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Invited Review

In vitro cultivation methods for coccidian parasite research

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ABSTRACT

The subclass Coccidia comprises a large group of protozoan parasites, including important pathogens of humans and animals such as *Toxoplasma gondii, Neospora caninum, Eimeria* spp., and *Cystoisospora* spp. Their life cycle includes a switch from asexual to sexual stages and is often restricted to a single host species. Current research on coccidian parasites focuses on cell biology and the underlying mechanisms of protein expression and trafficking in different life stages, host cell invasion and host-parasite interactions. Furthermore, novel anticoccidial drug targets are evaluated. Given the variety of research questions and the requirement to reduce and replace animal experimentation, in vitro cultivation of Coccidia needs to be further developed and refined to meet these requirements. For these purposes, established culture systems are constantly improved. In addition, new in vitro culture systems lately gained considerable importance in research on Coccidia. Well established and optimized in vitro cultures of monolayer cells can support the viability and development of parasite stages and even allow completion of the life cycle in vitro, as shown for *Cystoisospora suis* and *Eimeria tenella*. Furthermore, new three-dimensional cell culture models are used for propagation of *Cryptosporidium* spp. (close relatives of the coccidians), and the infection of three-dimensional organoids with *T. gondii* also gained popularity as the interaction between the parasite and host tissue can be studied in more detail. The latest advances in three-dimensional culture systems are organ-on-a-chip models, that to date have only been tested for *T. gondii* but promise to accelerate research in other coccidians. Lastly, the completion of the life cycle of *C. suis* and *Cryptosporidium parvum* was reported to continue in a host cell-free environment following the first occurrence of asexual stages. Such axenic cultures are becoming increasingly available and open new avenues for research on parasite life cycle stages and novel intervention strategies.

1. Introduction

The class Conoidasida consists of a large and diverse group of parasites that contains some of the most important protozoan pathogens of humans and animals, including members of the genus *Cryptosporidium* and the subclass Coccidia which encompasses *Eimeria*, *Toxoplasma*, *Cystoisospora, Sarcocystis* and *Neospora* (Long, 1990; Smith et al., 2002; Morrison, 2009). Some species cause major diseases that can impair human and/or animal health, as well as animal reproduction and growth which are linked to economic losses for the affected livestock (Long, 1990; Lillehoj and Trout, 1993). Scientific research on parasite biology and treatment options often requires intensive in vivo experiments (Müller and Hemphill, 2013). However, since the late 1950s the guiding principles for ethical use of animals in scientific research, the three Rs (Russell and Burch, 1959), are applied in the majority of research laboratories. They include efforts and principles for reduction, replacement and refinement of animal experiments. Methods that minimize the number of animals used per experiment or possibilities to obtain more information from the same number of animals are implemented for the reduction of animal experiments. Replacement aims to avoid the use of animals and seeks replacement of animal experiments by other methods, while refinement applies to all aspects of animal use, from housing and husbandry to the procedures performed (Fenwick et al., 2009; Graham and Prescott, 2015; Sneddon et al., 2017; Traversa and Joachim, 2018). In vitro methods are used to completely replace in vivo experiments or to reduce them, e.g. by completing in vitro product characterization prior to animal experimentation (Robinson, 2011; Tannenbaum and Bennett, 2015; Kirschner, 2021).

The term "in vitro" includes all experiments involving isolated cells, tissues or organs cultured in a nutritive medium under controlled sterile conditions. The classical in vitro culture system consists of cells derived from a multicellular organism. Many cell lines are routinely cultured and commercially available (Gorzalczany and Rodriguez Basso, 2021). In cell biology, ex vivo procedures often involve living cells or tissues taken from an organism and

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cultured under laboratory conditions with no further manipulations for up to 24 h. Experiments on living cells or tissue for longer time periods are typically considered to be in vitro (Stewart et al., 2016; Acosta Davila and Hernandez De Los Rios, 2019).

Due to the complex life cycle of coccidian parasites, often involving multiple hosts, organs and tissues (and, with this, specific growth conditions and interaction variables with the host cells), their in vitro cultivation has been challenging. However, ethical aspects of experimental animal use, as well as economic aspects, are driving research on inexpensive, reliable and (largely) animal-free research in parasitology, and the utilization of in vitro models for research on coccidians is constantly increasing. For this, already established in vitro culture systems are improved, making different parasite life stages more readily available, and novel cultivation methods that can bridge gaps in detailed parasite analysis are being developed and evaluated.

This review gives an overview on different in vitro systems for propagation of, and research on, coccidians, discusses the more recent developments, highlights the advantages and drawbacks of those systems, and pinpoints recent research work that would not have been possible without effective and robust in vitro culture systems. Recent classification of the Apicomplexa places *Cryptosporidium* outside of the subclass Coccidia (Schlegel and Hülsmann, 2007; Adl et al., 2012), however their development, in vitro cultivation and oocyst production resembles those of coccidian parasites, hence we decided to include them in this review.

2. In vitro cultivation methods: advantages and drawbacks

2.1. Ethical considerations

In accordance with the three Rs, in vitro approaches are favored over in vivo examinations for life science research. In vitro cultivation is used as an important adjunct to diagnosis, for the production of antigens, for pre-clinical screening of drugs and other important therapeutic agents, and to study the biochemistry, physiology and metabolism of pathogens (Visvesvara and Garcia, 2002). This methodology is especially relevant in cases where in vitro systems could partially or completely replace animal studies for research purposes (Leist et al., 2012). Ironically, where the in vitro system produces better reproducible data, this has been held against it as an inaccurate prediction of in vivo results (Hoffmann, 2015; Hartung, 2018). Another reason why in vitro approaches are favored is their cost effectiveness, as their setup is commonly less expensive than animal experimentation (Roach et al., 1989; Fontana et al., 2021). However, many medium components require filter sterilization and have relatively short shelf lives, which also boosts the amount of waste produced during the experiments (Visvesvara and Garcia, 2002). For many medium formulations, addition of fetal bovine serum (FBS), synonymous FCS, is required as a cell growth factor. The production of FBS frequently raises concerns about the value of in vitro techniques as replacement alternatives, since medium containing FBS cannot be considered completely animal experimentation-free (Jochems et al., 2002). However, the shared benefits of in vitro systems in toto distinctly outweigh those of animal experiments (Roach et al., 1989; Visvesvara and Garcia, 2002; García and Díaz-Castro, 2013).

2.2. Different cell/tissue types

Culturing cells and tissues are probably the easiest and least expensive option to reduce or even exclude the use of animals in research. For propagation of members of the Coccidia, in vitro culture systems using cell monolayers are well established, frequently catering for the specific needs of different parasite species (Table 1). The cell lines used also have the benefits of being genetically uniform (clonal) and often very well characterized, supporting reproducibility of the results (Ahmed, 2014). Yet, they might express unique protein patterns or metabolic profiles that are not representative of the primary host cells in vivo. To counteract this, the characteristics of cultured cells must be defined in detail and assessed periodically (Bowes et al., 2012). In monolayer cell cultures the structure of the three-dimensional (3D) and more variable environment of complex tissue is frequently missing, which may constitute a major disadvantage for studies on parasite biology and host-parasite interactions (Gorzalczany and Rodriguez Basso, 2021); however, reduction of complexity might be beneficial for other research goals. In recent decades, research focused on developing new systems that can mimic organ architecture and function more closely (Table 2), compensating for the disadvantages of the monolayer commonly used in in vitro culture (Bayir et al., 2019; Ramírez-Flores and Knoll, 2021; Yang and Liberali, 2021). These 3D organoids are usually generated using either a single cell type or a mixture of host cells to recreate organ structure (Visvesvara and Garcia, 2002; Roth et al., 2018; Ramírez-Flores et al., 2022). Although organoids are transplantable, improved versions of 2D models and comparatively easy to manipulate, and their production requires considerable expertise in cultivation techniques, making establishment and maintenance more difficult compared with many established systems (Díaz et al., 2020).

2.3. Axenic cultures

A culture that contains only one species, variety or strain of microorganism within a cultivation medium is called axenic. Usually this technique is useful in growing defined monospecific cultures of fungi, algae or bacteria (Taylor and Baker, 1968; Diamond, 1983; Jensen, 2018) or extracellular parasitic protozoa for scientific use. *Plasmodium* spp., malaria parasites which are related to coccidians, have also been propagated in extracellular axenic environments (Munderloh and Kurtti, 1985; Porter-Kelley et al., 2006). Trager and Williams (1992) concluded that, although *Plasmodium falciparum* will infect host cells during their development, host erythrocytes are not essential for the parasite's life cycle. Axenic cultures have two main advantages: their methodological simplicity and the possibility to exclude possible interference of host cells or host cell metabolites with parameter readouts. Despite that, the constant problem facing those who rely on axenic cultures of these organisms is their fastidiousness, which can significantly inhibit reproducibility or even prevent parasite growth after slight changes in medium or culture conditions (Diamond, 1983; Singh et al., 2013). Another disadvantage of axenic cultures of protozoan parasites is that the infection dose needed to start a culture is relatively high in comparison with the those used for monolayers (Zhang et al., 2009; Karanis and Aldeyarbi, 2011). Nevertheless, progress has been made for some parasite species, which will be outlined further below.

3. Historical overview of coccidian parasite cultivation

3.1. The development of monolayer cultures

The first in vitro cell culture for coccidians in an attempt to culture *Toxoplasma gondii* was described by Levaditi et al. (1929). After that, successful attempts to culture *T. gondii* (Cook and Jacobs, 1958) and *Sarcocystis* sp. (Scott, 1943) in a monolayer cell culture were described, and until the 1980s, other coccidians also were cultivated in vitro. Current and Long (1983) described the development of *Cryptosporidium* spp. in the endoderm cells of the

Table 1
In vitro systems used for selected coccidia and *Cryptosporidium*.

Species	Mono-layer	Organoids	Body-on-a-chip	Axenic culture	References
Toxoplasma gondii	✓	✓	✓	×	Levaditi et al. (1929); Sakurai et al. (1987); Weiss et al. (1995); Ramírez-Flores et al. (2022)
Cryptosporidium	✓	✓	×	✓	Upton et al. (1994); Nesterenko et al. (1997); Sato et al. (2009); Karanis (2018)
Cryptosporidium parvum	✓	×	×	✓	Griffiths et al. (1994); Boxell et al. (2008); Hijjawi et al. (2010)
Eimeria	✓	✓	×	×	Hofmann and Raether (1990); Augustine (2001); Nash et al. (2021)
Eimeria tenella	✓	×	×	×	Bussière et al. (2018); Marugan-Hernandez et al. (2020)
Sarcocystis neurona	✓	×	×	✓	MacGowen (1923); Scott (1943); Marsh et al. (1997)
Cystoisospora suis	✓	×	×	✓	Harleman and Meyer (1983); Worliczek et al. (2013); Feix et al. (2021)
Neospora caninum	✓	×	×	×	Lindsay and Dubey (1989); Cole et al. (1990)
Hammondia	✓	×	×	×	Gondim et al. (2015); Sokol et al. (2018)

Table 2
Advantages and drawbacks of the most popular cell lines used for selected members of the subclass Coccidia and the genus *Cryptosporidium*.

Parasite species	Cell line	Advantages	Drawbacks	References for cell line
Toxoplasma gondii	HFF HeLa	Fast growth, asexual parasite stages can be harvested continuously	Propensity for contamination	Gey et al. (1953); Khan and Grigg (2017)
Cryptosporidium parvum	HCT-8	Fast growth	Not all life stages are supported	Upton et al. (1994)
Eimeria tenella	CLEC-213	Supports full life cycle, high rate of cell invasion	Susceptible to cross-infection	Bussière et al. (2018)
Sarcocystis neurona	BT	Oocysts from culture can be used for continuous cultivation	Susceptible to cross-infection	Andrews et al. (1990)
Neospora caninum	VERO	Growth in a variety of suspension media	Different antigen expression	von Laufen et al. (2004)
Cystoisospora suis	IPEC	Fast growth	Limited culture time	Brosnahan and Brown (2012)

chorioallantoic membrane of chicken embryos. A year later Current and Haynes (1984) described the successful cultivation of *Cryptosporidium* in human fetal lung, primary chicken kidney, and porcine kidney cells, marking the start of increasing research interest in coccidian in vitro cultures. Presently, coccidian parasites can be cultivated in a multitude of different cell lines; the most popular are listed in Table 2.

Cryptosporidium can be cultivated in various cell lines, making it the most popular cultured species of Coccidia sensu lato worldwide (Karanis, 2018). Different cell lines can be used for different scientific approaches, as parasites grow in different cells at variable speeds, so different parts of the life cycle can be highlighted. *Cryptosporidium* can be cultured on cell lines of bovine, chicken, human, and rabbit origin. HCT-8 (a human adenocarcinoma cell line) is the preferred cell line for *Cryptosporidium* research, as it supports several *Cryptosporidium* spp. and grows well on both culture plates and slides (Nesterenko et al., 1997; Upton et al., 1994; Hijjawi et al., 2002; Wu et al., 2009). Furthermore, HCT-8 cells seem to support the growth of *Cryptosporidium* developmental stages under favorable culture conditions two-fold better than Madin-Darby bovine kidney (MDBK) or CaCo2 (colon cancer) cells (Boxell et al., 2008; Hijjawi, 2010). Studies on the human-derived cell line CaCo-2 showed that a *Cryptosporidium parvum* infection causes epithelial cell death and trans-monolayer permeability (Griffiths et al., 1994; Hijjawi, 2003). Although all cell lines used are suitable for the division and maintenance of asexual stages, the development of sexual stages or oocysts to finish the in vitro life cycle is not supported by most of those cell lines (Arrowood, 2002). However, an inoculation of COLO-680N (esophageal squamous-cell carcinoma cells) cultures with *C. parvum* produced a sufficient amount of infective oocysts, which would enable sustainable propagation of the parasite in tissue cell culture (Miller et al., 2018).

Eimeria asexual stages can be maintained in different host cell types, but only develop further at variable degrees, depending on cells derived from bovine, human, porcine or monkey intestinal epithelium (Augustine, 2001; Müller and Hemphill, 2013; Dubey et al., 2020). Monolayers of primary kidney cells of chicken (PCKC) enabled the routine development of all schizont generations as well as, in general, young oocysts (Hofmann and Raether, 1990), however it was not possible to show sexual stages of the parasite in culture. Both gametes and oocysts of *Eimeria tenella* could be observed in a chicken lung epithelial cell line model, CLEC-213, when infected with second-generation merozoites from wild type strains. This demonstrates that this avian cell line constitutes a useful tool for studying *Eimeria*-epithelial cell interactions and the effect of drugs on *E. tenella* invasion, merogony and gametogony (Bussière et al., 2018). MDBK cells allow for partial development through one round of parasite invasion and intracellular replication of *E. tenella*. This model, however, is more suited for qualitative and quantitative studies on sporozoite invasion than on intracellular development (Marugan-Hernandez et al., 2020). The establishment of in vitro systems for the class Mammalia, infection with *Eimeria* spp. is extremely difficult and hence not yet widely spread. However, the development of *Eimeria bovis* and *Eimeria ninakohlyakimovae* is possible on VERO (kidney epithelial cells extracted from an African green monkey) cells (Hermosilla et al., 2002; Taubert et al., 2006; Ruiz et al., 2010). Still primary Bovine Umbilical Endothelial Vein Cells (BUVECs) are the preferred culture system for mammalian-infecting *Eimeria* spp. (Hermosilla et al., 2006; Taubert et al., 2010; Velásquez et al., 2021).

First studies on the development of *Cystoisospora* spp. in cell cultures demonstrated that *Cystoisospora felis* of cats; Fayer and Thompson, 1974) and *Cystoisospora canis* of dogs; Fayer and Mahrt, 1972) sporozoites could infect mammalian cells in vitro. Later the development by endodyogeny of both *Cystoisospora* spp. was shown in Madin-Darby canine kidney (MDCK) cells (Lindsay, 2019). However, no sexual multiplication was found in those cell lines. First studies showed that *Cystoisospora suis* would develop in five different types of mammalian cell cultures, and asexual stages were comparable with previous in vivo studies, but oocysts did not sporulate in culture. The complete development of *C. suis* was demonstrated in vitro by Lindsay and Current (1984), but oocysts derived from this system could not successfully

sporulate. Worliczek et al. (2013) demonstrated that oocysts can be produced in vitro using porcine intestinal epithelial cells. This system has been further improved (Feix et al., 2020), and the complete development of *C. suis* can be maintained in vitro, allowing its application in new research topics.

Cell cultures of *Sarcocystis* have been established since 1934 (Scott, 1934); however, they mainly support the production of merozoites after successful infection with excysted sporozoites. For *Sarcocystis cruzi,* a continuous cultivation from the original sporozoite inoculum has been maintained for more than 1,320 days by subinoculating merozoites onto new cultures of calf pulmonary artery (CPA) cells (Andrews et al., 1990). Cells typically used in monolayers for *Sarcocystis* cultivation are bovine turbinate (BT) cells, which can be the host for *Sarcocystis neurona*, *Sarcocystis falcatula* (Marsh et al., 1997) and *Sarcocystis cruzi*. Sporozoites have also been cultivated in penetrated bovine monocytes (BM), CPA (cellosaurus cell line), MDBK cells (Speer et al., 1986), and equine dermal and kidney cells (Verma et al., 2017). In vitro cultivation of *Sarcocystis* spp. gamonts has been possible in various cell lines including MDCK, embryonic BT, and kidney cells (Verma et al., 2017). *Sarcocystis neurona* is probably the most investigated *Sarcocystis* sp. due to its economic and health impact, and a variety of cell lines have been used to culture the parasite in monolayers (Ellison et al., 2001). The successful propagation of *S. neurona* also had a great impact on the initiation of genomic studies in coccidian parasites (Howe, 2001; Howe et al., 2005; Müller and Hemphill, 2013).

Neospora caninum was initially cultivated in vitro in BM and CPA cultures (Lindsay and Dubey, 1989). Since then, it has been grown in MDBK, fetal mouse brain, and several other well established cell lines (Cole, R.A., Lindsay, D.S., Blagburn, B.L., Dubey, J. P. 1990. Neospora caninum (Protozoa:Apicomplexa): Comparison of in vitro development of NC-1 and NC-3 isolates in three cell lines and three primary cell cultures. Proceedings of Southeastern Society of Parasitologists, Boone, North Carolina, USA, April 18–20. Abstract No. 34.). More recent studies also showed a successful infection of human breast carcinoma cell 7 (MCF-7) (Lv et al., 2010) and CaCo2 cells, in which an anti-*N. caninum* antibody also inhibited parasite growth in vitro (Omata et al., 2005).

Hammondia spp. are close relatives of *Neospora* and *Toxoplasma*; however, members of this genus display a strict two-host life cycle. In vitro cultivation of *Hammondia* spp. is rarely described, but monolayer systems of primary bovine embryonic heart cells and feline kidney cells (CRFK) have been infected with *Hammondia hammondi* in the past (Rishi et al., 1995; Sokol et al., 2018). Also, *Hammondia heydorni* can infect primary bovine embryonic heart cells and rhesus monkey kidney cell cultures (Gondim et al., 2015; Sokol et al., 2018).

In vitro culture systems have been the grindstone of research on *Toxoplasma gondii*. In the past, cell culture methods for this parasite have been well established in several distinct cell lines, mainly using tachyzoites for the infection. The focus of most in vitro cultures has been the production of tachyzoites as an experimental in vitro model (Soldati and Meissner, 2004; Montazeri et al., 2017), genetic studies (Radke et al., 2005), biochemical pathway or drug studies (Gold et al., 2015; Guo et al., 2021). Traditionally human foreskin fibroblast (HFF) cells are widely used for the propagation and maintenance of *T. gondii* tachyzoites, as they allow a constant production of new tachyzoites, due to their fast growth (Khan and Grigg, 2017). Another popular cell line for propagating and producing large amounts of viable *T. gondii* tachyzoites is the transformed HeLa cell line, an 'immortal' cancer cell line named after its donor. It has the advantage of fast growth and offers the possibility of continuous tachyzoite production that is required for many experiments (Sakurai et al., 1987; Sağlam Metiner et al., 2019). *Toxoplasma* bradyzoites were first cultivated in vitro in human fibroblast cells, where their development was induced by pH changes in the medium (Weiss et al., 1995). The formation of typical *Toxoplasma* tissue cysts can be induced in several cell types, i.e. BM, human fetal lung or MDBK, if they are infected with either sporozoites, tachyzoites or bradyzoites (Lindsay et al., 1991). A recent study also demonstrated the in vitro maturation of *T. gondii* bradyzoites in human myotubes and showed that tissue cysts derived from these cultures are infectious to mice per os (Christiansen et al., 2022). As *T. gondii* infects a variety of different cell types and has a high and fast turnover of parasite stages, it is considered to be the ideal parasite for in vitro infection studies (Sutrave and Richter, 2021). Despite its high output of asexual stages in vitro, the continuation of the sexual cycle (with production of micro- and macrogametocytes) occurs exclusively in the intestinal epithelial cells of felids. Maybe due to the obstacles related to this host and tissue specificity, recent research on *T. gondii* focused primarily on the array of effector proteins in the pathways involved in the interaction between host and parasite (Matta et al., 2021), i.e parasite invasion strategies or growth (López-Yglesias et al., 2021; Márquez-Nogueras et al., 2021).

3.2. Transgenic and altered target cell lines

Most of the research on coccidian parasites has been performed using immortalized cell lines, such as cancer cell lines or non-transformed cell lines, as described in section 3.1. In many cases, these monolayers do not reproduce the natural environment of the parasite and they lack the architecture and properties of the cell populations found in the host which are required for the complete endogenous development, although these cell lines also have a number of advantages such as reliable growth and limited variability between infections of different cell batches (Mital and Ward, 2008).

A useful approach to analyze the parasités ability to invade and replicate is to use stem cells, genetically modified cells or chemically (enzymatically) altered host cells. Advances in stem cell technology have opened new directions for the development of in vitro cell systems that can be customized to provide more physiological environments for the culture of protozoan parasites. Various stem cell lines (ESC or iPS) have been created that can be expanded, cultured, stored, genetically edited and differentiated into any desired cell type (Al Abbar et al., 2020; Pance, 2021).

Recent studies reported the use of human neurons to study cerebral toxoplasmosis. The results of two studies using such cells derived from cellular reprogramming indicate that such human neuronal models provide a novel in vitro culture system to analyze the effects of *T. gondii* on neurons and neurological functions (Tanaka et al., 2016; Halonen, 2017).

Enteroid-derived epithelial sheets and monolayers, and cat and mouse intestinal organoids, have been use to study the sexual stages of *T. gondii* (Luu et al., 2019). A recent study has changed the current research of *T. gondii* by achieving the in vitro sexual cycle using mouse intestinal monolayers supplemented with linoleic acid and the chemical SC-26196 to inhibit the enzyme delta-6-desaturase. This took advantage of the fact that members of the feline family lack this enzyme which is responsible for the breakdown of linoleic acid, literally turning murine into feline intestines – at least form the view of *T. gondii* (Di Genova et al., 2019). It can be expected that a well stablished, standardized in vitro culture system for the sexual development of *T. gondii* in mouse intestinal organoids with a delta-6-desaturase enzyme knockout and linoleic acid supplementation will be available, and that other mechanisms of host specificity can be elicited and the results also be applied to create tailor-made in vitro culture systems for other species of coccidians.

3.3. Cultivation of 3D organoids

Three-dimensional in vitro systems have also been utilized to study the infection biology of *T. gondii* in various cell types, most notably a placental model for the study of congenital toxoplasmosis (Betancourt et al., 2019), an intestinal organoid model for the study of *T. gondii* sexual stages (Luu et al., 2019; Holthaus et al., 2021) and organoids representing brain structures to study cerebral toxoplasmosis (Seo et al., 2020). The novel 3D systems open up a plethora of new research purposes, but also shine light on the differentiated invasion, replication and egress mechanisms of the parasite during infection of the host. Although the rate of egress of the parasite is not significantly different between monolayer cultures and 3D organoids, the path of egress differs. While *T. gondii* egressed perpendicularly to the bottom of the cells in monolayers, it egresses radially in all directions in the 3D system (Danielson et al., 2018; Ramírez-Flores et al., 2022). Furthermore, the sexual stages of *T. gondii* were successfully cultured for the first time in vitro using mouse intestinal organoids infected with bradyzoites, comparable to previous studies on the completion of the sexual cycle of *T. gondii* in mice (Di Genova et al., 2019). Luu et al. (2019) developed three novel 3D organoid in vitro cultures of the small intestinal epithelium that are susceptible to, and support, an infection with *T. gondii*, most importantly a collagen-supported epithelial sheet model that allows for high-throughput infection studies. Another model induced pluripotent stem cell-derived neurons as a model for cerebral toxoplasmosis and allows study of the interaction of tachyzoites and bradyzoites in human neurons (Tanaka et al., 2016). Studies on 3D- rganoids also showed that a *T. gondii* infection has no detrimental effect on the transepithelial electrical resistance and tight junction integrity of murine organoid-derived monolayers (Holthaus et al., 2021).

For *Cryptosporidium* spp. the most popular model is an intestinal organoid from either mouse or human cells (Sato et al., 2009), but a *Cryptosporidium* infection in lung organoids has been used (Heo et al., 2018). Both systems showed extremely promising results, as the *Cryptosporidium* infection was supported for up to 28 days and oocysts generated from these 3D systems were also infectious to mice in vivo. However, the number of parasites in the culture diminished over time and the total number of parasites produced was much lower than in the established in vivo models. Mini-gut tubes derived from mouse intestinal organoids support the whole life cycle of *Cryptosporidiumparvum* in vitro. This system enables long- term host–microorganism cocultures and its scaffold is permeable to gases, nutrients, and macromolecules facilitating adhesion, proliferation, and differentiation of intestinal stem cells (DeCicco RePass et al., 2017; Gunasekera et al., 2020; Nikolaev et al., 2020).

The use of organoids in *Eimeria* research is still at the beginning and only a few systems are available to date. A very sophisticated method to propagate *Eimeria* using floating avian intestinal organoids derived from embryonic villi or mature chicken crypts has been developed which has two unique characteristics; first, the presence of leukocytes in tissue and second the apical brush border facing the medium. Thus, sporozoites were able to infect the epithelium and undergo both schizogony and gametogony (Nash et al., 2021).

3.4. Cultivation of the future: body-on-a-chip

Although organoids offer many advantages, they still do not completely represent a host organ. Therefore a relatively novel technique, the organ-on-a-chip, might be useful, as it represents in vivo factors that might be missing in organoid systems (Achberger et al., 2019). Within the organ-on-a-chip system, several organs have already been recreated from human cell lines, i.e. skin, spleen, intestine, liver, brain and eye (Rigat-Brugarolas et al., 2014; Jannasch et al., 2015; Kasendra et al., 2018; Achberger et al., 2019; Amirifar et al., 2022). Application of these systems in parasitology is still in its infancy, but it has been used for research purposes on *Plasmodium*, where the effects of anti-*Plasmodium* drugs were tested in an eye-on-a-chip system, showing a measurable reduction in parasite cell viability upon treatment (Peng et al., 2020; Manafi et al., 2021).

Although coccidian species have not yet been tested in 3D cultivation systems, this system offers advantages for mechanistic research on host-pathogen interactions, and for the general cultivation of all coccidians. Ramírez-Flores et al. (2022) propose that studies on *Toxoplasma gondii* in a brain-on-a-chip system can help solve the problem of genetic variation between animal models and human hosts in studies on neurological diseases. Similar studies on *Cryptosporidium*, *Cystoisospora* and *Eimeria* could be possible in intestine-on-a-chip models. Furthermore, the interrelationship between the gut microbiota and the parasite during endogenous development is thought to play an important role in the onset of clinical disease, its progression and successful treatment. However, this relationship is still poorly understood (Macdonald et al., 2017; Vieira et al., 2020; Lu et al., 2021; Orso et al., 2021). The intestine-on-a-chip system could be helpful in understanding the underlying mechanisms during the interaction of the parasite and the gut microbiome in the host.

3.5. Axenic in vitro systems

Interestingly, early attempts to cultivate various coccidian parasites used liquid culture medium which was free of host tissue. In the first report on this, *Sarcocystis tenella* was cultured in a 1% glucose solution (MacGowen, 1923). Two years later, merozoites of different *Eimeria* spp. were transferred to various nutrient-rich media and survived up to 9 days after transfer (Triffitt, 1925).

In vitro, stage conversion could so far only be demonstrated for some *Cryptosporidium* sp. and the porcine parasite *Cystoisospora suis*. In *Cryptosporidium. hominis*, all asexual stages as well as gamonts were propagated over a 9-day incubation period (Hijjawi et al., 2010) and the production of newly formed oocysts was observed. The complete development of *Cryptosporidium. parvum* in a host cell-free culture was also demonstrated (Boxell et al., 2008; Hijjawi et al., 2010); however, even the original authors of these works do not further apply this cultivation system to their research (Girouard et al., 2006; Karanis, 2018). Hence, the axenic propagation of *Cryptosporidium* still seems difficult to master, and other cultivations systems are preferred, due to their higher output of parasite stages and better use for transcriptomic and proteomic research. To date only Zhang et al. (2009) were able to show that the parasite undergoes a change in DNA level during development in the host cell-free environment, indicating cell division and multiplication at a cellular level, as well as morphological changes as previously shown.

Completion of the life cycle of *Cystoisospora suis* can be demonstrated in a host cell-free environment, for which the asexual stages of the parasite (merozoites) are harvested from an in vitro cell culture using intestinal porcine epithelial cells and transferred to a host cell-free environment (Feix et al., 2021). After this transfer, parasite development continues within the typical *C. suis* time frame to sexual stages. Unsporulated and sporulated oocysts can be harvested from these axenic cultures and used for further research, as the morphology of the stages does not differ greatly from cell-based *C. suis* cultivation systems which also provide infectious oocysts after cultivation (Harleman and Meyer, 1983, 1984; Worliczek et al., 2013), and the transcription profiles of selected sexual genes also match those of previous studies (Feix et al., 2020, 2021). As no genetic manipulation technique is cur-

rently available for *C. suis* to confirm the direct involvement of specific genes in the development of this parasite, this in vitro system opens up new possibilities for research on novel targeted control for *C. suis*. Hence, the host cell-free in vitro culture system for *C. suis* made it possible to evaluate the effects of culture conditions and an antibody against a tyrosine-rich protein on the development of merozoites to sexual stages and oocysts (Cruz-Bustos et al., 2022).

4. Aspects of in vitro cultivation relevant for coccidians

Coccidians are characterized by a complex life cycle, during which asexual multiplication, with sporogony and merogony, is followed by sexual development with two morphologically distinct cell types, which fuse to form a zygote. Zygotes will further develop into oocysts, so that the parasite can continue its life cycle (Long 1990; Taylor and Catchpole, 1994; Cruz-Bustos et al., 2021). Therefore coccidian cultivation techniques often include complex procedures, as each developmental step requires a specific environment. Still, monolayer cell cultures are the most frequent in vitro cultivation technique for obligate intracellular parasites such as coccidians. The host cell lines for coccidian parasites, however, differ greatly, as they have to cater for the specific needs of the respective parasite species. However, a general rule is that cultures for coccidian parasites are incubated at temperatures of 37 °C to 40 °C and that incubation conditions are usually microaerophilic (Jensen, 2018). The culture medium has to be chosen in accordance with the host cell line and includes various nutrients (Ahmed, 2014).

The host specificity of coccidians is primarily related to completion of the parasitic life cycle until the accumulation and excretion of the oocysts as a (future) environmental stage. *Cryptosporidium* and *Eimeria* spp. obviously prefer cell lines that are derived from their natural host species; however, in the case of *Cryptosporidium*, they often are also capable of developing in a "foreign" host in vivo. This might be due to the range of different species this coccidian genus caters to. Recent studies show that host switching is more frequent than originally believed and is an important driver in the evolution of host-parasite interactions (Mácová et al., 2018). Recent studies showed that *Eimeria necatrix* might have evolved from Coccidia that originally parasitized turkeys but switched to chickens during domestication of these birds (Ogedengbe et al., 2011, 2018). Despite seemingly frequent host switches in *Eimeria* spp., this is not utilized for in vitro cultivation because it would include a change in cell lines during several cultivated generations. Although *Cystoisospora* can also be cultivated in cell lines that are not close to their natural hosts, i.e cultivation of *C. suis* in chicken chorioallantoic membrane or swine testicular cells (Current and Long, 1983; Steger, A.M., 1997. Development and evaluation of an *Isospora suis* sporozoite neutralizing antibody assay in swine testicular cells. Doctoral dissertation, Drake University, Des Moines, IA, USA), they are generally considered highly host-specific with regard to their sexual development, as they have not undergone a host switch during their evolution (Andrews, 1927; Kogut, 1990).

The life cycles within the subclass Coccidia vary greatly and can follow a homoxenous or a facultative or obligatory heteroxenous (two-host) life cycle. In general, oocysts will be ingested by the (intermediate) host and sporozoites excyst within the digestive system. In in vitro cultures, the excystation protocol used already has an immediate effect on the viability and amount of sporozoites during their excystation process from oocysts (Jensen, 1983). Most excystation protocols for in vitro culture infections include a mechanical disruption of the oocyst wall, so that sporozoites can more easily evade the sporocyst/oocyst. Improved protocols for *Eimeria* and *Cystoisospora* combine enzymatic treatment of the oocyst wall with pepsin, trypsin and/or taurocholate in combination/after the mechanical disruption, which significantly shortens the time of excystation (Kowalik and Zahner, 1999; Worliczek et al., 2007; Kurth and Entzeroth, 2009; Hijjawi, 2010).

After a primary infection step, one or more rounds of asexual multiplication will take place either in the in the definitive (single) host or, after a host switch, in the intermediate host. Continuous propagation through asexual reproduction in intermediate hosts cells is possible for a number of coccidian parasites, and allows for an endless production of tachyzoites of *Toxoplasma gondii* (Dubey, 1998; Evans et al., 1999; Skariah et al., 2010) and *Neospora caninum* in vitro (Hemphill et al., 1996; von Laufen et al., 2004).

Regardless of the coccidian species, the formation of asexual stages is, however, a crucial step during their life cycle, as it provides a sufficient number of stages for infection of further host cells with or without a host switch. To simulate this process during successful maintenance of the culture, pH changes can be helpful, as those are thought to be important for both invasion and egress of parasite stages (Quist et al., 1993; Upton et al., 1994; Hijjawi, 2010). The average pH for coccidian culture media is between 7.0 and 8.0, and results in infection of host cells with the encysted sporozoites (Upton et al., 1995), as stage conversion in protozoan parasites can be considered as a response to alkaline pH stress (Naguleswaran et al., 2010). During their development coccidians are known to consume carbohydrates (Speer and Hammond, 1972; Fayer and Thompson, 1975), vitamins and amino acids (Doran and Augustine, 1978; Strout and Schmatz, 1990), which can be added to the culture medium to increase the amount of viable parasites.

Sexual reproduction of coccidian parasites occurs regardless of the number of intermediate hosts. After late asexual stages have effectively infected a host cell, a subpopulation undergoes gametogenesis and proceeds to differentiate into either micro- or macrogametes. This development, including syzygny resulting from gametogenesis, is necessary to complete the life cycle and form a zygote (Cornelissen and Overdulve, 1985; Cruz-Bustos et al., 2022). In cyst-forming coccidians, cells from reactivated tissue cysts will differentiate into sexual stages, resulting in the production of oocysts, while in intestinal coccidians the sexual reproduction occurs from dividing merozoites in the intestinal tissue of the host (Fig. 1). However, sexual reproduction and oocyst formation is a relatively quick developmental process, which makes in vitro cultivation more challenging (Frenkel and Smith, 2003; White et al., 2014; Sokol-Borrelli et al., 2020). Successful cultivation and harvest of sexual stages for further research is therefore currently only possible for selected coccidian species, i.e. *T. gondii*, *C. suis*, or *C. parvum*.

5. Recent applications of in vitro cultures of coccidians in research

A detailed discussion about the different research projects for coccidians applying in vitro cultivation would go far beyond the scope of this review, so we want to highlight only some approaches. Applications of in vitro cultures for coccidian research include detailed descriptions of parasite morphology and cellular function during motility (Meissner et al., 2002; Feix et al., 2020), mechanisms underlying host cell invasion (Periz et al., 2017, 2019; Whitelaw et al., 2017), protein trafficking (Hu et al., 2017; Ramakrishnan et al., 2017; Marugan-Hernandez et al., 2021), host-pathogen interaction (Sikorski et al., 2021; Smith et al., 2021) and the effects the parasites have on host cells (Lüder and Rahman, 2017). However, a detailed discussion would reach beyond the scope of this article, hence we will focus on the evaluation of potential drug targets.

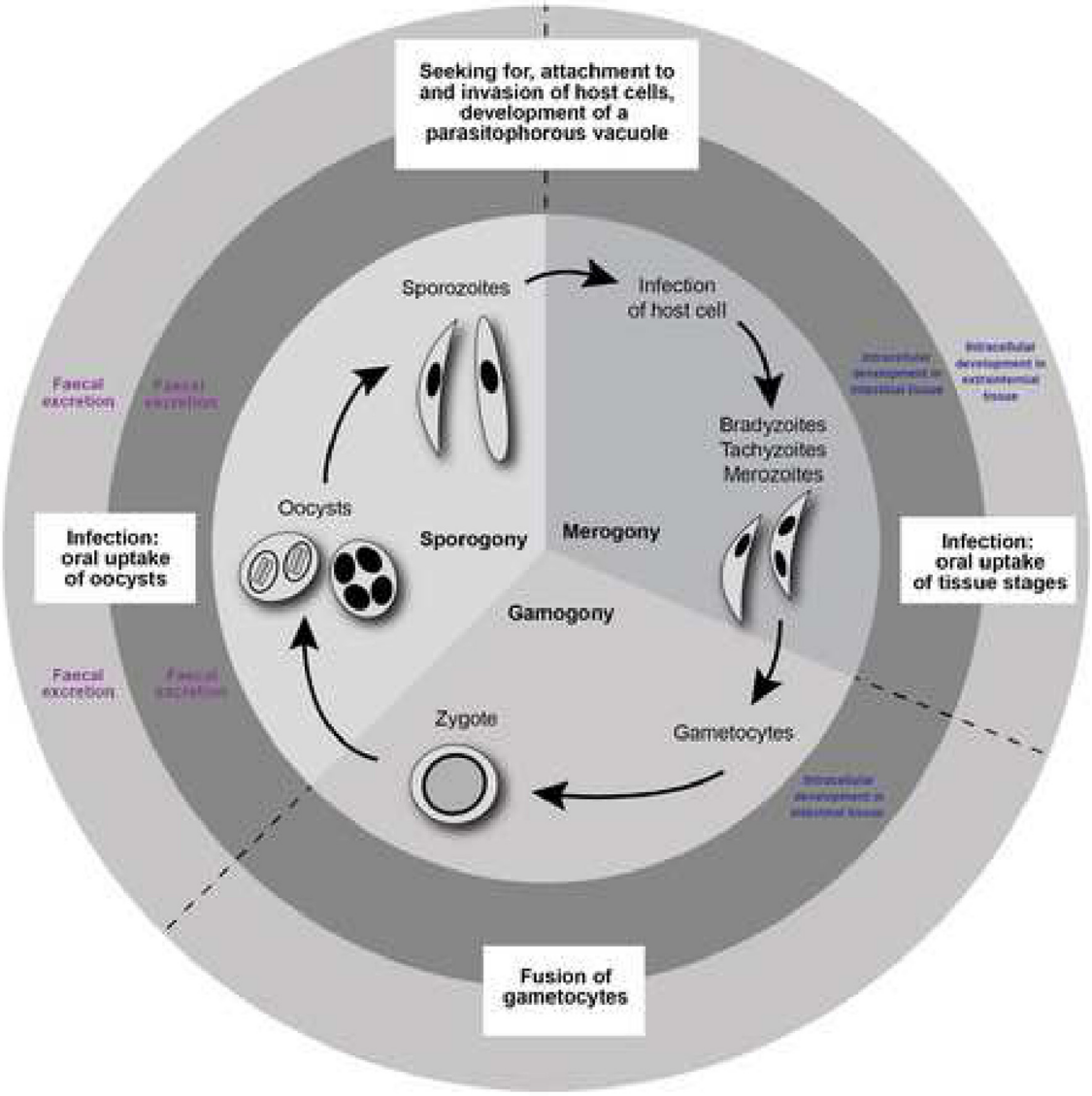

Fig. 1. Schematic life cycle of coccidian parasites, characterized by three distinct steps, sporogony, merogony and gamogony. The transmissible stages, sporozoites, seek a host cell for possible invasion after infection of the host. Merogony takes place in the intestinal tissue. The various stages develop taxon-specifically in different forms and numbers, represented by the different stages. The outer light grey circle represents the developmental steps of cyst-forming coccidia, where infection of tissue is possible in heteroxenous coccidia. The middle circle represents the life cycle of intestinal coccidia. Environmental oocysts can be found in unsporulated and sporulated forms after faecal excretion.

As coccidians mostly colonize the intestinal tract of the definitive host, recent research also discusses mechanisms by which coccidians interact directly or indirectly with the gut microbiota of the host. However, in vitro systems suitable for host-parasite interaction research relating to the gut microbiome are still lacking. In particular, the incorporation of microbiota is challenging because they require complex media to support the growth of diverse cell types and organisms (Smith et al., 2021). The development of high-throughput sequencing technology and in vitro culture systems which foster a host-like enteral environment and therefore support the maintenance of gut microbiota as well as parasite growth has enabled studies on that topic (Gaboriaud et al., 2021; Lu et al., 2021; Ramakrishnan and Smith, 2021). Studies that demonstrated that parasites enhance the growth of mucin-utilizing bacteria in vitro (Ramanan et al., 2016) and influence the well-being of microbiota by their own metabolic activities (Tailford et al., 2015) support the use of in vitro culture systems to study complex multi-organism interactions without the use of animal models.

5.1. Compound testing in vitro

Coccidiosis, both in animals and in humans, represents a considerable medical and economic burden worldwide. To prevent disease causing economic losses, various control measures including anticoccidial drugs, natural or synthetic feed compounds and vaccination strategies are constantly improved, tested and developed both in vitro and in vivo (Felici et al., 2021).

A number of methods have been applied to assess the disinfecting capacity of chemicals on coccidians of veterinary impact (Daugschies et al., 2002; Bogan, 2018). In vitro, disinfection efficacy can be directly evaluated by either oocyst destruction or sporulation inhibition (Daugschies et al., 2002). For example, under in vitro conditions, Neopredisan 135-1® destroyed all *C. suis* oocysts after a contact time of 90 min or more (Straberg and Daugschies, 2007). To evaluate inactivation of oocysts by other mechanisms, however, infection trials are necessary. Formerly, the efficacy to reduce infectivity by disinfection was tested in an

animal model of chicken infected with *E. tenella,* which required large numbers of animals for quantitative readouts and was neither cost-effective nor compatible with the 3R concept (Daugschies et al., 2013). The possibility of replacing the animal infection trials with infections of monolayers using *C. parvum* as a model for coccidians was tested by comparing a mouse model with a cell culture assay (Joachim et al., 2003). It was demonstrated to be better quantifiability in the cell culture model and this was further developed to a standard test system for disinfection efficacy (Shahiduzzaman et al., 2010; Delling et al., 2017; https://www.desinfektion-dvg.de).

Especially in avian *Eimeria,* the use of anticoccidial botanicals is on the rise, and a large number of different compounds have already been tested in vitro. For this, extracts from different plants are thought to target the exogenous stages of *Eimeria* spp., resulting in reduced oocyst production (Abbas et al., 2011; Felici et al., 2021). Artemisin reduced the calcium ATPase in the macrogamete endoplasmatic reticulum, which most likely lead to abnormal oocyst formation and hence an increased amount of dead oocysts and abnormal oocyst formation (Del Cacho et al., 2010; Dragan et al., 2010). Some garlic extracts such as propyl thiosulfinate, propyl thiosulfinate oxide, and allicin have been shown to have anticoccidial efficacy by influencing the invasion of *Eimeria* (Dkhil et al., 2011; Khalil et al., 2015; Chang et al., 2021). *Thonningia sanguinea*, an African medical plant, has also been shown to be useful as an anticoccidial treatment during the invasion of *E. tenella* and *E. necatrix* (Séverin, 2012). As a caveat, all these studies performed in vitro must be considered as preclinical screening of compounds, since efficacy testing must be evaluated in the context of host and parasite, considering absorption, tissue distribution, metabolization and possible toxic side effects. However, these tests show promise in reducing the use of laboratory animals and the possibility of large-scale compound screening.

Furthermore, the use of transgenic parasites constitutively expressing a measurable marker are often used to quantify parasite growth in vitro (Gubbels et al., 2002; Li et al., 2009). GFP-fluorescent parasites proved sensitive and highly reproducible for quantifying the growth-inhibitory activity of the tested compound (Wilson et al., 2020; Bowden et al., 2018; Chen et al., 2019). Hence, this system further allows in vitro drug testing at a high throughput.

5.2. Strategies to interrupt the parasite's life cycle by stage-specific blocking strategies

Blocking the transmission of parasite stages is considered an effective technique against coccidiosis and can be achieved either by specific antibodies inhibiting the sexual stages or by transmission-blocking drugs that affect specific stages (Hirai and Mori, 2010).

In this context, sexual stages are considered to be suitable targets. Studies on *T. gondii* showed that blocking the fusion of micro- and macrogametes can be a successful tool for intervention in the control of coccidial infections, as HAP2 knockout parasites failed to produce sporulated oocysts in vivo and in vitro (Ramakrishnan et al., 2019). Monoclonal antibodies against microgametocytes of *E. tenella* reduce oocyst formation in vitro more than 50% but the mechanism of inhibition was investigated in detail (Madden and Vetterling, 1977; Laxer et al., 1987; Suarez et al., 2017). As coccidians are characterized by the environmental stage, the oocyst, a blocking strategy targeting this stage or its emergence would most likely have the highest impact on interruption of transmission and, following multiplication and accumulation of infectious stages within a flock or herd, prevention of infections relevant to animal (or human) health. The formation of the resilient oocyst wall is an important time in coccidian development. In *E. maxima* two glycosylated tyrosine-rich proteins of the wall-forming bodies were shown to induce a strong antibody response to other *Eimeria* spp. as well (Belli et al., 2003; Mai et al., 2009). After a series of in vivo experiments, a commercial vaccine, CoxAbic® containing the native gametocyte antigens of *E. maxima* oocyst wall, Gam56 and Gam82, was developed and marketed for the vaccination of chickens in 2002 (Sharman et al., 2010). Since the production of this vaccine, several proteins associated with *E. maxima*, *E. tenella*, and *E. necatrix* gametocytes have been proposed as potential vaccine targets to induce immunity (Jang et al., 2010; Xu et al., 2013; Wiedmer et al., 2017a, 2017b; Rafiqi et al., 2019). Recent studies in *C. suis* propose several potential, highly expressed vaccination candidates both in merozoites (Palmieri et al., 2017) and the sexual stages of the parasite (Cruz-Bustos et al., 2022). Specifically, a gene coding for a tyrosin-rich protein, CSUI_001473, seems important for oocyst wall formation, and an in vitro assay showed that anti-rCSUI_001473 serum from chicken immunized with recombinant protein inhibited the late sexual stage development and the formation of oocysts by 75% in comparison to a non-treated control group (Cruz-Bustos et al., 2022), indicating that TyRP would be a viable candidate for a target-blocking vaccine for *C. suis*.

A group of enzyme inhibitors, Bumped Kinase Inhibitors (BKIs) that target calcium-dependent protein kinase 1 (CDPK1) in various apicomplexan parasites has shown promise in in vitro and in vivo trials (Choi et al., 2020). A wide range of BKIs have already been evaluated for efficacy against coccidian parasites both in vivo and in vitro, and no consistent side effects of BKIs have been observed in these trials (Winzer et al., 2015; Ojo et al., 2016; Schaefer et al., 2016; Van Voorhis et al., 2017). Various CDPK family members have already been identified in coccidians which are differentiated by the gatekeeper which enables binding for BKIs (Van Voorhis et al., 2017). Tests in vitro have covered a broad range of parasites and enabled the comparison of different BKIs for efficacy before in vivo trials were performed. BKI-1294 is one of the most efficient BKIs against coccidian parasites. The amount of parasite stages of *T. gondii* and *N. caninum* could be inhibited by 50% when treated with BKI 1294 in vitro and mouse infection experiments support these findings (Winzer et al., 2015). BKI-1294 also demonstrated impact on cryptosporiosis in vitro and in treated calves (Schaefer et al., 2016). Furthermore, BKI 1369 is effective against *C. suis* in vivo and in vitro (Shrestha et al., 2019). This shows that BKIs have great potential for therapeutic use in veterinary and possibly also in human medicine, due to a low number of side effects, and it highlights the usefulness of preclinical in vitro screening models for compound efficacy testing.

6. Concluding remarks

The replacement and reduction of animal experimentation, in accordance with the three Rs, with in vitro cultivation methods was an important step in research on coccidians. The constant development and improvement of in vitro culture systems for coccidian research ensures that more and more tools for research in various aspects of this important parasite group are and will be available as part of ethical considerations. Several well-established in vitro monolayer cultures are already available, allowing investigation of a variety of aspects of coccidian biology and parasite control. Although the complete life cycle of only a few species is supported in vitro, we are optimistic that constant methodological improvement will overcome the current obstacles. The continuous use of cell monolayers has been gradually improved to obtain high output of parasitic stages during culture and greatly increased our knowledge on coccidian biology, life

cycle and interaction with the host. Furthermore, the strategic focus on a significant reduction in animal experimentation has had an impact on the pharmaceutical industry as well as academic institutions regarding toxicological and compound efficacy testing, and the development and application of in vitro tests increased significantly.

Novel technologies will help to further improve research on coccidians. Three-dimensional technologies serve as a bridge between the traditional monolayer cultures and in vivo experiments. This specifically will aid studies on host-pathogen interactions, and although this method has already been used successfully for coccidian research, it has only been tested for a few coccidian species. Hence, the improvement in 3D culture techniques will greatly enhance research on coccidians (as well as in other fields of life sciences). Organ-on-a-chip technologies can be regarded as a direct development from other 3D organoids and allows compilation of data with greater biological accuracy without animal experimentation.

The use of axenic cultures in coccidian research is still in its infancy; however, the results shown for *Cryptosporidium* and *C. suis* are promising. Although the simplicity of this culture system is a clear advantage, it does not (yet) support the full coccidian life cycle so applications are limited to some research questions. Still, the applicability of host cell-free in vitro culture systems further supports the proposition that the last generation of asexual stages during coccidian development are already sexually committed and henceforward already include the information on which sexual stage they are going to develop into, and that these later asexual stages as well as the sexual stages do not require a host cell but can survive and progress extracellularly and therefore in axenic cultures.

In particular, in vitro culture systems which support the sexual stages of the parasites might be useful for the development of anticoccidial intervention targets. Fertilization (and, more broadly, sexual development) is a potential bottleneck in the life cycle progression of these parasites, and interfering with these crucial events to block further development will effectively preclude transmission of the parasite via infectious oocysts. Aaxenic and 3D in vitro techniques, especially, are extremely useful to study the quantitative effects concerning the affected life cycle stages and might be used more frequently in the future.

However, to date only selected species can be cultivated with a selection of in vitro systems. An extension to other coccidian species for successful cultivation also might have a considerable impact on research topics including drug screening for coccidians important for livestock health and production. In particular, in intensive poultry management resistance of different *Eimeria* spp. to commercially used compounds is increasing, and in vitro systems could improve validation of novel drug compounds. Furthermore, the development of high-hroughput pre-clinical screening methods validated for a number of coccidian species is still in early stages. Drugs against cyst forming coccidia of domestic animals are not commercially available, and for intestinal coccidiosis of humans by *Cystoisospora belli* information about effective control is still lacking. The different in vitro systems that are already available for coccidian "model species", however, should also be useful to tackle these problems in the future and thereby contribute directly to a One Health approach to coccidiosis.

References

Abbas, R.Z., Iqbal, Z., Blake, D., Khan, M.N., Saleemi, M.K., 2011. Anticoccidial drug resistance in fowl coccidia: The state of play revisited. Worlds. Poult. Sci. J. 67, 337–349. https://doi.org/10.1017/S004393391100033X.

Achberger, K., Probst, C., Haderspeck, J.C., Bolz, S., Rogal, J., Chuchuy, J., Nikolova, M., Cora, V., Antkowiak, L., Haq, W., Shen, N., Schenke-Layland, K., Ueffing, M., Liebau, Loskill, P., 2019. Merging organoid and organ-on-a-chip technology to generate complex multi-layer tissue models in a human retina-on-a-chip platform. Elife 8, 1–26. https://doi.org/10.7554/eLife.46188.

Acosta Davila, J.A., Hernandez De Los Rios, A., 2019. An overview of peripheral blood mononuclear cells as a model for immunological research of *Toxoplasma gondii* and other apicomplexan parasites. Front. Cell. Infect. Microbiol. 9, 1–10. https://doi.org/10.3389/fcimb.2019.00024.

Adl, S.M., Simpson, A.G.B., Lane, C.E., Lukeš, J., Bass, D., Bowser, S.S., Brown, M.W., Burki, F., Dunthorn, M., Hampl, V., Heiss, A., Hoppenrath, M., Lara, E., Gall, L.L., Lynn, D.H., McManus, H., Mitchell, E.A.D., Mozley-Stanridge, S.E., Parfrey, L.W., Pawlowski, J., Rueckert, S., Shadwick, L., Schoch, C.L., Smirnov, A., Spiegel, F.W., 2012. The revised classification of eukaryotes. J. Eukaryot. Microbiol. 59, 429–514. https://doi.org/10.1111/j.1550-7408.2012.00644.x.

Ahmed, N., 2014. Cultivation of parasites. Trop. Parasitol. 4, 80. https://doi.org/10.4103/2229-5070.138534.

Al Abbar, A., Ngai, S.C., Nograles, N., Alhaji, S.Y., Abdullah, S., 2020. Induced pluripotent stem cells: reprogramming platforms and applications in cell replacement therapy. Biores. Open Access 9, 121–136. https://doi.org/10.1089/biores.2019.0046.

Amirifar, L., Shamloo, A., Nasiri, R., de Barros, N.R., Wang, Z.Z., Unluturk, B.D., Libanori, A., Ievglevskyi, O., Diltemiz, S.E., Sances, S., Balasingham, I., Seidlits, S. K., Ashammakhi, N., 2022. Brain-on-a-chip: Recent advances in design and techniques for microfluidic models of the brain in health and disease. Biomaterials 285,. https://doi.org/10.1016/j.biomaterials.2022.121531 121531.

Andrews, J., 1927. Host-parasite specificity in the Coccidia of mammals. J. Parasitol. 13 (3), 183–194. https://doi.org/10.2307/3271500.

Andrews, C.D., Fayer, R., Dubey, J.P., 1990. Continuous *in vitro* cultivation of *Sarcocystis cruzi*. J Parasitol. 76 (2), 254–255. PMID: 2108236.

Arrowood, M.J., 2002. *In vitro* cultivation of *Cryptosporidium* species. Clin. Microbiol. Rev. 15, 390–400. https://doi.org/10.1128/CMR.15.3.390-400.2002.

Augustine, P.C., 2001. Cell: sporozoite interactions and invasion by apicomplexan parasites of the genus *Eimeria*. Int. J. Parasitol. 31, 1–8. https://doi.org/10.1016/S0020-7519(00)00150-8.

Bayir, E., Sendemir, A., Missirlis, Y.F., 2019. Mechanobiology of cells and cell systems, such as organoids. Biophys. Rev. 11, 721–728. https://doi.org/10.1007/s12551-019-00590-7.

Belli, S.I., Wallach, M.G., Luxford, C., Davies, M.J., Smith, N.C., 2003. Roles of tyrosine-rich precursor glycoproteins and dityrosine and in development of the oocyst wall in the coccidian parasite *Eimeria maxima*. Eukaryot. Cell 2, 456–464. https://doi.org/10.1128/EC.2.3.456.

Betancourt, E.D., Hamid, B., Fabian, B.T., Klotz, C., Hartmann, S., Seeber, F., 2019. From entry to early dissemination-*Toxoplasma gondii*'sInitial encounter with its host. Front. Cell. Infect. Microbiol. 9, 1–9. https://doi.org/10.3389/fcimb.2019.00046.

Bogan, J.E., 2018. Disinfection techniques for *Cryptosporidium*. J. Dairy Vet. Sci. 7, 7–10. https://doi.org/10.19080/jdvs.2018.07.555718.

Bowden, G.D., Land, K.M., O'Connor, R.M., Fritz, H.M., 2018. High-throughput screen of drug repurposing library identifies inhibitors of *Sarcocystis neurona* growth. Int. J. Parasitol. Drugs Drug Resist. 8 (1), 137–144. https://doi.org/10.1016/j.ijpddr.2018.02.002.

Bowes, J., Brown, A.J., Hamon, J., Jarolimek, W., Sridhar, A., Waldron, G., Whitebread, S., 2012. Reducing safety-related drug attrition: The use of *in vitro* pharmacological profiling. Nat. Rev. Drug Discov. 11, 909–922. https://doi.org/10.1038/nrd3845.

Boxell, A., Hijjawi, N., Monis, P., Ryan, U., 2008. Comparison of various staining methods for the detection of *Cryptosporidium* in cell-free culture. Exp. Parasitol. 120, 67–72. https://doi.org/10.1016/j.exppara.2008.04.023.

Brosnahan, A.J., Brown, D.R., 2012. Porcine IPEC-J2 intestinal epithelial cells in microbiological investigations. Vet Microbiol. 156 (3–4), 229–237. https://doi.org/10.1016/j.vetmic.2011.10.017.

Bussière, F.I., Niepceron, A., Sausset, A., Esnault, E., Silvestre, A., Walker, R.A., Smith, N.C., Quéré, P., Laurent, F., 2018. Establishment of an *in vitro* chicken epithelial cell line model to investigate *Eimeria tenella* gamete development. Parasit. Vectors 11, 1–8. https://doi.org/10.1186/s13071-018-2622-1.

Chang, L. yun, Di, K. qian, Xu, J., Chen, Y. fan, Xi, J. zhong, Wang, D.H., Hao, E. ying, Xu, L. jun, Chen, H., Zhou, R. yan, 2021. Effect of natural garlic essential oil on chickens with artificially infected *Eimeria tenella*. Vet. Parasitol. 300, 109614. https://doi.org/10.1016/j.vetpar.2021.109614.

Chen, H., Guo, Y., Qiu, Y., et al., 2019. Efficient genome engineering of *Toxoplasma gondii* using the TALEN technique. Parasites Vectors 12, 112. https://doi.org/10.1186/s13071-019-3378-y.

Choi, R., Hulverson, M.A., Huang, W., Vidadala, R.S.R., Whitman, G.R., Barrett, L.K., Schaefer, D.A., Betzer, D.P., Riggs, M.W., Doggett, J.S., Hemphill, A., Ortega-Mora, L.M., McCloskey, M.C., Arnold, S.L.M., Hackman, R.C., Marsh, K.C., Lynch, J.J., Freiberg, G.M., Leroy, B.E., Kempf, D.J., Choy, R.K.M., de Hostos, E.L., Maly, D.J., Fan, E., Ojo, K.K., Van Voorhis, W.C., 2020. Bumped Kinase Inhibitors as therapy for apicomplexan parasitic diseases: lessons learned. Int. J. Parasitol. 50, 413–422. https://doi.org/10.1016/j.ijpara.2020.01.006.

Christiansen, C., Maus, D., Hoppenz, E., Murillo-León, M., Hoffmann, T., Scholz, J., Melerowicz, F., Steinfeldt, T., Seeber, F., Blume, M., 2022. *In vitro* maturation of *Toxoplasma gondii* bradyzoites in human myotubes and their metabolomic characterization. Nat. Commun. 13, 1–15. https://doi.org/10.1038/s41467-022-28730-w.

Cole, R.A., Lindsay, D.S., Blagburn, B.L., Dubey, J.P. 1990. *Neospora caninum* (Protozoa:Apicomplexa): Comparison of *in vitro* development of NC-1 and NC-3 isolates in three cell lines and three primary cell cultures. Proceedings of

Southeastern Society of Parasitologists, Boone,North Carolina, April 18-20. Abstract No. 34.
Cook, M.K., Jacobs, L., 1958. Cultivation of *Toxoplasma gondii* in tissue cultures of various derivations. J. Parasitol. 44, 172.
Cornelissen, A.W.C.A., Overdulve, J.P., 1985. Sex determination and sex differentiation in coccidia: Gametogony and oocyst production after monoclonal infection of cats with free-living and intermediate host stages of *Isospora (Toxoplasma) gondii*. Parasitology 90, 35–44. https://doi.org/10.1017/S003118200004899X.
Cruz-Bustos, T., Feix, A.S., Ruttkowski, B., Joachim, A., 2021. Sexual development in non-human parasitic Apicomplexa: Just biology or targets for control? Animals 11, 1–18. https://doi.org/10.3390/ani11102891.
Cruz-Bustos, T., Feix, A.S., Lyrakis, M., Dolezal, M., Ruttkowski, B., Joachim, A., 2022. The transcriptome from asexual to sexual *in vitro* development of *Cystoisospora suis* (Apicomplexa : Coccidia). Sci. Rep. 1–17. https://doi.org/10.1038/s41598-022-09714-8.
Current, W.L., Long, P.L., 1983. Development of human and calf *Cryptosporidium* in chicken embryos. J. Infect. Dis. 148 (6), 1108–1113. https://doi.org/10.1093/infdis/148.6.1108.
Current, W., Haynes, T., 1984. Complete development of *Cryptosporidium* in cell culture. Science 224 (4649), 603–605. https://doi.org/10.1126/science.6710159.
Danielson, J.J., Perez, N., Romano, J.D., Coppens, I., 2018. Modelling *Toxoplasma gondii* infection in a 3D cell culture system *in vitro:* Comparison with infection in 2D cell monolayers. PLoS One 13, 1–13. https://doi.org/10.1371/journal.pone.0208558.
Daugschies, A., Bangoura, B., Lendner, M., 2013. Inactivation of exogenous endoparasite stages by chemical disinfectants: Current state and perspectives. Parasitol. Res. 112, 917–932. https://doi.org/10.1007/s00436-013-3324-4.
Daugschies, A., Böse, R., Marx, J., Teich, K., Friedhoff, K.T., 2002. Development and application of a standardized assay for chemical disinfection of coccidia oocysts. Vet. Parasitol. 103, 299–308. https://doi.org/10.1016/S0304-4017(01)00581-7.
DeCicco RePass, M., Chen, Y., Lin, Y., Zhou, W., Kaplan, D.L., Warda, H.D., 2017. Infection of *Cryptosporidium parvum*. Fungal Parasit. Infect. 85, 1–11.
Del Cacho, E., Gallego, M., Francesch, M., Quílez, J., Sánchez-Acedo, C., 2010. Effect of artemisinin on oocyst wall formation and sporulation during *Eimeria tenella* infection. Parasitol. Int. 59, 506–511. https://doi.org/10.1016/j.parint.2010.04.001.
Delling, C., Lendner, M., Müller, U., Daugschies, A., 2017. Improvement of *in vitro* evaluation of chemical disinfectants for efficacy on *Cryptosporidium parvum* oocysts. Vet. Parasitol. 245, 5–13.
Diamond, L.S., 1983. Lumen dwelling protozoa: Entamoeba, trichomonads, and Giardia. In: Jensen, J.B. (Ed.), *In vitro* cultivation of protozoan parasites. CRC Press, Boca Raton, FL, pp. 67–109.
Di Genova, B.M., Wilson, S.K., Dubey, J.P., Knoll, L.J., 2019. Intestinal delta-6-desaturase activity determines host range for *Toxoplasma* sexual reproduction. PLoS Biol. 17, 1–19. https://doi.org/10.1371/journal.pbio.3000364.
Díaz, L., Zambrano, E., Flores, M.E., Contreras, M., Crispín, J.C., Alemán, G., Bravo, C., Armenta, A., Valdés, V.J., Tovar, A., Gamba, G., Barrios-Payán, J., Bobadilla, N.A., 2020. Ethical considerations in animal research: The Principle of 3R's. Rev. Invest. Clin. 73, 199–209. https://doi.org/10.24875/RIC.20000380.
Dkhil, M.A., Abdel-Baki, A.S., Wunderlich, F., Sies, H., Al-Quraishy, S., 2011. Anticoccidial and antiinflammatory activity of garlic in murine *Eimeria papillata* infections. Vet. Parasitol. 175, 66–72. https://doi.org/10.1016/j.vetpar.2010.09.009.
Doran, D.J., Augustine, P.C., 1978. *Eimeria tenella*: vitamin require-ments for development in primary cultures of chicken kidney cells. J. Protozool. 25, 544–546.
Dragan, L., Titilincu, A., Dan, I., Dunca, I., Dragan, M., Mircean, V., 2010. Effects of *Artemisia annua* and *Pimpinella anisum* on *Eimeria tenella* (Phylum Apicomplexa) low infection in chickens. Rev. Sci. Parasitol. 11, 77–82.
Dubey, J.P., 1998. Advances in the life cycle of *Toxoplasma gondii*. Int. J. Parasitol. 28, 1019–1024.
Dubey, J.P., Lindsay, D.S., Jenkins, M.C., Bauer, C., 2020. Biology of intestinal coccidia. In: Dubey, J.P. (Ed.), Coccidiosis in livestock, poultry, companion animals, and humans. CRC Press, Boca Raton, pp. 1–36.
Ellison, S.P., Greiner, E., Dame, J.B., 2001. *In vitro* culture and synchronous release of *Sarcocystis neurona* merozoites from host cells. Vet. Parasitol. 95, 251–261. https://doi.org/10.1016/S0304-4017(00)00391-5.
Evans, R., Chatterton, J.M.W., Ashburn, D., Joss, A.W.L., Ho-Yen, D.O., 1999. Cell-culture system for continuous production of *Toxoplasma gondii* tachyzoites. Eur. J. Clin. Microbiol. Infect. Dis. 18, 879–884. https://doi.org/10.1007/s100960050423.
Fayer, R., Mahrt, J.L., 1972. Development of *Isospora canis* (Protozoa; Sporozoa) in cell culture. Z. Parasitenkünd. 38, 313–318. https://doi.org/10.1007/BF0032927816.
Fayer, R., Thompson, D.E., 1974. *Isospora felis*: development in cultured cells with some cytological observations. J. Parasitol. 60, 160–168. https://doi.org/10.2307/3278693.
Fayer, R., Thompson, D.E., 1975. Cytochemical and cytological observations on *Sarcocystis* sp. propagated in cell culture. J. Parasitol. 61, 466–475.
Feix, A.S., Cruz-Bustos, T., Ruttkowski, B., Joachim, A., 2020. Characterization of *Cystoisospora suis* sexual stages *in vitro*. Parasit. Vectors 13, 143. https://doi.org/10.1186/s13071-020-04014-4.
Feix, A.S., Cruz-Bustos, T., Ruttkowski, B., Mötz, M., Rümenapf, T., Joachim, A., 2021. Progression of asexual to sexual stages of *Cystoisospora suis* in a host cell-free environment as a model for Coccidia. Parasitology 148, 1475–1481. https://doi.org/10.1017/S0031182021001074.
Felici, M., Tugnoli, B., Piva, A., Grilli, E., 2021. *In vitro* assessment of anticoccidials: methods and molecules. Animals 11, 1–19. https://doi.org/10.3390/ani11071962.
Fenwick, N., Griffin, G., Gauthier, C., 2009. The welfare of animals used in science: How the "Three Rs" ethic guides improvements. Can. Vet. J. 50, 523–530.
Fontana, F., Figueiredo, P., Martins, J.P., Santos, H.A., 2021. Requirements for animal experiments: problems and challenges. Small 17, 1–8. https://doi.org/10.1002/smll.202004182.
Frenkel, J.K., Smith, D.D., 2003. Determination of the genera of cyst-forming Coccidia. Parasitol. Res. 91, 384–389. https://doi.org/10.1007/s00436-003-0969-4.
Gaboriaud, P., Sadrin, G., Guitton, E., Fort, G., Niepceron, A., Lallier, N., Rossignol, C., Larcher, T., Sausset, A., Guabiraba, R., Silvestre, A., Lacroix-Lamandé, S., Schouler, C., Laurent, F., Bussière, F.I., 2021. The absence of gut microbiota alters the development of the apicomplexan parasite *Eimeria tenella*. Front. Cell. Infect. Microbiol. 10, 1–9. https://doi.org/10.3389/fcimb.2020.632556.
García, Y., Díaz-Castro, J., 2013. Advantages and disadvantages of the animal models *in vitro* studies in iron metabolism: A review. Animal 7, 1651–1658. https://doi.org/10.1017/S1751731113001134.
Gey, G., Coffman, W.D., Kubicek, M., 1953. Tissue Culture Studies of the Proliferative Capacity of Cervical Carcinoma and Normal Epithelium. Cancer Res. 12 (1952), 264–265.
Girouard, D., Gallant, J., Akiyoshi, D.E., Nunnari, J., Tzipori, S., 2006. Failure to propagate *Cryptosporidium* spp. in cell-free culture. J. Parasitol. 92, 399–400. https://doi.org/10.1645/GE-661R.1.
Gold, D.A., Kaplan, A.D., Lis, A., Bett, G.C.L., Rosowski, E.E., Cirelli, K.M., Bougdour, A., Sidik, S.M., Beck, J.R., Lourido, S., Egea, P.F., Bradley, P.J., Hakimi, M.A., Rasmusson, R.L., Saeij, J.P.J., 2015. The *Toxoplasma* dense granule proteins GRA17 and GRA23 mediate the movement of small molecules between the host and the parasitophorous vacuole. Cell Host Microbe 17, 642–652. https://doi.org/10.1016/j.chom.2015.04.003.
Gondim, L.F.P., Meyer, J., Peters, M., Rezende-Gondim, M.M., Vrhovec, M.G., Pantchev, N., Bauer, C., Conraths, F.J., Schares, G., 2015. *In vitro* cultivation of *Hammondia heydorni*: Generation of tachyzoites, stage conversion into bradyzoites, and evaluation of serologic cross-reaction with *Neospora caninum*. Vet. Parasitol. 210, 131–140. https://doi.org/10.1016/j.vetpar.2015.03.028.
Gorzalczany, S.B., Rodriguez Basso, A.G., 2021. Strategies to apply 3Rs in preclinical testing. Pharmacol. Res. Perspect. 9, 1–9. https://doi.org/10.1002/prp2.863.
Graham, M.L., Prescott, M.J., 2015. The multifactorial role of the 3Rs in shifting the harm-benefit analysis in animal models of disease. Eur. J. Pharmacol. 759, 19–29. https://doi.org/10.1016/j.ejphar.2015.03.040.
Griffiths, J.K., Moore, R., Dooley, S., Keusch, G.T., Tzipori, S., 1994. *Cryptosporidium parvum* infection of CaCo-2 cell monolayers induces an apical monolayer defect, selectively increases transmonolayer permeability, and causes epithelial cell death. Infect. Immun. 62, 4506–4514. https://doi.org/10.1128/iai.62.10.4506-4514.1994.
Gubbels, M.J., Li, C., Striepen, B., 2002. High-throughput growth assay for *Toxoplasma gondii* using yellow fluorescent protein. Antimicrob Agents Chemother. 47 (1), 309–316. https://doi.org/10.1128/AAC.47.1.309-316.2003.
Gunasekera, S., Zahedi, A., O'dea, M., King, B., Monis, P., Thierry, B., Carr, J.M., Ryan, U., 2020. Organoids and bioengineered intestinal models: Potential solutions to the *Cryptosporidium* culturing dilemma. Microorganisms 8, 715. https://doi.org/10.3390/microorganisms8050715.
Guo, H., Gao, Y., N'da, D.D., Xuan, X., 2021. *In vitro* anti-*Toxoplasma gondii* efficacy of synthesised benzyltriazole derivatives. Onderstepoort J. Vet. Res. 88, 1–8. https://doi.org/10.4102/ojvr.v88i1.1898.
Halonen, S.K., 2017. Use of human neurons derived via cellular reprogramming methods to study host-parasite interactions of *Toxoplasma gondii* in neurons. Cells 6, 32. https://doi.org/10.3390/cells6040032.
Harleman, J.H., Meyer, R.C., 1984. Life cycle of *Isospora suis* in gnotobiotic and conventionalized piglets. Vet. Parasitol. 17, 27–39. https://doi.org/10.1016/0304-4017(84)90062-1.
Harleman, J.H., Meyer, R.C., 1983. *Isospora suis* infection in piglets. A review. Vet. Q. 5, 178–185. https://doi.org/10.1080/01652176.1983.9693894.
Hartung, T., 2018. Perspectives on *in vitro* to *in vivo* extrapolations. Applied *In Vitro* Toxicology 4, 305–316.
Hemphill, A., Gottstein, B., Kaufmann, H., 1996. Adhesion and invasion of bovine endothelial cells by *Neospora caninum*. Parasitology 112, 183–197. https://doi.org/10.1017/s0031182000084754.
Heo, I., Dutta, D., Schaefer, D.A., Iakobachvili, N., Artegiani, B., Sachs, N., Boonekamp, K.E., Bowden, G., Hendrickx, A.P.A., Willems, R.J.L., Peters, P.J., Riggs, M.W., O'Connor, R., Clevers, H., 2018. Modelling *Cryptosporidium* infection in human small intestinal and lung organoids. Nat. Microbiol. 3, 814–823. https://doi.org/10.1038/s41564-018-0177-8.
Hermosilla, C., Barbisch, B., Heise, A., Kowalik, S., Zahner, H., 2002. Development of *Eimeria boris in vitro*: Suitability of several bovine, human and porcine endothelial cell lines, bovine fetal gastrointestinal, Madin-Darby bovine kidney (MDBK) and African green monkey kidney (VERO) cells. Parasitol. Res. 88, 301–307. https://doi.org/10.1007/s00436-001-0531-1.
Hermosilla, C., Zahner, H., Taubert, A., 2006. *Eimeria bovis* modulates adhesion molecule gene transcription in and PMN adhesion to infected bovine endothelial cells. Int. J. Parasitol. 36, 423–431. https://doi.org/10.1016/j.ijpara.2006.01.001.

Hijjawi, N., 2010. *Cryptosporidium*: New developments in cell culture. Exp. Parasitol. 124, 54–60. https://doi.org/10.1016/j.exppara.2009.05.015.
Hijjawi, N., 2003. *In vitro* cultivation and development of *Cryptosporidium* in cell culture. Cryptosporidium From Mol. to Dis. 233–253. https://doi.org/10.1016/B978-044451351-9/50034-3.
Hijjawi, N., Estcourt, A., Yang, R., Monis, P., Ryan, U., 2010. Complete development and multiplication of *Cryptosporidium hominis* in cell-free culture. Vet. Parasitol. 169, 29–36. https://doi.org/10.1016/j.vetpar.2009.12.021.
Hijjawi, N.S., Meloni, B.P., Ryan, U.M., Olson, M.E., Thompson, R.C.A., 2002. Successful *in vitro* cultivation of *Cryptosporidium andersoni*: Evidence for the existence of novel extracellular stages in the life cycle and implications for the classification of Cryptosporidium. Int. J. Parasitol. 32, 1719–1726. https://doi.org/10.1016/S0020-7519(02)00199-6.
Hirai, M., Mori, T., 2010. Fertilization is a novel attacking site for the transmission blocking of malaria parasites. Acta Trop. 114, 157–161. https://doi.org/10.1016/j.actatropica.2009.08.005.
Hofmann, J., Raether, W., 1990. Improved techniques for the *in vitro* cultivation of *Eimeria tenella* in primary chick kidney cells. Parasitol. Res. 76, 479–486. https://doi.org/10.1007/bf00931053.
Hoffmann, S., 2015. LLNA variability: An essential ingredient for a comprehensive assessment of non-animal skin sensitization test methods and strategies. ALTEX 32, 379–383.
Holthaus, D., Delgado-Betancourt, E., Aebischer, T., Seeber, F., Klotz, C., 2021. Harmonization of protocols for multi-species organoid platforms to study the intestinal biology of *Toxoplasma gondii* and other protozoan infections. Front. Cell. Infect. Microbiol. 10, 1–18. https://doi.org/10.3389/fcimb.2020.610368.
Howe, D.K., 2001. Initiation of a *Sarcocystis neurona* expressed sequence tag (EST) sequencing project: A preliminary report. Vet. Parasitol. 95, 233–239. https://doi.org/10.1016/S0304-4017(00)00418-0.
Howe, D.K., Gaji, R.Y., Mroz-Barrett, M., Gubbels, M.J., Striepen, B., Stamper, S., 2005. *Sarcocystis neurona* merozoites express a family of immunogenic surface antigens that are orthologues of the *Toxoplasma gondii* surface antigens (SAGs) and SAG-related sequences. Infect. Immun. 73, 1023–1033. https://doi.org/10.1128/IAI.73.2.1023-1033.2005.
Hu, X., Binns, D., Reese, M.L., 2017. The coccidian parasites Toxoplasma and Neospora dysregulate mammalian lipid droplet biogenesis. J Biol Chem. 292 (26), 11009–11020. https://doi.org/10.1074/jbc.M116.768176.
Jang, S.I., Lillehoj, H.S., Lee, S.H., Lee, K.W., Park, M.S., Cha, S.-R., Lillehoj, E.P., Subramanian, B.M., Sriraman, R., Srinivasan, V.A., 2010. *Eimeria maxima* recombinant Gam82 gametocyte antigen vaccine protects against coccidiosis and augments humoral and cell-mediated immunity. Vaccine 28, 2980–2985. https://doi.org/10.1016/J.VACCINE.2010.02.011.
Jannasch, M., Groeber, F., Brattig, N.W., Unger, C., Walles, H., Hansmann, J., 2015. Development and application of three-dimensional skin equivalents for the investigation of percutaneous worm invasion. Exp. Parasitol. 150, 22–30. https://doi.org/10.1016/j.exppara.2015.01.005.
Jensen, J.B., 1983. In Vitro Cultivation of Protozoan Parasites. CRC Press, Boca Raton, Florida, pp.1–297.
Jensen, J.B., 2018. *In vitro* cultivation of protozoan parasites (1st ed.). CRC Press. https://doi.org/10.1201/9781351073455.
Joachim, A., Eckert, E., Petry, F., Bialek, R., Daugschies, A., 2003. Comparison of viability assays for *Cryptosporidium parvum* oocysts after disinfection. Vet Parasitol. 111, 47–57. https://doi.org/10.1016/s0304-4017(02)00329-1.
Jochems, C.E.A., Van der Valk, J.B.F., Stafleu, F.R., Baumans, V., 2002. The use of fetal bovine serum: Ethical or scientific problem? ATLA Altern. to Lab. Anim. 30, 219–227. https://doi.org/10.1177/026119290203000208.
Khan, A., Grigg, M.E., 2017. *Toxoplasma gondii*: Laboratory Maintenance and Growth. Curr Protoc Microbiol. 44. https://doi.org/10.1002/cpmc.26. 20C .1.1–20C.1.17.
Karanis, P., 2018. The truth about *in vitro* culture of *Cryptosporidium* species. Parasitology 145, 855–864. https://doi.org/10.1017/S0031182017001937.
Karanis, P., Aldeyarbi, H.M., 2011. Evolution of *Cryptosporidium in vitro* culture. Int. J. Parasitol. 41, 1231–1242. https://doi.org/10.1016/j.ijpara.2011.08.001.
Kasendra, M., Tovaglieri, A., Sontheimer-Phelps, A., Jalili-Firoozinezhad, S., Bein, A., Chalkiadaki, A., Scholl, W., Zhang, C., Rickner, H., Richmond, C.A., Li, H., Breault, D.T., Ingber, D.E., 2018. Development of a primary human small Intestine-on-a-Chip using biopsy-derived organoids. Sci. Rep. 8, 1–14. https://doi.org/10.1038/s41598-018-21201-7.
Khalil, A.M., Yasuda, M., Farid, A.S., Desouky, M.I., Mohi-Eldin, M.M., Haridy, M., Horii, Y., 2015. Immunomodulatory and antiparasitic effects of garlic extract on *Eimeria vermiformis*-infected mice. Parasitol. Res. 114, 2735–2742. https://doi.org/10.1007/s00436-015-4480-5.
Kirschner, K.M., 2021. Reduce, replace, refine—Animal experiments. Acta Physiol. 233. https://doi.org/10.1111/apha.13726.
Kogut, Michael, 1990. Host Specificity of the Coccidia, in: Long, P.L. (Ed.). (1990). Coccidiosis of Man and Domestic Animals (1st ed.). CRC Press. p. 43–62. https://doi.org/10.1201/9781351070744.
Kowalik, S., Zahner, H., 1999. *Eimeria separata*: Method for the excystation of sporozoites. Parasitol. Res. 85, 496–499. https://doi.org/10.1007/s004360050584.
Kurth, M., Entzeroth, R., 2009. Reporter gene expression in cell culture stages and oocysts of *Eimeria nieschulzi* (Coccidia, Apicomplexa). Parasitol. Res. 104, 303–310. https://doi.org/10.1007/s00436-008-1192-0.
Laxer, M.A., Healey, M.C., Youssef, N.N., 1987. Production of monoclonal antibodies specific for *Eimeria tenella* microgametocytes. Parasitology 73, 611–616.
Leist, M., Hasiwa, N., Daneshian, M., Hartung, T., 2012. Validation and quality control of replacement alternatives - Current status and future challenges. Toxicol. Res. 1, 8–22. https://doi.org/10.1039/c2tx20011b.
Levaditi, C., Sanchis-Bayarri, V., Lepine, P., Schoen, R., 1929. Etude sur l'encephalomyelite provoquee par le *Toxoplasma caniculi*. Ann. Inst. Pasteur, Paris 43, 673.
Li, W., Zhang, N., Liang, X., Li, J., Gong, P., Yu, X., Ma, G., Ryan, U.M., Zhang, X., 2009. Transient transfection of *Cryptosporidium parvum* using green fluorescent protein (GFP) as a marker. Mol Biochem Parasitol. 168 (2), 143–148. https://doi.org/10.1016/j.molbiopara.2009.07.003.
Lillehoj, H.S., Trout, J.M., 1993. Coccidia: A review of recent advances on immunity and vaccine development. Avian Pathol. 22, 3–31. https://doi.org/10.1080/03079459308418897.
Lindsay, D.S., 2019. *Cystoisospora* species insights from development *in vitro*. Front. Vet. Sci. 5. https://doi.org/10.2307/3279560.
Lindsay, D.S., Current, W.L., 1984. Complete development of *Isospora suis* of swine in chicken embryos. J. Protozool. 31, 152–155.
Lindsay, D.S., Dubey, J.P., 1989. *In vitro* development of *Neospora caninum* (Protozoa: Apicomplexa) from dogs. J. Parasitol. 75, 163–165.
Lindsay, D.S., Dubey, J.P., Blagburn, B.L., Toivio-Kinnucan, M., 1991. Examination of tissue cyst formation by *Toxoplasma gondii* in cell cultures. J. Parasitol. 77, 126–132.
Long, P.L., 1990. Coccidiosis of Man and Domestic Animals. CRC Press. https://doi.org/10.1201/9781351070744.
López-Yglesias, A.H., Burger, E., Camanzo, E., Martin, A.T., Araujo, A.M., Kwok, S.F., Yarovinsky, F., 2021. T-bet-dependent ILC1- And NK cell-derived IFN-γ mediates cDC1-dependent host resistance against *Toxoplasma gondii*. PLoS Pathog. 17, 1–17. https://doi.org/10.1371/journal.ppat.1008299.
Lu, C., Yan, Y., Jian, F., Ning, C., 2021. Coccidia-Microbiota Interactions and their effects on the host. Front. Cell. Infect. Microbiol. 11, 1–12. https://doi.org/10.3389/fcimb.2021.751481.
Luu, L., Johnston, L.J., Derricott, H., Armstrong, S.D., Randle, N., Hartley, C.S., Duckworth, C.A., Campbell, B.J., Wastling, J.M., Coombes, J.L., 2019. An open-format enteroid culture system for interrogation of interactions between *Toxoplasma gondii* and the intestinal epithelium. Front. Cell. Infect. Microbiol. 9, 1–19. https://doi.org/10.3389/fcimb.2019.00300.
Lüder, C.G.K., Rahman, T., 2017. Impact of the host on Toxoplasma stage differentiation. Microb Cell. 4 (7), 203–211. https://doi.org/10.15698/mic2017.07.579.
Lv, Q., Li, J., Gong, P., Xing, S., Zhang, X., 2010. *Neospora caninum*: *in vitro* culture of tachyzoites in MCF-7 human breast carcinoma cells. Exp. Parasitol. 126, 536–539. https://doi.org/10.1016/j.exppara.2010.06.006.
Macdonald, S.E., Nolan, M.J., Harman, K., Boulton, K., Hume, D.A., Tomley, F.M., Stabler, R.A., Blake, D.P., 2017. Effects of *Eimeria tenella* infection on chicken caecal microbiome diversity, exploring variation associated with severity of pathology. PLoS One 12, 1–17. https://doi.org/10.1371/journal.pone.0184890.
MacGowen, J.P., 1923. Some points relating to the morphology and the development of *Sacrocystis tenella*. Parasitology 15, 139.
Mácová, A., Hoblíková, A., Hypša, V., Stanko, M., Martinů, J., Kvičerová, J., 2018. Mysteries of host switching: Diversification and host specificity in rodent-coccidia associations. Mol. Phylogenet. Evol. 127, 179–189. https://doi.org/10.1016/j.ympev.2018.05.009.
Madden, P.A., Vetterling, J.M., 1977. Scanning electron microscopy of *Eimeria tenella* microgametogenesis and fertilization. J. Parasitol. 63, 607–610. https://doi.org/10.2307/3279559.
Mai, K., Sharman, P.A., Walker, R.A., Katrib, M., de Souza, D., McConville, M.J., Wallach, M.G., Belli, S.I., Ferguson, D.J.P., Smith, N.C., 2009. Oocyst wall formation and composition in coccidian parasites. Mem. Inst. Oswaldo Cruz 104, 281–289. https://doi.org/10.1590/S0074-02762009000200022.
Manafi, N., Shokri, F., Achberger, K., Hirayama, M., Mohammadi, M.H., Noorizadeh, F., Hong, J., Liebau, S., Tsuji, T., Quinn, P.M.J., Mashaghi, A., 2021. Organoids and organ chips in ophthalmology. Ocul. Surf. 19, 1–15. https://doi.org/10.1016/j.jtos.2020.11.004.
Márquez-Nogueras, K.M., Hortua Triana, M.A., Chasen, N.M., Kuo, I.Y., Moreno, S.N.J., 2021. Calcium signaling through a transient receptor channel is important for *Toxoplasma gondii* growth. eLife 10, 1–27. https://doi.org/10.7554/eLife.63417.
Marsh, A.E., Barr, B.C., Tell, L., Koski, M., Greiner, E., Dame, J., Conrad, P.A., 1997. *In vitro* cultivation and experimental inoculation of *Sarcocystis falcatula* and *Sarcocystis neurona* merozoites into budgerigars (*Melopsittacus undulatus*). J. Parasitol. 83, 1189–1192. https://doi.org/10.2307/3284386.
Marugan-Hernandez, V., Jeremiah, G., Aguiar-Martins, K., Burrell, A., Vaughan, S., Xia, D., Randle, N., Tomley, F., 2020. The growth of *Eimeria tenella*: Characterization and application of quantitative methods to assess sporozoite invasion and endogenous development in cell culture. Front. Cell. Infect. Microbiol. 10, 1–13. https://doi.org/10.3389/fcimb.2020.579833.
Marugan-Hernandez, V., Sanchez-Arsuaga, G., Vaughan, S., Burrell, A., Tomley, F.M., 2021. Do All Coccidia Follow the Same Trafficking Rules? Life 11 (9), 909. https://doi.org/10.3390/life11090909.
Matta, S.K., Rinkenberger, N., Dunay, I.R., Sibley, L.D., 2021. *Toxoplasma gondii* infection and its implications within the central nervous system. Nat. Rev. Microbiol. 19, 467–480. https://doi.org/10.1038/s41579-021-00518-7.
Meissner, M., Schlüter, D., Soldati, D., 2002. Role of *Toxoplasma gondii* myosin a in powering parasite gliding and host cell invasion. Science 298, 837–840. https://doi.org/10.1126/science.1074553.
Miller, C., Josse, L., Brown, I., Blakeman, B., Povey, J., Yiangou, L., Price, M., Cinatl, J., Xue, W.-F., Michaelis, M., Tsaousis, A., 2018. A cell culture platform for Cryptosporidium that enables long-term cultivation and new tools for the systematic investigation of its biology. Int. J. Parasitol. 48 (3–4), 197–201. https://doi.org/10.1016/j.ijpara.2017.10.001.

Mital, J., Ward, G., 2008. Current and emerging approaches to studying invasion in apicomplexan parasites. Subcell. Biochem. 47, 1–32. https://doi.org/10.1007/978-0-387-78267-6_1.

Montazeri, M., Sharif, M., Sarvi, S., Mehrzadi, S., Ahmadpour, E., Daryani, A., 2017. A systematic review of *in vitro* and *in vivo* activities of anti-*Toxoplasma* drugs and compounds (2006–2016). Front. Microbiol. 8, 25. https://doi.org/10.3389/fmicb.2017.00025.

Morrison, D.A., 2009. Evolution of the Apicomplexa: where are we now? Trends Parasitol. 25, 375–382. https://doi.org/10.1016/j.pt.2009.05.010.

Munderloh, U.G., Kurtti, T.J., 1985. Malarial parasites complete sporogony in axenic mosquitoes. Experientia 41, 1205–1207. https://doi.org/10.1007/BF01951731.

Müller, J., Hemphill, A., 2013. *In vitro* culture systems for the study of apicomplexan parasites in farm animals. Int. J. Parasitol. 43, 115–124. https://doi.org/10.1016/j.ijpara.2012.08.004.

Naguleswaran, A., Elias, E.V., McClintick, J., Edenberg, H.J., Sullivan, W.J., 2010. *Toxoplasma gondii* lysine acetyltransferase GCN5-a functions in the cellular response to alkaline stress and expression of cyst genes. PLoS Pathog. 6, 1–10. https://doi.org/10.1371/journal.ppat.1001232.

Nash, T.J., Morris, K.M., Mabbott, N.A., Vervelde, L., 2021. Inside-out chicken enteroids with leukocyte component as a model to study host–pathogen interactions. Commun. Biol. 4. https://doi.org/10.1038/s42003-021-01901-z.

Nesterenko, M.V., Woods, K.M., Upton, S.J., 1997. Effects of manganese salts on the AIDS-related pathogen, *Cryptosporidium parvum in vitro* and *in vivo*. Biol. Trace Elem. Res. 56, 243–253. https://doi.org/10.1007/BF02785297.

Nikolaev, M., Mitrofanova, O., Broguiere, N., Geraldo, S., Dutta, D., Tabata, Y., Elci, B., Brandenberg, N., Kolotuev, I., Gjorevski, N., Clevers, H., Lutolf, M.P., 2020. Homeostatic mini-intestines through scaffold-guided organoid morphogenesis. Nature 585, 574–578. https://doi.org/10.1038/s41586-020-2724-8.

Ogedengbe, J.D., Hanner, R.H., Barta, J.R., 2011. DNA barcoding identifies Eimeria species and contributes to the phylogenetics of coccidian parasites (Eimeriorina, Apicomplexa, Alveolata). Int. J. Parasitol. 41, 843–850. https://doi.org/10.1016/j.ijpara.2011.03.007.

Ogedengbe, M.E., El-Sherry, S., Ogedengbe, J.D., Chapman, H.D., Barta, J.R., 2018. Phylogenies based on combined mitochondrial and nuclear sequences conflict with morphologically defined genera in the eimeriid coccidia (Apicomplexa). Int. J. Parasitol. 48, 59–69. https://doi.org/10.1016/j.ijpara.2017.07.008.

Ojo, K.K., Dangoudoubiyam, S., Verma, S.K., Scheele, S., DeRocher, A.E., Yeargan, M., Choi, R., Smith, T.R., Rivas, K.L., Barrett, M.A., Fan, L.K., Maly, D.J., Parsons, M., Dubey, J.P., Howe, D.K., Van Voorhis, W.C., 2016. Selective inhibition of Sarcocystis neurona calcium-dependent protein kinase 1 for equine protozoal myeloencephalitis therapy. Int J Parasitol. 46 (13–14), 871–880. https://doi.org/10.1016/j.ijpara.2016.08.003.

Omata, Y., Kano, R., Masukata, Y., Kobayashi, Y., Igarashi, M., Maeda, R., Saito, A., 2005. Development of *Neospora caninum* cultured with human serum *in vitro* and *in vivo*. J. Parasitol. 91, 222–225. https://doi.org/10.1645/GE-3364-RN.

Orso, C., Stefanello, T.B., Franceschi, C.H., Mann, M.B., Varela, A.P.M., Castro, I.M.S., Frazzon, J., Frazzon, A.P.G., Andretta, I., Ribeiro, A.M.L., 2021. Changes in the ceca microbiota of broilers vaccinated for coccidiosis or supplemented with salinomycin. Poult. Sci. 100,. https://doi.org/10.1016/j.psj.2020.12.066 100969.

Palmieri, N., Shrestha, A., Ruttkowski, B., Beck, T., Vogl, C., Tomley, F., Blake, D.P., Joachim, A., 2017. The genome of the protozoan parasite *Cystoisospora suis* and a reverse vaccinology approach to identify vaccine candidates. Int. J. Parasitol. 47, 189–202. https://doi.org/10.1016/j.ijpara.2016.11.007.

Pance, A., 2021. The stem cell revolution revealing protozoan parasites' secrets and paving the way towards vaccine development. Vaccines 9, 105. https://doi.org/10.3390/vaccines9020105.

Peng, Z., Zhou, L., Wong, J.K.W., Chan, Y.K., 2020. Eye-on-a-chip (EOC) models and their role in the future of ophthalmic drug discovery. Expert Rev. Ophthalmol. 15, 259–261. https://doi.org/10.1080/17469899.2020.1788388.

Periz, J., Whitelaw, J., Harding, C., Gras, S., Del Rosario Minina, M.I., Latorre-Barragan, F., Lemgruber, L., Reimer, M.A., Insall, R., Heaslip, A., Meissner, M., 2017. Toxoplasma gondii F-actin forms an extensive filamentous network required for material exchange and parasite maturation. Elife 6,. https://doi.org/10.7554/eLife.24119 e24119.

Periz, J., Del Rosario, M., McStea, A., et al., 2019. A highly dynamic F-actin network regulates transport and recycling of micronemes in Toxoplasma gondii vacuoles. Nat Commun 10, 4183. https://doi.org/10.1038/s41467-019-12136-2.

Porter-Kelley, J.M., Dinglasan, R.R., Alam, U., Ndeta, G.A., Sacci, J.B., Azad, A.F., 2006. *Plasmodium yoelii*: Axenic development of the parasite mosquito stages. Exp. Parasitol. 112, 99–108. https://doi.org/10.1016/j.exppara.2005.09.011.

Quist, K.L., Taylor, R.L., Johnson, L.W., Strout, R.G., 1993. Comparative development of *Eimeria tenella* in primary chick kidney cell cultures derived from coccidia-resistant and -susceptible chickens. Poult. Sci. 72, 82–87. https://doi.org/10.3382/ps.0720082.

Radke, J.R., Behnke, M.S., Mackey, A.J., Radke, J.B., Roos, D.S., White, M.W., 2005. The transcriptome of *Toxoplasma gondii*. BMC Biol. 3, 1–18. https://doi.org/10.1186/1741-7007-3-26.

Rafiqi, S.I., Garg, R., Ram, H., Reena, K.K., Asari, M., Kumari, P., Kundave, V.R., Singh, M., Banerjee, P.S., 2019. Immunoprophylactic evaluation of recombinant gametocyte 22 antigen of *Eimeria tenella* in broiler chickens. Parasitol. Res. 118, 945–953. https://doi.org/10.1007/s00436-018-06198-2.

Ramakrishnan, C., Walker, R.A., Eichenberger, R.M., Hehl, A.B., Smith, N.C., 2017. The merozoite-specific protein, TgGRA11B, identified as a component of the Toxoplasma gondii parasitophorous vacuole in a tachyzoite expression model. Int J Parasitol. 47 (10–11), 597–600. https://doi.org/10.1016/j.ijpara.2017.04.001.

Ramakrishnan, C., Maier, S., Walker, R.A., Rehrauer, H., Joekel, D.E., Winiger, R.R., Basso, W.U., Grigg, M.E., Hehl, A.B., Deplazes, P., Smith, N.C., 2019. An experimental genetically attenuated live vaccine to prevent transmission of *Toxoplasma gondii* by cats. Sci. Rep. 9, 1474. https://doi.org/10.1038/s41598-018-37671-8.

Ramakrishnan, C., Smith, N.C., 2021. Recent achievements and doors opened for coccidian parasite research and development through transcriptomics of enteric sexual stages. Mol. Biochem. Parasitol. 243,. https://doi.org/10.1016/j.molbiopara.2021.111373 111373.

Ramanan, D., Bowcutt, R., Lee, S.C., Tang, M.S., Kurtz, Z.D., Ding, Y., Honda, K., Gause, W.C., Blaser, M.J., Bonneau, R.A., Lim, Y.A.L., Loke, P., Cadwell, K., 2016. Helminth infection promotes colonization resistance via type 2 immunity. Science 352, 608–612. https://doi.org/10.1126/science.aaf3229.

Ramírez-Flores, C.J., Knoll, L.J., 2021. Breakthroughs in microbiology made possible with organoids. PLoS Pathog. 17, 1–6. https://doi.org/10.1371/journal.ppat.1010080.

Ramírez-Flores, C.J., Tibabuzo Perdomo, A.M., Gallego-López, G.M., Knoll, L.J., 2022. Transcending dimensions in apicomplexan research: from two-dimensional to three-dimensional *in vitro* cultures. Microbiol. Mol. Biol. Rev. 86,. https://doi.org/10.1128/mmbr.00025-22 e0002522.

Rishi, H., Dardé, M.L., Bouteille, B., Leboutet, M.J., Pestre-Alexandre, M., 1995. *Hammondia hammondi* cysts in cell cultures. J. Parasitol. 81, 821–824 https://www.jstor.org{stable/3283990#metadata_info_tab_contents.

Rigat-Brugarolas, L.G., Elizalde-Torrent, A., Bernabeu, M., De Niz, M., Martin-Jaular, L., Fernandez-Becerra, C., Homs-Corbera, A., Samitier, J., Del Portillo, H.A., 2014. A functional microengineered model of the human splenon-on-a-chip. Lab Chip 14, 1715–1724. https://doi.org/10.1039/c3lc51449h.

Roach, H.I., Shearer, J.R., Archer, C., 1989. The choice of an experimental model. A guide for research workers. J. Bone Joint Surg. Br. 71, 549–553. https://doi.org/10.1302/0301-620x.71b4.2768295.

Robinson, R., 2011. Three Rs of Animal Testing for Regenerative Medicine Products. Sci. Transl. Med. 3, 112fs11. https://doi.org/10.1126/scitranslmed.3003394.

Roth, A., Maher, S.P., Conway, A.J., Ubalee, R., Chaumeau, V., Andolina, C., Kaba, S.A., Vantaux, A., Bakowski, M.A., Luque, R.T., Adapa, S.R., Singh, N., Barnes, S.J., Cooper, C.A., Rouillier, M., McNamara, C.W., Mikolajczak, S.A., Sather, N., Witkowski, B., Campo, B., Kappe, S.H.I., Lanar, D.E., Nosten, F., Davidson, S., Jiang, R.H.Y., Kyle, D.E., Adams, J.H., 2018. A comprehensive model for assessment of liver stage therapies targeting *Plasmodium vivax* and *Plasmodium falciparum*. Nat. Commun. 9, 1–16. https://doi.org/10.1038/s41467-018-04221-9.

Ruiz, A., Behrendt, J.H., Zahner, H., Hermosilla, C., Pérez, D., Matos, L., Muñoz, M. del C., Molina, J.M., Taubert, A., 2010. Development of *Eimeria ninakohlyakimovae in vitro* in primary and permanent cell lines. Vet. Parasitol. 173, 2–10. https://doi.org/10.1016/j.vetpar.2010.05.023.

Russell, W.M., Burch, R., 1959. Principle of Humane Experimental Technique. Methuen & Co., Ltd., London, UK.

Sağlam Metiner, P., Can, H., Ayyıldız Tamiş, D., Karakavuk, M., Kımız Geboloğlu, I., Gülçe İz, S., Atalay Şahar, E., Değirmenci Döşkaya, A., Gürüz, Y., Deliloğlu Gürhan, S.İ., Döşkaya, M., 2019. The use of *Toxoplasma gondii* tachyzoites produced in HeLa cells adhered to Cytodex 1 microcarriers as antigen in serological assays: an application of microcarrier technology. Cytotechnology 71, 91–105. https://doi.org/10.1007/s10616-018-0269-6.

Sakurai, H., Sakurai, H., Tegoshi, T., Takagi, T., Saito, A., Suzuki, N., Seitz, H.M., 1987. Long term *in vitro* suspension culture of *Toxoplasma* and HeLa cells. Zentralblatt fur Bakteriol. Mikrobiol. und Hyg. A 264, 468–477. https://doi.org/10.1016/S0176-6724(87)80070-6.

Sato, T., Vries, R.G., Snippert, H.J., Van De Wetering, M., Barker, N., Stange, D.E., Van Es, J.H., Abo, A., Kujala, P., Peters, P.J., Clevers, H., 2009. Single Lgr5 stem cells build crypt-villus structures *in vitro* without a mesenchymal niche. Nature 459, 262–265. https://doi.org/10.1038/nature07935.

Schaefer, D.A., Betzer, D.P., Smith, K.D., Millman, Z.G., Michalski, H.C., Menchaca, S. E., Zambriski, J.A., Ojo, K.K., Hulverson, M.A., Arnold, S.L.M., Rivas, K.L., Vidadala, R.S.R., Huang, W., Barrett, L.K., Maly, D.J., Fan, E., Van Voorhis, W.C., Riggs, M.W., 2016. Novel bumped kinase inhibitors are safe and effective therapeutics in the calf clinical model for cryptosporidiosis. J. Infect. Dis. 214, 1856–1864. https://doi.org/10.1093/infdis/jiw488.

Scott, J.W., 1943. Life history of Sarcosporidia, with particular reference to *Sarcocstis tenella*. Univ. Wyo. Agr. Exp. Sta. Bull. 259, 1.

Schlegel, M., Hülsmann, N., 2007. Protists - A textbook example for a paraphyletic taxon. Org. Divers. Evol. 7, 166–172. https://doi.org/10.1016/j.ode.2006.11.001.

Séverin, K., 2012. *In vitro* anticoccidial activity of *Thonningia sanguinea* extract on *Eimeria tenella* and *Eimeria necatrix* sporozoites cells. African J. Microbiol. Res. 6, 22–27. https://doi.org/10.5897/ajmr12.114.

Seo, H.H., Han, H.W., Lee, S.E., Hong, S.H., Cho, S.H., Kim, S.C., Koo, S.K., Kim, J.H., 2020. Modelling *Toxoplasma gondii* infection in human cerebral organoids. Emerg. Microbes Infect. 9, 1943–1954. https://doi.org/10.1080/22221751.2020.1812435.

Sharman, P.A., Smith, N.C., Wallach, M.G., Katrib, M., 2010. Chasing the golden egg: Vaccination against poultry coccidiosis. Parasite Immunol. 32, 590–598. https://doi.org/10.1111/j.1365-3024.2010.01209.x.

Shahiduzzaman, M., Dyachenko, V., Keidel, J., Schmäschke, R., Daugschies, A., 2010. Combination of cell culture and quantitative PCR (cc–qPCR) to assess disinfectants efficacy on *Cryptosporidium* oocysts under standardized conditions. Vet. Parasitol. 167 (1), 43–49.

Shrestha, A., Ojo, K.K., Koston, F., Ruttkowski, B., Vidadala, R.S.R., Dorr, C.S., Navaluna, E.D., Whitman, G.R., Barrett, K.F., Barrett, L.K., Hulverson, M.A., Choi,

R., Michaels, S.A., Maly, D.J., Hemphill, A., Van Voorhis, W.C., Joachim, A., 2009. Bumped kinase inhibitor 1369 is effective against *Cystoisospora suis in vivo* and *in vitro*. Int. J. Parasitol. Drugs Drug Resist. 10, 9–19. https://doi.org/10.1016/j.ijpddr.2019.03.004.

Sikorski, P.M., Commodaro, A.G., Grigg, M.E., 2021. A Protective and Pathogenic Role for Complement During Acute *Toxoplasma gondii* Infection. Front Cell Infect Microbiol. 11 (634610). https://doi.org/10.3389/fcimb.2021.634610.

Singh, S., Eldin, C., Kowalczewska, M., Raoult, D., 2013. Axenic culture of fastidious and intracellular bacteria. Trends Microbiol. 21 (2), 92–99. https://doi.org/10.1016/j.tim.2012.10.007.

Skariah, S., McIntyre, M.K., Mordue, D.G., 2010. *Toxoplasma gondii*: determinants of tachyzoite to bradyzoite conversion. Parasitol. Res. 107, 253–260. https://doi.org/10.1007/s00436-010-1899-6.

Smith, N.C., Sinden, R.E., Ramakrishnan, C., 2021. Editorial: get over the gut: apicomplexan parasite interaction, survival and stage progression in vertebrate and invertebrate digestive tracts. Front. Cell. Infect. Microbiol. 11,. https://doi.org/10.3389/fcimb.2021.680555 680555.

Smith, T.G., Walliker, D., Ranford-Cartwright, L.C., 2002. Sexual differentiation and sex determination in the Apicomplexa. Trends Parasitol. 18, 315–323. https://doi.org/10.1016/S1471-4922(02)02292-4.

Sneddon, L.U., Halsey, L.G., Bury, N.R., 2017. Considering aspects of the 3Rs principles within experimental animal biology. J. Exp. Biol. 220, 3007–3016. https://doi.org/10.1242/jeb.147058.

Sokol-Borrelli, S.L., Coombs, R.S., Boyle, J.P., 2020. A comparison of stage conversion in the coccidian apicomplexans *Toxoplasma gondii*, *Hammondia hammondi*, and *Neospora caninum*. Front. Cell. Infect. Microbiol. 10, 1–14. https://doi.org/10.3389/fcimb.2020.608283.

Sokol, S.L., Primack, A.S., Nair, S.C., Wong, Z.S., Tembo, M., Verma, S.K., Cerqueira-Cezar, C.K., Dubey, J.P., Boyle, J.P., 2018. Dissection of the *in vitro* developmental program of *Hammondia hammondi* reveals a link between stress sensitivity and life cycle flexibility in *Toxoplasma gondii*. Elife 7, 1–29. https://doi.org/10.7554/eLife.36491.

Soldati, D., Meissner, M., 2004. *Toxoplasma* as a novel system for motility. Curr. Opin. Cell Biol. 16, 32–40. https://doi.org/10.1016/j.ceb.2003.11.013.

Speer, C., Whitmire, W.M., Reduker, D.W., Dubey, J.P., 1986. *In vitro* cultivation of meronts of *Sarcocystis cruzi*. J. Parasit. 72, 677–683.

Speer, C.A., Hammond, D.M., 1972. Motility of macrogamonts of *Eimeria magna* (Coccidia) in cell culture. Science 178, 763–765. https://doi.org/10.1126/science.178.4062.763.

Steger, A.M., 1997. Development and evaluation of an *Isospora suis* sporozoite neutralizing antibody assay in swine testicular cells (Doctoral dissertation, Drake University, Des Moines, IA, USA). doi:10.1016/j.tim.2012.10.007.

Stewart, M.P., Sharei, A., Ding, X., Sahay, G., Langer, R., Jensen, K.F., 2016. *In vitro* and *ex vivo* strategies for intracellular delivery. Nature 538, 183–192. https://doi.org/10.1038/nature19764.

Straberg, E., Daugschies, A., 2007. Control of piglet coccidiosis by chemical disinfection with a cresol-based product (Neopredisan 135–1®). Parasitol. Res. 101, 599–604. https://doi.org/10.1007/s00436-007-0521-z.

Strout, R.G., Schmatz, D.M., 1990. *In vitro* culture of Coccidia/Biochemistry. In: Long, P.L. (Ed.), Coccidiosis of man and domestic animals. CRC Press, Boca Raton, FL, USA, pp. 221–234.

Suarez, C.E., Bishop, R.P., Alzan, H.F., Poole, W.A., Cooke, B.M., 2017. Advances in the application of genetic manipulation methods to apicomplexan parasites. Int. J. Parasitol. 47, 701–710. https://doi.org/10.1016/j.ijpara.2017.08.002.

Sutrave, S., Richter, M.H., 2021. Truman show for protozoan parasites: A review of *in vitro* cultivation platforms. PLoS Negl. Trop. Dis. 15, 1–21. https://doi.org/10.1371/journal.pntd.0009668.

Tailford, L.E., Crost, E.H., Kavanaugh, D., Juge, N., 2015. Mucin glycan foraging in the human gut microbiome. Front. Genet. 5. https://doi.org/10.3389/fgene.2015.00081.

Tanaka, N., Ashour, D., Dratz, E., Halonen, S., 2016. Use of human induced pluripotent stem cell-derived neurons as a model for cerebral Toxoplasmosis. Microbes Infect. 18, 496–504. https://doi.org/10.1016/j.micinf.2016.03.012.

Tannenbaum, J., Bennett, B.T., 2015. Russell and Burch's 3Rs then and now: The need for clarity in definition and purpose. J. Am. Assoc. Lab. Anim. Sci. 54, 120–132.

Taubert, A., Krüll, M., Zahner, H., Hermosilla, C., 2006. *Toxoplasma gondii* and *Neospora caninum* infections of bovine endothelial cells induce endothelial adhesion molecule gene transcription and subsequent PMN adhesion. Vet. Immunol. Immunopathol. 112, 272–283. https://doi.org/10.1016/j.vetimm.2006.03.017.

Taubert, A., Wimmers, K., Ponsuksili, S., Jimenez, C.A., Zahner, H., Hermosilla, C., 2010. Microarray-based transcriptional profiling of *Eimeria bovis* -infected bovine endothelial host cells. Vet. Res. 41. https://doi.org/10.1051/vetres/2010041.

Taylor A.E.R., Baker, J.R., 1968. The Cultivation of Parasites *in vitro*. Oxford Blackwell Scientific Publications, CABI International, Oxford, UK.

Taylor, M.A., Catchpole, J., 1994. Coccidiosis of domestic ruminants. Appl. Parasitol. 35 (2), 73–86. PMID: 8087156.

Traversa, D., Joachim, A., 2018. The 3Rs concept: time to change how we evaluate the efficacy of anthelmintics in companion animals. Trends Parasitol. 34, 41–52. https://doi.org/10.1016/j.pt.2017.09.002.

Trager, W., Williams, J., 1992. Extracellular (axenic) development *in vitro* of the erythrocytic cycle of *Plasmodium falciparum*. Proc. Nat. Acad. Sci. 89, 5351–5355.

Triffitt, M.J., 1925. Observations on *Gastrocystis gilruthi*, a parasite of sheep in Britain. Protozoology 1, 7–18.

Upton, S., Tilley, M., Brillhart, D.B., 1995. Effect of select medium supplements on *in vitro* development of *Cryptosporidium andersoni* in HCT-8 cells. Parasitol. Res. 33, 371–375. https://doi.org/10.1007/s00436-009-1576-9.

Upton, S.J., Tilley, M., Brillhart, D.B., 1994. Comparative development of *Cryptosporidium parvum* (Apicomplexa) in 11 continuous host cell lines. FEMS Microbiol. Lett. 118, 233–236. https://doi.org/10.1111/j.1574-6968.1994.tb06833.x.

Van Voorhis, W.C., Doggett, J.S., Parsons, M., Hulverson, M.A., Choi, R., Arnold, S.L.M., Riggs, M.W., Hemphill, A., Howe, D.K., Mealey, R.H., Lau, A.O.T., Merritt, E.A., Maly, D.J., Fan, E., Ojo, K.K., 2017. Extended-spectrum antiprotozoal bumped kinase inhibitors: A review. Exp. Parasitol. 180, 71–83. https://doi.org/10.1016/j.exppara.2017.01.001.

Velásquez, Z.D., López-Osorio, S., Waiger, D., Manosalva, C., Pervizaj-Oruqaj, L., Herold, S., Hermosilla, C., Taubert, A., 2021. *Eimeria bovis* infections induce G 1 cell cycle arrest and a senescence-like phenotype in endothelial host cells. Parasitology 148, 341–353. https://doi.org/10.1017/S0031182020002097.

Verma, S.K., Lindsay, D.S., Grigg, M.E., Dubey, J.P., 2017. Isolation, Vulture and Cryopreservation of *Sarcocystis* species. Curr. Protoc. Microbiol. 45. https://doi.org/10.1002/cpmc.32. 20D.1.1.–20D1.27.

Vieira, A.M., Teixeira Soratto, T.A., Cardinal, K.M., Wagner, G., Hauptli, L., Ferreira Lima, A.L., Dahlke, F., Netto, D.P., de Oliveira Moraes, P., Leal Ribeiro, A.M., 2020. Modulation of the intestinal microbiota of broilers supplemented with monensin or functional oils in response to challenge by Eimeria spp. PLoS One 15, 1–15. https://doi.org/10.1371/journal.pone.0237118.

Visvesvara, G.S., Garcia, L.S., 2002. Culture of Protozoan Parasites. Clin. Microbiol. Rev. 15, 327–329. https://doi.org/10.1128/CMR.15.3.327.

Von Laufen, N., Guetg, N., Naguleswaran, A., Müller, N., Björkman, C., Schares, G., Von Blumroeder, D., Ellis, J., Hemphill, A., 2004. *In vitro* induction of *Neospora caninum* bradyzoites in Vero cells reveals differential antigen expression, localization, and host-cell recognition of tachyzoites and bradyzoites. Infect. Immun. 72, 576–583. https://doi.org/10.1128/IAI.72.1.576-583.2004.

Weiss, L.M., Laplace, D., Takvorian, P.M., Tanowitz, H.B., Cali, A., Wittner, M., 1995. A cell culture system for study of the development of *Toxoplasma gondii* bradyzoites. J. Eukaryot. Microbiol. 42, 150–157. https://doi.org/10.1111/j.1550-7408.1995.tb01556.x.

White, M.W., Radke, J.R., Radke, J.B., 2014. *Toxoplasma* development - turn the switch on or off? Cell. Microbiol. 16, 466–472. https://doi.org/10.1111/cmi.12267.

Whitelaw, J.A., Latorre-Barragan, F., Gras, S., et al., 2017. Surface attachment, promoted by the actomyosin system of Toxoplasma gondii is important for efficient gliding motility and invasion. BMC Biol. 15, 1. https://doi.org/10.1186/s12915-016-0343-5.

Wiedmer, S., Alnassan, A.A., Volke, B., Thabet, A., Daugschies, A., Lendner, M., Kurth, M., 2017a. Passive immunization with *Eimeria tenella* gametocyte antigen 56 (EtGAM56) specific antibodies and active immunization trial with the epitope containing peptide. Vet. Parasitol. 247, 100–107. https://doi.org/10.1016/j.vetpar.2017.09.019.

Wiedmer, S., Erdbeer, A., Volke, B., Randel, S., Kapplusch, F., Hanig, S., Kurth, M., 2017b. Identification and analysis of *Eimeria nieschulzi* gametocyte genes reveal splicing events of gam genes and conserved motifs in the wall-forming proteins within the genus *Eimeria* (Coccidia, Apicomplexa). Parasite 24, 50. https://doi.org/10.1051/parasite/2017049.

Wilson, D.W., Crabb, B.S., Beeson, J.G., 2020. Development of fluorescent *Plasmodium falciparum* for i*n vitro* growth inhibition assays. Malar J. 9, 152. https://doi.org/10.1186/1475-2875-9-152.

Winzer, P., Müller, J., Aguado-Martínez, A., Rahman, M., Balmer, V., Manser, V., Ortega-Mora, L.M., Ojo, K.K., Fan, E., Maly, D.J., Van Voorhis, W.C., Hemphill, A., 2015. *In vitro* and *in vivo* effects of the bumped kinase inhibitor 1294 in the related cyst-forming apicomplexans *Toxoplasma gondii* and *Neospora caninum*. Antimicrob. Agents Chemother. 59, 6361–6374. https://doi.org/10.1128/AAC.01236-15.

Worliczek, H.L., Buggelsheim, M., Saalmüller, A., Joachim, A., 2007. Porcine isosporosis: infection dynamics, pathophysiology and immunology of experimental infections. Wien. Klin. Wochenschr. 119, 33–39. https://doi.org/10.1007/s00508-007-0859-3.

Worliczek, H.L., Ruttkowski, B., Schwarz, L., Witter, K., Tschulenk, W., Joachim, A., 2013. *Isospora suis* in an epithelial cell culture system - an *in vitro* model for sexual development in coccidia. PLoS One 8, e69797. https://doi.org/10.1371/journal.pone.0069797.

Wu, L., Chen, S.X., Jiang, X.G., Shen, Y.J., Lu, Z.X., Tu, G.H., Fu, X.L., Cao, J.P., 2009. Effect of select medium supplements on *in vitro* development of *Cryptosporidium andersoni* in HCT-8 cells. Parasitol. Res. 105, 1419–1424. https://doi.org/10.1007/s00436-009-1576-9.

Xu, J., Zhang, Y., Tao, J., 2013. Efficacy of a DNA vaccine carrying *Eimeria maxima* Gam56 antigen gene against coccidiosis in chickens. Korean J. Parasitol. 51, 147–154. https://doi.org/10.3347/kjp.2013.51.2.147.

Yang, Q., Liberali, P., 2021. Collective behaviours in organoids. Curr. Opin. Cell Biol. 72, 81–90. https://doi.org/10.1016/j.ceb.2021.06.006.

Zhang, L., Sheoran, A.S., Widmer, G., 2009. *Cryptosporidium parvum* DNA replication in cell-free culture. J. Parasitol. 95, 1239–1242. https://doi.org/10.1645/ge-2052.1.