In vitro screening of non-antibiotic components to mitigate intestinal lesions caused by Brachyspira hyodysenteriae, Lawsonia intracellularis and Salmonella enterica serovar

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**Abstract**

Swine dysentery, ileitis, and porcine salmonellosis are production-limiting diseases of global importance in swine production. They are caused by infection with *Brachyspira hyodysenteriae*, *Lawsonia intracellularis*, and *Salmonella enterica* serovar Typhimurium, respectively. Currently, the prevention, treatment, and control of these diseases still rely on antimicrobials. The goal of this study was to evaluate the effectiveness of four commercially available non-antimicrobial compounds in preventing lesions caused by the bacteria cited above using an *in vitro* intestinal culture model. A total of five pigs per pathogen were used and multiple compounds were evaluated. For compounds F (a fungal fermented rye), S (a blend of short and medium chain fatty acids) and P (a synergistic blend of short and medium chain fatty acids, including coated butyrates) a total of 4 explants/pig for each treatment were used, while for compound D (an extract of carob and thyme) only 12 explants/pig for each treatment were used. Explants were exposed to a combination of pathogen only (n = 4/compound/pig), compound only (n = 4/compound/pig) or pathogen and compound (n = 4/compound/pig) and sampled at two time-points. Histopathology and gene expression levels were evaluated to investigate the treatment effect on explants. Short and medium-chain fatty acids, and an extract of carob and thyme can mitigate lesions due to *B. hyodysenteriae* exposure. A fungal fermented prebiotic increased healthy epithelial coverage when explants were exposed to *L. intracellularis* or *S. Typhimurium*. These findings are a step towards finding alternatives to antimicrobials usage and control of swine dysentery, ileitis, and salmonellosis in pork production.

**Background**

Swine dysentery (SD), ileitis, and porcine salmonellosis are intestinal diseases of grower and finisher pigs that lead to major economic losses due to poor growth performance, and increased production costs associated with treatment (1–3). SD, characterized by mucohaemorrhagic diarrhea and colitis, is caused by *Brachyspira hyodysenteriae*. Recently, *B. hampsonii* and *B. suanatina* were found to be associated with a syndrome indistinguishable from SD (4,5). Diarrhea caused by *Lawsonia intracellularis* is characterized by two clinical presentations: PIA is the classic proliferative enteropathy and characterized by mucosal thickening at the chronic stage of disease, mainly affecting post-weaned pigs (between 6 and 20 weeks of age). PHE is the acute manifestation characterized by severe intestinal haemorrhage and melena during the acute stage, most commonly observed in young adult pigs (4 to 12 months of age) (1,6). *Salmonella enterica* serovar Typhimurium leads to enterocolitis and watery diarrhea mainly in grower and finisher pigs (2). Overtime several different vaccine development approaches have been explored for SD, such as bacterins (7–9), protein digests of whole cell bacterins (10,11) and reverse vaccinology (12). However, these attempts failed to induce a robust immune response, and currently there is no efficient vaccine against SD commercially available (9). In contrast, there are commercial vaccines for ileitis and salmonellosis (13–15). Live and inactivated *L. intracellularis* vaccines have their own practical barriers for implementation (16,17). *Salmonella* spp. vaccination programs are still a challenge due to the great diversity of serovars in commercial pigs, the lack of cross-protection between serovars,
and the fact that vaccination can interfere with serological monitoring programs (14,18,19). Therefore, treatment and control of these diseases under production settings still requires antimicrobial use.

The injudicious use of antimicrobials selects for resistant bacterial strains, imposing a risk for human and animal health (16,20–22). Restrictions imposed on antimicrobial drugs available for veterinary use demands improved on-farm management measures, biosecurity practices and the development of novel non-antimicrobial alternatives to treat and prevent infectious diseases (23–26). Organic acids (OA), being short chain fatty acids (SCFA) and medium chain fatty acids (MCFA), prebiotics, phytobiotics and enzyme inhibitors are being explored commercially as alternatives to antimicrobials (27–34).

The objective of this study was to evaluate the effect of five non-antimicrobial compounds (D - phytobiotic, F - prebiotic, P - blend of SCFA and MCFA, and S - blend of OA to prevent lesions following ex vivo infection of swine colon with \textit{B. hyodysenteriae}, \textit{L. intracellularis} or \textit{S. Typhimurium}.

**Results**

A summary of significant findings is presented in Table 1.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Compound</th>
<th>Early time-point</th>
<th>Late time-point</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{B. hyodysenteriae}</td>
<td>D</td>
<td>Increased epithelial coverage.</td>
<td>IL-1(\alpha), INF-(\gamma) and TNF-(\alpha) down-regulated.</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>Decreased epithelial coverage.</td>
<td>Decreased epithelial coverage TNF-(\alpha) down-regulated.</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>Decreased mucus layer thickness.</td>
<td>Decreased mucus layer thickness. TNF-(\alpha) down-regulated.</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>Decreased mucus layer thickness.</td>
<td>INF-(\gamma) up-regulated.</td>
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<td></td>
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<td></td>
<td>iNOS up-regulated.</td>
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<tr>
<td>\textit{L. intracellularis}</td>
<td>F</td>
<td>-</td>
<td>Increased epithelium coverage.</td>
</tr>
<tr>
<td>\textit{S. Typhimurium}</td>
<td>P</td>
<td>Increased epithelial coverage.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>IL-1(\alpha) up-regulated.</td>
<td></td>
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**Brachyspira hyodysenteriae**
**Early time-point**

Explants treated with Compound D (TG) trended towards higher epithelial coverage, when compared to PCG ($P = 0.06$). For compound F, explants from the PCG showed significant higher level of epithelial coverage when compared to either the CCG or treatment group (TG) (Fig. 1A). Compound S and treated explants (TG) showed a trend to reduced mucus layer thickness ($P = 0.06$, Fig. 1A) when compared to the PCG. Compound P significantly reduced mucus layer thickness ($P < 0.05$) compared to PCG and CCG. Treatment with compound F significantly down-regulated TNF-α mRNA expression, when compared to PCG (Fig. 1C). Up-regulation of iNOS mRNA expression was observed for compound S TG when compared to explants from the PCG (Fig. 1C). No difference was observed in mRNA expression for all genes investigated for compounds P and D.

**Late time-point**

Surprisingly, increased epithelial coverage was found in explants from the PCG, when compared to compound F TG samples (Fig. 1B). Compound P treated explants had significant higher epithelial coverage than the PCG and TG (Fig. 1B). Mucus layer thickness was significantly increased in the PCG when compared to TG for compound P (Fig. 1B). Explants treated with compound D had significantly lower levels of TNF-α, IL-1α, and INF-γ mRNA detected when compared to samples from the PCG. For compound P, TNF-α was found down regulated in TG samples, when compared to PCG (Fig. 1C) and INF-γ mRNA level trended towards downregulation ($P = 0.08$, Fig. 1C). Treatment of explants with compound S led to the up-regulation trend of IL-1α ($P = 0.06$, Fig. 1C) and significant up regulation of INF-γ (Fig. 1C), in relation to the PCG.

**Lawsonia intracellularis**

**Early time-point**

Healthy epithelium coverage was significant higher in compound F CCG than PCG (Fig. 2A). No differences in mRNA level were observed for any compound (Fig. 2B).

**Late time-point**

Epithelium coverage for compound F was higher in the TG than PCG samples (Fig. 2A). No other differences were observed for any gene-compound combination (Fig. 2B).

**Salmonella enterica serovar Typhimurium**

**Early time-point**

Explants treated with compounds F had a significant higher epithelium coverage in CCG compared to PCG. Compound P CCG maintained significant higher epithelial coverage than the PCG and TG samples.
For compound S, IL-1α mRNA level was higher in TG than PCG (Fig. 3B). Compound P treated explants had a trend to decrease levels of IL-1α mRNA (P = 0.07, Fig. 3B), when compared to the PCG.

**Late time-point**

No significant differences were seen for compound F in the late time-point. Compound P TG and PCG had a significant lower epithelial coverage compared to the CCG samples (P < 0.05; Fig. 3A). Explants in compound S TG had higher percentage of healthy epithelium coverage trend than the PCG (P = 0.07, Fig. 3A). No gene expression differences were observed (Fig. 3B).

**Discussion**

There is an increasing need for alternatives strategies to treat livestock bacterial diseases without the use of antimicrobials. In this study, we used *in vitro* porcine colon culture to evaluate the efficacy of non-antimicrobial compounds in preventing tissue damage following exposure to *B. hyodysenteriae*, *L. intracellularis* or *S. Typhimurium*. Compound P treatment, a blend of MCFA and SCFA, improved explant epithelial coverage, decreased the accumulation of mucus, and the expression of TNF-α mRNA following challenge with *B. hyodysenteriae* (Fig. 1A). A trend towards downregulation of IFN-γ mRNA expression following challenge was also observed (Fig. 1C). Compound S, a blend of OA decreased the accumulation of mucus, and reduced the expression of TNF-α and iNOS mRNA following challenge with *B. hyodysenteriae*. TNF-α and IFN-γ have a recognized role in tight junction regulation (35,36). Tight junction proteins, such as occludins, claudins and zonulae occludentes (ZO), are crucial for the maintenance of epithelial barrier integrity and to regulate the paracellular movement of ions and water (37,38). Fatty acids appear to modulate tight junction permeability and have an anti-inflammatory effect in the colon (39–41). Increased TNF-α and IFN-γ levels lead to the rearrangement of myosin molecules associated with tight-junction proteins, consequently increasing paracellular permeability (42–44). In our study, TNF-α and IFN-γ mRNA expression was down regulated when explants were treated with a blend of MCFA and SCFA, including butyrates (compound P), while an upregulation was observed in explants being treated with compound S, that does not contain butyrates. Similar responses were identified in weaned pigs supplemented with butyrate, and when culturing human colonic biopsies, human colonic cell lines and isolated lamina propria cells with butyrate (45–48). Intestinal epithelial cells exposed to TNF-α and IFN-γ have reduced cystic fibrosis transmembrane conductance regulator (CFTR) expression and chloride (Cl\(^-\)) secretion (49–51). This impairment of anion secretion affects the mucus layer integrity. Mucins require the interaction of bicarbonate (HCO\(_3^-\)) and Cl\(^-\) with calcium (Ca\(^{2+}\)) for proper release and expansion from goblet cells (52,53). A recent study indicated that host cytokines are not responsible for the impairment of anion channels, and that *B. hyodysenteriae* may directly cause the decrease in Cl\(^-\) secretion and which may lead to mucin aggregation and accumulation (54). In contrast, our findings suggest a relationship between reduced gene expression of TNF-α and IFN-γ and a reduction in mucus secretion following infection with *B. hyodysenteriae* and treatment with compound P. This link between host cytokines and
mucus secretory response in SD remains to be clarified. In addition, it is important to highlight that the fold changes observed in this study were quantitatively small, when compared to previously published data. This could be an effect of the model used and the biological significance remains to be explored.

Explants treated with compound F (prebiotic based on *Agaricus subrufescens* fermented rye) had higher epithelial coverage when challenged with *L. intracellularis* (Fig. 2A) than those untreated. Ribogluccans, β-glucans and glucomannans are examples of bioactive polysaccharides isolated from *A. subrufescens* (55). These molecules can act as a substrate for bacterial adherence, as they mimic the host glycocalyx (56). D-mannose, a prebiotic, reduced the adhesion of *Escherichia coli*, *Vibrio cholerae*, *Campylobacter jejuni*, and *S. Typhimurium* to HT-29 cells as per the concept described above (57). This effect was also observed in animal studies, when weaned piglets feed was supplemented with *Lentinus edodes* mycelium extracts, leading to reduced viable counts of *E. coli* and Streptococci in the digesta (stomach, jejunum) and mucosal scrapings of the small intestine (58). In vitro studies with ingredients in compound F have also proven binding affinity to *S. Typhimurium* and *S. Enteritidis*, and in vivo reducing peak and average shedding of these bacteria (59,60). However, our data revealed no significant effect of compound F in epithelial coverage or cytokine expression following *S. Typhimurium* challenge. To the best of our knowledge, this is the first report which evaluate the effectiveness of *A. subrufescens* rye fermentation against *L. intracellularis*.

A recent study (70) showed the potential immunomodulatory effect of compound F when supplementing piglets post-weaning, with a reduction of pro-inflammatory cytokine production in jejunum, ileum and colon. In our study, no significant differences in cytokine mRNA levels were observed after *L. intracellularis* challenge (Fig. 2B). This observation may be due to the short period of in vitro incubation which may lead to a low level of bacteria infecting and propagating inside the epithelial cells. Previous authors reported that the pathogen may take up to 12 hours to invade cells after oral inoculation, or 6 hours when ligated intestinal loops were infected directly with vaccine inoculum (61,62). The ability of the attenuated vaccine strain to induce such changes is also questionable, but it has been shown to do so in vivo (63). However, the inoculum concentration used in current study for *L. intracellularis* challenge would not be considered to cause clinical disease and lesions in natural infections, and therefore can explain the lack of effect between the PCG and the CCG or for almost all TGs challenged with *L. intracellularis*. Thus, further studies investigating the immunomodulatory role of compound F following infection with a virulent *L. intracellularis* using longer incubation periods are strongly suggested.

Surprisingly, a lower degree of epithelial coverage was observed in explants exposed to compound F alone than explants exposed to *B. hyodysenteriae* (Fig. 1A and B). It is known that colon explants harbor a microbiota compositionally similar to the donor pig prior to euthanasia (64). Thus, we postulate that compound F may have served as a substrate for the microbiota already present in the explants, leading to bacterial overgrowth. The lack of colonic peristalsis, may have further contributed to our observations.

Explants infected with *B. hyodysenteriae* and treated with compound D (phytobiotic) had increased epithelial coverage and decreased levels of IL-1α, TNF-α and IFN-γ, when compared to infected, untreated
explants (Fig. 1A and C). Thymol and carvacrol are present in the essential oils extracted from thyme (*Thymus vulgaris*), the active ingredients on compound D (65). Carvacrol was demonstrated to have a gastroprotective effect in a rodent model of gastritis (66, 67). It was associated with reduced colonic lesions in colitis induced by 2,4,6-trinitrobenzenesulfonic (TNBS) in rats (68) and in acetic acid-induced colitis in mice (69). The protective effect of carvacrol was associated with its ability to regulate cyclooxygenase-2 (COX-2) expression (70, 71). An *in vitro* T cell model also linked the reduction of IL-2 and IFN-γ expression to exposure to thymol and carvacrol (72). In contrast, IL-1β and TNF-α induce the expression of COX-2 (73). Mice treated with carvacrol had decreased TNF-α levels and milder lesions following acetic acid-induced colitis (69). Additionally to the effects of thyme, carob (*Ceratonia siliqua*, another ingredient in compound D) contains phenolic compounds such as flavonoids and gallotannins that also inhibit COX-2 (74). Thus, the effect of compound D was likely due to its anti-inflammatory effects associated with the inhibition COX-2 cascade.

In our study, no significant differences in cytokine mRNA levels were observed after *L. intracellularis* challenge. This observation may be due to the short period of *in vitro* incubation which may have led to a low level of bacteria infecting and propagating inside of the epithelial cells. Previous authors reported that the pathogen may take up to 12 hours to invade cells after oral inoculation, or 6 hours when ligated intestinal loops were infected directly with vaccine inoculum (71). The ability of the vaccine strain, at the same dose used in our study, to induce such changes is also questionable, but it has been shown to do so *in vivo* (73). Thus, further studies investigating the immunomodulatory role of compound F following infection with a virulent *L. intracellularis* during longer incubation periods are strongly suggested.

In conclusion, our findings suggest that the non-antimicrobial compounds studied may have a beneficial effect to the host based on the explant model data shown. Compound P supported epithelial survival and reduces mucus thickness when explants were exposed to *B. hyodysenteriae*. Compound D has an immune-modulating effect in explants challenged with *B. hyodysenteriae*. Compound F prevented epithelial death following *L. intracellularis* exposure. The authors warrant that further investigations are needed to verify compound effectiveness *in vivo*.

**Methods**

**Spiral colon collection and explant culture**

A total of 20 healthy, commercial crossbred male pigs from high health herds, with 6 weeks of age were used as tissue donors. Out of 20 animals, 5 were used for *B. hyodysenteriae*, 5 pigs for *L. intracellularis*, 5 pigs for *S. Typhimurium*. Additionally, 5 pigs were used for *B. hyodysenteriae* to screen for compound D only. Following euthanasia, distal spiral colon collection and culture followed the protocol previously described (75). For each pig, after gastrointestinal post-mortem examination, a lesion-free 10 cm segment of the spiral colon was aseptically collected and transported to a biosafety cabinet in a container with precooled (6°C – 10°C) Hank's balanced salt solution (HBSS, VWR, Sanborn, New York) within 10 minutes. Colon segments were washed with approximately 200 mL of the transport solution to remove
luminal contents. Next, separation of the colonic serosa from the mucosa was performed on a refrigerated surface. The mucosa containing the submucosa and the muscularis mucosa was preserved and it was further divided into multiple 2 cm x 2 cm segments (explants). Each explant was individually placed with the mucosa facing up on a 70 µm cell strainer (Fisher Scientific, Hanover Park, IL, USA) in a six-well plate (Millipore Sigma, St. Louis, MO, USA) containing 3 mL of culture media (KBM-Gold calcium and phenol-red free Bullet Kit, Lonza, Walkersville, MD) per well. The media volume dispensed could touch the bottom aspect of the cells strainer but not invade the inner aspect of the mucosa, therefore creating an air-liquid interface. Plates containing explants were incubated in a modular chamber (Billups Rothenberg INC, MIC101, San Diego, CA, USA) gassed for 2 minutes with 99% oxygen ($O_2$), 1% carbon dioxide ($CO_2$) gas mix. Finally, the chamber was incubated at 37°C.

**Inocula preparation**

The work described below was performed at the University of Saskatchewan. The study is reported in accordance with ARRIVE guidelines for in vitro studies. Glass vials (9 mL) with luria broth (LB) were used for culturing *S. Typhimurium* strain SL1344 at 37°C. *B. hyodysenteriae* isolated from a SD case was cultured in glass vials (9 mL) with JBS broth (brain heart infusion broth supplemented with 1% (w/v) glucose, 5% (v/v) deactivated fetal bovine serum, and 5% (v/v) defibrinated sheep blood) anaerobically incubated using a commercial gas pack system (Oxoid AnaeroGen, Thermo Scientific, Hanover Park, IL, USA) at 39°C with constant stirring. For *L. intracellularis*, a live vaccine strain capable of invading epithelial cells and inducing an immune response was used as inoculum (Enterisol Ileitis, Boehringer Ingelheim Vetmedica, Inc, St. Joseph, MO) (63). Immediately before inoculating explants, aliquots from each inoculum were collected for quantification kept frozen at −80°C until processing. Inocula averaged $3.2 \times 10^8$ CFU/mL for *S. Typhimurium*, $7.9 \times 10^7$ genome copies/mL for *B. hyodysenteriae*, and $1 \times 10^4$ cells/mL for *L. intracellularis*. *B. hyodysenteriae* and *S. Typhimurium* inocula were quantified using previously described methods (4, 76). *L. intracellularis* dose was provided by the vendor. Prior to inoculation, *B. hyodysenteriae* motility was checked as an indicator of bacteria viability using phase contrast microscopy.

For each pathogen, 1 mL of inoculum was centrifuged at 10,000 g for 5 minutes. Next, the supernatant was discarded, and the pellet was resuspended in 0.1 M, pH 7.0, sterile phosphate buffered saline (PBS). Explants in the PCG received 100 µl of inoculum of a given pathogen and explants in the CCG received 100 µl of compound only. The TG (explant co-exposure to a given pathogen-compound combination), received 50 µl of 2X bacterial inocula and 50 µl of 2X compound dilution. After the inoculum and the compound were prepared, both were mixed and then exposure to the explants.

Compounds were diluted following guidelines for *in vivo* use (3kg/1000kg of feed for compound F, P and S − 0.0042mg/g of explant); and 1 kg/1000kg of feed for compound D − 0.0028mg/g of explant). Dilutions were calculated based on explant weight to mimic the guidelines for use *in vivo* and confirmed to be innocuous to the mucosa by histopathology in preliminary experiments (data not shown).
Challenge trials

For *S. Typhimurium* and *L. intracellularis*, explants from five different tissue donors were evaluated and compounds F, S and P were tested. Due to logistical reasons, 10 different pigs were used to challenge explants with *B. hyodysenteriae*. 5 were used for compounds F, S and P, and 5 additional pigs were used for compound D. For compounds F, S and P a total of 4 explants/pig for each combination group were used (Supplementary Material, Table 1). Only for compound D a total of 12 explants/pig for each combination group were used (Supplementary material, Table 2). For all the compounds explants were randomly exposed to one of the following combination groups: 1) PCG; 2) CCG and 3) TG. To confine the inoculum within the luminal aspect of the explants, a polystyrene ring (1 cm diameter × 1 cm height) was attached to the mucosal side of each explant using a surgical-grade cyanoacrylate adhesive (3M Vetbond Tissue Adhesive, St. Paul, USA). Due to differences in pathogen ecology, explants were co-incubated with each pathogen for the following periods: *B. hyodysenteriae* and *L. intracellularis* explants for 2 hours and 8 hours, while *S. Typhimurium* explants were incubated for 45 minutes and 2 hours. Immediately after explant harvest at each time point, explants were fixed in 10% buffered formalin until processing for histopathology. The remaining explants per pathogen-compound combination were immersed in RNA-later (Qiagen, Germantown, MD, USA) at 4°C for 24 hours, then stored at -80°C until PCR analysis. To confirm the absence of ante-mortem lesions, explants were preserved immediately following preparation for culture (10 minutes after colon collection), as described above, for histopathology and RT-PCR analyses.

Histopathology analysis

Explants fixed in formalin were sectioned and stained using hematoxylin and eosin (H&E). An evaluator (MM) blinded to slide identification assessed the percentage of healthy epithelium and the mucus layer thickness (for *B. hyodysenteriae*-challenged explants only) covering explants. A digital image of each explant, covering its entire length, was analyzed using an image processing software (Image Pro, version 9.2, Media Cybernetics, Inc, Rockville, MD, USA). Healthy epithelium was defined as the superficial layer of cells covering the luminal aspect of the explants in a simple columnar fashion, without signs of metaplasia (abnormal cell shape), edema (increased intercellular space), or apoptosis and necrosis (picnotic or misshaped nuclei). One measurement of healthy epithelium covering the total length of each explant was obtained and data was reported as a percentage. Mucus layer thickness was measured at five evenly spaced locations along the length of the explant (far left, left, center, right, far right) and an average mucus layer thickness was reported for each explant.

Reverse transcriptase Real-Time PCR (RT-PCR) assays

Analyses of explant mRNA levels targeted the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping reference gene, TNF-α genes, IFN-γ (76) and IL-1α (77). iNOS was evaluated for *B. hyodysenteriae* (78) samples only. Total RNA load was extracted from explants preserved in RNA-later using a commercial kit (RNeasy Plus animal cell and tissue kit, Qiagen, Austin, Texas, USA). Complementary DNA (cDNA) was generated following a commercial kit instruction (QuantiTect Reverse
Transcription Kit, Qiagen, Germantown, MD, USA). cDNA samples were diluted with nuclease-free water to a final concentration of 500 ng/mL.

RT-PCR reactions were performed using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems®, ThermoFisher Scientific, Hanover Park, IL, USA). Each 20 µl reaction contained 10 µl of PowerUp SYBR Master Mix®, 1 µl of forward and 1 µl of reverse primers (10 µM each), 6 µl of nuclease-free water and 2 µl of cDNA template. Reactions were incubated at 50°C for 2 min and 95°C for 2 min, followed by 40 cycles of 15 s at 95°C, 30 s at 60°C and 1 min at 72°C and a melt curve step of 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. Every reaction was performed in duplicate, alongside negative extractions, and no-template controls in each run. Samples where duplicates differed by more than 1 Ct were re-analyzed. GAPDH expression levels was constant across all samples.

For evaluation of the effectivity of the challenge method and the screening methods used in this study, comparisons between explants from the pathogen control group (PCG) and combined compound control groups (CCG) (Compound D, F, P and S combined) for a given pathogen were performed regarding histopathology and RT-PCR assay analysis. Results are shown in the appendix, Supplementary Figs. 1 and 2.

**Statistical analysis**

Mucus layer thickness and percentage of healthy epithelium data were compared between challenge groups by generalized estimating equations (GEE) using an unstructured correlated working matrix while clustering by pig. The data followed a normal distribution. Statistical analysis was performed using IBM SPSS 21 (IBM Corporation, Armonk, NY, USA). Messenger RNA levels (Ct) were analyzed using the MCMC qPCR package (one-way design) with a naive statistical model (79) on R studio (version 1.1.463) (80).

**Abbreviations**

Ca$^{2+}$
Calcium

CCG
Compound control group

cDNA
Complementary DNA

CFTR
Cystic fibrosis transmembrane conductance regulator

cGMP
Cyclic guanosine monophosphate

Cl$^-$
Chloride

CO$_2$
Carbon dioxide
COX-2
Cyclooxygenase-2
GAPDH
Glyceraldehyde-3-phosphate dehydrogenase
GEE
Generalized estimating equations
H&E
Hematoxylin and eosin
HBSS
Hank's balanced salt solution
HCO³⁻
Bicarbonate
IACUC
Institutional Animal Care and Use Committee
IFN-γ
Interferon-γ
IL-1α
Interleukin-1α
LB
Luria broth
LPS
Lipopolysaccharides
MCFA
Medium chain fatty acids
O₂
Oxygen
OA
Organic acids
PBS
Phosphate buffered saline
PCG
Pathogen control group
PE
proliferative enteropathy
PIA
Porcine intestinal adenomatosis
SCFA
Short chain fatty acids
SD
Swine dysentery
TG
Treatment group

TNBS
2,4,6-trinitrobenzenesulfonic

TNF-α
Tumor necrosis factor-α

ZO
Zonula occludens

Declarations

Ethics approval and consent to participate

The experiment was conducted following approval by the Institutional Animal Care and Use Committee (IACUC) from the University of Minnesota (Protocol # 1906-37179) and was in accordance with the Canadian Council for Animal Care, being approved by the University of Saskatchewan Committee on Animal Care and Supply (Protocol # 20180051). The study is reported in accordance with ARRIVE guidelines for in vitro studies.

Consent for publication

Not Applicable (NA)

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceived and designed the experiments: Matheus de O. Costa, Nienke de Groot. Performed the experiments: Mariana Meneguzzi and Barbara de Souza. Analyzed the data: Mariana Meneguzzi and Matheus Costa. First manuscript draft: Mariana Meneguzzi and Matheus Costa. Reviewed the manuscript: Nienke de Groot, Matheus de O. Costa. Funding: Nienke de Groot and Matheus de O. Costa.

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**Figures**
Figure 1

Microscopic changes and gene expression data from explants collected from 10 pigs and challenged with *B. hyodysenteriae*. Histopathology assessment including the percentage of healthy epithelium covering explants and mucus layer thickness at early (A) and late (B) time-points. Horizontal lines represent group mean, and whiskers depict ± standard deviation from the mean. C) Gene expression data is reported as fold change from TG samples using the PCG as reference. Compound control group (CCG), Pathogen control group (PCG), Treatment group (TG). Bars represent mean mRNA levels; whiskers depict standard deviation from the mean. Star denotes significant difference ($P \leq 0.05$) and two stars denote $P = 0.06$. 
Microscopical changes and gene expression data from explants challenged with *L. intracellularis*. A & C) Histopathology assessment. Horizontal lines represent group mean, and whiskers depict standard deviation from the mean. B & D) Gene expression data is reported as fold change from TG samples using the PCG as reference. Compound control group (CCG), Pathogen control group (PCG), Treatment group (TG). Bars represent mean mRNA levels; whiskers depict standard deviation from the mean. Star denotes significant difference (*P* ≤ 0.05).
Figure 3

Microscopical changes and gene expression data from explants challenged with S. Typhimurium. A & C) Histopathology assessment. Horizontal lines represent group mean, and whiskers depict standard deviation from the mean. B & D) Gene expression data is reported as fold change from TG samples using the PCG as reference. Compound control group (CCG), Pathogen control group (PCG), Treatment group (TG). Bars represent mean mRNA levels; whiskers depict standard deviation from the mean. Star denotes significant difference ($P \leq 0.05$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- 2022deGrootetal.Appendix.docx
- 2022deGrootetal.SupplementarymaterialNdG.docx