated oocyst, and then the sporozoites are released in to the intestinal lumen. The free-released *E. bovis* sporozoites must traverse the gut epithelium in order to invade endothelial cells of the central lymph capillaries of the ileum villi, where they undergo the first merogony (Hammond et al. 1946). During this period in the extracellular space, the sporozoites are susceptible to be eliminated by professional immune cells. It has been reported that PMN-mediated innate

immune response play an important role in the early immune response to *E. bovis* infections in calves (Jan Hillern Behrendt et al. 2010; J H Behrendt et al. 2004). PMN respond to pathogens with diverse mechanism, which include phagocytosis, the release of oxidative radicals as a result of the oxidative burst reaction and the production of immuno-modulatory molecules, such as cytokines or chemokines, contributing to initiate acquired immune responses. After exposure to *E. bovis* sporozoites, PMN response include enhance in transcription of IL-6, MCP-1, GROa, TNF-a, and iNOS genes. Stimulation with merozoite-antigen, in addition, upregulated IL-8, IP-10 and IL-12 gene transcription. Furthermore, enhanced *in vitro* oxidative burst and phagocytic activities were observed after contact of PMN with viable sporozoites (Jan Hillern Behrendt et al. 2010). Additionally, PMN-mediated killing of pathogens can be achieve by forming neutrophil extracellular traps, as has been described previously (Jan Hillern Behrendt et al. 2010). PMN derived NET-like structures firmly attached to *E. bovis* sporozoites have been described. SEM analyses suggested immobilization of the parasites which may have a preventive effect on host cell invasion (Jan Hillern Behrendt et al. 2010). The *Eimeria*-triggered NETosis is dependent on ROS generated by NADPH oxidase (classical NETosis pathway). Furthermore, sporozoites co-cultured with neutrophils that undergo NETosis, show reduce infectivity in BUVEC cells. Munoz-Caro et al described that CD11b receptor of neutrophils (an integrin component of complement receptor 3) was implicated in this NETosis. In addition, NETosis is also dependent on calcium mobilization from store-operated calcium entry (SOCE), as well as on NE and MPO activities (Muñoz-Caro et al. 2015).

Furthermore, two mechanism of NET release have been described so far, “suicidal” and “vital” NETosis. NET secretion by cell death is a slow process (120-240 min) and depend on the classic pathways [suicidal NETosis] (Hakkim et al. 2011). On the contrary, an alternative rapid process (5-60 min) for NET formation has been reported [vital NETosis] (Pilsczek et al. 2010). This latter process was described as ROS-independent in response to some pathogens (Byrd et al. 2013; Rochael et al. 2016). Additionally, vital NETosis compromise vesicular DNA

movement from the nucleus to the extracellular space (Pilsczek et al. 2010). This pathway maintains the integrity of the membranes, and it does not require the death of the PMN (Clark et al. 2007). Until know, there is no information about the type of NETosis occurring in bovine PMN after exposure to *E. bovis* (Li and Tablin 2018).

Autophagy is also an essential intracellular degradation system described in neutrophils. First evidences suggest that autophagy is necessary and can make PMN to undergo NET formation (Zhou et al. 2019). In the case of *Besnoitia besnoiti* (another apicomplexan parasite in bovine) it has been described the simultaneous NET formation with autophagosome in *B. besnoiti* tachyzoite-exposed bovine PMN. Autophagy is a lysosomal degradation pathway that is essential for survival, differentiation, development, and homeostasis (Levine and Kroemer 2008). Autophagy is regulated by the metabolic sensor molecule AMP activated kinase α (AMPKα) and by the mechanistic target of rapamycin (mTOR) (Laplante and Sabatini 2012). This process has been shown to play a crucial role in regulating early innate cell-associated effector mechanisms against pathogens, such as phagocytosis (Levine, Mizushima, and Virgin 2011), and NET formation (Remijsen et al. 2011). Some evidences suggest that autophagy is necessary for PMN to undergo NET formation (Park et al. 2017; Skendros, Mitroulis, and Ritis 2018). Nevertheless, the potential role of autophagy in parasite-triggered NET formation is unclear. Here we aimed to analyze autophagosome formation in potentially NET-forming PMN being exposed to *E. bovis* sporozoites and generate the first evidence on vital NETosis in *E. bovis*-stimulated PMN.

Materials and methods

Ethics statement

The experiments were conducted in accordance to Justus Liebig University Giessen Animal Care Committee Guidelines. Protocols were approved by Ethic Commission for Experimental Animal Studies of Federal State of Hesse (Regierungspräsidium Giessen; A9/2012; JLU-No.521_AZ) and in accordance to

European Animal Welfare Legislation: ART13TFEU and current applicable German Animal Protection Laws.

Parasites

For *Eimeria bovis* oocyst production, two 8-weeks-old calves, kept in isolation conditions without *Eimeria* spp. exposure, were infected orally with 3×10^4 sporulated *E. bovis* oocysts. Then, oocysts were isolated from faeces beginning at 18-20 days p. i. according to Jackson, 1964 and described elsewhere (Silva et al. 2015; Hamid et al. 2015; C. Hermosilla et al. 2002). For the obtention of viable *E. bovis* sporozoites, the following modified excystation protocol was used (Silva et al., 2015). Sporulated *E. bovis* oocysts stock solution was added to 4 % (v/v) sodium hypochlorite solution and magnetically stirred on ice for 20 min. After mixing in the vortex for 20 s, oocyst solution was centrifuged (300×g, 5 min) and supernatant was mixed with bi-distilled water (1:1). Washed oocyst solution was then filtered through 40 and 10 µm sieves (PluriSelect) to remove remaining debris. Then, oocyst were centrifugated 15 min at 600×g, and the pellet was resuspended in 0.02 M L-cysteine/0.2 M NaHCO3 (Merk) solution and incubated in a 100 % CO2 atmosphere (37 °C, 20 h). Afterwards, oocysts were re-suspended in the following excystation medium: Hank's balanced salt solution (HBSS, Gibco) containing 0.4 % (w/v) trypsin (Sigma) and 8 % (v/v) sterile filtered bovine bile (obtained from the local slaughterhouse). Afterwards, oocysts were incubated up to 3 h (37 °C, 5 % CO2 atmosphere). Every hour, excystation progress was checked under an inverted microscope (IX81, Olympus®) to estimate the number of freereleased sporozoites. Freshly released sporozoites of *E. bovis* were filtrated througth 5 µm (PluriSelect) and then washed two times (600×g, 15 min) with cell culture medium 199 (M199, Gibco) supplemented with 2 % (v/v) foetal calf serum (FCS, Gibco) and 1 % penicillin (v/v, 500 U/ml; Sigma-Aldrich) and streptomycin (v/v, 500 µg/ml; PS; Sigma-Aldrich) and finally suspended in RPMI 1640 medium (Sigma-Aldrich) (2×10^6 sporozoites/ml).

Isolation of bovine PMN

Healthy adult dairy cows (n = 9) served as blood donors. Animals were bled by puncture of jugular vein and 30 ml blood was collected in heparinized sterile plastic tubes (Kabe Labortechnik). 20 ml of heparinized blood were diluted in 20 ml sterile PBS with 0.02% EDTA (SigmaAldrich), layered on top of 12 ml Biocoll separating solution (density = 1.077 g/l; Biochrom AG) and centrifuged (800× g, 45 min). After removal of plasma and mononuclear cells, the cell pellet was suspended in 27 ml bi-distilled water and gently mixed during 30 s to lyse erythrocytes. Osmolarity was rapidly restored by adding 3 ml of 10 × Hanks balanced salt solution (Biochrom AG). For complete erythrocyte lysis, this step was repeated twice and PMN were later suspended in sterile RPMI 1640 medium (Sigma-Aldrich). PMN counts were analyzed in a Neubauer haemocytometer. Finally, freshly isolated bovine PMN were allowed to rest at 37◦C and 5% CO2 atmosphere for 30 min until further use.

For live cell and cell-parasite interaction experiments (i. e. live cell 3D holotomography with Nanolive ®), the protocol for erytrocyte lysis was modified. In brief, after the Biocol gradient, plasma and buffy coat were aspirated. The remaining red blood cells and PMN pellet were suspended in Hank's balanced salt solution (HBSS). The red blood cells were removed by flash hypotonic lysis using a cold phosphate-buffered water solution (5.5 mM NaH2PO4, 8.4 mM HK2PO4, pH 7.2). After 1 minute of incubation, a hypertonic phosphate-buffered solution (5.5 mM NaH2PO4, 8.4 mM HK2PO4, 0.46 M NaCl, pH 7.2) was use to return the isotonicity. Then, the cells were centrifuged at 600g for 10 min. The lysis step was repeated twice (or until the complete lysis of erytrocytes was achieved) (Conejeros et al. 2012). The remaining PMN pellet was then washed with HBSS. PMN counts were analyzed in a Neubauer haemocytometer. Finally, freshly isolated bovine PMN were allowed to rest at 37◦C and 5% CO2 atmosphere for 30 min until further use.

Live cell 3D holotomographic microscopy

To evidenciate the early interaction of the PMN with the *E. bovis* sporozoites, 3D holotomographic video was recorded. In total, 5 x 10^5 PMN were seeded into

35 mm tissue culture µ-dish (Ibidi®) in imaging medium [RPMI 1640 lacking phenol red and serum, with Sytox Green (Life Technologies) and DRAQ 5^{TM}]. After 30 minutes of incubation in the ibidi Stage Top Incubation System, *E. bovis* sporozoites were added (1:1, 5 x 10^5). Then, holotomographic video was obtained by using 3D cell-explorer microscope (Nanolive 3D) equipped with a 60x magnification (λ = 520 nm, sample exposure 0.2 mW/mm2) and a depth of field of 30 µm. FITC channel was used to visualize extracellular DNA (present in NETs and death cells), and the TRITC channel was used for nucleus vizualization. The video was analysed using STEVE software (Nanolive).

Autophagosome detection by immunofluorescence analysis

Analysis of autophagosome formation in PMN was performed according to Itakura and McCarty (Itakura and McCarty 2013). Bovine PMN (n = 3) were deposited on poly-L-lysine (0.01%) pre-treated coverslips (15 mm diameter, Thermo-Fisher scientific). In addition, pretreatment of PMN with rapamycin (50 nM) or wortmannin (50 nM) for 30 min was performed before being exposed to *E. bovis* sporozoites at a 1:4 PMN:sporozoites ratio for 2 h. After incubation, cells were fixed with 4% paraformaldehyde (10 min), permeabilized by ice cold methanol treatment (3 min at 4◦C) and blocked with blocking buffer (5% BSA, 0.1% Triton X-100 in sterile PBS; all Sigma-Aldrich) for 60 min at RT. Thereafter, cells were incubated overnight at 4◦C in anti-LC3B antibody solution (cat N° 2775 Cell Signaling Technology) diluted 1:200 in blocking buffer. After incubation, samples were washed thriee times with PBS 1X and incubated 30 min in the dark and RT in a 1:500 dilution of goat anti-rabbit IgG conjugated with Alexa Fluor 488 (Invitrogen). After three washes in PBS 1X, samples were mounted in Prolong Anti-fading reagent with DAPI (Invitrogen) on glass slides and images were taken applying confocal microscopy (Zeiss LSM 710). To estimate LC3B-positive cells, the background fluorescence signal was determined in control conditions for FITC (green) and DAPI (blue) channels. Image processing was carried out with Fiji ImageJ R using Z-project and merged channel plugins and restricted to overall adjustment of brightness and contrast.

Assessment of different types of NETs

NET structures were described referring to their appearance as “diffuse” NETs (diffNETs) [globular and compact form with a size of 25–28 nm diameter], “spread” NETs (sprNETs) [smooth and elongated web- like structures with thin fibers with a diameter of 15–17 nm] and “aggregated” NETs (aggNETs) [large clusters with size larger than 50 μ m in diameter] (Muñoz-Caro et al. 2015, Schauer et al. 2014 and Hakkim et al. 2011).

Statistical analysis

Statistical significance was defined by a p value <0.05. p value were determined by applying non-parametric analyses: One-way analysis of variance was performed followed by Dunnett's Multiple Comparison Test. All graphs (mean ± SD) and statistical analyses were generated by the use of Graph Pad software (v. 7.03).

Results

Vital NETosis ocurred in bovine PMN after 13 min exposure with the E. bovis *sporozoites.*

A holotomographic analysis with 3D Cell Explorer (Nanolive) of the interaction between rested bovine PMN and live *E. bovis* sporozoites was performed. The interaction between PMN and sporozoites was followed with a live cell video during 100 minutes. Figure 1 shows the time lapse of the co-cultivation. The nuclei of PMN were stained with DRAQ5 (red), and the extracellular DNA (as a marker of NETosis) with Sytox green. Six PMN lobulated nuclei are clearly stained red at the beggining of the video, and one death *E. bovis* sporocyst was marked with green. At 13 min, an extrusion of a DNA spread-web like structure was observed in a PMN (white arrow, green staining), which was in closely contact with sporozoites. This spread NET become larger and was seen swimming close to the sporozoites during all the incubation period. The PMN which released the NET (yellow arrow) was alive during the process, and died at minute 45 (32 minutes after NETosis), suggesting a procces of vital NETosis.

Some of the sporozoites which where in contact with the NET died after 20 min (blue arrow). An increase on Sytox green signal was observed after 35 minutes of co-cultivation, and was correlated with the DRAQ5 signal of the nuclei, suggesting that *E. bovis* was able to trigger NETosis after 35 minutes post incubation. Expansion of the nuclei of the PMN were observed after 40 minutes.

Interestly, at minute 9, one sporozoite invade one PMN (Video 1, Fig. 1, red arrow), this process took 36 seconds. The parasite moves inside the citoplasm (the deformation of the cell can be clearly seen) for less than a minute and then tried to scape, but when half of the body was outside, the process stopped, and then a slow retrograde movement of the sporozoite back into the cell was seen, a process that took 7.5 minutes. The cell began its nuclear expantion and death at minute 35, with the parasite inside. If we consider the time, apparently this process was not an active movement of the sporozoite, instead an action of the cell.

E. bovis *sporozoites induced mainly aggregated NETs*

PMN stimulated with *E. bovis* sporozoites were abble to produce NETs detected by immunofluorescence (Fig. 2.A,B). *E. bovis* sporozoites triggered most the formation of aggrNETS, which is the most robust type of NETs. This type of NETs, consists of rigid clusters of NET-like structures of >20 µm in diameter, which is necessary for the immobilization of large parasites. The parasite also trigger the formation of some diffNETs, which are composed of a complex of extracellular decondensed chromatin, decorated with antimicrobial proteins with globular and compact form, defined by a size of 15-20 µm diameter. Additionally, *E. bovis* induced few sprNETs, which consist of smooth and elongated web-like structures of decondensed chromatin and antimicrobial proteins within fibers with a diameter of 15-17 µm (Fig. 2C).

Autophagy on E. bovis-*stimulated NET formation in bovine PMN*

mTOR-mediated autophagy inducer rapamycin (Itakura and McCarty 2013) and the PIK3-mediated autophagy inhibitor wortmannin were used to asses the effects of autophagy on *E. bovis*-triggered bovine NET formation (Itakura and

McCarty 2013; Blommaart et al. 1997). Adittionally, we used an antibody against LC3B as a marker of autophagy to investigate the effect of *E. bovis* sporozoite exposure to PMN-derived autophagy (Fig. 3A). During autophagy, the cytosolic form of microtubule-associated protein 1A/1B-light chain 3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-II which allows LC3 to become associated with autophagic vesicles (Tanida et al. 2008). Exposure to *E. bovis* sporozoites led to significant autophagosome formation in exposed bovine PMN ($p<0.05$) (Fig.3B). However, parasite-mediated autophagosome formation was neither affected by rapamycin nor by wortmannin treatments (Fig. 3B). Furthermore, cells undergoing autophagy also showed NET formation against *E. bovis* sporozoites, which were firmly entrapped in chromatin structures (Fig. 2D).

Discussion

E. bovis sporozoites become potential targets for professional phagocytes when they search for adequate host cells in the lymph vessels. Early innate immune reactions against cattle *Eimeria* spp. have been little investigated, however, the first contact between parasite and the innate immune system its considered to be decisive for the presentation of the clinical disease (Taubert et al. 2009). Thanks to the 3D live cell imaging, we were abble to document for the first time the releasing of vital NETosis in PMN stimulated with *E. bovis* sporozoites. This novel tool allowed us to capture in real time the release of a DNA structure from a vital PMN, that was directed againgst the parasites after short time of exposure. Neutrophil extracellular traps (NETs) released by bovine PMN exposed to *E. bovis* sporozoites have been reported previously (Jan Hillern Behrendt et al. 2010; Muñoz-Caro et al. 2015). But so far, no data are available on *E.* vital NETosis or *bovis* induced LC3B-related autophagosome formation in parallel to NET formation in bovine PMN.

Rapid release of a DNA structure from a vital PMN (no positive stained to SYTOX green) allows us to suggest that vital NETosis was triggered after exposure with *E. bovis* sporozoites. For another apicomplexan parasite (*Toxoplasma gondii*) it has been described the process of NETosis as suicidal, and just for some

bacterias and fungi the vital NETosis has been reported (Byrd et al. 2013; Clark et al. 2007; Li and Tablin 2018). Further research should focus on quantify the number of cells doing vital NETosis and the co-ocurrence of suicidal and vital NETosis during the stimulation with the sporozoites, and also try to elucidate the stimuli necessary for the PMN to choose each pathway.

In the other hand, to detect autophagy in *E. bovis* sporozoite-exposed PMN, autophagosome formation was visualized by LC3B-based immunostaining. Autophagosomes are double-membraned vesicles formed during autophagy, which represent characteristic markers of autophagy (Zhou et al. 2019). Confocal microscopy showed that confrontation of PMN with sporozoites caused a significant increase of autophagosome formation. Additionally, we observed that autophagic PMN also performed NET formation. However, neither rapamycin nor wortmannin pre-treatments had any influence on PMN-derived autophagosome formation, suggesting that these processes were mTOR-independent. Our results agreed with those obtained by Zhou et al., in bovine PMN (Zhou et al. 2019). They also found correlation between NETs and autophagosome formation in *B. Besnoiti* exposed PMN and autophagy in a mTOR-independent pathway. Nevertheless, in other studies, mTOR pathway has been described as a key role in NET formation via regulation of autophagy pathways (Itakura and McCarty 2013). We assume that early sporozoite-triggered NET formation is linked to autophagy in bovine PMN. Furthermore, the fact that formation of LC3B-positive autophagosomes was observed in bovine PMN while casting NETs supported the potential role of autophagy in PMN-derived responses against tachyzoite stages (Zhou et al. 2019).

Addittionally, *E. bovis* sporozoite invasion of a PMN was documented. This invasion of a leukocyte by *E. bovis* was previously reported in monocytes (Taubert et al. 2009). Nevertheless, this parasite invasion has been reported as a rare event, as the development of sporozoites, neither in a bovine macrophage cell line nor in primary bovine macrophages were possible(Taubert et al. 2009).

In summary, the current data provide first evidence on *E. bovis* sporozoites-induced vital NETosis and simultaneous formation of autophagosomes.

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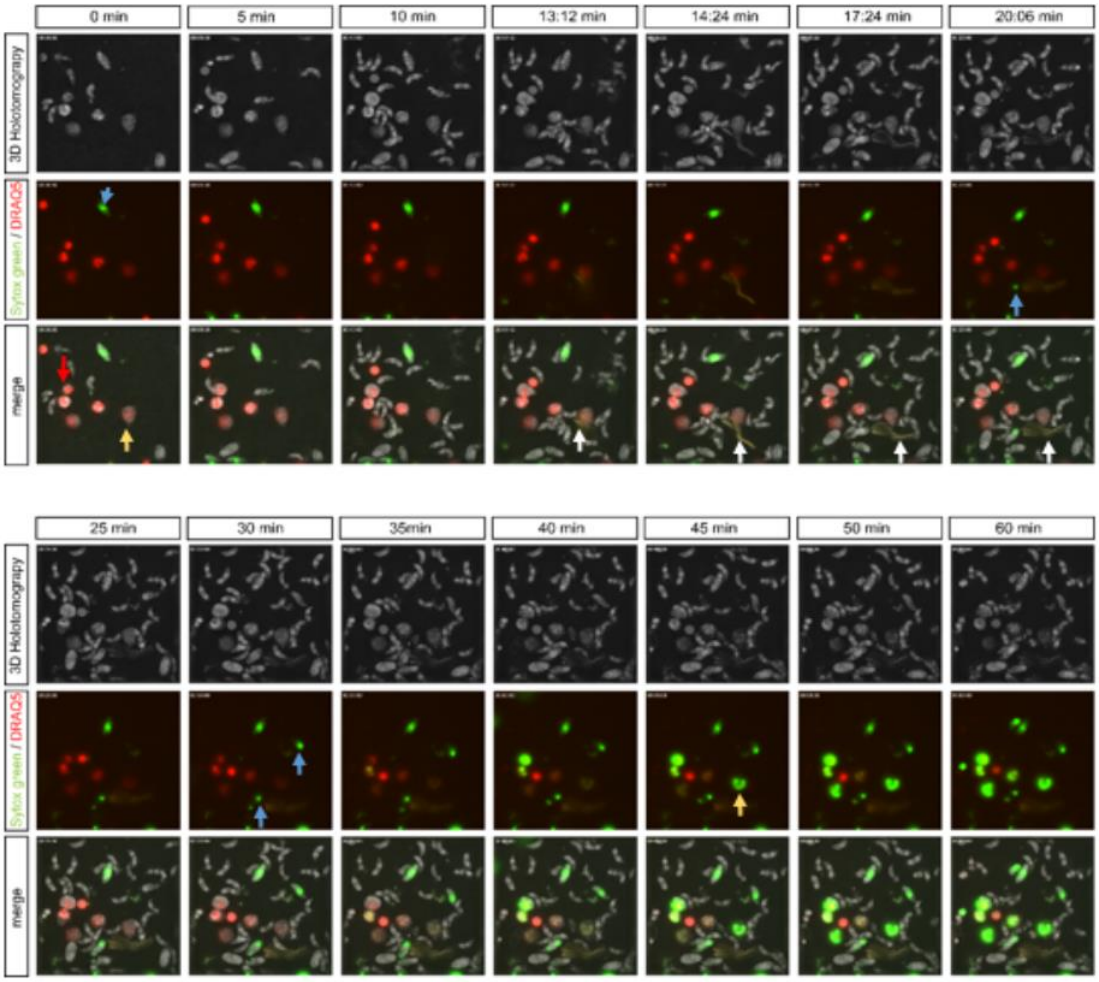
Figures

Fig. 1. Real time formation of vital NETosis in *E. bovis*-exposed PMN.

Time lapse of holotomographic video with 3D Cell Explorer (Nanolive) of the interaction between bovine PMN and live *E. bovis* sporozoites. The interaction between PMN and sporozoites was followed with a live cell video during 100 minutes. The panel shows the 3D holotomography, green-red channel and the merge. The nuclei of PMN were stained with DRAQ5 (red), and the extracellular DNA (as a marker of NETosis) with Sytox green. Red arrow: PMN invaded by *E. bovis* sporozoite. Yellow arrow: PMN which release NET. White arrows: NET structure. Blue arrow: dead sporozoite. Orange arrow: nuclear expansion, death of the PMN.

Fig. 2. Autophagy and NET formation occurs simultaneously in *E. bovis*-exposed PMN. Bovine PMN were exposed to *E. bovis* sporozoites for 2 h. Samples were fixed and permeabilized for LC3B-based immunostaining to determine autophagosome formation by microscopy. (A) Panel showing the staining for LC3B (green), DAPI (blue) and merge. (B) Percentage of NETotic cells was also calculated. (C) Types of NETs triggered by *E. bovis* sporozoites in bovine PMN. (D) Zoom-in. Entrapped *E. bovis* sporozoite.

Fig. 3. Analysis of autophagosome formation (by expresion of protein LC3B) in PMN exposed to *E. bovis* sporozoites compared with non- exposed by confocal microscopy. Panel showing the staining for LC3B (green), DAPI (blue) phase contrast (gray scale) and merge (A). In addition, PMN were pretreated with rapamycin or wortmannin (50 nM for 30 min) and then exposed to *E. bovis*. After 2 h of incubation, the samples were stained for LC3B and the number of autophagosome-positive cells was determined (B).


0 min
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3D Holotomography
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3D Holotomography
Sytox green / DRAQ5
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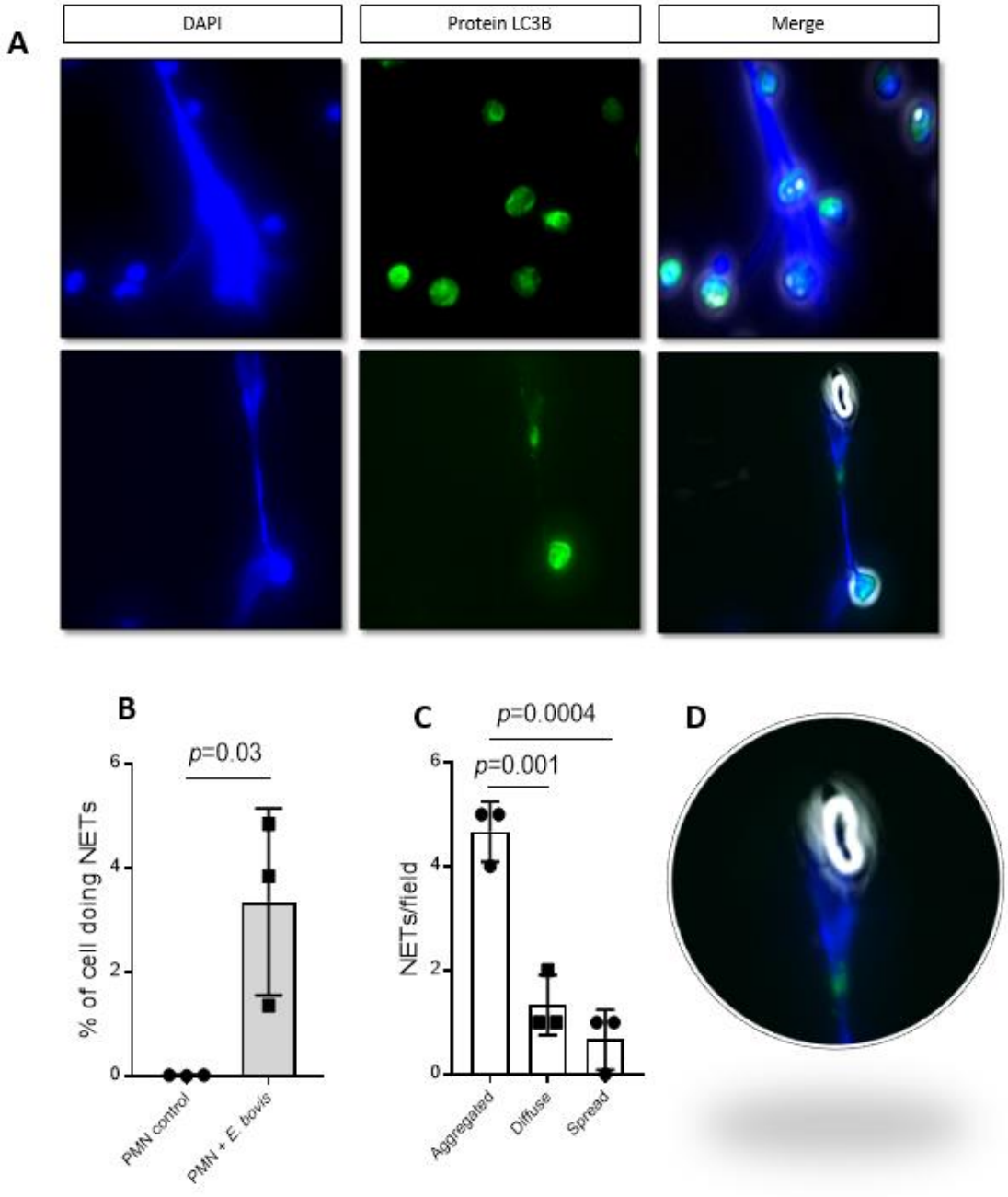
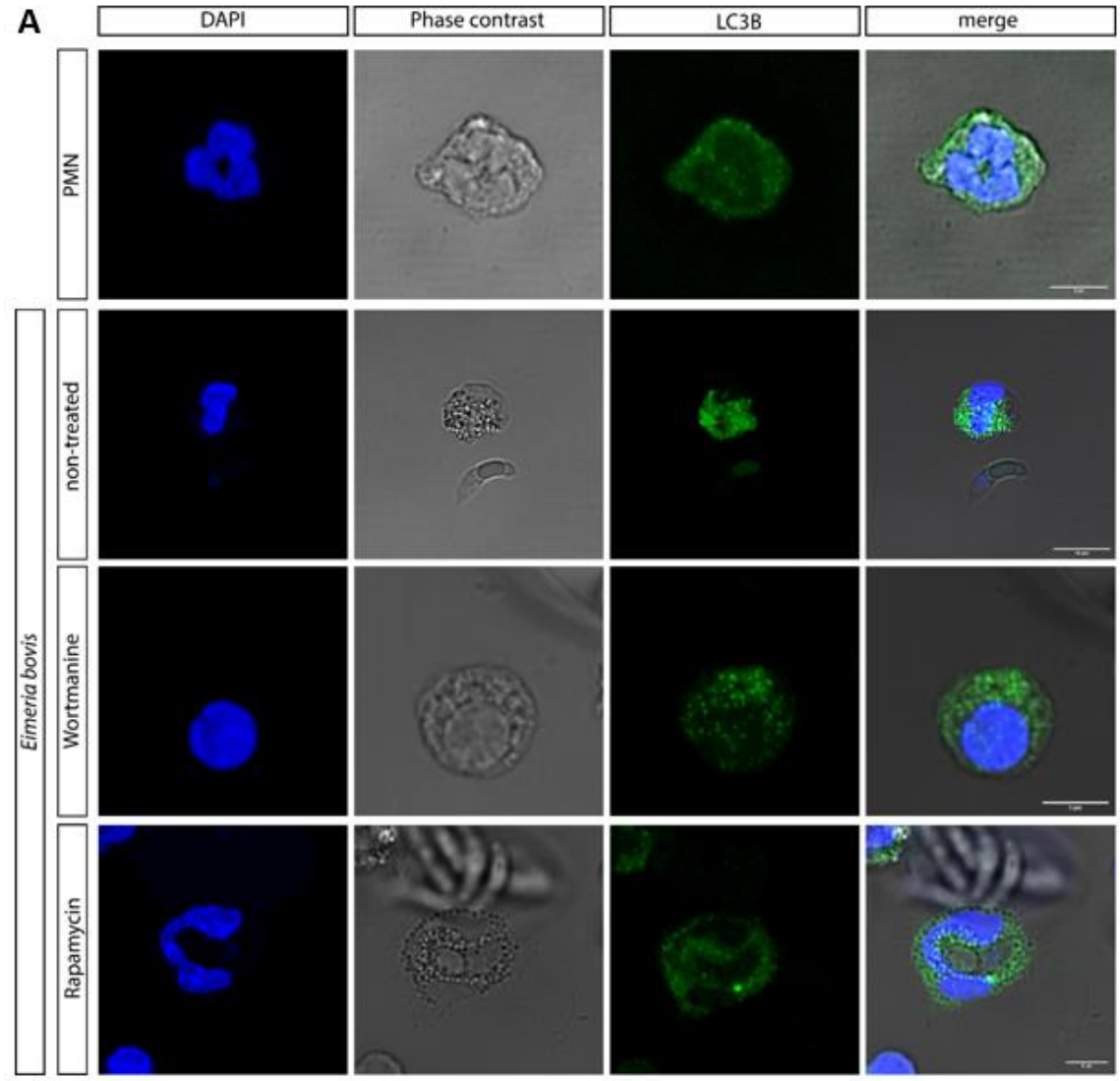


Fig. 2. Autophagy and NET formation occurs simultaneously in *E. bovis*-exposed PMN.


A
DAPI
Phase contrast
LC3B
merge
PMN
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Wortmanine
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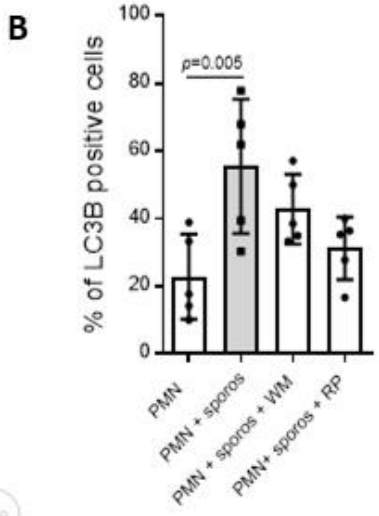
Eimeria bovis


B
% of LC3B positive cells
p=0.005
100
80
60
40
20
0
PMN
PMN + sporos
PMN + sporos + WM
PMN+ sporos + RP

9. Chapter: discussion and outlook

During the past decades, the understanding of cattle coccidiosis has considerably improved by using suitable *in vitro* systems for some *Eimeria* species (Daugschies and Najdrowski, 2005). Furthermore, attention in *Eimeria* spp. infections seems to have increased among veterinary practitioners and farmers raising further questions related to the epidemiology, biology, treatment and control measurements. Hereby we present the first large-scale investigation on epidemiological aspects of bovine *Eimeria*-infections carried out in Colombia. These results were justified by reports of various researchers in many countries of the world (Matjila and Penzhorn, 2002; Tomczuk *et al.*, 2015). *Eimeria* species identified in this study have previously been reported worldwide (Rehman *et al.*, 2012). *E. bovis* and *E. zuernii* were recorded as the highest prevalent coccidian species. According to Deplazes et al. (2016), these two species are the most pathogenic ones in bovine coccidian parasites. This is the main reason for focusing in these two species, together with caprine *E. arloingi*, which is also considered as highly pathogenic species (Silva *et al.*, 2017). The prevalence of *Eimeria* spp. infection in cattle and goats is generally high and can reach 100% in calves/goat kids (Daugschies and Najdrowski, 2005; Silva *et al.*, 2014).

Although we presented here first approach on epidemiological status of *Eimeria* spp. in young cows, epidemiological data on *Eimeria* infections of Colombian goats still remain unknow, and thus further studies on caprine *Eimeria* spp. prevalences and associated risk factors should be conducted. The breeding of goats in Colombia began as a cultural and gastronomic tradition nevertheless Colombian goat industry has been growing in the past years. Currently, more than 1 million goats are in the national territory. Since they provide food and represent sustainable alternative production of meat and milk, goats have taken an important social role for the rural population and indigenous communities of

the Colombian territory[1]. Consequently, it seems necessary to improve sanitary status of small ruminants in Colombia, and further to develop future control of enteropathogens such as *Eimeria* spp. alongside with other parasitic infections. As such, Colombian calves seemed to be not only affected by coccidian parasites but also by various relevant gastrointestinal parasites including nematodes and cestodes. We found *Dictyocaulus viviparus* in 1.3% of the samples evaluated with Baerman technique. Furthermore, 54.1% were positive to Trichostrongylidae-type eggs. Identification of L3 demonstrated the presence of *Cooperia* spp. (42.9%), *Ostertagia* spp. (41.7%), *Trichostrongylus* spp. (33.3%), *Haemonchus* spp. (13.1%), *Cooperia oncophora* (8.3%), *Bunostomum* spp. (3.6%) and *Oesophagostomum* spp. (1.2%). All these parasites are also responsible for disease in young animals and should be studied more in detail to determine the real impact on the catte industry of Colombia (Chaparro *et al.*, 2016, 2017; Villar *et al.*, 2018). On the other hand, to achieve control of coccidiosis in herds associated risk factors should be assessed particularly the ones with respect to hygiene, feeding, animal density and floor type. Installation of slatted floors that allow less accumulation of faeces in the pens significantly reduces coccidiosis (Daugschies and Najdrowski, 2005). Nevertheless, if conditions of animal husbandry are not improvable, treatment is inevitable.

Treatment options for coccidiosis in calves and goats are limited and include just few compounds to use as efficient chemotherapy. The last is certainly a viable choice, but the compounds should interfere with more than just one stage of the life cycle by exhibiting selective anti-parasitic toxicity. This is why knowledge on suitable targets for intervention against *Eimeria* must be generated and candidate compounds must be thoroughly characterised. For such studies, *in vitro* culture systems must be applied (Müller and Hemphill, 2013b). As it has been described before, the pathogenesis of apicomplexan parasites is related to intracellular stages of their life cycle. Nowadays, animal experimentation has been almost

[1] Informe: Sector ovino-caprino, un gremio que pisa fuerte en Colombia Por: Andrés Moncada Montenegro
27 de Marzo 2015. https://www.contextoganadero.com/ganaderia-sostenible/informe-sector-ovino-caprino-un-gremio-que-pisa-fuerte-en-colombia

replaced by suitable *in vitro* culture systems in order to investigate the molecular features of parasite stages and the mechanisms that lead to differentiation or stage conversion (Müller and Hemphill, 2013b). Currently, *in vitro* systems for ruminants *Eimeria* spp. are not common. Unlike, as for e. g. *Eimeria* in poultry, where a large reservoir of definitive host intestines is available for research activities, intestinal or particularly lymphatic endothelial cells from goats and calves as a source for primary cells are not easy to obtain. Furthermore, to obtain large number of parasites (oocyst) from bovines and goats is difficult and more expensive than to obtain oocysts from other minor species (i. e. chickens, rabbits or rodents). Therefore, only a limited number of studies have been carried out using bovine/caprine primary host endothelial cells isolated from umbilical veins or arterias for ruminant *Eimeria* replication (Hermosilla et al., 2012).

In order to obtain more information on most pathogenic and prevalent species of cattle and goats, we used the *in vitro* system based on primary endothelial host cells. Key points for successful *in vitro* culture of *Eimeria* spp. include: *i)* to obtain viable sporulated oocysts, and *ii*) to obtain large amount of viable free sporozoites after a successful excystation. These two points were studied as part of this doctoral thesis: an improved method for excystation of sporulated oocysts and collection of infective sporozoites of *E. bovis/E. zuernii* and *E. arloingi* for *in vitro* systems was described. Successful excystation of *Eimeria* spp. oocysts is an important step to acquire large numbers of viable sporozoites to be later used in *in vitro* experiments for the study of such fascinating and complex host cell-parasite interactions. Here, we document for the first time *in vivo* respiration of *E. bovis* oocyst sporogony using live cell imaging microscopy techniques, which allow a non-invasive 3D-holotomographic characterization under physiological conditions. However, there is still lack of information on all the process and key metabolic pathways for these parasites in this exogenous phase that could be used for new oocyst control approaches. Additional studies should be directed towards the better understanding of sporogony phase of coccidians and their aerobic-dependent metabolic demands as fully sporulated oocysts are the infective stages of all *Eimeria* species.

For their intracellular replication, apicomplexan parasites need energy, building blocks, and high level of cholesterol for offspring development. It is known that apicomplexan parasites in general are unable to synthesize *de novo* cholesterol and purines so they use various "scavenger" routes to obtain these nutrients from infected host cells or tissue environment (Furlong, 1989; Gero and O'Sullivan, 1990; Matias *et al.*, 1990; I Coppens, Sinai and Joiner, 2000; Kouni and Mahmoud, 2003; Chaudhary *et al.*, 2004; Bansal, Bhatti and Sehgal, 2005; Labaied *et al.*, 2011a; Ehrenman *et al.*, 2013). It is clear that many mechanisms used by parasites to gain access to nutrients, energy and blocking units are still unknown and so becoming evident the pivotal role on characterizing these pathways to find specific inhibitors for parasite replication that may be used as alternative treatments. As we learn more about the parasite metabolism and pathways involved, we will be able to develop more effective anticoccidial therapies/drugs for coccidiosis control not only in ruminants but also in chickens.

As it has been already described, these parasites are generally considered as defective for *de novo* cholesterol synthesis and needing to scavenge this molecule from their host cells (e. g. *T. gondii*, *N. caninum*, *C. parvum*, *E. bovis* and *Plasmodium* spp.) (Grellier *et al.*, 1994; I. Coppens, Sinai and Joiner, 2000; Labaied *et al.*, 2011b; Ehrenman *et al.*, 2013; Hamid *et al.*, 2015; Nolan *et al.*, 2015). For instance, an infected-host cell can enhance its endogenous *de novo* synthesis or upregulate LDL-mediated cholesterol uptake from extracellular sources to provide the parasite with sufficient cholesterol. Hamid et al (2015), demonstrated an increase in total cholesterol contents for *E. bovis*-infected host endothelial cells (Hamid *et al.*, 2015). Hereby, we suggest that the uptake of cholesterol via SR-B1 is required for the production of merozoite membranes during the first merogony while using BLT-1, specific inhibitor of SR-B1, inhibited completely the production of merozoites I, but not formation and growing of the macromeronts. However, it is necessary to characterize the mechanisms associated with these pathways and calculate the real consumption of different cholesterol types and lipids in *Eimeria*-infected cells. There are still many questions unsolved related with the metabolism demands of *Eimeria*, including

carbohydrates, lipids, nucleic acids and protein metabolism, that should be the focus of further research.

On the other hand, *E. bovis* and *E. arloingi* sporozoites become potential targets for professional phagocytes when they search for adequate host cells in the lymph vessels. NETs released by bovine PMN exposed to *E. bovis* sporozoites have been previously reported (Behrendt et al., 2010). So far, no data are available on metabolic requirements of *E. bovis* sporozoite-triggered NETosis. Therefore, here we determined the relevance of distinct PMN-derived metabolic pathways via pharmacological inhibition experiments. Our results indicate a key role of ATP, pyruvate- and lactate-mediated metabolic pathways for proper sporozoite-mediated NETosis release. Additionally, *E. bovis* induced LC3B-related autophagosome formation in parallel to NET formation in exposed bovine PMN. In summary, the current data provide first evidence on carbohydrate-related metabolic pathways and energy supply to be involved in *E. bovis* sporozoite-induced NETosis and simultaneous formation of autophagosomes. Nevertheless, metabolic signatures of triggered NETosis are still unknow, and more experiments related to this effector mechanism are needed to elucidate these pathways.

Conclusions

Apicomplexan parasites have specific pathways to evade host immune system and to successfully obtain their required nutrients for obligate intracellular replication. Nevertheless, many of these mechanisms remain unknown. Elucidation of these metabolic routes in detail is necessary to target specific pathways in order to develop new inhibitors or even anticoccidial drug candidates. As we learn more about the parasite's metabolism and pathways involved in these scavenger routes, we will be able to develop more effective therapies for parasite control strategies. The same is true for the better understanding of innate immune reactions as this knowledge will also help to improve immunoprophylaxis as already successfully achieved for avian coccidiosis.

10. Abstract

Coccidiosis is an intestinal disease caused by parasite species of the genus *Eimeria* and has high economic impact on livestock worldwide. Oocysts of *Eimeria* spp. are found ubiquitously in the environment, making the infection almost inevitable. The massive replication of the parasite leads to the destruction of intestinal cells thereby producing diarrhoea and sometimes causing death. Furthermore, because of massive intestinal damage through obligate intracellular replication of *Eimeria* spp., the digestion and absorption of nutrients become affected and even without clinical symptoms animal performance is compromised, causing economical losses in cattle and goat industry. Although understanding of coccidiosis (mainly in chicken) has improved during the past decades, there is still a lack of information specifically in the field ruminant coccidiosis. Advanced tools for research on epidemiology, immunology, biology or diagnosis are now available opening new perspectives for modern research in this field. As part of this thesis, a large-scale cross-sectional epidemiological study was conducted to evaluate prevalence, species diversity and associated risk factors of *Eimeria* spp. infections in 55 cattle farms across seven states of Colombia. In total, 1333 fecal samples from young animals (<one year of age) were examined. The overall *Eimeria* prevalence was 75.5%, with no difference observed between age categories. In total, 13 different *Eimeria* species were here identified. The most prevalent species was *E. bovis* (33.5%). Analysis of extrinsic associated risk factors revealed floor type, feeding system, watering system and herd size as significant ($p < 0.05$) risk factors for *Eimeria* infections. On the other hand, a Colombian *E. zuernii* strain was isolated and a corresponding suitable *in vitro* culture was established. The new *E. zuernii* (strain A) will allow detailed *in vitro* investigations on metabolism and on host innate/adaptive immunity for this specific bovine species. Additionally, we used novel tools in microscopy (e. g. 3D-holotomography analysis) to study exogenous sporogony of *E. bovis* and farther improved the excystation method to facilitate the work with ruminant *Eimeria* strains. Overall, three different *E. bovis* sporogony phases were documented: *i)* sporoblast/sporont transformation into sporogonial stages, *ii)* cytokinesis followed

by nuclear division, and *iii)* formation of fully developed four sporocysts each with two developed sporozoites. Moreover, we generated first evidence on carbohydrate-related metabolic pathways and energy supply to be involved in *E. bovis* sporozoites-induced NETosis and simultaneous formation of autophagosomes. *E. bovis*-induced cell-free and anchored NETs. Both phenotypes were significantly diminished via inhibitor pretreatments of some metabolic of exposed PMN, thereby indicating a key role of ATP, pyruvate- and lactate-mediated metabolic pathways for proper sporozoite-mediated NETosis. Interestingly, *E. bovis* additionally induced LC3B-related autophagosome formation in parallel to NET formation in bovine PMN. Finally, we explored the metabolism of lipids during *in vitro* of caprine *E. arloingi* first merogony resulting in macromeront formation within host endothelial cells. The complete inhibition of merozoite I production in *E. arloingi*-treated host endothelial cells suggested that the uptake of lipids via SR-B1 is necessary for parasite replication within host cell. Although all this novel data leads to a better understanding of parasite-host/host cell interactions, we still need more investigation thoroughly on parasite-induced modulation of different cellular functions (e. g. apoptosis, autophagy, cytoskeleton, cholesterol metabolism) which may be altered during endogenous infection and taking particular care of adequate highly specific host cells. Thus, ruminant *Eimeria* spp.-related investigations *in vitro* should be conducted mainly in primary host cells corresponding to the ones parasitized *in vivo*. As such, ruminant pathogenic *Eimeria* species, all of them resulting in macromeront formation, replicate *in vivo* in lymphatic endothelial cells of the small intestinal villi.

Key words: Bovine coccidiosis in Colombia, cattle eimeriosis, caprine eimeriosis, *in vitro* culture, cholesterol, metabolic pathways, NETosis, bovine PMN.

Resumen

La coccidiosis es una enfermedad intestinal causada por parásitos del género *Eimeria* y que tiene un alto impacto económico en los sistemas ganaderos a nivel mundial. Los ooquistes de *Eimeria* spp. se encuentran de forma ubicua en el medio ambiente, lo que hace que la infección sea casi inevitable. La replicación masiva del parásito conduce a la destrucción de las células intestinales, lo que produce diarrea y en algunas ocaciones la muerte. Además, debido al daño intestinal masivo debidoa la replicación intracelular obligada de *Eimeria* spp., la digestión y la absorción de nutrientes se ven afectadas e incluso sin síntomas clínicos, el rendimiento del animal se ve comprometido, causando pérdidas económicas en la industria bovina y caprina. Aunque la comprensión de la coccidiosis (principalmente en pollos) ha mejorado en las últimas décadas, todavía hay falta de información específicamente en el campo de la coccidiosis de rumiantes.Hoy en día están disponibles herramientas avanzadas para la investigación en epidemiología, inmunología, biología o diagnóstico,abriendo nuevas perspectivas para la investigación moderna en este campo. Como parte de esta tesis, se realizó un estudio epidemiológico transversal a gran escala para evaluar la prevalencia, la diversidad de especies y los factores de riesgo asociados a la infeccion con *Eimeria* spp. en 55 fincas ganaderas de siete estados de Colombia. En total, se examinaron 1333 muestras fecales de animales jóvenes (<1 año de edad). La prevalencia general de *Eimeria* fue del 75,5%, sin diferencias observadas entre las categorías de edad. En total se identificaron 13 especies diferentes de *Eimeria*. La especie más prevalente fue *E. bovis* (33.5%). El análisis de los factores de riesgo asociados reveló que el tipo de piso, el sistema de alimentación, el sistema de riego y el tamaño del rebaño son factores de riesgo significativos ($p < 0.05$) para las infecciones por *Eimeria*. Por otro lado, se aisló una cepa de *E. zuernii* colombiana y se estableció cultivo *in vitro* adecuado. La nueva *E. zuernii* (cepa A) permitirá investigaciones *in vitro* detalladas sobre el metabolismo y sobre la inmunidad innata / adaptativa del huésped para esta especie que afecta a bovinos específicamente. Además, utilizamos nuevas herramientas en microscopía (por ejemplo, análisis

holotomográfico 3D) para estudiar la esporogonía exógena de *E. bovis* y mejoramos aún más el método de exquistación para facilitar el trabajo con las cepas de *Eimeria* de rumiantes. En general, se documentaron tres fases diferentes de esporogonía de *E. bovis*: *i*) transformación de esporonte en esporoblasto, *ii*) citoquinesis seguida de división nuclear, y *iii*) formación de cuatro esporoquistes completamente desarrollados, cada uno con dos esporozoitos desarrollados. Además, generamos la primera evidencia sobre las rutas metabólicas relacionadas con los carbohidratos y el suministro de energía para participar en la NETosis inducida por esporozoitos de *E. bovis* y la formación simultánea de autofagosomas. *E. bovis* indujo NETs libre de células y ancladas. Ambos fenotipos disminuyeron significativamente con los pretratamientos de PMN con inhibidores de algunas rutas metabólicas, lo que indica un rol clave de las vías mediadas por ATP, piruvato y lactato para la NETosis inducida por esporozoitos. En paralelo a la formación de NET en PMN bovinos, *E. bovis* indujo adicionalmente la formación de autofagosomas relacionados con LC3B. Finalmente, exploramos el metabolismo de los lípidos durante la merogonía de *E. arloingi in vitro*, lo que resultó en la formación de macromeronte dentro de las células endoteliales. La inhibición completa de la producción de merozoitos I en células endoteliales hospederas tratadas con BLT-1 sugirió que la captación de lípidos a través de SR-B1 es necesaria para la replicación del parásito dentro de la célula. Aunque todos estos datos conducen a una mejor comprensión de las interacciones parásito-huésped / célula-huésped, todavía necesitamos profundizar en la modulación inducida por parásitos en diferentes funciones celulares (por ejemplo, apoptosis, autofagia, citoesqueleto, metabolismo del colesterol) que pueden alterarse durante infección endógena, teniendo cuidado particular en la selección de células del huésped adecuadas. Por lo tanto, las investigaciones relacionadas con *Eimeria* spp. de los rumiantes deben ser realizadas en las células hospedadoras primarias correspondientes a las parasitadas *in vivo*. Como tal, las especies de *Eimeria* patógenas de los rumiantes, se replican *in vivo* en células endoteliales linfáticas de las vellosidades del intestino delgado, todas resultando en la formación de macromeronte.

Palabras clave: coccidiosis bovina en Colombia, eimeriosis bovina, eimeriosis caprina, cultivo *in vitro*, colesterol, vías metabólicas, NETosis, PMN bovina.

Zusammenfassung

Kokzidiose ist eine Darmkrankheit, die durch Parasiten der Gattung *Eimeria* verursacht wird und weltweit einen hohen wirtschaftlichen Einfluss auf das Vieh hat. Oozysten von *Eimeria* spp. sind in der Umwelt allgegenwärtig und machen die Infektion fast unvermeidlich. Die massive Replikation des Parasiten führt zur Zerstörung der Darmzellen, wodurch Durchfall und manchmal Tod verursacht werden. Aufgrund massiver Darmschäden durch obligat intrazelluläre Replikation von *Eimeria* spp. wird außerdem die Verdauung und Absorption von Nährstoffen beeinträchtigt. Auch ohne klinische Symptome ist die Leistung der Tiere beeinträchtigt, was wirtschaftliche Verluste in der Rinder- und Ziegenindustrie zur Folge hat. Obwohl sich das Verständnis für Kokzidiose (hauptsächlich bei Hühnern) in den letzten Jahrzehnten verbessert hat, mangelt es immer noch an Informationen speziell im Bereich der Wiederkäuer-Kokzidiose. Weiterentwickelte Methoden für die Erforschung von Epidemiologie, Immunologie, Biologie oder Diagnose stehen jetzt zur Verfügung und eröffnen neue Perspektiven für die moderne Forschung auf diesem Gebiet. Im Rahmen dieser Arbeit wurde eine groß angelegte epidemiologische Querschnittsstudie durchgeführt, um die Prävalenz, die Artenvielfalt und die damit verbundenen Risikofaktoren von *Eimeria* spp.-Infektionen in 55 Rinderfarmen in sieben Staaten in Kolumbien zu bewerten. Insgesamt wurden 1333 Kotproben von Jungtieren (<1 Jahr) untersucht. Die allgemeine *Eimeria*-Prävalenz betrug 75,5%, wobei kein Unterschied zwischen den Alterskategorien beobachtet wurde. Insgesamt wurden hier 13 verschiedene *Eimeria*-Arten identifiziert. Die am häufigsten vorkommende Art war *E. bovis* (33,5%). Die Analyse der extrinsisch assoziierten Risikofaktoren ergab, dass Bodentyp, Fütterungssystem, Bewässerungssystem und Herdengröße signifikante ($p < 0,05$) Risikofaktoren für *Eimeria*-Infektionen sind. Andererseits wurde ein kolumbianischer *E. zuernii*-Stamm isoliert und eine entsprechende geeignete *in vitro*-Kultur etabliert. Der neue *E. zuernii* (Stamm A) wird detaillierte i*n-vitro* Untersuchungen zum Metabolismus und zur angeborenen/adaptiven Immunität des Wirts für diese spezifische Rinderspezies ermöglichen. Zusätzlich verwendeten wir neuartige

Werkzeuge in der Mikroskopie (z. B. 3D-Holotomographie-Analyse), um die exogene Sporogonie von *E. bovis* zu untersuchen und die Exzystierungsmethode weiter zu verbessern, um die Arbeit mit *Eimeria*-Wiederkäuerstämmen zu erleichtern. Insgesamt wurden drei verschiedene *E. bovis*-Sporogonie-Phasen dokumentiert: i) Sporoblasten/Sporont-Transformation in sporogoniale Stadien, ii) Zytokinese mit anschließender Kernteilung und iii) Bildung von vier voll entwickelten Sporozysten mit jeweils zwei entwickelten Sporozoiten. Darüber hinaus haben wir erste Hinweise auf kohlenhydratbezogene Stoffwechselwege und die Energieversorgung für die Beteiligung an der durch *E. bovis*-Sporozoiten induzierten NETose und der gleichzeitigen Bildung von Autophagosomen erhalten. *E. bovis*-induziert zellfreie und verankerte NETs. Beide Phänotypen wurden durch Inhibitor-Vorbehandlung einiger Metaboliten von exponiertem PMN signifikant verringert, was auf eine Schlüsselrolle der ATP-, Pyruvat- und Lactat-vermittelten Stoffwechselwege für eine korrekte Sporozoiten-vermittelte NETose hinweist. Interessanterweise induzierte *E. bovis* zusätzlich die LC3B-bedingte Autophagosomenbildung parallel zur NET-Bildung bei Rinder-PMN. Schließlich untersuchten wir den Metabolismus von Lipiden während der in vitro-Untersuchung der ersten Merogonie von caprinen *E.-arloingi*, die zur Makromont-Bildung in Endothelzellen des Wirts führte. Die vollständige Hemmung der Merozoit I-Produktion in mit *E. arloingi* behandelten Wirtsendothelzellen legte nahe, dass die Aufnahme von Lipiden über SR-B1 für die Parasitenreplikation in der Wirtszelle notwendig ist. Obwohl all diese neuen Daten zu einem besseren Verständnis der Wechselwirkungen zwischen Parasiten und Wirtszellen führen, müssen wir die durch Parasiten verursachte Modulation verschiedener zellulärer Funktionen (z. B. Apoptose, Autophagie, Zytoskelett, Cholesterinstoffwechsel), die sich während der endogenen Infektion ändern können, noch eingehender untersuchen und besondere Sorgfalt auf adäquate hochspezifische Wirtszellen legen. Daher sollten *in-vitro*-Untersuchungen im Zusammenhang mit Wiederkäuern von *Eimeria* spp. hauptsächlich in primären Wirtszellen durchgeführt werden, die den *in vivo* parasitierten Zellen entsprechen. Als solche replizieren sich pathogene *Eimeria*-

Spezies von Wiederkäuern, die alle zur Makromontbildung führen, *in vivo* in lymphatischen Endothelzellen der Dünndarmzotten.

Schlüsselwörter: Rinder-Kokzidiose in Kolumbien, Rinder-Eimeriose, Ziegen-Eimeriose, In-vitro-Kultur, Cholesterin, Stoffwechselwege, NETosis, Rinder-PMN.

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13. Declaration

I hereby declare that I have completed the submitted doctoral thesis independently and without any unautorised outside help and with only those financial forms of support mentioned on this work. All the analyses conducted in this work, followed the principles of good scientific practice, as the stated in the Statute of Justus Liebig University Giessen for ensuring good scientific practices. All the texts that have been quoted verbatim or by analogy from published and non-published writings and all details based on verbal information have been identified as such.

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