Host cell response analysis during *Giardia duodenalis* infection

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Thesis abstract

Giardia duodenalis is a common intestinal protozoan that infects various vertebrates, including humans causing significant damage in their intestinal epithelium. The infection is associated with diarrhea, dehydration, abdominal pain, bloating, weight loss, and malabsorption. Despite a wealth of research, the virulence factors of parasite isolates and their effects in host pathophysiological responses are still incompletely defined. This thesis examined the cellular gene responses in the rat intestinal epithelial cell line (IEC-6) to co-culture with various *G. duodenalis* isolates from assemblage A (P-1, WB, NF) and assemblage B (GS/M), or their excretory/secretory proteins, following 2 or 6hrs incubation *in vitro*.

RNA-sequencing of total mRNA derived from IEC-6 following co-culture with trophozoites of two assemblage A isolates (P-1 and WB) was performed. RNA-seq data showed significant differences between the assemblages A isolates, P-1 and WB, in terms of the number of expressed genes at 2hrs. However, these differences were not consistent between these isolates after 6hrs incubation with the IEC-6. The analysis of gene transcription identified many early changes with the potential to affect the regulation of several pathways in host responses to parasitism, characterised by inflammatory responses including the modulation/upregulation of pro-inflammatory cytokines, interleukins, chemokines and TNFSF members.

The *NOS2* transcript was highly upregulated, suggesting a potential role of NO via L-arginine catabolism as an immune defence mechanism against early giardiasis. Induction of *NOX1* transcript also suggested that the parasitised IEC-6 might produce moderate levels of NOX1-generated ROS as one of the host defence mechanisms used in response to early giardiasis.

This study also suggests that early in the course of giardiasis (2/6hrs) both parasite attachment and parasite secreted molecules disturb the intestinal epithelial barrier leading to the upregulation of gene transcription of the apical junction complex (AJC) proteins, such as *CLDN-1*.

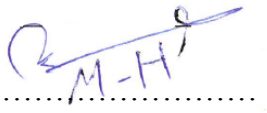
The overall RNA-seq data suggested that clear differences in early gene responses between the assemblage A isolates were detected and it was hypothesised this could influence variations in the pathogenicity level of these isolates on their host cells during infection.

Quantitative real time-polymerase chain reaction (qRT-PCR) analyses was also used to quantify expression levels of key host cell adhesion and apoptosis genes following the 2 and 6hrs induction with either trophozoites or their excretory/secretory products (ESP) of the four isolates. qRT-PCR and a biochemical assay confirmed the RNA-seq data, in that the IEC-6 did not undergo apoptosis at 2 or 6hrs. Whilst host cells increased rates of FASR transcription, and may become more sensitive to FAS induced apoptosis, they did not undergo apoptosis as as a consequence of the upregulation of transcripts of IAPs (e.g. *CFLAR*, *BIRC2*, and *BIRC3*) and the pro-survival factor (NF-kB). Other studies implicating apoptosis as an important response to *G. duodenalis* infection, indicate apoptosis occurs after an extended (>6 hrs) period of infection.

Overall, this thesis has provided a more comprehensive insight into *G. duodenalis* induced early host gene responses of epithelial cells.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Signature: 

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Conferences / workshops

Type	Year	Details
Conference	2015	Mohammad Al-Hasnawy, Mark Sandeman, David Piedrafita, Phillip Brook-Carter (2015), Host-parasite interactions in the pathogenesis of *Giardia duodenalis* infection in mammals, participation by a poster at Microbiology@Monash 3rd Plenary Meeting – 3 December 2015.
Workshop	2016	Mohammad Al-Hasnawy (2016). EuPathDB Parasite Genomics, the Walter Eliza Hall, Institute of Medical Research. Melbourne, Australia February 15-19, 2016.
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Chapter 1: Literature Review

1.1. Introduction

Giardia, a flagellated unicellular eukaryotic protozoan parasite, was first described in 1681 by the Dutch microscopist Antonie van Leeuwenhoek, who found the trophozoites in his own diarrheal stool (Dobell, 1920; Boutros, 1985; Adam, 2001; Koehler *et al.*, 2014). *Giardia duodenalis* (synonyms *Giardia lamblia* and *Giardia intestinalis*) is the most common waterborne parasitic infection of the human intestine worldwide, ubiquitious in children presenting with diarrohea and the most common cause of diarrhea in travelers presenting to clinics. With an estimated 280 million infections every year, particularly in resource poor settings, the disease has been included in the World Health Organisation (WHO) Neglected Diseases Initiative (reviewed in Savioli *et al.*, 2006; Bartelt and Sartor, 2015; Buret *et al.*, 2015). The disease is considered a zoonotic pathogen as *G. duodenalis* can infect both humans and animals (Adam, 1991; Thompson, 2000; Feng and Xiao, 2011; Thompson and Monis, 2012; Ryan and Cacciò, 2013). *G. duodenalis* causes symptomatic acute or chronic giardiasis, the symptoms of which include; diarrhoea, abdominal pain, bloating, dehydration, weight loss, and malabsorption (Buret and Cotton, 2011). Nevertheless, asymptomatic infections are also reported in different hosts, including in farm animals (Geurden and Olson, 2011). Although *Giardia* is considered a neglected tropical disease in resource poor settings, paradoxically, it is also considered as a re-emerging infectious disease in industrialised countries (Thompson, 2000; Savioli *et al.*, 2006; Thompson and Monis, 2012; Bartelt *et al.*, 2013).

1.2. *Giardia duodenalis* life cycle

G. duodenalis has a direct life cycle, which includes two main forms, the trophozoite and cyst. The infectious cycle (figure 1) begins when the cysts are ingested with 10-100 cysts generally considered an infective dose (Di Genova and Tonelli, 2016). Following exposure of the cyst to the acidity of the stomach, excystation will occur in the duodenum of the small intestine and the trophozoite stage of the parasite emerges (Rendtorff, 1954; Gardner and Hill, 2001). The trophozoite reproduces by mitotic division in the small intestine following attachment to the epithelial surface via a ventral disc (Cotton *et al.*, 2011). Following exposure to biliary fluid in the jejunum, many trophozoites undergo encystation, to produce environmentally resistant cysts. These newly produced cysts are excreted with the faeces where they are able to infect

another host through various transmission modes (Adam, 2001; Huang and White, 2006; Feng and Xiao, 2011). These include the faecal-oral route via animal-to-human, animal-to-animal, human-to-human, or indirectly by ingestion of contaminated food and water (Adam, 2001; Cacciò, 2015). These routes are more prevalent in developing countries that have less hygiene infrastructure and provide a more suitable environment for the endemic establishment of giardiasis (Savioli *et al.*, 2006). The cysts remain viable for weeks, depending on several factors including temperature and humidity (Erickson and Ortega, 2006).

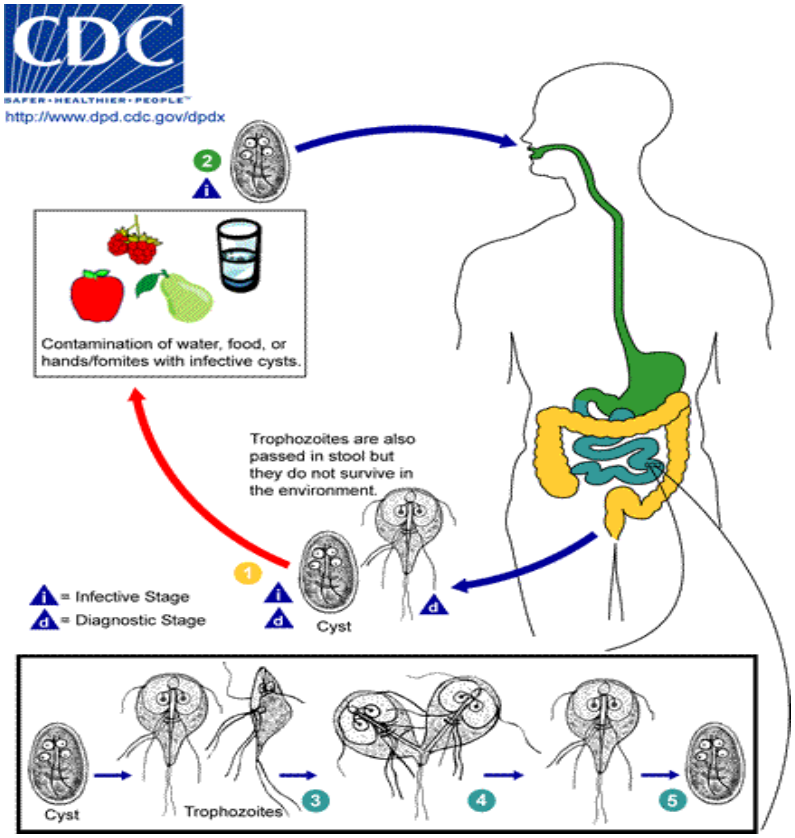


Figure 1: Life cycle of *G. duodenalis*; "Cysts are resistant forms and are responsible for transmission of giardiasis. Both cysts and trophozoites can be found in the faeces (diagnostic stages)1. The cysts are hardy and can survive several months in cold water. Infection occurs by the ingestion of cysts in contaminated water, food, or by the faecal-oral route (hands or fomites)2. In the small intestine, excystation releases trophozoites (each cyst produces two trophozoites)3. Trophozoites multiply by longitudinal binary fission, remaining in the lumen of the proximal small bowel where they can be free or attached to the mucosa by a ventral sucking disk4. Encystation occurs as the parasites transit toward the colon. The cyst is the stage found most commonly in nondiarrheal faeces 5. Because the cysts are infectious when passed in the stool or shortly afterward, person-to-person transmission is possible." Taken from: http://www.dpd.cdc.gov/dpdx.

1.3. *G. duodenalis*: The parasite, epidemiology, diagnosis and treatment

1.3.1. *G. duodenalis* morphology

The trophozoite is a motile flagellate microorganism that causes host pathology, while the cyst is a non-motile infective stage. These stages are structurally and biochemically different (Adam, 2001).

1.3.1.1. Trophozoite structure

The trophozoite (Figure 2a) is a pear-shaped organism containing two claw-shaped median bodies and is approximately 12-15µm in length and 5-9 µm in width (Thompson *et al.*, 2000). It has four pairs of flagella and two nuclei that appear identical, as well as an adhesive disc on the ventral surface for attachment to host cells (Adam, 1991, 2001; Monis *et al.*, 2009). Although there is an absence of a typical Golgi apparatus, peroxisomes and mitochondria in *G. duodnelis*, there is an endoplasmic reticulum (ER) which can form secretory vesicles (Luján *et al.*, 1997; Lujan and Touz, 2003; Gottig *et al.*, 2006); trophozoites also have several vesicles and tubules (the peripheral vacuoles or PVs) that act as endosomes and lysosomes. These components are located in the cytoplasm associated with the plasma membrane suggesting they play a role in endocytosis (Gottig *et al.*, 2006; Touz *et al.*, 2012). The adhesive disk consists of α- and β-tubulin, contractile proteins, and cytoskeletal proteins called giardins, which are connected to the plasma membrane by short fibers (Elmendorf *et al.*, 2003; Palm *et al.*, 2005).

1.3.1.2. Cyst structure

The *G. duodenalis* cyst is oval and non-motile with a length of 8-12 µm and width of 7-10 µm (Figure 2b). It has a wall and an inner layer that are formed from two membranes. Flagellar axonemes, vacuoles, ribosomes and fragments of the ventral disk are located inside the cyst wall (Sheffield and Bjorvatn, 1977; Erlandsen and Feely, 1984; Erlandsen *et al.*, 1989). The cyst has four tetraploid nuclei and the surface of the cyst lacks the adhesive disc and the flagella which are apparent on surface of the trophozoite stage (Adam, 1991; Monis *et al.*, 2009; Ankarklev *et al.*, 2010).

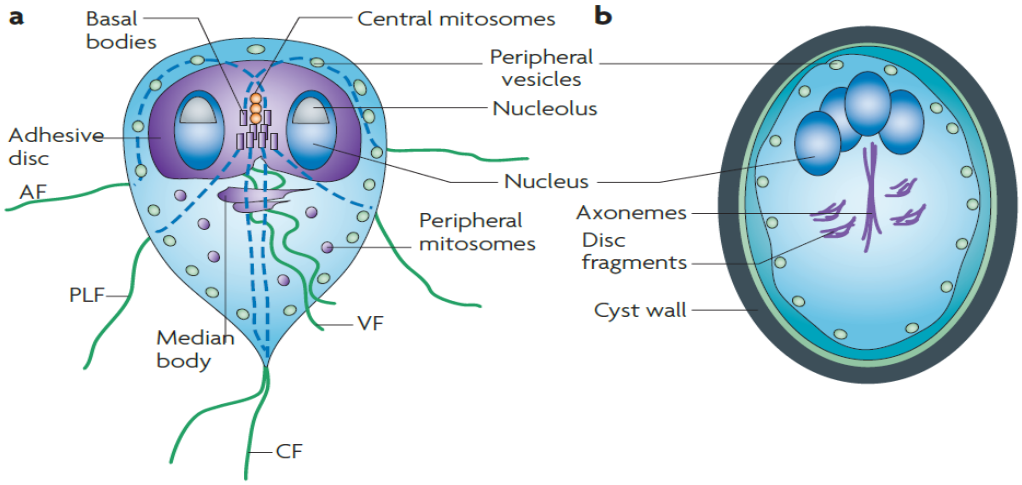


Figure 2: Morphological structures of the motile trophozoite (a) and immotile cyst (b) of *G. duodenalis*. (Adapted from Ankarklev *et al.*, 2010)

1.3.2. *G. duodenalis* genotypes

Different genotypes and sub genotypes of *G. duodenalis* have been identified in mammals, including humans. To date, eight assemblages have been identified within *G. duodenalis*, including A, B, C, D, E, G and H (Ryan and Cacciò, 2013) (Table 1). Assemblages A and B infect humans (Cacciò and Ryan, 2008; Lasek-Nesselquist *et al.*, 2010), although, these assemblages can infect other animals such as cattle, sheep, horses, pigs, rats, dogs and cats (Cacciò and Ryan, 2008). In addition, both assemblages A and B are zoonotic and have wide host specificity (Thompson *et al.*, 2008). Dogs are infected by assemblages C and D, assemblage E infects domestic ruminants and pigs, assemblage F infects cats, and assemblages G and H infect rats and marine mammals, respectively (Ryan and Cacciò, 2013; Cacciò, 2015). However, recent reports have documented that humans can be infected by assemblage E (Foronda *et al.*, 2008; Helmy *et al.*, 2014; Abdel-Moein and Saeed, 2016; Fantinatti *et al.*, 2016; Scalia *et al.*, 2016; Zahedi *et al.*, 2017), assemblage C and D (Broglia *et al.*, 2013; Liu *et al.*, 2014; Štrkolcová *et al.*, 2015), and assemblage F (Gelanew *et al.*, 2007). Assemblage A has been grouped into three sub-assemblages (AI, AII and AIII) and Assemblages B into four sub-assemblages (BI, BII, BIII and BIV), based on allozyme and phylogenetic sequence analyses. Nevertheless, BIII and BIV have not been supported by DNA sequence analyses yet (Table 1) (Monis *et al.*, 2003; Read *et al.*, 2004; van der Giessen *et al.*, 2006; Robertson *et al.*, 2007; Cacciò *et al.*, 2008; Sprong *et al.*, 2009; Ryan and Cacciò, 2013).

Table 1: Assemblages and sub-assemblages of *G. duodenalis* and their hosts (Ryan and Cacciò, 2013).

G. duodenalis Assemblage	Sub-assemblage	Host distribution
A	AI, AII, AIII	Humans and primates (AI & AII), livestock (AI, AI & AIII), dogs (AI & AII), cats (AI, AII & AII), pig (A I& AII), horse (AI & AII), marine animals (AI or AII) and some species of wild hoofed animals (AIII)
B	BI, BII, BIII, BIV	Humans (BIII & BIV) and primates (BI & BII), dogs (BI), cats, sheep, pig, horse, rabbit, marine animals and some species of wild mammals
C	CI, CII, CIII	Dogs and other canids
D	-	Dogs and other canids
E	EI, EII, EIII	Hoofed livestock, sheep (EI), cattle (EII), pig (EIII)
F	-	Cats
G	-	Rats
H	-	Marine mammals (pinnipeds)

Species-specific isolates have also been differentiated on biological or epidemiological characteristics (Plutzer *et al.*, 2010), including variations in metabolism, biochemistry, *in vitro* growth rates, infectivity, duration of the infection, susceptibility to infection with a dsRNA virus, drug sensitivity and/or pH preference among different isolates (Thompson, 2004; Cacciò *et al.*, 2005; Monis *et al.*, 2009).

1.3.3. Epidemiology

The potential for persistent and recurrent *G. duodenalis* infections is high due to the hardy cysts and an infection that is capable of propagating through multiple hosts. Zoonotic transmission between animals and humans has been assumed for multiple animal species such as cattle, dogs, and cats which can harbor both zoonotic and host-specific *G. duodenalis* species (Traub *et al.*, 2004; Traub *et al.*, 2009; Feng and Xiao, 2011; Khan *et al.*, 2011; Uehlinger *et al.*, 2011; Budu-Amoako *et al.*, 2012; Inpankaew *et al.*, 2014). However, further epidemiological studies are needed to provide certainty of the host-specificity and transmission of giardiasis (Cacciò, 2015).

Presently, the most common outbreaks of giardiasis occur in low-resource settings with poor hygiene and healthcare (Cacciò and Sprong, 2011; Bartelt and Sartor, 2015). Furthermore, *Giardia* infection is ubiquitous in children aged 1–4 years and observed in adults 30-40 years such as mothers in contact with their infected children (Cacciò and Sprong, 2011).
Large historical infections of giardiasis in developed countries, such as the 1954 outbreak in Oregon, USA causing 50,000 human cases, have been commonly reported (Karanis *et al.*, 2007). With improved standards of water treatment now common in developed countries such outbreaks are rare (Baldursson and Karanis, 2011). However, there is still a potential health risk, as it has been shown that *G. duodenalis* is the main etiological agent of traveller's diarrhoea and is common among immigrants/refugees (Angel *et al.*, 2010; Baldursson and Karanis, 2011; Cacciò and Sprong, 2011; Ross *et al.*, 2013; Cacciò, 2015).

Management and prevention measures are necessary to achieve a reduction in infection rates and break the parasite's life cycle (Geurden and Olson, 2011). These measures include effective sanitation and improvement of water supplies. In fact, boiling or filtering water collected from water supplies is the simplest effective procedure to kill/decontaminate water of *Giardia* cysts. Other procedures such as chlorine disinfection are not entirely effective as this does not always kill the cysts (Jarroll *et al.*, 1981; Tangtrongsup and Scorza, 2010). To avoid zoonotic transmission, management of animals should also be taken in the consideration, where animal housing must be effectively cleaned and disinfected regularly (Bomfim *et al.*, 2005; Maddox-Hyttel *et al.*, 2006).

1.3.4. Diagnosis

Monitoring outbreaks and efficient treatment programs requires accurate surveillance data. Several organizations in different countries (e.g. United States, Sweden, Japan, United Kingdom, Canada and some European Countries) have been set up to improve waterborne disease outbreak surveillance. In 1990, the National Notifiable Diseases Surveillance System (NNDSS) was created in Australia (Kirk *et al.*, 2003; Baldursson and Karanis, 2011).

Conventional light microscopy is the most common method employed for the detection of cysts and trophozoites of *Giardia* species. Identification is improved by use of staining to differentiate *Giardia* from other microorganisms, and/or after enrichment of the parasite in duodenal, faecal, tissue and water specimens (Ament and Rubin, 1972; Thornton *et al.*, 1983;

Adam, 1991; Wolfe, 1992; Zajac *et al.*, 2002; Schuurman *et al.*, 2007; Garcia, 2009). However, microscopy of trophozoites or cysts cannot be used to differentiate *Giardia* species (Adam, 2001; Koehler *et al.*, 2014).

1.3.5. Treatment

Multiple antimicrobial drugs (Table 2) are available for the treatment of *G. duodenalis* infection, but some cause side effects in patients and are not always effective due to the increasing development of drug resistance by the parasite. Up to 20% of cases have a recorded failure of treatment during first-line therapy (Abboud *et al.*, 2001; Upcroft and Upcroft, 2001; Watkins and Eckmann, 2014). Members of the nitroimidazole family such as metronidazole and tinidazole are the most common drugs used worldwide. Metronidazole is the first choice and is given in three divided oral doses daily (250 mg for 5 – 10 days), and efficacy has been reported at 80 - 95 % (Samuelson, 1999; Gardner and Hill, 2001; Watkins and Eckmann, 2014). Recently, the USA Food and Drug Administration (FDA) has approved tinidazole as the best choice of drug in the USA for giardiasis, due to its efficacy (90%), with few patient side effects and only requiring a single dose (Watkins and Eckmann, 2014).

Table 2: Classes of antimicrobial drugs used for the treatment of giardiasis (adopted from Watkins and Eckmann, 2014).

Compound class	Examples	Mechanism of action
5-Nitroimidazoles	Metronidazole, tinidazole	Adduction and protein/DNA inactivation
5-Nitrothiazoles	Nitazoxanide	Adduction and protein/DNA inactivation
5-Nitrofurans	Furazolidone	Adduction and protein/DNA inactivation
Acridins	Quinacrine	DNA intercalation, inhibition of DNA synthesis
Benzimidazoles	Albendazole, mebendazole	Tubulin binding, interference with cytoskeleton
Quinolines	Chloroquine	Unknown
Aminoglycosides	Paromomycin	Unknown; possibly inhibition of protein synthesis

1.4. Host-parasite interactions, pathophysiology and immunity

Trophozoites are responsible for the clinical signs and pathogenicity of symptomatic giardiasis and interact with three potential host barriers; the intestinal microbiota, the mucus layer and the

epithelial cells upon infection of the host gut (Adam, 2001; Einarsson *et al.*, 2016; Allain *et al.*, 2017). During an interaction between host cells and *G. duodenalis*, many factors may be synthesized and/or released by host cells and the parasite (Cotton *et al.*, 2011). Both parasite factors and the host immune response are proposed to play a key role in the pathogenesis and/or protective responses following infection with *G. duodenalis*, although the pathophysiological mechanisms underlying symptomatic giardiasis are not fully understood (Figure 3) (Cotton *et al.*, 2011; Geurden and Olson, 2011).

Pathophysiological changes following *G. duodenalis* infection, include intestinal barrier dysfunction, damage to the enterocyte epithelium with increased rates of apoptosis, increased intestinal chloride secretion, disaccharidase enzyme deficiencies and accelerated small intestine transit (Buret *et al.*, 1990; Scott *et al.*, 2002; Chin *et al.*, 2002; Panaro *et al.*, 2007; Koh *et al.*, 2013). The infection also leads to shortening of brush border microvilli with or without coinciding villous atrophy, deficiencies of luminal enzymes, malabsorption of electrolytes, fats, D-xylose, lactose, vitamin A, and vitamin B12 (Hjelt *et al.*, 1992; Buret, 2007; Cotton *et al.*, 2011). Furthermore, *G. duodenalis* trophozoites can alter enterocyte tight junctions and lead to breakage or re-arrangement of the proteins that form the junctions (Adam, 2001; Buret *et al.*, 2003; Buret and Cotton, 2011; Humen *et al.*, 2011; Cacciò, 2015). It was also shown that *G. duodenalis* causes increased eosinophilic infiltration in duodenal biopsies in children, suggesting that mucosal inflammation can be observed in these patients (Koot *et al.*, 2009). *G. duodenalis* trophozoites have been proposed to induce apoptosis in host cells (Panaro *et al.*, 2007; Koh *et al.*, 2013), cause intestinal barrier dysfunction and tight junction disruption (Chen *et al.*, 2013; Buret and Cotton, 2011; Maia-Brigagão *et al.*, 2012), T cell-mediated epithelial cell damage (Scott *et al.*, 2000, 2004) and effects on host microbiota (Chen *et al.*, 2013) (Figure 3).

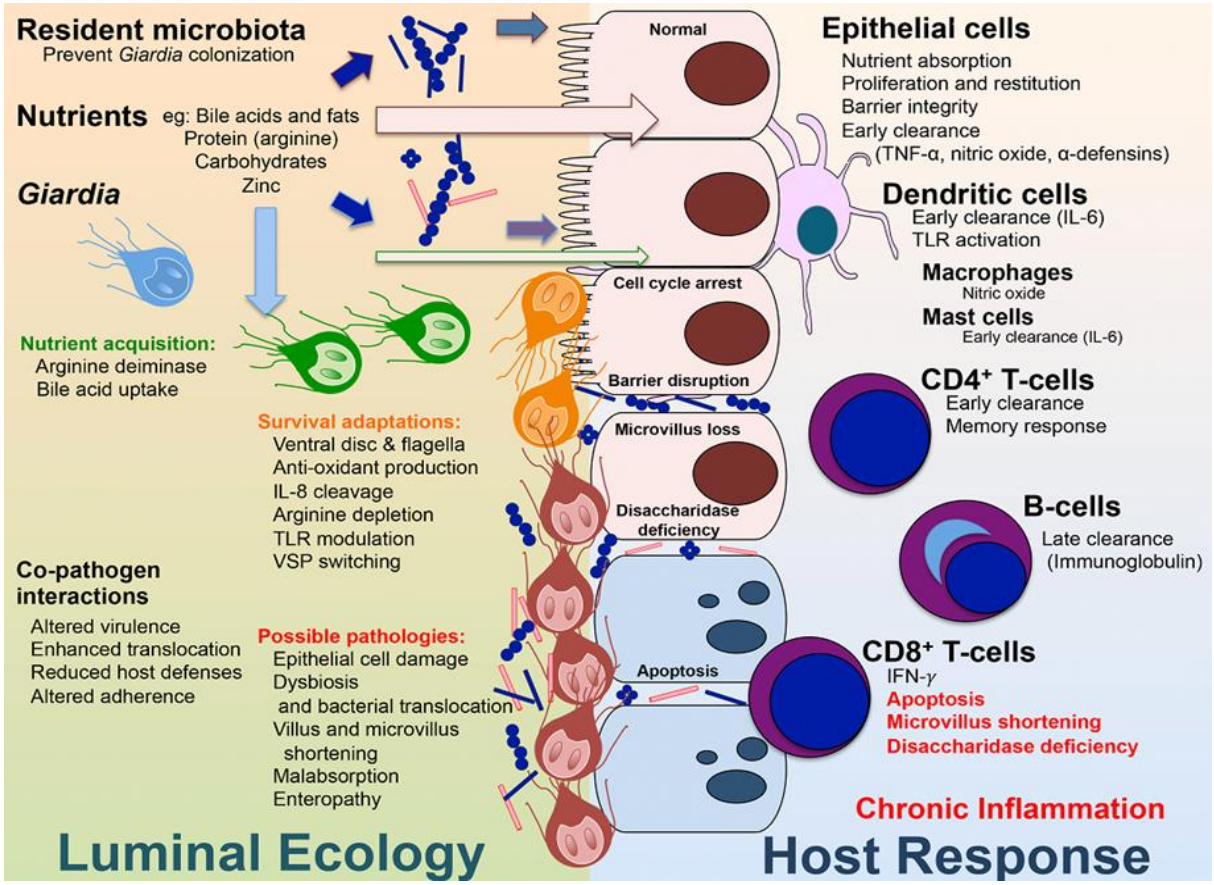


Figure 3: Complex interactions between microbiota, nutrients, *Giardia* strain, co-enteropathogens, and host molecular responses in the luminal and mucosal environment likely influence *Giardia* infectivity and disease outcomes. (Left) Resident microbiota impede colonization of *Giardia* parasites. *Giardia* uses, and potentially sequesters, nutrients such as bile, arginine and zinc in order to survive, replicate, and evade microbiota and host defenses. Flagella and the ventral disc aid attachment and adherence to intestinal epithelial cells (IEC). *Giardia* uses functional virulence factors to evade host inflammatory responses through antioxidant production, cleavage of interleukin-8 (IL-8), arginine depletion via arginine deiminase (ADI) and shifts in variant surface protein (VSP) expression. Effects of *Giardia* on epithelial cells (that is cell-cycle arrest, impaired proliferation, tight-junction disruption and apoptosis) may be strain dependent and either direct or indirect. Subsequent changes in nutrient availability, microbial composition, inflammatory defenses and epithelial cell pathogen attachment sites may secondarily alter disease manifestations of co-infecting enteropathogens. (Right) A range of mucosal immune responses promote *Giardia* clearance early (epithelial cell nutrient uptake for host fitness, barrier function maintenance and pro-inflammatory molecules; IL-6 derived from dendritic cells and mast cells; and CD4+ and CD8+ T cells) and later in disease progression (CD4+ T-cell memory and B cells). CD4+ T cells induce memory responses but also contribute to chronic inflammation and may promote disaccharidase deficiency. CD8+ T cells mediate apoptosis, microvillus shortening, and disaccharidase deficiency. Epithelial cell damage may persist beyond parasite clearance, allowing sustained translocation of microbiota and microbial products. The altered mucosal homeostasis and inflammation (enteropathy) and microbiota composition may further impede nutrient uptake and contribute to prolonged sequelae, including impaired growth and cognitive development. (Adopted from Bartelt and Sartor, 2015).

The occurrence and severity of *G. duodenalis* infection is, in part based on the strong attachment and high mobility of the trophozoite to the intestinal epithelial cells of the host (Cacciò, 2015). Attachment is established via a unique structure, the ventral adhesive disc and the flagella. Parasite surface molecules such as giardins (alpha, beta, delta and gamma) are involved in this attachment. In addition, a number of contractile proteins plays a vital role in trophozoite

adhesion although, the involvement of these molecules in pathogenesis and/or development of immunity by the host is not well-defined (Holberton *et al.*, 1988; Peattie *et al.*, 1989; Jiménez-Cardoso *et al.*, 2009).

Both innate and adaptive host immune responses are proposed to limit the pathogenesis of *Giardia* infection including phagocytosis by macrophages, secretion of defensins, nitric oxide (NO) and mucin by epithelial cells as well as the recruitment and activation of mucosal mast cells (Smith, 1985; Eckmann, 2003; Roxström-Lindquist *et al.*, 2006; Solaymani-Mohammadi and Singer, 2010). Early in *G. duodenalis* infection, host control is mediated by B-cell independent mechanisms that involve NO, ROS, defensins, lactoferrins, cytokines and immune cells such as mast cells, dendritic cells and phagocytes. This is followed by an antibody-dependent mechanism (Eckmann, 2003; Roxström-Lindquist *et al.*, 2006). *G. duodenalis* proteins such as variant-specific surface proteins (VSPs), the adhesive disk proteins and various secreted proteins are antigenic and can be distinguished by the sera of *Giardia*-infected humans or mice. In murine models, it has been suggested that IgA antibodies play an important role in protective immunity against *G. duodenalis* infection (Solaymani-Mohammadi and Singer, 2010). However, recovery from *G. duodenalis* infection has been shown even in mice that are unable to produce antibodies (Singer and Nash, 2000). Conversely, T-cells are important in the control of *G. duodenalis* infections, since T-cell–deficient mice develop chronic giardiasis (Singer and Nash, 2000).

Up-regulation of inflammatory cytokines (e.g. *IL-6, IL-8, IFN-γ, TNF-α, CCL20*) has not been observed in rat IEC-6 cell monolayers in response to *G. duodenalis* infections (Ma'ayeh, 2013). It has been suggested that the *FAU* and *TGFβ-1* genes inhibit such cytokine up-regulation which may explain the absence of inflammation during giardiasis (Ma'ayeh, 2013). In contrast, a significant upregulation in genes coding chemokines (CCL2, CCL20, CXCL1, CXCL2, and CXCL3) was shown in epithelial cells in response to *G. duodenalis* assemblage A (Roxström-Lindquist *et al.*, 2005) suggesting their role in the recruitment of immune cells to infection sites. *G. duodenalis* trophozoites have strategies to escape the effective immune response of the host. VSPs cover the surface of trophozoites and variation of these major antigens is a proposed mechanism for immune avoidance (Carranza and Lujan, 2010). Previous studies have shown no identical VSPs among all *G. duodenalis* assemblages (Franzén *et al.*, 2009; Jerlström-Hultqvist *et al.*, 2010) but a single VSP was found to be expressed on four *G. duodenalis* isolates

of different assemblages (GS/M, P-1, NF and WB) after incubation with murine intestinal epithelial cells. In addition, these isolates also up-regulated genes encoding high cysteine membrane proteins (HCMPs), oxygen defence proteins and glycolytic enzymes, suggesting their importance in the variety of symptoms and in virulence of the disease (Ma'ayeh and Brook-Carter, 2012).

A considerable amount of literature has been published on the participation or effect of *Giardia* on arginine metabolism, which interferes with host immune responses. NO is among the important antimicrobial products that are synthesised by intestinal epithelial cells, with potential microbiostatic and microbicidal effects on diverse pathogens, including giardiasis (Fang, 1997; Fernandes and Assreuy, 1997; Tako *et al.*, 2013). *G. duodenalis* can enzymatically catabolise and use the host metabolite arginine (the precursor for NO production by the host) as its main energy source, leading to inhibition of host NO production (Edwards *et al.*, 1992; Knodler *et al.*, 1994; Eckmann *et al.*, 2000; Stadelmann *et al.*, 2012). Three enzymes in *Giardia's* arginine catabolism include; arginine dihydrolase, arginine deiminase (ADI) and ornithine carbamoyltransferase (OCT) (Schofield *et al.*, 1990; Ringqvist *et al.*, 2008). Secretion of ADI, OCT and enolase on contact with host epithelial cells inhibits host innate immune responses, including the production of nitric oxide (NO) (Ringqvist *et al.*, 2008; Rópolo and Touz, 2010). In addition, up-regulation of the NO-degrading parasite protein, flavohemoglobin was detected in response to host NO production and T-cell proliferation (Stadelmann *et al.*, 2013). The corollary of these findings is that NO production by the host is as an important molecule in host defense against *Giardia* infection (Stadelmann *et al.*, 2013).

Tissue repair or restitution, via regulatory peptides, growth factors and cytokines (including TGF-β and insulin-like growth factor II) plays a key role in the recovery from superficial epithelial tissue damage, contributing to the integrity of epithelial cell-cell contact via a rapid self-sealing process (Mammen and Matthews, 2003; Krafts, 2010; O'Dell and Day, 1998; Sturm and Dignass, 2008). TGFβ-1 is considered one of the main growth factors in the gastrointestinal tract and has a critical role on mucosal healing of the small intestine (Yamada *et al.*, 2013). The TGF-β-dependent pathway, promotes several growth factors and cytokines, including EGF, VEGF, HGF, GLP-2, various FGF peptides, IL-1, IL-2 and IFN-γ which promote epithelial cell repair (Dignass *et al.*, 1994; Bulut *et al.*, 2006; Sturm and Dignass, 2008). Restitution of rat intestinal epithelial cell monolayers was characterized by the up-regulation of gene transcripts

involved in tissue repair, including those encoding proteins of the extracellular matrix (ECM) and the cell cytoskeleton (Ma'ayeh, 2013). Further investigations are needed to better identify and understand the role of host responses in repair and recovery.

Although limited studies have been carried out to date, the proceeding section highlights there are significant changes in secreted proteins and gene expression levels in *G. duodenalis* trophozoites and in host intestinal epithelial cells exposed to these parasites, which require further detailed studies (Müller *et al.*, 2008; Ringqvist *et al.*, 2008; Ringqvist *et al.*, 2011; Li *et al.*, 2012; Stadelmann *et al.*, 2013; Cotton *et al.*, 2014a; Raj *et al.*, 2014).

1.5. Key pathological consequences of *G. duodenalis* infection on the host

1.5.1. Intestinal epithelial cell apoptosis

Programmed cell death (apoptosis) is an important mechanism in animal development and throughout life. It is a process for safely eliminating individual cells to control cell numbers (homeostasis) and for fighting infections and cancer (Hall *et al.*, 1994). Sequential activation of proteolytic cysteinyl asparate proteases (caspases) is considered a significant modulator in programmed cell death (Elmore, 2007). These caspases are divided into two subgroups: initiator caspases (caspase-2, -8, -9 and -10) and executioner caspases (caspase-3, -6 and -7) (Figure 4) (Lüthi and Martin, 2007).

Apoptosis is initiated through two activation mechanisms, the intrinsic (mitochondria-mediated) pathway and the extrinsic (receptor-mediated) pathway (Elmore, 2007; Fuchs and Steller, 2015). The intrinsic pathway begins via intracellular signals generated when cells are stressed and the release of pro-apoptotic proteins from the mitochondria, leading to activation of caspase proteases, which trigger apoptosis (Degterev *et al.*, 2003; Fulda *et al.*, 2010). There are two main branches in the extrinsic pathway, tumor necrosis factor (TNF)-initiated apoptosis and Fas-Fas ligand-mediated apoptosis (Zhang *et al.*, 2016). The activation of death receptors (DRs) and binding to their ligands leads to recruitment and activation of the adaptor molecule Fas-associated death domain (FADD) and procaspase-8, which then induce apoptosis through direct cleavage of downstream effector caspases (Niederfellner, 2012). Additionally, several proteins are essential players in the apoptosis pathway, including: c-Jun N-terminal kinase

(JNK); mitogen-activated protein kinase kinase (MEKK); p53 upregulated modulator of apoptosis (Puma); TNF receptor-associated death domain protein (TRADD); TNF receptor-associated factor 2 (TRAF2); tumor necrosis factor receptor 1 (TNFR1); apoptotic protease activating factor-1 (Apaf-1); B-cell lymphoma-2 (Bcl-2); BH3 interacting domain death agonist (Bid); second mitochondria-derived activator of caspase (Smac/DIABLO); cellular inhibitor of apoptosis proteins (cIAPs); death-inducing signaling complex (DISC) [Figure 4] (Zhang *et al.*, 2016).

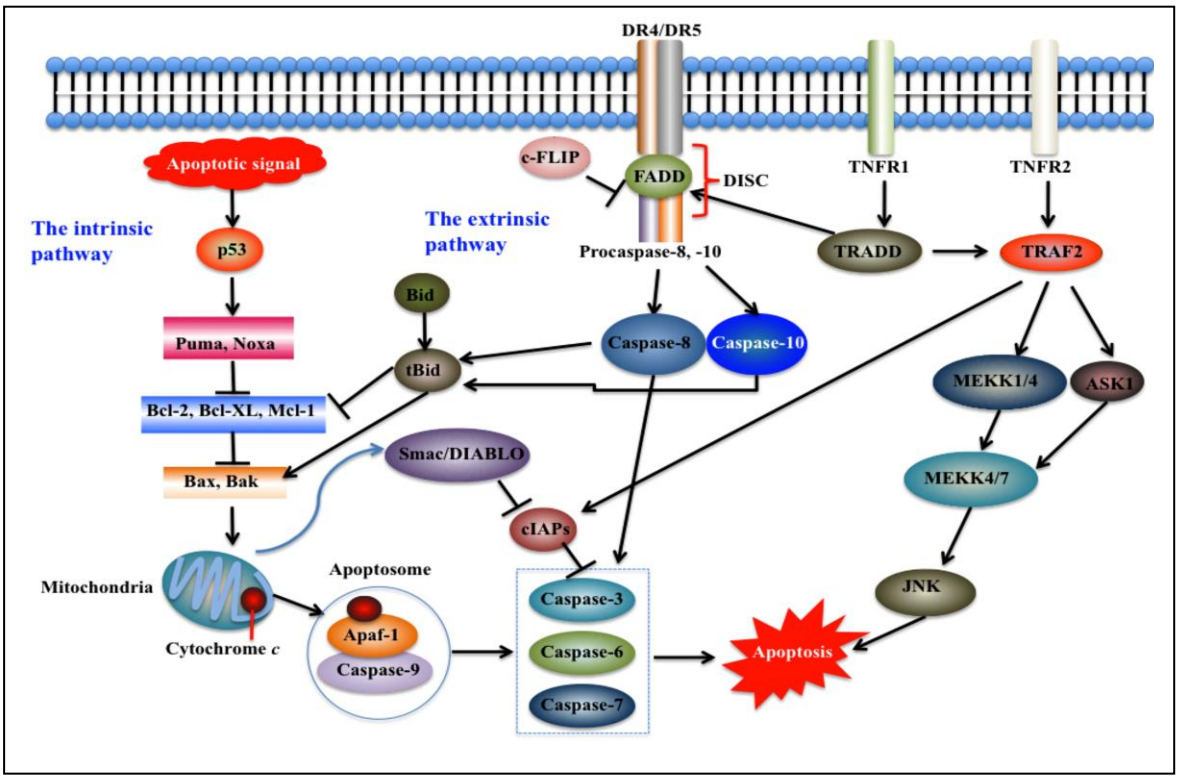


Figure 4: Intrinsic and extrinsic pathways of apoptosis in host cells. Adopted from (Zhang *et al.*, 2016).

It has been suggested that *G. duodenalis* causes caspase-3-dependent apoptosis which is proposed to be a pathogenic mechanism in giardiasis, in that the death of intestinal epithelial cells increases the permeability of the epithelial barrier thus leading to the production of leaky flux diarrhoea (Chin *et al.*, 2002; Panaro *et al.*, 2007; Cotton *et al.*, 2011; Koh *et al.*, 2013). Variation in the mediation of apoptosis between parasite isolates has been noted, as have differences in the pathogenicity between assemblages A and B in clinical disease, suggesting an association between apoptosis and the severity of clinical disease (Chin *et al.*, 2002; Haque *et al.*, 2005; Panaro *et al.*, 2007; Breathnach *et al.*, 2010; Yang *et al.*, 2010; Koh *et al.*, 2013; Asher *et al.*, 2014). In previous *in vitro* studies, apoptosis was detected in epithelial cells after 24hrs and 48hrs co-culture with *Giardia* NF and S2 isolates, but apoptosis was not observed

following co-cultures with WB or PB isolates (Chin *et al.*, 2002). Another study suggested that *Giardia*-induced apoptosis in the intestinal epithelial cell line HCT-8 through DNA fragmentation, while western blotting analysis showed down-regulation of Bcl-2 and up-regulation BAX after 16hrs of interaction (Panaro *et al.*, 2007). Additionally, mixed *Giardia* isolates from assemblages A, B and E induced apoptosis in epithelial cells after 24hrs co-culture, but surprisingly, co-culture of cells with assemblages A or B alone did not induce apoptosis (Koh *et al.*, 2013). Furthermore, increased epithelial apoptosis was observed in 13 duodenal biopsies isolated from patients with chronic giardiasis (Troeger *et al.*, 2007). It was proposed that physiological changes in human derived enteric cells were mediated by secreted proteins of *G. duodenalis* trophozoites. Such proteins could have an impact in host-pathogen interactions, as well as be potential markers for virulence strain differentiation (Dubourg, 2014). Interestingly, co-incubation of host cells with sonicated parasites induced apoptosis of non-transformed human duodenal epithelial cells suggesting proteins on the surface or in the excretory/secretory products (ESP) released from the parasite could mediate apoptosis (Chin *et al.*, 2002). Although both host and parasite factors may mediate apoptosis in host cells, the apoptosis inducing factors (AIFs) involved remain undefined (Cotton *et al.*, 2011).

Significant apoptosis in calves infected with assemblage E has been demonstrated (Dreesen *et al.*, 2012). It was postulated that sodium-glucose linked transporter-1 (SGLT-1)-mediated glucose uptake played a vital role in preventing lipopolysaccharide (LPS)-induced apoptosis and epithelial barrier disorders by increasing concentrations of anti-apoptotic proteins (Yu *et al.*, 2005). Another study also suggested that *Giardia*-induced apoptosis was inhibited in Caco-2 cells via the SGLT-1-dependent glucose uptake mechanism in the presence of high glucose (Yu *et al.*, 2008).

G. duodenalis-mediated cell apoptosis is incompletely understood and may vary between isolates and/or assemblages. Taken together, experimental studies to date suggest *G. duodenalis*-induced apoptosis in host cells is variable and many extrinsic factors may play a role, such as the period of co-culture or the *G. duodenalis* assemblages or their isolates and the type of host cells used (Fisher *et al.*, 2013).

1.5.2. Epithelial barrier function

The intestinal epithelium consists of a single-cell layer that acts as a selectively permeable barrier between the intestinal lumen and the external environment. It functions to transport and absorb nutrients, electrolytes and water, as well as playing a vital role inhibiting the entry of antigens, enteric flora and toxins. The barrier mechanically connects neighbouring cells and seals the intracellular space via a network of proteins forming three adhesive complexes; tight junctions (TJ), adherens junctions (AJ) and desmosomes as shown in Figure 5 (Groschwitz and Hogan, 2009; Suzuki, 2013). TJ and AJ collectively form the Apical Junctional Complex (AJC). The AJC consists of the tight junction, which includes interactions between proteins such as claudins, zonula occludens 1 (ZO1), occludin and F-actin. While E-cadherin, α-catenin, β-catenin and F-actin interact to form the adherens junctions. Desmosomes are formed by interactions between desmoglein, desmocollin, desmoplakin and keratin filaments and their location is beneath the apical complex (O'Hara and Buret, 2008; Turner, 2009; Ulluwishewa *et al.*, 2011).

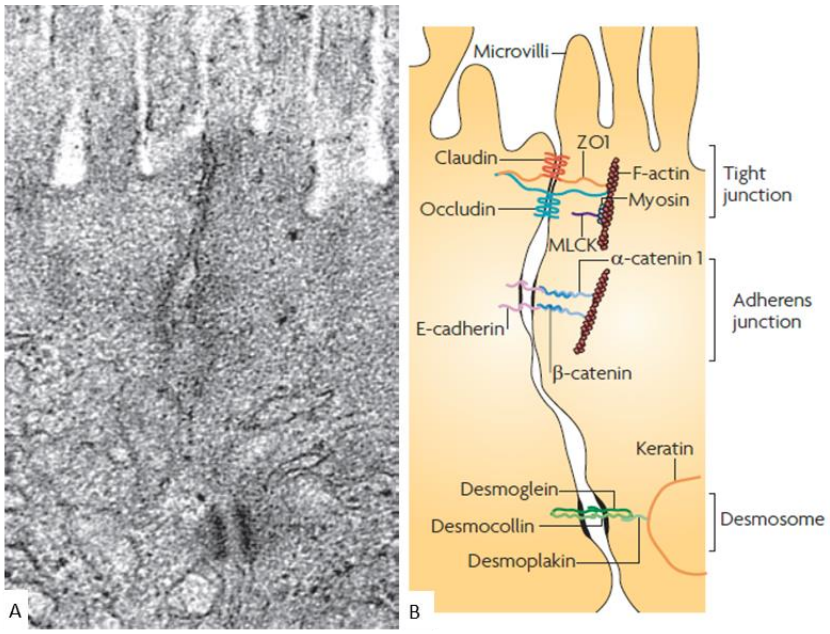


Figure 5: A- an electron micrograph of the junctional complex of an intestinal epithelial cell. B- Line drawing of the junctional complex of an intestinal epithelial cell. Below the base of the microvilli, the tight junction consists of claudins, zonula occludens 1 (ZO1), occludin and F-actin interact. The adherens junction is formed by E-cadherin, α-catenin 1, β-catenin, catenin δ1 and F-actin interact. Myosin light chain kinase (MLCK) is associated with the perijunctional actomyosin ring. Desmosomes are formed by interactions between desmoglein, desmocollin, desmoplakin and keratin filaments. Adopted from (Turner, 2009).

In the gastrointestinal tract the proteins comprising the AJC function to separate the intestinal lumen from underlying host tissues (Shen, 2012). In giardiasis, it has been suggested that

changes or dysfunction of AJC leads to alterations of trans-membrane permeability resulting in a diarrheal efflux (Chin *et al.*, 2002; Scott *et al.*, 2002; Troeger *et al.*, 2007; Maia-Brigagão *et al.*, 2012). Nevertheless, understanding whether the direct effects of different *Giardia* isolates or indirect parasite effects cause alterations in host luminal dynamics and mucosal homeostasis is still critical (Bartelt and Sartor, 2015).

Chin *et al*. (2002) investigated the possible relationship between enterocyte apoptosis and the increased permeability of epithelial cell membranes induced by the *Giardia* isolate NF. In this work, the authors found NF induced enterocyte apoptosis within the monolayers and damaged tight junction ZO-1 proteins; however, pre-treatment of host cells by a caspase-3 inhibitor prevented these disorders. Thus, in a caspase-3-dependent manner, *Giardia* NF had a negative impact on tight junction ZO-1 and increased permeability as well as causing a loss of epithelial barrier function (Chin *et al.*, 2002).

Furthermore, there was a down-regulation of claudin-1 and increased epithelial apoptosis from duodenal biopsy specimens of patients with chronic giardiasis (Troeger *et al.*, 2007). While a previous study found that trophozoites promoted an adhesion dependent decrease in transepithelial electrical resistance (TER) accompanied by a rearrangement of occludin, and delocalization of claudin-1 (Humen *et al.*, 2011). Another study using laser scanning confocal microscopy, 3D reconstruction, showed that the WB strain caused a rearrangement of junction proteins (tight and adherens) in Caco-2 cells, although immunoblotting assays did not show changes in the expression of these proteins (Maia-Brigagão *et al.*, 2012). Additionally, *Giardia* infection causes modifications of the apical TJ, including disruption of ZO1, cytoskeletal filamentous (F)-actin, claudin-1, and α-actinin, which lead to increased intestinal permeability (Scott *et al.*, 2002; Troeger *et al.*, 2007; Cotton *et al.*, 2011). A recent *in-vitro* study suggested that *G. duodenalis* assemblages A, B, and E cause disruption of epithelial TJ and induce different degrees of apoptosis in transformed human epithelial cells (Koh *et al.*, 2013).

The epithelial barrier function can be influenced through the activation and interference of various signalling molecules such as protein kinase C (PKC), mitogen-activated protein kinases (MAPKs), myosin light chain kinase, protein kinase A (PKA), phosphoinositide 3-kinase (PI3-kinase) and the Rho kinase family (González-Mariscal *et al.*, 2008). TJ barrier integrity is regulated by the interaction of TJ with the actomyosin ring (Madara, 1987). Myosin light chain

kinase (MLCK) is a critical mediator of tight junction barrier function, as it induces contraction of the perijunctional actomyosin ring through myosin II regulatory light chain phosphorylation as such it has a significant role in the small intestine epithelial barrier function (Chen *et al.*, 2012; Cunningham and Turner, 2012). It has also been proposed that increased epithelial permeability occurs as a result of the activation of MLCK by *Giardia* trophozoites and that MLCK phosphorylates the myosin light chain (MLC) and disrupts cytoskeletal and tight junction elements in enterocytes (Scott *et al.*, 2002).

Rho family GTPases regulate the reorganisation of the actin cytoskeleton (Raftopoulou and Hall, 2004). The protein encoded by the *RHOA* gene is considered a core molecule involved in actin stress fibres induction which regulates cell adhesion via reconstructing the cytoskeleton in response to extracellular stimuli (Ridley and Hall, 1992; Bishop and Hall 2000). More importantly, reorganisation of actin cytoskeleton is mediated by Rho-ROCK signaling that activates LIM kinase and stabilises actin filaments through the phosphorylation and inactivation of cofilin which is necessary for actin filament turnover (Maekawa *et al.*, 1999; Du *et al.*, 2016). Previous data suggested that caspase-3 may cause cleavage of both MLCK and ROCK in apoptotic cells, leading to the relocation of F-actin and ZO-1 (Mills *et al.*, 1998; Coleman *et al.*, 2001; Sebbagh *et al.*, 2001). In giardiasis, it remains to be defined whether *G. duodenalis* trophozoites catalyse the Rho kinase (ROCK)-mediated disruption of intestinal epithelial tight junctional proteins or if this process is also dependent upon caspase-3 (Cotton *et al.*, 2011). In addition, investigating whether the activation of ROCK or MLCK is mediated by *G. duodenalis* trophozoites or their parasitic products, either via caspase-3 dependent or independent mechanisms, could be important in understanding the mechanisms that mediate the disorders in the structure and function of epithelial tight junctions (Troeger *et al.*, 2007; Buret and Cotton, 2011).

1.6. Studying the host-*Giardia* relationship

Despite these studies showing that barrier and intestinal permeability disorders can be explained by both apoptosis and changes in the production and degradation of junctional proteins at cell-cell junctions, it is still not understand which is more important to the parasite or host and how these processes are regulated (Chin *et al.*, 2002; Troeger *et al.*, 2007; Förster, 2008; Groschwitz and Hogan, 2009; Humen *et al.*, 2011; Maia-Brigagão *et al.*, 2012). Further studies are required

to provide insights into *Giardia's* effects on the host intestinal epithelial barrier and the timing of these effects relative to that of apoptosis. The following sections highlight the current model systems used to study giardiasis and the most promising techniques available to study host pathophysiological protein and gene modulation.

1.6.1. *In vivo*/*in vitro* models

(i) Animal models: Over recent decades, *in vivo* models, such as mice and gerbils, have been used to study *G. duodenalis*/host cells interactions (Fernandes and Assreuy, 1997; Scott *et al.*, 2002; Bienz *et al.*, 2003; Zhou *et al.*, 2003; Li *et al.*, 2004; Chen *et al.*, 2013; Reynoso-Robles *et al.*, 2015). However, limitations have been observed in using these models. In a mouse model, most clinical *Giardia* isolates can cause infection in neonatal mice (Hill *et al.*, 1983), while only one isolate (H7/1 clone of GS) was able to infect adult mice (Byrd *et al.*, 1994). However, it has been shown that the neonatal mouse model is a robust model for the pre-clinical development of antigiardial agents (Abraham *et al.*, 2018).

Gerbils have also been suggested to be an experimental model for investigating the development and pathogenesis of *Giardia* infections, as a result of their high susceptibility to infection (Belosevic *et al.*, 1983; Amorim *et al.*, 2010). Most recently, a study using a gerbil model found that trophozoites invaded the intestinal mucosa and submucosa of infected gerbils, suggesting this finding may help clarify *G. duodenalis* pathogenic mechanisms (Reynoso-Robles *et al.*, 2015). Nevertheless, lack of genetic information in gerbils, as well as antibody reagents against defined gerbil proteins will be an impediment for this model.

(ii) *Ex-situ*/ *In vitro models: Giardia* trophozoites have been axenically cultured for over four decades (Karapetyan, 1962). In 1978, TYI-S-33 medium was manufactured for *Entamoeba* and trichomonad species (Diamond *et al.*, 1978), and that medium was suggested to support axenic growth of *Giardia* trophozoites (Visvesvara *et al.*, 1980). Later, modifications were added to Diamond's medium to improve axenic culture of *G. duodenalis*, and this medium was named "complete modified TYI-S-33" (Keister, 1983). Modified TYI-S-33 is commonly used to propagate most *Giardia* strains/species (Davids and Gillin, 2011). *In vitro*, the *G. duodenalis* isolates used for investigation of giardiasis are GS/M, isolated in the USA from a patient who had "typical symptoms" of giardiasis (Nash *et al.*, 1987), the WB isolate, isolated from a patient with chronic giardiasis who failed to respond to several courses of metronidazole treatment

(Smith *et al.*, 1982), the P-1 isolate, isolated from a patient with "typical symptoms" of giardiasis (Meyer, 1976) and the NF isolate, isolated from drinking water during a *G. duodenalis* outbreak in Newfoundland in 1991 (Yanke *et al.*, 1998). To date, most studies have used these isolates to investigate *Giardia* biology and gain a better comprehension of the disease process (Buret *et al.*, 1990; Chin *et al.*, 2002; Roxström-Lindquist *et al.*, 2005: Ringqvist *et al.*, 2008; Ma'ayeh and Brook-Carter, 2012; Stadelmann *et al.*, 2013).

In vitro cultures of these isolates incubated with cell lines have been used to study the cellular and molecular mechanisms by which *G. duodenalis* alter the structure and function of host cells (Plutzer *et al.*, 2010; Duell *et al.*, 2011; Liévin-Le Moal, 2013). Several types of cell lines have been used including, human colonic adenocarcinoma cell lines (Caco-2) (Chin *et al.*, 2002; Roxström-Lindquist *et al.*, 2005; Müller *et al.*, 2006; Fisher *et al.*, 2013; Stadelmann *et al.*, 2013), Human ileocecal adenocarcinoma HCT-8 (Panaro *et al.*, 2007), and rat intestinal epithelial cells (IEC-6) (McCabe *et al.*, 1991; Ringqvist *et al.*, 2008, 2011; Ma'ayeh and Brook-Carter, 2012). The IEC-6 is a non-transformed cell line derived from rat intestinal epithelium, which differentiates in culture after 3 or 4 days in a manner similar to that *in vivo* (Quaroni *et al.*, 1979; Ametani *et al.*, 1993; Ametani *et al.*, 1996). Other cell lines (i.e. Caco-2 and HCT-8) are ileocecal adenocarcinoma cells requiring 14-21 days after confluence to differentiate the morphologic characteristics of normal enterocytes (Barbat *et al.*, 1998; Engle *et al.*, 1998; Roxström-Lindquist *et al.*, 2005; Panaro *et al.*, 2007; Lea, 2015).

1.6.2. Host response analysis

In general, protein or gene expression responses have been used to study the host-*Giardia* relationship (Emery *et al.*, 2015; Ansell *et al.*, 2016, 2017; Ma'ayeh *et al.*, 2015; 2017). Gene expression analysis conducted by several high-throughput methods has been used to identify the dysregulation in host molecular mechanisms during various infections and pathogenic conditions (Wu *et al.*, 2013). A variety of these techniques (hybridization or sequence-based technologies) have been applied to quantify and deduce differential gene expression in giardiasis, such as representational difference analysis (RDA), microarrays, serial analysis of gene expression (SAGE), quantitative reverse transcription PCR (qRT-PCR), and RNA sequencing (RNA-seq) (Roxström-Lindquist *et al.*, 2005; Ringqvist *et al.*, 2011; Ma'ayeh and Brook-Carter, 2012; Stadelmann *et al.*, 2013; Cotton *et al.*, 2014a; Raj *et al.*, 2014; Emery *et al.*, 2015; Maloney *et al.*, 2015; Ansell *et al.*, 2017; Ma'ayeh *et al.*, 2017).

qRT-PCR has been commonly used to quantify and analyse messenger RNA (mRNA) expression patterns obtained from different biological conditions, showing considerable sensitivity, and sequence-specificity with a large dynamic range (Jozefczuk and Adjaye, 2011). It can also be shown that this technique is able to produce a large amount of data during short times using small amounts of RNA (Ginzinger, 2002). However, using qRT-PCR for comparing levels across different experiments requires complicated normalization methods (Costa *et al.*, 2013). In addition, variations in the quality of qRT-PCR data can make the technique time consuming with more costs for replicating the analysis (Costa *et al.*, 2013; Bustin, 2010). It continues to be the most common technique for validating gene expression profiles generated from different assays, such as RNA-seq (Wang *et al.*, 2009; Thornton and Basu, 2011; Ma'ayeh *et al.*, 2017).

RNA sequencing (RNA-seq) is the latest sequence-based technology that has made a revolution in transcriptomics investigations, where it potentially provides full-genome coverage compared to microarray and tag-based techniques (Wang *et al.*, 2009). This approach (Figure 6) relies on next-generation sequencing (NGS) platforms, where cDNA is prepared from RNA molecules by reverse transcription. Subsequently, single-end or pair-end sequencing is applied in a high-throughput manner using a variety of sequencing systems such as Illumina (Marioni *et al.*, 2008; Mortazavi *et al.*, 2008), Applied Biosystems SOLiD (Cloonan *et al.*, 2008) and Roche 454 life Science (Emrich *et al.*, 2007; Vera *et al.*, 2008). Following the sequencing reaction, a reference genome is needed to map the obtained sequence reads in order to conclude the structure and/or expression state of a transcript (Westermann *et al.*, 2012). The RNA-seq technology provides several significant advantages compared to other existing methods. For instance, it is not limited to detecting transcripts that match to an annotated genomic sequence (Vera *et al.*, 2008; Wang *et al.*, 2009). Another advantage is that sequence variations, including single-nucleotide polymorphisms (SNPs) in the transcribed regions can also be identified using this technology (Cloonan *et al.*, 2008). Compared to the qRT-PCR, it has been found to be highly accurate for quantifying expression levels (Nagalakshmi *et al.*, 2008). Equally importantly, this technology reveals results with high levels of reproducibility (Cloonan *et al.*, 2008; Nagalakshmi *et al.*, 2008). Collectively, RNA-seq is considered the first sequencing technique that has a very high-throughput and is quantitative enabling examination of the entire transcriptome, while providing expression analysis and single-base resolution for annotation (Wang *et al.*, 2009). More recently, this technology has been used for the investigation the effects of *Giardia*

trophozoites on host cells (Ansell *et al.*, 2016, 2017; Ma'ayeh *et al.*, 2015; Ma'ayeh *et al.*, 2017).

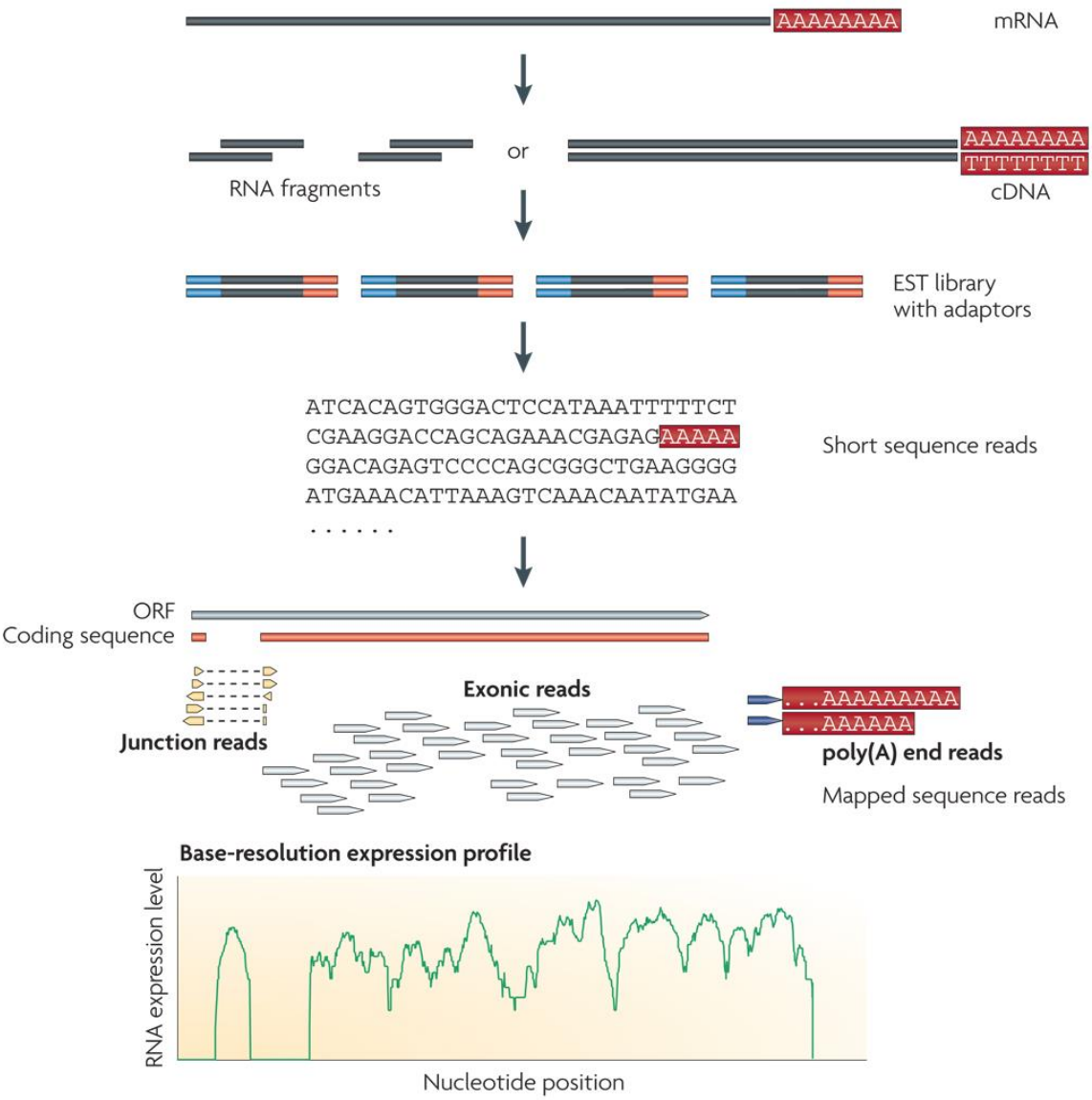


Figure 6: A typical RNA-seq experiment work-flow chart. Briefly, long RNAs are first converted into a library of cDNA fragments through either RNA fragmentation or DNA fragmentation (see main text). Sequencing adaptors (blue) are subsequently added to each cDNA fragment and a short sequence is obtained from each cDNA using high-throughput sequencing technology. The resulting sequence reads are aligned with the reference genome or transcriptome, and classified as three types: exonic reads, junction reads and poly (A) end-reads. These three types are used to generate a base-resolution expression profile for each gene, as illustrated at the bottom; a yeast ORF with one intron is shown. (Adopted from Wang *et al.*, 2009).

1.7. Unknown factors in *G. duodenalis* pathogenesis

Recent research has extended our understanding of *G. duodenalis*/host interactions but a clear understanding of the pathogenesis of the disease remains incompletely understood (Ankarklev

et al., 2010; Cotton *et al.*, 2011; Di Genova and Tonelli, 2016; Allain *et al.*, 2017). For instance, recent genomic and proteomic studies have shown significant variations among assemblage A isolates (Ankarklev *et al.*, 2015; Emery *et al.*, 2015), but it is not known how these variations are significant in terms of their effects on host cells (Buret *et al.*, 2015).

The mechanisms by which *G. duodenalis* causes disruption to the intestinal epithelial barrier (Allain *et al.*, 2017) are uncertain and while the same is true of apoptosis (Chin *et al.*, 2002; Panaro *et al.*, 2007; Koh *et al.*, 2013), it remains uncertain of the importance of each of these mechanisms in pathogenesis. *Giardia*-induced apoptosis in host cells is also variable and many extrinsic factors may play a role such as the period of co-culture or the infection, *G. duodenalis* assemblages or their isolates used and type of host cells (Fisher *et al.*, 2013). It has been postulated that intestinal epithelial cell apoptosis can be modulated by host and parasite factors but it remains unclear which parasite/host products are responsible (Cotton *et al.*, 2011). These differences may relate to the variable clinical findings for these different isolates but there are few correlations between pathogenic mechanisms and clinical outcomes currently known (Allain *et al.*, 2017).

Innate and adaptive immunity are key processes for the induction of effector mechanisms for control of giardiasis presumably by the induction of host's pro-inflammatory cytokines (Cotton *et al.*, 2015). However, despite increased levels of pro-inflammatory cytokines, little or no inflammation was shown during *G. duodenalis* infection (Jung *et al.*, 1995; Oberhuber *et al.*, 1997; Eckmann *et al.*, 2000; Scott *et al.*, 2004; Cotton *et al.*, 2015; Lopez-Romero *et al.*, 2015). It is also unclear how *G. duodenalis* antigens can induce a protective immune response or how *G. duodenalis* infection may break mucosal tolerance (Lopez-Romero *et al.*, 2015). For example, does the change in host mucosal homeostasis occur due to a direct physical attachment of trophozoites or indirectly through their ESP (Bartelt and Sartor, 2015; Di Genova and Tonelli, 2016).

The clinical symptoms of giardiasis are the result of a combination of both host and parasite factors (Cotton *et al.*, 2011). Key host responses to early infection are likely to determine host protective or disease exacerbation outcomes. These early responses are poorly defined and would be of likely value in understanding host/parasite interaction and clinical disease outcome.

This thesis attempted to gain a more comprehensive insight into early host gene responses of epithelial cells to *G. duodenalis*.

1.8. Aims of study

This thesis attempted a comprehensive analysis of the host cell responses using RNA-seq technology and targeted confirmation of selective gene responses using qRT-PCR to improve our understanding of the parasite's effects on the host and the timing of those effects in the first few hours of interaction. Three hypotheses were addressed: (i) to analyse the differential effects of each parasite isolate on the initial host cell response, (ii) the effect of parasite isolates on epithelial cell integrity and apoptosis and finally, (iii) whether the physical presence of trophozoites or just their products were necessary to modulate host epithelial cell integrity or apoptosis.

Specifically:

Hypothesis 1: Is there a variation in host cell responses during early infection with different *G. duodenalis* isolates of assemblage A?

The hypothesis was investigated as follows;

a) Co-culture of IEC-6 with two *G. duodenalis* isolates (P-1 or WB) for 2 and 6hrs.
b) Broad transcriptome profiling and cellular pathway analysis using RNA-seq analysis.

Hypothesis 2: Does intestinal epithelial cell dysfunction occur in early infection because of either direct physical interaction or indirect interaction of excretory/secretory products (ESP) of *G. duodenalis* isolates, or a combination of both?

The hypothesis was investigated as follows;

a) Co-culture of IEC-6 with two *G. duodenalis* isolates (P-1 or WB) for 2 and 6hrs, followed by,
b) Transcriptome profiling of genes involved in the integrity of the intestinal epithelial barrier in IEC-6 using RNA-seq data.
c) Co-culture of IEC-6 with each *G. duodenalis* isolate (GS/M, NF, P-1 or WB) and/or ESP for 2 and 6hrs, followed by,

d) Quantification of specific genes involved in the integrity of the intestinal epithelial barrier in IEC-6 using qRT-PCR.

Hypothesis 3: Do host intestinal epithelial cells undergo apoptosis during early infection because of either direct physical interaction or interaction of excretory/secretory products (ESP) of *G. duodenalis* isolates, or a combination of both?

The hypothesis was investigated as follows;

a) Co-culture of IEC-6 with two *G. duodenalis* isolates (P-1 or WB) for 2 and 6hrs, followed by,
b) Transcriptome profiling of genes involved in the apoptotic gene pathway in IEC-6 using RNA-seq data.
c) Co-culture of IEC-6 with each *G. duodenalis* isolate (GS/M, NF, P-1 or WB) and/or ESP for 2 and 6hrs, followed by,
d) Quantification of specific genes involved in the apoptotic pathway in IEC-6 using qRT-PCR.
e) Detection of apoptosis in IEC-6 using a biochemical assay (cell death detection).

Chapter 2

Materials and Methods

Chapter 2: Materials and Methods

2.1. Propagation of cells, parasites and collection of parasite ESP

2.1.1. Propagation of IEC-6 cell monolayer cultures

The non-transformed rat Intestinal Epithelial Cell line (IEC-6) (ECACC, Sigma-Aldrich, catalogue number 88071401, passage number 29) was used throughout these studies. IEC-6 is a characteristic intestinal epithelial cell line used in numerous *Giardia*/host cell interaction studies (McCabe *et al.*, 1991; Ortega-Barria *et al.*, 1994; Hausen *et al.*, 2011; Ma'ayeh and Brook-Carter, 2012). The cells are used as they show morphologic, immunologic, and functional features similar to intestinal epithelial cells *in-vivo* and are a non-transformed cell line (Quaroni *et al.*, 1979; Quaroni, 1985; Conteas and Majumdar, 1987; Ametani *et al.*, 1993; Ametani *et al.*, 1996).

IEC-6 was cultured to confluence in Dulbecco's Modified Eagles Medium (DMEM) supplemented with L-glutamine (29.2mg/ml), antibiotics (100U penicillin, 10µg streptomycin) and 10% Fetal Calf Serum (FCS) (Life Technologies) in 25cm^2 Corning® canted neck, vented cap cell culture flasks (Sigma- Aldrich MO, USA). Cell cultures were maintained under standard conditions (37°C, 5% CO_2). The number and viability of cells were evaluated by phase-contrast light microscopy using the trypan blue assay according to the manufacturer's instructions (Sigma Chemical Co, USA). Where viability of culture was >90% cells were used for co-culture experiments as described previously. Cells were also stored in liquid nitrogen (LN) in labelled cyto-protective ampules (3 x10^6 cell/ml) using a freezing medium protocol (Sigma Chemical Co, USA) for subsequent experiments.

2.1.2. Axenic cultures and harvesting of *Giardia* trophozoites

Four (4) *G. duodenalis* isolates were purchased from the American Type Culture Collection (ATCC), including NF (ATCC® 203332™), P-1 (ATCC® 30888™) and WB (ATCC® 30957™) all assemblage A; and GS/M (ATCC® 50580™) assemblage B, which were maintained in the laboratory for use in experiments. The NF isolate is described as having been cultured from drinking water during a *Giardia* outbreak in Newfoundland in 1991 (Yanke *et al.*, 1998). The P-1 isolate cultured from a patient reported to have typical symptoms of giardiasis (Meyer,

1976). The WB isolate was cultured from a patient with chronic giardiasis who failed to respond to several courses of metronidazole treatment (Smith *et al.*, 1982). Finally, the GS/M isolate was cultured from a patient reported to have typical symptoms of giardiasis in the USA (Nash *et al.*, 1987).

Each isolate was propagated axenically in TYI-S-33 Keister's medium (Life Technologies) supplemented with penicillin (100 U/ml), streptomycin (100 mg/ml) and gentamycin (50µg/ml), as previously described (Keister, 1983; Davids and Gillin, 2011), in 50ml conical tubes (Becton Dickinson, NJ, USA). Briefly, following three to four days of the incubation (at 37°C, 5% CO_2), *G. duodenalis* trophozoites formed a dense, adherent mass on the surface of culture tubes. Trophozoites were detached from the tube walls by cold shock (20 min on ice) and free trophozoites were harvested by centrifugation (2000g, 4°C, 10 mins). These were then resuspended and washed twice in ice cold Dulbecco's phosphate-buffered saline (DPBS). A total of $9x10^6$ trophozoites (of each isolate at the same passage number in independent cultures) were resuspended in 5ml of DMEM (pH7). To each sample of trophozoites, L-cysteine hydrochloride and ascorbic acid both at 11.4mM (Sigma-Aldrich, MO, USA) were added to support the attachment of the trophozoites to cells, and avoid potential oxygen damage of trophozoites (Gillin and Diamond, 1981; Gillin and Reiner, 1982; Rodríguez-Fuentes *et al.*, 2006; Ma'ayeh and Brook-Carter, 2012; Ferella *et al.*, 2014). Trophozoites of each isolate were subcultured for 72 hrs according to ATCC protocols (Keister, 1983). The number and viability of the trophozoites were evaluated by phase-contrast light microscopy using the trypan blue assay according to manufacturer's instructions (Sigma Chemical Co, USA).

Where the viability of the trophozoites was >90% the purified trophozoites (of each isolate) were used in subsequent experiments (as detailed below in each experimental outline). Samples of these same trophozoites were also stored in labelled cytoprotective ampules ($2 x10^7$ trophozoite/ml) in liquid nitrogen (LN) using a freezing medium protocol (ATCC, USA).

2.1.3. *Giardia* enriched excretory/secretory products (ESP)

G. duodenalis enriched excretory/secretory products (ESP) were collected after co-culture of $9x10^6$ trophozoites (of each isolate independently) with $2.8x10^6$ IEC-6 cells for 2 and 6 hrs,

respectively. The supernatant (the culture medium including any proteins secreted by *G. duodenalis*) was collected by cold shock (20 mins on ice) and centrifugation (2000g, 4°C, and 10mins) from each of the different co-cultures and placed in 15ml conical tubes (Becton Dickinson, NJ, USA). Each tube was adjusted to pH 7.2 with 1N NaOH and then filtered via a 0.2-micron syringe filter (Corning Incorporated, Germany) into a 10ml tube to remove any trophozoites or cells present. The ESP were stored at -80°C until required for experimentation. Before addition of the ESP to incubations (section 2.3.2), aliquots were thawed in a water bath at 37°C for approximately 15 minutes. The same process was carried out to prepare supernatants from control cultures (IEC-6 without *Giardia*) after 2 and 6hrs incubation times.

2.2. IEC-6 total mRNA gene expression using RNA-seq

Following incubation with or without *G. duodenalis* trophozoite isolates *in vitro (*Figure 1; section 2.2.1), host cell (IEC-6) mRNA gene expression was prepared and analysed (Figure 2; sections 2.2.2 and 2.2.7) as described below.

2.2.1. *Giardia* trophozoite and IEC-6 co-culture

Preparation of mRNA and transcriptome profiling of IEC-6 was carried out following culture with or without each *G. duodenalis* assemblage A isolates, P-1 or WB, at each time point (2 and 6 hrs). Co-culture was carried out in DMEM under standard conditions (37°C, 5% CO_2) in 25cm^2 Corning® canted neck, vented cap cell culture flasks ((Sigma- Aldrich MO, USA) (Figure 1). A ratio of *Giardia*: IEC-6 of approximately 3:1 (9x10^6 trophozoites: 2.8x106 cells) was used for all co-culture experiments.

Following co-culture for 2 or 6hrs, trophozoites were detached by cold shock for 20 minute on ice, and the supernatant containing the trophozoites was removed. The IEC-6 cell monolayer remaining in the culture flask, verified trophozoite free by microscopy, was washed twice with cold PBS, and then lysed with 350µl lysis buffer (Bioline Aust. Pty Ltd.), containing 3.5µl of 2-β-mercaptoethanol (Sigma Chemical Co, USA) and the resultant lysate collected for total RNA extraction.

IEC-6 were incubated (2 and 6hrs) in DMEM, without trophozoites, as comparative controls and lysed as described above. The collected cells were then stored at -20°C until nucleic acid extraction.

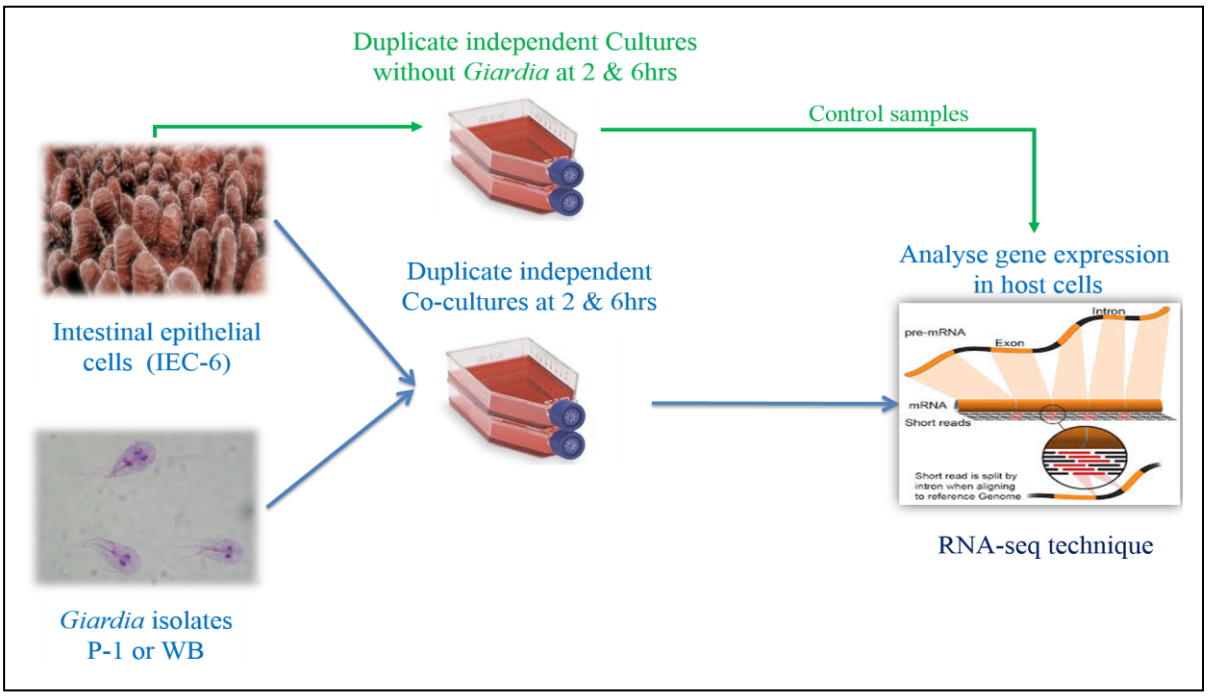


Figure 1: Co-culture experimental design for RNA-seq analyses. In duplicate, IEC-6 infected with two *G. duodenalis* isolates P-1 or WB at 2 and 6hrs *in vitro*. Duplicate IEC-6 cultures without *Giardia* were also carried out as control samples to these experiments. (Photos were modified from open sources materials).

2.2.2. RNA-seq data generation

The integrity of the total RNA extracted initially from IEC-6 was assessed by agarose gel electrophoresis (1.2%) and quantified using a Qubit® fluorometer RNA assay kit (Invitrogen™) according to the manufacturer's instructions. RNA samples which showed high quantity and integrity were then sent for sequencing at Micromon (Next-Generation Sequencing and Genomics, Department of Microbiology, Monash University, Australia) followed by analysis of the results. A workflow of this procedure is summarised in Figure 2.

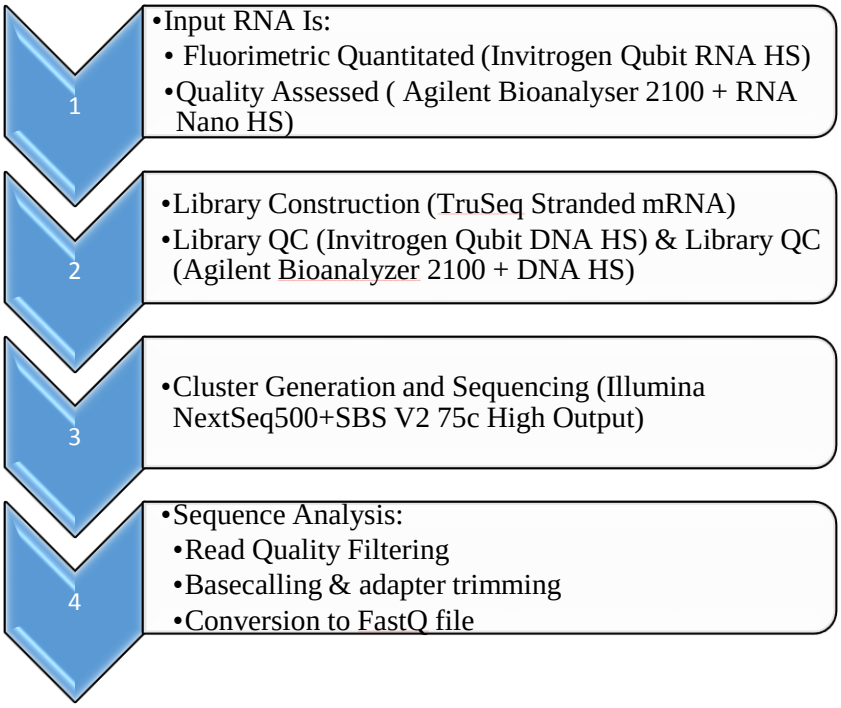


Figure 2: RNA-seq generation Flow-chart. **1**. Total RNA extracted from samples were subjected to QC analysis. **2**. High quality RNA was used to create cDNA libraries for sequencing, which were in turn subjected to QC analysis. **3**. High quality libraries were sequenced using NextSeq500 6. **4**. All sequence reads were filtered to remove low-quality reads and non-target sequences, to produce the, fastq file containing the sequence reads.

2.2.3. Total RNA quantification

The Invitrogen Qubit and associated chemistry (Invitrogen, Carlsbad CA, USA) was used to quantitate the RNA samples. The integrity of the samples was measured using the Agilent Bioanalyzer 2100 microfluidics device, in conjunction with the associated hardware and chemistry (Agilent Technologies, Waldbronn, Germany).

2.2.4. Libraries construction and sequencing

Sequencing of the entire complement of IEC-6 mRNA from each experimental condition was carried out in duplicate on an Illumina NextSeq500+SBS V2 75C High Output platform at Micromon as per the manufacturer's instructions (Illumina TruSeq Stranded mRNA Sample Prep Guide 15031047 Rev. E). A total of 4.0µg mRNA was used as input to the Illumina TruSeq Stranded mRNA kit per library and 12 RNA-seq sequencing libraries representing individual experimental conditions and controls (listed in Table 1) were produced.

Table 1: Samples used to create libraries for Next Generation Sequencing (NGS). As shown, duplicate RNA-seq experiments were carried out in IEC-6, following 2 and 6hrs incubation with *G. duodenalis* isolates P-1 or WB. IEC-6 cultured in the absence of trophozoites for 2 and 6hr cultures was used as control samples in these experiments.

Libraries	Source
1 and 2	Non-infected IEC-6 post 2hrs incubation without *G. duodenalis* (C-2hrs)
3 and 4	Non-infected IEC-6 post 6hrs incubation without *G. duodenalis* (C-6hrs)
5 and 6	IEC-6 (I) post 2hrs incubation with *G. duodenalis* isolate P-1 (IP-1 2hrs)
7 and 8	IEC-6 (I) post 6hrs incubation with *G. duodenalis* isolate P-1 (IP-1 6hrs)
9 and 10	IEC-6 (I) post 2hrs incubation with *G. duodenalis* isolate WB (IWB 2hrs)
11 and 12	IEC-6 (I) post 6hrs incubation with *G. duodenalis* isolate WB (IWB 6hrs)

All libraries were quantified using the Qubit DNA HS kit (Invitrogen, Carlsbad CA, USA). Quality control of the libraries were carried out including sizing and adapter contamination testing using an Agilent Bioanalyzer 2100 microfluidics device in conjunction with Agilent DNA HS kits and chemistry (Agilent Technologies, Waldbronn, Germany). Single end sequencing was carried out using the Illumina NextSeq500 platform with High-Output SBS v2 sequencing chemistry, which gives an average read length of 75 bases.

2.2.5. Mapping to the reference genome and differential gene expression analysis

Specific bioinformatics software was used to analyse the IEC-6 sequence data generated from each library. The RNAsik pipeline (a web tool generated by the Monash Bioinformatics Platform) was used as this incorporates a number of source code algorithms required to analyse the sequence data files. The "STAR" aligner algorithm (Dobin *et al.*, 2013), first aligns sequence data in the FASTQ files (target sequence files) to the reference rat ensemble genome (Rnor_6.0 ensemble genome, release 84). The "Feature Counts" code was used to count reads to the annotated features in the ensemble rat GFF file, which contains feature annotation (e.g. coordinates of your genes, exons and other genomic features). These feature counts were then used as input into "Degust" (formerly DGE-Vis), version: 3.1.0, which is an interactive web tool for visualising differential gene expression data. This tool has been written by David R. Powell and is supported by the Monash Bioinformatics Platform, Monash University, Clayton. Using Degust, the gene expression changes resulting from the exposure of host IEC-6 to *G.*

duodenalis isolates P-1 or WB, for 2 and 6hrs were analysed. A filter for minimum gene CPM (counts per million) of 1 CPM for both duplicates was set. The Voom/limma method was used to perform differential expression analyses following conversion of data to log2-counts-per-million [logCPM] (Law *et al.*, 2014). Linear Models for Microarray and RNA-seq Data (limma) is principally designed to analyse different experimental conditions in complex projects, as well as simultaneously analysing comparisons between many RNA targets (Ritchie *et al.*, 2015). Equally importantly, Voom is a precision weights approach that is used to model the mean-variance relationship in RNA-seq data (Law *et al.*, 2014). A Benjamini-Hochberg (BH) multiple test was also performed, this is a false discovery rate (FDR) statistical analysis, allowing correction for the rate of type I errors (incorrect rejection of a true null hypothesis) in multiple comparison analyses (Benjamini and Hochberg, 1995). The BH test based on adjusted *P* value of ≤ 0.05 was used to determine differentially expressed (DE) transcripts.

2.2.6. Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis

To analyse and understand the functions of expressed genes in *G. duodenalis* -induced host cells compared to controls, KEGG pathway analysis was used via the DAVID Bioinformatics Resources 6.8 [National Institute of Allergy and Infectious Diseases (NIAID), NIH] (Huang *et al.*, 2008). The KEGG pathway is a group of pathway maps constructed from known gene product functions and interactions. It is a powerful way in which to visualise and analyse the molecular interaction, reaction and relationship networks of complex systems such as metabolism, genetic information processing, environmental information processing, cellular processes, organismal systems, human diseases, and drug development (Kanehisa and Goto, 2000). Differentially expressed genes whose expression levels had altered (up- or down) by log2 fc, were used to identify KEGG pathways whose operation may have been altered by the presence of the parasite, or parasite derived products. Analysis and a *P* value was adjusted according to the Benjamini and Hochberg method (1995) with an FDR cut-off of 0.05.

2.2.7. Gene ontology (GO) analysis

The GO is a dynamic ontology-based resource that relies on many computational tools to provide comprehensive biological knowledge of molecular systems through the investigation of the functional activities of specific genes (Blake, 2013). GO analysis ascribes particular gene sequences to three main domains: Molecular Function (MF), Biological Process (BP), and

Cellular Component (CC). Collectively these three domains describe core information about protein and gene mechanisms based on the experimental data (Hill *et al.*, 2013). In the present analysis, some individual genes were functionally identified according to GO analysis using **G**ene **O**ntology en**RI**chment ana**L**ysis and visua**L**iz**A**tion tool [***GO****RILLA*] (Eden *et al.*, 2007, 2009). Three functional categories: MF, BP and CC were analysed for up or down regulated gene expression. A *P* value was adjusted according to Benjamini-Hochberg method and the false discovery rate (FDR) cut-off of 0.05 indicated significantly up- or down-regulated genes (Benjamini and Hochberg, 1995).

2.3. IEC-6 gene expression using qRT-PCR

2.3.1. *Giardia* trophozoites and IEC-6 co-culture

To determine the effects of *G. duodenalis* trophozoites on expression levels of specific host cell genes we also used the qRT-PCR technique. In this instance we used four different *G. duodenalis* isolates co-cultured with IEC-6 in triplicate at two time points (2 and 6 hrs) under standard conditions (5% CO_2, 37°C) using 25cm^2 Corning® cell culture flasks, canted neck, vented cap (Sigma- Aldrich MO, USA) (Figure 3). A ratio of *Giardia*: IEC-6 of approximately 3:1 (9x10^6 trophozoites: 2.8x10^6 cells) was used throughout co-culture. The collected cells were then stored at -20°C until nucleic acid extraction. Total RNA was extracted from host cells as described previously (2.2.1).

2.3.2. *Giardia* enriched ESP and IEC-6 co-culture

In order to determine the effects of *G. duodenalis* ESP alone on host cells, ESP from each previous co-culture (2.1.3; 2.3.1) was incubated with IEC-6 in triplicate for two time points (2 and 6hrs) (Figure 3). If the collected ESP was from a 2 hrs co-culture of cells and a *G. duodenalis* isolate, then it was incubated for a further 2 hrs with cells alone; similarly, if the collected ESP was from a 6 hrs co-culture of cells and a *G. duodenalis* isolate, then it was incubated for a further 6 hrs with cells alone. A ratio of 5mls of ESP: 2.8x10^6 IEC-6 was used throughout, unless stated otherwise. Each of the co-cultures was incubated for the required time (2 or 6hrs) under standard conditions (5% CO_2 37°C) using 25cm^2 Corning® cell culture flasks, canted neck, vented cap (Sigma- Aldrich MO, USA). Following co-culture, the supernatant was drawn off placed into a new 10ml tube; all tubes were labelled and stored at -80°C. The IEC-6

was washed twice with cold PBS (life technologies), collected by scraping then homogenised in 350µl lysis buffer (Bioline Aust. Pty Ltd.), 3.5µl 2-β-mercaptoethanol (Sigma Chemical Co, USA) and the lysate collected for total RNA extraction. IEC-6 was incubated without ESP (2 and 6hrs) in DMEM, as comparative controls and lysed as described above. All non-infected and E/S treated IEC-6 were stored at -20°C until nucleic acid extraction.

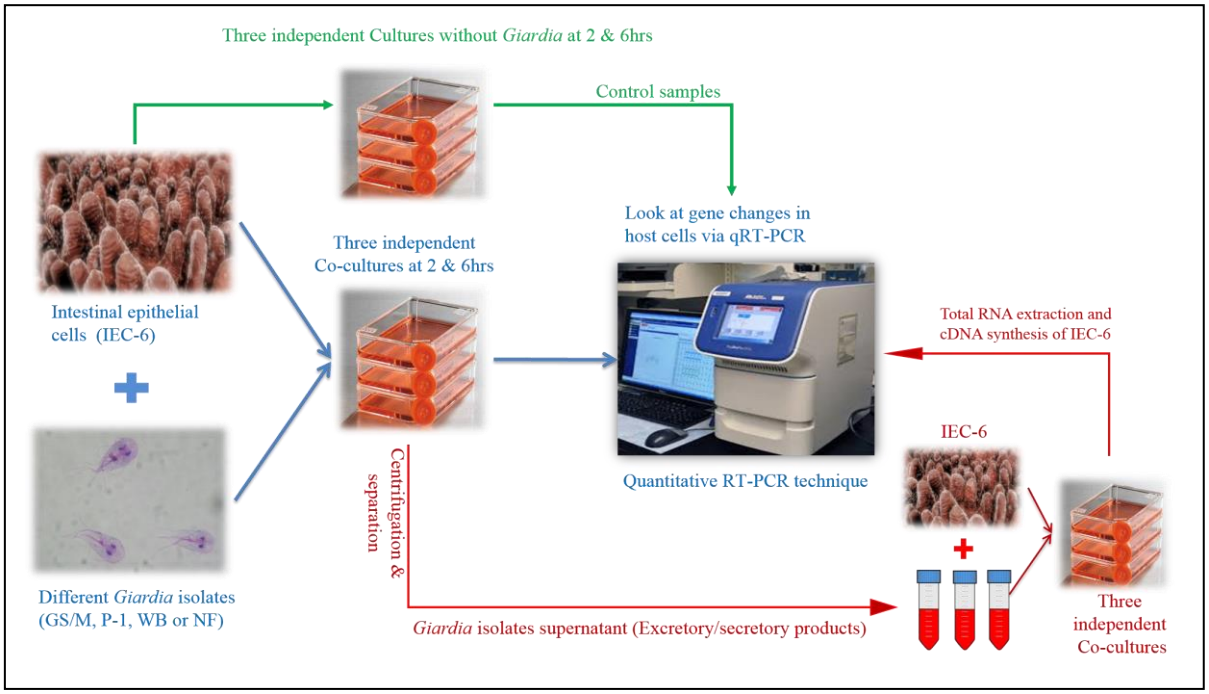


Figure 3: Co-culture experiment design for qRT-PCR analyses. IEC-6 was infected in triplicate with each of four *G. duodenalis* isolates GS/M, P-1, WB or NF for 2 and 6hrs. Triplicate IEC-6 were cultured without *Giardia* as control samples for these experiments. In addition, the supernatant at the end of each incubation was collected for ESP and then individually incubated with IEC-6 for a further 2 and 6hrs (Photos were modified from open sources materials).

2.3.3. Total RNA quantification and complementary DNA synthesis

Following treatment with either *G. duodenalis* trophozoites or their ESP, total RNA was extracted from all IEC-6 monolayer cell lysates using the ISOLATE II RNA Mini Kit (Bioline Aust. Pty Ltd., NSW, Australia) according to the manufacturer's instructions. RNA quality was assessed by agarose gel electrophoresis (1.2%) and quantified using a Qubit® fluorometer RNA assay kit (Invitrogen™)[section 5.2.2.1]. cDNA was prepared from 2µg of total RNA from each experimental cell line (and controls) using a Tetro© polyA-primed cDNA Synthesis Kit (Bioline Aust. Pty Ltd., NSW, Australia), according to manufacturer's instructions. cDNA integrity was assessed by conventional PCR amplification and agarose gel electrophoresis of amplicons of

the rat *GAPDH* gene (Yamada *et al.*, 1997; Buehlmeyer *et al.*, 2007; Wang *et al.*, 2010; Teerapornpuntakit *et al.*, 2014) using the My Taq HS Mix kit (Bioline Aust. Pty Ltd., NSW, Australia).

2.3.4. Quantitative RT-PCR primer design and validation

Target genes for analysis by qRT-PCR were initially chosen from reported literature (Panaro *et al.*, 2007; Ma'ayeh, 2013, unpublished data) as having altered levels of gene expression in the presence of *G. duodenalis* trophozoites. Primer pairs complementary to target rat cDNA were designed and prepared for qRT-PCR analysis. In addition, the ubiquitously expressed genes *GAPDH* and *PPIB* were used to normalise gene expression levels and determine target fold change (fc). Sequence starting approx. 150bp to approx. 800bp from the 3'-end was selected for primer design. The selected span avoids the inclusion of the poly-A tail and will result in an average amplicon length of 600bp. Primers used to amplify select gene products (amplicons) were designed using Primer-Blast (**N**ational **C**entre for **B**iotechnology **I**nformation, **NCBI**). Accession numbers of target gene cDNA sequences used in the design of primers, primer sequences and the predicted product sizes for each primer pair are detailed in Table 2. All primers were purchased from GeneWorks Pty Ltd, Australia.

Table 2: Rat (*Rattus norvegicus*) gene qRT-PCR target selection. The table shows the gene, the accession number, forward (F:) and reverse (R:) primer sequence, the exons spanned by the primers, the expected amplicon (bp) and the reference.

Gene	Accession No.	Forward and Reverse primers 5'-3'	Spans Exon Junction	Amp. size (Bp)	Ref.
GAPDH	NM_017008.4	F: CCTGGAGAAACCTGCCAAGTATGA R: AGCATCAAAGGTGGAAGAATGGG	Exon 6/7	148	This study
PPIB	NM_022536.2	F: CCAATGGCTCCCAGTTCTTCATA R: ACCTTCCGTACCACATCCATGC	Exon 4/5	106	This study
CASP-3	NM_012922.2	F: GAG AAG ATG GTT TGA GCC GGA R: TTT GCA GGA GCT TCT GAT CTG G	Exon 7	144	This study
BAX	NM_017059.2	F: CGG GTG GTT GCC CTT TTC TAC R: AGG AGG TCC AGT GTC CAG CC	Exon 4/5	107	This study
FAS-R	NM_139194.2	F: GGA CAA ACC CAG TAG CGT TCA G R: AAC TCC TCA AAG TAG GCA CAG G	Exon 9	89	This study
BCL-2	NM_016993.1	F: TGG ATG ACT GAG TAC CTG AAC C R: CAG AGA CAG CCA GGA GAA ATC A	Exon 1/2	126	This study
TGFB-1	NM_021578.2	F: GATACGCCTGAGTGGCTGTC R: AGCGAAAGCCCTGTATTCCG	Exon 3/4	85	This study
P38	NM_031020.2	F: GACGAAATGACCGGCTACGTG R: GCAGCCCACGGACCAAATATC	Exon 8/9	105	This study
SMAD-2	NM_001277450.1	F: TCTTTGTGCAGAGCCCCAAC R: ACTGAGCCAGAAGAGCAGCA	Exon 10/11	126	This study
CLDN-1	NM_031699.2	F: CTGGCTTCGCTGGGATGGAT R: TATCTGCCCGGTGCTTTGCG	Exon 1	150	(Wang *et al.*, 2012)
OCLN	NM_031329.2	F: GTGGCAGGAGTCATAAAACGG R: CAATGGGCACACCCTGATACT	Exon 9	120	This study
ZO-1	NM_001106266.1	F: TCTGAGATGTTTGATGTGTTCAGG R: GGCTCAAGAGGTACAGGAGAG	Exon 27	160	This study

2.3.5. Standard PCR and Sequencing

All primer pairs designed were used in conventional PCR to validate their ability to uniquely amplify a single product. The primer pair testing was carried out using My Taq HS Mix kit

(Bioline Aust. Pty Ltd., NSW, Australia) according to the manufacturer's instructions and analysed by 1.2% agarose gel electrophoresis and Sanger sequencing. A 50µl cPCR reaction mix: 200ng cDNA, 20µM each primer 25µl 2x My Taq HS Mix, nuclease-free water up to 50µl was used. Cycle times of: 1x 95°C, 1 minute, 35 cycles of; 95°C, 15s, 60 °C, 15s and 72°C, 10s) were used on an Applied Biosystems, thermal cycler (Life Technologies, Australia). Amplicons were purified using the QIAquick® PCR purification kit (Qiagen) according to the manufacturer's instructions and sequenced at the Australia Genome Research Facility Ltd (AGRF), Melbourne. Primer efficiency of each primer pair was analysed by the standard curve method using five serial 10-fold dilutions of template cDNA. Subsequent qRT-PCR reactions were performed only with primer pairs demonstrating efficiencies of 90% or higher.

2.3.6. Gene expression analyses using Comparative C_T method ($\Delta\Delta C_T$ method)

All qRT-PCR was conducted using the SensiFAST SYBR Hi-ROX Mix (Bioline Aust. Pty Ltd., NSW, Australia) according to manufacturer's instructions, on an Applied Biosystems, 7300 StepOne™ Plus, thermal cycler (Life Technologies, Australia). Gene expression levels of target genes in IEC-6 host cells were quantified by qRT-PCR following co-culture with one of four different *G. duodenalis* isolates as previously described (sections 2.3.1 and 2.3.2). Quantitation of gene expression was based on the comparative C_T method [$\Delta\Delta C_T$ method] (Schmittgen and Livak, 2008), and was applied to analyse the fold change for each gene tested. The levels of expression of the ubiquitously expressed genes *GAPDH* and *PPIB* were used to normalise experimental levels and non-infected IEC-6 was considered as control (basal) gene expression.

2.4. Apoptosis assay

2.4.1. *Giardia* trophozoites and IEC-6 co-culture

Cell death detection experiments were carried to determine *G. duodenalis* -induced apoptosis of host IEC-6 *in-vitro.* For these experiments each of the four *G. duodenalis* isolates was co-cultured with IEC-6 in duplicate for each time point (2 and 6hrs) under standard conditions (5% CO_2 37°C) using 24 well, flat bottom, low evaporation lid tissue culture plates (Becton Dickinson, NJ, USA) (Figure 4). A *Giardia*: IEC-6 cell ratio of approximately 3:1 ($3x10^6$

trophozoites: $1x10^6$ cells) was used. Following co-culture, for the appropriate time, all samples were subjected to cold shock by ice for 20 min to detach the trophozoites. The media (containing the free-floating trophozoites) was drawn off and trophozoites collected by centrifugation (2000g, 4°C, 10 minutes). The IEC-6 was washed twice with cold PBS (life technologies) then harvested as in section 2.3.2. IEC-6 grown in DMEM without *Giardia* for 2 and 6hrs was prepared as controls. Additionally, 4µg/ml camptothecin (CAM) (Sigma Chemical Co, USA) was incubated concurrently in separate IEC-6 co-cultures at 2 and 6hrs to induce apoptosis (positive control) (Chin *et al.*, 2002).

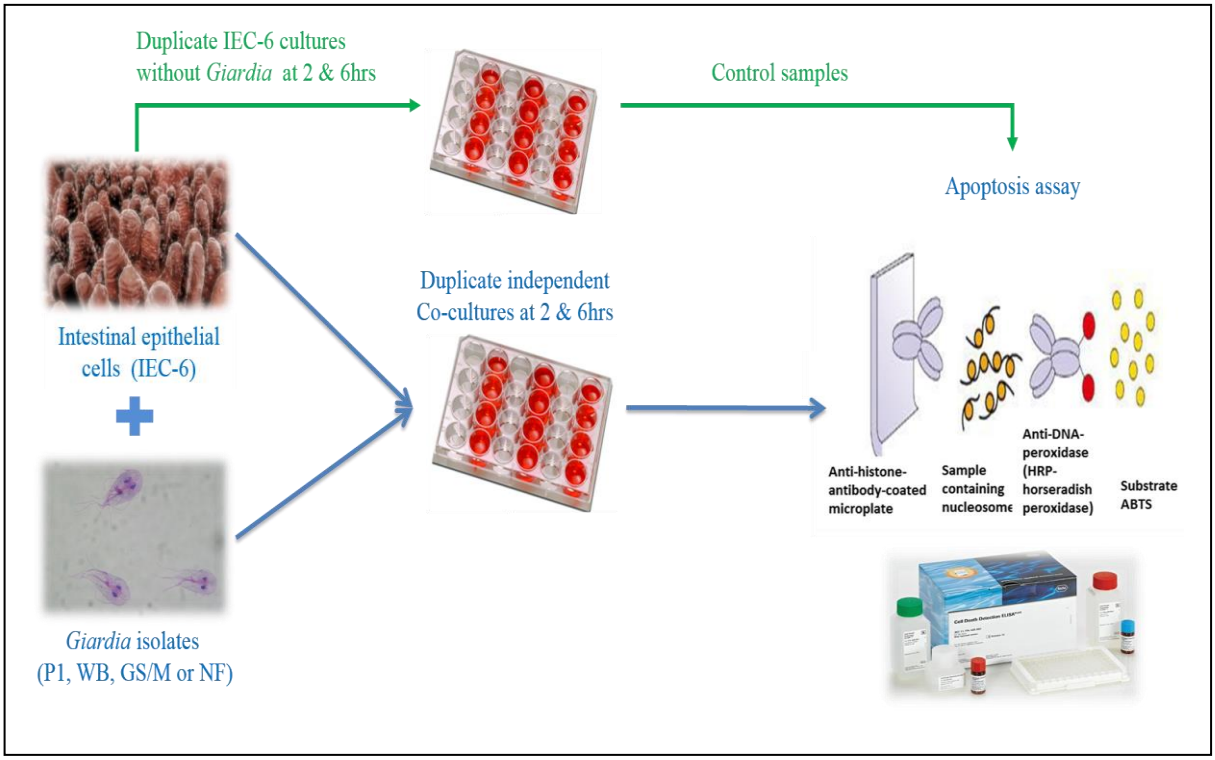


Figure 4: Experimental design for cell death detection analyses in co-cultures. IEC-6 was infected in duplicate with each of four *G. duodenalis* isolates GS/M, P-1, WB, or NF at 2 and 6hrs. IEC-6 cultures without *Giardia* were carried out as control samples. (Photos were modified from open sources materials)

2.4.2. Cell death detection

The ELISA^{PLUS} photometric enzyme immunoassay kit (Sigma Chemical Co, USA) was used for the apoptosis assay. This kit enables quantitative determination of mono- and oligo-nucleosome fragments, which are hallmarks of apoptosis. Following incubation with or without *Giardia* or CAM (section 2.4.1) IEC-6 was lysed by adding 200µl of a lysis buffer and then incubated for 30 minute at 15 - 25°C (cell lysis). Infected and non-infected cell lysates were

measured photometrically with the anti-histone conjugated-biotin antibody to quantify the concentration of mono- and oligo nucleosomes in the cells. A BMG FLUOstar OPTIMA Microplate reader was used to read OD at a wavelength of 405 nm.

2.5. Data analysis

R, a well-developed program for statistical computing using a command-driven language was used to analyse RNA-sequencing data. The R program was initially applied on raw FASTQ files to the list of differentially expressed transcripts, through the mapping of the reads to the rat reference genome and statistical analysis using the limma package (Law *et al.*, 2014; Ritchie *et al.*, 2015; Phipson *et al.*, 2016). Subsequently, a CSV file of read counts for each replicate was uploaded to Degust. Comparison analyses of the infection timeframes: 2hrs vs control, 6hrs vs control, and 2hrs vs 6hrs were carried out. Transcripts displaying altered levels of expression of the logarithm two of fold change [log2 fc] (Love *et al.*, 2014) at an FDR cut off ≤ 0.05 from the control group were analysed using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995).

For qRT-PCR and the biochemical assay analyses, Graph Pad Prism 6 software® was used. Data met all required assumptions for completing ANOVA (One Way ANOVA and Two-Way ANOVA) which were performed for comparison among the co-culture groups compared to controls and significant differences determined by Bonferroni's multiple comparisons test. All results were reported as mean ± standard deviation and $P \leq 0.05$ was used as the threshold for statistical significance.

Chapter 3
Transcriptional profiling of responses to assemblage A *Giardia duodenalis* isolates

Chapter 3: Transcriptional profiling of responses to assemblage A *Giardia duodenalis* isolates

3.1. Introduction

G. duodenalis can cause disease as an acute, self-limited dysenteric form (≤14 days) and a chronic form (≥30 days) with long-term effects in different hosts (Di Genova and Tonelli, 2016). The hallmarks of giardiasis, diarrhoea and abdominal pain have both been reported to be associated with pathological injury and changes to the surface of the gastrointestinal tract (Adam, 2001; Cotton *et al.*, 2011). Several parasite-mediated pathogenic mechanisms have been postulated for both infection establishment and variability in infection severity such as in acute and chronic infections (Bartelt and Sartor, 2015). Nevertheless, the pathogenic mechanisms of early giardiasis are still incompletely understood. Differences in the pathogenicity between assemblages A and B and their genotypes within each assemblage have been reported in clinical disease for many years (Homan and Mank, 2001; Haque *et al.*, 2005; Breathnach *et al.*, 2010; Yang *et al.*, 2010; Feng and Xiao, 2011; Asher *et al.*, 2014). These differences suggest that variations in virulence levels between these assemblages or genotypes underlies these findings.

With the development of more efficient and accurate sequencing methods, researchers have been able to address questions of the genetic similarity (or differences) between assemblages and genotypes. Two papers based on open reading frame and phylogenetic analyses found that assemblage A subassemblages AI and AII were substantially similar (Adam *et al.*, 2010; Adam *et al.*, 2013). Furthermore, whole-genome sequencing suggested that assemblage A and B isolates are different and may be independent species (Adam *et al.*, 2013). A genomic study of different *G. duodenalis* genotypes of the same assemblage (A) revealed considerable genomic variation with respect to chromosome size and gene content, surface protein repertoire and gene polymorphisms (Ankarklev *et al.*, 2015). Additionally, a proteomics study, demonstrated significant variation in the proteome of host cells induced by eight *G. duodenalis* assemblage A zoonotic genotypes (Emery *et al.*, 2015). This implies not only different host cell responses to different assemblages but also to different genotypes within the same assemblage(Laishram *et al.*, 2012; Broglia *et al.*, 2013; Liu *et al.*, 2014; Wang *et al.*, 2014).

As neither genomic nor protein based studies have been more conclusive in understanding apparent assemblage and isolate specific responses in host cells researchers have investigated *G. duodenalis* and host cell transcriptome responses to co-culture. Gene expression studies conducted between 2005 and 2014, identified a large number of genes whose expression levels altered in the presence of assemblage A isolates (Roxström-Lindquist *et al.*, 2005; Ringqvist *et al.*, 2011; Ma'ayeh and Brook-Carter, 2012; Stadelmann *et al.*, 2013; Ferella *et al.*, 2014). One of these studies in which both assemblage A and B isolates were examined, showed an assemblage specific difference in the types of host genes responding to the presence of the trophozoites (Ma'ayeh and Brook-Carter, 2012). In that study, three assemblage A genotypes (WB, P-1, and NF) and one assemblage B genotype (GS/M) were determined to have induced different gene expression responses in the same host cell type at the same time points. The study detected significantly increased transcription rates of genes encoding a group of high cysteine membrane proteins (HCMPs) in response to assemblage A genotype, but not the assemblage B genotype (Ma'ayeh and Brook-Carter, 2012).

Taken together, these studies have highlighted similarities and differences in genomes, transcriptomes and proteomes of different *Giardia* assemblages and genotypes within an assemblage. Research has also identified differences in pathological effects of assemblages during interactions with host cells. Nevertheless, there are many unanswered questions relating to assemblage and genotype differences, the causes of different host cell responses and whether different assemblages display differing levels of virulence (Adell *et al.*, 2014; Bartelt and Sartor, 2015; Buret *et al.*, 2015).

Several parasite-mediated pathogenic mechanisms have been postulated for both infection establishment and variability in infection severity such as acute and chronic infections (Bartelt and Sartor, 2015). Nevertheless, the pathogenic mechanisms of early giardiasis are still incompletely understood.

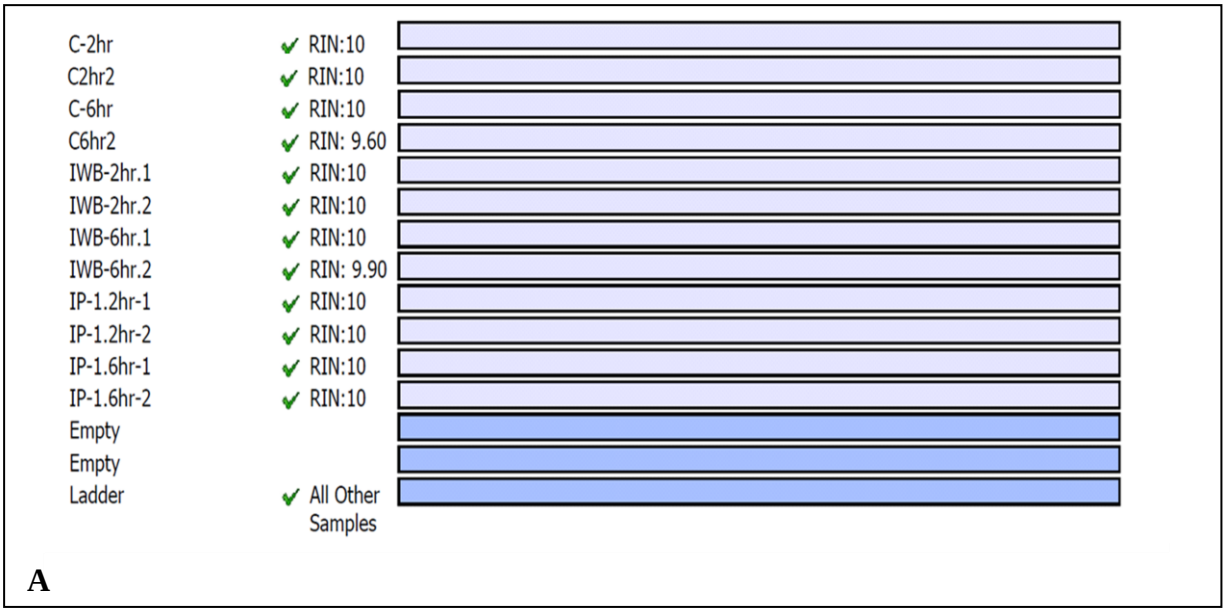
RNA-seq is a modern technology utilising the concept of deep-sequencing (Hrdlickova *et al.*, 2017). It is our current best technology for high throughput RNA sequencing and, as with all NGS technologies, can sequence multiple targets multiple times per run. The sequence data provided can be used for a large number of applications (Wang *et al.*, 2009). RNA-seq data can

be analysed in many ways to uncover patterns of expression not seen using gene-by-gene approaches. Applications such as Gene Ontology (GO) and cellular pathway analysis, can provide greater understanding of comprehensive functional activities of expressed genes and assist in unravelling the complexity of genome-wide expression studies (Huang *et al.*, 2009; Young *et al.*, 2010; Blake, 2013). It is evident that advanced transcriptomic and proteomic approaches, including RNA-seq technology can be used to unravel the issues of the mechanisms of virulence in *G. duodenalis* (Ma'ayeh *et al.*, 2015; Ansell *et al.*, 2016; 2017). To date, there are few studies of host cell responses to *G. duodenalis* using RNA-seq. RNA-seq was used in this study to provide further insights into cellular responses of intestinal epithelium following 2 or 6hrs infections with two different *G. duodenalis* assemblage A isolates.

3.2. Results

RNA-seq was carried out to identify global changes in the transcriptome profile of rat intestinal epithelial cells (IEC-6) during early infection (2 or 6hrs) with two *G. duodenalis* isolates, P-1 or WB. Four experimental groups (IP-1 2hrs, IP-1 6hrs, IWB 2hrs, and IWB 6hrs) were created and analysed relative to control groups at 2 or 6hrs.

High quality RNA as judged by Micromon quality control (QC) analysis showed high integrity values in total RNAs used in these experiments. Micromon applies an RNA integrity number (RIN) to all samples supplied for library construction and sequencing. All RNA samples had RIN values of between 9.6 -10, predicating good quality data (Figure 1).



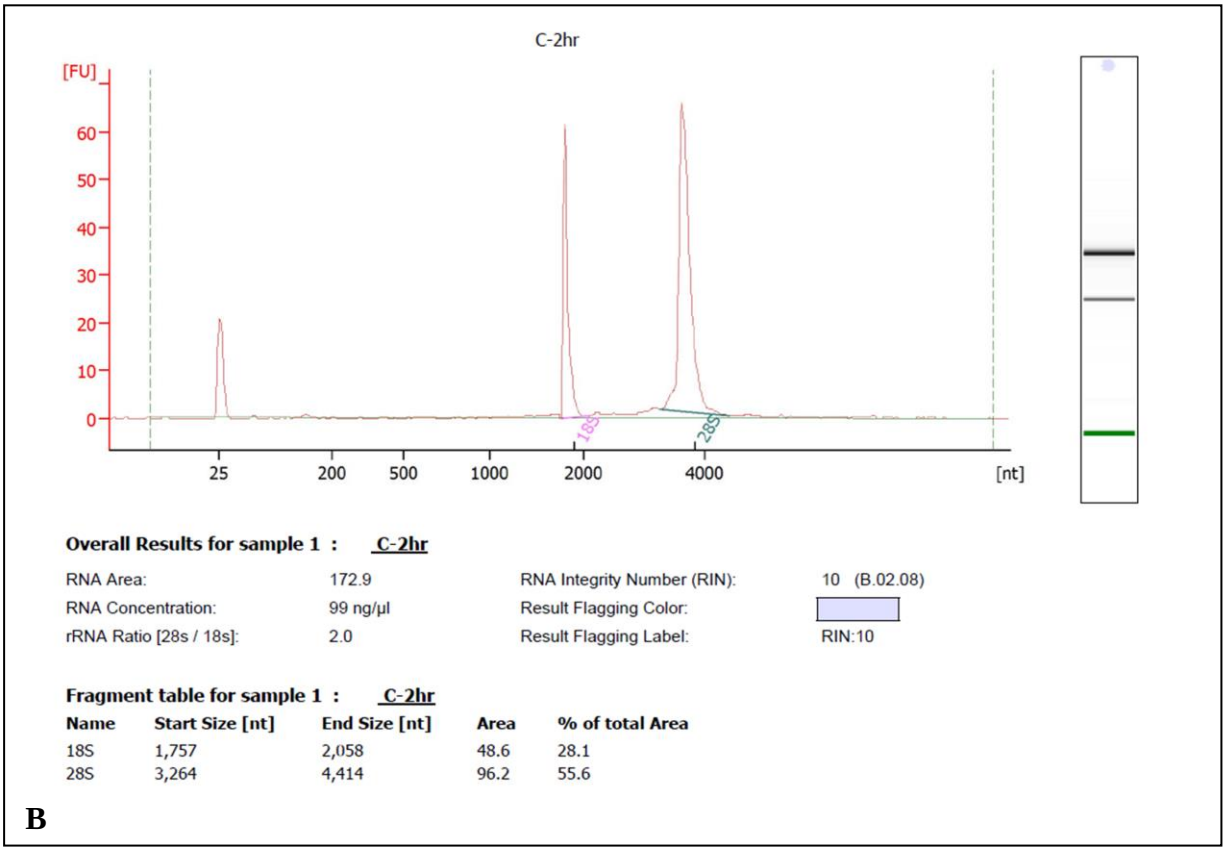


Figure 1: A. Electrophoresis File Run Summary (Chip Summary) shows the RNA integrity number (RIN) to all samples which were used for the library construction and sequencing of RNA-seq experiments. As shown, all samples had a great value of RIN ranging 9.6-10. B. Electropherogram Summary diagram which displays the overall QC results of sample 1(control 2hrs 1) as example.

Boxplot analysis of all libraries created from RNA samples supplied showed that they all (sequencing libraries) had a similar distribution of per-gene read counts per sample, indicative of a high degree of inter sample similarity. The distribution ranging in each sequencing library

was approximately from 3 to 6 log2- count per million (CPM), where the average expression level among these libraries was around 5 CPM (Figure 2A). Multidimensional scaling (MDS) was used to visualise the relationships between the individual replicates of experimental datasets. The MDS plot (Figure 2B) demonstrated that biological replicates from the same experimental conditions grouped closely together for both the control and infected samples.

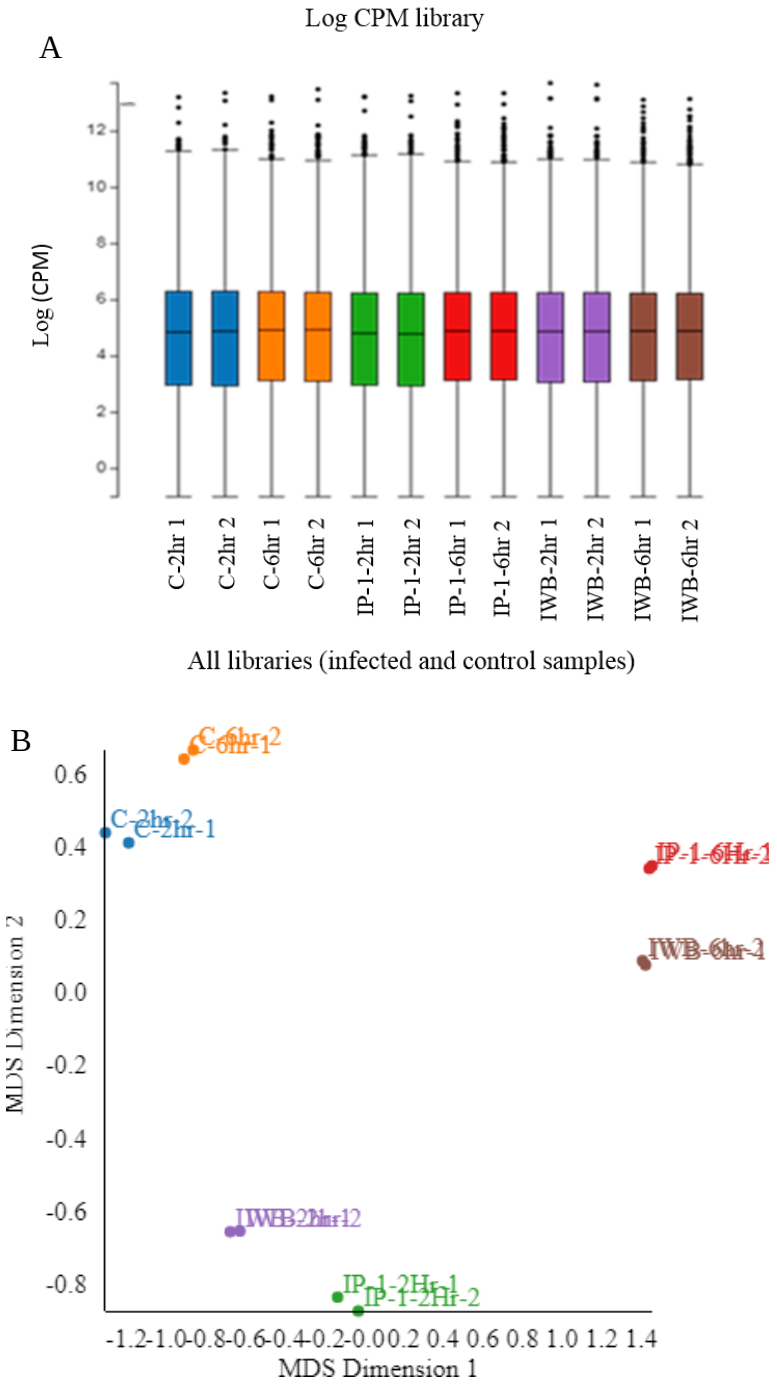


Figure 2: Quality control (QC) of RNA-seq datasets. A. boxplot of log- counts per million (CPM) values showing expression distributions for normalised data for each sample in the dataset. C-2hr and C-6hr represent the control samples (non-infected IEC-6) at 2 and 6hrs, respectively. IP-1 co-culture (at 2and6hrs) denotes IEC-6 infected by *G. duodenalis* isolate P-1. IWB co-culture (at 2and6hrs) represents IEC-6 infected by *Giardia* isolate WB. B. Multidimensional scaling (MDS) plot shows the level of similarity of individual replicates of the dataset (in duplicate) in the RNA-seq experiment.

The heatmap (Figure 3) of 50 random expressed transcripts representing all experimental conditions also demonstrated the close clustering and similarity between the biological replicates. In particular, the clustering of 6hrs infected samples (IP-1 co-culture (black boxes)

and IWB co-culture (red boxes)) was the most similar to each other. The heatmap clearly indicates the global difference in gene expression levels of the selected genes following infection for either time period between the control and infected samples.

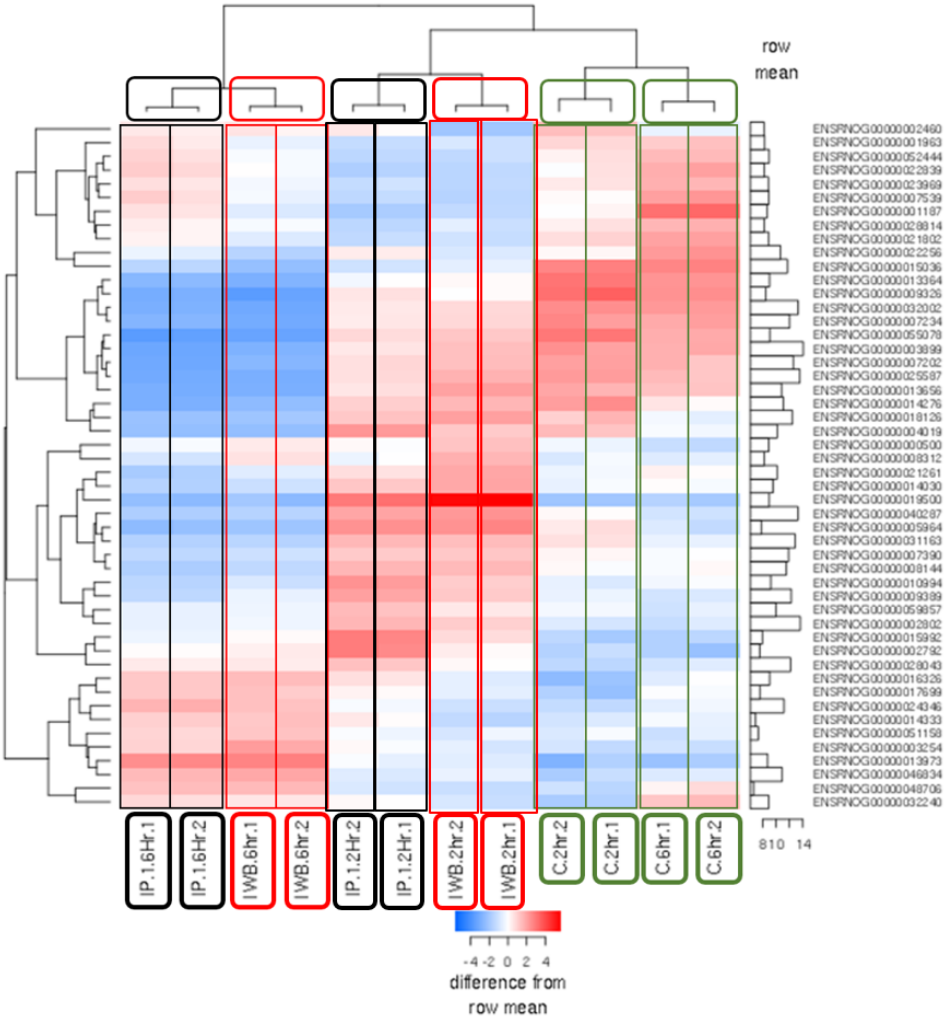


Figure 3: Heatmap of hierarchical clustering analysis of 50 random expressed genes representing all experimental conditions. This analysis shows the pattern of expression levels (Red= upregulation, Blue= downregulation) of each of the chosen transcripts in individual samples. Clustering and similarity between the biological replicates is clear. C-2hrs and C-6hrs (green boxes) represent the control samples (non-infected IEC-6) at 2 or 6hrs, respectively. IP-1 2 or 6hrs (black boxes) and IWB 2 or 6hrs (red boxes) represent co-culture of respective *G. duodenalis* isolates and time points. IP-1 represents IEC-6 (I) infected by *Giardia* isolate P-1, IWB denotes IEC-6 (I) infected by *Giardia* isolate WB.

3.2.1. Transcriptome profiling of parasitised IEC-6

Analysis of the RNA-seq data was carried out to determine gene transcription level changes in IEC-6 infected by either *G. duodenalis* P-1 or WB isolates through the first six hours of

infection. Transcripts identified as having altered expression levels with a log2 fc (with respect to the control) and a false discovery rate (FDR) cut-off ≤ 0.05 only were initially considered as input into this analysis. The FDR approach was used to correct the p-value for multiple testing in this data based on the Benjamini and Hochberg (1995) method, where an FDR adjusted p-value of 0.05 indicates that 5% of significant tests will appear to be false positives, which is considered a small false positives outcome.

The overall results obtained from this analysis demonstrated that the expression levels of a substantial number of transcripts were altered in the IEC-6 in response to both isolates at both time-points. In addition, significant differences were observed in terms of the number of expressed genes responding to an individual genotype. The following sections clarify statistical differences in the findings of transcriptional changes analyses according to infection time-points.

3.2.1.1. Transcriptome profiling at 2hrs

Following a 2hrs infection, IEC-6 showed a significant difference in the number of transcripts whose expression was altered in response to the isolate WB compared to the P-1 isolate. Some 5503 and 3395 transcripts showed statistically significant alteration of expression level to these isolates, respectively. 2641 expressed transcripts were found to be shared between two co-cultures at this time of the infection (Figure 4).

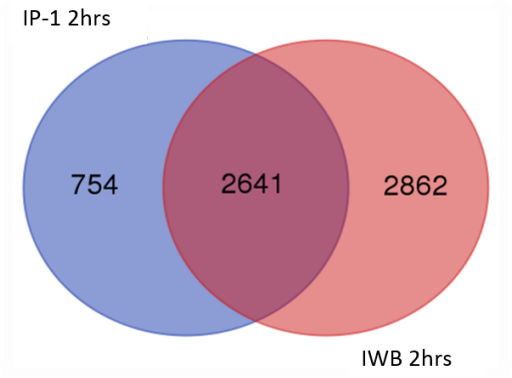


Figure 4: Transcripts whose expression levels were significantly altered (log2 fc and FDR cut-off ≤0.05) in IEC-6 induced by *G. duodenalis* isolates (P-1 or WB) at 2hrs. The Venn diagram shows the distribution of total transcripts whose expression was altered either downregulated or upregulated in response to each isolate, as well as indicating shared transcripts between each co-culture at 2hrs.

3.2.1.2. Transcriptome profiling at 6hrs

Following 6hrs induction, the parasitised cells showed fewer variations in the number of transcripts responding to either genotype. As shown in the figure 5, 6011 and 6096 transcripts responded (altered expression level) to the P1 and WB isolates, respectively. The analysis also revealed that 4808 transcripts were expressed in both the 6hrs co-cultures.

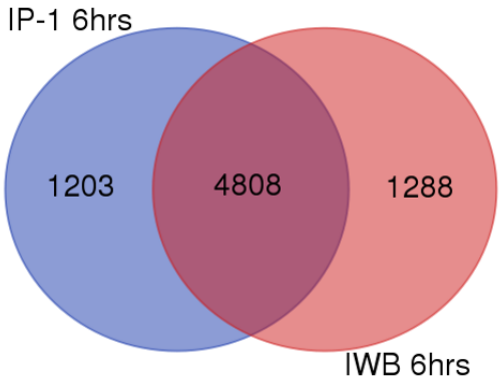


Figure 5: Transcripts whose expression levels were significantly altered (log2 fc and FDR cut-off ≤0.05) in IEC-6 induced by *G. duodenalis* isolates (P-1 or WB) at 6hrs. The Venn diagram shows the distribution of total genes whose expression was altered either downregulated or upregulated in response to each isolate, as well as indicating shared genes between each co-culture at 6hrs.

Closer analysis as shown in Figure 6 B and C, showed that of the 5502 transcripts responding to the WB isolate, a roughly similar number (2951 and 2551) were up or down regulated, respectively. While the total number of genes responding to the P1 isolate was lower (3394) than the WB-induced number of transcripts, the distribution of up or downregulated transcripts (1629 and 1765, respectively) was roughly similar.

The Venn diagram A in Figure 6 showed that 558 transcripts out of the total number responded to both isolates. Expression levels of 193 transcripts were altered in response to the P1 isolate only whilst expression of 1255 transcripts were altered in response to the WB isolate only. Gene regulation events can be both stimulatory (upregulation) and inhibitory (downregulation) and we further analysed the data to determine the numbers and identities of up or downregulated transcripts. The data (Figure 6) shows 152 and 1034 transcripts whose expression levels were specifically downregulated in response to P-1 and WB, respectively. The expression of 494 transcripts was found to be downregulated in response to both isolates.

There was a significant difference in the number of transcripts upregulated in response to the isolate P-1 compared to the WB isolate (Figure 6C) with 161 and 992 transcripts upregulated, respectively. Increased expression levels were detected in 387 transcripts in response to both isolates.

Furthermore, the analysis at 6hrs displayed that of the 6011 transcripts responding to P-1, 3161 transcripts were downregulated, and 2851 were upregulated. The total number of transcripts responding to WB (6096) was similar to that of the P-1 response, and the distribution of down and upregulated transcripts (3221 and 2877, respectively) was relatively similar to the P-1 isolate response (Figure 6B and C).

The data showed that 1822 transcripts responded to both isolates (Figure 6A), whilst 650 transcripts responded to P-1 only and 577 responded to WB only. There were 519 and 380 genes whose expression levels were specifically downregulated in response to P-1 and WB, respectively (Figure 6B), while 1315 transcripts were downregulated in response to both isolates. Upregulation events (Figure 6C) also showed similar variations with 501 and 336 genes upregulated in response to the isolates P-1 and WB, respectively. There were 1146 transcripts significantly upregulated in response to both isolates.

3.2.1.3. Transcriptome profiling of 2hrs and 6hrs

A direct comparison of the numbers of transcripts displaying altered levels of transcription at the 2 and 6hrs time points revealed a significant difference in response of IEC-6 to infection between different isolates. A considerable variance was noted between the two time points in response to each individual isolate (Figure 6 A). There were 51 transcripts which responded to P-1 only at both time points and 325 transcripts which responded to WB only at both time points. A total of 1584 transcript responded to both isolates at both time points.

As shown in the table in Figure 6 the 2hrs induction indicated considerable variation in the number of expressed transcripts responding to both isolates while the 6hrs analysis showed much less variation in terms of either the total number of transcripts, or the number of down and upregulated transcripts in response to the isolates P-1 and WB.

The total number of transcripts responding to P-1 at 6hrs was roughly double the number of transcripts responding at 2hrs. The number of transcripts responding to WB at 2 and 6hrs was relatively similar. The implications of the transcript regulation data were that the WB isolate induced a high level of host cell response from the earliest time point, whilst the P-1 isolate induced a smaller response at 2hrs but by 6hrs the level of response was similar to that for the WB isolate. Taken together, these findings indicated a difference in the speed of the host cell response to these isolates at 2hrs with a similar level response 4hrs later. Analysis of the identities of the transcripts that responded and whether they were up or downregulated should allow clarification of these responses in the developing pathology of giardiasis.

Number of expressed transcripts (Down and upregulated) in parasitised IEC-6

Co-culture	Total No. of transcript	Downregulated transcripts	Upregulated transcripts
IP-1- 2hrs	3394	1765	1629
IP-1- 6hrs	6011	3161	2851
IWB- 2hrs	5502	2951	2551
IWB- 6hrs	6096	3221	2877

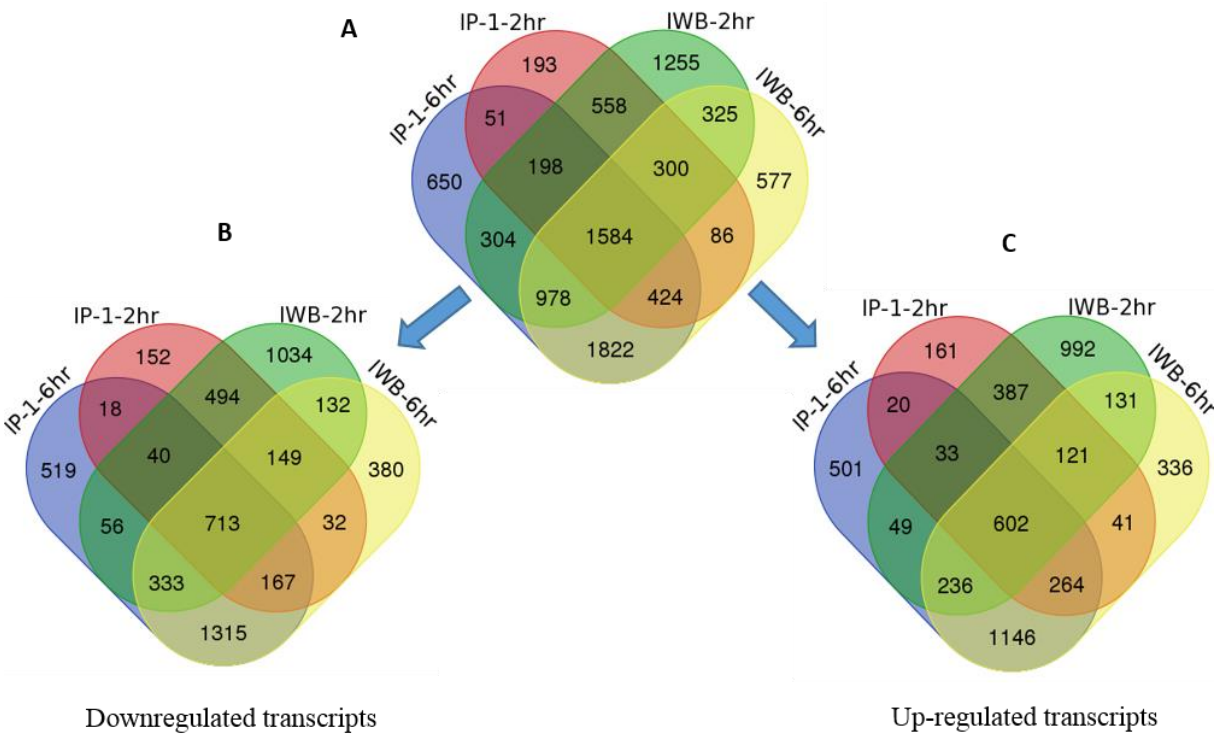


Figure 6: Transcripts whose expression levels were significantly altered (log2 fc and FDR cut-off ≤0.05) in IEC-6 induced by *G. duodenalis* isolates (P-1 or WB) at 2 or 6hrs. IP-1 represents IEC-6 (I) infected by *Giardia* isolate P-1, IWB denotes IEC-6 (I) infected by *Giardia* isolate WB. The table above shows the total number of transcripts whose expression levels were altered (downregulated and upregulated) in the parasitised IEC-6 in response to each isolate (P-1 or WB) following the 2 and 6hrs induction. The Venn diagram A shows the distribution of total transcripts whose expression was altered either downregulated or upregulated in response to each isolate, as well as indicating shared genes between each co-culture at different times. The Venn diagrams B and C denote numbers of transcripts which were only down regulated and those upregulated, respectively, in each co-culture at both time points.

3.2.1.4. Transcriptome profiling at 6hrs relative to 2hrs

During the 6hrs incubation time, P-1-induced slightly more transcripts than induced by WB (Figure 7). In response to P-1, 7,658 transcripts displayed significantly altered transcription rates, with 3,977 and 3,681 down and up-regulated, respectively. The total number of transcripts responding to the WB isolate was 6,901, with 3,521 and 3,380 of those being down and upregulated, respectively.

The data showed 5,424 transcripts responded to both isolates with 2,234 responding to P-1 only and 1,477 to WB only. The difference in the number of expressed transcripts at this time is indicative of a variable response to these isolates.

2 vs 6hrs	Total No. of expressed transcripts	Downregulated transcripts	Upregulated transcripts
IP-1	7658	3977	3681
IWB	6901	3521	3380

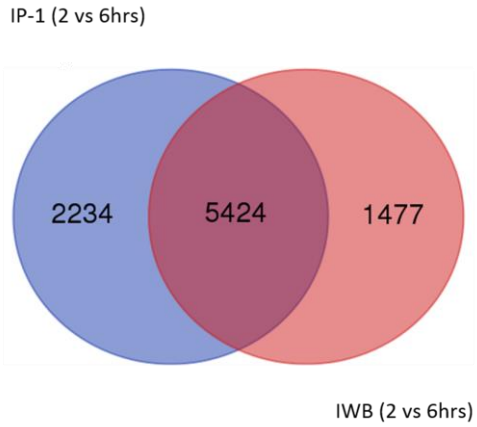


Figure 7: Transcripts whose expression level was significantly altered (log2 fc and FDR cut-off ⩽0.05) in IEC-6 (I) induced by *G. duodenalis* isolates (P-1 or WB) at 6hrs compared the response at 2hrs. The table above explains the total number of expressed transcripts responding to each isolate, as well as the number downregulated or upregulated. The Venn diagram shows the distribution of the total number of transcripts whose expression level altered or was shared between the isolates at 2 or 6hrs infection.

3.2.2. Cellular pathway and Gene Ontology analyses

3.2.2.1. Overview of induced cellular pathways

In order to identify and investigate cellular processes and signalling pathways that were statistically associated with expressed genes in the parasitised cells, a KEGG pathways analysis was applied using DAVID Bioinformatics Resources 6.8, NIAID/NIH (section 2.5.4). Several pathways were found to be significantly induced in IEC-6 during early giardiasis including: immune response-associated pathways, apoptosis, arginine, cell adhesion-related pathways, glycan biosynthesis and lipid metabolism, and multiple signalling pathways (Table 1 and 2).

At 2hrs (Table 1) signalling pathways representing various cellular activities were the most common pathways induced. These pathways included: TNF signalling, chemokines, PI3K-

AKT, MAPK, NOD-like receptor, FoxO, and TGF-beta signalling. The analysis also revealed differences between each isolate, for instance apoptosis, Jak-STAT, NF-kappa B, HIF-1, Toll-like receptor and B cell receptor signalling pathways were induced in response to P-1 but not WB. In contrast, focal adhesion, ECM-receptor interaction, Rap1, Wnt, and thep53 signalling pathway were only shown in response to WB.

Table 1: KEGG pathways enriched in IEC-6 (I) induced by *G. duodenalis* isolate P-1 and WB at 2hrs. KEGG pathway analysis using DAVID Bioinformatics Resources 6.8 (National Institute of Allergy and Infectious Diseases (NIAID), NIH) identified significant pathways that were identified in the parasitised host cells (*P* value ≤ 0.05). Transcripts whose mRNA level altered log2 fc were used as input into pathways enriched analyses. The table shows relevant significant pathways, number of expressed transcripts involved to these pathways, and *P* value. ND denotes not detected.

Induced pathways	No of transcripts in IP-1 at 2hrs	p-Value	No of transcripts in IWB at 2hrs	p-Value
Cytokine-cytokine receptor interaction	36	2.40E-16	25	1.20E-05
TNF signalling pathway	31	5.90E-21	24	8.80E-11
Chemokine signalling pathway	21	4.90E-07	15	1.70E-02
PI3K-Akt signalling pathway	22	1.90E-03	29	4.50E-04
MAPK signalling pathway	20	4.10E-04	22	2.90E-03
NOD-like receptor signalling pathway	11	6.90E-06	7	2.90E-02
TGF-beta signalling pathway	7	5.00E-02	10	9.80E-03
Jak-STAT signalling pathway	16	1.70E-05	ND	ND
NF-kappa B signalling pathway	13	1.70E-05	ND	ND
FoxO signalling pathway	9	6.00E-02	ND	ND
HIF-1 signalling pathway	9	1.50E-02	ND	ND
Toll-like receptor signalling pathway	8	3.00E-02	ND	ND
Apoptosis	6	4.20E-02	ND	ND
Rap1 signalling pathway	ND	ND	19	4.30E-03
Hippo signalling pathway	ND	ND	18	2.20E-04
Focal adhesion	ND	ND	15	5.90E-02
Wnt signalling pathway	ND	ND	15	2.30E-03
ECM-receptor interaction	ND	ND	9	3.30E-02
p53 signalling pathway	ND	ND	8	2.70E-02

In addition to the pathways induced at 2hrs additional inductions at 6 hrs included the regulation of actin cytoskeleton, calcium signalling pathway, and fc gamma R-mediated phagocytosis. The analysis identified cell adhesion molecules (CAMs) and the glucagon-signalling pathway as

induced in response to P-1 but not WB, while MAPK, the cGMP-PKG signalling pathway and ECM-receptor interaction responded only to WB.

Table 2: KEGG pathways induced in IEC-6 (I) by *G. duodenalis* isolate P-1 and WB at 6hrs. KEGG pathway analysis using DAVID Bioinformatics Resources 6.8 (National Institute of Allergy and Infectious Diseases (NIAID), NIH) identified significant pathways (P value ≤ 0.05). Transcripts whose mRNA level altered log2 fc were used for further analyses. The table shows significant pathways, the number of expressed transcripts, and *P* value. ND denotes not detected.

Induced pathways	No of transcripts in IP-1 at 6hrs	p-Value	No of transcripts in IWB at 6hrs	p-Value
Metabolic pathways	117	3.40E-03	120	6.70E-03
PI3K-Akt signalling pathway	47	1.40E-05	52	8.80E-07
Cytokine-cytokine receptor interaction	39	1.40E-07	43	5.90E-09
TNF signalling pathway	31	5.10E-11	31	1.70E-10
Chemokine signalling pathway	28	1.20E-04	26	1.40E-03
Toll-like receptor signalling pathway	13	4.20E-02	13	5.70E-02
NF-kappa B signalling pathway	13	2.30E-02	15	6.00E-03
NOD-like receptor signalling pathway	10	1.60E-02	10	2.20E-02
Jak-STAT signalling pathway	19	7.10E-03	21	2.40E-03
Fc gamma R-mediated phagocytosis	12	3.80E-02	12	5.10E-02
Rap1 signalling pathway	25	1.90E-02	26	1.90E-02
Ras signalling pathway	29	3.70E-03	30	3.90E-03
HIF-1 signalling pathway	19	3.50E-04	18	1.70E-03
cAMP signalling pathway	22	3.70E-02	24	1.90E-02
B cell receptor signalling pathway	10	5.50E-02	11	3.10E-02
Calcium signalling pathway	24	6.00E-03	26	2.70E-03
Regulation of actin cytoskeleton	25	2.10E-02	27	1.20E-02
Focal adhesion	22	7.10E-02	31	4.20E-04
Apoptosis	11	1.10E-02	13	1.90E-03
Arginine and proline metabolism	11	2.80E-03	9	3.40E-02
Adherens junction	ND	ND	11	4.40E-02
ECM-receptor interaction	ND	ND	12	6.70E-02
MAPK signalling pathway	ND	ND	28	4.80E-02
AMPK signalling pathway	ND	ND	15	9.20E-02
Cell adhesion molecules (CAMs)	22	1.00E-02	ND	ND
Glycolysis / Gluconeogenesis	11	2.60E-02	ND	ND
Glucagon signalling pathway	12	9.80E-02	ND	ND

It is evident that a significant number of cellular pathways show changes in gene expression in response to *G. duodenalis*. A number of pathways were chosen to be investigated in greater

depth to highlight their response to individual isolates. Cytokine-cytokine receptor interaction, chemokine and TNF signalling pathways comprised the majority of immune response transcripts reacting to both isolates during the six hours of the infection. Equally importantly, some transcripts were found to be involved in the extrinsic and intrinsic apoptosis pathways. Finally, a significant number of expressed genes were involved in cell adhesion assembly, in metabolic pathways and in hypoxia-related pathways. Thus, it was decided to concentrate on these pathways and their interrelating processes in an attempt to clarify the molecular events in parasitised cells exposed to each isolate.

3.2.2.3. Cytokine and Interleukin transcripts induction

Upregulation of transcripts was observed in many target genes representing interleukins, colony-stimulating factors, TNF superfamily members and chemokines. The following sections detail these changes.

3.2.2.3.1. Interleukin expression

At 2hrs, the expression of *IL-1A, IL-23A* and *IL-6* transcripts were significantly upregulated in response to the both isolates (Table 3). Significant downregulation was observed in the *IL-15* transcript. The *IL-7* transcript was slightly downregulated upon interaction with the WB isolate. At 6hrs, the results were very similar with the *IL-1A, IL-23A* and *IL-6* transcripts upregulated with both isolates. But significant downregulation in the *IL-7* transcript compared to the 2hr culture. The expression level of the *IL-15* transcript did not show any significant change at 6hrs.

Table 3: Pro-inflammatory cytokine transcripts whose expression level altered in IEC-6 (I) infected by either *G. duodenalis* isolate P-1 or WB at 2 and 6hrs. The mRNA level of transcripts that expressed log2 FC at FDR cut off ⩽0.05 from the uninfected IEC-6 were considered using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). The table below displays transcript name, transcript symbol and fold change of each transcript in response to the isolates P-1 or WB at 2 or 6hrs. ND denotes not detected.

Cytokine	Symbol	Gene transcription (log2 fold change) in co-cultures			
		2hrs IP-1	2hrs IWB	6hrs IP-1	6hrs IWB
interleukin 1 alpha(Il1a)	*IL-1A*	4.47	1.99	3.56	3.59
interleukin 23 subunit alpha(Il23a)	*IL-23A*	3.73	1.64	6.66	7.86
interleukin 6(Il6)	*IL-6*	4.15	2.03	3.47	3.55
interleukin 15(Il15)	*IL-15*	-1.43	-1.22	ND	ND
interleukin 7(Il7)	*IL-7*	ND	-0.76	-1.85	-1.70

3.2.2.3.2. Colony-stimulating factor (CSF) Induction

Giardia isolates induced the transcripts (*CSF1, CSF2* and *CSF3*) encoding the three colony-stimulating factors in IEC-6 at both infection times (Table 4). At 2hrs, the expression level of these transcripts was significantly upregulated in response to the P-1 isolate, while the WB isolate only induced significant upregulation in the mRNA levels of *CSF1* and *CSF3*. At 6hrs the expression level of the transcripts *CSF1, 2* and *3* were all upregulated with both isolates.

Table 4: Colony-stimulating factor (CSF) cytokines transcripts whose expression level changed in IEC-6 (I) induced by either *G. duodenalis* isolate P-1 or WB at 2 and 6hrs. The mRNA level of transcripts that expressed log2 fc at FDR cut off ≤0.05 from the uninfected IEC-6 was considered using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). The table below shows transcript name, transcript symbol, and fold change level of each transcript in response to the isolates P-1 or WB at 2/6hrs. ND denotes not detected.

CSFs	Symbol	Gene expression (log2 fold change) in co-cultures			
		2hrs IP-1	2hrs IWB	6hrs IP-1	6hrs IWB
colony stimulating factor 2(Csf2) also called (GM-CSF)	*CSF2*	5.27	ND	5.55	5.34
colony stimulating factor 3(Csf3) also called G-CSF	*CSF3*	5.98	3.93	7.94	7.76
colony stimulating factor 1(Csf1) also called M-CSF	*CSF1*	1.86	0.84	1.43	1.48

3.2.2.3.4. Chemokine induction

The most striking result to emerge from the data was that the mRNA levels of most chemokines were significantly upregulated throughout the infection (Table 5). The transcripts of *CCL20, CXCL2, CX3CL1, CCL9, CXCL3, CCL7, CXCL1,* and *CCL2* all showed substantial changes in expression. The expression of *CXCl16* and *CXCL12* were only moderately affected while the expression of *CXCR3* was downregulated. Transcripts that changed their responses during the culture period included *CCR7* and *CXCL13* which were significantly upregulated at 2hrs but did not show any significant change at 6hrs. In contrast, the *CCL5* transcript was not significantly changed at 2hr, but was upregulated at 6hrs. The expression of *CCL6* was only significantly induced in response to P-1, while *CCL27* expression only changed with WB.

Table 5: Chemokine transcripts whose expression level altered in IEC-6 (I) induced by either isolate P-1 or WB at 2 and 6hrs. The mRNA level of genes that expressed log2 fc at FDR cut off ⩽0.05 from the uninfected IEC-6 was considered using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). The table below illustrates transcript name, transcript symbol, and fold change level of each transcript in response to the isolates P-1 or WB at 2 or 6hrs. ND denotes not detected.

Chemokines and receptors	Symbol	Gene transcription (log2 fold change) in co-cultures			
		2hrs IP-1	2hrs IWB	6hrs IP-1	6hrs IWB
C-C motif chemokine ligand 20(Ccl20)	*CCL20*	6.84	4.08	4.69	5.21
C-X-C motif chemokine ligand 2(Cxcl2)	*CXCL2*	5.92	2.58	5.36	5.67
C-X3-C motif chemokine ligand 1(Cx3cl1)	*CX3CL1*	5.92	2.58	5.36	5.67
chemokine (C-C motif) ligand 9(Ccl9)	*CCL9*	4.94	2.70	4.68	4.69
chemokine (C-X-C motif) ligand 3(Cxcl3)	*CXCL3*	4.48	2.53	4.18	4.16
C-X-C motif chemokine ligand 1(Cxcl1)	*CXCL1*	4.19	2.95	3.47	3.59
C-C motif chemokine ligand 2(Ccl2)	*CCL2*	3.81	1.60	2.98	3.44
C-X-C motif chemokine ligand 6(Cxcl6)	*CXCL6*	2.63	ND	5.43	6.05
C-C motif chemokine receptor 7(Ccr7)	*CCR7*	2.57	3.18	ND	ND
C-C motif chemokine ligand 7(Ccl7)	*CCL7*	2.50	0.86	1.79	1.86
C-X-C motif chemokine ligand 13(Cxcl13)	*CXCL13*	1.72	2.75	ND	ND
C-X-C motif chemokine ligand 10(Cxcl10)	*CXCL10*	1.86	ND	ND	-1.19
C-X-C motif chemokine ligand 16(Cxcl16)	*CXCL16*	1.02	0.36	1.59	1.43
C-X-C motif chemokine ligand 12(Cxcl12)	*CXCL12*	0.91	0.44	0.40	0.70
C-X-C motif chemokine receptor 3(Cxcr3)	*CXCR3*	-1.91	-1.51	-1.53	-1.82
chemokine (C-C motif) ligand 6(Ccl6)	*CCL6*	-2.56	ND	1.96	ND
C-C motif chemokine ligand 27(Ccl27)	*CCL27*	ND	-0.65	ND	-0.92
C-C motif chemokine ligand 5(Ccl5)	*CCL5*	ND	ND	1.62	0.84

3.2.2.3.3. TNF superfamily induction

Transcription of the tumor necrosis factor superfamily members was affected in *G. duodenalis* - induced IEC-6 at 2 and 6hrs (Table 6). There was variation in responses at 2hrs with the *TNFSF10* transcript not responding to the P-1 isolate while *TNFSF18, TNFSF-15* and *TNFRSF11B* did not respond to WB. *TNFRSF21* and *TNFRSF1A* transcripts showed some upregulation at 2hrs but their expression was downregulated at 6hr with both isolates.

Table 6: TNF superfamily transcripts altered in IEC-6 (I) induced by either *G. duodenalis* isolate P-1 or WB at 2 and 6hrs. The expression level of transcripts that changed log2 fc at FDR cut off ≤ 0.05 from the uninfected IEC-6 was considered using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). The table below indicates transcript name, transcript symbol, and fold change level of each transcript in response to the isolates P-1 or WB at 2 or 6hrs. ND denotes not detected.

Cytokines and receptors	Symbol	Gene transcription (log2 fold change) in co-cultures			
		2hrs IP-1	2hrs IWB	6hrs IP-1	6hrs IWB
TNF receptor superfamily member 21(Tnfrsf21)	*TNFRS-21*	0.37	0.23	-0.75	-0.85
TNF receptor superfamily member 1A(Tnfrsf1a)	*TNFRSF-1A*	0.37	0.23	-0.75	-0.85
TNF receptor superfamily member 9 (Tnfrsf9)	*TNFRSF-9*	1.76	1.68	2.86	3.71
tumor necrosis factor superfamily member 15(Tnfsf15)	*TNFSF-15*	2.44	ND	2.88	2.88
tumor necrosis factor superfamily member 18(Tnfsf18)	*TNFSF-18*	1.85	ND	2.79	3.13
TNF receptor superfamily member 11B(Tnfrsf11b)	*TNFRSF-11B*	1.38	ND	4.59	4.70
tumor necrosis factor superfamily member 10(Tnfsf10)	*TNFSF-10*	ND	2.24	1.94	2.21
Fas cell surface death receptor (Fas)	*FASR*	3.74	2.32	1.84	2.37

3.2.3.4. Arginine pathway induction

Arginine metabolism was significantly altered following the 2 and 6hrs co-incubation with both isolates (Table 7). The nitric oxide synthase 2 (*NOS2*) transcript in particular showed considerable change in the parasitised IEC-6 throughout the interaction.

At the 2hrs, the expression level of *NOS2* was significantly upregulated in IEC-6 in response to both isolates with 5.06 and 2.34 log2 fold changes for P1 and WB, respectively. The *ARG1* transcript showed a slight upregulation in response to WB but not P1. No significant differences were found in the expression of the *ARG2* transcript. Expression of the *ODC1* transcript was either constant or slightly downregulated to both isolates. In contrast, the expression of *AZIN2* was remarkably upregulated in the presence of both isolates.

At 6hrs the *NOS2* transcript remained at similar levels to 2hrs for the P1 isolate but showed a significant increase from 2hrs for the WB isolate. *ARG1* showed no significant expression,

while the *ARG2* transcript was significantly upregulated with both isolates. Furthermore, the expression level of the *ODC1* transcript was triggered in response to both isolates. The upregulation of the *AZIN2* transcript was observed at both 2 and 6hrs with both isolates.

Table 7: Arginine pathway transcripts induced in IEC-6 (I) infected by either *G. duodenalis* isolate P-1 or WB at 2 and 6hrs. The expression level of genes that expressed log2 fc at FDR cut off ≤0.05 from the uninfected IEC-6 was considered using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). The table below shows transcript name, transcript symbol, and fold change level of each transcript in response to the isolates P-1 or WB at 2 or 6hrs. ND denotes not detected.

Arginine pathway-involved transcripts	Symbol	Gene transcription (log2 fold change) in co-cultures			
		2hrs IP-1	2hrs IWB	6hrs IP-1	6hrs IWB
Nitric oxide synthase 2(Nos2)	*NOS2*	5.06	2.34	5.73	4.88
Arginase 1(Arg1)	*ARG1*	ND	0.38	ND	ND
Arginase 2(Arg2)	*ARG2*	ND	ND	2.09	2.00
Ornithine decarboxylase 1(Odc1)	*ODC1*	ND	-0.23	0.52	0.36
antizyme inhibitor 2(Azin2)	*AZIN2*	2.50	1.79	2.17	2.38

3.2.3.5. HIF-1 signalling pathway induction

G. duodenalis infection significantly induced the expression of hypoxia-inducible (HIF) transcripts, (transcripts involved in response to hypoxia, host defence, and signalling pathways) in IEC-6 (Table 8).

It is apparent from this table that the gene transcription change of *HIF1A* was significantly upregulated in response to both isolates, with expression increasing at both times. Simultaneously, significant downregulation was observed in the expression of the *PHD3* transcript at 6hrs with both isolates. The metabolic transcripts *SLC2A1* and *HK2* were also significantly upregulated in response to both isolates particularly at the 6hrs induction.

The mRNA levels of *NOX1* and *NOS2* transcripts were significantly induced in the parasitised IEC-6 at 2hrs in response to P1, *NOX1* however, did not show significant changes in response

to WB. At 6hrs the expression of both genes was upregulated with both isolates. The *NFKB1* gene transcript was consistently upregulated with both isolates at 2 and 6hr. Furthermore, mRNA levels of some transcripts encoding cytokines (i.e. IL-6, IL-6R, and IFNGR2), growth and translational factors (i.e. EGFR and PRKCG) and signalling pathways such as PI3K-AKT and MAPK were significantly induced and are involved in the HIF-1 pathway (Table 8).

Table 8: HIF-1 pathway transcripts whose expression level altered in IEC-6 (I) induced by either *G. duodenalis* isolate P-1 or WB at 2 and 6hrs. The mRNA level of transcripts that expressed log2 fc at FDR cut off ⩽0.05 from the uninfected IEC-6 was considered using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). The table below reveals transcript name, transcript symbol, and fold change level of each transcript in response to the isolates P-1 or WB at 2 or 6hrs. ND denotes not detected.

HIF-1 signalling pathway	Symbol	Gene transcription (log2 fold change) in co-cultures			
		2hrs IP-1	2hrs IWB	6hrs IP-1	6hrs IWB
hypoxia inducible factor 1 alpha subunit(Hif1a)	*HIF1A*	0.71	0.17	1.25	1.12
egl-9 family hypoxia-inducible factor 3(Egln3)	*EGLN3 (PHD3)*	ND	ND	-1.06	-0.78
von Hippel-Lindau tumor suppressor(Vhl)	*VHL*	ND	ND	0.31	0.55
solute carrier family 2 member 1(Slc2a1)	*SLC2A1 (GLUT1)*	0.26	0.77	1.18	0.68
hexokinase 2(Hk2)	*HK2*	0.90	ND	1.93	1.74
NADPH oxidase 1(Nox1)	*NOX1*	2.61	ND	4.23	4.34
nitric oxide synthase 2(Nos2)	*NOS2 (iNOS)*	5.06	2.34	5.73	4.88
interleukin 6(Il6)	*IL-6*	4.15	2.03	3.47	3.55
interleukin 6 receptor(Il6r)	*IL-6R*	1.11	0.94	ND	0.30
interferon gamma receptor 2(Ifngr2)	*IFNGR2*	1.36	0.56	1.36	1.65
epidermal growth factor receptor(Egfr)	*EGFR*	0.17	0.16	1.54	1.27
TEK receptor tyrosine kinase(Tek)	*TEK*	ND	ND	-0.67	-0.90
nuclear factor kappa B subunit 1(Nfkb1)	*NFKB1*	1.87	0.73	1.64	1.74
protein kinase C, gamma(Prkcg)	*PRKCG*	ND	ND	0.78	0.55
phosphoinositide-3-kinase regulatory subunit 3(Pik3r3)	*PIK3R3*	-0.99	-1.10	-0.42	-0.63
phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit delta(Pik3cd)	*PIK3CD*	-0.89	-0.58	-0.37	-1.15
phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit beta(Pik3cb)	*PIk3CB*	ND	-0.90	1.67	1.43
phosphoinositide-3-kinase, regulatory subunit 5(Pik3r5)	*PIK3R5*	ND	ND	4.95	4.96
mitogen activated protein kinase 3(Mapk3)	*MAPK3*	0.3	0.34	0.24	0.47
MAP kinase-interacting serine/threonine kinase 2(Mknk2)	*MKNK2*	ND	0.18	-0.51	-0.45
mitogen activated protein kinase kinase 1(Map2k1)	*MAP2K1*	ND	-0.28	0.63	0.60
ribosomal protein S6 kinase, polypeptide 2(Rps6kb2)	*RPS6KB2*	ND	ND	-0.44	-0.52
eukaryotic translation initiation factor 4E binding protein 1(Eif4ebp1)	*EIF4EBP1*	ND	0.35	1.99	1.85

3.3. Discussion

Recently, it has been suggested that strain-specific pathogenicity in *Giardia* may be elucidated through advanced comparative genomics studies (Bartelt and Sartor, 2015). A comparative genomic study has demonstrated that significant differences in terms of gene polymorphisms, chromosome size, surface protein repertoire and gene content are present in assemblage A isolates (Ankarklev *et al.*, 2015). Nevertheless, little is known about the transcriptome profile of host cells in response to assemblage A isolates. In this chapter, RNA-seq technology was used to provide insights into the transcriptome of IEC-6 infected with *G. duodenalis* assemblage A isolates P-1 or WB for 2 and 6hrs. Initial quality control (QC) metrics for both total RNA and prepared libraries showed outstanding integrity and concentration, giving confidence in the validity of the subsequent sequence data.

Analysis of the NGS data revealed no significant variations between biological replicates in each experimental condition, resulting in equivalent average expression levels (CPM) of expressed genes in control and infected samples. QC is a crucial step in RNA-seq experiments because sequencing and subsequent analyses essentially rely on the high quality of source RNA (Wang *et al.*, 2009).

3.3.1. Transcriptome profiling induced in the parasitised IEC-6

RNA-seq identified a large number of expressed transcripts in the parasitised IEC-6 at both incubation times with both isolates. This wealth of data gave a holistic picture of host cell responses to early giardiasis. Comparison with the limited data sets available in the literature suggested that the some results are in agreement with a previous study which used a hybridization method (microarray) in differentiated Caco-2 cells following 1.5, 6, and 18hrs induction with *G. duodenalis* isolate WB-A11 (Roxström-Lindquist *et al.*, 2005). For instance, the findings of both studies detected a significant increase in the mRNA level of several chemokines in response to the infection. However, the current study identified considerably more transcripts whose expression levels were altered compared to the microarray method. The difference might be ascribed to either the different techniques used or the different cells tested. Recently in our laboratory, the transcriptome of *G. duodenalis* infected IEC-6 was investigated using representational difference analysis (RDA). The results of this study revealed 132 and

106 transcripts upregulated at 2 and 6hrs incubation (Ma'ayeh, 2013, unpublished data). The current study identified 3394 and 5502 transcripts in response to P-1 and WB isolates at 2hrs, while 6011 and 6096 transcripts responded to these isolates at 6hrs, respectively. RNA-seq is thus a highly sensitive and high throughput technology providing a more representative transcriptome than other hybridization-based approaches available to date, including microarray (Wang *et al.*, 2009).

At 2hrs the WB isolate induced more changes in gene transcription (up and downregulated) than P-1. Additionally, a large number of unique genes (1255) were specifically affected by WB, whilst only 193 transcripts responded to P-1. These observations might suggest an ability of WB to modulate molecular events in the parasitised cell more quickly than P-1. This might be due to differences in pathology, growth rate or adaptation to culture conditions.

The transcriptome analysis of the 6hr infection relative to 2hrs displayed increased numbers of expressed transcripts in response to both isolates with only slight differences in these numbers. The increase in the number of transcripts with altered expression at 6hrs suggests increased effects of both isolates on the cells probably due to growth in the numbers of parasites and/or the development of defensive responses against the infection. In previous studies, it has been reported that hosts develop immune responses to parasite antigens during infection (Faubert, 2000). Another study stated that the difference in clinical manifestations could be determined by factors such as; the virulence of the *G. duodenalis* strain, the state of the host immune system at the time of infection, the number of cysts ingested and the age of the host (Aggarwal and Nash, 1987; Nash *et al.*, 1987).

3.3.2. Cellular pathway analysis

The most obvious finding to emerge from this analysis was that of the induction of important cellular pathways, such as cytokine-cytokine receptor interaction, TNF and chemokine signalling, arginine, HIF-1 signalling, cell-cell adhesion, and apoptosis. Furthermore, the general observations showed that various signalling pathways were the most commonly altered at the level of gene transcription in the parasitised cells. The number of different pathways affected suggests their relative significance and emphasises the multifunctional impact of *G.*

duodenalis on early responses of the host cell (Fernandes and Assreuy, 1997; Chin *et al.*, 2002; Scott *et al.*, 2002; Roxström-Lindquist *et al.*, 2005; Fulda, 2013; Shirley and Micheau, 2013; Tako *et al.*, 2013; Lopez-Romero *et al.*, 2015).

3.3.2.1. Cytokine induction

The increases in mRNA levels of the pro-inflammatory cytokines transcripts *IL-1A, IL-23A,* and *IL-6* may be attributed to the role of enterocytes in mediating immune responses through recruitment of immune cells at sites of the infection. A previous study noted an important role for *IL-6* in the early control of acute *G. duodenalis* infections in wild-type mice (Zhou *et al.*, 2003), which was confirmed by the significant effects on protective immune responses to *G. duodenalis* GS/M-83-H7 in IL-6-deficient mice (Bienz *et al.*, 2003). Another study suggested that IL-6 secreted by mast cells probably has a vital role in *Giardia* infection control (Li *et al.*, 2004, Muñoz-Cruz *et al.*, 2010). Thus, the result for IL-6 expression in IEC-6 cultured with *Giardia duodenalis* would be expected.

Although very little was found in the literature concerning the role of *IL-23A* and *IL-1A* in giardiasis, a recent study showed that increased secretion of IL-23 was observed in dendritic cells induced by *Giardia* in the presence of Toll-like receptor 2 ligands (Obendorf *et al.*, 2013). This study showed the expression of the toll-like receptor 2(*TLR2*) transcript was highly upregulated in *G. duodenalis*-induced IEC-6 together with increased levels of IL-23. Previous research documented the production IL-23 by dendritic cells and macrophages during bacterial infections and Toll-like receptor engagement. Although the biological impact of IL-23 is incompletely understood (Langowski *et al.*, 2006), it is suggested that the combination of TLR2 and IL23 found here is likely an early event in immune response initiation.

IL-1A is a primary cytokine that initiates inflammatory and immune responses (Bankers-Fulbright *et al.*, 1996), thus increases in IL-1A mRNA levels in the current study would be expected to play a key role in the response to *G. duodenalis* infection. However, this *IL-1A* outcome is contrary to that of the Roxstrom-Lindquist *et al.* who did not find significant induction in gene transcription of *IL-1* in intestinal epithelial cells (Caco-2) during giardiasis using oligonucleotide microarrays (Roxström-Lindquist *et al.*, 2005). Inconsistent findings

might be ascribed to the use of different techniques and different cell types, with RNA-seq technology having a higher sensitivity to survey the transcriptome, compared with hybridization-based methods, including microarray (Wang *et al.*, 2009). In addition, these inconsistent results might be ascribed to the difference in the cell line used, where Caco-2 cells are transformed (Engle *et al.*, 1998; Lea, 2015) whilst IEC-6 was a non-transformed cell line (Quaroni *et al.*, 1979; Ametani *et al.*, 1993).

The downregulation in expression of the *IL-15* and *IL-7* transcripts observed in our data might suggest an inhibitory role for *G. duodenalis* trophozoites against these responses. These transcripts are known to be important for the development of protective CD8+ T cell immune response against coccidian parasites (Berard *et al.*, 2003; Bhadra *et al.*, 2010). These cytokines may be involved in host cell defence against early giardiasis, and their inhibition may thus be important to parasite survival. Further analysis of the role of these cytokines in vivo and the direct effects of the parasite may be worthwhile.

The upregulation of the *CSF1*, *CSF2*, and *CSF3* transcripts is interesting as these cytokines are classified as immuno-stimulatory factors involved in the regulation and production of immune cells (Hamilton, 2008; Seruga *et al.*, 2008). In a recent investigation, the expression level of *CSF2* was found to be upregulated in individuals infected with giardiasis (Saghaug *et al.*, 2016) and it has been suggested that this cytokine mediates cell survival, as well as neutrophil recruitment and activation (Annunziato *et al.*, 2015). CSF3 promotes granulopoiesis and granulocytic differentiation (Numata *et al.*, 2005). In the experiments reported here it may be supposed that *G. duodenalis* induces these inflammatory cytokines as part of the host cell response to initiate and direct immune and inflammatory responses against the parasite.

The results in Table 5 showed the greatest upregulation in the expression of transcripts for chemokines *CCL20*, *CXCL2*, *CXCL3*, *CXCL1* and *CCL2* at 2 and 6hrs of infection. This is in agreement with previous studies which reported these chemokines were induced in differentiated human colon carcinoma (Caco-2) cells on infection with *G. duodenalis* assemblage A and B isolates, suggesting their role to recruit neutrophils, dendritic cells, T and B cells to infection sites (Roxström-Lindquist *et al.*, 2005; Ma'ayeh *et al.*, 2017).

In the current data a new set of chemokines including; *CX3CL1*, *CCL9*, *CXCL6*, *CCL7*, *CXCL13*, *CXCL16*, *CXCL12*, *CCL6*, *CCL27*, and *CCL5* was also detected. Some of these chemokines play important roles in other conditions and diseases, including protozoal infections. For instance, *CX3CL1* and *CXCL16* attach to the surface of their origin cells, and mediate the adhesion and rolling of leucocytes to endothelial cells (Bryant and Slade, 2015). The expression level of *CX3CL1* transcript is upregulated in epithelial cells upon *Cryptosporidium parvum* infection, suggesting a role in mucosal defence via immune cell recruitment (Zhou, Gong, *et al.*, 2013). The chemokine CCL9 plays a key role in CD11b+ DC recruitment to the dome regions of mouse Peyer's patches (Zhao *et al.*, 2003). While the upregulation in mRNA levels of the *CXCL6* transcript was observed in IBD-infected patients (Gijsbers *et al.*, 2004) suggesting a role in inflammation. Furthermore, the chemokines *CCL5*, *CCL2* and *CXCL10* have been found to be produced in enterocytes infected by *C. parvum* to initiate an intestinal inflammatory response (Lacroix-Lamandé *et al.*, 2002; Pantenburg *et al.*, 2008). Finally, increased levels of the *CXCL13* together with *CXCL8* and *CXCL2* were detected in bacteria - induced dendritic cells (DCs), indicating a potential chemotactic role for *CXCL13* in B cells recruitment (Vissers *et al.*, 2001; Vermi *et al.*, 2006). Thus the role of these chemokines in most gut infections is in the recruitment of various cells to the site of infection and their activation in this study would be consistent with such a role.

The transcriptome of TNF superfamily (TNFSF) members was significantly stimulated in the IEC-6 by both *G. duodenalis* isolates (Table 6). The literature reports that these proteins are cytokines and cytokine receptors with important impacts on the regulation of cellular homeostasis, innate and adaptive immunity and the induction of cell death (Locksley *et al.*, 2001; Hehlgans and Pfeffer, 2005; Croft *et al.*, 2012). It has been suggested that inflammatory responses in epithelial cells and fibroblasts can be driven through the TNFSF cytokines; but the functions of many TNFSF proteins are incompletely understood (Croft and Siegel, 2017). The mRNA level changes in *TNFSF10*, *TNFRSF1A* and *FASR* in this data are suggested to be key players in the cellular extrinsic apoptosis pathway during *G. duodenalis* infection (Chapter 6). In addition, these transcripts may participate in cellular immune responses against this infection with previous research suggesting these proteins are pro-inflammatory mediators depending on the target cell type (Croft and Siegel, 2017). For example, the activation of *TNFRSF1A* in *G. duodenalis* infected intestinal cells suggests a role in promotion of leukocyte binding (Roxström-Lindquist *et al.*, 2005).

The upregulation the receptor *TNFSF11B*, in particular at 6hrs might be critical to maintenance and survival of the IEC-6 with previous studies revealing that TNFSF11B (also called osteoprotegerin (OPG)) has a crucial impact in the inhibition of apoptosis (Baud'huin *et al.*, 2013). Thus, the *TNFSF11B* gene was found to be secreted in human ileal mucosa and its expression level is upregulated upon early *Cryptosporidium* infection, where it prevented the binding between TRAIL and death-inducing receptors to suppress the apoptotic signalling cascade and give a survival advantage to the cells (Castellanos-Gonzalez *et al.*, 2008). Another study revealed that *NF-kB* regulates *TNFSF11B* in rat intestinal epithelial cells, where its upregulation was again linked to inhibition of apoptosis (Toruner *et al.*, 2006).

Increased mRNA levels of the *TNFSF15* transcript in the parasitised IEC-6 suggests a role as a co-stimulator of cytokine activity. Both *in vitro* and i*n vivo* investigations suggested that *TNFSF15* is a co-stimulator that increases IL-2 responsiveness and the secretion of pro-inflammatory cytokines (Migone *et al.*, 2002). Other research found that the binding between *TNFSF15* and its receptor has a role in inflammatory bowel disease, where upregulation was significant in inflamed intestinal tissues (Siakavellas and Bamias, 2015). Taken together, analysis of the expression of mRNA levels in the TNF superfamily suggests roles at least in immune induction and inhibition of cell death.

The main goal of the current analysis was to determine the specifics of the induction of pro-inflammatory cytokines in intestinal epithelial cells on exposure to different *G. duodenalis* assemblage A isolates. The RNA-seq data supports a conclusion of significant alterations in mRNA levels of a wide range of cellular cytokines following the 2 and 6hrs induction. Most of these cytokines have a pro-inflammatory function and target specific cell types often in common with other protozoan infections of the gut. In addition, a number are involved in controlling intracellular responses including the inhibition of cell death responses which is investigated further in this thesis (Chapter 6). Differences in the response between the two isolates tested were also found and though these are limited they are consistent with the pathological and epidemiological variation that characterises these isolates. Future studies are required to confirm that the observations of changes in mRNA expression are accompanied by protein production and the direct biological effects of these proteins both on the enterocytes, other cells and on the infecting trophozoites.

3.3.2.2. Arginine pathway induction

Arginase and inducible nitric oxide synthase (iNOS or NOS2) play key roles in the metabolism of arginine, leading to the production of a range of radically different biological compounds (Bernard *et al.*, 2001; Morris, 2004). Previous studies have suggested that *G. duodenalis* trophozoites consume cellular arginine as an energy source through three arginine-consuming enzymes (arginine dihydrolase, arginine deiminase (ADI), and ornithine carbamoyl transferase (OCT) (Schofield *et al.*, 1990; Ringqvist *et al.*, 2008). The parasite depletes the amino acid arginine of the host leading to inhibit of NO production, so escaping from one of the main host defences (Schofield *et al.*, 1990; Edwards *et al.*, 1992; Knodler *et al.*, 1994; Eckmann *et al.*, 2000; Ringqvist *et al.*, 2008; Stadelmann *et al.*, 2012).

Mammalian arginase (the final enzyme of the urea cycle), has two distinct isoforms (arginase 1 and 2) encoded by independent transcripts which differ in regulation of expression, molecular and immunological characteristics (Jenkinson *et al.*, 1996). In our current data, the *ARG1* transcript did not generally show significant expression upon infection with the exception of small but significant upregulation was observed in response to the isolate WB at 2hrs. The slight upregulation in mRNA levels of this transcript to this isolate suggests a response to reduced arginine or ornithine, the catalyst involved in conversion of arginine to ornithine and urea. Host cells may be attempting to maintain a homeostatic balance of metabolites. Ornithine is a precursor of proline, supporting cellular regeneration, repair, and cell proliferation (Morris *et al.*, 1998; Bronte and Zanovello, 2005). In recent research, the mRNA level of the *ARG1* transcript was found to be upregulated in mouse small intestine tissue infected with *G. duodenalis* isolate GS, though it did not show significant expression in duodenal macrophages of infected mice (Maloney *et al.*, 2015). The upregulation to WB might be indicative of faster growth or more specific targeting of this isolate to host arginine stores, compared with the absence of *ARG1* induction in response to the isolate P-1. Thus, this finding might also indicate the difference in pathogenicity levels between these two isolates.

Our investigation did not detect significant changes in *ARG2* transcript expression at 2hr. However, its expression was obviously upregulated at 6hrs with both isolates. The research to date has tended to focus on ARG1 rather than ARG2 during giardiasis, except one study which investigated the gene transcription of different arginine-metabolizing genes including *ARG1*

and *2* in intestinal epithelial cells in response to three different *G. duodenalis* isolates WB, GS, and P15. The findings showed that most transcripts, including *ARG1 and 2* were either constant or downregulated, independent of the parasite isolate (Stadelmann *et al.*, 2013). In a different study, upregulation was observed in mRNA and protein levels of ARG2 upon a bacterial infection, with an implication in immune evasion suggested through arginine depletion leading and inhibition of NO (Lewis *et al.*, 2010). Although, this study showed the function of arginine 2 in infected macrophages of mice, bacterial infections are not appropriate references for the comparison with giardiasis. Therefore, the role of arginine 2 upon giardiasis remains to be investigated.

NOS2 produces NO, an important chemical of the innate immune response and toxic to a wide range of different pathogens (Popovic *et al.*, 2007). The current study identified changes in transcripts involved in the cellular arginine pathway and NO production pathway following *Giardia* infection. The most interesting finding was that the expression level of the *NOS2* gene was greatly upregulated in response to both isolates at both time points. This finding is in agreement with a previous study which demonstrated that inducible NO synthase (iNOS) gene transcription was upregulated within 6hrs of *G. duodenalis* infection (Stadelmann *et al.*, 2013). It is also consistent with an *in vivo* study conducted by Maloney *et al.* (2015) who showed that *NOS2* gene expression increased in mouse intestine infected with *G. duodenalis* using qRT-PCR and immunohistochemistry techniques. This study also found that NO inhibits *G. duodenalis in-vitro* and *in-vivo*. Thus increased iNOS induction might be associated with *G. duodenalis*-arginine consumption in host cells (Stadelmann *et al.*, 2013), or with NOS2 triggered NO production involved in host defence against giardiasis (Fernandes and Assreuy, 1997; Popovic *et al.*, 2007; Tako *et al.*, 2013).

Other transcripts involved in arginine utilisation were also induced in the parasitised IEC-6. For instance, slight upregulation was detected in the expression of the Ornithine decarboxylase (*ODC1)* transcript at 6hrs. The *ODC1* gene encodes the rate-limiting enzyme of the polyamine biosynthesis pathway which catalyses ornithine to putrescine (tetramethylenediamine). Putrescine is produced by the breakdown of amino acids typically in dead or dying tissue and has a characteristic foul odour, it is also a signalling molecule linked to the cell cycle and cell growth (Alm and Oredsson, 2009). This finding is in agreement with Stadelmann *et al*, who

found that this gene was upregulated after 3 and 6hrs of the incubation with three different *G. duodenalis* isolates (Stadelmann *et al.*, 2013). Another study indicated a high level in the *ODC* gene transcription in host cells after the 24hrs interaction with *G. duodenalis*, suggesting that this parasite causes polyamine depletion leading to cell cycle suspension (Stadelmann *et al.*, 2012). Previous data reported that the ODC is an important enzyme that is present in different protozoal infections, including *Giardia*, and has been validated as drug target for *Trypanosoma brucei* infection (Bacchi *et al.*, 1980; Birkholtz *et al.*, 2011).

AZIN2 is another transcript significantly upregulated at the both times and in response to both isolates. It has been reported that this antizyme inhibitor (AZIN) is an ODC-related protein that effectively negates the antizymes activity, by contrast it positively regulates the ODC activity and polyamine uptake (Kanerva *et al.*, 2008). Thus, induction in *ODC1* gene transcription in this data might be ascribed to an initial effect of *G. duodenalis* on IEC-6 cell proliferation. In addition, it has been thought that the parasitised IEC-6 responded to the infection by increasing mRNA levels of *AZIN2* transcript to support the ODC mechanism, which in turn maintains cellular proliferation during this time of the infection.

In this investigation, the aim was to assess whether *G. duodenalis* assemblage A induces arginine pathway genes in intestinal epithelial cells following short-term co-cultures. The RNA-seq data revealed significant changes in the expression of these transcripts, in particular the *iNOS* transcript, a major source of NO production. The data supports suggestions by previous studies that host cells produce NO as an effective factor in control of giardiasis. It is recommended that further research be undertaken to understand the biological role of the other arginine pathway transcripts significantly expressed during giardiasis, such as *ARG2*.

3.3.2.3. HIF-1 induction

Significant clusters of genes associated with hypoxia and its immune defence displayed altered expression in these cultures. For instance, the expression level of the *HIF1A* transcript which encodes one of the main hypoxia inducible factors, was upregulated in the infected cells. This gives strong support to the suggestion that *G. duodenalis* induces hypoxia in these intestine epithelial cells. HIF-1, HIF-2, and HIF-3 are known to represent the transcriptional response

that mediates hypoxia (Ratcliffe *et al.*, 2017). The current finding supports those of Roxstrom-Lindquist *et al.* (2005), who found increased levels of hypoxia inducible factor 2 (HIF2) in host cells upon *Giardia* infection. Ma'ayeh, (2013) also showed the upregulation of expression of *HIF-1α* and other hypoxia transcript in the IEC-6 after 6hrs interaction with *G. duodenalis*. Expression levels of these transcripts is also upregulated in host cells during toxoplasmosis infection and the suggestion was made that activation of HIF-1 was necessary for growth and survival the parasite (Spear *et al.*, 2006). A role for hypoxia either as part of the parasite's survival strategy or the host cell response is suggested by the downregulation in expression of the *PHD3* transcript and the stability of *HIF-1 α* in the present study at 6hrs. During hypoxia, prolyl hydroxylase domain proteins (PHDs) are inhibited and HIF-α factors become stable leading to them to associate with HIF-β subunits inside the nucleus which in turn activates transcription factors that bind hypoxia responsive elements (HREs) and trigger various pathway genes (Wenger *et al.*, 2005; Mole *et al.*, 2009; Semenza, 2010). The actual role of hypoxia in giardiasis of not illuminated by these results but its existence is confirmed. Further studies would be useful to determine the mechanism of parasite hypoxia induction and its effects on parasite and host cell survival and growth.

The upregulation in expression levels of various metabolic transcripts such as *SLC2A1 (GLUT1)* and *HK2* are triggered by HIF-1 pathway and this was observed in our cultures. This not only suggests reduced oxygen consumption but also glucose starvation. The protein encoded by *SLC2A1* gene is a major facilitative glucose transporter, whose mRNA level is upregulated by reduced glucose levels (Wang, 2013). HK2 also plays a critical role in glucose metabolism, as it significantly promotes ATP production needed for energy (Ahn *et al.*, 2009; Palmieri *et al.*, 2009; Shan *et al.*, 2014). Ma'ayeh, (2013) found upregulation of both these transcripts in IEC-6 during *Giardia* infection. Furthermore, previous studies have shown that HK2 and HK3 can be regulated by hypoxia (Wyatt *et al.*, 2010), while overexpression of HK2 has cytoprotective effects in cells through prevention of the binding of BAX-VDAC channel which releases cytochrome c from the mitochondria membrane during apoptosis (Pastorino *et al.*, 2002). Thus, the induction of hypoxia and cellular metabolic pathways suggests that the host cells are under significant stress from the infection at an early time point and suggest direct parasite effects on these pathways. The role of such host cell perturbations in parasite growth and survival is conjecture but further work on these mechanisms might be worthwhile is understanding early parasite establishment and pathology.

As discussed briefly above the upregulation of the expression of *NOX1* and *NOS2* transcripts leads to production of reactive oxygen species (ROS) and NO, respectively. Previous investigations suggest that NO and ROS play important roles in the maintenance of different biological functions and in immune responses to various pathogens (Bedard and Krause, 2007; Jiang *et al.*, 2007; Sorci and Faivre, 2009; Pavanelli *et al.*, 2010; Liu *et al.*, 2016).

Hypoxia leads to increased generation of ROS (Waypa *et al.*, 2001; Guzy *et al.*, 2005; He *et al.*, 2005) and although excessive levels of ROS cause cellular protein damage, production in low or moderate amounts play a key role in physiological processes such as killing of invading pathogens, tissue repair and wound healing (Rao *et al.*, 1999; Bhattacharyya *et al.*, 2014).

The production of NO is mainly mediated by NOS2 in epithelial, endothelial and inflammatory cells (Stuehr, 1999; Popovic *et al.*, 2007; Lundberg *et al.*, 2008). It has been recently suggested that NO may modulate the cellular response to hypoxia, where it regulates HIF-1 through modulating the activity of the oxygen-sensor enzymes PHDs and FIH-1 (Liu *et al.*, 1998; Huang *et al.*, 1999; Callapina *et al.*, 2005; Kozhukhar *et al.*, 2006; Berchner-Pfannschmidt *et al.*, 2010). Thus, induction of NO in the present study might also be a cellular defence mechanism induced by hypoxia stress.

The current findings are in agreement with Roxström-Lindquist *et al.*, (2005) who found increased levels of *NOX1* and *NOS2* transcripts using microarray analysis, suggesting that induction of host innate immune defences (ROS and NO) might be ascribed to stress in *Giardia*-infected cells. A more recent study did not find significant upregulation in neither *NOX1* nor *NOS2* in IEC-6 using the RDA technique (Ma'ayeh, 2013). The differences in these findings are likely to be due the different techniques used and a lack of sensitivity in the RDA method (Chapter 4).

In summary, the present study was designed to evaluate alterations in gene transcription in rat intestine epithelial cells at 2 and 6hrs after infection with two *G. duodenalis* assemblage A isolates. RNA-seq technology was used and identified a large number of transcripts displaying altered expression (downregulation and upregulation) over the study period. The most notable

changes occurred in genes coding for pro-inflammatory cytokine and chemokines many of which have not been previously described in responses to *Giardia*. In addition, transcriptional regulation of several cellular pathways was shown including apoptosis, hypoxia/stress, arginine metabolism, cell adhesion, and other immune and stress pathways. The data also confirmed differences in response between the WB isolate, which caused more gene transcription changes compared with P-1 at 2hrs, suggesting that these isolates might have different pathogenic effects in their hosts. Biochemical and metabolomics studies on these pathways may not only confirm these results but also allow insights into the earliest events of establishment of the parasite and defence initiation in the host.

Chapter 4

Comparative RNA-Sequencing (RNA-seq) and representational difference analysis (RDA)

Chapter 4: Comparative RNA-Sequencing (RNA-seq) and representational difference analysis (RDA)

4.1. Introduction

Representational difference analysis (RDA) is a technique that relies on the creation of 'representations' of the transcriptome by PCR of cDNA, followed by subtractive hybridization to identify specific differences between two complex genomes (populations), as well as analysing gene expression in eukaryotes (Lisitsyn and Wigler, 1993; Lewis *et al.*, 1997; Bowler *et al.*, 1999; Bugni and Drinkwater, 2003). The technique has been commonly used to identify genes responsible for a particular phenotype, as well as their downstream products (Hubank and Schatz, 1994). A reported key limitation of this technique is the low sensitivity (Hubank and Schatz, 1994). In addition, RDA can produce difference products from fragments throughout the cDNA region; as the majority of sequences in databases represent the 3'- or 5'-ends of the cDNA, some amplified fragments may be efficiently enriched, but these fragments do not necessarily represent gene targets (Hubank and Schatz, 1999).

RNA-seq is a more recent high-throughput sequencing technology that allows direct sequencing of transcripts, producing data for whole-genome transcriptome profiling (Mortazavi *et al.*, 2008; Mutz *et al.*, 2013). Furthermore, the design of probes and genome annotation are not required for this technology. This makes RNA seq highly sensitive and capable of detecting novel genes and genetic variants (Wang *et al.*, 2009; Zhao *et al.*, 2014). RNA-seq is reported to have high accuracy for quantification of transcript expression levels of the entire genome and providing excellent reproducibility (Cloonan *et al.*, 2008; Nagalakshmi *et al.*, 2008; Wang *et al.*, 2009). However, novel algorithmic and logistical challenges have faced RNA-seq data analysis. Various computational methods have been applied for read mapping and quantification of genes and/or transcripts (Garber *et al.*, 2011) and these methods can show significant variability (Zhao *et al.*, 2014).

Both the RDA and RNA-seq have been used in experiments to generate transcriptome profiles of IEC-6 host cells during *G. duodenalis*-host cell interaction. RDA identified several differentially expressed genes in each of four *G. duodenalis* isolates following a 2 and 6 hrs incubation with rat intestinal epithelial cells (IEC-6); however, this technique was reported to

only capture upregulated levels of expression (Ma'ayeh and Brook-Carter, 2012). RNA-seq has also been applied to explore both transcriptome and proteomic profiling of *G. duodenalis*. For instance, it was applied to study transcriptome changes of two *G. duodenalis* trophozoites in response to oxidative stress, indicating significant up and downregulated transcripts (Ma'ayeh *et al.*, 2015). Other RNA-seq studies have been carried out to understand the pathogenic mechanisms of *G. duodenalis* (Franzén *et al.*, 2009; Ansell *et al.*, 2015; 2016, 2017), as well as investigating proteomics in *G. duodenalis* (Emery *et al.*, 2015; 2016).

Although previous studies have suggested limitations in hybridization-based technologies, including the RDA method, especially in comparison to sequence-based methods such as RNA-seq (Hubank and Schatz, 1994; Hubank and Schatz, 1999; Zhao *et al.*, 2014), both technologies have been suggested as valid tools to study transcriptome profiling or as complementary analytical tools (Liu *et al.*, 2007). Here, we performed a transcriptome comparison between RNA-seq and RDA techniques from separate experiments in which the same host cell line (IEC-6) was incubated with the same *G. duodenalis* isolates (P-1 or WB) for identical time periods (2 and 6 hrs co-culture) to determine whether these disparate techniques would suggest common pathway/transcript changes in the host cell transcriptome.

4.2. Results

The RNA-seq data generated during this study was compared to the RDA data which had previously been generated in our laboratory (Ma'ayeh and Brook-Carter, 2012; Ma'ayeh, 2013, unpublished data); the parasite isolates, cell line and incubation time periods were common to both experiments. As the RDA technique selectively detected up-regulated difference products (Ma'ayeh and Brook-Carter, 2012), a comparison with the upregulated levels of gene expression from the RNA-seq was carried out.

4.2.1. Total number of upregulated transcripts at 2 or 6hrs

RDA identified 486 and 399 transcripts as difference products in the IEC-6 cell line after a 2hr incubation with the isolate P-1 or WB, respectively. In contrast, RNA-seq identified 1629 and 2551 transcripts upregulated in IEC-6 in response to the same isolates (Table 1). Additionally,

the RDA results suggested that P-1 induced a greater number of difference products compared to WB, whilst the RNA-seq data showed that WB induced a greater number of upregulated transcripts in IEC-6.

At 6hrs a similar trend to that observed at 2hrs was seen in gene expression patterns in IEC-6 using RDA or RNA-seq. RNA-seq again identified a much larger number of upregulated transcripts (P1:2851 and WB: 2877) in IEC-6 when compared to the number of difference products (P1:322 and WB:380) identified by RDA (Table 1). Within each technique, differences in the number of expressed transcripts in IEC-6 was similar for both parasite isolates.

Table1: Transcriptome profiling changes in IEC-6 (I) co-cultured with either *G. duodenalis* isolate P-1 or WB. The table displays results of two techniques (RDA and RNA-seq) in terms of the number of transcripts whose expression level was upregulated during the 2 and 6hrs induction.

Technique	No. of upregulated transcripts detected in co-culture at 2hrs		No. of upregulated transcripts detected in co-culture at 6hrs	
	IP-1	IWB	IP-1	IWB
RDA	486	399	322	380
RNA-seq	1629	2551	2851	2877

4.2.3. Transcriptome profiling at 2hrs

Up-regulated transcripts in IEC-6 were assigned to cellular pathways and several were common to both techniques following a 2hrs incubation with the parasite. These pathways included cell cytoskeleton, ECM, apoptosis, response to hypoxia, metabolism, signalling and vesicular trafficking (Table 2). Differences were also observed between these techniques in terms of the genes identified and in the number of gene transcripts detected per pathway with RNA-seq always greater than RDA. There were also multiple pathways (tight junction, adherens junctions, cytokine-cytokine receptor interaction, chemokine and TNF) identified with increased transcript expression by the RNA-seq analysis, but not by RDA. Overall RNA-seq

detected more target pathways and usually a larger number of transcripts in those pathways than RDA.

Table 2: Comparison of the main cellular pathways and their expressed transcripts identified by either RDA or RNA-seq techniques in IEC-6 following 2hrs co-culture with *G. duodenalis.*

Main induced cellular pathways	Detected by RDA and/or RNA-seq at 2hrs	Target transcripts identified by RDA	Target transcripts identified by RNA-seq
Tight junction	RNA-seq	ND	*CLDN1, TJP2, ICAM1, JAM2*
Adherens junction	RNA-seq	ND	*CDH1, CTNND1, AFDN, CSNK2A1, PTPN1*
Cell cytoskeleton	RDA and RNA-seq	*ACTG*, ***ACTB***, *CTNNB1*, ***VIM***, *ACTG1*, ***VCAN***, *ARPC1B*	*ROCK1, ROCK2, MYLK, ACTN1,* *VCL, EZ,* ***VIM***, ***VCAN***, *LIMK2, EZR,* ***ACTB***, *ACTN1, CFL2*
Extracellular matrix (ECM)	RDA and RNA-seq	***COL3A1, COL1A1***, ***BGN***, ***LAMA3***, ***MMP2, MMP24, ICAM1***	***COL1A1, COL3A1***,*COL4A1, COL4A2,COL5A3, COL6A6,* ***LAMA3***, *LAMB1, LAMC1, ITGB6, SDC4, SDC1, TNC,* ***ICAM1***, ***BGN***, ***MMP2, MMP24***
Apoptosis	RDA and RNA-seq	*BCL2,* ***BIRC7, NOL3***, *CTSD,* ***GADD45G***	*FASR, TNFRSF1, p53, CASP-3, BAX, BID, CLFAR, BIRC2, BIRC3, NF-kB, GADD****45G, BIRC7, NOL3***
Response to hypoxia	RDA and RNA-seq	***FTH1, MT-ND3***, ***HIF1A*** *SAT2, SELK, CLCA1*	***HIF1A***, *SLC2A1, HK2, NOX1, NOS2,* ***FTH1***, ***MT-ND3***
Metabolism	RDA and RNA-seq	***HK2***, *ENOL,* ***ALDOA,*** *TPI1, APOE, G6PD*	***ALDOA***, ***HK2***, *CYP1B1, INSIG2, GPC3, SLC40A1, NOX1, NOS2, IPPK,*
Signalling and vesicular trafficking	RDA and RNA-seq	*ERK1/2, MAPK, PI3k/Akt, RASL11B, ARHGAP5*	*Complete pathways, such as PI3K-Akt, Rap1, Ras, p53, TGFβ, Toll-like receptor, NOD-like receptor, MAPK, Wnt*
Cytokine-cytokine receptor interaction	RNA-seq	ND	*IL-1A, IL-6, IL-23A, CSF3, CSF1*
Chemokine pathway	RNA-seq	ND	*CCL20, CXCL2, CX3CL1, CCL9, CXCL3, CXCL1, CCL, CXCL1*
Tumor necrosis factor (TNF)	RNA-seq	ND	*TNFRSF-1A, TNFRSF-9, TNFSF-15, TNFSF-10, FASR*

Cellular pathways in bold were identified by both RDA and RNA-seq techniques; genes in bold were identified as up-regulated by both techniques. ND, not detected

4.2.4. Transcriptome profiling at 6hrs

Table 3 illustrates the comparison in cellular pathway induction between RDA and RNA-seq in IEC-6 at 6hrs after infection. As with the 2hrs results, common pathway identification by both techniques were evident, but the number of gene transcripts detected per pathway was greater using RNA-seq. There were also multiple immune related pathways activated (cytokine-cytokine receptor interaction, chemokine and TNF) identified with increased transcript expression by the RNA-seq analysis, but not by RDA. As seen with the 2hrs data, overall RNA-seq detected more target pathways and usually a larger number of transcripts in those pathways than RDA.

Table 3: Comparison of the main cellular pathways and their expressed transcripts identified by either RDA or RNA-seq techniques in IEC-6 following 6hrs co-culture with *G. duodenalis.*

Main induced cellular pathways	Detected by RDA or/and RNA-seq at 6hrs	Target transcripts identified by RDA	Target transcripts identified by RNA-seq
Tight junction	RNA-seq	ND	*CLDN1, CLDN2, TJP2, TJP3, ICAM1, JAM2, JAM3, MAGI1*
Adherens junction	RNA-seq	ND	*CDH1*, ***CTNNB1,*** *CTNND1, AFDN, FYN, PTPRB*
Cell cytoskeleton	RDA and RNA-seq	***EZR****, CLDN4, ACTG, BGN, VIM,* ***CTNNB1, ACTB****, ARPC1B,* ***WASF2***	*ROCK1, ROCK2, MYLK, ACTN1,* ***ACTB,*** *VCL, LIMK, RHOA, MYLK2, MYL12, MYLK3, CFL2,* ***EZR, VIM****,* ***WASF2, ARPC1B***
Extracellular matrix (ECM)	RDA and RNA-seq	*COL3A1,* ***COL1A1****, COl11A2, COL5A2 , COL10A1*	*COL24A1,* ***COL1A1****, COL4A2, COL6A6, COL5A3, LAMA2, LAMA5, LAMB1, LAMB3, FN1, SDC4, SDC1, ITGA1, ITGA2, ITGA5, ITGA7, ITGB8, ITGB3*
Apoptosis	RDA and RNA-seq	***CDKN1A, GADD45G, CASP3, BNIP3****, TNFRSF10B,* ***FASR***	***FASR****, TNFRSF1, p53,* ***CASP-3****, BAX, BID, CLFAR, BIRC2,* ***GADD45G****, BIRC3, NF-kB, CASP12, CASP7, TNFSF10, BCL2L1, APAF1, TP53, BCL2, CYCS,* ***BNIP3, CDKN1A***
Response to hypoxia	RDA and RNA-seq	***DDIT4, FTH1 and MT-ND3****, CYP1B1,* ***HIF1A****, OXSR1, SAT2*	***DDIT4, MT-ND3, FTH1, HIF1A****, SLC2A1, HK2, NOX1, NOS2, PHD3, VHL, TEK,*
Metabolism	RDA and RNA-seq	***HK2****, ENOL,* ***ALDOA****,* ***TPI1, ALDH3A1, ODC1****, ACSS1,* ***INSIG1, CYP1A1, LDHA***	***HK2****, NOS2, ARG2,* ***ODC1,*** *AZIN2, ENO3,* ***ALDOA****,* ***ALDH3A1,*** *ALDH1A3, ALD1A1,* ***CYP1A1****, SAT1,* ***ODC1****,* ***TPI1****, ACSS2,* ***INSIG1, LDHA***
Signalling and vesicular trafficking	RDA and RNA-seq	*ERK1/2, MAPK, PI3k/Akt, RAB9A, RAB11A, RHOG, RAB5A*	Complete pathways, include *PI3K-Akt, Rap1, Ras, p53, TGFβ, Toll-like receptor, NOD-like receptor, MAPK, NF-kappa B, cAMP, Jak-STAT*
Cytokine-cytokine receptor interaction	RNA-seq	ND	*IL-1A, IL-6, IL-7, IL-23A, CSF3, CSF1, CSF2*
Chemokine pathway	RNA-seq	ND	*CCL20, CXCL2, CX3CL1, CCL9, CXCL3, CXCL1, CXCL6, CCL5, CCL7,CCL2*
TNF	RNA-seq	ND	TNFRSF-1A, TNFRSF-9, TNFSF-10, FASR, TNFRSF-11B, TNFSF-15, TNFSF-18

Cellular pathways in bold were identified by both RDA and RNA-seq techniques; genes in bold were identified as up-regulated by both techniques. ND, not detected.

4.3. Discussion

Over recent decades, several hybridization or sequence-based technologies have been used to conduct large-scale studies of transcriptome profiling, including the use of RDA (Wang *et al.*, 2009). However, limitations have been found in such techniques as background hybridization levels can restrict the accuracy of expression measurements and probes can vary in their hybridization properties (Hubank and Schatz, 1994; Okoniewski and Miller, 2006; Zhao *et al.*, 2014). RNA-seq is a recent technique purported to be a rapid and high-throughput sequencing technology that allows direct sequencing of transcripts, producing high-quality data for whole-genome transcriptome profiling (Mortazavi *et al.*, 2008; Mutz *et al.*, 2013). RNA-seq is considered a powerful technology that can chart transcriptome dynamics in various tissues or conditions without complex normalization procedures for data samples (Cloonan *et al.*, 2008; Mortazavi *et al.*, 2008; Wilhelm and Landry, 2009). Importantly, unlike RDA (Hubank and Schatz, 1994), RNA-seq can provide analysis of both up and down-regulated transcript expression. Our laboratory carried out both techniques with the same experimental parameters (cell-line, *Giardia* isolates, and incubation times). Therefore, the purpose of the present study was to compare RNA-seq with RDA data (Ma'ayeh, 2013, unpublished data) to determine whether RNA-seq would provide a more detailed transcriptome profile in rat intestinal epithelial cells (IEC-6) co-cultured with *G. duodenalis* trophozoites.

Although RNA-seq identified a large number of down-regulated genes (presented in chapter 3) no comparison was able to be carried out as RDA only detected up-regulated transcripts from a comparison of the two test populations (Ma'ayeh and Brook-Carter, 2012). Comparative gene analysis of up-regulated transcripts between these two methods demonstrated RNA-seq detected significantly higher numbers of transcripts compared with RDA. This is in agreement with studies suggesting that RNA-seq is a more sensitive technology in analysing changes in transcripts expression of the entire transcriptome (Cloonan *et al.*, 2008; Nagalakshmi *et al.*, 2008; Wang *et al.*, 2009). Given that RNA-seq also detects down regulated transcripts, this technique is clearly more sensitive at detecting modulation of gene transcripts. However, it was of interest to determine whether each technique would identify common up-regulated transcripts and by inference, common pathways.

Both techniques did identify common pathways at both time points and some of these pathways had common upregulated transcripts. However, several gene transcripts differed within a common pathway between the two techniques. Both sets of data highlight changes in the cellular cytoskeleton and extracellular matrix components, and signalling suggesting parasite effects on maintenance and establishment of the epithelial barrier integrity and a restitution process (tissue repair) (Florian *et al.*, 2002; Hopkins *et al.*, 2007). Many growth factors (e.g. EGF, IGF1and2, FGF and TGFB) (Dignass *et al.*, 1994; Sturm and Dignass, 2008) and signalling pathways are involved in intestinal epithelial wound repair, including PI3K/Akt- and MAPK/ERK kinase (MEK)/ERK1/2 (El-Assal and Besner, 2005; Iizuka and Konno, 2011). The actin cytoskeleton plays a vital role in the mediating several cellular structures (Bailly and Condeelis, 2002) while the ECM plays a vital role in cell adhesion, cell-to-cell communication and differentiation (Abedin and King, 2010). These two pathways showed increased expression of some common transcripts, such as *COL1A1, EZR, VIM* and *ACTB*. However, RDA showed upregulation of the *ACTB* transcript while its expression was significantly downregulated at both times using RNA-seq. This transcript encodes F-actin protein which has been found to be disorganised in host cells following *Giardia* infection (Teoh *et al.*, 2000; Humen *et al.*, 2011). Further research is needed to confirm whether this gene transcription and/or its protein is induced or inhibited in host cells during giardiasis.

Both RDA and RNA-seq suggested that apoptosis was inhibited in the parasitised cells. The RDA data showed the upregulation of anti-apoptotic transcripts (e.g. *BCL2*), as well as lack of upregulation in the expression of other pro-apoptotic transcripts at this time. RNA-seq data showed significant upregulation in mRNA levels of inhibitors of apoptotic proteins (IAPs) such as BIRC3, as well as upregulation of NF-kB transcript expression and downregulation in expression levels of *CASP3* transcript. The protein BIRC3 is a major inhibitor of apoptosis and exerts negative effects on initiator and executioner caspases in both the extrinsic and intrinsic pathways (Saleem *et al.*, 2013). In addition, NF-κB has been found to act as a promoter for cell survival through activating cytokines, growth factors, anti-apoptotic proteins (e.g. Bcl-2 family) and other survival genes (Bubici *et al.*, 2006). Furthermore, induction of apoptosis was suggested by RDA at 6hrs with upregulation of key apoptotic transcripts (e.g. *FAS* and *CASP-3*). RNA-seq data showed upregulation of *FAS* transcript expression, but downregulation of *CASP3* and significant upregulation of IAPs (e.g. *CLFAR* & *BIRC3*) and NF-kB as discussed

briefly in section 4.3.1 (for a full discussion see chapter 6). The RNA-seq data enabled a much more detailed analysis of these pathways during *G. duodenalis* infection.

The response to hypoxia was the third common pathway shown in the current comparison. Both RDA and RNA-seq showed activation of the ROS pathway which is involved in different cellular activities including host defence and oxygen redox signalling (Geiszt and Leto, 2004). Although excessive levels of ROS generation cause cellular protein damage, its production in low or moderate amounts play a key role in various physiological processes, such as killing of invading pathogens, tissue repair and wound healing (Rao *et al.*, 1999; Bhattacharyya *et al.*, 2014). RDA data suggested that increased mRNA levels of the *FTH1* gene in parasitised cells might lead to ROS production in response to oxidative stress in the infected cells. In RNA-seq data the level of *FTH1* showed a slight upregulation, while significant upregulation in the *NOX1* transcript suggesting that this is the main source of ROS production in these cells. Additionally, RNA-seq data showed increased levels of *HIF-1 α*, which is one of the hypoxia-inducible factors (HIFs) that respond to hypoxia in the cellular environment (Smith *et al.*, 2008; Wilkins *et al.*, 2016). Epithelial cells are known to release NADPH oxidase 1 (Nox1) to produce ROS which have antimicrobial effects in response to various pathogens (Bedard and Krause, 2007; Jiang *et al.*, 2007; Sorci and Faivre, 2009; Liu *et al.*, 2016). Previous research using microarray analysis found *NOX1, HIF2* and many other transcripts involved in stress and/or hypoxia and cellular proliferation were induced by *G. duodenalis*-infected cells, suggesting that these cells produced ROS as a result of stress and proliferation responses (Roxström-Lindquist *et al.*, 2005). Thus, a more complete picture was obtained through RNA-seq.

Other common pathways identified in this analysis were signalling pathways, including MAPK and PI3K/AKT. These pathways play in important role in cellular proliferation and apoptosis (Martelli *et al.*, 2006; Zhang *et al.*, 2016).

Comparison within the two techniques at 2 and 6hrs generally identified the same pathways. Expression of metabolic transcripts was confirmed, including those associated with glucose starvation and transport (e.g. *SLC2A1 and HK2),* suggesting hypoxia, reduced glucose levels (Wang, 2013) and promotion of ATP production (Ahn *et al.*, 2009; Palmieri *et al.*, 2009; Shan

et al., 2014). *HK2* might also support the finding of inhibition of apoptosis as it prevents release of cytochrome c by suppression of the binding of BAX-VDAC channel (Pastorino *et al.*, 2002). Furthermore, RNA-seq data, but not RDA again suggested increased NO production through induction of *NOS2 (iNOS)* transcript with known effects against infection including *Giardia* (Clark and Rockett, 1996; Fang, 1997; Fernandes and Assreuy, 1997; Krause, 2004; Pavanelli *et al.*, 2010; Tako *et al.*, 2013).

In addition to these relative similarities, RNA-seq identified cellular pathways that were not shown by the RDA at either time point. Thus, pathways involved in cell adhesion mechanisms (tight and adherens junctions) and which play a significant role in host intestinal epithelial barrier function (Groschwitz and Hogan, 2009; Suzuki, 2013). These pathways include transcripts such as *CLDN-1*, with its upregulation one of the most prominent findings throughout the infection suggesting a requirement for on-going maintenance of the epithelial barrier (for a full discussion see chapter 5). A previous study suggested that its protein, claudin1, is required to prevent the paracellular diffusion of small molecules through tight junctions, as well as maintain a normal barrier function (Kirschner *et al.*, 2013). The key role of claudin1 in cell-cell junctions (Troeger *et al.*, 2007; Kirschner *et al.*, 2013) would not have been implicated by the RDA analysis. Significant variation was also noticed between RDA and RNA-seq such that, the intercellular adhesion molecule 1 (*ICAM1*) transcript did not show significant changes in RDA at 6hrs, while its expression was significantly upregulated in RNA-seq data at both infection times. This transcript is a cell surface transmembrane glycoprotein (Hopkins *et al.*, 2004) and is induced by various stimuli, including pro-inflammatory cytokines in intestinal epithelial cells (Kelly *et al.*, 1992; Huang *et al.*, 1996; Omagari *et al.*, 2009). The RNA-seq finding is consistent with a previous microarray study which found upregulation of the *ICAM1* transcript in host cells following the 6hrs infection with *G. duodenalis* (Roxström-Lindquist *et al.*, 2005).

RNA-seq but not RDA analysis found increased mRNA levels of several cytokines, including interleukin, chemokine, colony-stimulating factors (CSFs), all highly upregulated. Transcripts for *IL-1A, IL-23A,* and *IL*-6 are all upregulated and might play vital roles in mediating immune response at sites of the infection. A previous study noted the important role of *IL-6* for early control of acute *G. duodenalis* infections in wild-type mice (Zhou *et al.*, 2003). Moreover, the

induction of a variety of chemokines is consistent with Roxstrom-Lindquist *et al.,* (2005) who found a novel chemokine profile induced in host cells following *G. duodenalis* infection. The RDA did not show cytokine induction at 2 or 6hrs. Several previous studies support the RNA-seq findings, suggesting a more reliable and robust indication of the cellular responses against giardiasis (Zhou *et al.*, 2003; Roxström-Lindquist *et al.*, 2005; Bénéré *et al.*, 2012: Lee *et al.*, 2012; Obendorf *et al.*, 2013; Chen *et al.*, 2013).

In summary, it is evident that RDA and RNA-seq differ in their detection of the total number of expressed transcripts at each time point of the infection, as well as identification of cellular pathways. Taken together, RNA-seq was able to identify all cellular pathways that were also detected by RDA. In addition, RNA-seq identified more transcripts compared to RNA within a common pathway and designated increased gene expression to pathways not detected by the RDA analysis. Given RNA-seq data can determine both up and down regulation of target transcripts, unlike RDA, RNA-seq provides a more holistic picture of transcriptome changes in *G. duodenalis*-induced host cells.

Chapter 5

Epithelial barrier function in intestinal epithelial cells: transcriptome profiling of early responses to *G. duodenalis*

Chapter 5: Epithelial barrier function in intestinal epithelial cells: transcriptome profiling of early responses to *G. duodenalis*

5.1. Introduction

The intestinal epithelium consists of a single layer of cells that acts as a selectively permeable barrier between the intestinal lumen and the external environment. It functions to transport and absorb nutrients, electrolytes and water, as well as playing a vital role preventing entry of antigens, enteric flora and toxins. The mechanical connection between neighbouring cells seals the intracellular space via a complex network of proteins forming three adhesive complexes; tight junctions (TJ), adherens junctions (AJ) collectively known as the Apical Junctional Complex (AJC), and the desmosomes (Figure 1) (Groschwitz and Hogan, 2009; Suzuki, 2013). The TJ complex is formed by the interaction of three types of transmembrane proteins; occludin, claudin and junctional adhesion molecules (JAMs), as well as cytoplasmic plaque scaffolding proteins called Zonula occludens (ZO-1, ZO-2, and ZO-3) which link the cytoplasmic parts of TJ to the actin cytoskeleton (Tsukita *et al.*, 2008; Anderson and Van Itallie, 2009; Turner, 2009). Cytoskeletal regulation of TJ barrier integrity is regulated by the interaction of TJ with actomyosin ring, where myosin light chain (MLC) regulates the peripheral contraction and tension of the perijunctional actomyosin ring (Madara, 1987). The contraction of the actomyosin ring occurs through MLC phosphorylation via kinases such as myosin light chain kinase (MLCK) and Rho-associated kinase (ROCK) (Walsh *et al.*, 2001; Cunningham and Turner, 2012). Together, different signal transduction cascades regulate the intestinal epithelial barrier, including MLCK and ROCK which are required for TJ barrier arrangement (Benais-Pont *et al.*, 2003; Wang *et al.*, 2005; Du *et al.*, 2016).

Epithelial cadherin (E-cadherin) is the primary cell adhesion molecule (CAM) at AJ which form via cadherin-catenin interactions (Groschwitz and Hogan, 2009). These interactions bind actin filaments between adjacent cells, whereby catenins (α and β catenin) bind cadherin to the cytoskeletal network either directly by F-actin or indirectly through other adaptor proteins (e.g. afadin) (Vasioukhin *et al.*, ; Pokutta and Weis, 2007). In general, alterations in the phosphorylation status of specific proteins, such as ZO-1 or occludin lead to increased permeability of ZO-1 and disturbed AJC assembly. Moreover, the epithelial barrier function

can be influenced through the activation and interference of signal pathways such as protein kinase C (PKC), protein kinase A (PKA), phosphoinositide 3-kinase (PI3-kinase) and Rho signalling pathways (González-Mariscal *et al.*, 2008) which also affect phosphorylation.

Desmosomes are complexes that consist of transmembrane adhesion proteins of the cadherin family, connecting neighbouring cells through their cytoskeletons (Nekrasova and Green, 2013). In addition, the extracellular matrix (ECM) provides an important physical scaffolding and biochemical support for the cellular components during homeostasis, differentiation, and tissue morphogenesis (Bruckner, 2009; Van Agtmael and Bruckner-Tuderman, 2009; Piña-Vázquez *et al.*, 2012).

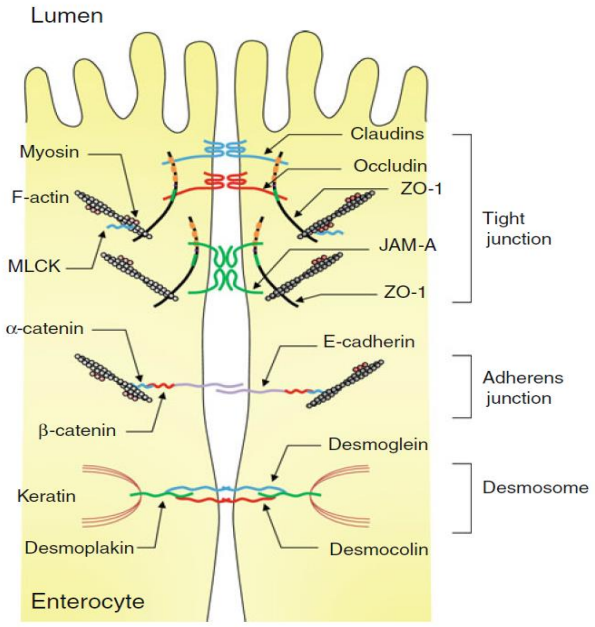


Figure 1: The molecular structure of the intercellular junction of intestinal epithelial cells. See text for explanation of terms and function. (Adopted from Suzuki, 2013).

Barrier and intestinal permeability disorders may also be observed upon changes in the expression and degradation of various junctional proteins (Förster, 2008; Groschwitz and Hogan, 2009). The change in expression levels of AJC proteins is an important feature of giardiasis since several authors have found suggested increased intestinal permeability of the parasitised cells associated with AJC disruption (Chin *et al.*, 2002; Scott *et al.*, 2002; Troeger *et al.*, 2007 ; Humen *et al.*, 2011; Maia-Brigagão *et al.*, 2012).

Giardia trophozoite interactions with host epithelial cells involves attachment via a ventral adhesive disk (Adam, 2001), interaction via surface molecules such as giardins (alpha, beta, delta and gamma) and a complex network of contractile proteins (Jiménez-Cardoso *et al.*, 2009) and the release of ESP (Nash *et al.*, 1983). It has been proposed that adhesion of *Giardia* trophozoites can alter enterocyte tight junctions and lead to breakage or re-arrangement of the proteins that form the junctions (Adam, 2001; Chin *et al.*, 2002; Cotton *et al.*, 2011; Cacciò, 2015). It has been also suggested that attachment to cells *in vitro* via the adhesive disc, is sufficient to cause cell injury and disrupt intercellular TJ and AJ (Humen *et al.*, 2011; Maia-Brigagão *et al.*, 2012). ESP is directly and indirectly involved in the mechanism of *Giardia* pathogenesis (Lee *et al.*, 2012) and include a range of proteins, such as cysteine proteases, immune evasion and anti-inflammatory factors and other proteins affecting host cell processes (Rodríguez-Fuentes *et al.*, 2006; Ringqvist *et al.*, 2008; Cotton *et al.*, 2014a).

Previous studies have suggested that increased epithelial permeability occurs as a result of the activation of MLCK by *G. duodenalis* trophozoite products, which then phosphorylates the myosin light chain (MLC) and disrupts cytoskeletal and TJ elements in enterocytes(Scott *et al.*, 2002). Chin *et al.*, (2002) proposed that *Giardia* isolate NF had a negative impact on tight junction ZO-1 and increased permeability with a loss of epithelial barrier function in a caspase-3-dependent manner. The down-regulation of claudin-1 was also associated with increased epithelial apoptosis in duodenal biopsy specimens of patients with chronic giardiasis (Troeger *et al.*, 2007). Rearrangement of occludin and delocalisation of claudin were shown by Humen *et al* (2011) with an associated decrease in transepithelial electrical resistance (TER). Finally, isolate WB caused a rearrangement of junctional proteins without down-regulation of expression in Caco-2 cells, with disturbance of the TJ, AJ and desmosomes (Maia-Brigagão *et al.*, 2012). Previous studies have also suggested that the proteolytic activity of *Giardia* trophozoites and their ESP could degrade collagens, which are the main fibrous ECM proteins (Knaippe, 1990; Coradi and Guimarães, 2006; de Carvalho *et al.*, 2008). Thus, disruption of intracellular junctions' results from adherence of the trophozoite and a range of interactions with the host cell.

Despite studies that show specific effects of *G. duodenalis* on the cellular epithelial barrier, the mechanisms by which *Giardia* causes diarrhoea are not entirely understood. Additionally, little

is known about the expression of proteins in the intestinal cellular epithelial barrier of host cells during infection. Thus, the specific objectives of this study were to offer insights into changes in mRNA levels of proteins that regulate the host intestinal epithelial barrier during early giardiasis using RNA-seq. Transcriptome profiling was carried out on 1. Tight junctions (TJ), 2. Adherens junctions (AJ) 3. Regulation of actin cytoskeleton, and 4. The extracellular matrix (ECM). Additionally, gene transcription changes of three TJ transcripts in response to *Giardia* trophozoites or their ESP was undertaken using qRT-PCR.

5.2. Results

5.2.1. Transcriptome profiling of the parasitised IEC-6 epithelium barrier using RNA-seq

5.2.1.1. Tight junctions (TJ) induction

Analysis of expression levels of transcripts involved in TJ assembly (Table 1) revealed that expression of most of the transcripts involved were significantly altered in IEC-6 following 2 or 6hrs incubation with either of the *G. duodenalis* isolates P-1 and WB. At 2hrs, the expression of *CLDN1, JAM2* and *ZO-3* transcripts were upregulated in response to both isolates. Other transcripts such as *CLDN15* and *ZO-2* were slightly downregulated in both isolates. The expression of *ZO-1, MAGI1, and MAGI3* was downregulated in response to WB, but not P-1.

Following 6hrs incubation, more transcripts displayed altered expression than at 2hrs. *CLDN1, CLDN15*, and *ZO-2* expression was similar to the 2hrs findings. The *ZO-3* mRNA was upregulated, whereas the levels of *MAGI1* and *MAGI3* were downregulated in response to both isolates. The *ZO-1* transcript, showed slight change except in response to P-1. Some transcripts were only expressed at this time-point, including *CLDN2* upregulated, while *CLDN18, JAM3, and SHROOM4* were downregulated in both isolates. Several transcripts encoding kinases/phosphatases which act as TJ regulators were significantly induced in the parasitised cells at both times of the infection.

Table 1: Tight junctions (TJ) transcripts differentially expressed in IEC-6 (I) during *G. duodenalis* isolate P-1 or WB at 2 and 6hrs. The DE transcripts that expressed log2 fc at FDR cut off 0.05 from the uninfected IEC-6 were considered using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). ND denotes not detected.

Tight junctions (TJ) transcripts	Symbol	Functional annotation	Gene transcription (log2 fold change) in co-cultures			
			2hrs IP-1	2hrs IWB	6hrs IP-1	6hrs IWB
claudin 1(Cldn1)	*CLDN1*	binding and structural	2.15	1.14	1.85	1.81
claudin 2(Cldn2)	*CLDN2*	binding and structural	ND	ND	3.56	3.33
claudin 15(Cldn15)	*CLDN15*	binding and structural	-0.21	-0.35	-0.67	-0.70
claudin 18(Cldn18)	*CLDN18*	binding and structural	ND	ND	-0.67	-0.56
junctional adhesion molecule 2(Jam2)	*JAM2*	Heterodimerisation activity	0.60	ND	2.45	2.55
junctional adhesion molecule 3(Jam3)	*JAM3*	Homodimerization activity and integrin binding	ND	ND	-0.48	-0.49
zonula occludens-1 (ZO-1)	*ZO-1*	TJ adaptor protein (binding)	ND	-0.14	0.14	NA
zonula occludens-1 (ZO-2)	*ZO-2*	TJ adaptor protein (binding)	-0.34	-0.62	-0.60	-0.66
zonula occludens-1 (ZO-2)	*ZO-3*	TJ adaptor protein (binding)	ND	0.60	2.97	3.27
shroom family member 4(Shroom4)	*SHROOM4*	actin filament binding	ND	ND	-0.94	-1.26
membrane associated guanylate kinase, WW and PDZ domain containing 1(Magi1)	*MAGI1*	alpha-actinin binding	ND	-0.32	-1.25	-1.05
membrane associated guanylate kinase, WW and PDZ domain containing 3(Magi3)	*MAGI3*	Frizzled binding and guanylate kinase activity	ND	-0.32	-0.86	-0.86

5.2.1.2 Adherens junction (AJ) induction

Transcriptome profiling of IEC-6 following 2 and 6hrs incubation with *G. duodenalis* isolates P-1 or WB showed significant changes in the expression of AJ transcripts (Table 2). Following 2hrs infection, cadherin-catenin complex transcripts such as *CDH1* and *CTNND1* were slightly downregulated, while the mRNA level of the *AFDN* transcript showed a slight upregulation. In addition, the expression of *MET* and *CSNK2A1* was downregulated, while *SRC* and *PTPN1* were upregulated in response to both isolates at this time of the infection.

At 6hrs the *CDH1* and *CTNND1* transcripts were downregulated, while *CTNNB1* showed significant upregulation in both isolates. Transcripts encoding tyrosine proteins, which regulate the cadherin-catenin complex, *SRC, PTPRB, and MET* were downregulated, whereas *FYN* and *PTPN1* were upregulated. Additionally, the expression of *AFDN* increasingly upregulated at this time compared with the 2hrs induction.

Table 2: Adherence junctions (AJ) transcripts differentially expressed in IEC-6 (I) infected with *G. duodenalis* isolate P-1 or WB at 2 and 6hrs. The DE transcripts showing expression changes of log2 fc at FDR cut off 0.05 from the uninfected IEC-6 were considered using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). ND denotes not detected.

Adherence junctions (AJ) transcripts	Symbol	Functional annotation	Gene expression (log2 fold change) in co-cultures			
			2hrs IP-1	2hrs IWB	6hrs IP-1	6hrs IWB
cadherin 1 (Cdh1)	*CDH1*	calcium ion binding	-0.19	-0.30	-0.92	-0.97
catenin beta 1 (Ctnnb1)	*CTNNB1*	transcription factor activity	ND	ND	0.66	0.75
catenin delta 1 (Ctnnd1)	*CTNND1*	binding and receptor binding	-0.17	-0.33	-0.27	-0.34
afadin, adherens junction formation factor (Afdn)	*AFDN (Mllt4)*	cadherin binding	0.77	0.23	1.55	1.86
casein kinase 2 alpha 1 (Csnk2a1)	*CSNK2A1*	protein tyrosine kinase activity	-0.28	-0.26	ND	ND
SRC proto-oncogene, non-receptor tyrosine kinase (Src)	*SRC*	Protein tyrosine kinase activity.	0.54	0.66	-0.48	-0.57
FYN proto-oncogene, Src family tyrosine kinase(Fyn)	*FYN*	protein tyrosine kinase activity	ND	ND	1.53	1.08
MET proto-oncogene, receptor tyrosine kinase (Met)	*MET*	protein tyrosine kinase activity	-0.27	-0.22	-0.76	-1.16
protein tyrosine phosphatase, non-receptor type 1 (Ptpn1)	*PTPN1*	enzyme binding	1.51	0.84	0.92	0.74
protein tyrosine phosphatase, receptor type, B (Ptprb)	*PTPRB*	Transmembrane receptor protein tyrosine phosphatase activity.	ND	ND	-1.79	-2.16

5.2.1.3. Actin cytoskeleton induction

The transcriptome profile of genes involved in the actin cytoskeleton (Table 3) showed that several transcripts displayed significantly altered expression levels in IEC-6 during *G. duodenalis* infection. At 2hrs, transcripts encoding kinases such as MAPKs and ROCKs were generally upregulated and downregulated, respectively. The *MAPK3* transcript was upregulated while the *MAP2K1* transcript was downregulated in response to WB. *ROCK1* and *ROCK2* transcripts were downregulated in both isolates. The MLCKs were induced, while the *MYLK* transcript was considerably upregulated, and *MYLK3* was downregulated but only in response to isolate WB. Analysis of the downstream transcripts showed that expression of *LIMK2* was downregulated, whereas mRNA levels of *CFL1* and *CFL2* were slightly decreased and increased, respectively, in response to WB. The *ACTN1* and *ACTN4* transcripts that encode essential cytoskeletal proteins were upregulated and downregulated, respectively, in both isolates. *ACTB* which encodes another key protein was downregulated. Expression of the *VCL* gene was downregulated and the *EZR* transcript was slightly upregulated in both isolates.

At 6hrs the expression of the *MAPK3* transcript was again upregulated, as was the *MAP2K1* transcript. The expression of *ROCK1* and *ROCK2* transcripts was continually downregulated in response to both isolates. Transcripts encoding GTPase proteins of the Rho family were altered, while *RHOA* was upregulated in both isolates. The MLC and MLCKs transcripts were upregulated, but *MYLK2* and *MYL12A* were downregulated. *LIMK2* was also downregulated with P-1 at this time, but without significant change with WB. Furthermore, *CFL1* and *CFL2* were downregulated and upregulated, respectively. *ACTN1* was upregulated with P-1 and downregulated with WB. Other downstream genes such as *ACTN4, ACTB, VCL*, and *EZR* were downregulated in both isolates. Importantly, various transcripts encoding G-protein–coupled receptors (GPCRs), growth factors, and integrins that activate signal transduction pathways were significantly induced, suggesting a response to dysregulation of cell adhesion in early giardiasis.

Table 3: Regulation of actin cytoskeleton transcripts differentially expressed in IEC-6 (I) infected with *G. duodenalis* isolate P-1 or WB at 2and 6hrs. Cytoskeleton transcripts expressed at log2 fc at FDR cut off 0.05 from the uninfected IEC-6 were considered using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). ND denotes not detected.

Regulation of actin cytoskeleton transcripts	Symbol	Functional annotation	Gene transcription (log2 fold change) in co-cultures			
			2hrs IP-1	2hrs IWB	6hrs IP-1	6hrs IWB
mitogen-activated protein kinase 3(Mapk3)	*MAPK3*	protein tyrosine kinase	0.30	0.34	0.24	0.47
mitogen-activated protein kinase kinase 1(Map2k1)	*MAP2K1*	protein tyrosine kinase	ND	-0.28	0.63	0.60
Rho-associated coiled-coil containing protein kinase 1(Rock1)	*ROCK1*	protein tyrosine kinase	-0.29	-0.52	-0.17	-0.22
Rho-associated coiled-coil containing protein kinase 2(Rock2)	*ROCK2*	protein kinase	-0.25	-0.66	-0.42	-0.53
ras homolog family member A(RhoA)	*RHOA*	GTP binding and myosin binding	ND	ND	-0.59	-0.64
myosin light chain kinase(Mylk)	*MYLK*	protein tyrosine kinase	0.85	1.08	0.86	1.32
myosin light chain kinase 3(Mylk3)	*MYLK3*	protein tyrosine kinase	ND	-0.77	0.80	0.66
myosin light chain kinase 2(Mylk2)	*MYLK2*	protein tyrosine kinase	ND	ND	-1.54	-2.23
myosin light chain 12A (Myl12a)	*MYL12A*	calcium ion inding and glutamate receptor binding	ND	ND	-0.21	-0.22
LIM domain kinase 2(Limk2)	*LIMK2*	protein tyrosine kinase	-0.59	-0.78	-0.17	ND
cofilin 1(Cfl1)	*CFL1*	actin binding	ND	-0.16	-0.42	-0.40
cofilin 2(Cfl2)	*CFL2*	actin binding	ND	0.25	0.28	0.41
actinin, alpha 1(Actn1)	*ACTN1*	actin filament binding	0.61	0.59	0.23	-0.25
actinin alpha 4(Actn4)	*ACTN4*	actin filament binding	-0.22	-0.28	-0.59	-0.74
actin, beta(Actb)	*ACTB*	identical protein binding	-0.42	-0.40	-0.55	-0.74
vinculin(Vcl)	*VCL*	actin binding	-0.72	-0.65	-0.85	-1.63
ezrin(Ezr)	*EZR*	Protein domain specific binding.	0.48	0.24	-0.36	-0.29

5.2.1.4. Extracellular matrix (ECM) induction

The effects of *Giardia* on ECM transcripts in IEC-6 is shown in Table 4. The 2 and 6hrs infections revealed a large number of transcripts encoding fibrous ECM proteins that changed

expression levels. For example, most transcripts of collagen family members, laminin, and fibronectin were upregulated. The transcripts encoding proteoglycans (PGs) such as thrombospondin 1, 2 and tenascin C, which form the ECM were induced and mRNA levels of several ECM receptors include, Integrins and syndecans were changed in the infected cells. Among the collagen family transcripts, *COL6A6* showed the highest upregulation in both isolates.

Table 4: Extracellular matrix (ECM) transcripts differentially expressed in IEC-6 (I) infected with *G. duodenalis* isolates P-1 or WB at 2 and 6hrs. ECM transcripts expressed at log2 fc at FDR cut off 0.05 compared with uninfected IEC-6 were considered using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). ND denotes not detected.

Extracellular matrix (ECM) transcripts	Symbol	Functional annotation	Gene transcription (log2 fold change) in co-cultures			
			2hrs IP-1	2hrs IWB	6hrs IP-1	6hrs IWB
collagen type I alpha 1 chain(Col1a1)	*COL1A1*	extracellular matrix structural constituent	0.38	0.83	ND	0.34
collagen type III alpha 1 chain(Col3a1)	*COL3A1*	integrin binding and SMAD binding	0.30	0.77	-0.80	-0.18
collagen type IV alpha 1 chain(Col4a1)	*COL4A1*	extracellular matrix structural constituent	0.65	1.34	0.48	1.25
collagen type IV alpha 2 chain(Col4a2)	*COL4A2*	extracellular matrix structural constituent	0.62	1.05	ND	0.95
collagen type IV alpha 5 chain(Col4a5)	*COL4A5*	extracellular matrix structural constituent	0.15	ND	-0.38	-0.31
collagen type V alpha 2 chain(Col5a2)	*COL5A2*	Extracellular matrix structural constituent and SMAD binding.	0.31	0.47	ND	0.32
collagen type V alpha 3 chain(Col5a3)	*COL5A3*	extracellular matrix structural constituent	0.45	1.0	0.37	0.78
collagen type VI alpha 6 chain(Col6a6)	*COL6A6*	proteinaceous extracellular matrix	1.58	ND	2.38	2.97
collagen type XXIV alpha 1 chain(Col24a1)	*COL24A1*	extracellular matrix structural constituent	ND	ND	0.93	1.36
collagen type XXVII alpha 1 chain(Col27a1)	*COL27A1*	extracellular matrix structural constituent	ND	ND	1.14	1.74
laminin subunit alpha 2(Lama2)	*LAMA2*	receptor binding and structural molecule	0.50	0.71	0.35	0.67
laminin subunit alpha 3(Lama3)	*LAMA3*	transcription factor & signal transducer	0.84	1.60	ND	ND
laminin subunit alpha 5(Lama5)	*LAMA5*	receptor binding and integrin binding	-0.16	-0.45	0.31	0.29
laminin subunit beta 1(Lamb1)	*LAMB1*	enzyme binding and integrin binding	0.61	0.88	0.87	0.98
laminin subunit beta 2(Lamb2)	*LAMB2*	Structural molecule & integrin binding.	ND	ND	-0.30	-0.54
laminin subunit beta 3(Lamb3)	*LAMB3*	structural molecule & protein complex binding	ND	0.54	0.40	0.63
laminin subunit gamma 1(Lamc1)	*LAMC1*	extracellular matrix structural constituent	0.27	0.31	ND	ND

laminin subunit gamma 3(Lamc3)	*LAMC3*	structural molecule activity	ND	3.44	ND	ND
fibronectin 1(Fn1)	*FN1*	protease binding	0.27	ND	1.31	0.85
thrombospondin 1(Thbs1)	*THBS1*	calcium ion binding	1.12	-1.28	0.73	0.85
thrombospondin 2(Thbs2)	*THBS2*	calcium ion binding	ND	0.90	ND	0.91
tenascin C(Tnc)	*TNC*	syndecan binding	0.26	0.83	-0.88	-0.54
syndecan 1(Sdc1)	*SDC1*	glycoprotein binding	0.37	0.85	0.18	0.45
syndecan 4(Sdc4)	*SDC4*	Cytoskeletal protein binding and fibronectin binding.	1.35	0.52	0.84	0.94
integrin subunit alpha 1(Itga1)	*ITGA1*	receptor binding and collagen binding	-0.37	-0.86	1.19	0.96
integrin alpha 2(Itga2)	*ITGA2*	integrin binding	ND	ND	2.51	1.54
integrin subunit alpha 3(Itga3)	*ITGA3*	integrin binding	-0.24	-0.82	-0.46	-1.14
integrin subunit alpha 5(Itga5)	*ITGA5*	integrin binding	0.50	ND	1.64	1.64
integrin subunit alpha 6(Itga6)	*ITGA6*	protein complex binding and laminin binding	NA	0.20	-0.63	-0.91
integrin subunit alpha 7(Itga7)	*ITGA7*	cell adhesion molecule binding	ND	1.94	1.88	1.68
integrin subunit alpha 11(Itga11)	*ITGA11*	collagen binding and collagen receptor activity	ND	ND	-3.18	-2.45
integrin subunit beta 3(Itgb3)	*ITGB3*	protein binding and protease binding	0.52	0.79	0.76	0.39
integrin subunit beta 4(Itgb4)	*ITGB4*	G-protein coupled receptor binding	ND	-0.25	-1.06	-1.10
integrin subunit beta 6(Itgb6)	*ITGB6*	receptor activity	0.54	0.65	-0.67	ND
integrin subunit beta 8(Itgb8)	*ITGB8*	extracellular matrix protein binding	-0.66	-1.39	1.22	1.07

5.2.2. Gene expression changes using qRT-PCR

In the qRT-PCR experiments, another two *G. duodenalis* isolates (GS/M or NF) were also used with the P-1 or WB isolates for quantifying mRNA levels of three target TJ genes in the parasitised IEC-6. The aim of these analyses was to investigate whether trophozoites or their ESP of these four isolates alter the gene transcription of TJ during a short-term infection (2 and 6hrs), as well as highlighting if there is a variation in cellular responses to each individual isolate.

5.2.2.1. RNA extraction and cDNA synthesis

All IEC-6 monolayer cells induced by four *G. duodenalis* isolates (GS/M, P-1, WB or NF) and non-infected IEC-6 were successfully extracted at 2 and 6hrs (Figure 2). The results showed that RNAs were purified and had high concentrations.

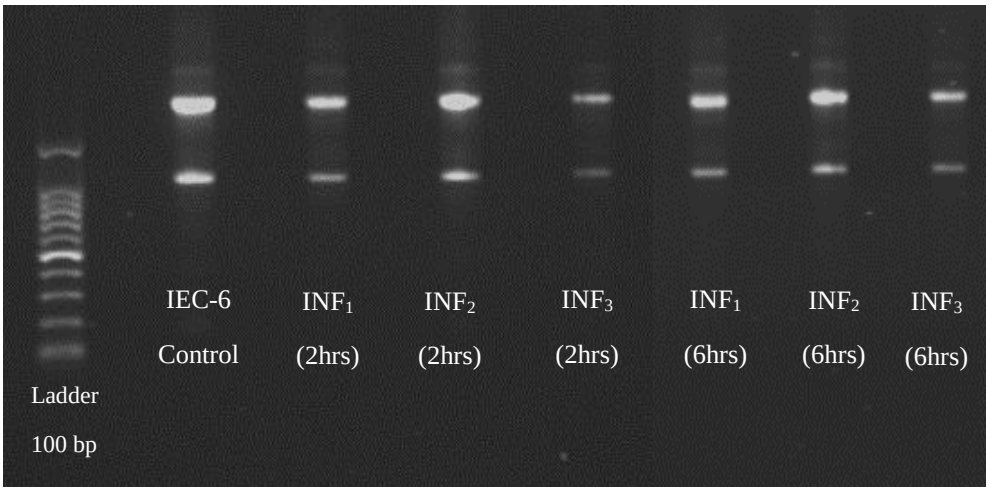


Figure 2: Extracted RNAs of triplicate IEC-6 monolayer cells induced by *G. duodenalis* (NF isolate) at 2 and 6hrs on a 1.2% agarose gel. The results show RNAs as defined bands and have high concentrations after the measurement by Qubit® fluorometer RNA assay kit (Invitrogen™).

High representative complementary DNA (cDNA) was prepared for all the extracted RNAs. The size range of cDNA was determined by agarose gel electrophoresis was between 300bp and 1000bp (Figure 3). These results allowed selection of an appropriate location within each chosen gene for optimal primer design.

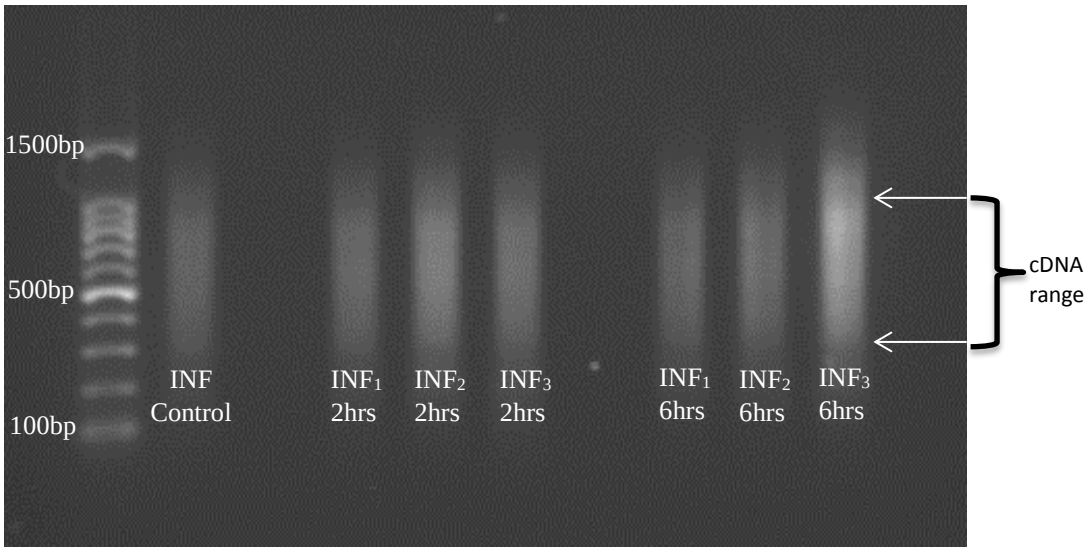


Figure 3: Determination of the size range of cDNA by 1.2% agarose gel electrophoresis based on 1500bp.

The results also revealed that the integrity of cDNA in each sample with defined and single distinct bands corresponding to the expected size (Figure 4).

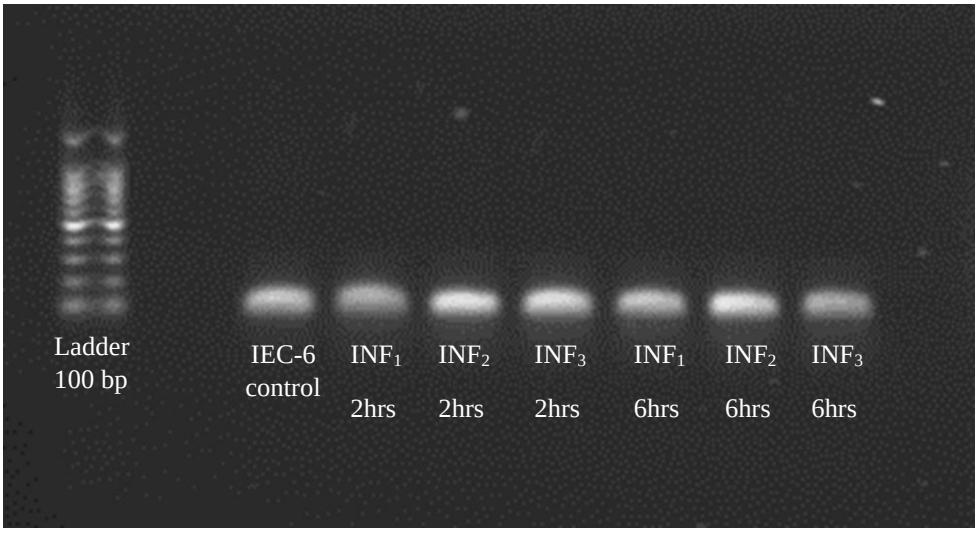


Figure 4: The assessment of cDNA integrity of triplicate INF co-culture at 2 and 6hrs using rat housekeeping gene *GAPDH* on a 2 % agarose gel. The results show defined and single distinct bands in each sample.

5.2.2.2. qRT-PCR primer efficiency of the target genes

Primer efficiency assessment was done for the selected primers using qRT-PCR based on the standard curve method. The results showed that the primer efficiency of all the selected primer pairs was approximately 90-100% as shown as 95.59 % for *CLDN-1* gene (Figure 5).

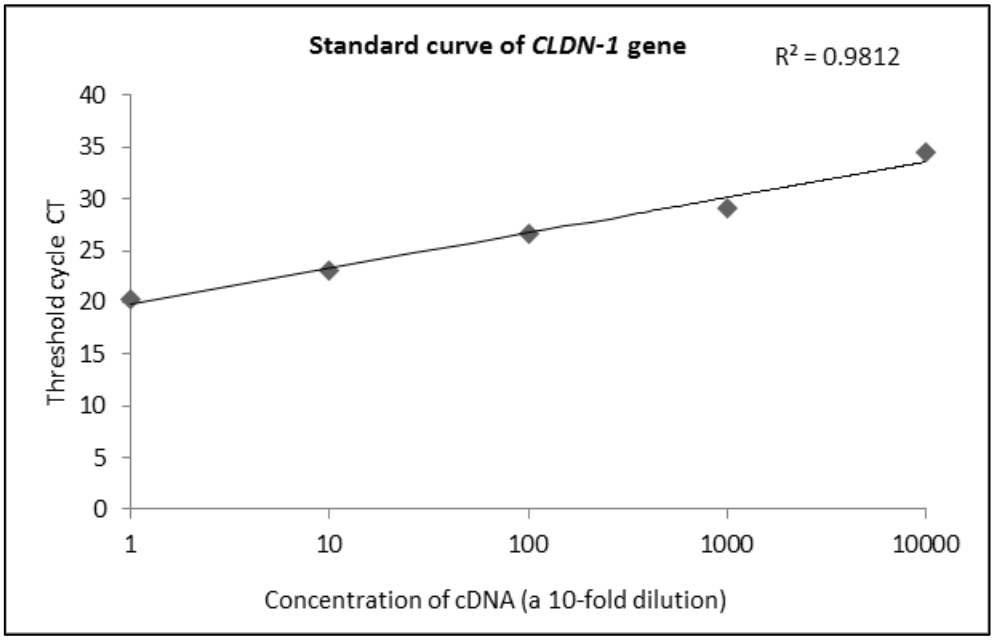


Figure 5: Primer efficiency for the *CLDN-1* gene using qRT-PCR based on a standard curve method using five serial dilutions of 100ng/µl cDNA. The results show that the efficiency of the primers was 95.59 % according to a slope value (-3.4323). The correlation coefficient (R2 value) is 0.9812. Briefly, the Y axis represents the PCR amplification value of each cDNA concentration at a certain threshold cycle, while the X axis represents five serial dilutions of cDNA (neat; 1-10; 1-100; 1-1000 and 1-10000).

5.2.2.3. qRT-PCR Based Relative Quantitation of Gene Expression

In this study, mRNA levels of three target genes representing cellular tight junctions (TJ), i.e. *CLDN-1, OCLN,* and *ZO-1* genes were assessed in the parasitised IEC-6 following the infection with each one of four *G. duodenalis* isolates (GS/M, P-1, WB or NF) or their ESP at 2 and 6hrs. In general, control experiments with IEC-6 alone showed no significant differences in expression of all the genes, as well as between 2 and 6hrs of culture. While, most of the genes were significantly expressed whether at 2 or 6hrs of the infections with the *G. duodenalis* isolates. The following sections clarify the results in detail.

5.2.2.3.1. TJ gene expression in response to *Giardia* trophozoites

At 2hrs, some significant changes were shown in TJ genes with most of the isolates, where mRNA levels of the *CLDN-1* gene was clearly upregulated with all the isolates. However, its upregulation with NF isolate was not statistically significant. For tight junction protein *ZO-1* gene, the results did not show a significant change with all the isolates. In contrast, *OCLN* gene expression was significantly downregulated with all isolates.

At 6hrs *CLDN-1* gene expression was also upregulated in response to GS/M, P-1 and WB, but upregulation in response to GS/M decreased at this time compared with the 2hrs induction. The NF isolate *CLDN-1* gene showed a similar expression at 2hrs and 6hrs co-culture. The mRNA level of *ZO-1* gene was significantly upregulated in response to all the isolates, except with NF isolate. The mRNA level of *OCLDN* gene did not show a significant change with GS/M and P-1, while its expression with WB and NF remained significantly downregulated.

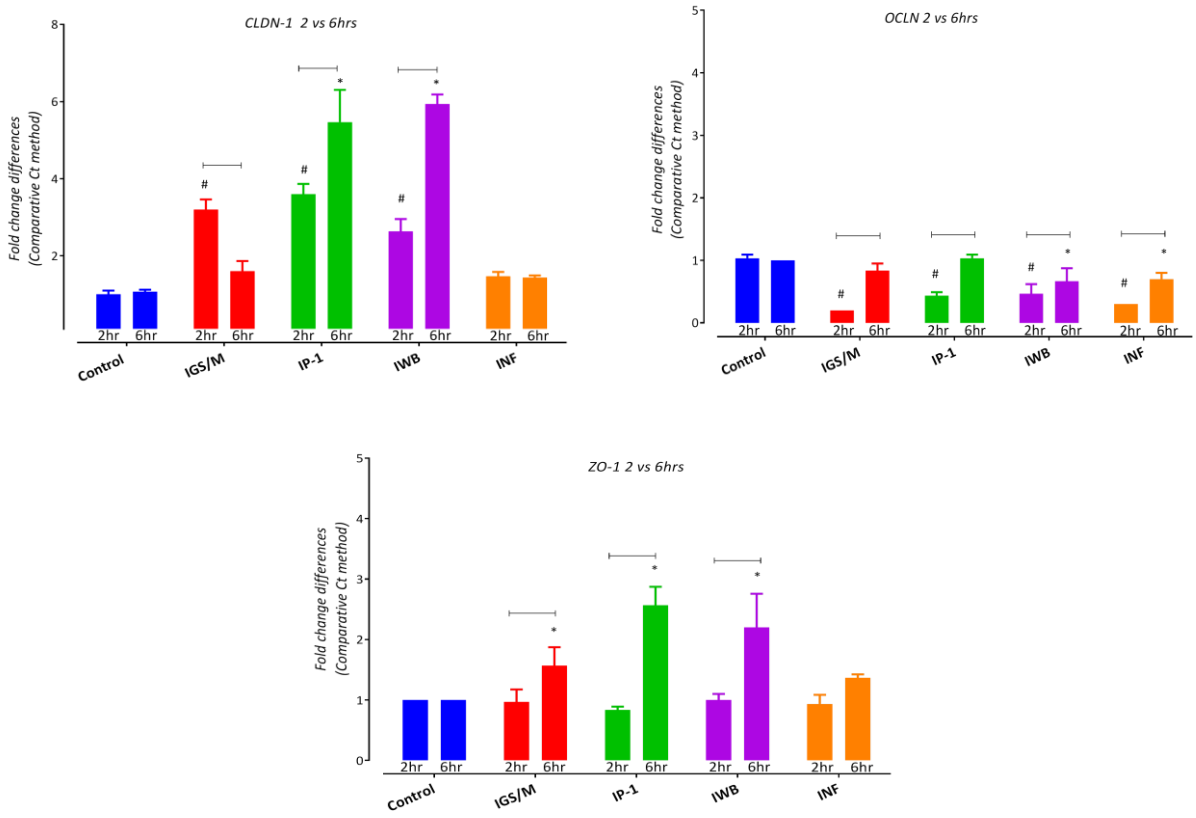


Figure 6: Gene expression changes using qRT-PCR in the TJ genes (*CLDN-1*, *ZO-1*, and *OCLN*) in IEC-6 (I) induced by the trophozoites of the *G. duodenalis* isolates (GS/M, P-1, WB or NF) after 2 and 6hrs infection. Analysis was by Two-way ANOVA and significance determined by Bonferroni's multiple comparisons test Data: Mean± S.D. P≤0.05. # denotes a significant difference between 2hrs vs control. * denotes a significant difference between 6hrs vs control. ── denotes a significant difference between 2 vs 6hrs.

5.2.2.3.2. TJ gene expression in response to *Giardia* ESP

ESP of the *G. duodenalis* isolates (GS/M, P-1, WB or NF) was cultured with the IEC-6 at 2 and 6hrs. Many genes were significantly expressed in all isolates at either 2 or 6hrs (Figure 7). The mRNA levels of *CLDN-1* and *ZO-1* genes were significantly upregulated with all isolates, while expression of *OCLN* was significantly downregulated with GS/M, P-1 and NF. *CLDN-1* and *ZO-1* gene expression in particular were greater in response to the *Giardia* ESP compared to trophozoites co-cultures. At 6hrs, significant upregulation was observed in the expression of *CLDN-1* and *ZO-1* genes in response to all *G. duodenalis* isolates, but not in the *OCLN* gene expression.

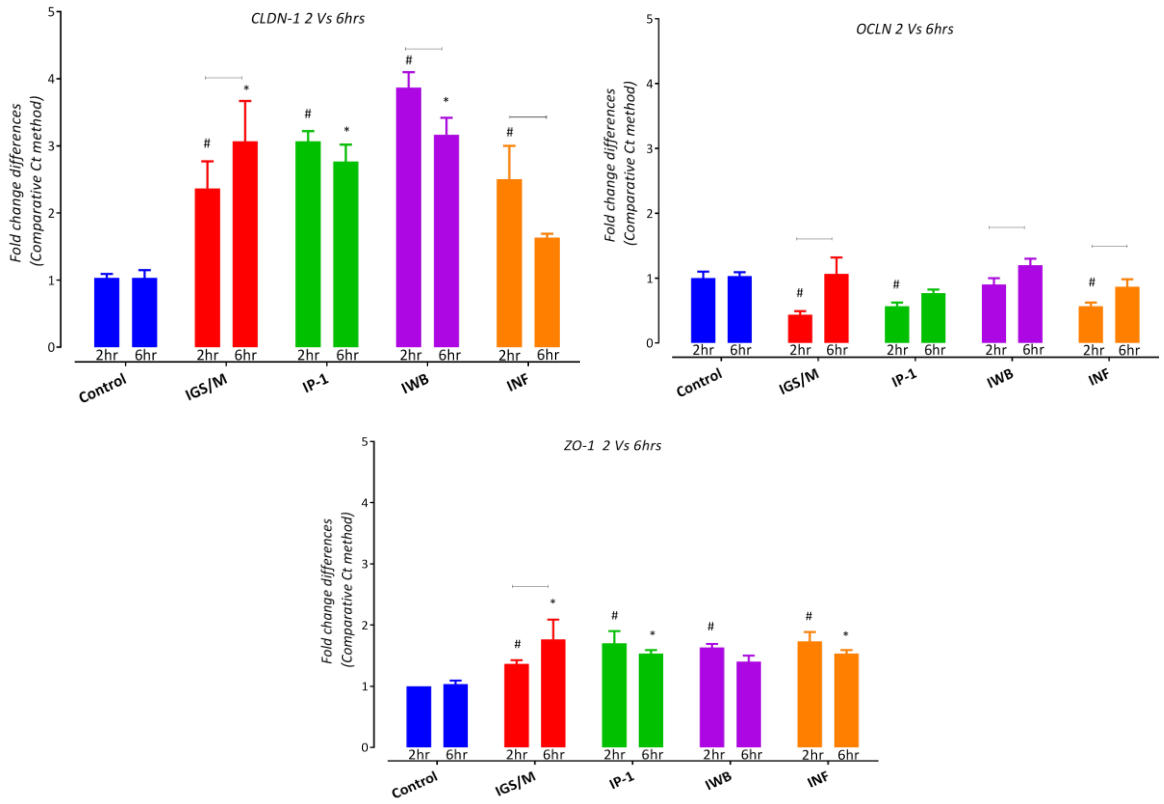


Figure 7: Gene expression changes using qRT-PCR in the TJ genes (*CLDN-1*, *ZO-1*, and *OCLN*) in IEC-6 (I) induced by the ESP of the *G. duodenalis* isolates (GS/M, P-1, WB or NF) after 2 and 6hrs infection. Analysis was by Two-way ANOVA and significance determined by Bonferroni's multiple comparisons test Data: Mean± S.D. P≤0.05. # denotes a significant difference between 2hrs vs control. * denotes a significant difference between 6hrs vs control. ── denotes a significant difference between 2 vs 6hrs.

5.2.2.3.3. Trophozoites vs ESP 2hrs Co-cultures

In IGS/M co-culture, the *CLDN-1* gene showed a significant fold change difference in IEC-6 between the trophozoites (T) and ESP (Figure 8). In IP-1, a difference was found in the fold change of the *ZO-1* gene in IEC-6. Whilst the change of *CLDN-1* and *ZO-1* in IWB and INF co-cultures were statistically different. Overall, despite some differences in the fold change, cells responded in a similar manner to both trophozoites and their ESP at 2hrs co-culture.

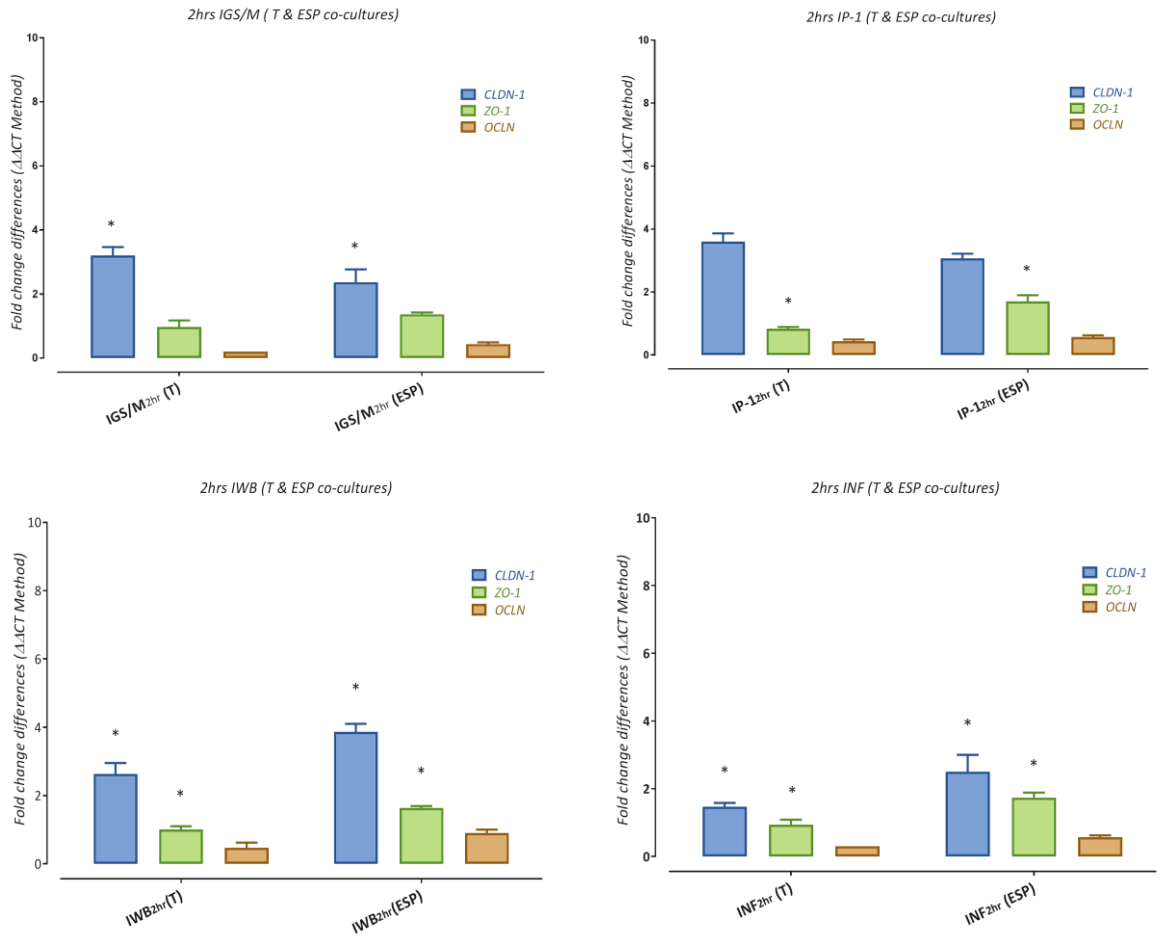


Figure 8: Gene expression changes using qRT-PCR in the TJ genes (*CLDN-1*, *ZO-1*, and *OCLN*) in IEC-6 (I) induced by trophozoites or ESP of *G. duodenalis* isolates (GS/M, P-1, WB or NF) at 2hrs culture. An analysis was done by using Two-way ANOVA and significance determined by Bonferroni's multiple comparisons test Data: Mean ± S.D. P ≤0.05. * denotes a significant difference between trophozoites (T) and ESP co-cultures.

5.2.2.3.4. Trophozoites vs ESP 6hrs Co-cultures

The levels of *CLDN-1* and *ZO-1* induction were significantly different with all isolates except NF (Figure 9). In the IGS/M co-culture, the expression level of *CLDN-1* was significantly different between T and ESP co-cultures. It was also noted that *CLDN-1* and *ZO-1* showed significant fold changes between the T and ESP in both IP-1 and IWB co-cultures. The INF co-culture showed no significant differences in any of the genes between T or ESP co-cultures. ESP alone induced consistent significant changes in TJ throughout the infection timepoints and these changes were consistent across isolates.

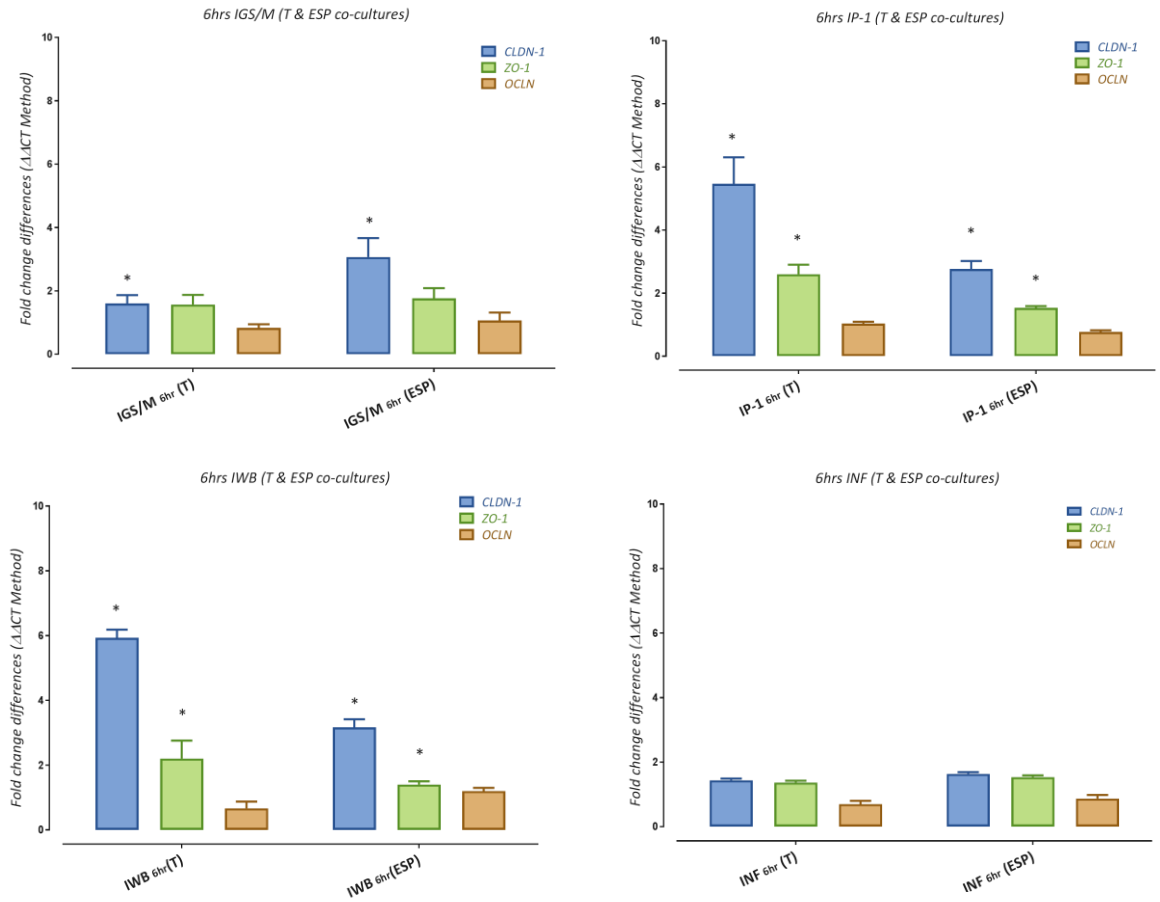


Figure 9: Gene expression changes using qRT-PCR in the TJ genes (*CLDN-1*, *ZO-1*, and *OCLN*) in IEC-6 (I) induced by trophozoites or ESP of *G. duodenalis* isolates (GS/M, P-1, WB or NF) at 6hrs culture. An analysis was done by using Two-way ANOVA and significance determined by Bonferroni's multiple comparisons test Data: Mean ± S.D. P≤0.05. * denotes a significant difference between trophozoites (T) and ESP co-cultures.

5.3. Discussion

5.3.1. RNA-Seq Transcriptome profiling

Very little data is available on gene transcription events related to cell adhesion during *G. duodenalis* infection. This study involved transcriptome profiling of the intestinal epithelial barrier assembly (tight junctions, adherens junctions, regulation of actin cytoskeleton, and extracellular matrix transcripts) during early giardiasis using RNA-sequencing. Results suggest significant alterations in the expression of TJ proteins particularly after 6hrs of infection. The alterations in gene expression of the claudins (e.g. claudin-1 and claudin-2) suggests a major effect on intracellular barriers by *Giardia* infection. This claudin family of proteins form paracellular barriers and pores, disruption of which can contribute to increased permeability of epithelial barriers (Gunzel and Yu, 2013). Changes in paracellular permeability have been

observed during overexpression, knockdown or knockout of claudins (Bazzoni, 2006; Vandenbroucke *et al.*, 2008). It has been proposed that loss of TJ in giardiasis may result from a reduced expression of junctional components and through their relocation or reorganisation under external stimuli (Di Genova and Tonelli, 2016). The present results are consistent with studies which found that disruptions in tight junction proteins, cause losses to the integrity of the epithelial barrier (Scott *et al.*, 2002; Troeger *et al.*, 2007; Koh *et al.*, 2013).

The upregulation of *CLDN-1* transcript was the most significant result in IEC-6 during the infection. *CLDN-1* has been shown to prevent paracellular diffusion of small molecules through tight junctions, and is required to maintain normal barrier function (Kirschner *et al.*, 2013). In chronic giardiasis, downregulation of *CLDN-1* gene expression and increased apoptosis were observed during epithelial barrier dysfunction (Troeger *et al.*, 2007). In another study, giardiasis caused decreases in transepithelial electrical resistance (TER) with rearrangement of functional occludin and delocalization of claudin-1 (Humen *et al.*, 2011). Upregulation of the *CLDN-2* transcript expression in the 6hrs infection suggests that *Giardia* trophozoites disturb TJ assembly, which might lead to the cell-cell leakage. It was previously proposed that the overexpression of *CLDN-2* gene was associated with leaky intestinal crypts (Rahner *et al.*, 2001). In addition, overexpression of *CLDN-2* can lead to increased paracellular water permeability in a leaky kidney epithelial cell line (Rosenthal *et al.*, 2010). The downregulation of the *CLDN-15* gene expression also suggests permeability changes, as issues such as Na^+ deficiency and glucose malabsorption were shown in the mouse small intestine following the knockout of Claudin15 (Tamura *et al.*, 2011).

The current study has shown induction of transcripts encoding the junctional adhesion molecule family members (JAM-2 and JAM-3) upon the infection. The JAMs family constitutes integral membrane proteins that regulate functional TJ and cell-cell border formation, as well as being involved in leukocyte-endothelial cell adhesion (Bazzoni *et al.*, 2000; Babinska *et al.*, 2002; Babinska *et al.*, 2002). Other transcripts (*ZO-1*, *-2*, *-3*, *SHROOM4*, *MAJI 1*, and *2*) were induced in response to both isolates at 6hrs. These transcripts encode a network of intracellular proteins that link TJ to the actin cytoskeleton. Previous studies suggested that increased cell permeability was associated with the disruption of the TJ protein (ZO-1) in host cell lines infected with *G. duodenalis* (Chin *et al.*, 2002; Buret *et al.*, 2003; Koh *et al.*, 2013). It was proposed that ZO-1

disruption was linked to caspase-3-dependent apoptosis in the infected cells (Chin *et al.*, 2002 ; Koh *et al.*, 2013). Although, apoptosis is a downstream response to loss of cellular barrier proteins in some diseases the fact of its occurrence in giardiasis remains to be defined (Di Genova and Tonelli, 2016) and is discussed in chapter 6 of this thesis.

Gene transcription related to adherens junctions (AJ) was significantly changed in IEC-6, particularly at 6hrs. It has been supposed that the alteration in these transcripts may be a result of the increased need/demand for AJ forming proteins presumably as a consequence of the effect of the parasite on the AJ. AJ assemblies play vital roles in the stabilisation of cell-cell adhesion and regulation of the actin cytoskeleton, with E-cadherin, p120-catenin, β-catenin, and α-catenin considered the major proteins (Hartsock and Nelson, 2008). E-cadherin binds to the cadherin of adjacent cells through its extracellular region (Shapiro and Weis, 2009), whereas the intracellular area of E-cadherin binds to the actin cytoskeleton through catenins (Perez-Moreno and Fuchs, 2006). In the present research, downregulation of the E-cadherin (*CDH1*) transcript expression throughout the infection indicates significant effects on AJs, possibly including dissociation of the E-cadherin bridge linking neighbouring cells. Downregulation of E-cadherin is associated with weakness of cell adhesion in intestinal epithelium leading to disturbances in intestinal epithelial proliferation and migration (Hermiston and Gordon, 1995). During giardiasis, modification in the distribution of E-cadherin was not observed by Humen *et al.*, (2011), although, Maia-Brigagão *et al.*, (2012) mentioned that *Giardia* caused significant changes in the distribution of tight and adherens junction proteins, even though their expression did not alter in parasitised Caco-2 cells.

In this study induction of the β-catenin transcript (*CTNNB1*) at 6hrs, downregulation of *CSNK2A1* transcript and induction of several transcripts encoding tyrosine proteins strengthens the case for dissociation of the cadherin-catenin complex (Table 2). Previous studies have suggested that phosphorylation of the cadherin/β-catenin complex determines the structural integrity of the complex, which ultimately maintains the integrity of AJ (Tian *et al.*, 2011). Equally importantly, casein kinase CK II can phosphorylate E-cadherin or β-catenin, leading to stabilisation of complex integrity (Lickert *et al.*, 2000; Bek and Kemler, 2002). In contrast, tyrosine phosphorylation of β-catenin leads to disturbance of the complex (Behrens *et al.*, 1993; Taddei *et al.*, 2002) causing cell-cell junction adhesion disorders (Hulsken *et al.*, 1994). The

induction of the *AFDN* transcript in this study might also have a substantial impact on the integrity of AJ. Afadin is an F-actin-binding protein that plays a vital role in adherens junctions (Mandai *et al.*, 1997). Although, this protein is not important for the assembly of the individual AJs, it was shown that afadin is required for arrangement cell-cell contacts in epithelial cells (Zhadanov *et al.*, 1999; Ikeda *et al.*, 1999). Previous studies also reported that cell-cell adhesion and the association of E-cadherin with F-actin and catenin delta 1 were damaged in afadin knockdown cells (Lorger and Moelling, 2006; Sato *et al.*, 2006). Thus, its induction during *Giardia* infection might be associated with attempts to reorganise or repair the AJC.

The third area of interest concerned expression of actin cytoskeleton transcripts. The actin cytoskeleton plays a vital role in mediating the integrity of many cellular structures (Bailly and Condeelis, 2002). In cell-cell adhesion there is a physical association between cadherin and the actin cytoskeleton (Han and Yap, 2012). The present data showed that the *ACTB* transcript encoding the F-actin protein, was downregulated, which is consistent with previous studies showing disturbance of actin cytoskeleton organisation during *Giardia* infection (Teoh *et al.*, 2000; Humen *et al.*, 2011). In addition, transcripts encoding the myosin light chain (MLC) and its kinases (MLCKs) were considerably altered in the present study, again suggesting an effect on actin cytoskeleton stabilisation. Disruption of F-actin and ZO-1 has been demonstrated in enterocytes exposed to *G. duodenalis* sonicates following phosphorylation by myosin light chain kinase in the myosin light chain at the actomyosin ring (Scott *et al.*, 2002). Finally, the expression of Rho family of GTPases were induced upon the infection in IEC-6. The downregulation of *RHOA, ROCKs* and their downstream transcripts might again lead to disturbance of the actin cytoskeleton. Rho family GTPases regulate the reorganisation of the actin cytoskeleton (Raftopoulou and Hall, 2004). The *RHOA* transcript encodes molecules involved in the induction of stress fibres which regulate cell adhesion via reconstruction of the cytoskeleton in response to extracellular stimuli (Bishop and Hall 2000). More importantly, reorganisation of the actin cytoskeleton is mediated by Rho-ROCK through the LIM kinase, which stabilises actin filaments via phosphorylation and inactivation of cofilin (Maekawa *et al.*, 1999; Du *et al.*, 2016). Thus, activation of the Rho family may again be a response to disruption of the actin cytoskeleton and or effects on cell-cell adhesion.

The regulation of the extracellular matrix (ECM) transcripts in IEC-6 during *Giardia* infection is reported in Table 4. A significant number of collagens, laminins, and integrin genes showed changes in response suggesting significant effects on the dynamics and integrity of the ECM. The ECM plays a vital role in cell adhesion, cell-to-cell communication and differentiation (Abedin and King, 2010). The extracellular matrix is considered a primary barrier to extracellular and intracellular protozoa, where the mechanism of its degradation may be a priority for these parasites' survival (Piña-Vázquez *et al.*, 2012). Results of a previous study demonstrated that *Giardia* trophozoites degraded collagen on the apical surface of the epithelium following attachment to confluent MDCK cell monolayers and type I collagen films (Knaippe, 1990). The collagenolytic activity of *G. duodenalis* was identified with collagenase activity found in trophozoite lysates (Coradi and Guimarães, 2006). Another study revealed that *Giardia* trophozoites release ESP that have proteolytic activity against collagen type I (de Carvalho *et al.*, 2008). The induction of several integrins in the present study might result from the effect of *Giardia* on cell adhesion and cell-to-cell communication. Integrins are cell-surface proteins that bind cells to ECM structures, such as fibronectin and laminin and to integrin proteins on the surface of other cells (Wergin, 2005). Collectively, these observations suggested that early events in giardiasis might include the disruption and reorganisation of these assemblies, probably leading to cell-cell junction leakage. These findings support previous studies and provide basic data on a range of gene expression events associated with *Giardia* infection.

5.3.2. TJ gene expression using qRT-PCR

It has been suggested that understanding whether the direct effects of different *G. duodenalis* isolates or indirect parasite's effects cause alterations on dynamic luminal ecology and host mucosal homeostasis is an important step (Bartelt and Sartor, 2015). Therefore, this investigation was conducted to quantify mRNA level changes of three target TJ genes in the IEC-6 induced by either trophozoites of four different *G. duodenalis* isolates (GS/M, P-1, WB or NF) or their ESP at 2 and 6hrs. These isolates were selected because they have been used worldwide in several laboratories for investigating host- *G. duodenalis* interactions (Buret *et al.*, 1990; Chin *et al.*, 2002; Roxström-Lindquist *et al.*, 2005; Ringqvist *et al.*, 2008; Ma'ayeh and Brook-Carter, 2012; Stadelmann *et al.*, 2013; Ferella *et al.*, 2014; Maloney *et al.*, 2015; Ma'ayeh *et al.*, 2015). In *G. duodenalis* axenic culture a variation in growth rates among isolates

was observed throughout sub-culturing of trophozoites every 72hrs. The GS/M isolate showing a slower growth rate compared to other isolates used. This result was in agreement with previous findings that found a difference in growth rates of human origin *G. duodenalis* isolates (Thompson and Monis, 2011). Additionally, the difference in culture conditions was shown in particular assemblages, where assemblage A isolates tend to have a selective advantage in axenic cultures compared to Assemblage B. While, *in vivo* (suckling mice) the findings of this variation appear the reverse (Binz *et al.*, 1992; Thompson and Lymbery, 1996; Andrews *et al.*, 2009).

Gene transcription analyses revealed that TJ genes in host cells were significantly expressed during the trophozoites infection, which upregulation shown in *CLDN-1* and *ZO-1*, whereas *OCLN* gene levels tended to be downregulated in response to all isolates. This short-term infection result might indicate that *G. duodenalis* trophozoites disturb the mechanism of TJ assembly. Simultaneously, increased levels of *CLDN-1* gene might be indicative of effective cellular responses to repair or reorganise cell-cell tight junctions. Disturbance in cellular TJ was assumed in previous investigations which illustrated how *G. duodenalis* could change the levels of some AJC proteins and lead to increase intestinal permeability (Chin *et al.*, 2002; Scott *et al.*, 2002; Troeger *et al.*, 2007; Maia-Brigagão *et al.*, 2012; Koh *et al.*, 2013). In biochemical studies, *G. duodenalis* trophozoite were found to promote an adhesion dependent decrease in transepithelial electrical resistance (TER) accompanied by a rearrangement of occludin, and delocalization of claudin-1 (Humen *et al.*, 2011). It was also observed that trophozoites of the WB isolate led to a rearrangement of junctional proteins of Caco-2 cells, but without down-regulation events, even though the disturbance was shown at the tight junction, as well as the adherence and desmosomes junctions (Maia-Brigagão *et al.*, 2012).

Previous investigations have suggested that Casein kinase 2 (CK2) plays an important role in the regulation of TJ protein interactions, where CK2 inhibition leads to promote transepithelial resistance by reducing paracellular cation flux (Raleigh *et al.*, 2011; Shen, 2012). Interestingly, our RNA-seq data indicated that transcripts encoding casein kinase 2 alpha 1 (Csnk2a1) and claudin-2 (CLDN-2) were significantly downregulated and upregulated, respectively in response to both isolates P-1 and WB. It was found a stable complex forms at the TJ by occludin, ZO-1, and claudin-2 in the absence of CK2 activity, which ultimately protects barrier

function (Raleigh *et al.*, 2011). The qRT-PCR findings also revealed that significant changes in mRNA levels of *CLDN-1*, *OCLN* and *ZO-1* genes, which might be ascribed to IEC-6 responses to the physical effect of trophozoites attachment that occurs during the interaction. It was previously explained that *Giardia* trophozoites strongly attach to host intestinal epithelial cells using the adhesive disk and flagella and this attachment could cause the structural and physiological changes (Müller and von Allmen, 2005; Ankarklev *et al.*, 2010; Humen *et al.*, 2011; Maia-Brigagão *et al.*, 2012). Hence, induction of these TJ proteins and inhibition of CK2 may have an essential impact in epithelial restitution mechanisms due to *G. duodenalis* damage.

Furthermore, the ESP co-cultures (without trophozoites) also induced significant changes in the expression of these genes. Significant upregulation in *CLDN-1* and *ZO-1* genes expression might clarify that there was a greater level of response to the *Giardia* ESP compared to trophozoites co-cultures. These findings might be indicator that ESP of *G. duodenalis* have an important impact on cell-cell tight junction mechanisms through disturbing structural proteins and triggering intestinal inflammatory cytokines responses. It was previously reported that a number of pro-inflammatory cytokines and chemokines such as tumour necrosis factor α (TNF-α) and Interleukin-8 (CXCL8) were activated via ESP of *G. duodenalis* assemblage B trophozoites (Lee *et al.*, 2012). It has been suggested that this is first in this study showing that ESP of four *Giardia* isolates (assemblage A and B) significantly induced gene expression levels of TJ proteins. These changes might be ascribed to attempts by the infected IEC-6 to maintain or restore TJ assemblies. mRNA level changes in these TJ proteins appear to be mediated by either pathophysiological or physical attachment mechanisms. This data derived from qRT-PCR showed that all genes showed a similar expression pattern in response to the four isolates with some variations of fold change levels. Thus, there were no significant difference between any of the four *Giardia* isolates on host cells throughout the infection time points.

To summarise this chapter, the RNA-seq data allowed insights into transcriptional changes concerning the intestinal epithelial barrier in IEC-6 during short-term infection with *G. duodenalis*. This data detected that transcripts involved in TJ, AJ, actin cytoskeleton and ECM assemblies were significantly altered during 2 and 6hrs infections with trophozoites. These findings suggest that early giardiasis may cause a dysregulation in the intestinal epithelial barrier assembly, probably leading to a cell-cell leakage during a prolonged period of the

infection. Furthermore, the present research using qRT-PCR suggested that both trophozoites and ESP of *G. duodenalis* significantly altered the expression of TJ genes, suggesting that the ESP of *Giardia* may be key players for disturbance or reorganisation of these genes during infection. Future work could be usefully focussed on the differences between *Giardia* isolates and their effects on host cells. Further investigations will also be needed to confirm the expected changes in relevant protein levels and the effects on the infection process.

Chapter 6

Lack of *Giardia duodenalis*-mediated apoptosis in intestinal epithelial cells *in-vitro*

Chapter 6: Lack of *G. duodenalis*-mediated apoptosis in intestinal epithelial cells *in-vitro*

6.1. Introduction

Apoptosis is a well understood process of programmed cell death involved in many developmental and pathological events (Figure.1) (Hall *et al.*, 1994; Jacobson *et al.*, 1997; Fuchs and Steller, 2015). The main morphological features of cells undergoing apoptosis include; blebbing of the external membrane, cell surface display of phosphatidylserine, shrinkage of cytoplasm, nuclear fragmentation, and chromatin condensation with the persistent integrity of cellular membrane and organelles (Kerr *et al.*, 1972; Elmore, 2007). Several cysteinyl aspartic proteases (caspases) regulate the mechanism of apoptosis, these caspases are differentiated into either initiator caspases (i.e. caspase-2, -8, -9 and -10) which when activated trigger the executioner caspases including caspase-3, -6 and -7 (Elmore, 2007 Lüthi and Martin, 2007). In addition, it has been recently documented that apoptotic cells release signals such as, mitogens and death factors which are key players in the control of the cellular environment (Fuchs and Steller, 2015). Apoptosis is activated either through an intrinsic pathway involving mitochondrial damage which activates the initiator caspases or via an extrinsic pathway through cell surface receptor binding (TNFR or FasR death receptors) and activation of the Fas-Associated Death Domain (FADD) pathway leading to caspase activation (Figure 1).

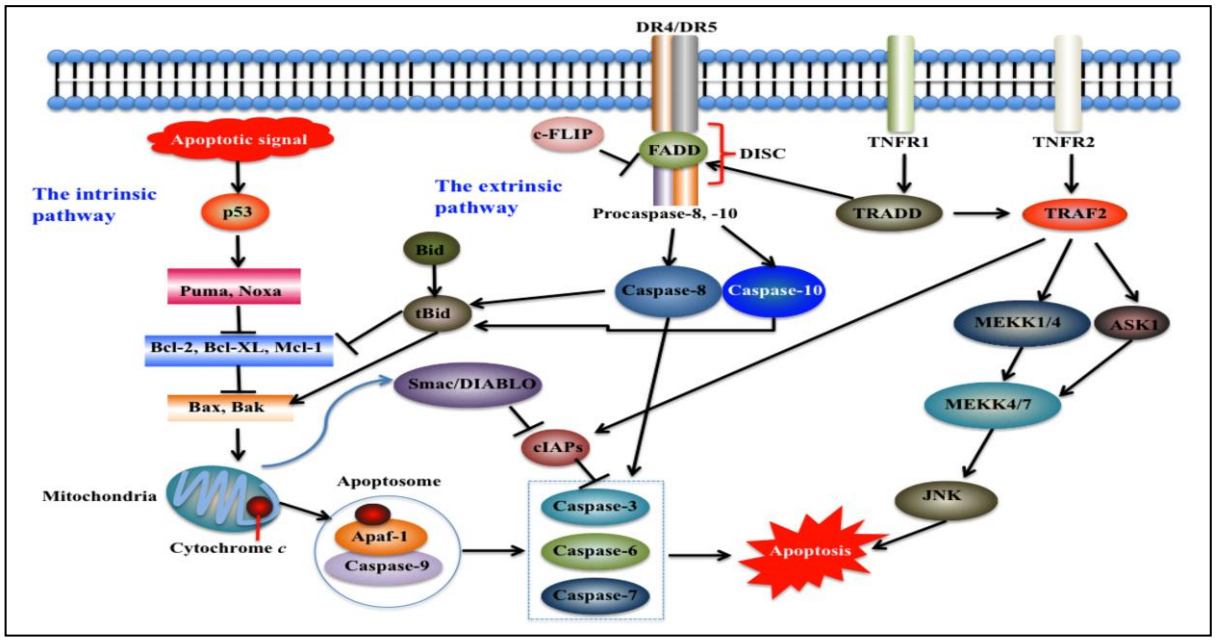


Figure 1: Intrinsic and extrinsic pathways of apoptosis. Adopted from (Zhang *et al.*, 2016). Abbreviations are explained in the text.

Apoptosis has been proposed as a cellular response to *G. duodenalis* parasitism as the death of intestinal epithelial cells can lead to the increased permeability of the epithelial barrier leading to a key symptom of giardiasis i.e. the production of leaky flux diarrhoea (Elmore, 2007; Panaro *et al.*, 2007; Cotton *et al.*, 2011; Ringqvist *et al.*, 2011). However, the exact role of *Giardia* trophozoites in epithelial cell apoptosis remains inconclusive. Differences in pathogenicity of assemblages A and B in clinical disease and variation in the degree of apoptotic induction between parasite isolates has been reported (Homan and Mank, 2001; Haque *et al.*, 2005; Breathnach *et al.*, 2010; Yang *et al.*, 2010; Asher *et al.*, 2014; Di Genova and Tonelli, 2016). Some authors have concluded that this data association suggests variation in *G. duodenalis* - mediated apoptosis induced by different isolates signals a significant contribution of apoptosis to clinical disease (Chin *et al.*, 2002; Panaro *et al.*, 2007; Koh *et al.*, 2013; Di Genova and Tonelli, 2016).

An *in-vitro* study conducted by Chin *et al* (2002), detected apoptosis in the non-transformed human duodenal epithelial cell line (SCBN) after 24hrs and 48hrs co-culture with NF and S2 *Giardia* isolates but not with WB or PB isolates. Another *in-vitro* study found *G. duodenalis* induced apoptosis in the transformed human intestinal epithelial cell line HCT-8 through DNA fragmentation, as well as western blotting analysis which showed down-regulation of Bcl-2 and up-regulation BAX after 16hrs of interaction (Panaro *et al.*, 2007). In chronic giardiasis, increased epithelial apoptosis was observed in 13 duodenal biopsy specimens using the TUNEL assay (Troeger *et al.*, 2007). Additionally, mixed *Giardia* isolates from assemblages A, B and E induced apoptosis in the human ileocecal adenocarcinoma cell line (HCT-8) after 24hrs co-culture, but surprisingly, co-culture of cells with assemblages A or B alone did not induce apoptosis in these experiments (Koh *et al.*, 2013). Less certain is an association of apoptosis with pathogenicity and strain virulence and currently any parasite derived factors that may mediate apoptosis are undefined (Cotton *et al.*, 2011). Taken together, the results of various experiments testing the ability of *Giardia* isolates to induce apoptosis have shown significant discrepancy, although these findings do suggest that apoptosis is stimulated in host epithelial cells by interaction with *Giardia* trophozoites at least over longer culture periods and with specific isolates.

However, studies have contested this conclusion with an *in-vivo* study using fluorescence microscopy on tissue slides stained with the TUNEL system showing no significant apoptosis in calves infected with *G. duodenalis* assemblage E (Dreesen *et al.*, 2012). In other parasites, it has been suggested that programmed cell death pathways of mammalian cells can be inhibited by intracellular protozoa (e.g. *Toxoplasma gondii* and *Leishmania donovani*) as an effective mechanism for their survival (Goebel *et al.*, 2001; Graumann *et al.*, 2015; Giri *et al.*, 2016). While *Giardia* is an extracellular protozoan that colonises and reproduces in the small intestine, it remains unclear if any of the *Giardia* surface proteins or their excretory/secretory products (ESP) play a role in activation or inhibition of cellular apoptosis; or whether apoptosis may be a consequence of cell activation due to stress of the infection rather than a specific *Giardia* activated event. Further research investigating the activation of apoptosis is clearly indicated.

Despite the suggested role of apoptosis in the pathophysiology of giardiasis the responses of host cells to *Giardia*-induced apoptosis are incompletely defined (Di Genova and Tonelli, 2016). The present study was carried out to identify apoptosis related genes that may play a significant role in the mechanism of apoptosis in intestinal epithelial cells during the initial infection (2/6hrs) response. The aims of this study were to:

a. Identify and analyse changes in the transcription level of genes known to be associated with apoptosis, using non-transformed IEC-6 host cells induced by *G. duodenalis* isolates P-1 or WB.
b. To determine if any of the four isolates (P-1, WB, NF, or GS/M) or their enriched ESP, representing assemblages A and B of *G. duodenalis* show significant differences in induction of specific genes known to be associated with apoptosis (*FASR, BCL2, BAX, and CASP3*) in IEC-6.
c. Quantitate the rate of apoptosis in IEC-6 following initial exposure to trophozoites of *G. duodenalis* isolates (P-1, WB, NF, or GS/M).

6.2. Results

6.2.1. Transcriptome profiling of the parasitised IEC-6 using RNA-seq

RNA-seq analyses resulted in the expression of several transcripts involved in regulating the extrinsic and intrinsic apoptotic pathways, were significantly altered (up-or down-regulated) in IEC-6 infected with *G. duodenalis* isolates P-1 or WB after 2 and 6hrs co-cultures (Figures 2 and 3).

6.2.1.1. Transcriptome changes at 2hrs co-culture

The 2hrs findings resulted in the *FASR* death receptor transcript expression upregulation (3.76 and 2.37 fc) in response to P-1 and WB isolates, respectively. Additionally, the mRNA level of the TNF receptor superfamily member 1A (*TNFRSF1A*), another receptor mediating extrinsic apoptosis, was up-regulated 0.58 and 0.72 fc following P-1 and WB co-cultures. Expression of ligands for both receptors showed no change in level in response to either isolate within the 2hrs timeframe. The expression of the tumor necrosis factor superfamily member 10 (*TNFSF10*), TNF-related apoptosis-inducing ligand (*TRAIL)* was upregulated 2.17 fc in response to the WB co-culture only, but the expression of its receptors (*DR4* and *5*) did not increase. On the basis of changes in gene transcription level it appears unlikely that the presence of either isolate (P1 or WB) triggered the extrinsic apoptotic pathway in these cultures. However, both isolates may have "sensitised" the cell to extrinsic apoptosis by the upregulation of expression of the *FASR* and *TNFR* transcripts. In the absence of Fas and TNF producing immune cells in these cultures upregulation of the receptors alone would be insufficient to trigger apoptosis.

In the intrinsic apoptotic pathway cleavage of the pro-apoptotic *BID* (BH3 interacting-domain death agonist) protein can trigger apoptosis through interaction with Bcl2 family members such as *BAX* and *BAK*. Although, the expression of *BID* was slightly upregulated (0.66 fc) in response to P-1, expression of the *BAX* transcript was unchanged with P1 and downregulated (-0.27fc) with WB. The *BAK* transcript did not show any changes. Release of cytochrome c from mitochondria is an essential step in activating the intrinsic pathway via the apoptotic peptidase activating factor 1 (*Apaf1*) triggering conversion of procaspase 9 to caspase-9, which goes on to trigger an effector caspase cascade. The RNA-seq results showed that cells

responded to the WB isolate with downregulation of both the cytochrome c (*CYCS)* and *APAF1* transcripts (0.6 and 0.32 fc, respectively), whilst *CASP9* expression slightly increased in response to WB only (0.47 fc). Expression of downstream effector caspases 7 and 3 were also altered (0.33 and -0.66 fc, respectively). However, in response to the P1 isolate neither *BAX*, *APAF1*, *CASP9* nor *CASP 7* transcripts showed any change in expression, *CASP3* transcript expression alone was downregulated (- 0.51 fc). Whilst expression of caspase transcripts themselves is not required to induce either the intrinsic or extrinsic apoptotic pathways, their downregulation has implications for the long-term ability of IEC-6 to induce apoptosis.

An important finding is that the expression of baculoviral IAP repeat-containing 2 and 3 (*BIRC2* and *BIRC3*) transcripts were considerably upregulated in response to P-1 or WB as shown in Figures 2 A and B. These genes are inhibitors of apoptotic proteins (IAPs) and can suppress apoptosis by inhibiting activation of caspases. Additionally, a variety of expressed genes involved in the PI3K/AKT signalling pathway were significantly induced in response to both isolates as shown in Figures 2A and B. These findings might indicate that the (PI3k-Akt) signalling pathway play a key role in the cell's fate in response to *G. duodenalis*.

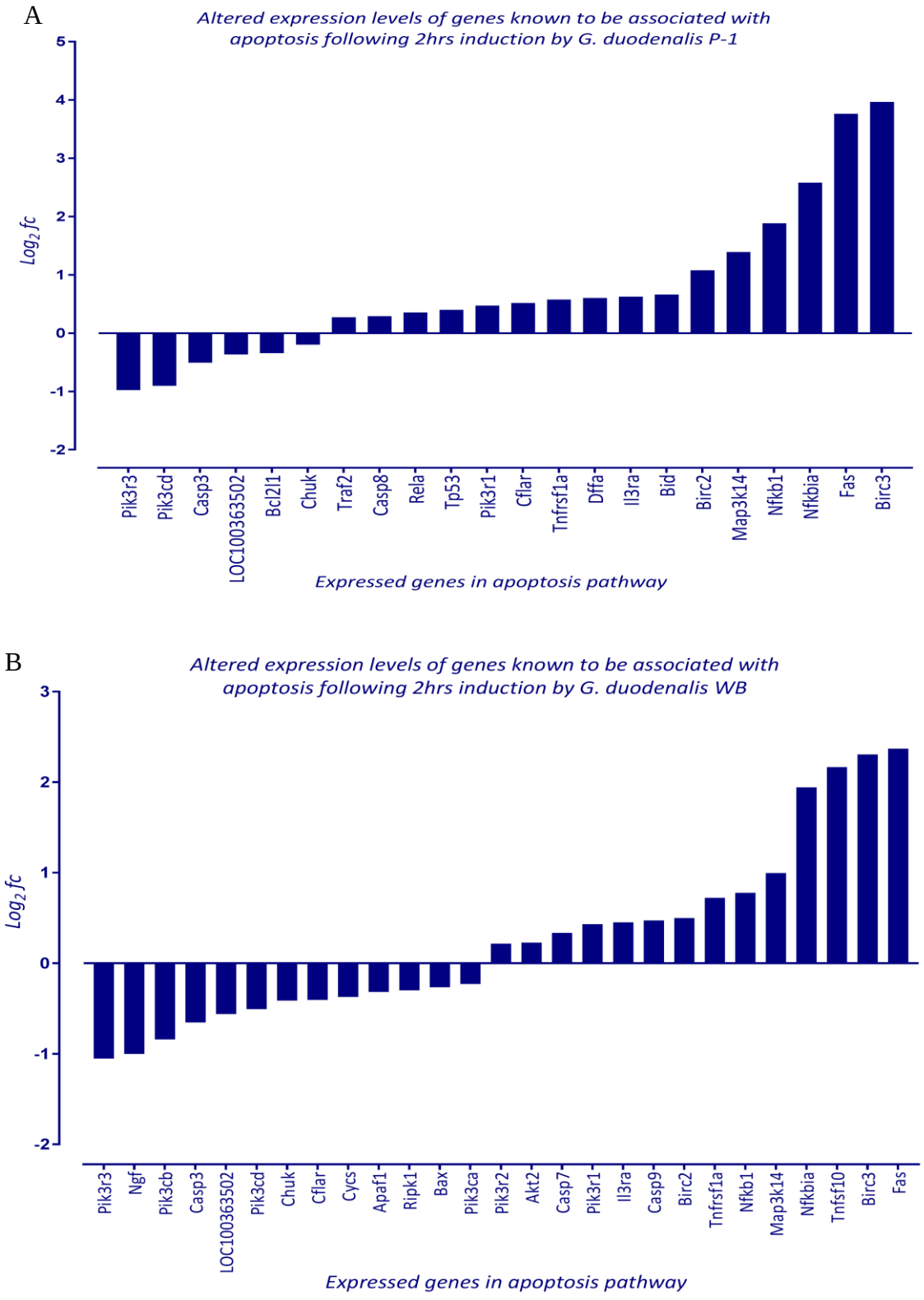


Figure 2: Expressed transcripts of the apoptotic pathways in IEC-6 induced by *G. duodenalis* isolates P-1 or WB at 2hrs. Transcripts whose expression level altered log2 fc at FDR cut off 0.05 from the uninfected IEC-6 were considered using the Benjamini and Hochberg method. A. In response to the isolate P-1, the analyses showed that 22 transcripts displayed significant altered levels of expression (6 downregulated and 16 upregulated). B. Responding to the isolate WB, 27 transcripts were significantly altered (13 downregulated and 14 upregulated).

6.2.1.2. Transcriptome changes at 6hrs co-culture

At 6hrs mRNA levels of the external death receptor *FASR* and the ligand *TNFSF10* transcripts were upregulated in both co-cultures, but there was no activation of either the ligands or receptors throughout this time (Figures 2 A and B). Thus, the adaptor protein Fas-Associated protein with Death Domain (FADD) did not trigger or recruit initiator caspase 8, the activator of caspase 3. *TNFRSF1A* (also called TNF-R1) was induced and triggers TNFRSF1A associated via death domain (TRADD), which in turn recruits TNF receptor-associated factor 2(*TRAF2*) to activate the NF-κB pathway, subsequently releasing the transcriptional factor (NF-κB) which promotes cell survival. It is important to mention that the expression of CASP8 and FADD-like apoptosis regulator (*CFLAR*) transcript was significantly upregulated in the P-1 (1.5 fc) and WB (1.53fc) induced host cells. This gene is an inhibitor of Fas-mediated apoptosis with upregulation associated with a block to activation of initiator caspases such as caspase 8.

In regards to the intrinsic pathway, the analyses showed that mRNA levels of the pro-apoptotic transcript (*BID*) was significantly upregulated (~ 1.9 fc) in both co- cultures, and the level of anti-apoptotic transcript (*Bcl2*) was significantly downregulated (-1.41 fc) in WB, but not P-1 induced host cells. Nevertheless, the expression of other pro-apoptotic transcripts such as *BAD* and *BAX* were considerably downregulated (-0.69 and -0.31 fc, respectively). The apoptosis inhibitor Bcl2-like 1 (*BCL2L1*) transcript, was induced at 0.39 & 0.32 fc in P-1 and WB, respectively. The level of cytochrome c, somatic (*CYCS*) was significantly downregulated -0.59 P-1 induced host cells, while the level of the apoptotic peptidase activating factor 1 (*APAF1*) was slightly upregulated 0.48 and 0.68 fc in both IP-1 and IWB, respectively. The tumor suppressor protein p53 (*TP53*) was upregulated in P-1 (0.54 fc) and WB (0.69 fc) induced host cells. Additionally, the expression of caspase-7 was significantly upregulated (0.87 fc) in response to both isolates. Finally, despite the various gene transcription changes in the intrinsic pathway, neither caspase 9 nor caspase 3 expression were changed at 6hrs.

The data detected changes in transcripts involved in endoplasmic-reticulum (ER) stress, another intrinsic apoptosis activation mechanism. For instance, increased levels of *CASP-12* transcript were seen at 1.25 and 1.24 fc in P1 and WB induced host cells, respectively and downregulation of calpain 1 and 2 (*CAPN 1 and 2*), ~ -0.47 and 0.58 fc in P-1 and WB induced host cells, respectively.

The expression of the *BIRC2* and *BIRC3* transcripts, whose products have key roles in inhibiting initiator caspase-9 and executioner caspases-3 and -7, were continuously upregulated in P-1 and WB induced host cells (Figures 3 A and B). Furthermore, significant upregulation of Nfkb1 and Nfkbia both involved in the NF-kB signalling pathway may indicate apoptosis inhibition in both co-cultures at 6hrs. Finally, this data highlighted the overexpression of phosphoinositide-3-kinase, regulatory subunit 5 (Pik3r5) gene transcription in response to the both isolates (4.94 fc and 4.99 fc). Collectively these gene transcription events may be seen as a *Giardia* induced high level of apoptotic suppression in host cells in early giardiasis.

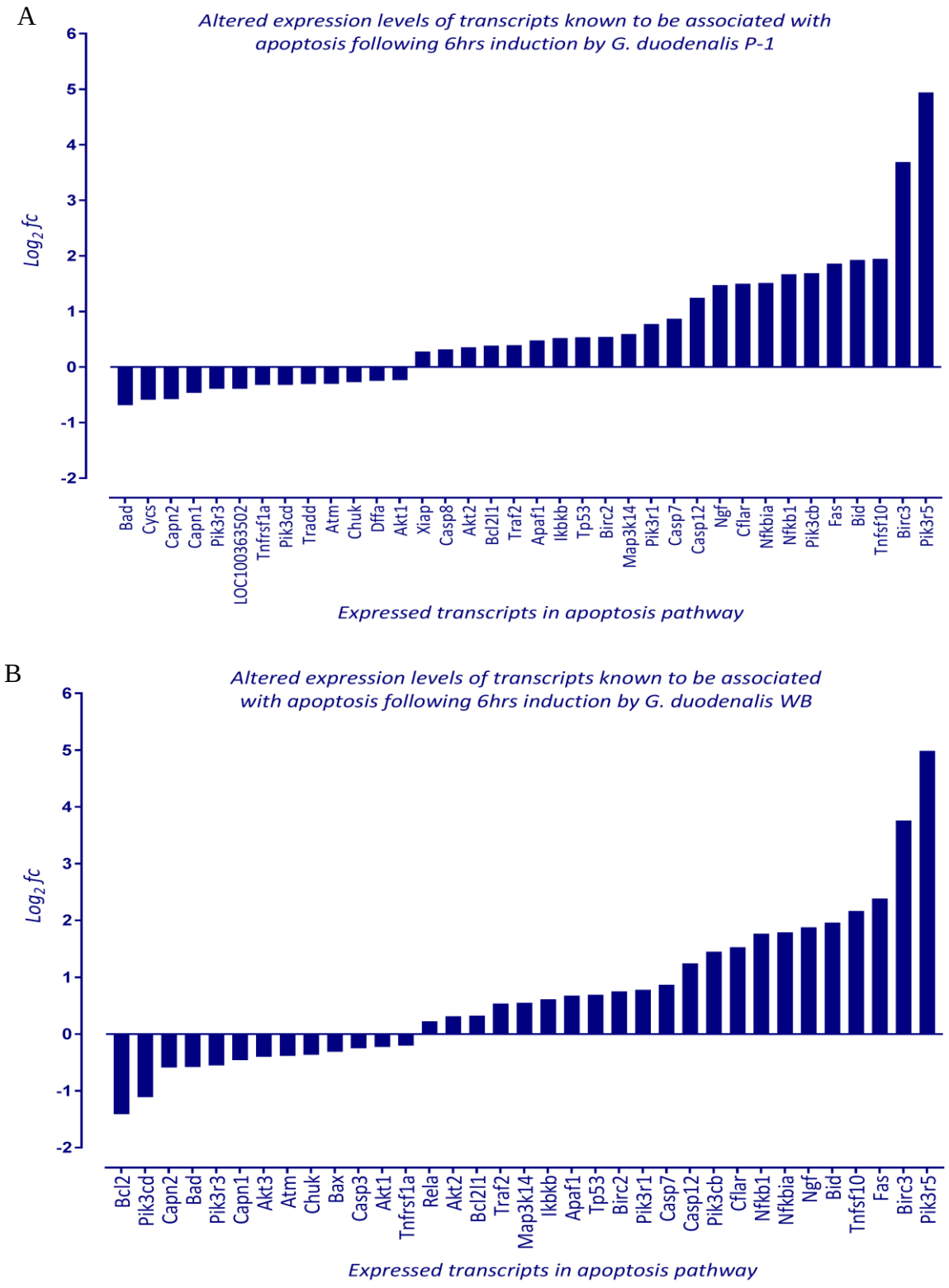


Figure 3: Expressed transcripts of apoptotic pathways in IEC-6 induced by *G. duodenalis* isolates P-1 or WB at 6hrs. Transcripts whose expression level changed log2 fc at FDR cut off 0.05 from the uninfected IEC-6 were considered using the Benjamini and Hochberg method. **A.** in response to the IP-1, the analyses showed that 36 transcripts were significantly altered (13 downregulated and 23 upregulated). **B.** in response to the IWB, the mRNA level of 37 transcripts was significantly altered (13 downregulated and 22 upregulated).

6.2.1.3. 2 vs 6hrs transcriptome changes in P-1 induced host cells

The number of transcripts that displayed significantly altered levels of expression in parasitised IEC-6 was higher at 6hrs (36 transcripts) than 2hrs (22 transcripts) (Figures 1A and 2A). In relation to apoptosis the most significant findings are that mRNA levels of *BIRC3, FAS-R, NFKBIA, NFKB1*, and *MAP3K14*, transcripts were significantly upregulated at both time points while the level of *PIK3R3* and *PIK3CD* were consistently downregulated.

CASP3 and *IL3RA* were downregulated and upregulated, respectively, at 2hrs, but their expression were not significantly different at 6hrs. The expression of *TNFRSF1A* and *DFFA* were both upregulated at 2hrs but downregulated at 6hrs. Furthermore, the expression of *CFLAR* and *BID* significantly increased between the 2 and 6hrs, with fold changes of 0.52and 0.66 at 2hrs to 1.50 and 1.93 at 6hrs, respectively. Some transcripts were significantly altered at 6hrs only. For example, the levels of *BAD*, *CAPN 1* and *2* transcripts was considerably downregulated, while the level of *CASP-7, CASP-12, NGF, TNFSF10 and PIK3R5* were significantly upregulated.

6.2.1.4. 2 vs 6hrs transcriptome changes in WB induced host cells

In this co-culture, 27 and 37 transcripts had significantly altered expression in the parasitised cells at 2 and 6hrs, respectively (Figures 1B, 2B). Transcripts showing consistent change at both time points included PIK3R-3, *PIK3CD, CYCS and CASP-3* which were all downregulated, while levels of *FASR, BIRC3, NFKBIA, NFKB1, TNFSF10, MAP3K14*, and *CASP-7* were upregulated. Other transcripts showed differential changes, thus *PIK3CB, APAF1, NGF* and *CFLAR* transcripts were downregulated at 2hrs but upregulated at 6hrs. *CASP-9* transcript expression was slightly upregulated at 2hrs but it did not show a significant change at 6hrs. Finally, a number of transcripts showed responses at 6hrs but not at 2hrs. For example, the expression of *BCL-2, CAPN1, CAPN2*, and *BAD* were downregulated, while *PIK3R-5, BID, CASP-12* and *TP53* transcripts were upregulated at 6hrs.

6.2.2. qRT-PCR analysis of specific genes known to be associated with apoptosis

The qRT-PCR was used to confirm some RNA-seq results and to examine whether ESP of different *G. duodenalis* isolates alone could induce apoptosis in IEC-6. The effect of the

presence of *Giardia* trophozoites on specific genes as quantitated by qRT-PCR (Figure 4) followed a similar trend as the RNA-seq data. qRT-PCR of the *FASR* gene showed significant upregulation in the presence of all *Giardia* isolates as early as 2hrs and continuing to 6hrs. The anti-apoptotic gene *BCL2* increased expression over 2hrs, but by 6hrs had decreased in all isolates. The expression level of the pro-apoptotic *BAX* gene showed no significant changes throughout culture with either isolate. Finally, the expression of the *CASP-3* was downregulated at the 2hrs but was not significantly different to the control levels by 6hrs (Figure 4). At 2hrs the mRNA level of *TGFβ-1* were significantly upregulated in co-cultures with GS/M, P-1 and WB isolates, with the highest expression with GS/M, while there was no a significant change with the NF isolate. The expression of the *p38* gene was significantly upregulated with all the isolates, while *SMAD2* transcript levels did not change. At 6hrs, the expression of *TGFβ-1* was lower with all isolates, while *p38* gene expression was significantly upregulated and SMAD-2 again remained unchanged.

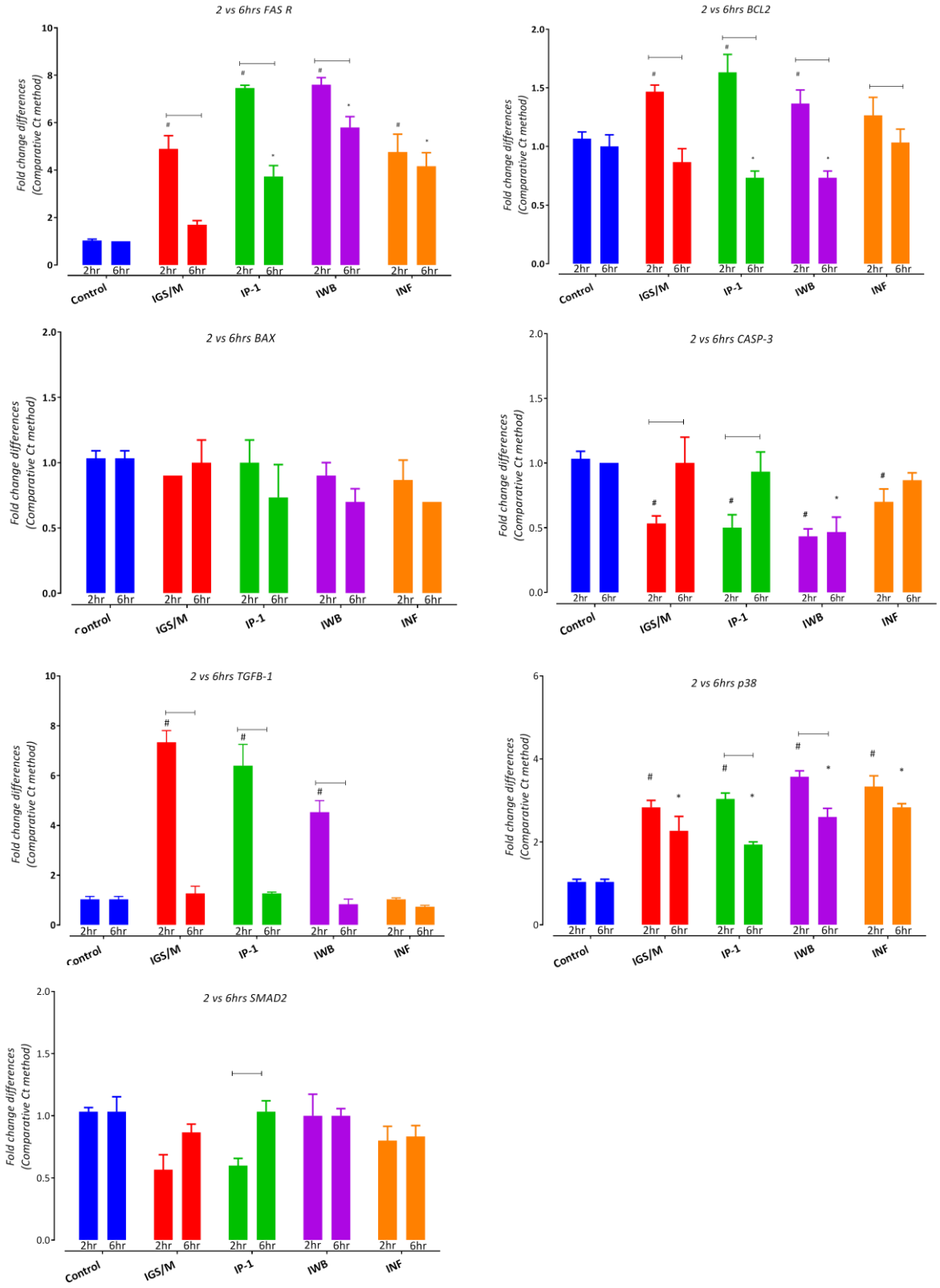


Figure 4: qRT-PCR analysis of fold change differences in IEC-6 induced by trophozoites of four *G. duodenalis* isolates (GS/M, P-1, WB or NF) at 2 and 6hrs. An analysis was done by using Two-way ANOVA and significance determined by Bonferroni's multiple comparisons test Data: Mean ± S.D. P ≤0.05. # denotes a significant difference between 2hrs vs control. * indicates a significant difference between 6hrs vs control. − indicates a significant difference between 2 vs 6hrs.

The ESP caused some genes to change expression in a similar manner to trophozoites with variations in terms of fold change levels while others responded quite differently (Figure 5). *FASR* expression was upregulated at 2 and 6hrs, while *BCL-2* was only upregulated at 2hrs. *BAX* gene expression again did not change while *CASP-3* was similar to its expression in the control group except it was downregulated at 2hrs in 2 cultures.

There was a significant decrease in expression of *TGFβ-1* and *p38 at* 2hrs in P-1, WB and NF but not with the GS/M isolate. The mRNA levels of *SMAD2* were significantly downregulated with P-1, WB and NF, but not with GS/M. At 6hrs, the expression of *TGFβ-1* and *p38* genes were significantly decreased with all isolates except *p38* with P-1 which did not show a significant change compared to the control. Finally, there was no substantial change in *SMAD2* with GS/M, P-1 and WB, but a significant downregulation in co-cultures with the NF isolate.

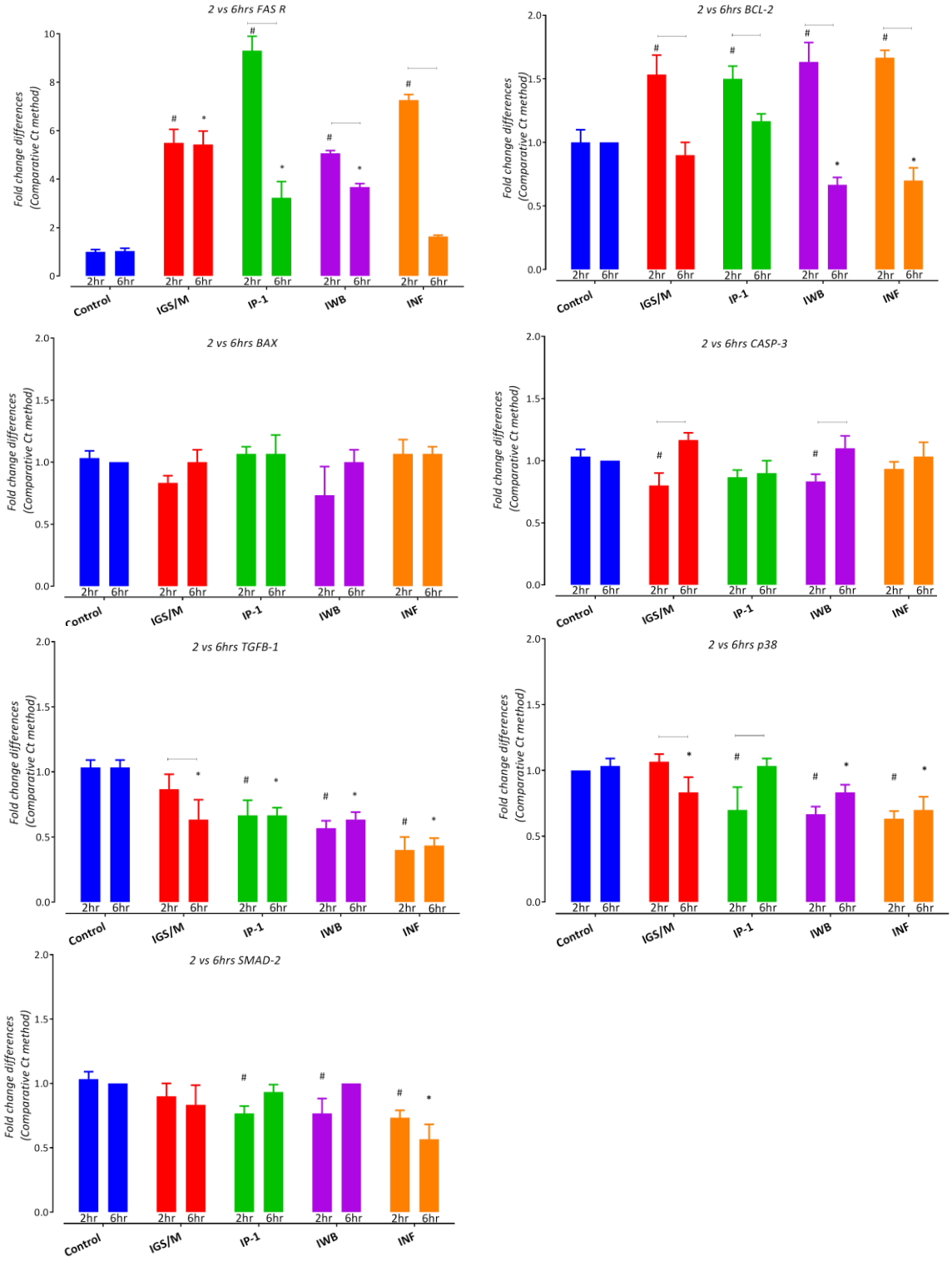


Figure 5: qRT-PCR analysis of fold change differences in IEC-6 induced by ESP of four *G. duodenalis* isolates (GS/M, P-1, WB or NF) at 2 and 6hrs. An analysis was done by using Two-way ANOVA and significance determined by Bonferroni's multiple comparisons test Data: Mean ± S.D. P ≤0.05. # denotes a significant difference between 2hrs vs control. * indicates a significant difference between 6hrs vs control. − indicates a significant difference between 2 vs 6hrs.

6.2.3. *In-vitro* assay of apoptotic response of IEC-6

A cell death assay was used to determine whether the RNA-seq and qRT-PCR data indicating a lack of apoptosis in early giardiasis with the four different isolates (GS/M, P-1, WB or NF), would be confirmed by a biological assay. The cell death detection assay was carried out using the ELISA^{PLUS} kit to detect DNA fragmentation consistent with apoptosis activation following each time point. There was no evidence of apoptosis in the parasitised IEC-6 at either 2 or 6hrs of culture (Figures 6 A, B).

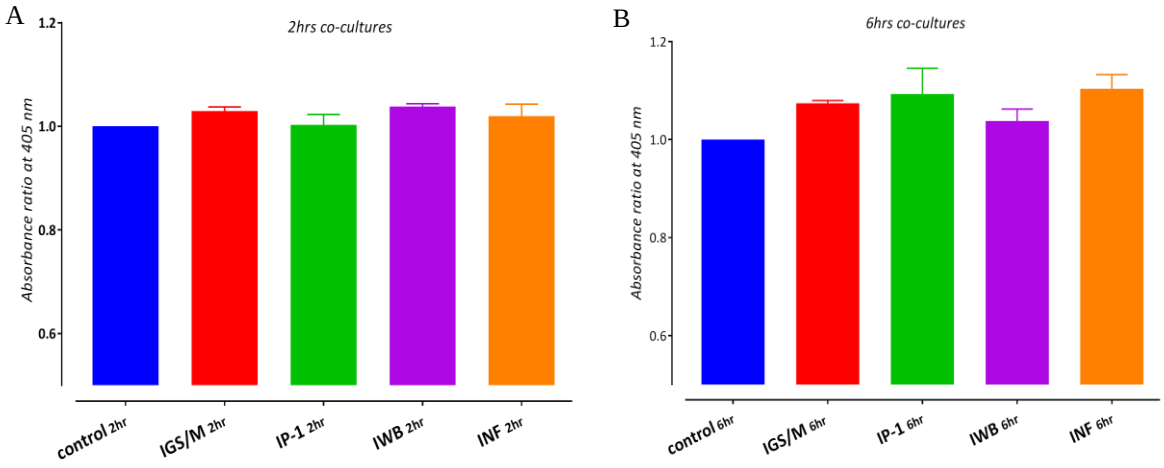


Figure 6: Apoptosis rates in IEC-6 following incubation with the four different *G. duodenalis* isolates (GS/M, P-1, WB or NF) or DMEM culture media alone (controls) at 2 and 6hrs *in vitro*. A and B. At 2 and 6hrs, respectively, no significant level of apoptosis was seen in infected cells compared to the control. Data was analysed based by ONE-WAY ANOVA and significance was determined by Bonferroni's multiple comparisons test (Mean ± SD $p \leq 0.05$).

6.3. Discussion

Previous studies evaluating whether *G. duodenalis* isolates induce significant apoptosis in their host cells have observed inconsistent results (Chin *et al.*, 2002; Panaro *et al.*, 2007; Koh *et al.*, 2013). Explanations for these results include factors such as *Giardia* isolate, type of host cell line, and time of the infection and trophozoite density (Fisher *et al.*, 2013). Thus, the main question in this study sought to determine whether different *Giardia* isolates induced apoptosis genes in non-transformed intestinal epithelial cells (IEC-6) during the early infection period (2/6hrs).

RNA-seq data provided a comprehensive picture of gene transcription in the extrinsic and intrinsic apoptosis pathways during early giardiasis. The data showed significant upregulation in mRNA levels of cell death receptors, including *FASR* and *TNFRSF1A* in response to isolates P-1 and WB; nevertheless, expression of their cognate ligands did not show any significant changes. In addition, the *TNFSF10* ligand increased in response to WB; but expression of its receptor was not changed. These findings might indicate that the IEC-6 was increasing their death receptor density in response to the parasite and thus are more sensitive to apoptosis ligands. It has been documented that the expression of Fas is pronounced in normal intestinal epithelial cells, while Fas ligand is more restricted, with expression mostly shown in hematopoietic cells (Iwamoto *et al.*, 1996; Sayani *et al.*, 2004; Fernandes *et al.*, 2014). It is noteworthy that the IEC-6 also showed increased levels of *CFLAR* in response to both isolates, especially at 6hrs which supports inhibition of the extrinsic apoptosis pathway (Micheau, 2003; Shirley and Micheau, 2013; Fulda, 2013).

In terms of the intrinsic pathway, NF-kB and BCl2 family members are the major regulators. Pro-apoptotic members include Bax, Bak, Bad, Bcl-xS, Bid, Bik and Bim, while anti-apoptotic members include Hrk, Bcl-2, Bcl-xL, Bcl-W, Bfl-1 and Mcl-1 (Tait and Green, 2010; Kalimuthu and Se-Kwon, 2013; Zhang *et al.*, 2016). RNA-seq data detected inconsistent induction in mRNA levels of both anti and pro-apoptotic genes. Upregulation of *BID* and downregulation of *BCL2* transcripts at 6hrs suggests that the balance of pro-apoptotic proteins is increasing which may ultimately lead to the apoptosis of host cells (Fuchs and Steller, 2015). However, other pro-apoptotic transcripts, such as *BAD* and *BAX* were downregulated, while the anti-apoptotic transcript *BCL2l1* was upregulated, which might indicate the integrity of mitochondrial membrane (Tait and Green, 2010). A slight downregulation of cytochrome c and upregulation of *APAF1* transcript were observed in response to both isolates at 6hr again suggesting a shift in the balance of apoptosis controlling proteins which may pre-dispose the host cell to apoptosis in the event of intrinsic or extrinsic induction of the caspase cascade (Liu *et al.*, 1996; Kluck *et al.*, 1997; Yang *et al.*, 1997; Ow *et al.*, 2008). However, there was no significant change in *CASP9* levels arguing against an increased preparedness for intrinsic apoptosis to occur. Lack of caspase-9 activation again suggests apoptosis inhibition (Tait and

Green, 2010). Finally, consistent significant downregulation of *CASP-3* in response to both isolates at both infection times suggests neither apoptosis pathway is active (Salvesen, 2002; Devarajan *et al.*, 2002; Elmore, 2007; Ghavami *et al.*, 2009).

Importantly, previous *in vitro* and in *vivo* studies documented that no significant apoptosis could be detected in host cells in response to four *Giardia* isolates at 8hrs (Chin *et al.*, 2002) or calves infected with *G. duodenalis* assemblage E (Dreesen *et al.*, 2012). This data was in agreement with the present findings, probably suggesting that *Giardia* trophozoites may produce proteins that modulate cellular apoptosis mechanisms for supporting their survival during early parasitism. Research conducted on some intracellular protozoa has demonstrated anti-apoptotic survival mechanisms used to inhibit cellular apoptosis, ultimately enhancing survival in hosts (Heussler *et al.*, 2010; Graumann *et al.*, 2015; Giri *et al.*, 2016). In this study persistent upregulation of the anti-apoptotic *BIRC2* and *BIRC3* genes suggests general inhibition of the activation of caspases (Deveraux and Reed, 1999; Saleem *et al.*, 2013), and thus suppression of intrinsic and extrinsic apoptosis. The action of Birc3 is triggered by transcriptional factor dimers that interact with the TNF and NF-$_k$B signaling pathways. Beginning with activation of the *TNFRSF1A*, TNF receptor-associated factor 2 (*TRAF-2*) or receptor interacting serine/threonine kinase 1 (*RIPK1*), and *MAP3K14* (*NIK*) genes that subsequently induce NF-kB signaling pathway genes such as IkB kinase (*IKK*), NFKB inhibitor alpha (*NFKBIA*), and *NFKB1*. Ultimately, *NFKB1* induces pro-survival genes, including *CFLAR* (Rothe *et al.*, 1996; Saleem *et al.*, 2013). Furthermore, BIRC3 is an E3 ubiquitin-protein ligase whose normal targets include: *RIPK1, RIPK2, RIPK3, RIPK4, CASP3, CASP7, CASP8, IKBKE, TRAF1,* and *BCL10,* proteins that are ubiquinated by BIRC3 and then targeted for destruction by the proteasome (Bertrand *et al.*, 2011; Zhou *et al.*, 2013). Thus, Birc2 and Birc3 might be the main proteins targeted by *Giardia* trophozoites to influence on cellular apoptosis at this stage of the infection.

Alternative pathways for inhibition of apoptosis in the IEC-6 are suggested by the upregulation of the transcriptome factor NF-kB which is a key player in *CFLAR* induction, as well as blocking the activation of caspase-8, and thus the extrinsic pathway (Wang *et al.*, 1998;

Micheau *et al.*, 2001). In addition, TNF can induce apoptosis in NF-kB-deficient intestinal epithelial cells; indicating a role for NF-kB in inhibition of TNF-induced apoptosis (Wullaert *et al.*, 2010). Furthermore, transcripts involved in the PI3k-Akt signalling pathway, which inhibits apoptosis through the *BAD* transcript (Datta *et al.*, 1997; Peso *et al.*, 1997; Martelli *et al.*, 2006) were also induced in IEC-6, possibly assisting cells survival through inhibition of the intrinsic pathway. Significant upregulation of *PIK3R1* and *PIK3R5* transcripts were observed and recently both genes have been proposed to be important factors in apoptosis, in particular *PIK3R5* (Xu *et al.*, 2017). This data might also support a hypothesis suggesting that the role of *Giardia* trophozoites to activate these transcripts, which ultimately involved in inhibition of apoptosis. Therefore, further studies are required to confirm the role of these proteins in cells during giardiasis and the balance in the cell between activation and inhibition of apoptosis over the infection period.

Contrary to expectations, this study found significant upregulation of *CASP-12* transcript, which has been suggested to be a key mediator of endoplasmic-reticulum (ER) stress-induced apoptosis (Nakagawa *et al.*, 2000; Szegezdi *et al.*, 2003). The inhibition of *CAPN -1,-2* transcripts in this data was also observed at 6hrs, where calpain is a cytoplasmic calcium-activated protease which can cleave and activate caspase-12 in response to calcium flux from the ER (Nakagawa and Yuan, 2000). Nevertheless, caspase 12 is expressed in rodents, additionally caspases associated with ER stress-induced apoptosis are incompletely defined (Fischer *et al.*, 2002; Szegezdi *et al.*, 2006). Together, these pathways might suggest ER-stress induced intrinsic activation related to the stress of the infection except that the activation of *BIRC2* and *BIRC3* transcripts at the time points studied would suppress the main executioner caspases -3 and -7 (Roy *et al.*, 1997; Huang *et al.*, 2001; Salvesen and Duckett, 2002).

The qRT-PCR data was similar to the RNA-seq data for the same genes. The transcript level of the *FASR* gene was significantly upregulated by trophozoites or ESP at both infection times. This finding is in agreement with (Ma'ayeh, 2013), indicating an increased sensitivity to Fas induced apoptosis. There were no significant differences found between isolates in the

upregulation of *FASR* gene expression following co-culture with either trophozoites or ESP. The consistent finding of upregulation of the FASR gene in this study may indicate that intestinal epithelial cells are primed to apoptosis induction via the extrinsic pathway. In the presence of cells secreting/releasing Fas *in-vivo* there may be increased levels of apoptotic induction (Peter *et al.*, 2007). Although FasR is a well-studied and recognised cell death receptor that has an essential impact in extrinsic apoptosis, it is also involved in non-apoptotic functions (Wajant, 2002; Peter *et al.*, 2007) including NF-kB activation and cell proliferation (Wajant *et al.*, 2003; Peter *et al.*, 2015). While there was no evidence for gene expression changes in the intrinsic pro-apoptotic gene (*BAX*) at either the 2 or 6hrs with trophozoites or ESP, there was significant upregulation of anti-apoptotic gene *Bcl-2* at 2hrs, with inconsistent downregulation at 6hrs.

Overall, this suggests that the intrinsic apoptotic pathway is unlikely to be active in the IEC-6 in either set of co-cultures at either time points tested. In contrast other studies have reported induction of the intrinsic apoptotic pathway. Panaro and colleagues found that the *Giardia* isolate WB clone C6, induced apoptosis in the transformed human intestinal epithelial cell line HCT-8 through DNA fragmentation, with western blotting showing down-regulation of *BCL-2* and up-regulation of BAX after 16 hrs of interaction (Panaro *et al.*, 2007). The differences found in these studies may be ascribed to the early time points analysed in the current study (up to 6hrs) which is supported by Chin *et al.*, (2002) who did not observe apoptosis in cells incubated with *Giardia* for 8hrs.

The key apoptotic gene *CASP-3* is involved in both the extrinsic and intrinsic pathways at the final step of DNA fragmentation (Youle and Strasser, 2008). Changes in the expression of this gene were inconsistent in the current study with low levels of downregulation or no significant changes observed at the 2 and 6hrs. Propagation of the apoptosis signal arising from the intrinsic or extrinsic pathways require the direct activation of caspase-3 (CASP-3) which cleaves DNA, producing the cellular and biochemical events of apoptosis (Thornberry and Lazebnik, 1998; Slee *et al.*, 2001; Elmore, 2007). Whilst production of new procaspase 3 is not required for

apoptosis to occur if induced, the lack of change (up-regulation) of expression of the gene argues against an apoptotic cell fate. In the present study, the lack of observed DNA fragmentation confirms the absence of apoptosis even by already available caspase 3 in these experiments (Chin *et al.*, 2002; Koh *et al.*, 2013). These qRT-PCR findings differ compared to previous RDA which suggested upregulation of *CASP-3* expression at 6hrs co-culture with any of the four isolates of *Giardia* (Ma'ayeh, 2013, unpublished data). Chin *et al* reported caspase-3-dependent apoptosis of SCBN cells following incubation with the NF and S2 isolates, but not with WB and PB isolates, following 24 and 48 hrs of co-culture (Chin *et al.*, 2002). Variation between these studies may be ascribed to the infection timeframes where host cells following 24/48 hrs might be under more stress as a result of the parasites' mechanisms or be exhausted due to the environment of *in-vitro* co-culture.

It has been documented that the transforming growth factor-β (TGF-β) signaling pathway has an important role in several cellular processes (Nagaraj and Datta, 2010). TGFB-1 is a multifunctional protein that is involved in pathways including growth, proliferation, differentiation and apoptosis (Taherkhani *et al.*, 2009; Ikushima and Miyazono, 2010; Ma'ayeh, 2013; Yamada *et al.*, 2013). In the current study, mRNA levels of the *TGFB-1* gene increased in response to trophozoite culture at 2hrs. This is in an agreement with Ma'ayeh, (2013) and induction particular at 2hrs might be ascribed to the involvement of *TGFB-1* in signalling networks involved in cell survival. Previous studies also suggested that TGFβ-1 has a key role in regulating the proliferation and differentiation of intestinal epithelial cells as well as wound repair (Beck *et al.*, 2003; Bulut *et al.*, 2006; Sturm and Dignass, 2008; Ikushima and Miyazono, 2010; Yamada *et al.*, 2013). mRNA levels of *TGFβ-1* was significantly upregulated, suggesting that TGFβ-1 might have a key role in reconstitution of epithelial integrity, promoting rapid healing in mucosal injury of IEC-6 (Ciacci *et al.*, 1993). Therefore, upregulation of *TGFB-1* gene expression in response to *Giardia* infection, in particular at 2hrs might be involved in reconstitution processes as a result of the physical attachment of trophozoites.

Further to this finding, one of the key proteins of the TGFβ-1 signalling pathway is p38 whose mRNA level was significantly upregulated in this study. Mitogen-activated protein kinases (MAPKs), including p38 are protein kinases that regulate cell functions including proliferation, differentiation, physical stresses, inflammatory cytokines, cell survival, and apoptosis (Pearson *et al.*, 2001; Kyosseva, 2004). Mammalian p38 is activated in response to inflammatory cytokines as well as by various cellular stresses (Cuenda and Rousseau, 2007). Another study revealed that *G. duodenalis* induced Interleukin 8 (IL-8) production that leads to activate p38, ERK1/2 MAPK, NF-κB and AP-1 (Lee *et al.*, 2012). Thus, it is suggested that the upregulation of p38 in the current study might be in response to inflammatory cytokines that are secreted in response to the physical attachment of *Giardia* trophozoites on the host cells.

Following incubation of IEC-6 with *Giardia* ESP, the expression of *TGFβ-1* and *p38* genes was significantly different than observed after incubation with *Giardia* trophozoites. Thus, expression was either significantly downregulated or without change at 2 and 6hrs. This finding might support the suggestion that physical attachment of the trophozoites directly activates the TGFβ-1 signalling networks of host cells.

Overall, short term co-culture of intestinal epithelial cells with *Giardia* may preclude the detection of apoptosis as suggested here and by Chin *et al*., (2002) who did not find apoptosis after 8hrs incubation with *Giardia* strains; NF, S2, WB, or PB using Hoechst fluorescence staining, ELISA or electron microscopy. Cell apoptosis has been detected after incubation for 24 and 48hrs with NF and S2 isolates (Chin *et al.*, 2002). In chronic giardiasis, increased epithelial apoptosis was observed in duodenal biopsy specimens using the TUNEL assay (Troeger *et al.*, 2007). Recently, apoptosis was also observed in human enterocytes using a cell death detection ELISA after 24hrs interaction with mixed *Giardia* isolates from assemblages A, B and E; but not with assemblages A or B alone (Koh *et al.*, 2013). Thus, comparison of the current data with previous findings suggests, either that differences in infection timeframes might influence apoptosis induction *in-vitro*, or *Giardia* trophozoites may be able to secrete

products that activate inhibitors of apoptosis (IAPs) to prolong the survival of intestinal cells. Further studies are needed to investigate and clarify these suggestions.

Taken together the data implies that whilst there may be an increased sensitivity to FAS ligand induced apoptosis, there is no evidence of further activation of apoptotic pathways. Obviously, protein studies may allow more certain conclusions as to the state of the apoptosis pathway in these cells but at the time points studied apoptosis is not occurring in these cultures.

Chapter 7
General discussion, conclusions and future studies

Chapter 7: General discussion, conclusions and future studies

7. General discussion

This thesis set out with the fundamental aim of assessing cellular gene responses in the rat intestinal epithelial cell line (IEC-6) in response to the initial interaction with various *G. duodenalis* isolates (P-1, WB, NF, or GS/M) *in-vitro*. A considerable number of studies have been carried out in an attempt to understand the pathogenicity of *G. duodenalis,* however, little is known regarding host factors and cellular responses to this protozoan (Cotton *et al.*, 2011).

Epidemiological and genetic studies have shown significant prevalence and genomic variations within and between isolates of *G. duodenalis* assemblages A and B (Homan and Mank, 2001; Haque *et al.*, 2005; Breathnach *et al.*, 2010; Adam *et al.*, 2013; Asher *et al.*, 2014; Ankarklev *et al.*, 2015; Emery *et al.*, 2015). Despite a wealth of research the virulence factors of parasite isolates and their effects in host pathophysiological responses are still incompletely defined (Buret *et al.*, 2015). Studies using animal models have revealed the importance of the early transcription of genes coding for components of intestinal mucosal immunity, indicating the impact of changes in transcription in understanding of *Giardia*/host interactions (Bienz *et al.*, 2003; Zhou *et al.*, 2003; Dreesen *et al.*, 2014; Di Genova and Tonelli, 2016). Despite the findings, the early transcriptome profiling of *G. duodenalis* -induced cells remains to be fully investigated.

RNA-seq and qRT-PCR techniques were used to quantify and analyse the transcriptomes of parasitised IEC-6 at 2 and 6hrs. Additionally, biochemical analysis of cell death was conducted to specifically determine whether cells *in-vitro* are induced to undergo apoptosis. The overview of RNA-seq data has shown significant differences between the assemblages A isolates P-1 and WB in terms of the number of expressed genes at 2hrs, which might be ascribed to variations in the pathogenicity level of these isolates on their host cells during an early stage of the infection. However, these differences were not consistent between these isolates at 6hrs induction. qRT-PCR and biochemical assays did not reveal significant differences between the

same isolates or the GS/M and NF isolates on the IEC-6, with all isolates showing a similar pattern of gene activation except some variability in the qRT-PCR results. Thus, at these time points in the cellular response to trophozoites there appear to be few differences between *G. duodenalis* isolates although further analysis of the variations that were observed would be useful and may point to pathological or immunological pathways that become important later in the development of the infection.

The analysis of gene transcription did identify many early changes with the potential to affect the regulation of several pathways in host responses to parasitism. Increased mRNA levels of transcripts involved in responses to stress and hypoxia clearly illustrate an early cellular response to the parasites. Another clear set of responses were those concerned with immune defence and the early signalling events that generate such responses. Dysfunction in the epithelial barrier between host cells was highlighted with responses from an interrelating network of structural and signalling transcripts involved in cell-cell junctions. Finally, a significant finding was the identification of transcripts involved in the regulation of both the extrinsic and intrinsic apoptotic pathways during the first six hours of the infection.

The major outcome of the current thesis is a comprehensive profile of IEC-6 transcription in response to *G. duodenalis* isolates over the first six hours of infection. This extensive catalogue of cellular responses will require a large amount of further research to identify and clarify the importance of each specific gene or induced pathways. Biochemical and proteomic studies will be required to confirm the protein levels of these transcripts and understand the outcome of each response to the infection outcome.

7.1. Immune responses to giardiasis

Innate and adaptive host responses to *Giardia* infection have been investigated by numerous researchers in attempts to provide a better understanding of host/parasite interactions (Bienz *et al.*, 2003; Eckmann, 2003; Zhou *et al.*, 2003; Roxström-Lindquist *et al.*, 2006; Solaymani-

Mohammadi and Singer, 2010). Nevertheless, the nature of the immune responses and the role of immune modulators produced by the host cell in response to infection remain unclear (Lopez-Romero *et al.*, 2015). Current data shows that mRNA levels of many immune-response transcripts (cytokines and chemokines) were upregulated over the six hours of infection. Thus, host epithelial cells actively elicit cellular immune responses within 2 hours of contact with trophozoites.

Cytokines and chemokines modulate cellular activities such as growth, survival, differentiation and inflammation. Cytokines act as both pro- and anti-inflammatory factors whilst chemokines can act as chemotactic attractants for different immune cell types. Many immune modulator proteins have multifunctional roles, IL-6, IL-12 and IL-17 for example, are involved in the activation of both innate and adaptive immune responses (Khader *et al.*, 2009; Scheller *et al.*, 2011). Whilst it has been recently proposed that both innate and adaptive immune mechanisms are required for host clearance of giardiasis (Lopez-Romero *et al.*, 2015), the exact nature of the recruitment and action of immune cells is unclear. It is also unclear which host inflammatory responses would be host and/or parasite-protective; or indeed which responses lead to host pathology.

Previous studies have provided inconsistent results on whether *Giardia* infection induces host pro-inflammatory intestinal responses (Cotton *et al.*, 2015). It has been proposed that little or no mucosal inflammation accompanies giardiasis since trophozoites do not invade the epithelial layer of the small intestine (Oberhuber *et al.*, 1997; Campbell *et al.*, 2004; Scott *et al.*, 2004). Other studies however, suggest that intestinal inflammatory responses might be observed during the infection (Roxström-Lindquist *et al.*, 2005; Hanevik *et al.*, 2007; Bénéré *et al.*, 2012: Lee *et al.*, 2012; Chen *et al.*, 2013). Furthermore, data collected from *in-vivo* and *in-vitro* studies suggest that intestinal pro-inflammatory responses were induced by *Giardia* assemblage B isolates, but not with assemblage A (Cotton *et al.*, 2015). Our RNA-seq data identified a variety of mostly pro-inflammatory factors (cytokines and chemokines) which displayed significantly altered transcription profiles in IEC-6 in response to assemblage A trophozoites very early in

the infection. These results agree with a previous microarray analysis which showed increased mRNA levels of chemokines in host cells in response to assemblage A (Roxström-Lindquist *et al.*, 2005). Thus, this data supports findings of previous studies that *G. duodenalis* assemblage A infection can induce pro-inflammatory gene responses. Whether these are translated into metabolic consequences are still to be determined.

Transcription of interleukins was significantly increased in IEC-6 in response to both isolates. Thus, the pro-inflammatory cytokine IL-6 which is involved in inflammation and infection responses and acts on epithelial and immune cells (Grivennikov *et al.*, 2009; Scheller *et al.*, 2011). Many cell types produce IL-6 in response to various stimuli and pathogens (Akira *et al.*, 1993; Tanaka *et al.*, 2014). In addition to its inflammatory role (McGee *et al.*, 1992; Scheller *et al.*, 2011), IL-6 plays a vital role in T-cell responses and in the ability of IEC to act as Antigen Presenting Cells (APCs) (Mayer *et al.*, 1990), as well as enhancing Peyer's patch B cells to secrete IgA (Beagley *et al.*, 1989). The presence of IgA antibodies has been identified as a requirement for eliminating giardiasis and may involve epithelial cells acting as APCs to elicit the T helper response (Langford *et al.*, 2002). In other studies, IL-6 was found to be involved in early control and clearance of *Giardia* infection (Bienz *et al.*, 2003; Zhou *et al.*, 2003; Li *et al.*, 2004; Muñoz-Cruz *et al.*, 2010). Thus, induction of the IL-6 transcript in the present study confirms previous investigations showing its influence in host immune defence against *Giardia* infection. Further studies might investigate inflammatory and cellular responses *in vitro* to determine the comparative role of these defences against the trophozoite through analysis of their timing and the trophozoite response.

The transcription profile of several chemokines was altered in the parasitised cells at both sampling times. Chemokines create chemotactic gradients along which immune cells migrate to the site of chemokine production. They are involved in various biological activities, including homeostasis and immune activation and regulation (Bryant and Slade, 2015). The current results demonstrate alteration of the host cell chemokine profile similar to that identified by microarray analysis (Roxström-Lindquist *et al.*, 2005). Thus, increased mRNA levels of the

pro-inflammatory chemokines *CCL20*, *CCL2*, *CXCL1*, *CXCL2*, and *CXCL3* transcripts were identified in response to assemblage A isolates. It has been documented that CCL20 is strongly chemotactic for lymphocytes and weakly attracts neutrophils (Baba *et al.*, 1997; Hieshima *et al.*, 1997). The *CCL20* gene is expressed in the gut under normal circumstances, but its expression is increased in inflamed epithelium (Smith *et al.*, 2013). It is suggested here that the parasitised cells upregulate production of this chemoattractant in order to recruit lymphocytes to the site of infection. It is interesting to note that CCL20 expression is suggested to be strongly controlled by NF-kB, with overexpression of the p65/RelA subunit of NF-kB demonstrated to significantly increase CCL20 mRNA levels (Sugita *et al.*, 2002). In the present study, inconsistent upregulation was detected in the transcription level of the *RelA* gene, with low levels of induction in response to P-1 and WB at 2 and 6hrs, respectively. This finding might indicate that *CCL20* gene transcription is not dependent on NF-κB activity in this study.

Recent research reported that the inflammatory cytokines CCL2, CCL3, CCL5, CXCL1, CXCL2 and CXCL8 are significantly up-regulated during injury, insult, or infection of tissues (Graham and Locati, 2013). CCL2 is a monocyte chemoattractant protein 1 (MCP1) that recruits monocytes, memory T cells, and dendritic cells to sites of inflammation (Carr *et al.*, 1994; Xu *et al.*, 1996). CXCL1 and CXCL2 are small cytokines belonging to the CXC chemokine family, which are expressed by macrophages, neutrophils and epithelial cells (Iida and Grotendorst, 1990; Becker *et al.*, 1994; Ohno *et al.*, 1997). A recent study has shown that CXCL1/CXCL2 control the early phase of neutrophil recruitment during inflammation (De Filippo *et al.*, 2013). Taken together, the current data has suggested the induction of early inflammation by chemotaxis of monocytes, neutrophils and T cells plus induction of APCs. This general inflammation and immune induction presumably leads to protective responses under some circumstances. Further research following the pathways highlighted by this analysis may well allow discrimination of the specific pathways and circumstances that allow control of *Giardia.*

Tumour necrosis factor (TNF) is a pro-inflammatory cytokine that plays an essential role in immunity and cellular homeostasis in mammalian cells (Brenner *et al.*, 2015). It has been also documented that the TNF superfamily (TNFSF) and the TNF receptor superfamily (TNFRSF) are involved in inflammation, cell death, survival, and proliferation (Gravestein and Borst, 1998; Aggarwal *et al.*, 2012; Croft *et al.*, 2012; Croft and Siegel, 2017). The present study revealed mRNA responses for a variety of ligands and receptors from the TNFSF in parasitised IEC-6.

Recently TNFRs have been shown to promote mucosal repair in Inflammatory Bowel Disease (IBD) through Wnt/β-catenin signalling (Bradford *et al.*, 2017). Previous studies had also suggested that TNF is a positive regulator of Wnt signaling through transcription control (e.g. NF-kB and PI3K signaling), again promoting β-catenin activation in collaboration with Wnt (He *et al.*, 2004; Barrett, 2012). In this study, Wnt/β-catenin signalling was significantly induced in response to both *Giardia* isolates. This finding suggests the induction of TNFSF and a possible role in mucosal repair during giardiasis. Furthermore, the alteration in transcription level of these transcripts is again indicative of activation of inflammatory immune responses to *Giardia* infection. Previous studies have found that impairment in the host defence is a consequence of the absence of TNF (Kato *et al.*, 1989; Hauser *et al.*, 1990). TNF plays a key role in stimulating chemokine and cell adhesion molecule production, leading to mononuclear cell accumulation (Hauser *et al.*, 1990; Roach *et al.*, 2002). Current data revealed consistent upregulation in the expression of the *TNFSF15* transcript in response to both isolates at both times. Previous research suggested that the TNFSF15 cytokine (TL1A) has a role in initiating or promoting Th1 responses via enhancing IFN gamma production (Prehn *et al.*, 2004). There are also various ligands and receptors in the TNFSF and the TNFRSF which promote pro-inflammatory signalling (Aggarwal *et al.*, 2012; Brenner *et al.*, 2015). Nevertheless, the functions of the TNFSF proteins remain to be fully defined (Croft and Siegel, 2017).

Taken together, the above observations suggest that the early infection in IEC-6 particularly in response to *G. duodenalis* assemblage A is characterised by inflammatory responses via pro-inflammatory cytokines, interleukins, chemokines and TNFSF members. Thus, unsurprisingly,

host cells respond to defend themselves and recruit immune cells to clear the parasite. Further work is required to confirm the various protein responses and the specific roles for each of these mediators during early giardiasis.

The role of Nitric oxide (NO) is a major area of interest for its microbiostatic and microbiocidal effects against many pathogens, including *G. duodenalis* (Fang, 1997; Fernandes and Assreuy, 1997; Liew *et al.*, 1997; Tako *et al.*, 2013). In the current study, the mRNA level of the *NOS2* transcript was highly upregulated throughout the infection implying the increased production of NO by the parasitised IEC-6 from the earliest time of the infection. The *NOS2* isoform of the NO-synthases utilises L-arginine as the major substrate for NO synthesis in epithelial, endothelial and inflammatory cells (Tepperman *et al.*, 1993; Stuehr, 1999; Popovic *et al.*, 2007; Lundberg *et al.*, 2008). Previous studies have shown that *Giardia* enzymes can efficiently and preferentially utilise arginine and that this can prevent the host cell from utilising the NOS-2 pathway to produce NO (Schofield *et al.*, 1990; Knodler *et al.*, 1994; Eckmann *et al.*, 2000; Ringqvist *et al.*, 2008; Stadelmann *et al.*, 2012). Although these studies explained arginine depletion by *Giardia*, indicating that host cells are less likely to have a ready supply of L-arginine for conversion to NO, other *in vitro* and *in vivo* findings observed that growth inhibition in *G. duodenalis* excitation and encystation through NO (Eckmann *et al.*, 2000) and NO produced by Nos2 plays important roles in the control of giardiasis (Tako *et al.*, 2013).

Our present study observed no significant downregulation of host arginine metabolism gene transcription and a significant and consistent upregulation in *NOS2* expression during 2 and 6hrs of the infection. Previous studies demonstrated that NOS2 expression can be activated through cytokines (e.g. interferon response factor-1 (IRF1)), indicating that their role in the stimulating of NO production through NOS2 transcription in immune responses (Kamijo *et al.*, 1994; Gao *et al.*, 1997). Our data also revealed that the expression of *IRF1* transcript was significantly upregulated throughout the infection times in response to both isolates, suggesting that NO production might be triggered via IRF1-dependent activation of the inducible NOS.

Taken together, these findings indicate that the parasitised cells may attempt to produce NO via L-arginine catabolism and if substrate levels are high the cell may well produce NO as an immune defence mechanism against early giardiasis. Further research is required to confirm whether NO is produced during the first six hours in response to giardiasis.

Significant upregulation was found in the *NOX1* transcript which encodes one of the NADPH oxidase family of enzymes responsible for catalytic electron transfer of oxygen to generate reactive oxygen species (ROS) such as superoxide or hydrogen peroxide. NOX1-generated ROS are involved in many cellular activities, such as host defence, epithelial restitution, redox signalling involved oxygen sensing and apoptosis, but their exact physiological role is still incompletely defined (Geiszt and Leto, 2004; Bedard and Krause, 2007; Jiang *et al.*, 2007; Kato *et al.*, 2016). The upregulation in *NOX1* implies that host cells increase production of antimicrobial ROS in response to *Giardia* infection. In addition, increases in transcription of the hypoxia-inducible factor 1A (*HIF1A)* transcript might be ascribed to hypoxia/stress caused by *Giardia* trophozoites. It has been previously shown that *NOX1* and many other transcript involved in stress and/or hypoxia and cellular proliferation such as *HIF 2* were induced in *Giardia* infected host cells (Roxström-Lindquist *et al.*, 2005). This suggests that ROS production is part of a general response to stress. Alternatively, parasitised IEC-6 may produce ROS as a host defence mechanism and it is likely that ROS are also involved in the epithelial restitution processes in response to trophozoite-induced damage. It has been documented that low or moderate levels of ROS play a vital role in killing invading pathogens and in promoting the mucosal repair processes (Bhattacharyya *et al.*, 2014; Kato *et al.*, 2016).

Although excessive levels of ROS can induce apoptosis through both the extrinsic and intrinsic pathways (Ozben, 2007; Scherz-Shouval and Elazar, 2007), cells use a scavenging system to control ROS levels by balancing the generation of ROS with their elimination (Winterbourn, 2008). An antioxidant system mediates cellular redox when superoxide is dismutated to H_2O_2 by the superoxide dismutase (SOD2) in the mitochondrial matrix, while cytosolic SOD (SOD1) dismutates superoxide to H_2O_2 in the cytosol (Nogueira and Hay, 2013). The present study

showed that the *SOD2* transcript was increasingly upregulated throughout the early infection. The protein encoding this transcript has an anti-apoptotic impact against oxidative stress, inflammatory cytokines, and ionizing radiation (Becuwe *et al.*, 2014). It acts by transforming superoxide, a toxic by-product of the mitochondrial electron transport chain, into hydrogen peroxide and diatomic oxygen, which allows mitochondrial clearance of reactive oxygen species (ROS), downregulating the potential for apoptosis to occur (Pias *et al.*, 2003).

The antioxidant system also comprises of glutathione compounds, considered the major non-enzymatic components of intracellular antioxidant defences and with the ability to maintain redox balance through regulation of intracellular levels of NADPH (Turrens, 2003; Nogueira and Hay, 2013). The current results showed a significant downregulation in the transcription level of peroxiredoxins (PRX), which are one group of antioxidants in the glutathione system involved in regulating H_2O_2 levels (Rhee *et al.*, 2005). H_2O_2 detoxification by glutathione peroxidase (GPx) and PRX plus fatty-acid synthesis are considered the main consumers of intracellular NADPH (Schafer and Buettner, 2001).

This data suggests that the parasitised IEC-6 might produce moderate levels of NOX1-generated ROS through the SOD2-dependent scavenging system and this production might be one of the host defence mechanisms used in response to early giardiasis. More research is needed to confirm the associated protein responses and to better understand the importance of these responses to *G. duodenalis* infection.

7.2. Epithelial Barrier Dysfunction and Giardiasis

In giardiasis, epithelial dysfunctions have been reported both in acute and chronic infections (Buret, 2007; Troeger *et al.*, 2007; Einarsson *et al.*, 2016). *Giardia* trophozoites do not invade the intestinal epithelial barrier (Oberhuber *et al.*, 1997), although recent studies have suggested that *Giardia* trophozoites may invade the sub-epithelial space, between the enterocytes or at the base of goblet cells (Martínez-Gordillo *et al.*, 2014; Reynoso-Robles *et al.*, 2015; Allain *et al.*,

2017). This finding may indicate another *G. duodenalis* pathogenic mechanism, but the role of this finding in the pathophysiology and survival of *G. duodenalis* remains to be confirmed (Reynoso-Robles *et al.*, 2015).

Analysis of the intracellular junction transcripts in the present study found significant alteration of gene transcription in most pathways forming the epithelial barrier in IEC-6 (TJ, AJ, actin cytoskeleton, and extracellular matrix). Several studies have previously reported that *Giardia* infection causes Apical Junctional Complex (AJC) disruption, possibly leading to increased intestinal permeability in the parasitised cells (Chin *et al.*, 2002; Scott *et al.*, 2002; Troeger *et al.*, 2007; Humen *et al.*, 2011; Maia-Brigagão *et al.*, 2012; Koh *et al.*, 2013). These changes might be ascribed to the physical effect of trophozoite attachment to host intestinal epithelial cells or to chemical activities. *G. duodenalis* trophozoites strongly attach to host intestinal epithelial cells using the adhesive disk and flagella and this attachment could cause the structural and physiological changes and induce gene transcription changes (Adam, 2001; Müller and von Allmen, 2005; Ankarklev *et al.*, 2010; Humen *et al.*, 2011; Maia-Brigagão *et al.*, 2012).

However, our qRT-PCR data indicates for the first time that incubation with trophozoite ESP can cause changes in gene transcription associated with dysregulation in TJ assemblies. Thus, ESP incubated with IEC 6 cells for 2 and 6 hrs resulted in upregulation of the transcripts *CLDN-1* and *ZO-1* while the transcript *OCLN* was downregulated at 2hrs. Previous studies with ESP have suggested the presence of cysteine proteases (Lee *et al.*, 2012; Jiménez *et al.*, 2014) which might account for an effect on TJ integrity but no analysis has found such an effect to date. The results presented in this work are especially noteworthy as they suggest a new mechanism for *Giardia* pathology and thus a new avenue for ameliorating the infection outcome. Further research is required to prove that the observed gene induction is due to the disruption of TJ integrity or some other effect. The exact nature of the effect of the *G. duodenalis* ESP on IEC-6 is unknown in this study. It should be noted that as *G. duodenalis* ESP was collected from co-cultures of IEC-6 and trophozoites, the subsequent effect of *G. duodenalis* ESP supernatants

alone on IEC-6 could have resulted from the direct effects of the *G. duodenalis* ESP on the IEC-6 or indirectly by host soluble factors (e.g. cytokines) within the supernatant which were enriched along with the *G. duodenalis* ESP. The logic of collecting *G. duodenalis* ESP from co-cultures of trophozoites and IEC-6 was to attain *G. duodenalis* ESP resulting from this host-parasite interaction which was surmised to be more representative of biological relevant interactions than isolating ESP from trophozoites alone in the absence of IEC-6 where key ESP proteins may or may not be expressed.

It has been documented that barrier function in intact epithelia is maintained by tight junction integrity (Odenwald and Turner, 2016). The RNA-seq and qRT-PCR findings presented here identified increased levels of TJ gene transcription (e.g. *CLDN-1*) possibly as a response to disturbances of TJ integrity at both 2 and 6hrs. Claudin-1 is required to seal and prevent paracellular diffusion of small molecules through tight junctions, as well as maintaining normal barrier function (Inai *et al.*, 1999; Kirschner *et al.*, 2013). Downregulation of Claudin-1 and increased apoptosis was reported by Troeger *et al.*, in a chronic giardiasis case suggesting a loss of TJ integrity during infection (Troeger *et al.*, 2007). Previous studies have also suggested that the overexpression of the *CLDN-2* gene was linked to leaky intestinal crypts and increased paracellular water permeability (Rahner *et al.*, 2001; Rosenthal *et al.*, 2010). The present findings showed a significant upregulation of *CLDN-2* transcription at 6hrs infection which is strongly suggestive of an early ability of the parasite to cause damage to TJ, leading to cell-cell leakage.

Collectively, this study suggests that early in the course of giardiasis (2/6hrs) both parasite attachment and parasite secreted molecules disturb the intestinal epithelial barrier leading to the upregulation of gene transcription of the AJ, TJ and APC transcripts as the cell attempts to repair these junctions. A prolonged period of infection would be characterised by leakage across the epithelial barrier due to both the physical and pathophysiological effects of *G. duodenalis* trophozoites.

7.3. Apoptosis and infection establishment

The RNA-seq and biochemical assay data presented in this study have shown that the IEC-6 host cells did not undergo apoptosis at 2 or 6hrs when cultured with *G. duodenalis*. The lack of apoptosis maybe controversial as a number of *in-vitro* studies have suggested that host cells undergo apoptosis in response to *Giardia* infection (Chin *et al.*, 2002; Troeger *et al.*, 2007; Panaro *et al.*, 2007; Koh *et al.*, 2013; Fisher et al., 2013). An *in-vivo* study in 2012, did not detect significant apoptosis in calves infected with *G. duodenalis* assemblage E (Dreesen *et al.*, 2012). Research conducted on intracellular protozoa have revealed that other parasites use anti-apoptotic survival mechanisms to inhibit cellular apoptosis, ultimately enhancing survival in hosts (Graumann *et al.*, 2015; Giri *et al.*, 2016); although these mechanisms have not been identified in giardiasis which is not known to have an intracellular form.

Closer examination of previous *in-vitro* studies suggests important differences from the current analysis. Perhaps the most important is the use of different *Giardia* isolates (Di Genova and Tonelli, 2016) which may suggest variations in the ability of different isolates to cause apoptotic activity. Other variations include the use of transformed or non-transformed cell lines which may differ considerably in their response to parasite factors. The period of the cell cultures is longer in previous studies which may give more time and opportunity for the induction of apoptosis. The current data did not detect significant apoptosis in IEC-6 at up to 6hrs, which is in agreement with a lack of apoptosis in parasitised non-transformed human duodenal epithelial cells (SCBN) at 8hrs (Chin *et al.*, 2002). While, significant apoptosis was shown in the transformed human intestinal epithelial cell line (HCT-8) at 16hrs (Panaro *et al.*, 2007), 24 and 48hrs (Chin *et al.*, 2002; Koh *et al.*, 2013) in an isolate/assemblage-dependent manner. Nutrient levels also determine and support cell survival and growth processes and where there are insufficient nutrients cells will undergo cell cycle arrest, which can lead to apoptosis (Mason and Rathmell, 2011). Most recently, it has been documented that medium composition is a key factor in *Giardia*/host cell interaction (Kraft *et al.*, 2017). Hence, sufficient nutrients in co-culture media might reduce cellular stress and support cell survival at least during the early culture period.

Despite a lack of apoptosis, it is apparent that the extrinsic apoptosis mechanism in the parasitised IEC-6 became more sensitive at 2 and 6hrs with the upregulation of FASR transcription. In previous *in-vivo* studies such a result may increase the chances of initiating FasL induced apoptosis (Wajant, 2002; Elmore, 2007), however the present study showed that IEC-6 transcription of the FasL transcript did not accompany the increase in FASR transcription reducing the probability of auto activation of the extrinsic pathway. In addition, induction of the anti-apoptotic protein (*CFLAR*) strongly suggests inhibition of activation of extrinsic apoptosis (Micheau, 2003; Shirley and Micheau, 2013; Fulda, 2013). The significant upregulation of IAPs transcripts such as BIRC2 and BIRC3 supports this inhibition. It is known that IAPs regulate cell death and survival pathways and their expression can be induced in response to cellular stress such as hypoxia, endoplasmic reticular stress and DNA damage (Dubrez-Daloz *et al.*, 2008; Marivin *et al.*, 2012; Saleem *et al.*, 2013). Equally importantly, IAPs inhibit the early stages of caspase-dependent and independent cell death (Marivin *et al.*, 2012; Vasudevan and Ryoo, 2015). Whether such effects are induced by the host cell as part of its stress response or perhaps through parasite mechanisms to assist cell survival and thus parasite reproduction is not indicated by this research although it has been previously discussed (Marivin *et al.*, 2012; Vasudevan and Ryoo, 2015).

7.4. Comparative analysis of RNA-seq and RDA data

Multiple genomics technologies have been used by researchers to gain an understanding of the complex interactions between cells and internal or external parasites. These technologies have not only garnered detailed insights into the "war" between host and parasite but also provide significant quantitative biological information (Baginsky *et al.*, 2010). However, limitations have been identified in all methods and in particular with hybridization based methods (Hubank and Schatz, 1994; Okoniewski and Miller, 2006; Zhao *et al.*, 2014). Data generated in our laboratory is derived from two methods, one relying on cDNA preparation of mRNA followed by PCR and rounds of subtractive hybridisation (RDA) the other on sequence library preparation from cDNA followed by sequencing. We were therefore able to carry out a comparative analysis between the RDA and RNA-seq technologies.

The main difference highlighted was that RDA could not detect downregulated levels of transcription (Hubank and Schatz, 1994; Ma'ayeh and Brook-Carter, 2012), while RNA-seq is able to identify both upregulated and downregulated transcripts with high-quality data (Cloonan *et al.*, 2008; Nagalakshmi *et al.*, 2008; Wang *et al.*, 2009; Ansell *et al.*, 2017). This limitation of RDA undoubtedly leads to an incomplete picture of cellular responses to giardiasis. For instance, transcriptional down-regulation of cell adhesion transcripts (e.g. *JAM-3, ZO-2, CDH-1, CTNND-1, ROCK,* and *RHOA*) was identified by RNA-seq data whilst RDA was not able to provide this level of detail.

Another difference is in the sensitivity and reproducibility of the two technologies, where RNA-seq was more reproducible, sensitive and accurate, as noted by other authors (Cloonan *et al.*, 2008; Nagalakshmi *et al.*, 2008; Wang *et al.*, 2009; Zhao *et al.*, 2014). A clear difference was observed in the total number of transcriptional changes detected by RNA-seq compared to RDA in response to *Giardia* infection (section 4.2). Furthermore, RNA-seq was able to classify and clarify several cellular pathways that were significantly induced during *Giardia* infection, providing a comprehensive picture of the IEC-6 cell responses. Induction of various cytokines and chemokines gives an example of pathways which were not shown by RDA. Another example was in the cell adhesion assembly transcripts, such as TJ, AJ, actin cytoskeleton and ECM, while these pathways were poorly classified by RDA which missed data related to transcripts including *CLDN-1, CLDN-2, ZOs, JAMs, CDH-1, ROCKs* and *RHOA*. Taken together, RNA-seq gave a more complete understanding of gene transcription changes in IEC-6 in response to *G. duodenalis*.

7.5. Conclusions and Future studies

The purpose of the current study was to evaluate and understand host cellular responses against early giardiasis by direct comparison of the transcript profiles of parasitised and non-parasitised IEC-6 in response to different *G. duodenalis* isolates at 2 and 6hrs *in-vitro*. Transcript profile analysis using RNA-seq identified a large number of transcripts whose mRNA levels changed in a statistically significant manner at both infection times. The most obvious finding to emerge

from this data is a large increase in production of transcripts for a variety of cytokines and chemokines indicative of an attempt by the host cell to raise an immune response against infection. Some of these cytokines have a pro-inflammatory function, which suggests attempts to recruit immune cells. An increase in transcription of genes responsible for ROS and NO production indicates cell defence responses against early giardiasis. The present study has also suggested that both trophozoites and their ESP cause disruption to the intestinal epithelial barrier, as evidenced by the alteration of the transcription rates of target transcripts involved in tight junctions, adherens junctions, the actin cytoskeleton, and extracellular matrix. There is also evidence for attempts by parasitised cells to restore and repair this damage during the first six hours of the interactions. Another important finding derived from both RNA-seq and a biochemical assay is that whilst host cells may become more sensitive to FAS induced apoptosis they do not readily undergo apoptosis as evidenced by the upregulation of transcripts of IAPs and their survival during these short-term cultures. Taken together, the findings suggest that early transcriptional changes in host cells in response to giardiasis concern setting up the immune response, as well as immediate defence, responses to stress and then repair mechanisms as the parasite establishes.

This study has generated RNA-seq transcription data identifying significant differences in transcript levels in response to *G. duodenalis* assemblage A isolates. The results have provided a comprehensive picture of the alteration of the transcriptome caused by parasitism, and have created more questions than it has provided answers. For instance at 2hrs, a significant difference in the number of transcripts whose levels altered was seen between isolates P-1 or WB. Our hypothesis that there is a difference in host cell response depending upon the infecting isolate was not fully answered. There were clear differences in the number of genes experiencing altered levels of transcription in the host cell for the P1 and WB isolates within the first 2hrs of infection; however, by 6hrs the size of the difference between the two was statistically insignificant. Further studies are needed to determine whether these differences truly constitute a variation in parasite pathogenicity or cell response to these isolates.

This data also showed significant upregulation in target transcripts of cytokines (interleukin, CSF, chemokine, and TNFSF) key players in immune responses against early giardiasis. Confirmation that transcription levels represent functional proteins can be clarified by proteomic studies. Equally important, biochemical research needs to be carried out to confirm the transcriptome changes of pro-inflammatory cytokines and chemokines (e.g. IL-6, IL-1A, IL-23A, CSF2, CCL20, CCL2, CXCL1, CXCL2, and CXCL3) and confirm that *G. duodenalis* assemblage A induces inflammatory responses in host cells. In addition, further work to confirm findings of the Fas receptor transcript, where a FAS sensitivity assay could determine whether this protein and its ligand are activated during 2/6hrs interaction with giardiasis. Another possible area of future research would be to confirm the levels of overexpression of the transcripts *NOS2* and *NOX1* and estimate whether NO and ROS are produced in response to early infection.

In regards to the second hypothesis in this study, the effect of physical or/and ESP of *Giardia* trophozoites on the mechanism of cell-cell adhesion is partially clarified. Our data strongly indicates that host cells undergo restitution and repair of the TJ and AJ epithelial barrier assemblies, nevertheless, the finding is limited by the lack of information on the alteration in protein levels of the genes. Moreover, as yet there is no clear indication of the trigger(s) that initiate repair and restitution. Proteomic and biochemical assays are required to provide a complete picture of the cell adhesion mechanism. Equally important, this study has demonstrated that *G. duodenalis* ESP might have a significant effect on TJ assembly of IEC-6. Previous studies have suggested that the ESP was able to induce immune responses in the infected host cells (Kaur *et al.*, 1999; Jiménez *et al.*, 2004; Lee *et al.*, 2012; Jiménez *et al.*, 2014). Thus, more research is needed to better understand the effect of ESP on the host intestinal epithelial barrier, which may add more knowledge about the mechanisms by which *Giardia* causes diarrhoea.

This is the first study using molecular and biochemical analyses to investigate the effect of early giardiasis (2/6hrs) on host cell apoptosis. The findings of this investigation complement those

of earlier studies (Chin *et al.*, 2002; Panaro *et al.*, 2007; Koh *et al.*, 2013). In terms of the original research hypotheses that asked whether intestinal epithelial cells undergo apoptosis early in the course of the infection and whether the cellular response can be associated to the virulence of each *Giardia* isolate we are able to be more definitive. The current work clearly demonstrates that neither of the two isolates of assemblage A used caused any significant apoptosis. Indeed there was strong evidence that host cell apoptosis was being actively prevented by the upregulation of host apoptotic inhibitor genes. More research with the same cells and isolates used here need to be performed to determine the effect of longer-term co-culture of IEC-6 and trophozoites. Longer co-culture of 12-48hrs could be used in order to estimate whether apoptosis occurs over a longer term of infection. This type of experiment would require careful planning and control to ensure that any apoptosis seen was a product of the *Giardia* trophozoites and not of other factors such as worsening culture conditions or cell nutrient deprivation. Another avenue would be to use different cell types in the same conditions to understanding whether cell type influences the outcomes.

There is a definite need for *in-vivo* studies since many of the pathways up-regulated in the host cell initiate immune responses. Whilst protein analysis of *in-vitro* studies can determine whether immune modulators identified are actually expressed and released by the parasitised host they cannot determine if there is inflammation and recruitment of immune cells.

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