Investigating the effect of butyrate treatment on resistance to *Toxoplasma gondii* infection using 3D model cultures

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This study investigated whether treatment with butyrate, a breakdown product from dietary materials in the large intestine, could protect the small intestine from toxoplasmosis; a disease in humans and animals that is caused by a parasite that invades the gut. In pigs, the disease can cause inflammation of the heart and brain. Disease spreads from animals to humans after eating undercooked infected meat or consuming contaminated food and water. Infection causes inflammation of the brain in people with a weakened immune system. If infection occurs during pregnancy, the disease is passed to the baby and causes problems with the nervous system and vision. Pig intestine models were established and either treated with butyrate or left untreated. The protein quantities that were present in butyrate treated and untreated cultures were analysed. Following butyrate treatment, cultures were infected with the parasite that causes toxoplasmosis and the percentage of invaded intestinal cells was calculated. Treatment with butyrate increased the production of proteins associated with joining cells in the small intestine closer together and the immune system. On the other hand, proteins that are important in the gut barrier and immunity were decreased in response to butyrate. The results showed that butyrate treatment reduced the number of cells in the small intestine that were invaded by the parasite. In conclusion, further studies are needed to understand if farmers should add butyrate to their pig feed to prevent toxoplasmosis.

Abstract

This study investigated whether treatment with butyrate, a short-chain fatty acid that increases protein abundance within tight junctions, could increase resistance to *Toxoplasma gondii* infection by altering the porcine intestinal epithelium. Toxoplasmosis is a disease caused by *T. gondii*, an intracellular protozoan parasite, which induces severe health implications on livestock such as encephalitis and myocarditis in sows. Zoonotic transfer occurs following ingestion of undercooked infected meat or food and water contaminated with oocysts. Infection causes encephalitis in immunocompromised individuals, as well as ocular and neurological defects via congenital transmission. Here we established 3D *in vitro* cultures of porcine intestinal epithelium, named organoids, that were treated with butyrate at concentrations between 0.5-1.0 mM for 22-24 hours, or left untreated. Quantitative label-free proteomics were then used to determine any differences in the protein abundance of butyrate treated and untreated organoids. Following butyrate treatment, cultures were infected with *T. gondii* (Pru-GFP) and flow cytometry analysis was conducted to assess parasite invasion of epithelial cells. Butyrate treatment upregulated protein expression of galectin-1 and selenium-binding protein 1 which are involved in cell-cell adhesion and the immune response. Contrarily, polymeric immunoglobulin receptor, serine protease 8, nectin-2, heat shock protein family A (Hsp70) member 4 and complement component 3 were downregulated in response to butyrate treatment and are important in immune functioning and the gastrointestinal barrier. We observed decreased invasion of intestinal epithelial cells by *T. gondii* following butyrate treatment. In conclusion, further studies are needed to determine whether butyrate supplements would be beneficial in preventing porcine toxoplasmosis.

Introduction

The ability to study and thoroughly understand enteric infections of domestic livestock is becoming of increased importance as they are accountable for outbreaks of abortion (1), worldwide economic losses (2) and the zoonotic transfer of harmful pathogens, resulting in major impacts on human health (3).

Toxoplasma gondii is an intracellular protozoan parasite which infects livestock (4) (Fig. 1). Toxoplasmosis usually develops via environmental transmission, where food or water contaminated with the infective stage oocysts are ingested (5). Oocysts are resistant to disinfecting practices (6), resulting in difficulty of disease eradication.

Toxoplasmosis induces severe health implications in livestock, such as encephalitis and myocarditis in sows, alongside piglet abortion (7). *T. gondii* is a zoonotic pathogen which causes toxoplasmic encephalitis in immunocompromised individuals (8), as well as ocular and neurological defects if infection occurs during pregnancy (9). The annual foodborne illness cost of *T. gondii* in the United States is estimated around $3 billion (10), highlighting the need to target livestock to reduce zoonotic transfer and thus prevalence of human disease. There are currently limited treatments for toxoplasmosis (11), therefore improved *in vitro* models are required to better understand host-pathogen interactions and enable the development of novel therapeutics.

The intestinal epithelium uses multiple mechanisms as a defence against invading enteric pathogens. Goblet cells secrete mucus which contains sticky binding sites to capture microbes (12) and paneth cells secrete large granules containing protective molecules which control microbial populations (13). Tight junction complexes contain proteins which enclose spaces between adjacent intestinal epithelial cells to increase the integrity of the intestinal barrier (14) and prevent paracellular entry of microbes (15). Understanding how different factors affect gastrointestinal barrier function has the potential to drive the development of specific methods that could promote the defensive mechanisms of the intestinal epithelium (16). For example, probiotic bacteria induce an anti-inflammatory effect which could reduce protozoal disease (17).

Butyrate is a short-chain fatty acid by-product of microbial fermentation in the large intestine (18). It can be used as a feed supplement in poultry and livestock to control enteric pathogens (19) because it increases the abundance of the main constituents of tight junctions (20). Butyrate also upregulates the expression of antimicrobial peptides which prevents pathogen colonisation in the gastrointestinal tract (21). Currently, intestinal epithelial cell monolayers (22) and epithelial cell lines (23) are used to study host-pathogen interactions. However, these model systems lack the cellular diversity found within the intestinal epithelium and are therefore not representative. Whole intestinal crypts or intestinal stem cells can be grown in a Matrigel matrix to produce 3D cultures of the intestinal epithelium, named organoids. 3D models allow cells to grow and interact in all directions unlike 2D models where growth is limited to flat surfaces (24). These models are advantageous because they contain cell lineages that would be observed *in vivo* and host-pathogen interactions can be examined in a physiologically relevant context (Fig. 2) (25).

There is currently no research on the effects of butyrate on toxoplasmosis using porcine organoids. The aim of this study was to understand how application of butyrate alters the porcine intestinal epithelium using quantitative label-free proteomics and assess the effects of butyrate on intestinal epithelial resistance to *T. gondii* using flow cytometry to investigate if butyrate supplements have the potential to reduce prevalence of porcine toxoplasmosis (Fig. 3).

Methods

Resuscitation of porcine organoids

Cryopreserved porcine organoids were transferred from storage in liquid nitrogen to a waterbath at 37°C. The solution was pipetted into a centrifuge tube and 5 ml IntestiCult (Stem Cell Technologies) was added. The organoid suspension was centrifuged (300 x g, 10 minutes, 4°C) to form a pellet. Supernatant was removed and the organoid pellet was resuspended in a mixture containing a 70:30 ratio of Matrigel (Corning) to IntestiCult. The organoid mixture was pipetted into a 24-well plate and incubated (37°C, 5% CO_2, 20 minutes). IntestiCult was added to each well and Dulbecco's phosphate buffered saline (PBS) was added to surrounding wells to prevent evaporation.

Passage of porcine organoids

Organoids were resuspended in PBS and transferred to a centrifuge tube. The organoid suspension was centrifuged (200 x g, 5 minutes, 4°C). Supernatant was removed and the pellet was resuspended in 70:30 Matrigel/IntestiCult solution. The mixture containing passaged organoids was pipetted into a 24-well plate and incubated (37°C, 5% CO_2, 20 minutes). IntestiCult was added to each well and PBS was added to surrounding wells before incubation (37°C, 5% CO_2). Organoids were passaged every three to seven days.

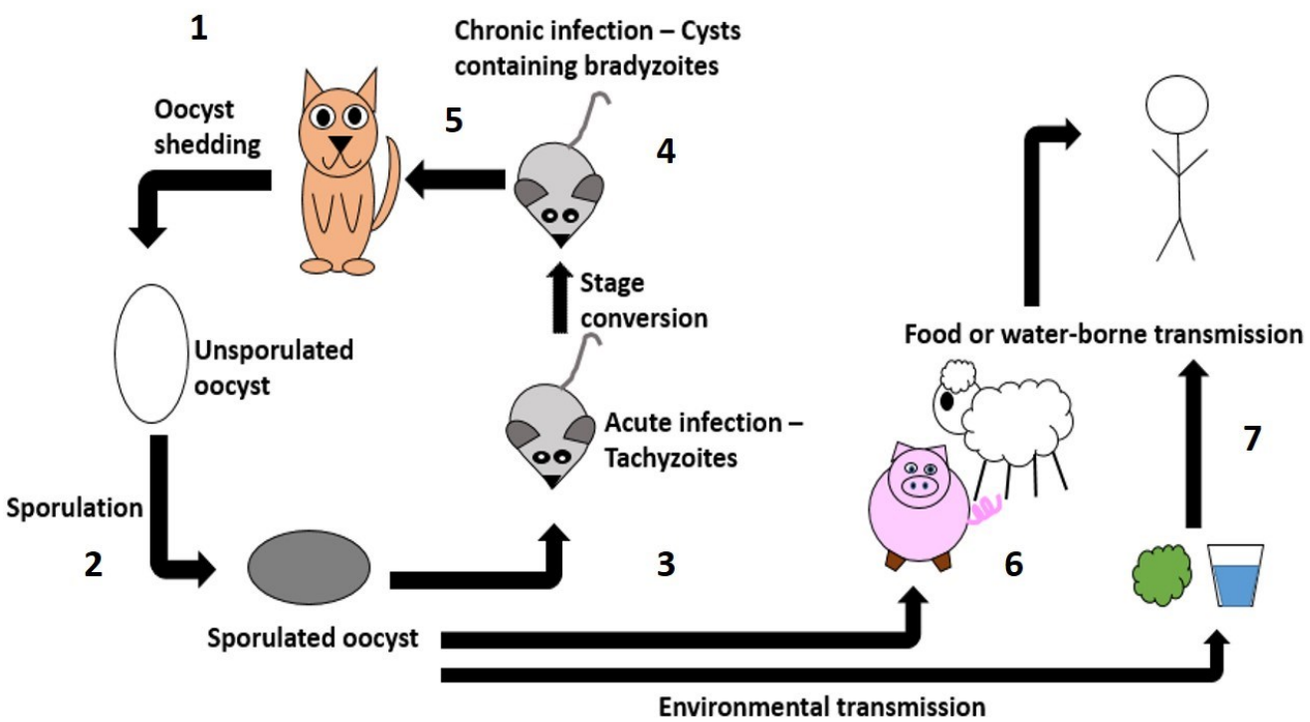

Figure 1. *Toxoplasma gondii* life cycle. (1) Sexual replication of *T. gondii* occurs inside the small intestine of members of the Felidae family which are the definitive hosts of the life cycle. Unsporulated oocysts are released into the environment in cat faeces; (2) Unsporulated oocysts undergo sporulation to become infective stage sporulated oocysts; (3) Rodents ingest soil, food or water containing sporulated oocysts and tachyzoites disseminate throughout the rodent, causing acute infection; (4) Tachyzoites differentiate into bradyzoites which form cysts within the tissues, resulting in chronic infection; (5) Sexual replication of the parasite within the cat begins again following the ingestion of rodents with encysted muscle tissue; (6) Sheep and pigs are intermediate hosts of the life cycle and become infected via environmental transmission; (7) Ingestion of fruit and vegetables or water contaminated with sporulated oocysts causes cysts to form in their tissues. Humans become infected by ingesting food or water contaminated with infective stage oocysts or by eating undercooked meat comprising tissue cysts. *T. gondii* can cause defects to the brain, eye and the heart of immunocompromised individuals and can also cause damage to a foetus via congenital transmission. Figure adapted from (43).

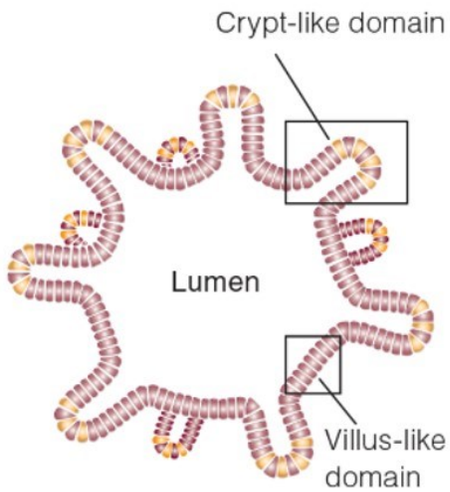

Figure 2. Organoid structure. Small intestinal organoid cultures have a central lumen and distinct crypt-villus domain that contain all the differentiated intestinal epithelial cell lineages. Figure adapted from (46).

Treating porcine organoids with butyrate

Sodium butyrate (Millipore Speciality Media) was added to IntestiCult to produce a solution with a butyrate concentration between 0.5 - 1.0 mM. Cultures were treated with butyrate or left untreated. Butyrate treatment lasted 22 - 24 hours.

Harvesting butyrate treated and untreated organoids

Butyrate treated and untreated organoids were harvested 22 hours post-treatment. Organoids were resuspended in PBS. Treated and untreated resuspended organoids were added to separate centrifuge tubes. Organoid suspensions were centrifuged three times (300 x g, 5 minutes, 4°C). After centrifugation, supernatant was removed and both tubes were placed in a -80°C freezer. This was repeated three more times to produce four replicates of butyrate treated and untreated organoids.

Harvesting *T. gondii* for porcine organoid infection

Vero cells infected with *T. gondii* (Pru-GFP) tachyzoites were scraped from flasks and centrifuged (2000 rpm, 10 minutes, room temperature [RT]). Supernatant was removed before PBS was added and the parasite mixture was centrifuged (2000 rpm, 5 minutes, RT). A blunt 5ml needle was used to release intracellular *T. gondii* and moved through a PD-10 desalting column. 10µl parasite suspension was added to a haemocytometer and the total number of *T. gondii* parasites were calculated. The remaining solution was centrifuged (2000 rpm, 10 minutes, RT), resuspended in the volume of IntestiCult required for passage and put on ice.

Infecting porcine organoids with *T.gondii*

Butyrate treated and untreated organoids were resuspended in PBS and separately transferred to two centrifuge tubes, filled with PBS and centrifuged (200 x g, 5 minutes, 4°C). Supernatant was removed and the organoid pellets were resuspended in the Toxoplasma/ IntestiCult suspension before incubation (37°C, 5% CO_2, 1hr). Following incubation, the volume of Matrigel needed for passage was added to the treated and untreated organoid/Toxoplasma suspensions and pipetted into a 24-well plate. 1 x 10^7 *T. gondii* (Pru-GFP) infected each well. The plate was incubated (37°C, 5% CO_2) for 20 minutes. IntestiCult was added to each well and PBS was added to surrounding wells. Infected and uninfected organoids were incubated (37°C, 5% CO_2) for three days.

Fixing butyrate treated and untreated organoids infected with *T. gondii* and uninfected untreated organoids

T. gondii infected organoids treated with butyrate were resuspended and transferred into two centrifuge tubes. This was repeated for the infected organoids with standard IntestiCult and the uninfected untreated organoids. PBS was added to each tube and centrifuged (100 x g, 3 minutes, 4°C). Supernatant was discarded to remove any extracellular *T. gondii*. Gentle Cell Disassociation Reagent (Stem Cell Technologies) was added to create a single cell suspension. Suspensions were centrifuged (1400 rpm, 5 minutes, 4°C) and supernatant removed. 4% paraformaldehyde was added and organoids were fixed for three hours in a refrigerator.

Proteomic analysis

Four experiments involved treating cultures with butyrate or leaving cultures untreated. These organoids were washed before RapiGest (Waters) was added. Cultures were sonicated on ice and then heated (10 minutes, 80ºC). Dithiothreitol (Sigma) was added (10 minutes, 60ºC) for protein reduction and iodoacetimde (Sigma) was added (30 minutes in the dark at RT) for protein alkylation. Trypsin (Sigma) was added at a 1:50 ratio of trypsin:peptide and incubated (37ºC, 5% CO_2) overnight. Tricholoroacetic acid was added to total 1% of the overall concentration, incubated for 2 hours (37ºC, 5% CO_2) and centrifuged (12,000 g, 4ºC, 1hr). Peptides were desalted using C18 spin tips and resuspended in acetonitrile with 5% trifluoroacetic acid.

Proteins were analysed using NanoLC-MS/MS. Samples were transferred to a trap column attached to a nano-electrospray emitter and connected to a Q-Exactive mass spectrometer. Thermo RAW files were uploaded to Progenesis LC-MS proteomics data analysis software. Peptide intensities for treated and untreated cultures were compared to determine differences in protein expression. Spectral data were exported using PEAKS Studio for protein identification. Tandem mass spectrometry data were searched against the Sus scrofa predicted proteome (Uniprot release Aug 2018).

Flow cytometry

After organoids were fixed, PBS was added to each of the six centrifuge tubes – two containing uninfected untreated organoids, two containing *T. gondii* infected organoids with butyrate treatment and two containing *T. gondii* infected untreated organoids. Each single cell organoid suspension was strained and transferred into a fluorescence-activated cell sorting tube. Flow cytometry analysis was performed using a MACSQuant Analyser to detect parasite GFP inside host epithelial cells. Gates were used to determine whether invading parasites were single or replicating. Data was analysed using FlowJo software.

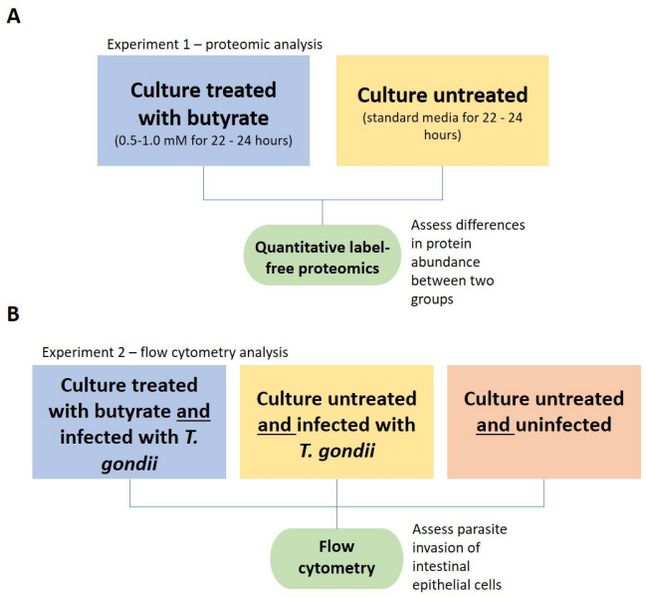

Figure 3. Experimental overview flowchart.

Results

Butyrate treatment significantly upregulates and downregulates protein expression

Exclusion criteria of *P*<0.1 and max fold change >1.5 were used to identify 7 upregulated proteins and 33 downregulated proteins following butyrate treatment. The low *P*-value and max fold change cut off points that were used reflects the small proportion of significantly up- or down-regulated proteins that were observed in the cultures.

Modulation of proteins associated with intestinal epithelial barrier function and immune function occur in butyrate treated and untreated cultures

Butyrate treatment upregulated three proteins that are of importance in this research (Table 1). Galectin-1 had the highest increased protein expression following application of butyrate and plays an important role in cell-cell adhesion and T cell homeostasis. Similarly, selenium-binding protein 1 is associated with the immune response and was upregulated in the butyrate treated cultures. 3-Hydroxy-3-Methylglutaryl-CoA Synthase 1 expression was also increased in organoids treated with butyrate and is an intermediate of cholesterol synthesis. Butyrate treatment led to the decreased expression of five proteins (polymeric immunoglobulin receptor, serine protease 8, nectin-2, heat shock protein family A (Hsp70) member 4 and complement component 3) with functions relating to gastrointestinal barrier integrity and the immune response.

Butyrate treatment induces slight intestinal epithelial resistance to *T. gondii*

This study aimed to assess if butyrate treatment had an effect on resistance to *T. gondii* infection. Organoids were treated with 1.0 mM butyrate for 24 hours or left untreated. Cultures were then infected with *T. gondii* parasites expressing green fluorescent protein and flow cytometry was used to detect invaded intestinal epithelial cells.

Flow cytometry analysis demonstrated that cultures that were treated with butyrate showed slightly higher intestinal epithelial resistance to *T. gondii* than cultures that were untreated (Fig. 4). The total percentage of cells invaded with *T. gondii* (Pru-GFP) in the control cultures on average was 3.62%, which is slightly higher than that shown in the butyrate treated cultures which on average was 2.35%. Similarly, the percentage of cells that were invaded with replicating and single parasites was generally higher in the untreated organoids compared to organoids treated with butyrate. Comparing the ratio of replicating parasites to single parasites, the butyrate treated organoids had a slightly lower ratio on average (0.70) compared to that of the untreated organoids (0.74), however the range was very large for the control cultures.

Conclusions

Butyrate is a short-chain fatty acid that can be used as a feed additive in an attempt to control enteric pathogens (19) because it increases the abundance of the main constituents of tight junctions (20) and prevents colonisation of pathogens within the gastrointestinal tract (21). However, there is currently limited in-depth research on the effect of butyrate on the porcine intestinal epithelium and whether this could be implemented as a potential treatment to reduce prevalence of porcine toxoplasmosis and therefore prevent the zoonotic transmission of *T. gondii* into human populations. In this study, 3D cultures of porcine intestinal organoids were effectively established and treated with butyrate to investigate molecular changes within the intestinal epithelium. In addition, this study has

Gene name	Protein name	Function/Importance	Highest mean treatment	Max fold change	Anova (p)	Unique peptides
LGALS1	Galectin-1	Cell-cell adhesion, T cell homeostasis	Butyrate treated	4.9	0.0005	2
SELENBP1	Selenium-binding protein 1	Immune response	Butyrate treated	1.7	0.0416	2
HMGCS1	3-Hydroxy-3-Methylglutaryl-CoA Synthase 1	Intermediate in cholesterol synthesis	Butyrate treated	1.7	0.0801	4
PIGR	Polymeric immunoglobulin receptor	Facilitates IgA transport	Control	2.3	0.0248	29
PRSS8	Protease serine S1 family member 8	Intestinal barrier function	Control	1.6	0.0519	2
PRR2	Nectin-2	Cell-cell adhesion	Control	1.5	0.0731	2
HSPA4L	Heat shock protein family A	Immune response	Control	2.0	0.0865	2
C3	Complement C3	Component of the complement system	Control	1.5	0.0884	65

Table 1. Modulation of proteins associated with intestinal epithelial barrier function and immune function occur in butyrate treated and untreated organoids. Cultures were treated with 0.5 mM Sodium butyrate for 22 hours or left untreated and used as controls. This was repeated to produce four replicates of butyrate treated and untreated organoids. Cultures were analysed using nanoflow liquid chromatography-tandem mass spectrometry and proteomic analysis was performed to show the highest mean treatment, max fold change and *P*-value of all proteins present within the cultures. Exclusion criteria of *P*<0.1 and max fold change >1.5 were used to identify significant changes in protein expression. The function of each protein was determined using the PubMed database.

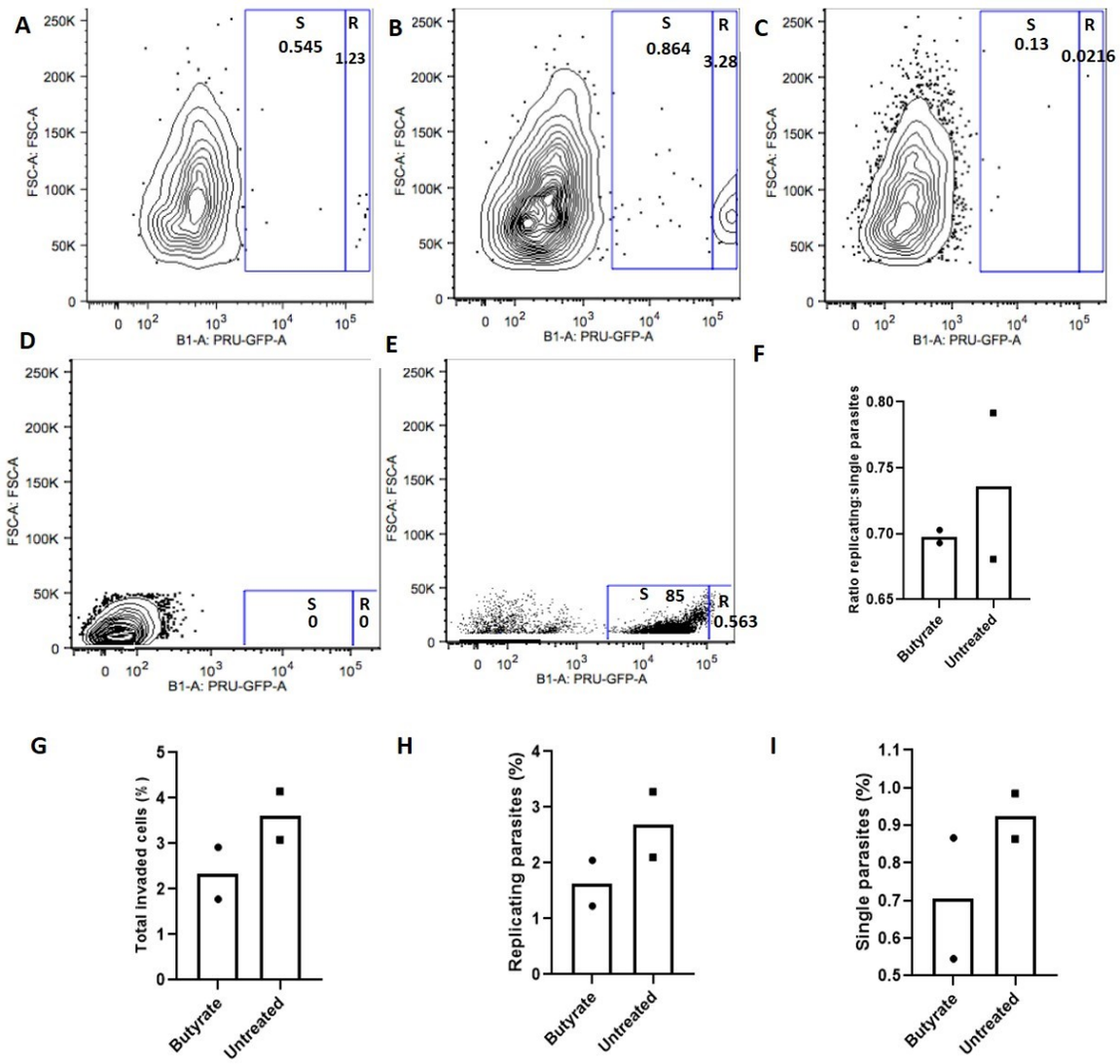

Figure 4. Butyrate treatment induces slight intestinal epithelial resistance to *T. gondii*. Cultures were treated with 1.0 mM Sodium butyrate (Millipore Speciality Media) for 24 hours or left untreated and used as controls. Butyrate treated and untreated cultures were infected with *T. gondii* (Pru-GFP) for three days. Additional cultures remained untreated and uninfected. Flow cytometry analysis was performed using FlowJo software to detect the proportion of green fluorescence parasites that had invaded intestinal epithelial cells. Gating was used to determine whether invaded *T. gondii* were single or replicating parasites. Comparison of parasite invasion was made between butyrate treated cultures infected with *T. gondii* (A), untreated cultures infected with *T. gondii* (B) and untreated uninfected cultures (C). Flow cytometry analysed the amount of extracellular *T. gondii* debris in uninfected cultures (D) and butyrate treated *T. gondii* infected cultures (E). Butyrate treated and untreated cultures infected with T. gondii were compared on the ratio of replicating parasites to single parasites (F), the total % of invaded cells (G), % of invaded cells with replicating parasites (H) and the % invaded cells with single parasites (I). S represents the percentage of invaded intestinal epithelial cells that contained single parasites and R represents the percentage of invaded intestinal epithelial cells that contained replicating parasites.

also shown that *in vitro* porcine organoids can be successfully infected with *T. gondii* (Pru-GFP) in order to determine whether application of butyrate has the ability to significantly reduce the invasion *of T. gondii* into porcine intestinal epithelial cells.

The results have demonstrated that abundance of galectin-1 is drastically increased in porcine organoid cultures following treatment with butyrate. Galectin-1 is a member of β-galactoside binding lectins which promotes cell-cell and cell-matrix adhesions (26) by directly binding to or increasing protein expression of cell-adhesion molecules (27). In addition to its importance in barrier function, galectin-1 plays a key role in regulation of enterocyte turnover within the villus of the small intestine which regulates homeostasis of the gastrointestinal tract during physiological stress (28). The upregulation of galectin-1 expression as a result of butyrate treatment is suggested to increase the integrity of the intestinal epithelial barrier and modulate gut homeostasis which could explain the slight increase of resistance to *T. gondii* following application of butyrate to organoid cultures.

Porcine organoids that were treated with butyrate also had increased expression of a protein important in the synthesis of cholesterol named 3-hydroxy-3-methyglutaryl coenzyme A synthase 1 (29). *T. gondii* exploits cholesterol from its host and transports it into its parasitophorous vacuole which allows the parasite to survive (30). Luu *et al.* (31) demonstrated that treatment of organoids with statins that blocked enzymes important in the synthesis of cholesterol could effectively inhibit

T. gondii replication, this further suggests that cholesterol is essential for parasitic infection. Therefore, butyrate treatment may be counterproductive as it encourages upregulation of the cholesterol biosynthesis pathway which may subsequently promote *T. gondii* infection as the parasite has a larger source of cholesterol to scavenge from driving its survival within the host.

Application of butyrate led to the decreased protein expression of protease serine S1 family member 8, a serine protease that inserts a protein named occludin, one of the main constituents of tight junctions, into the intestinal epithelium. Previous literature demonstrates that transepithelial electrical resistance of intestinal epithelial cell lines increases following application of serine proteases which indicates increased integrity of the gastrointestinal barrier (32). Due to the fact that this serine protease is associated with proper functioning of tight junction complexes and its expression was decreased in cultures that were treated with butyrate, it is suggested that butyrate does not have a significant effect on intestinal epithelial resistance to *T. gondii* due to the downregulation of protease serine S1 family member 8. Therefore, it is possible that treatment with butyrate may increase the ability of the parasite to transmigrate between adjacent intestinal epithelial cells which may cause injury to surrounding tissue (33).

Another suggestion as to why butyrate treatment did not significantly increase resistance of porcine organoids to *T. gondii* is that the parasite burden was too intense for application of butyrate to have a large impact. The proposed increased integrity of the gastrointestinal barrier due to the elevated abundance of galectin-1 may not have been strong enough to overcome the $1x10^7$ *T. gondii* (Pru-GFP) parasites that each well were infected with. In order to distinguish the difference between whether butyrate is ineffective at preventing infection or whether the parasite burden was too intense, future research should involve compounds from existing literature that have already been demonstrated to significantly reduce *T. gondii* invasion, such as atorvastatin (31).

Although organoid models of the intestinal epithelium are advantageous, a main weakness is the absence of an immune system. As discussed previously, this study showed that the expression of galectin-1 was upregulated in porcine organoid cultures following butyrate treatment. Alongside the increased intestinal epithelial integrity that galectin-1 induces on the gastrointestinal tract, it also plays a specific role in regulation of immune responses (34). Galectin-1 binds to complement receptor CR3 which is significant in the innate immune response (35). Zúñiga *et al.* (36) demonstrated that high concentrations of galectin-1 drives apoptosis of murine macrophages infected with *Trypanosoma cruzi*, suggesting that this protein is capable of instructing macrophages to control intracellular parasitic infections. Galectin-1 also has importance in T cell homeostasis by promoting apoptosis of neutrophils in order to inhibit the release of toxic contents which may destroy neighbouring tissue (37).

Butyrate treatment also upregulates the expression of another protein that has key roles in immune function called selenium-binding protein 1. Suitable levels of selenium are required to promote the immune system, as well as control inflammation and modulate excessive immune responses (38). Low levels of selenium can induce immunosuppression which subsequently results in increased susceptibility to disease (39). Previous literature demonstrates that organic selenium fed to pigs enhanced their immune response (40). In relation to parasitic infection, addition of selenium to rats that were subject to *T. cruzi* infection displayed reduced parasitic burden (41), suggesting that increased selenium as a result of butyrate treatment may have similar effects on *T. gondii* infection. Due to the fact that galectin-1 and selenium-binding protein 1 both drive multiple immune response mechanisms that are important in controlling parasitic infections, if butyrate was applied *in vivo* where an immune system was present, then a more significant increase in resistance to infection may have been observed.

On the other hand, the proteomic analysis from this study may suggest that the application of butyrate *in vivo* will not increase intestinal epithelial resistance to *T. gondii*, even though an immune system is present. This is because the expression of several proteins that are important in the immune response were downregulated in treated cultures. Our results showed that polymeric immunoglobulin receptor had decreased protein expression following butyrate application. Polymeric immunoglobulin receptor is an Fc receptor which interacts with IgA to reduce the immunoglobulin's susceptibility to digestion, before transporting it into the lumen of the intestine to reduce parasitic burden (42). If butyrate treatment was conducted in a model where immune cells were present, the downregulation of polymeric immunoglobulin receptor would reduce the transport of IgA into the intestinal lumen to control *T. gondii* infection.

In conclusion, this study demonstrates the potential of butyrate to protect the intestinal epithelium against *T. gondii* invasion, however further research is required to determine whether butyrate supplements should be recommended as a preventative measure for porcine toxoplasmosis. Future experiments should trial different concentrations and treatment periods of butyrate to thoroughly understand the molecular and structural changes that it induces on the porcine intestinal epithelium to determine whether treatment with butyrate has the ability to increase resistance to infection.

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