Chapter 24
Probiotics as Anti-*Giardia* Defenders: Overview on Putative Control Mechanisms

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Abstract *Giardia intestinalis* is a protist intestinal parasite responsible for giardiasis, a disease whose impact is recognized in public health. After ingestion of *Giardia* cysts from either contaminated food or water, the trophozoite proliferative form, responsible for pathogenic effects, develops in the proximal small intestine of the host where it coexists with gut microbiota. Several studies have revealed the importance of this gut ecosystem and/or some probiotic bacteria in providing protection against *G. intestinalis* infections through partially known mechanisms (Travers et al. Journal of Parasitology Research, 2011). In the last years, our team has shown, using biological and biochemical approaches, that some probiotic strains of *Lactobacillus*, in particular *L. johnsonii* La1 and *L. gasseri* CNCM-I 4884, display anti-*Giardia* effects both in vitro and in vivo (Travers et al. Frontiers in Microbiology 2016; Allain et al. Frontiers in Microbiology 8:2707, 2018a, Frontiers in Microbiology 9:98, b). Our investigations have demonstrated that the supernatant of these strains contains Bile-Salt-Hydrolase (BSH)-like activities mediating toxic effects on *Giardia*. This effect is not directly, but by converting non-toxic components of bile (conjugated bile salts) into bile salts deconjugates proved to be highly toxic to the parasite. These anti-*Giardia* effects could be mimicked in vitro by treating *Giardia* cultures with either commercially available BSH bacterial enzymes (Travers et al. 2016) or two *L.*

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johnsonii La1 BSH enzymes produced and purified from recombinant *Escherichia coli* strains (Allain et al. 2018a), in the presence of bile, or even directly with some deconjugated bile salts (Travers et al. 2016). Currently, we are focusing on understanding the mechanism of action (MoA) of toxic metabolites generated by these BSH activities on the parasite itself using imaging and RNA sequencing methods in order to explore the changes in gene expressions in *Giardia.* Altogether, these data pave the way for new approaches for the treatment of this widespread neglected infectious disease.

Keywords *Giardia intestinalis* · *Lactobacillus* · Bile salt hydrolase · Deconjugated bile salts · Anti-*Giardia* activity

Abbreviations

BSH	Bile Salt Hydrolase
C	Cholic acid
CDC	Chenodeoxycholic acid
CNCM	Collection Nationale de Cultures de Microorganismes (National Collection of Microrganisms Cultures)
DAPI	4′,6-diamidino-2-phenylindole
DC	Deoxycholic acid
FAO	Food and Agriculture Organization
FCS	Fetal Calf Serum
FDA	Food and Drug Administration
GC	Glyco-cholic acid
GCDC	Glyco-chenodeoxycholic acid
GDC	Glyco-deoxycholic acid
GRAS	Generally Recognized As Safe
IC50	Inhibitory Concentration yielding 50% inhibition
LAB	Lactic Acid Bacteria
MoA	Mechanism of Action
TC	Tauro-cholic acid
TCDC	Tauro-chenodeoxycholic acid
TDC	Tauro-deoxycholic acid
WGA	Wheat-Germ Agglutinin
WHO	World Health Organization

Giardia

Giardia intestinalis (also called *G. lamblia* or *G. duodenalis*) is a parasitic protozoa belonging to the order Diplomonadida of the super-group Excavata (Adl et al. 2019). It is responsible for giardiasis, an acute or chronic intestinal disorder, characterized by malabsorption, diarrhea, weight loss, dehydration, and abdominal pain in humans and a variety of vertebrates (Cotton et al. 2011). Giardiasis is a public health issue mainly in developing countries but also in developed countries, since outbreaks have been associated with drinking water contaminations with a low infectious dose (10 cysts) resulting from runoffs of contaminated soils by rainfalls, agricultural practices, and sewage treatment plant dysfunctions (Mons et al. 2009; Baldursson and Karanis 2004; Rendtorff 1954). Widely distributed in the environment as resistant cysts, *G. intestinalis* infects many mammals including humans by fecal-oral transmission. Following ingestion, cysts differentiate during their gastrointestinal transit into the motile and replicative flagellated form known as trophozoites (responsible for the pathogenic effects), before their release with the host feces into the environment as infective cysts (Ankarklev et al. 2010). Trophozoite forms proliferate in the host gut lumen, where they transiently adhere to the gut epithelium and coexist with the gut microbiota (Allain et al. 2017). Recent data show that this microbiota and/or some probiotic strains can protect hosts against *Giardia* infections, but the protective mechanisms involved in these effects are poorly understood (Travers et al. 2011; Burgess et al. 2017). *Giardia* belongs to a complex of species currently composed of eight different genotypes (called "assemblages") with variable host and host range specificities. Moreover, assemblages A and B display a wide host diversity infecting both human and animals and are thus considered zoonotic, contrary to other assemblages which have reduced or even specific host tropisms (Cacciò et al. 2018). Thus, assemblages C and D are mainly observed in dogs, assemblage F in cats and assemblage G in rodents (Cacciò et al. 2018).

Fighting against giardiasis is actually possible by using anti-infectious molecules such as metronidazole, tinidazole and benzimidazoles (Leitsch et al. 2011). However, treatments based on these drugs have their limits due to the emergence of strains resistant to these compounds, that are now becoming general for most infectious agents (Kirk et al. 2010). In light of these limitations, the development of new "therapeutic" strategies does also concern the intestinal parasite *G. intestinalis* itself, for which the use of probiotics, for the prevention or the treatment of giardiasis, is becoming an active emerging field, although the molecular mechanisms involved remain poorly described (Travers et al. 2011; Vitetta et al. 2016).

Probiotics

The last update on the definition of probiotics by the Food and Agriculture Organization/World Health Organization (FAO/WHO), dating back to October 2013, states: "*live microorganisms that, when administrated in adequate amounts, confer a health benefit to the host"* (Hill et al. 2014). An ideal probiotic, which is always defined at a strain level, should be able to positively modulate host intestinal microbiota, stabilizing resident microorganisms and restraining colonization by pathogens (Bakhtiar et al. 2013). Moreover, as beneficial microorganisms, probiotics should respond to a list of criteria (Ouwehand and Salminen 1992), detailed below under the prism of more recent data. Notably, once identified and selected, probiotic strains of interest should be characterized for their MoA since probiotic properties are also dependent on conditions of use and doses (Bakhtiar et al. 2013). Nowadays, an increasing number and diversity of commercial probiotic strains are available on market. The commercial and those under studies mostly target bacterial infectious diseases, however, little is known for fighting against parasitic and viral illness (Berrilli et al. 2012; Liévin-Le Moal and Servin 2014; Zare Mirzaei et al. 2018; Kiousi et al. 2019).

Lactic Acid Bacteria (LAB) are commonly used as probiotics as some strains are Generally Recognized As Safe (GRAS) for humans, according to the US Food and Drug Administration (FDA) and fulfill criteria of the Qualified Presumption of Safety (QPS) according to the European Food Safety Authority (EFSA). Among LAB, lactobacilli and bifidobacteria have been extensively studied by scientists and industrials for their potential as probiotics (Azad et al. 2018). Probiotic strains belonging to *Lactobacillus* genus have been until now the focus of our work on *Giardia*. *Lactobacillus* spp. are non-sporulating facultative anaerobic or microaerophilic Gram-positive bacteria (Holzapfel et al. 2001). Their fermentative metabolism is characterized by the production of lactic acid that has been implicated in lactobacilli ability to inhibit intestinal pathogens development, these latter ones being mostly documented as bacterial pathogens (Vandenbergh 1993). In addition to lactic acid production, the inhibitory effect of *Lactobacillus* spp. on these bacterial pathogens relies on the production of antimicrobial peptides (bacteriocins), the competition for mucosal site adhesion and nutrients consumption and also the modulation of the immune system (Figueroa-González et al. 2011). Probiotics ingestion has been also suggested to modulate the gut microbiota composition (Isolauri et al. 2012). For the screening procedure, as the FAO/WHO has recommended, selected probiotic strains should provide (i) resistance to gastric acidity and bile salts, (ii) adhesive properties to mucus and intestinal epithelial cells and finally (iii) anti-microbial and antagonism activities against potentially pathogenic microorganisms (Markowiak et al. 2017). *Lactobacillus* spp. consumption present no risk of mortality to humans, and side effects following their administration are scarce (0.05%−0.4% of cases) (Gasser 1994). Thus, *Lactobacillus* spp. have a well-reported history of safety and are awarded GRAS by the FDA (Sorokulova et al. 2008).

Characterization of *Giardia*-Probiotic Crosstalk

Since probiotic microorganisms provide health benefits to their hosts through the protection against pathogens and the modulation of both innate and adaptive immunity at local and systemic levels (Cebra 1999; Haller et al. 2000; Isolauri et al. 2001), trials have been exerted aiming at exploring whether these organisms could also be used to treat *Giardia* infections. Certainly, colonization of the intestine by *Giardia* strongly depends on the intestinal microbiota and its susceptibility (Singer and Nash 2000; Torres et al. 2000). In addition, *Giardia* infection, in its turn, may exert changes in the composition of the host microbiota and its diversity (Burgess et al. 2017). Since lactobacilli are one of the most common bacteria in the human duodenum (Mitsuoka 1992), several studies have focused on the ability of *Lactobacillus* probiotic strains to shield the host from the detrimental effects mediated by *Giardia* infections (Pérez et al. 2001; Humen et al. 2005; Goyal et al. 2013; Shukla et al. 2008, 2019; Vivancos et al. 2018). It was shown that *Lactobacillus casei* MTCC 1423 strain is effective in eliminating *Giardia* in mice (Shukla and Sidhu 2011). Moreover, in 2001, Perez and collaborators found that the culture supernatant of *L. johnsonii* La1 was able to control *G. lamblia* (*intestinalis*) growth in vitro, an effect that was strain-dependent since six other strains of *Lactobacillus acidophilus* (tested in parallel), did not show any noticeable effect on the parasite (Pérez et al. 2001). This effect was confirmed in vivo using a gerbil model that evaded *Giardia* colonization when treated with *L. johnsonii* La1 administrated by gavage, in addition to a reinforcement of the host immune response against the parasite (Humen et al. 2005). Based on available literature, *L. johnsonii* La1 appeared as a good model of choice, to study the molecular crosstalk between *Giardia* and probiotic bacteria, with, in addition, well-known genomes for both partners (Pridmore et al. 2004; Morrison et al. 2007; Franzén et al. 2009). Other non-lactobacilli probiotics have demonstrated anti-giardial effects such as *Enterococcus faecium* SF68, and Slab51 (Benyacoub et al. 2005; Perrucci et al. 2019). Beyond bacteria, it must be noted that trials have used yet other probiotics microorganisms including yeasts, as for example *Saccharomyces boulardii*, that have shown promising results in the protection against giardiasis with a decreased number of parasite cysts in feces from patients treated with a combination of *S. boulardii* and metronidazole *versus* patients treated only with metronidazole (Besirbellioglu et al. 2006). Quite recently, this observation was further supported by another study showing that *S. boulardii* could be also used as a co-adjuvant in giardiasis treatment since it shows a reduction in intestinal damages caused by *Giardia* with an approximate reduction of 70% of the parasite load in vivo model of infected gerbil mice (Ribeiro et al. 2018).

We have been interested in studying these probiotic-parasite interactions for prophylactic and/or therapeutic purposes, applied to *G. intestinalis,* and focused our initial interests on the probiotic strain *L. johnsonii* La1, based on the promising results cited above (Pérez et al. 2001; Humen et al. 2005; Pridmore et al. 2004; Morrison et al. 2007; Franzén et al. 2009). By combining biological, biochemical and metabolomic approaches, we have discovered that the MoA of *L. johnsonii* La1

against *G. intestinalis* is partially indirect, and involves Bile Salt Hydrolase (BSH) type enzyme activities produced by this bacterium, which provoke the death of the parasites by converting bile components (identified as being conjugated bile salts, non-toxic to *Giardia*) into toxic compounds (identified as deconjugated bile salts) (Travers et al. 2016). The deleterious action of deconjugated bile salts on parasites growth in vitro was then directly confirmed (Travers et al. 2016), and the recombinant BSH enzymes of this probiotic strain, produced in *E. coli* (currently 2 of the 3 encoded by its genome: BSH-47 and BSH-56), also allowed to block the parasite proliferation in vitro (parasites in culture) in the presence of bile and in vivo in a murine model of giardiasis: newborn mice, line OF1 (Allain et al. 2018a). Several in vitro tests reflected the potential benefit provided by BSH enzymes although displaying different substrate specificities: indeed, BSH-47 and BSH-56 (from *L. johnsonii* La1), have distinct substrate specificities—BSH-56 mainly hydrolyzing Tauro-conjugates but also Glyco-conjugates, whereas BSH-47 hydrolyzes mostly Tauro-conjugates—both being active in vitro in a dose-dependent manner and in vivo (BSH-47) (Allain et al. 2018a). These BSH effects, potentially distinct, are important to know since it is well established that bile composition differs dramatically according to the host (Farthing et al. 1985; Aguiar Vallim et al. 2013). In parallel, we tested 29 lactobacilli strains, isolated from a variety of environments, for their "anti-*Giardia*" and "BSH activity" properties in vitro (Allain et al. 2018b). These studies allowed establishing (1) a positive correlation between these two properties, making it possible to propose a screen of potentially anti-*Giardia* strains based on their BSH activities (Allain et al. 2018b). In addition, (2) it allowed to discover the novel *L. gasseri* CNCM I-4884 probiotic strain, that proved to be as active as *L. johnsonii* La1 strain in vitro but much more active in vivo, in the murine model of the newborn infant mouse OF1 (Allain et al. 2018b). Indeed, while *Giardia* trophozoite loads were reduced in infected mice by gavages with either probiotics, *Giardia* cyst loads were significantly more highly reduced by using *L. gasseri* CNCM I-4884 gavages as compared to using *L. johnsonii* La1 gavages (Allain et al. 2018b). The mechanism responsible for this higher activity of *L. gasseri* CNCM I-4884 compared to *L. johnsonii* La1 at controlling *Giardia* in vivo is not yet established, but we are currently investigating it (Fig. 24.1).

To our knowledge, the sum of these studies, that lead to the discovery of a possible MoA of probiotics on the development of *G. intestinalis* parasite involving BSH activities of lactobacilli (Travers et al. 2016; Allain et al. 2018a, b), remain rather unique in the emerging field of *Giardia*-probiotics cross-talk exploration. It would be obviously interesting to also test the specificity of this control mechanism of lactobacilli against the different assemblages of *G. intestinalis.* Hence, to go deeper, it is interesting to understand what is happening at the parasite level, especially the MoA of toxic metabolites that are generated by active BSH or present in lactobacilli supernatants, in both in vitro and in vivo models.

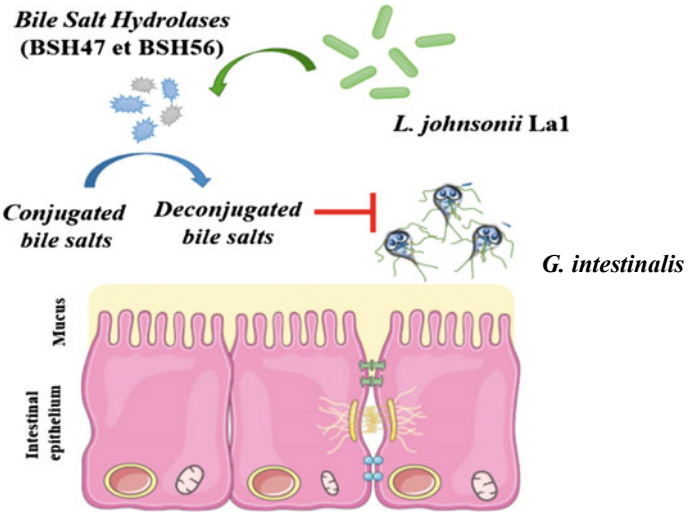

Fig. 24.1 Our data suggest a possible MoA by which the probiotic strain of *Lactobacillus johnsonii* La1, by secreting/releasing BSH-like enzymes in an environment where bile is present and abundant, can fight the *Giardia* parasite through the conversion of conjugated bile salts (non-toxic to *Giardia*) to deconjugated bile salts (toxic to *Giardia*)

The Direct Effect of Bile Active Compounds on the *Giardia* Parasite

Giardia culture medium has been historically supplemented with bile in order to promote the parasite in vitro growth; indeed, parasites have been documented to use bile lipids as metabolites or source of phospholipids for membrane biosynthesis (Farthing et al. 1985; Halliday et al. 1995; Das et al. 1997). However, in the presence of high concentrations of bile and bile salts, growth reduction rate is observed (Farthing et al. 1983, 1985; Gillin 1987; Gillin et al. 1989). Which mechanisms happen behind these observations, i.e., which of the bile components are beneficial/detrimental to *Giardia* remains incompletely explored, as only a few of these bile components have been tested on *Giardia* growth and survival (Farthing et al. 1983, 1985; Gillin 1987; Gillin et al. 1989). However, this remains challenging due to the complexity of bile composition, both in terms of metabolites and of their respective concentrations, depending on their biological sources (Aguiar Vallim et al. 2013; Farthing et al. 1985). Interestingly, intestine bile salts have been shown to be potent antimicrobial agents, involved in innate defenses (Sung et al. 1993; Itoh et al. 1999; Begley et al. 2005). Sannasiddappa and collaborators have found that bile salts exert an antibacterial effect on *Staphylococcus aureus* (Sannasiddappa et al. 2017); however, the potential antiparasitic activity exerted by bile salts on *G. intestinalis* is still poorly understood. Their exploration might however hold potential as recently, bile salts have been indeed considered as therapeutic agents (Donker et al. 2019). Since our

studies have clearly pointed towards modifications of bile composition, mediated by lactobacilli BSH enzymes, as important drivers of *G. intestinalis* development in vitro and in vivo (Travers et al. 2016; Allain et al. 2018a, b), a logical follow-up of this discovery was to focus on these bile salts.

Experiments have been conducted to study individually a series of pure tauro- and glyco-conjugated bile salts (tauro- and glyco-cholic (TC, GC), tauro- and glyco-deoxycholic (TDC, GDC), tauro- and glyco-chenodeoxycholic (TCDC, GCDC) acids) as well as their deconjugated counterparts: cholic-acid (C), deoxycholic acid (DC) and chenodeoxycholic (CDC) acids, on *Giardia* (Travers et al. 2016; Allain et al. 2018a). Results have shown that glycine or taurine conjugated bile salts have no toxic impact on *G. intestinalis* growth in vitro. However, the addition of a recombinant BSH enzyme from *Clostridium perfringens* to the culture medium, in presence of these conjugated bile salts, led to a remarkable parasite growth inhibition within the 24 h of the assay (Travers et al. 2016). Moreover, treating directly *G. intestinalis* trophozoites with pure deconjugated bile salts (C, DC and CDC) have also shown a toxic dose-dependent effect of notably DC and CDC on *Giardia* growth (IC50 of 132 μM and 147 μM respectively), which was not observed by using cholic acid (IC50 > 400 μM) or, as mentioned above, conjugated bile salts (Travers et al. 2016). Interestingly, the killing effect of deconjugated bile salts on *Giardia* have been correlated with their hydrophobicity properties, no inhibition being observed with the most hydrophilic salt cholate contrary to the more hydrophobic salts, deoxycholate and chenodeoxycholate (Travers et al. 2016). This could explain also the non-toxic effect of conjugated bile salts since deconjugated bile salts are more hydrophobic than their conjugated counterparts (Ridlon et al. 2016). However and very importantly, the IC50 values of these active deconjugated bile salts are much lower than their critical micellar concentrations, which means that the parasite killing effect is not simply related to their surfactant properties (Critical micellar concentrations of C ~ 14 mM, DC ~ 1.4 mM and CDC > 7 mM, based on the manufacturers). Based on these findings, comparative studies of deconjugated bile salts effects on *Giardia*, have been designed using cholic acid as negative control. Microscopic observations of treated and untreated *Giardia* cells using scanning electron microscopy, revealed altered morphology and plasma membrane disruptions under both recombinant bile-salts BSH treatment in presence of bile (Allain et al. 2018a), and pure DC treatment (0.1 g/L i.e. 232 μM) in absence of bile (Allain et al. 2018a; Fig. 24.2), compared to controls.

Current Investigations on the Toxic Effects of Bile Salts

Although numerous studies have converged towards the existence of a beneficial effect provided by different types of probiotics to control giardiasis, few have provided hints on the molecular MoA behind these effects (Amer et al. 2014; Allain et al. 2018a, b). Currently, our studies aim to determine the mechanism of killing of these *Lactobacillus* defenders on *Giardia* parasite itself since no data in this concern

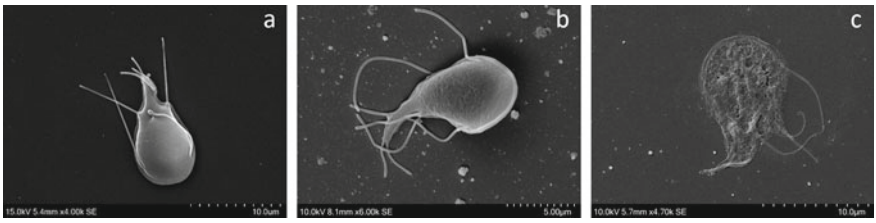

Fig. 24.2 Morphological alterations following in vitro treatments of *G. intestinalis* WB6 strain trophozoites by deoxycholic (DC) acid. **a** Modified TYI-S-33 medium control (+10% FCS) without bile. **b** Modified TYI-S-33 medium control (+10% FCS) with bile (bovine bile 0.6 g/L) showing the characteristic pear-shaped of *G. intestinalis* WB6 strain trophozoites in vitro. **c** Modified TYI-S-33 medium control (+10% FCS) with DC (0.1 g/L) displays alterations and a disruption of plasma membrane exposing cell interior. Scale bar = 5 μm (**b**) or 10 μm (**a**, **c**). See also Allain et al. (2018a)

have been published. Our hypothesis was that this detrimental effect—mediated by some deconjugated bile salts—could be due to their ability to disrupt essential membrane functions and bioenergetic processes, as shown for *Staphylococcus aureus* and *Clostridium difficile* (Thanissery et al. 2017; Sannasiddappa et al. 2017).

The main primary bile acids, produced by the human liver, are cholic acid and chenodeoxycholic acid, mostly conjugated to taurine and glycine (Donkers et al. 2019). Under intestinal microbiota deconjugation and by dehydroxylation at C7, primary bile acids are converted into secondary bile acids resulting in deoxycholic acid and lithocholic acid, respectively. In the intestine, bile salts are considered as digestive molecules helping in lipid digestion as well as important innate defenses and potent antibacterial agents due to their soap-like character (Sung et al. 1993; Itoh et al. 1999; Begley et al. 2005). Bile salts are known to inhibit bioenergetic processes by intracellular acidification, dissipation of the proton motive force, and induction of DNA damage and protein denaturation (Kurdi et al. 2006; Merritt and Donaldson 2009). Interestingly, Pérez and collaborators suggested that extracellular factors of *L. johnsonii* La1 arrest the in vitro growth of *G. intestinalis* at the G1 phase indicating that this bacterium may directly affect parasite replication (Pérez et al. 2001). Note that the suspected bacterial metabolite (< 1 kDa) (Pérez et al. 2001) is of a different nature than the BSH (> 30 kDa) we have identified. Thus, the interaction between *Giardia* and human bile salts is an important factor in its ability to colonize the host intestine. Despite this importance, the MoA of these bile salts on *Giardia* is not fully understood. Several experiments established in our laboratory, using fluorescent microscopy, have shown that *Giardia* trophozoites treated for 22 h with DC (0.1 g/L) and CDC (0.1 g/L) turn into roundish cells with some disassembled flagella, compared to untreated and C-treated conditions (Fig. 24.3). These results recall those recently obtained by Sievers and collaborators on a different biological model, the bacterium *C. difficile,* in which less flagellated cells were observed in the presence of DC and CDC but no influence on flagella was induced by C, cholic acid (Sievers et al. 2019). These findings also recall morphological observations accompanying *Giardia* encystation program, experimentally induced

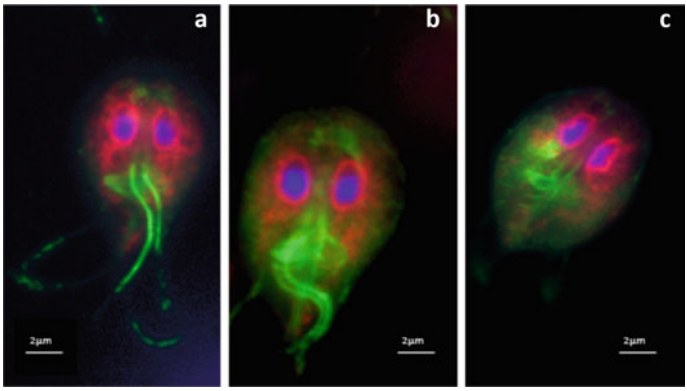

Fig. 24.3 Fluorescence microscopy images of *Giardia intestinalis* trophozoites labeled with DAPI (blue), anti-Tubulin antibody (green, Woods et al. 1989) and Wheat-Germ Agglutinin (WGA, red, Ratner et al. 2008) after 22 h of culture in vitro. **a** Modified TYI-S-33 medium control (+10% FCS, no bile), **b** Modified TYI-S-33 medium control (+10% FCS, no bile), with cholic acid (Sigma, 0.1 g/L), **c** Modified TYI-S-33 medium control (+10% FCS, no bile), with deoxycholic acid (Sigma, 0.1 g/L). Scale bar = 2 µm (**a**, **b**, **c**)

in vitro by modifying bile (and bile components?) supply as well as pH in the culture conditions (Lujan and Svärd 2011). We have described phenotypic *Giardia* growth alterations upon (1) *L. johnsonii* La1 supernatant culture challenge in the presence of bile, (2) rBSH supplementation in the presence of bile and (3) pure deconjugated bile salts treatments (Travers et al. 2016; Allain et al. 2018a, b), but no information on the adaptation of gene expression patterns is available so far in these various conditions. As a mean to get further insight into the molecular mechanisms involved, we have designed to use a transcriptomic approach (RNA sequencing) to explore the gradation of these responses, when *Giardia* cells are exposed to these three challenging but progressively simplified conditions.

As a starting point, we logically choose to focus on the least complex configuration: (3), i.e. pure deconjugated bile salts treatments, using DC (0.1 g/L) in vitro culture. Preliminary results, from triplicated cultures in presence and absence of DC sampled at T0, and after 7 and 16 h of treatments, have shown that there is a part of the parasite transcriptome that is altered due to the deconjugated bile salt treatment. Some genes, documented to be involved in encystation (Einarsson et al. 2016) seems to be altered in their transcriptomic profile, suggesting that *Giardia* cells in presence of DC have a tendency to encystation. This response is also accompanied with a deregulated cell cycle and translational phase. In addition, some morphological alterations of *Giardia* trophozoites under DC treatment (Fig. 24.3) could be paralleled with some changes at transcriptomic level. Genes encoding proteins participating in cytoskeleton components were modified after 16 h exposure to DC, in comparison to control, untreated, conditions.

Certainly, studying the "simple" treatment of *Giardia* parasites with deconjugated bile salts individually might not be sufficient to accurately reflect the more complex

experimental situation (Sannasiddappa et al. 2017), but it forms a simple model to explore individual bile acids activities on *Giardia*. Next, the higher levels of experimental complexity will be progressively explored as mentioned above such as (1) the combination of recombinant BSH enzymes in presence of bile, and (2) *Lactobacillus* culture supernatants in presence of bile, for their effects on *Giardia*. In both conditions, mixtures of deconjugated bile salts of several types (Cholic acid, deoxycholic acid, chenodeoxycholic acids and others) are expected to be produced, the combined effect of which on *Giardia* remains to be explored. Moreover, in the latter condition (probiotic supernatants), additional active principles may potentially be also present as previously demonstrated (Travers et al. 2016), which may further influence the biological response on the parasite.

Finally, in order to carefully understand how probiotics exert their defence on *Giardia* in vivo, it will be necessary to investigate the molecular mechanisms exerted on *Giardia* parasite following either probiotic gavages of murine models of giardiasis, or, ultimately, patients treatments by these probiotics.

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