INVESTIGATION OF THE EFFECTIVENESS OF NON-ANTIMICROBIAL COMPOUNDS AGAINST BRACHYSPIRA HYODYSENTERIAE, LAWSONIA INTRACELLULARIS, AND SALMONELLA ENTERICA SEROVAR TYPHIMURIUM

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Dedication

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General introduction

Brachyspira hyodysenteriae, *Lawsonia intracellularis,* and *Salmonella enterica* serovar Typhimurium are the causative agent of swine dysentery (SD), proliferative enteropathy (PE), and salmonellosis in pigs, respectively. SD is characterized by mucohemorrhagic diarrhea in grower and finisher pigs (Alvarez-Ordóñez et al., 2013). There is no efficient vaccine available for SD, and antibiotics are the only approach to prevent, control, and treat it in swine herds. In 2013, it was estimated that SD adds US$11.70-$17.50 for each diseased pig that reaches market weight (McKean and Burrough, 2013). In 2015, the World Organization for Animal Health (OIE) identified *B. hyodysenteriae* and *L. intracellularis* as agents for which high or moderately high amounts of antibiotic was still used for treatment, and could benefit from novel preventative approaches (World Organization for Animal Health, 2015). Disease caused by *L. intracellularis* in pigs is characterized as two syndromes: porcine intestinal adenomatosis (PIA) and proliferative hemorrhagic enteropathy (PHE) (Vannucci and Gebhart, 2014). PIA is observed after weaning and clinical manifestations are anorexia, diarrhea, and poor growth performance (Roberts et al., 1980). PHE mainly affects finisher pigs, gilts, and boars, resulting in mucosal thickening in the ileum, the classic hose-pipe gut lesion (Vannucci and Gebhart, 2014). In 1996, an Australian study suggested that PE costs in additional AUD$ 0.80-$7.57 per affected pig (Holyoake et al., 1996). Salmonellosis is an One Health issue. In the United States of America (USA), *Salmonella* infection was the second most common cause of foodborne illness in 2018 (CDC, 2018). In pigs, *S.* Typhimurium infection is associated with watery diarrhea and enterocolitis during the grower-finisher phases (Patterson et al., 2016). Each pig diseased with *Salmonella* was

estimated to cost an additional €1.55 to producers, due to the impact in daily weight gain and costs with antimicrobial therapy (Gavin et al., 2018).

The three diarrheal diseases described above significantly impact the pork industry due to sub-optimal performance of pigs while affecting animal welfare. Treatment, control, and eradication of these infections still rely on the use of antimicrobial agents (Alsop, 2005; Hampson et al., 2019; Wattanaphansak et al., 2019). However, the misuse of antimicrobial drugs raised concerns associated with the identification of multi-drug resistant bacterial strains. In 2020, the World Health Organization (WHO) considered antimicrobial resistance (AMR) one of the top 10 global public health threats facing humanity (World Health Organization, 2020). AMR affects our ability to control and treat bacterial infections in humans and animals. According to the 2019 Antibiotic Resistance Threats Report from the USA CDC, more than 2.8 million antibiotic-resistant infections occur in the USA yearly, and more than 35,000 people die as a result of these infections (CDC, 2019). Furthermore, AMR infections impacts the economy through death, disability, and cost of hospital stays and medicine (World Health Organization, 2020). As a measure to minimize this problem, the use of antimicrobials is now strictly regulated in several countries. In the European Union (EU), for example, the use of antibiotics as a growth promoter was prohibited in January 2006 (Castanon, 2007). Brazil has restricted the use of colistin, tylosin, lincomycin, and tiamulin (Diário Oficial da União, 2016, 2020; Skov and Monnet, 2016). Additionally to AMR concerns, the use of antimicrobials in animal production may lead to residues in meat products. Carbadox, for example, a drug used to treat enteric infection in pigs, results in carcinogenic residues in pork. Therefore, its use was banned from use in food animals by the EU and

Canada (Mathew et al., 2007), whereas the USA is studying proposed proposal to remove it from the market (U.S. Food & Drug Administration, 2016; U.S. Food and Drug Administration, 2020). In light of the decrease in bacterial susceptibility to antimicrobials and the increasingly stringent regulations, the livestock industry is actively seeking for novel alternatives to aid in control and to treat intestinal infections. Prebiotics, organic acids, and phytogenics are some optional tools that have been applied in swine production up to now (Thacker, 2013; Lillehoj et al., 2018). Enzyme inhibitors, although still not used in swine medicine, are potential tools yet to be investigated. Overall, this thesis work aimed to investigate the effectiveness of novel compounds to mitigate the diseases caused by *B. hyodysenteriae*, *L. intracellularis,* and *S.* Typhimurium *in vitro*.

CHAPTER 1: Literature review

1.1 *Brachyspira hyodysenteriae* infection in swine

1.1.1 Etiology and epidemiology

Brachyspira hyodysenteriae is a motile, Gram-negative, aerotolerant spirochete. It is the causative agent of SD, characterized by fibrinonecrotic typhlocolitis and mucoid and blood-stained diarrhea (Dong Kyun Suh, 2005). Although it was successfully reproduced experimentally in the 1920s, it was not until fifty years later that *B. hyodysenteriae* was identified as the etiologic agent of SD (Whiting et al., 1921; Harris et al., 1972). Interestingly, *Brachyspira hampsonii* and *Brachyspira suanatina* were also recently associated with a syndrome indistinguishable from SD (Råsbäck et al., 2007; Chander et al., 2012; Rubin et al., 2013; Mirajkar et al., 2016). SD is present in all major pork-producing countries, leading to significant economic losses due to high morbidity, poor growth rates and increased expenses associated with treatments (Alvarez-Ordóñez et al., 2013). *B. hyodysenteriae* is mainly transmitted by the ingestion of feces containing the bacteria, and outbreaks of SD may occur through the introduction of carrier animals to naive herds (Burrough, 2017). Noteworthy, the use of molecular diagnostic techniques associated with anaerobic culture has led to the identification of *B. hyodysenteriae* in samples of apparently healthy pigs (Hampson et al., 2016; La et al., 2016). Outbreaks can also occur when vectors, such as rodents, birds, humans or other fomites carry the agent to a susceptible population (Hampson, D. et al., 1991; Jansson et al., 2004; Backhans et al., 2010). It has been described that the pathogen persists in the environment for long periods at low temperatures, especially in the presence of organic matter (Boye et al., 2001).

1.1.2 Pathogenesis

Although it has been decades since the association of *B. hyodysenteriae* with SD, the pathogenesis of the disease is still not totally understood. Clinical SD develops influenced by several factors, such as virulence of individual strains, host microbiota, diet, and other factors beyond our current understanding (Olson, 1974; Whipp et al., 1979; John et al., 1996). *B. hyodysenteriae* colonizes the large intestine of pigs and is usually detected on the luminal surface and within crypts (Burrough, 2017). Both chemotaxis and motility are known to be important factors that allow *B. hyodysenteriae* to penetrate and move through the mucus layer into the crypts (Bellgard et al., 2009). In a murine infection model, inoculation with a mutant *B. hyodysenteriae* strain lacking flagella genes (*flaA* and *flaB*) resulted in reduced pathogen virulence and colon colonization (Rosey et al., 1996; Kennedy et al., 2006). *B. hyodysenteriae* employs NADH oxidase (*nox*) to reduce oxygen allowing its survival close to the intestinal epithelium where oxygen levels are higher than in the lumen (Stanton and Sellwood, 1999). *B. hyodysenteriae* mutants lacking the *nox* gene are less virulent to pigs (Stanton et al., 1999). After penetrating the mucus layer, *B. hyodysenteriae* causes a massive mucus induction and disorganization of the mucus layer molecular structure. There are an increased *de novo* production of MUC5AC and increased secretion levels of MUC2, both are glycoproteins secreted gel-forming mucins produced by goblet cells and epithelial cells (Wilberts et al., 2014; Quintana-Hayashi et al., 2015). Besides mucus, vivid blood is also found in the feces of affected pigs. The mechanism leading to bloody diarrhea is still not fully understood. Using ligated colon loops from germ-free pigs and rats, previous findings suggested that the release of hemolysins by *B. hyodysenteriae* may contribute to

its cytotoxic effect to enterocytes (Lysons et al., 1991). A recent work hypothesized that nitric oxide (NO) may play a role in tissue inflammation and the release of red blood cells to the lumen (Welle et al., 2017). NO leads to endothelial hyperpermeability, vasodilation, and chloride secretion associated with increased cytoplasmatic cyclic guanosine monophosphate (cGMP) levels (Brown et al., 1992; Tamai and Gaginella, 1993; Fan et al., 1996, 1998; Mourad et al., 1999). However, SD has been previously characterized as malabsorptive diarrhea, with abolished Na^+, Cl^- absorptive capacity in the colon without an effect on secretion (Argenzio et al., 1980; Schmall, L., Argenzio, R., Whipp, 1983). Indeed, a recent work by Enns et al (2020) confirmed these findings, adding further evidence through the observation of the downregulation of Na^+/H^+ exchanger 3 (NHE3) gene expression, an important Na^+ channel in the colon.

1.1.3 Diagnosis

A definitive diagnosis of *B. hyodysenteriae* infection is based on the observation of clinical signs in association with gross lesions, and bacterial culture or polymerase chain reaction (PCR) detection of the pathogen (Burrough, 2017). Clinically affected animals initially have soft feces that may progress to watery or not, but will eventually develop mucoid, bloody diarrhea. SD lesions are limited to the cecum and colon, macroscopic lesions include hyperemia and edema of the colonic wall, mesentery and, mesenteric lymph nodes. Typically, the large intestine mucosa is covered by mucus, fibrin, and blood flecks (Burrough, 2017). Pathogen growth in culture takes 2-8 days in selective media agar at 42°C under anaerobic conditions (Songer et al., 1976; Kunkle and Kinyon, 1988). The production of strong beta-hemolysis zones allows growth verification on solid

culture media (Calderaro et al., 2005). Although it was reported that culture is highly sensitive, *B. hyodysenteriae* is fastidious leading to long turnaround times (Burrough, 2017). Also, if pigs received antimicrobials therapy prior to sampling or the sample received at the lab was collected after 2-3 days, isolation will often fail to recover the bacterium, leading to a decrease in diagnostic sensitivity of culture. Therefore, PCR protocols for detection and identification of *B. hyodysenteriae* DNA have been designed (La et al., 2003, 2006; Råsbäck et al., 2006; Nathues et al., 2007; Song and Hampson, 2009). Recently, a real-time PCR for detection of *L. intracellularis* and *B. hyodysenteriae* showed satisfactory results in identifying both pathogens in subclinical or acute infected pigs (Zmudzki et al., 2012). Fluorescent *in situ* hybridization (FISH) has also been described for the identification of *B. hyodysenteriae* in formalin-fixed tissues and pig feces (Boye et al., 1998a; Wilberts et al., 2015).

1.1.4 Treatment and control

Unfortunately, to date, there are no effective commercial vaccines to prevent SD (Burrough, 2017). Knowledge gaps regarding disease pathogenesis impair the development of effective control measures. Thus, the disease has been prevented, controlled, and treated solely by antibiotic therapy and the implementation of biosecurity practices (Hampson et al., 2019). The most common antibiotics used to treat and control SD are tiamulin, valnemulin, tylosin, and lincomycin (Alvarez-Ordóñez et al., 2013). However, the use of some of these agents in animal production is restricted in some countries, for example tylosin and lincomycin are banned EU for the treatment, prevention and control of *Brachyspira* associated disease. While valnemulin is banned in

North America and Canada (Kulathunga and Rubin, 2017). Despite the limitations in drug use, *Brachyspira* susceptibility to these antimicrobials has been reducing worldwide (Hampson et al., 2019) alongside the emergence of multidrug-resistant strains (Duinhof et al., 2008; Šperling et al., 2011; Massacci et al., 2018). In 2015 the OIE classified SD a disease to which vaccine-development is a priority to reduce the need for antibiotic use in swine (World Organization for Animal Health, 2015). Due to country-specific legislation, as described above, antimicrobials previously used for treatment and prevention of SD are no longer allowed to be used in swine. In addition to antimicrobials, biosecurity has been applied to prevent SD. Strict external protocols focus on quarantining and testing pigs when introducing new stock, controlling unnecessary personnel visits, vehicles, and preventing the contact of pigs with other domestic or wild animal species. The latter is done through physical barriers such as fences, combined with pharmacological approaches to control pest population (Robertson et al., 1992; Alvarez-Ordóñez et al., 2013). Trucks play a role in spreading *B. hyodysenteriae* between farms. A total of 90.9% of trucks that visited more than 21 farms were found positive for *B. hyodysenteriae* (Giacomini et al., 2018). Intestinal swab from crows found in proximity to two commercial pig farms resulted in the isolation of *B. hyodysenteriae*. Genetic analyses showed that the same strain was present in pigs from a nearby farm (Zeeh et al., 2018). Besides vehicles and birds, wild rodents can shed *B. hyodysenteriae* in feces, with the potential to contaminate food and water (Hampson, D. et al., 1991; Backhans et al., 2010; Pearson et al., 2016). These reports reinforce the role of animal and human vectors in transmitting *B. hyodysenteriae* to pigs, and the importance of external biosecurity to control SD. Internal biosecurity is crucial to prevent the spread of SD to uninfected pigs

within a herd (Nations, 2010). The establishment of all-in/all-out flow (AIAO) disrupts transmission between production stages and consecutive reared batches. Along with AIAO, cleaning and disinfection procedures should be performed when rooms are emptied, before it is refilled with the next batch of pigs (Alvarez-Ordóñez et al., 2013). These measures cited above may help to decrease the infection levels in the farm or prevent clinical signs, but they are not generally sufficient to eliminate the pathogen from the herd on a long term (Neirynck et al., 2020). Thus, protocols including depopulation and repopulation, partial depopulation, herd closured and medication, and whole-herd medication without herd closure can help to eradicate the pathogen from the farm (Speiser et al., 2011; Figi et al., 2014; Neirynck et al., 2020). The term depopulation refers to the removal of the entire breeding herd and restocking with negative animals for the specific pathogen (Holst et al., 2015; Leary et al., 2019). Advantages of this protocol include the opportunity to improve genetics while eliminate more than one disease. However, depopulation and repopulation premises is difficult and expensive since there is a complete loss of production from the time the breeding herd is removed until the new herd is replaced (Nations, 2010; Holst et al., 2015). Therefore elimination protocols can be implemented for two reasons: 1) when control measures have been unsuccessful or 2) elimination of the pathogen from a herd is desired (Holst et al., 2015). The partial depopulation method consists in three main steps: 1) remove all animals less than 10 months of age from the herd; 2) discontinue farrowing for at least two weeks and 3) medicate the remaining animals with antimicrobials. It is important to note that all the strategies protocols for herd elimination of any specific pathogen should be combined

with improvement of farm management practices and biosecurity to effectively work as a long-term control in infected farms (Neirynck et al., 2020).

1.2 *Lawsonia intracellularis* infection in swine

1.2.1 Etiology and epidemiology

Lawsonia intracellularis is a Gram-negative, anaerobic, obligate intracellular bacterium and the cause of porcine proliferative enteropathy (PE) (Kroll et al., 2005). Lesions associated with PE were first described in the 1930s (Biester and Schwarte, 1931). However, it was not until the 1990s that the bacterium was isolated from pigs and cultured in rat enterocyte cell culture (Lawson et al., 1993). After that, the bacterium was determined to be of a new genus that was named in honor of Dr. Lawson (Gebhart et al., 1993; McOrist et al., 1995a). PE is an important disease in countries with significant pork production, being considered endemic in North America (Marsteller TA, Armbruster G, 2003; Paradis et al., 2007), South America (Chiriboga et al., 1999; Resende et al., 2015), Europe (Arnold et al., 2019), Asia (Wu et al., 2014) and Oceania (Holyoake et al., 2010). Economic losses due to PE are associated with its negative impact on weight gain, morbidity and mortality rates, costs with prevention, control, and treatment measures (McOrist et al., 1997; McOrist, 2005). Subclinical PE is also suggested to be costly as it leads to reduced average daily gain and feed efficiency (Kroll et al., 2005; McOrist, 2005). Transmission of *L. intracellularis* occurs by the fecal-oral route from infected feces, contaminated fomites and surfaces (Kroll et al., 2005). There are two major clinical manifestations of PE in pigs: the acute form of the disease, called proliferative hemorrhagic enteropathy (PHE), is associated with bloody diarrhea and high mortality;

porcine intestinal adenomatosis (PIA), the chronic form of PE, is characterized by non-hemorrhagic diarrhea with thickening of the ileal mucosa (Vannucci and Gebhart, 2014).

1.2.2 Pathogenesis

Knowledge on the early events involved in *L. intracellularis* infection of the intestinal epithelium is limited (Boutrup et al., 2010). *In vitro*, the bacterium was observed interacting closely with host cells followed by cellular uptake within 3 hours post-inoculation (McOrist et al., 1995b). *In vivo,* it was demonstrated that the enterocyte-bacterium interaction occurs as early as 12 hours post-infection (Boutrup. et al., 2010). *L. intracellularis* has a single polar flagellum likely involved in penetration of the mucus layer to reach the apical membrane of enterocytes (Lawson and Gebhart, 2000; Josenhans and Suerbaum, 2002). The *L. intracellularis* flagellum is lost after attachment to host cells and is not synthesized again (Smith and Lawson, 2001). The attachment requires a specific host-bacterium interaction, but the process of invasion does not depend on the viability of the *L. intracellularis* cell. This was proved by the internalization of formalin-killed bacteria in a cell (Lawson et al., 1995). Internalization occurs in a membrane-bound vacuole linked to host cytoskeleton changes (Smith and Lawson, 2001). Then the pathogen escapes this vacuole, replicating and lying free in the cell cytoplasm by the apical side. Intracellular replication of *L. intracellularis* results in the proliferation of immature enterocytes in the crypt, the main histological change associated with PE (Vannucci and Gebhart, 2014). Replication is then followed by the release of bacteria to adjacent cells (McOrist et al., 1995b).

1.2.3 Diagnosis

Antemortem diagnosis of *L. intracellularis* includes the use of serum for indirect immunofluorescent antibody test (IFAT) and immune peroxidase monolayer assay (IPMA), and feces for PCR (Guedes et al., 2002a; Boesen et al., 2005; Corzo et al., 2005; Wattanaphansak et al., 2008). Ancillary diagnostics should be associated with clinical signs, including sudden death with hemorrhagic diarrhea in the acute form of PE or moderate watery diarrhea and reduced weight gain in the chronic form (Pedersen et al., 2010). Detection of intracellular curved bacteria in fixed tissue by Warthin-Starry (WS) silver stain (Huerta et al., 2003; Van der Heijden et al., 2004), immunohistochemistry (IHC) (Guedes et al., 2002b; Szczotka et al., 2011), or *in situ* hybridization (Boye et al., 1998b; Weissenböck et al., 2007) directly associates *L. intracellularis* with the lesions.

1.2.4 Treatment and control

Currently, live attenuated and inactivated *L. intracellularis* vaccines are commercially available for the prevention of PE. Live attenuated vaccines are administered via drinking water but require an antibiotic-free window of at least 3 days before and after vaccination. This practice prevents killing the vaccine strain but can favor the emergence of other diseases (Holyoake et al., 2009). The use of an inactivated vaccine, on the other hand, allows the simultaneous use of antibiotics. However, it is laborious to individually vaccinate each pig (Karuppannan and Opriessnig, 2018). *In vitro* sensitivity of isolates from Brazil and Thailand demonstrated that carbadox (for Thai strains), tiamulin, and valnemulin were the most active compounds against *L. intracellularis.* (Wattanaphansak et al., 2019). North American and European isolates were susceptible to carbadox,

tiamulin and valnemulin, and resistant to lincomycin (Suphot Wattanaphansak, Randall S. Singer, 2009). Alternatives to antimicrobials such as prebiotics and phytogenic compounds to treat PE have been developed and tested in pigs (Karuppannan and Opriessnig, 2018). The addition of *Origanum vulgaris* (oregano) and *Allium sativum* (garlic) in pig feed reduced the load of *L. intracellularis* cells in the intestine and the clinical signs of PE in pigs (Papatsiros et al., 2009). A phytogenic feed additive, with a proprietary formula (mix of essential oil blend and plant extract), was investigated to control *L. intracellularis* in weaned pigs. Lower fecal excretion of the bacteria in the treatment group associated with decreased pathogen load in the ileum was observed. Additionally, pigs treated with the phytogenic had higher feed conversion ratio when compared to control animals (Draskovic et al., 2018). Pigs fed a fermented liquid feed showed a delay in *L. intracellularis* excretion detected by qPCR and decreased microscopical lesion scores following inoculation (Boesen et al., 2004). Since *L. intracellularis* is transmitted mainly by feces, cleaning and disinfection of premises reduces environmental contamination levels, and the incidence of PE (Kroll et al., 2005). Strict cleaning to remove feces from pens and disinfection with quaternary compounds or the combination of quaternary compounds with aldehydes and oxidizing agents, has been shown to inactivate *L. intracellularis* (Wattanaphansak, 2010; Collins, 2013).

1.3 *Salmonella enterica* serovar Typhimurium infection in swine

1.3.1 Etiology and epidemiology

Salmonella enterica serovar Typhimurium is a Gram-negative, facultative anaerobic and intracellular bacterium (Ryan et al., 2017). In pigs, *S.* Typhimurium causes diarrhea and enterocolitis (Boyen et al., 2008a). A portion of infected animals become carriers of *S.*

Typhimurium and shed the bacterium when stressed (e.g. after transportation to the slaughterhouse) (Isaacson et al., 1999). Contamination of pork with *Salmonella* products occurs as carcasses come in contact with feces during slaughter. In humans, *S.* Typhimurium is a well-recognized foodborne pathogen and represents an important public health issue in both developing and developed countries (Crump et al., 2004). Between 1998 and 2015, there were 288 human foodborne outbreaks attributed to pork in USA. Of these, 163 were associated with *Salmonella*, being 72 (44%) caused by serovar Typhimurium (Self et al., 2017). It was estimated that foodborne illness from *Salmonella* in 2013 cost approximately US$3,6 billion in the USA, including non-hospitalized and hospitalized cases and also post-hospitalization outcomes (Economic Research Service, 2014). The fecal-oral route is the most likely way for *S*. Typhimurium to infect pigs, but nasal discharges of infected animals also allow dissemination of the pathogen (Oliveira et al., 2007). *Salmonella* can also infect pigs through contaminated feed, insects, birds, and rodents (Binter et al., 2011; Wang et al., 2011; Andrés-Barranco et al., 2014).

1.3.2 Pathogenesis

S. Typhimurium has a large number of genes encoding virulence factors. Most of these genes are located in genomic regions known as *Salmonella* pathogenicity islands (SPI). A total of 21 SPI have been identified in *Salmonella* spp.. *S*. Typhimurium has 12 SPIs: SPI-1 to 6, 9, 11, 12, 13, 14 and 16 (López et al., 2012). Before infecting epithelial cells, *S*. Typhimurium needs to overcome innate immunity components such as antimicrobial peptides, the acid pH of the stomach, and the gastrointestinal mucus layer (Fàbrega and Vila, 2013). Then, the infection process involves the following steps: adhesion, invasion,

maturation of *Salmonella*-containing vacuoles (SCV), and replication. The bacterium uses different types of fimbriae to attach to different cell types in the intestinal epithelium. For example, long polar fimbriae (*Lpf*) attach to M cells (Bäumler et al., 1996). Six SPIs are involved in adhesion to host cells. SPI-3 encodes the protein *MisL*, which allows *S.* Typhimurium to bind to fibronectin (Dorsey et al., 2005). SPI-4 harbors the *siiE* gene that encodes for a nonfimbrial adhesion factor, and the *siiABCDEF* operon encodes for the type one secretion system (T1SS), that aids in injecting the adhesin into the host cells (López et al., 2012). After the pathogen is attached, invasion of the mucosa can occur in two ways: *Salmonella* can actively invade enterocytes, or be directly taken up by dendritic cells or microfold cells (Manon et al., 2012). Either way, type III and type IV secretion systems (T3SSs and T4SSs) located at SPI-1 are associated with changes in the host cell to facilitate invasion (Byndloss et al., 2017). In a mechanism call Trigger, at least 15 effector proteins are translocated by T3SS-1 (McGhie et al., 2009). Out of these 15 proteins, four (*SopE*, *SopE*2, *SopB*, and *SipA* initiate the process of *Salmonella* invasion by inducing actin rearrangements (Zhou and Galán, 2001; Patel and Galán, 2005; Ibarra and Steele-Mortimer, 2009). These proteins promote changes in the cell cytoskeleton and lead to actin remodeling and membrane ruffling. These cytoskeletal rearrangements disrupt the normal epithelial brush border and induce the consequent internalization of the bacteria in large vesicles called SCVS (Lamas et al., 2018). Inside the SCVS, *S*. Typhimurium expresses a second T3SS located in the SPI-2. Maturated SCVS is further engulfed by phagocytes, where the bacteria survives and replicates. This process is associated with the production of tumor necrosis factor-alpha (TNF-α) and interleukin-8 (IL-8) (Hobbie et al., 1997; Ohl and Miller, 2001). Finally, a pyroptotic host

cell death occur, which is a rapid form of programed cell death that possesses both apoptotic and necrotic features and is followed by dissemination of *Salmonella* cells (López et al., 2012). As the host cells death occur, the cytokines released by them recruit neutrophils, increase vascular permeability, all contributing to the onset of inflammatory diarrhea (Hurley et al., 2014). As a consequence of bacterial invasion, tissue injury with multifocal or coalescing erosions and ulcers covered with fibrinonecrotic debris due to the neutrophil activity is observed in affected animals (Orr et al., 1977).

1.3.3 Diagnosis

Diagnosis of *S*. Typhimurium infection is based on the association of clinical signs, compatible lesions, and bacterial culture from affected sites. Watery diarrhea is the main clinical sign observed in affected pigs, associated with reduced feed intake, anorexia, and dehydration (Alsop, 2005). Gross lesions of acute salmonellosis cases include diffuse catarrhal colitis associated with a removable fibrin necrotic membrane. In mature lesions, deep coalescent ulcers also known as button ulcers are observed. In both cases mesenteric lymph nodes are edematous (Wilcock et al., 1976). Microscopically, focal necrosis of cryptal and surface enterocytes is observed associated with numerous macrophages in the lamina propria (Oliveira et al., 2005). Other laboratory methods such as PCR can be performed to support bacterial culture findings. Serological assays such as ELISA using meat juice or serum are useful methods for herd screening programs but are neither sensitive nor specific for be used in individual animal diagnosis (Alsop, 2005; Vico and Mainar-Jaime, 2011).

1.3.4 Treatment and control

Control of *S*. Typhimurium in swine herds is an One Health issue. It is focused on reducing the exposure of pigs to the bacteria and prevent shedding that may contaminate pork products (Alsop, 2005). Biosecurity measures such as AIAO management, single source groups, low stocking density, and reduced number of farms on a determined area will decrease the risk of *Salmonella* exposure to the herd (Alsop, 2005; Andres and Davies, 2015). Vaccines for *S*. Typhimurium can be used to control clinical disease or to reduce subclinical shedding (Wales and Davies, 2017). Currently, killed *Salmonella* whole-cell and attenuated live vaccines are commercially available for pigs (de la Cruz et al., 2017). Since *S*. Typhimurium is a facultative intracellular bacterium, the live attenuated vaccine leads to improved cellular immunity response compared to the killed cell vaccine, with the advantage that inoculation can be performed by drinking water or aerosol. However, killed vaccines have the advantage of being safe as there is no potential to reverse virulence (de la Cruz et al., 2017; Wales and Davies, 2017). Overall, the diversity of *Salmonella* serovars in commercial swine operations makes it difficult to induce herd immunity through vaccines, and vaccination interference with serological tests troubles surveillance (Wallis, 2001; Wales and Davies, 2017). Antimicrobials such as amikacin, gentamicin, apramycin, ceftiofur, and trimethoprim/sulfonamide can be used to control *Salmonella*. Anti-inflammatory drugs such as flunixin meglumine and meloxicam can also be administrated to seriously ill animals to combat the effects of endotoxin (Alsop, 2005; Pairis-Garcia et al., 2013, 2015). Non-antimicrobial compounds against *Salmonella* infection in pigs are being investigated. Recently, a feed additive blend (fermented rye with *Agaricus subrufescens* combined with formic acid and lactic

acid) induced a reduction in the peak and average *S.* Typhimurium shedding (Fabà et al., 2020). A study using *in vitro* and *in vivo* assays found that coated fatty acids decreased the invasion capacity of *S.* Typhimurium (Boyen et al., 2008b).

1.4 *In vitro* intestinal culture

The intestinal mucosa is composed of several cell types organized in a complex structure that is responsible for nutrient absorption and waste secretion (Jankowski et al., 1994; Turner, 2009). A model that resembles the *in vivo* features of the intestinal mucosa is desirable for the study of infectious disease since the use of animal models has several limitations: 1) it is challenging to obtain samples from the early stages of infection prior to clinical sigs. There is limited access for imaging, there are confounding variables and limited throughput (Shanks et al., 2009). 2) Animals require extensive care and proper facilities, leading to costly projects that limit the number of replications and repetitions (Hartung, 2008). 3) The use of animals in research is ethically questionable (Singh et al., 2016). The moral principles of animal welfare should guide every experiment combined with the application of the 3Rs: reduction, refinement, and replacement of animals in research when possible (Barré-Sinoussi and Montagutelli, 2015).

1.4.1 Development of intestinal culture

In 1911 the first embryonic organs were cultured in a strictly aseptic environment by Carrel & Burrows (Carrel and Burrows, 1911). However, it was only in 1914 when Thomson and colleagues were able to improve the technique from Carrel & Burrows by minimizing the aseptic standards from the initial method and successfully performed tissue culture in an ordinary laboratory (David Thoms, 1914). Thereafter, the application

of *in vitro* organ and tissue culture was limited to fetal and embryological explanted tissues. Due to their small size, embryonic tissues could be cultured intact and seemed to tolerate the hypoxic and anoxic culture conditions better than mature organs (Randall et al., 2011). The culture of adult tissue began in 1959 when Trowell developed an aluminum chamber with a built-in gas reservoir to maintain alive sections of organs harvested from rats and mice. The culture medium was contained in a glass dish that also had a metal grid on. Organs or tissues were placed on the metal grid in a way that the medium did not submerge the sample. Although mature organs (ureter, ductus deferens, uterus, trachea, arteries, salivary glands, mammary gland, prostate, seminal vesicle, lung, thyroid, parathyroid, pituitary, pineal, ovary, skin, white adipose tissue, lymph nodes, sympathetic ganglia) were successfully cultured for up to 9 days, due to the possibility of infection Trowell did not attempt to culture intestine (Trowell, 1959). Ten years later, Browning and Trier successfully cultured a small section of intestinal mucosa obtained from the biopsy of an adult human based on a modified version of Trowell`s technique. Their method consisted of placing 3 cm biopsies (explants) with the mucosa sided up on a stainless-steel wire mesh. The tissue was maintained in an air-liquid interface (ALI), with enough culture medium to bath the serosal aspect of the biopsy without submerging the apical side. The apparatus was placed in a sealed jar with 95% O_2 and 5% CO_2 at 32°C. The jar was re-gassed at 6 hours intervals to prevent tissue anoxia, a key aspect for the success of the method. (Browning and Trier, 1969). Since the ALI technique was established, variations of the method were later used for other mature organs such as the skin, bronchus, and cornea (Jacobs et al., 2002; Seeber et al., 2008; Zimmermann et al., 2009). Adaptations include culture systems where the culture media moves to bath the

explant. Explants of human jejunum were placed in culture tubes with a small amount of medium and subjected to revolutions using a drum roller (Mitchell et al., 1974). In another variation, explants were placed on one edge of a culture dish and incubated on a rocking platform with a medium covering the mucosa surface intermittently (Autrup et al., 1978). Nietfeld et al., (1991) cultured small intestine mucosal explants from weaned pigs and obtained the best results when explants were cultured using the rocking platform (Nietfeld et al., 1991). Recently Tsilingiri et al., (2012) developed a mucosa explant culture model in which an apical to basolateral polarity was preserved during stimulation with bacteria. The authors attached a hollow cylinder to the apical side of the mucosa with surgical glue to mimic the *in vivo* exposure. Explants were placed in a culture chamber containing 99% O_2, and epithelial architecture was maintained for up to 36 hours to evaluate the effectiveness of probiotics in healthy and diseased explants (Tsilingiri et al., 2012).

1.4.2 Advantages of in vitro organ culture

In vitro intestinal culture offers multiple advantages over other *in vitro* methods such as two-dimension (2D) cell culture (e.g immortalized and primary cells) and three-dimension (3D) culture systems (e.g stem-cell organoids). The intestinal epithelium is constituted of stem cells at the base of the crypts giving rise to transient proliferative cells that then differentiate and migrate towards the lumen (Allaire et al., 2018). Therefore, an ideal intestinal model should include the cellular diversity and complexity observed *in vivo* (Costa and Ahluwalia, 2019). Simplistic 2D monocultures have the advantage of being of easy maintenance and low-cost (Yin et al., 2016). However, they lack the interaction between different tissues and the cellular diversity of organs, failing to mimic

cellular functions and signaling pathways present *in vivo* (Ripken and Hendriks, 2015). The identification of intestinal stem cells that differentiate into all intestinal epithelial cells gave rise to the so-called "mini guts" or organoids (Barker et al., 2007). Intestinal organoids self-organize in 3D structures that recapitulate major features of the intestinal tissue, however, there are limitations. Their spatial arrangement limits drug or pathogen access to the luminal compartment, which is needed for microbe-epithelium interactions, drug, and feed additives screening studies (Fatehullah et al., 2016; Hannan et al., 2018). Such studies also require a continuous and intact epithelial layer, which is absent in the model. In addition, the organoid system is limited when mimicking the inflammatory response, since it lacks immune cells, thus not ideal for host-pathogen studies (Yin et al., 2016; Hannan et al., 2018). *In vitro* intestinal culture, on the other hand, recapitulates the physiological processes and preserves the cellular diversity and anatomical architecture as *in vivo* (Udden et al., 2017; Costa et al., 2018). Besides, it allows the reproduction of the inflammatory response while supporting an organ-specific microbiota (Costa et al., 2016, 2020; Russo et al., 2016). Furthermore, the intestinal explant culture offers a better representation of the *in vivo* host defense responses (Udden et al., 2017). Additionally, *in vitro* tissue or organ culture is in line with the principles of 3Rs: reducing the use of animals in research as one animal may donate dozens of organ replicates (Costa et al., 2018).

1.4.3 Applications of in vitro intestinal culture

Understanding the pathogenesis of enteric pathogens is vital to control infection. Examples of studied pathogens using the *in vitro* intestinal culture model with relevance to veterinary medicine include *Brachyspira* spp., *Salmonella,* and *Escherichia coli*

(Robertson et al., 2000; Girard et al., 2005; Costa et al., 2017, 2020; Welle et al., 2017). Recently, a protocol for porcine colon *in vitro* culture using an ALI system has been developed, and explants were cultured for up to 5 days (Costa et al., 2016). The same authors later applied this model to investigate the early stages of *B. hampsonii* interaction with the colon and the host metabolomic response to *B. hyodysenteriae* infection. Both studies focused on understanding the pathophysiology mechanisms triggered by the spirochetes that led to mucohemorrhagic diarrhea (Costa et al., 2017; Welle et al., 2017). Similar approaches have been used to study the pathogenesis of *S. enterica* and *E. coli*. Robertson et al., (2000) used rat ileal explants to compare the adhesional ability of *S. enterica* serovar Enteritidis with multiple fimbriae and flagella gene deletions. The authors concluded that fimbriae were not essential for the attachment to ileal explants. Ileal explants modeled *E. coli* infection *in vitro*, including microvilli effacement known as attaching-effacing (A/E) lesions (Zhu et al., 1995; Girard et al., 2005). The effects of phytic acid on the intestinal mucosa were also investigated using explants. Phytic acid can decrease the toxic effects induced by mycotoxins (deoxynivalenol and fumonisin B1) (da Silva et al., 2014; Silva et al., 2019). In addition to these applications, *in vitro* intestinal culture has the potential to be used in the screening of novel non-antimicrobial compounds. Even though it is still not extensively used for that, it has great potential to decrease the number of animals used and produced reliable results. A plant extract (*Moringa oleifera*) effect against *Cryptosporidium parvum* infection was evaluated using buffalo ileal explants. Findings suggested that the extract is effective in preventing parasitism (Aboelsoued et al., 2019). Porcine intestinal explants were used to determine whether the presence of *L. intracellularis* in enterocytes modifies fosfomycin penetration

into the cell. No difference was observed for the penetration of the antibiotic in healthy or *L. intracellularis*-colonized enterocytes (Pérez Gaudio et al., 2018).

1.5 Alternatives to antimicrobials in swine production

Antibiotics played an important role in the treatment, control, and prevention of several swine diseases for more than 50 years (Cromwell, 2002; Thacker, 2013). Previously, antibiotics were used to improve the rate and growth efficiency in pigs (Cromwell, 2002). In the USA, between 2017 and 2018, domestic sales and distribution of medically important antimicrobials approved for use in food-producing animals increased by 9%. Swine are the second most treated species, receiving 2,374,348 kg of antibiotics per year (U.S Food & Drug administration, 2019). Possible drug residues in pork products and the potential contribution to the emergence of multi-drug resistant strains led to several countries imposing restrictions on the use of antibiotics to treat livestock, including pigs (Hayes et al., 2001; Turner et al., 2001; Cromwell, 2002; Diário Oficial da União, 2016; U.S. Food & Drug Administration, 2016; Rana et al., 2019). This led to a need for alternatives to antibiotics for treatment and prevention of infections (Vondruskova et al., 2010).

1.5.1 Organic acids

Organic acids (OAs) contain a carboxyl group (COOH), which confers its acidic characteristic (Dibner and Buttin, 2002). Common names used to describe this group include fatty, volatile fatty, lipophilic, weak, or carboxylic acids (Cherrington et al., 1991). OAs can be classified according to the carbon-chain length: short-chain fatty acids (SCFA) with maximum 6 carbon atoms; medium-chain fatty acids (MCFA) with 7 to 10 carbon atoms; and long-chain fatty acids (LCFA), which consist of more than 11 carbon

atoms (Dittoe et al., 2018). SCFA, such as acetic, propionic, and butyric acids, are produced in the large intestine under anaerobic conditions by microbial fermentation of fiber (Kim et al., 2014). The efficacy of a given acid to inhibit microbial growth is dependent on its dissociation status, which is described by its pKa value (the pH at which half of the available molecules are dissociated). The lower its pKa, the stronger the acid, and the greater is its ability to decrease the pH of a given environment (Pearlin et al., 2020). OAs in their undissociated form are lipophilic and can freely cross cellular membranes. Once inside the bacterial cytoplasm, the higher pH results in the acid dissociation, leading to the release of protons (H^+), decrease bacterial cytoplasm pH, and impairment of glycolysis enzymatic reactions and nutrient transportation (Dibner and Buttin, 2002), resulting in bacterial cell membrane disruption, enzymatic and energy deprivation (Cherrington et al., 1991). Additionally, the process of transporting free protons out of the cell requires energy, contributing to decrease the energy available for reproduction, invariably leading to a bacteriostatic effect (Russell, 1992). The efficacy of OAs in reducing bacterial growth also depends on the bacterial cell wall. Gram-positive bacteria are mainly susceptible to MCFA, while Gram-negative bacteria, due to lipopolysaccharide (LPS), are resistant to the lipophilic nature of MCFA (Sheu and Freese, 1973). Traditionally, OAs are used as feed preservatives. Their inclusion in swine feed has gained importance due to enhanced growth performance and intestinal microbiota modulation (Tugnoli et al., 2020).

1.5.2 Prebiotics

The intestinal microbiota plays a role in the outcome of pathogen colonization and shedding in pigs (Burrough, 2017; Kim and Isaacson, 2017; Argüello et al., 2019).

Therefore, microbiota modulation through nutritional interventions could be a tool to improve animal health (Lallès et al., 2007; Xiong et al., 2019). The concept of prebiotics has been discussed and refined several times since it was first introduced by Gibson and Roberfroid in 1995 (Gibson and Roberfroid, 1995). Currently, the definition of prebiotic, as proposed by the International Scientific Association for Probiotics and Prebiotics (ISAPP), refers to:

"a substrate that is selectively utilized by host microorganisms conferring health benefits to the host" (Gibson et al., 2017).

Such effects include enriching for beneficial taxa, inhibition of pathogen growth, and host immune modulation. Prebiotics resist digestion in the upper gastrointestinal tract serving as a subtract for colonic bacteria for fermentation (Sarao and Arora, 2017). Overall, prebiotics have three main mechanisms of action against pathogens in the intestine:

1) to promote growth of beneficial intestinal bacteria. These can form biofilms on the mucosa and prevent the adhesion of pathogenic bacteria;

2) to mimic host receptors and act as decoys, preventing pathogen binding and promoting excretion of pathogens from the intestine;

3) end products of prebiotic fermentation can inhibit the expression of bacterial adhesins or downregulate virulence factors, hampering pathogen growth (Tran et al., 2016).

1.5.3 Phytobiotics

Phytobiotics include plant-derived products classified based on the source, including herbs, spices, essential oils, and oleoresins (Windisch et al., 2008). Also known as

phytogenic feed additives, they recently gained increased interest in swine nutrition as an alternative tool to antimicrobials (Mohammadi Gheisar and Kim, 2018). The active compounds vary depending on several factors: the part of the plant used, the harvest season, and the geographical region where it was planted, and the technique used for the processing (e.g., cold expression, steam distillation, extraction with nonaqueous solvents) (Windisch et al., 2008; Subha, 2013). The use of phytobiotics in animal production has been exploited due to its antioxidant, antimicrobial properties, and beneficial impact on growth rate (Silva Júnior et al., 2020). However, knowledge about their mode of action is still rather limited (Jacela et al., 2010). Thyme (*Thymus vulgaris*) produces thymol and carvacrol, two phenolic compounds (Fani and Kohanteb, 2017). Thymol addition to feed in weaned piglets improves the jejunal barrier function by regulating tight junction gene expression and protein synthesis (Van Noten et al., 2020). Stress from weaning or LPS from gram-negative bacteria can induce inflammation of the gastrointestinal tract, which in turn may decrease the expression levels of tight junction proteins such as claudins, occluding and ZO-1 in the intestine. This was counteracted by administration of thymol (Park et al., 2010; Wei et al., 2017; Wang and Ji, 2019). The gastric mucosa of weaned pigs exposed for 12 hours to thymol demonstrated increased expression of genes that positively influenced mitosis and its regulation (Colombo et al., 2014). Carvacrol was shown to accelerate healing of chronic gastric ulcers induced by acetic acid in mice (Silva et al., 2012). In a mouse model of colitis, carvacrol (50 or 100 mg/kg) reduced macroscopic and microscopic lesions, which was linked to the modulation of antioxidant enzymes such as catalase, superoxide dismutase, and peroxidase (de Santana Souza et al., 2017). It was also identified that carvacrol regulates the expression of cyclooxygenase-2

(COX-2), associating this compound with an anti-inflammatory effect (Landa et al., 2009; Hotta et al., 2010). *In vitro* pretreatment of intestinal porcine epithelial cells (IPEC-J2) with thymol (50 µM) mitigated the negative effects of LPS-induced damage to the epithelium (Omonijo et al., 2018). *In vivo* supplementation of carvacrol-thymol mixture did not affect mRNA levels of occludin and ZO-1, when compared with non-supplemented piglets (Wei et al., 2017). Flavonoids and gallotannin from Carob (*Ceratonia siliqua*) are known to have an antioxidant and anti-inflammatory effect by scavenging free radicals, inhibiting COX, inducible nitric oxide synthase (iNOS) and poly(ADP-ribose) polymerase (PARP) (Havsteen, 2002; Lee et al., 2003; Erdèlyi et al., 2005; Luo and Lee Kraus, 2012; Ke et al., 2019). Administration of carob extract following ethanol-induced oxidative stress in rats revealed a protective effect on the stomach mucosa, preventing lesions (Rtibi et al., 2015). In dextran sulfate sodium (DSS) induced ulcerative colitis, pretreatment of rats with carob pods aqueous extract increased superoxide dismutase (SOD) activity and protected the mucosa against epithelial damage (Rtibi et al., 2016). Overall, the inclusion of phytobiotics in the swine diets has the potential to control infections while improving animal performance due to its anti-inflammatory and antioxidant properties.

1.5.4 Enzyme inhibitors

Enzyme inhibitors modulate, prevent, or alter the target enzyme catalytic functions in a reversible or irreversible way and in a competitive or non-competitive mode. From a therapeutic perspective, pathways associated with pathological processes can be therefore stopped, preventing disease. Enzyme inhibitors can be applied as therapeutic agents for bacterial, fungal, viral, and parasitic diseases as well as cancer, neurodegenerative,

immunological, and cardiovascular diseases (Walpole and Wrigglesworth, 1989; Manivasagan et al., 2015). In swine medicine, there are no reports of enzyme inhibitors being used as a bacterial virulence-blocker drug. These molecules have the potential to become an alternative to antibiotics to mitigate clinical signs of enteric infections by suppressing the physiological processes exacerbated during disease.

General hypothesis

The current policies to restrict the use of antimicrobials in animal production associated with an increase in multi-resistant bacterial strains have instigated the research for novel non-antimicrobials compounds. Thus, the work described in this thesis was structured based on two hypotheses, First, that the simultaneous exposure of porcine colon explants to non-antimicrobials compounds leads to a reduced degree of inflammation and mitigates microscopical lesions caused by the pathogen. Second, that oral L-NAME administrated to healthy mice does not affect the gastrointestinal tract.

Overall objective

The overall aim of this thesis was to investigate novel alternative compounds to mitigate lesions caused by *B. hyodysenteriae*, *L. intracellularis,* and *S.* Typhimurium.

Specific objectives

- To investigate the effectiveness of five non-antimicrobial compounds to mitigate lesions associated with *B. hyodysenteriae*, *L. intracellularis* and *S.* Typhimurium using a porcine colon culture technique.

- To evaluate the safety of oral delivery of a nitric oxide enzyme blocker (L-NAME) to healthy mice.

CHAPTER 2: *In vitro* screening of feed additives to mitigate intestinal lesions caused by *Brachyspira hyodysenteriae*, *Lawsonia intracellularis* and *Salmonella enterica* serovar Typhimurium

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Author contributions

Conceived and designed the experiments: Matheus de O. Costa. Performed the experiments: Mariana Meneguzzi and Barbara de Souza. Analyzed the data: Mariana Meneguzzi and Matheus Costa. Wrote the paper: Mariana Meneguzzi and Matheus Costa.

2.1 Preface

Swine dysentery, ileitis, and porcine salmonellosis are production-limiting diseases of pigs in commercial farms globally. They are caused by infection with *Brachyspira hyodysenteriae*, *Lawsonia intracellularis,* and *Salmonella enterica* serovar Typhimurium. Prevention, treatment, or control of these infections relies on vaccines and heavily on antimicrobials. The goal of this study was to evaluate the effectiveness of five non-antimicrobial compounds in preventing lesions caused by the bacteria cited above in an *in vitro* intestinal culture model. A total of five pigs per pathogen were used. For compounds F, S, L and P a total of 4 explants/pig for each combination group were used. Only for compound D a total of 12 explants/pig for each combination group were used. The combination groups were pathogen control group (PCG), compound control group (CCG), and treatment group (TG). Histopathological lesions and host-response gene expression levels were evaluated from the colon explants. Our results suggest that short and medium-chain fatty acids, a nitric oxide synthase inhibitor 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* and *S.* Typhimurium. These findings are a step towards finding alternatives to antimicrobials usage and control of SD, PE and salmonellosis in pork production.

Key words: swine, *in vitro* organ culture (IVOC), alternative, pig, antimicrobial, compounds, feed additive.

2.2 Introduction

Swine dysentery (SD), porcine intestinal adenomatosis (PIA), proliferative enteropathy (PE) and porcine salmonellosis are bacterial infections of pigs that lead to major economic losses due to poor growth performance, and increased production costs associated with treatment (Vannucci and Gebhart, 2014; Patterson et al., 2016; Burrough, 2017). SD, characterized by mucohaemorrhagic diarrhea and colitis, is caused by *Brachyspira hyodysenteriae.* Recently, *B. hampsonii* and *B. suanatina* were found to cause a syndrome indistinguishable from SD (Rubin et al., 2013; Rohde et al., 2018). Diarrhea caused by *Lawsonia intracellularis* is characterized by two clinical presentations: porcine intestinal adenomatosis (PIA) characterized by mucosal thickening at the chronic stage of disease, mainly affecting post-weaned pigs (between 6 and 20 weeks of age), and proliferative hemorrhagic enteropathy (PHE), associated with intestinal hemorrhage and melena during the acute stage, most commonly observed in young adult pigs (4 to 12 months of age) (Lawson and Gebhart, 2000; Vannucci and Gebhart, 2014). *Salmonella enterica* serovar Typhimurium leads to enterocolitis and watery diarrhea mainly in grower and finisher pigs (Patterson et al., 2016). Several different vaccine development approaches have been explored for SD, such as bacterins (Hampson et al., 1993; Diego et al., 1995; Mahu et al., 2017), protein digests of whole cell bacterins (Waters et al., 1999a, 1999b) and reverse vaccinology (Song et al., 2009). However, these methods induced either partial protection or no protection, and currently there is no efficient vaccine against SD commercially available (Mahu et al., 2017). In contrast, commercial vaccines are available for ileitis and salmonellosis (Mcorist, n.d.; de la Cruz et al.,

2017; Roerink et al., 2018). Live and inactivated *L. intracellularis* vaccines have their own barriers for implementation. The live attenuated vaccine is administered via drinking water or liquid feed and requires a medication-free window of three days before and after vaccination to avoid inactivation of the vaccine strain. In parallel, the *L. intracellularis* bacterin is administered intramuscularly, thus requiring significant human resources for herd immunization (Holyoake et al., 2009; Karuppannan and Opriessnig, 2018). *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 (de la Cruz et al., 2017; Wales and Davies, 2017; Hoelzer et al., 2018). Therefore, treatment and control of these diseases still require antimicrobial use.

The injudicious use of antimicrobials selects for resistant bacterial strains, imposing a risk for human and animal health (Jia et al., 2006; Holyoake et al., 2009; Coculescu et al., 2014; Mirajkar et al., 2016). Restrictions imposed on antimicrobial agents 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 (Hayes et al., 2001; Turner et al., 2001; Official Diary of the Union, 2016; U.S. Food & Drug Administration, 2016). Organic acids (OA), prebiotics, phytobiotics and enzyme inhibitors are alternatives to antimicrobials being explored. OA are compounds with the ability to share a proton, including fatty acids (Dibner and Buttin, 2002). OA are able to cross and disrupt the bacterial cell membrane, changing cytoplasmic pH, affecting enzymes and nutrient transport

systems, with both bacteriostatic and bactericidal effects (Dibner and Buttin, 2002; Tugnoli et al., 2020). Fatty acids, such as short chain fatty acids (SCFA) and medium chain fatty acids (MCFA), are a major energy source for colonocytes, stimulating epithelial cell proliferation while acting as immune-modulators (Cook and Sellin, 1998; Schönfeld and Wojtczak, 2016). Prebiotics are selectively fermented ingredients that modulate the gut microbiota composition to benefit host health through competitive exclusion of pathogens or the production of health-promoting metabolites (Bindels et al., 2015; Gibson et al., 2017). Phytobiotics are plant-derived products often referenced to as essential oils, depending on the process used to extract the biologically active ingredients (Gadde et al., 2017). Finally, enzyme inhibitors are a more recent alternative that has not yet been used in swine production. These compounds suppress host physiological processes exacerbated during infection, mitigating tissue damage (Keyser et al., 2008).

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

2.3 Materials and methods

Ethics statement

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).

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 against *B. hyodysenteriae* to screen for compound D only. Following euthanasia, distal spiral colon collection and culture followed the protocol previously described by Costa et al (2018). 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 (6ºC - 10ºC) 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 with the mucosa facing up was individually placed 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 do not invade the inner aspect of the mucosa, to create 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. 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) (Riber et al., 2015). Immediately before inoculating explants, aliquots from each inoculum were collected for quantification kept frozen at −80 °C until processing). Inocula averaged 3.2×10^8 CFU/mL for *S.* Typhimurium, 7.9×10^7 genome copies/mL for *B. hyodysenteriae,* and 1×10^4 cells/mL for *L. intracellularis*. 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 pathogen control group (PCG) received 100 µl of inoculum of a given pathogen and explants in the compound control group (CCG) received 100 µl of compound only. The treatment group (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; 0.714kg/1000kg for compound L, and 1 kg/1000kg of feed for compound D). 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, L and P, and 5 additional pigs were used for compound D. For compounds F, S, L and P a total of 4 explants/pig for each combination group were used (Table 2.8.1). Only for compound D a total of 12 explants/pig for each combination group were used (Table 2.8.2). For all the compounds explants were randomly exposed to one of the following combination groups (Figure 2.7.1) 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, housekeeping reference gene), tumor necrosis factor-α (TNF-α) genes, interferon-γ (IFN-γ) (Pasternak et al., 2020), interleukin-1α (IL-1α) (Duvigneau et al., 2005). Inducible nitric oxide synthase (iNOS), was evaluated for *B. hyodysenteriae* (Bernardini et al., 2012) 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.

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 (Matz et al., 2013) on R studio (version 1.1.463) (R Core Team, 2020).

2.4 Results

2.4.1 *Exposure to pathogens induced microscopic changes in colon explants*

The findings below represent the comparison between all explants from the PCG and CCG groups for a given pathogen.

Brachyspira hyodysenteriae

No significant difference was observed for the percentage of healthy epithelium (Figure 2.7.2; A). In contrast, a significantly thicker mucus layer was found in the PCG (early time-point and late time-point, Figure 2.7.2; B) and significant higher levels of TNF-α, IL-1α and INF-γ mRNA expression were found at the late time-point (Figure 2.7.3; A).

Lawsonia intracellularis

No differences between groups in epithelial coverage or gene expression were observed at either time-point (Figure 2.7.2; C, and 2.7.3; B).

***Salmonella enterica* serovar Typhimurium**

CCG explants had significant more healthy epithelial coverage than the PCG samples at both time-points (Figure 2.7.2; D). Late time-point PCG samples had increased IL-1α mRNA expression (Figure 2.7.3; C).

2.4.2 *Challenge trials*

Brachyspira hyodysenteriae

Early time-point

A summary of our findings is presented in Figure 2.7.4. Unexpectedly, for compound F, explants from the PCG showed significant high level of epithelial coverage when compared to either the CCG or TG (Figure 2.7.4; A). Compound L and P TG samples had significantly decreased mucus layer thickness, when compared to the PCG (Figure 2.7.4; A). Compound S treated explants showed a trend to reduced mucus layer thickness ($P = 0.06$, Figure 2.7.4; A) when compared to the PCG. Compound D TG explants trended towards higher epithelial coverage, when compared to PCG ($P = 0.06$).

Treatment with compound F significantly down-regulated TNF-α mRNA expression, when compared to PCG (Figure 2.7.4; C). Up-regulation of iNOS mRNA expression was observed for compound S TG when compared to explants from the PCG (Figure 2.7.4; C). No difference in mRNA expression for all genes investigated was observed for compounds L, P and D.

Late time-point

A summary of our findings is presented in Figure 2.7.4. Mucus layer thickness was significantly increased in the PCG when compared to TG for compounds L and P

(Figure 2.7.4; B). Surprisingly, increased epithelial coverage was found in explants from the PCG, when compared to compound F TG samples (Figure 2.7.4; B). Compound P treated explants had significant higher epithelial coverage than the PCG (Figure 2.7.4; B).

For compound P, TNF-α was found down regulated in TG samples, when compared to PCG (Figure 2.7.4; C) and INF-γ mRNA level trended towards downregulation (*P*= 0.08, Figure 2.7.4; C). Treatment of explants with compound S led to the up-regulation trend of IL-1α (*P*= 0.06, Figure 2.7.4; C) and significant down regulation of INF-γ (Figure 2.7.4; C), in relation to the PCG. 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.

Lawsonia intracellularis

Early time-point

Results are summarized in Figure 2.7.5. The percentage of healthy epithelium coverage was significant higher in compound F CCG than the PCG (Figure 2.7.5; A). No differences in mRNA level were observed for any compound (Figure 2.7.5; B).

Late time-point

Epithelium coverage for compound F was higher in the TG than PCG samples (Figure 2.7.5; A). No other differences were observed for any gene-compound combination (Figure 2.7.5; B).

***Salmonella enterica* serovar Typhimurium**

Early time-point

Explants treated with compounds F and P maintained significant higher epithelial coverage than the PCG samples (Figure 2.7.6; A). For compound S, IL-1α mRNA level was higher in the TG than the PCG (Figure 2.7.6; B). Compound P treated explants had a trend to decrease levels of IL-1α mRNA ($P = 0.07$, Figure 2.7.6; B), when compared to the PCG.

Late time-point

Explants in the compound S TG had higher percentage of healthy epithelium coverage trend than the PCG ($P = 0.07$, Figure 2.7.6; A). No gene expression differences were observed (Figure 2.7.6; B).

2.5 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. A summary of our significant findings is presented in Table 2.8.3.

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* (Figure 2.7.4; A). Together with a trend to downregulate INF-γ mRNA expression following challenge with *B. hyodysenteriae* was also observed (Figure 2.7.4; C). TNF-α and IFN-γ have a recognized role in tight junction regulation (Al-Sadi et al., 2009; Capaldo and Nusrat, 2009). Tight junction proteins, such as occludins, claudins and zonula occludens (ZO), are crucial for the

maintenance of epithelial barrier integrity and to regulate the paracellular movement of ions and water (Rescigno, 2011; Chelakkot et al., 2018). Fatty acids appear to modulate tight junction permeability and have an anti-inflammatory effect in the colon (Ohata et al., 2005; Liu, 2015; Yan and Ajuwon, 2017). Increased TNF-α and IFN-γ levels lead to the rearrangement of myosin molecules associated with tight-junction proteins, consequently increasing paracellular permeability (Stoplen et al., 1986; Madara and Stafford, 1989; Kita Arai et al., 1999). In our study, TNF-α and IFN-γ mRNA expression was down regulated when explants were treated with a blend of MCFA and SCFA (compound P). Similar responses were identified in weaned pigs supplemented with butyrate, and when human colonic biopsies, human colonic cell lines and isolated lamina propria cells were cultured with butyrate (Kvale and Brandtzaeg, 1995; Segain et al., 2000; Yin et al., 2001; Wen et al., 2012). Intestinal epithelial cells exposed to TNF-α and IFN-γ have reduced cystic fibrosis transmembrane conductance regulator (CFTR) expression and chloride (Cl^-) secretion (Hiribarren et al., 1993; Fish et al., 1999; Resta-Lenert and Barrett, 2006). 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 (Bansil and Turner, 2018; Rajendran et al., 2018). 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 (Enns et al., 2019). In contrast, our findings suggest a relationship between the down regulation of TNF-α and a trend to down regulation of IFN-γ in explants and the reduction in

mucus secretion following infection with *B. hyodysenteriae*. This link between host cytokines and mucus secretory response in SD remains to be further clarified.

Explant treatment with compound L led to decreased mucus layer thickness following *B. hyodysenteriae* exposure (Figure 2.7.4; A and B). Oxidation of L-arginine by nitric oxide synthase (NOS) results in NO release, which is crucial for the preservation of normal intestinal permeability, motility, mucus production, and epithelial fluid secretion (Zingarelli et al., 1999; Kolios et al., 2004). However, excessive production of NO, mainly by the inducible NOS (iNOS) isoform, is associated with mucosal damage as observed during colitis in humans (Gochman et al., 2012; Soufli et al., 2016). L-citrulline, a product of NOS, was found to accumulate in the porcine colon following *B. hyodysenteriae* challenge (Welle et al., 2017). Additionally, up-regulation of the iNOS signaling pathway was recently reported in explants infected with *B. hampsonii*, which causes a syndrome undistinguishable from SD (Costa and Harding, 2020). In our study, inhibition of NO production by compound L could have resulted in decreased mucus accumulation in explants following *B. hyodysenteriae* inoculation. Mucoid feces is one of the main features of SD (Burrough, 2017). NO directly interacts with increased cytosolic levels of cyclic guanosine monophosphate (cGMP), which has been implicated in mucus release by intestinal epithelial cells (Brown et al., 1992, 1993). Thus, pharmacological manipulation of NO production is suggested to decrease mucus secretion during NO-associated inflammation (Grisham et al., 1994; Rachmilewitz et al., 1995; Tanaka et al., 2001; Rajagopal et al., 2014). Taken together, our results indicate that compound L mitigates the effect of *B. hyodysenteriae in vitro*, independently from changes in iNOS mRNA expression.

Explants treated with compound F (prebiotic based on *Agaricus subrufescens* fermented rye) were associated with higher healthy epithelium coverage when challenged with *L. intracellularis* (Figure 2.7.5; A). Riboglucans, β-glucans and glucomannans are examples of bioactive polysaccharides isolated from *A. subrufescens* (Ariandi et al., 2015). These molecules can act as a substrate for bacterial adherence, as they mimic the host glycocalyx (Shoaf et al., 2006). 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 (Wang et al., 2015). 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, jejum) and mucosal scrapings of the small intestine (Van Nevel et al., 2003). To the best of our knowledge, this is the first report which evaluate the effectiveness of *A. subrufescens* rye fermentation against *L. intracellularis*. To enter the host cytoplasm by zipper and trigger endocytosis, the pathogen attachment mechanism requires direct host-bacterium interaction (Lawson et al., 1995; Smith and Lawson, 2001; Vannucci and Gebhart, 2014). Yeast cell wall polysaccharides are mainly constituted of β-glucans, which can act as an immunoregulatory (Førland et al., 2010; Lima et al., 2011; Borchani et al., 2016). β-glucans receptors are present on immune and non-immune cells such as monocytes, macrophages, eosinophils, natural killer cells and endothelial cells fungal (Brown and Gordon, 2003; Barreto-Bergter and Figueiredo, 2014). Mice fed β-glucan from a fungal culture showed enhanced phagocytic activity and IL-1 production of peritoneal macrophages obtained after more than 10 days of consecutive supplementation (Suzuki et al., 1990). *In vitro*,

lymphocytes from pigs supplemented with β-glucan produced less IL-6 and TNF-α following lipopolysaccharides (LPS) challenge (Li et al., 2005). Pigs fed for two weeks after weaning with fungal β-glucans were less susceptible to $F4^+$ enterotoxigenic *E. coli* infection, with reduced F4-specific serum antibody response (Stuyven et al., 2009).

In our study, no significant differences in cytokine mRNA levels were observed after *L. intracellularis* challenge (Figure 2.7.5; B). 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 (Boutrup et al., 2010; Boutrup. et al., 2010). The ability of the vaccine strain to induce such changes is also questionable, but it has been shown to do so *in vivo* (Riber et al., 2015). Thus, further studies investigating the immunomodulatory role of compound F following infection with a virulent *L. intracellularis* during longer incubation periods are strongly suggested.

Explants exposed to the compound F only had an increase in the percentage of healthy epithelium compared to explants challenged with *S.* Typhimurium (Figure 2.7.6; A). *In vitro*, rye overgrown with mycelium decreased *S.* Typhimurium growth over time. In an animal trial, the fungal fermented compounds reduced *Salmonella* shedding and improved weight gain and feed efficacy in pigs (Allaart et al., 2017). In accordance, when nursery pigs were fed fermented rye in combination with organic acids,

increased body weight, lower frequency of diarrhea and reduced *S.* Typhimurium shedding was observed (Fabà et al., 2020).

Surprisingly, a lower degree of epithelial health was observed in explants exposed to compound F alone than explants exposed to *B. hyodysenteriae* (Figure 2.7.4; A and B). It is known that colon explants harbor a microbiota compositionally similar to the donor pig prior to euthanasia (Costa et al., 2020). 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, and thus clearing of bacteria, may also 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 (Figure 2.7.4; A and C). Thymol and carvacrol are present in the essential oils extracted from thyme (*Thymus vulgaris*) (Fachini-Queiroz et al., 2012). Carvacrol was demonstrated to have a gastroprotective effect in a rodent model of gastritis (Oliveira et al., 2012; Silva et al., 2012). Carvacrol was associated with reduced colonic lesions in colitis induced by 2,4,6-trinitrobenzenesulfonic (TNBS) in rats (Dundar et al., 2008) and in acetic acid-induced colitis in mice (de Santana Souza et al., 2017). The protective effect of carvacrol was associated with its ability to regulate cyclooxygenase-2 (COX-2) expression (Landa et al., 2009; Hotta et al., 2010). An *in vitro* T cell model also linked the reduction of IL-2 and IFN-γ expression to exposure to thymol and carvacrol (Gholijani et al., 2015). In contrast, IL1β and TNF-α induce the expression of COX-2 (Cunha et al., 1992). Mice treated with carvacrol had decreased TNF-α

levels and milder lesions following acetic acid-induced colitis (de Santana Souza et al., 2017). Additionally to the effects of thyme, carob (*Ceratonia siliqua*, another ingredient of compound D) contains phenolic compounds such as flavonoids and gallotannins that also inhibit COX-2 (Lee et al., 2003). Thus, the effect of compound D was likely due to its anti-inflammatory effects associated with the inhibition COX-2 cascade.

In conclusion, our findings suggest that some non-antimicrobial compounds may have a beneficial effect in this explant infection model. Compound P, a blend of SCFA and MCFA and compound L, a NOS inhibitor, decreased the accumulation of mucus, and compound D, a phytobiotic, supported epithelial survival when explants were exposed to *B. hyodysenteriae*. Compound F, a fungal fermented prebiotic, prevented epithelial death following *L. intracellularis* when comparing CCG vs PCG and TG vs PCG. Besides, compound F increased the percentage of healthy epithelium when explants were exposed to compound only comparing to explants exposed only to *S.* Typhimurium. The authors warrant that further studies are needed to verify compound effectiveness *in vivo*.

2.6 Acknowledgement

Thanks to Roman Nosach for technical support with tissue collection, Champika Fernando for assistance with *B. hyodysenteriae* culture, Barbara Souza for help with project execution and Talita Pilar Resende for knowledge exchange with RT-PCR assays.

2.7 Figures

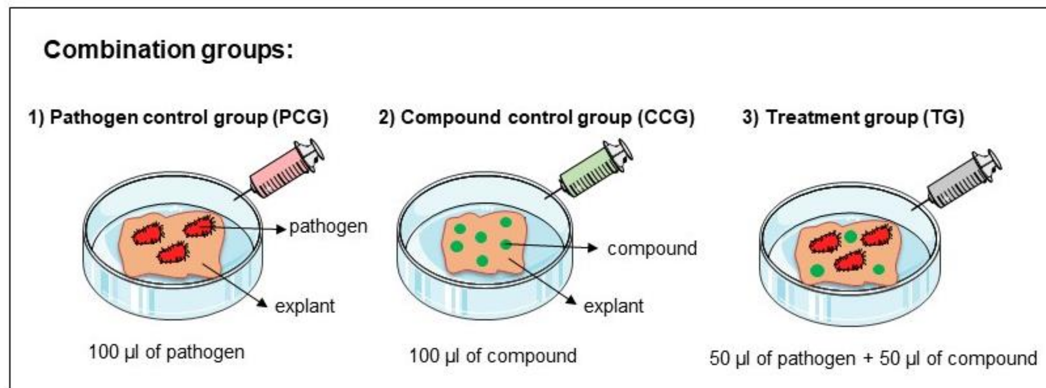


Figure 2.7.1. Description of combination groups used for each pathogen (*B. hyodysenteriae*, *L. intracellularis* and *Salmonella enterica serovar* Typhimurium).

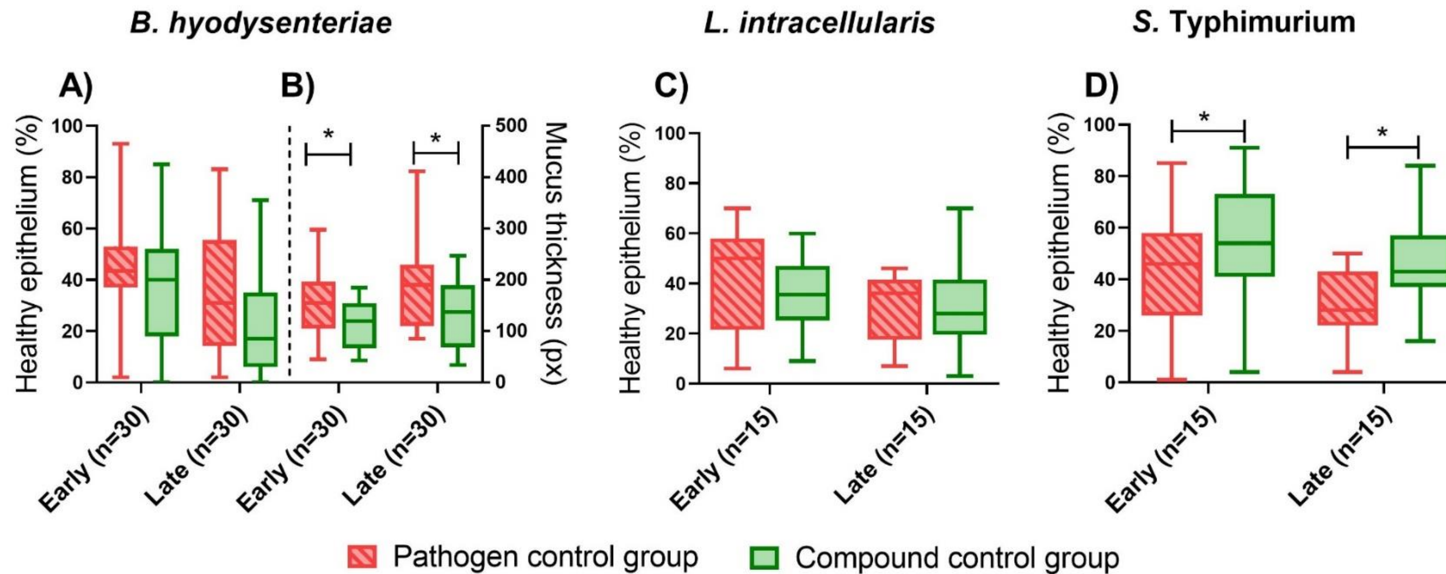
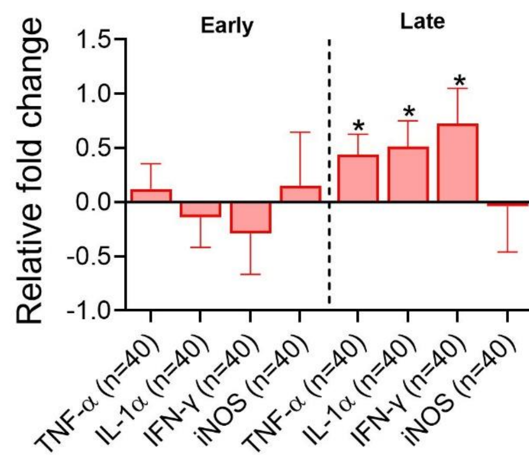
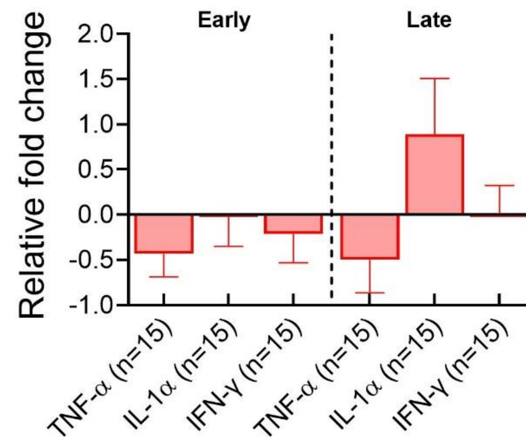


Figure. 2.7.2 Microscopic explant health comparison between the compound control group (CCG) and pathogen control group (PCG). Percentage of the healthy epithelium (A) and mucus layer thickness (B) in explants harvested from 10 pigs and exposed to *B. hyodysenteriae* (PCG) or controls (CCG). A total of 5 explants for each compound (F, L, P and S) were use, adding up 20 explants per time-point. For compound D a total of 2 explants for every 5 pigs were used adding up 10 explants per time-point. Sum up a total of 30 explants per time-point. C) Percentage of healthy epithelium on explants from 5 pigs exposed to *L. intracelullaris* (PCG*)* or controls (CCG). D) Percentage of healthy epithelium on explants from 5 pigs exposed to *S.* Typhimurium (PCG*)* or controls (CCG). For *L. intracellularis* and *S.* Typhimuirum, a total of 5 explants for each compound (F, P and S) were used, adding up 15 explants per time-point. Boxplots depict the median ± standard deviation of the median Whiskers depict the minimum and maximum values. Stars denote a significant difference between groups ($P \leq 0.05$).





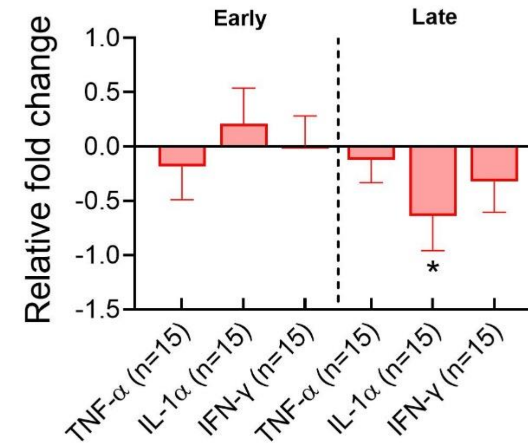


Figure. 2.7.3 Change in mRNA levels between the compound only (CCG) and pathogen only (PCG) groups. CCG was used as the reference group. A) Gene expression levels for explants from 10 pigs exposed *B. hyodysenteriae*. A total of 5 explants for compounds F, L, P, and S were use, adding up 20 explants per time-point. For compound D a total of 4 explants for every 5 pigs were used adding up 20 explants per time-point. Sum up a total of 40 explants per time-point. B) Gene expression levels for explants from 5 pigs exposed *L. intracellularis.* C) Gene expression levels for explants from 5 pigs exposed to *S*. Typhimurium. For *L. intracellularis* and *S.* Typhimuirum, a total of 5 explants for each compound (F, P, and S) were used, adding up 15 explants per time-point. Each bar represents mean fold-change, whiskers represent the standard deviation from the mean. Star denotes significant difference ($P \leq 0.05$).

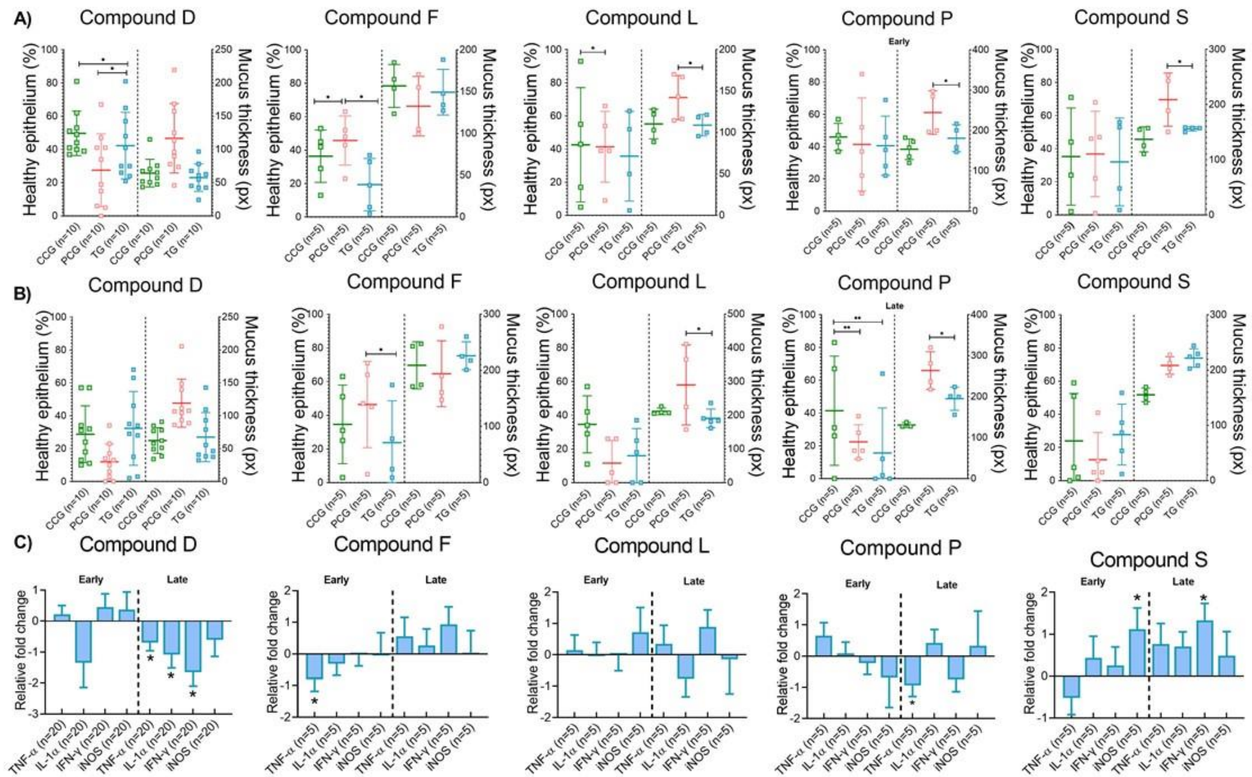


Figure. 2.7.4 Microscopic changes and gene expression data from explants collected from 10 pigs and challenged with *B. hyodysenteriae*. A & B) Histopathology assessment including the percentage of healthy epithelium covering explants and mucus layer thickness at early (A) and late (B) time-points. For compound D a total of 2 explants/pig were used adding up 10 explants per time-point. For compounds F, L, P, and S a total of 1 explant/pig was use, adding up 5 explants per time-point. 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. For compound D a total of 4 explants/pig were used adding up 20 explants per time-point. For compounds F, L, P, and S a total of 1 explant/pig was use, adding up 5 explants per time-point. A total of 5 pigs were used for compound D only and other 5 pigs were used for compound F, L, P, and S. Bars represent mean mRNA levels; whiskers depict standard deviation from the mean. Star denotes $P \leq 0.05$ and two stars denote $P = 0.06$. Compound control group (CCG) Pathogen control group (PCG). Treatment group (TG).

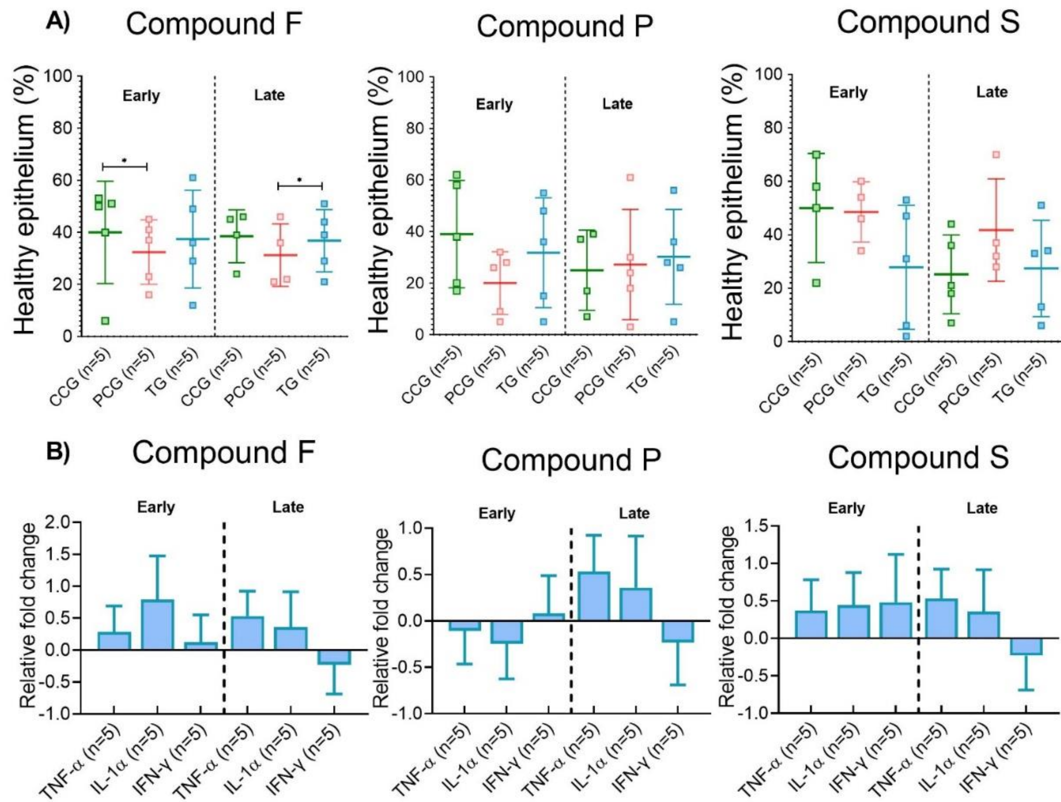


Figure. 2.7.5 Microscopical changes and gene expression data from explants challenged with *L. intracellularis*. A & C) Histopathology assessment. Horizontal lines represent group mean, and whiskers depict standard deviation from the mean. Compound control group (CCG) Pathogen control group (PCG). Treatment group (TG). B & D) Gene expression data is reported as fold change from TG samples using the PCG as reference. For histopathology and gene expression, a total of 5 pigs were used and 1 explant/pig was made up for each compound (F, P, and S) adding up 5 explants per time-point. Bars represent mean mRNA levels; whiskers depict standard deviation from the mean. Star denotes $P \leq 0.05$.

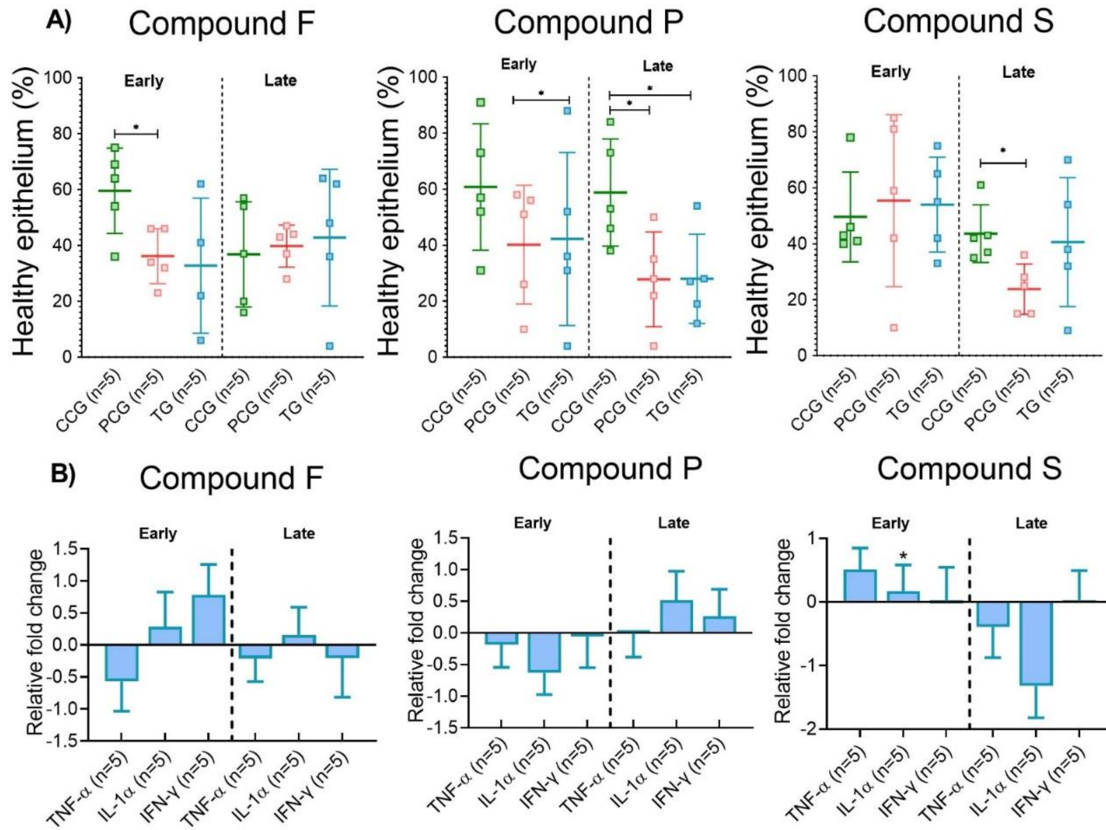


Figure. 2.7.6 Microscopical changes and gene expression data from explants challenged with *S.* Typhimurium. A & C) Histopathology assessment. Horizontal lines represent group mean, and whiskers depict standard deviation from the mean. Compound control group (CCG) Pathogen control group (PCG). Treatment group (TG). B & D) Gene expression data is reported as fold change from TG samples using the PCG as reference. For histopathology and gene expression, a total of 5 pigs were used and 1 explant/pig was made up for each compound (F, P, and S) adding up 5 explants per time-point. Bars represent mean mRNA levels; whiskers depict standard deviation from the mean. Star denotes $P \leq 0.05$.

2.8 Tables

Table 2.8.1 Description of the total number of explants used per animal for each compound (F, L, P, and S) and pathogen (*B. hyodysenteriae*, *L. intracellularis* and *S.* Typhimurium).

Number of animals	Combination groups	RT-PCR		Histopathology		Total of explants per combination groups
		Early	Late	Early	Late	
5	Pathogen control group (PCG)	1	1	1	1	4
	Compound control group (CCG)	1	1	1	1	4
	Treatment group (TG)	1	1	1	1	4

Table 2.8.2 Description of the total number of explants used per animal for compound D only tested against *B. hyodysenteriae*.

Number of animals	Combination groups	RT-PCR		Histopathology		Total of explants per combination groups
		Early	Late	Early	Late	
5	Pathogen control group (PCG)	4	4	2	2	12
	Compound control group (CCG)	4	4	2	2	12
	Treatment group (TG)	4	4	2	2	12

Table 2.8.3 Summary of significant findings when comparing the Treatment Group (TG) vs Pathogen Control Group (PCG) across the different pathogen-compound combinations.

Pathogen	Compound	Early time-point	Late time-point
	D	Increased epithelium coverage.	IL-1α, INF-γ and TNF-α down regulated.
	F	TNF-α down regulated.	-
B. hyodysenteriae	L	Decreased mucus layer thickness.	Decreased mucus layer thickness.
	P	Decreased mucus layer thickness.	Decreased mucus layer thickness. TNF-α down regulated.
L. intracellularis	F	-	Increased epithelium coverage.

CHAPTER 3: Effect of oral administration of a NOS-inhibitor compound on the intestinal mucosa of healthy mice

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Author contributions

Conceived and designed the experiments: Matheus de O. Costa and Mariana Meneguzzi. Performed the experiments: Mariana Meneguzzi. Analyzed the data: Mariana Meneguzzi and Matheus Costa. Wrote the paper: Mariana Meneguzzi and Matheus Costa.

3.1 Preface

Swine dysentery (SD) is a production-limiting disease associated with colitis in pigs. It is caused by *Brachyspira hyodysenteriae* or *B. hampsonii* and is associated with significant economic losses due to poor animal performance and costs associated with disease mitigation. To date, treatment and control of SD rely solely on antimicrobial therapy. Overexpression of nitric oxide (NO) has been implicated in the pathogenesis of colonic inflammatory diseases in humans, such as Crohn`s disease and ulcerative colitis. NO was also identified to play a role in the pathophysiology of SD. *In vitro* data revealed that inhibition of nitric oxide synthase (NOS), an enzyme that catalyzes NO production, reduced lesions following *B. hyodysenteriae* infection of colon explants. This study aimed to investigate the safety of the oral administration of a NOS inhibitor in mice. Eight female mice were assigned to control (n=4) or treatment (n=4) groups and treated with water or a NOS inhibitor (L-NAME, 0.1 mg/mL) for seven days. Body weight, clinical and fecal scores and feed and water intake were recorded daily for both groups. Animals were euthanized at the end of the experimental period and histopathology and gene expression analyses were performed. Neuronal NOS mRNA level was higher in L-NAME treated mice. No other differences in *ante* or *post-mortem* data were observed between the two groups. At the given dose, oral L-NAME administration did not affect the colonic mucosa or cause any clinical signs, suggesting it is safe to mice *per os*.

Key words: NOS, L-NAME, nitric oxide, murine, colitis, oral.

3.2 Introduction

Nitric oxide (NO) is a gaseous messenger molecule generated through the reaction of L-arginine with oxygen. In mammals, this process is catalyzed by a family of enzymes known as NO synthase (NOS), which consists of three isoforms: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS). Reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and (6*R*-)5,6,7,8-tetrahydrobiopterin (BH4) are cofactors in this reaction (Förstermann and Sessa, 2012). During homeostasis, nNOS and eNOS are regulated by intracellular calcium/calmodulin levels and expressed at a relatively constant rate. Together nNOS and eNOS are referred to as constitutive NOS (cNOS, (Gantner et al., 2020)). eNOS product leads to vasodilatation through stimulation of guanylyl cyclase and increased cyclic guanosine monophosphate (cGMP) in smooth muscle cells (Forstermann et al., 1986; Loscalzo, 2013). NO prevents leukocyte and platelet adherence to endothelium, (Radomski et al., 1987) and plays a critical role in angiogenesis post-ischemia (Murohara et al., 1998). NO produced by nNOS has been implicated in retrograde neuronal communications and regulation of synaptic plasticity, playing a role in learning, memory, and neurogenesis (Hölscher, 1997; Posada and Clarke, 1999; Zhou and Zhu, 2009). It is also involved in the central regulation of blood pressure, as smooth muscle cells can express low levels of nNOS, aiding with the maintenance of vasodilatation (Togashi et al., 1992; Schwarz et al., 1999). In the gastrointestinal tract, NO produced by cNOS regulates mucosal blood flow, mucus secretion, intestinal motility, water and electrolyte transport (Alican and

Kubes, 1996; Price et al., 1996; Martin et al., 2005). Differently from cNOS, iNOS is calcium/calmodulin-independent and is expressed upon activation of nuclear factor κB, cellular exposure to lipopolysaccharides (LPS), interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α) or oxidative stress (Nathan, 1997; Guzik et al., 2003; Stanek et al., 2008). The release of NO and NO-derived oxidants creates a hostile environment for most living cells, including pathogens and tumors (Knewtson et al., 2018; Barros et al., 2019). Overproduction of NO by iNOS has been implicated in the development of detrimental inflammatory processes (Morelli et al., 1997) such as gastric ulcer, ulcerative colitis, necrotizing enterocolitis, and Crohn`s disease (Middleton et al., 1993; Rachmilewitz et al., 1995b; Singer et al., 1996; Kimura et al., 1997; Vallance, 2003). In colitis, peroxynitrite (the result of NO reaction with a superoxide anion) causes DNA damage and consequently activates the nuclear enzyme poly (ADP-ribose) synthetase (PARS). This enzyme stops the cellular electron transport chain and adenosine triphosphate (ATP) formation, resulting in cellular dysfunction or apoptosis, and increased epithelial permeability (Colgan, 1998; Kennedy et al., 1998; Szabó and Dawson, 1998). Extensive enterocyte apoptosis was associated with peroxynitrite in infants with necrotizing enterocolitis (Ford et al., 1997).

Swine dysentery (SD), caused by *Brachyspira hyodysenteriae* and *B. hampsonii,* results in mucohemorrahegic diarrhea associated with fibrino-necrotic colitis in susceptible pigs (Alvarez-Ordóñez et al., 2013; Rubin et al., 2013). SD control and treatment rely solely on biosecurity measures and antimicrobial treatment. No commercial vaccine for either pathogen is available (Burrough, 2017). This limitation

in developing preventative methods is linked to a knowledge gap in the pathogenesis of SD. Recent studies pointed out the role of NO in the disease pathophysiology. Accumulation of L-citrulline, a by-product of NOS, and the up-regulation of the iNOS signaling pathway on the colonic mucosa after *Brachyspira* spp. challenge *in vitro* was reported (Welle et al., 2017; Costa and Harding, 2020). Furthermore, the emergence of multidrug-resistant *Brachyspira* strains together with the global trend to reduce the use of antimicrobials for animal protein production are evidence of an immediate need for alternatives to antimicrobials to control and treat SD (Šperling et al., 2011; Massacci et al., 2018; U.S. Food and Drug Administration, 2020).

Analogs of L-arginine compete with the L-arginine as substrates for the active site of NOS (Silverman et al., 1997). N-methyl-L-arginine (L-NMA), NG-nitro-L-arginine (L-NNA), and Nomega-nitro-L-arginine methyl ester hydrochloride (L-NAME) are examples of L-arginine analogs capable of inhibiting the reaction catalyzed by NOS. While these analogs are not selective for any NOS isoform, they were found to be safe to mammals, inducing minor adverse effects (Muscará and Wallace, 1999). Adverse effects of chronic administration of L-NAME via drinking water and by gavage was previously demonstrated to cause hypertension and glomerular injury (Gardiner et al., 1990; Arnal et al., 1992; Baylis et al., 1992). It has been suggested to be selective towards the cNOS isoforms, thus abolishing their homeostatic function (Furfine et al., 1993; Southan and Szabó, 1996; Flinspach et al., 2004). In some studies, L-NAME oral delivery following induction of colitis in rodents and mice offered no advantage over untreated groups (Kankuri et al., 1999; McCafferty et al., 1999) or even

exacerbated intestinal injury (Pfeiffer and Qiu, 1995; Takeuchi et al., 1997). In contrast, there are multiple reports demonstrating the beneficial effect of L-NAME to the gastrointestinal mucosa of rodents in 2,4,6-trinitrobenzenesulphonic acid (TNB) and dextran sulfate sodium (DSS) induced colitis (Rachmilewitz et al., 1995a; Pilichos et al., 2004; Aoi et al., 2008), for the treatment of megacolon, and ulcerative colitis (Green et al., 2000; Schwörer et al., 2001). The mechanism of L-NAME protection during colitis is likely linked to maintenance of tight junctions and decreased oxidative stress mediated by intracellular calcium (Samak et al., 2015; Gangwar et al., 2017).

Recent work by our group revealed that porcine colon explants challenged with *B. hyodysenteriae* and treated with L-NAME *in vitro* decreased mucus accumulation, a hallmark sign of SD (see Chapter 2, Compound L). Thus, to verify the safety of L-NAME *in vivo*, this study aimed to investigate the effects of the compound on the gastrointestinal tract of healthy mice.

3.3 Materials and methods

Ethical approval of the study protocol

This experiment was conducted after approval by the Institutional Animal Care and Use Committee (IACUC) from the University of Minnesota (Protocol number 2002-37903A).

Experimental design

Eight 4-week old female CF-1 mice (Charles River Laboratories, Kingston, NY) with no gastrointestinal clinical signs were assigned to two groups while blocking for body

weight: control (n= 4) and treatment (n=4, Table 3.8.1). Animals were housed in two group-designated cages (n=4 mice/cage) with aspen shavings and allowed to acclimate for 7 days. After this period, mice were given the appropriate treatment for another 7 days. Control animals received tap water without any compound, and the treatment group received tap water containing 0.1 mg/mL of L-NAME (Cayman Chemical Company, Ann Arbor, Michigan, USA). The concentration of L-NAME used (0.1 mg/mL) followed prior work, where colonic lesions in a rat model of colitis were mitigate after L-NAME treatment (Rachmilewitz et al., 1995a). L-NAME was in a powder presentation and dilution was performed in water, one time, on the first day of the experimental period. The compound was diluted in water and dispensed in a bottle of water (400 mL) which was then offered to the animals. All animals in both groups had access to normal rodent diet, Teklad Global 18% protein (Envigo, Madison WI) and water *ad libitum* throughout the experimental period.

Ante-mortem assessments

Mice were monitored by a veterinarian twice daily (09:00h and 15:00h) when they were also scored for clinical signs (Table 3.8.2) and fecal consistency (Table 3.8.3). A heat map (Figure 3.7.1) during the 7-days experimental period describing the fecal consistency scores for each day and the clinical sings as active (+) or inactive (-) related to aspects of clinical assessment from table 3.8.2 was performed. Fecal samples were collected every day in the morning following a brief period of individual mouse manipulation. Individual mouse weight and cage feed intake were recorded daily. Due to logistical reasons, it was not possible to measure the water

intake daily, thus the amount of liquid in each bottle was measured on the first and last day of the 7-days experimental period.

<u>Post-mortem</u> assessments

On day 7, all mice were euthanized by carbon dioxide asphyxiation. Immediately after euthanasia, a necropsy was performed focusing on the gastrointestinal tract. Samples from cecum (n=2/mouse) and colon (n=2/mouse) were collected and fixed in 10% buffered formalin until processing for histopathology. Scoring was performed by an observer blinded to slide identity. Histological changes were semi-quantitatively assessed as previously described by Ek et al., 2017; catarrhal inflammation score: 0 = no notable changes; 1 = mucosa with occasional sloughed off epithelial cells on the lumen; 2= focal to multifocal groups of sloughed cells on the surface or in the lumen; 3 = showing one or more layers of sloughed cells on the lumen. Neutrophilic inflammation: 0 = no notable changes; 1= only notable in crypts or occasional strands in the lumen; 2 = neutrophils are frequently in the lamina propria and/or submucosa; 3 = neutrophils are observed frequently in the lamina propria and/or submucosa together with present neutrophils in the crypts and/or lumen. Epithelial regeneration: 0 = no notable changes; 1 = thickened mucosa with frequent mitotic figures. Digital images of 2 colon sections/mouse and 2 cecum sections/mouse were also analyzed (Image Scope 12.1, Wetzlar, Germany) to determine the percentage of healthy epithelium covering the mucosa and the mucus layer thickness (measured at five evenly spaced locations along the length of the tissue section).

Fresh 2 cm samples from colon (n=2/mouse) and cecum (n=2/mouse) were also collected and immersed in RNA-later (Qiagen, Germantown, MD, US) at 4°C for 24 hours and then stored at -80°C until reverse transcriptase real-time PCR (RT-PCR) was performed.

Reverse transcriptase real-time PCR (RT-PCR) assays

Total RNA from cecum (n=2/mouse) and colon (n=2/mouse) samples was extracted using a commercial kit (RNeasy animal cell and tissue kit, Qiagen, Austin, Texas, US) and quantified by spectrophotometry (Winooski, Vermont, USA). Complementary DNA (cDNA) was generated following a commercial kit (QuantiTect Reverse Transcription Kit, Qiagen, Germantown, MD, US). cDNA load for all samples was normalized 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, US). 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 duplicates, alongside no-cDNA template controls in each run. Samples where duplicates differed by more than 1 Ct were re-analyzed. Messenger RNA (mRNA) level for the following genes was investigated: iNOS F (5- CAG CTG GGC TGT ACA AAC CTT-3) and R (5- CAT TGG AAG TGA AGC GTT TCG-3) (Overbergh et al., 1999), eNOS F (5-

GCTG GTACATGAGTTCAGAGATTG-3) and R (5- TTCCACCGCTGCCTTGTC-3), nNOS F (5-TGTCCTTAAAGCCATCCAGC-3) and R (5- CGGTTGT CATCCCTCAGC-3) (Morimoto et al., 2011) and GAPDH F (5- TCA AGA AGG TGG TGA AGC AGG-3) and R (5- TAT TAT GGG GGT CTG GGA TGG-3) (Resende et al., 2019). After RT-PCR reactions, samples that showed undetermined values were reported as a missing value. Probably the start quantity of cDNA for these samples was low which hampered the gene expression.

Statistical analysis

Water and feed intake are reported as the difference between initial and final volumes. Differences in mRNA levels (Cts) between groups were analyzed using Mann-Whitney Rank Test. Daily animal weight was performed using generalizing estimating equation (GGE). Statistical analysis was performed using SPSS v24 (IBM Corporation, Armonk, NY, USA). Mucus layer thickness, percentage of healthy epithelium and average daily weight difference were compared between control and treatment groups using unpaired t-test on R studio (version 1.1.463) (R Core Team, 2020). Group differences were considered statistically different if $P \leq 0.05$.

3.4 Results

Ante-mortem

A summary of findings for body weight, water, and feed intake is shown in Table 3.8.1 and Figure 3.7.2. No differences were observed between groups. In addition, during the 7-days experimental period no notable changes in fecal consistency or clinical behavior scores were found (Figure 3.7.1). Estimated daily L-NAME

consumption was 12.85 mg/kg per mouse in the treatment group and 13.57 mg/kg per mouse in the control group.

Post-mortem

A summary of the semi-quantitative histopathological scores from cecum and colon sections is shown in Table 3.8.4. No microscopical lesions were observed in mice from either group. Catarrhal inflammation score 1 was observed in a colon section from one L-NAME-treated mouse (#4). A summary of epithelial coverage and mucus layer thickness data is shown in Figure 3.7.3. mRNA expression level for nNOS was lower in the control group, when compared to L-NAME treated mice ($P = 0.002$, Figure 3.7.4). No other significant differences were observed in gene expression.

3.5 Discussion

Infectious colitis in pigs is often caused by *B. hyodysenteriae* and *B. hampsonii*, known as SD (Burrough, 2017). The recently described role of NO in the pathophysiology of SD prompted the investigation of strategies to prevent clinical signs through modulation of this molecule (Welle et al., 2017; Costa and Harding, 2020). Here we showed that administration of an oral NOS inhibitor does not affect healthy mice. This is the first step towards the *in vivo* use of L-NAME to treat SD in pigs.

L-NAME oral administration for 7 days did not affect the integrity of the colonic mucosa of healthy mice. No date available regarding water stability of L-NAME.

When in a solid form, L-NAME is stable and should be stored at -20 ºC. The L-NAME concentration used in this study (0.1 mg/ml) followed prior work by Rachmilewitz et al., 1995a, who observed decreased colonic injury in rats after induction of colitis and oral treatment with L-NAME. The same dose of L-NAME was given for 7 days to guinea pigs, *per os*, resulting in ileitis associated with increased ileal myeloperoxidase activity (Miller et al., 1993, 1994). It was previously postulated that the lack of specificity of L-NAME for iNOS could abolish the protective function of eNOS and nNOS in non-inflammatory states, thus leading to lesions (Furfine et al., 1993; Southan and Szabó, 1996; Flinspach et al., 2004). In contrast, when the effect of aminoguanidine (iNOS selective enzyme) and L-NAME were compared following TNBS and DSS induced colitis in rats, both inhibitors attenuated the colonic inflammation (Pilichos et al., 2004; Aoi et al., 2008). Previous work suggested that only calcium-dependent NOS (nNOS and eNOS) were expressed in the colon (Salter et al., 1991). Conversely, others suggest that iNOS can also be constitutively expressed at low levels in the normal colonic mucosa (Perner et al., 2002). iNOS has also been associated with baseline colonic mucus secretion, indicating a regulatory function of this enzyme during homeostasis (Schreiber et al., 2013). We found no difference in mucus layer thickness between L-NAME-treated and control animals. Similarly, L-NNA (10 mg/kg bolus followed by 3 mg/kg/h), a nonselective NOS inhibitor, had no effect on the colonic mucus layer thickness in healthy rats with colitis (Schreiber et al., 2013). Our findings further evidence that NOS inhibition by L-NAME does not affect colonic mucosa integrity.

Fecal consistency scores were statistically the same in control and L-NAME-treated mice during the experimental period. Once NO is generated, it binds to the heme group of soluble guanylyl cyclase, which catalyzes the conversion of guanosine triphosphate (GTP) to the second messenger cGMP, leading to an intracellular increase of cGMP concentration (Epstein et al., 1993). This guanylyl cyclase-cGMP signaling axis has been implicated in mucus secretion, but also stimulates chloride (Cl^-) and bicarbonate (HCO^{-3}) efflux into the intestinal lumen through the cystic fibrosis transmembrane conductance regulator (CFTR) (Tamai and Gaginella, 1993; Rappaport and Waldman, 2018). Additionally, cGMP signaling inhibits the apical Na^+/H^+ exchanger 3 (NHE3), preventing sodium (Na^+) absorption from the lumen (Foulke-Abel et al., 2016; Ahsan et al., 2017). These interactions ultimately lead to water accumulation into the intestinal lumen and diarrhea (Rappaport and Waldman, 2018). Most laxatives require the production of intestinal NO to result in their secretory effects (Izzo et al., 1998). Interestingly, in patients with collagenous colitis, which is characterized by a secretory colonic state, N^G-monomethyl-L-arginine (L-NMMA , a NOS inhibitor) reduced fluid secretion, while L-arginine (NOS substrate) increased it (Perner et al., 2001). In rats, L-NAME administration delayed the onset and intensity of diarrhea following magnesium sulfate ingestion (Izzo et al., 1994). Thus, findings from previous studies corroborate our observation of no differences in fecal consistency scores between groups, further adding to the body of evidence that L-NAME can be safely administrated to healthy subjects.

Feed, water intake and average daily gain were similar in control and L-NAME treated mice. It was suggested that NO plays a modulatory role in the development of obesity-related insulin resistance (Morley and Flood, 1994; Joost and Tschöp, 2007). L-NAME (100 mg/kg/day) oral administration for 12 weeks was reported to attenuated body weight gain of mice fed either a standard diet or a high-fat diet (Tsuchiya et al., 2007). In our study, even though no statistical difference was observed for the average daily weight, surprising the treatment group had a higher feed conversion comparing to control group. As previously described, administration of L-NAME in drinking water is a practical and effective method of delivering the compound to rodents (Kirk and Gardiner, 2000; Víteček et al., 2012; Yasukawa et al., 2012). While we could not verify individual mouse water intake, group data and the lack of clinical and *post-mortem* findings suggestive of dehydration provided evidence that water was ingested at similar, physiologic rates by mice in both groups.

Higher nNOS mRNA levels were found in the cecum samples from L-NAME treated mice comparing to control animals. NO produced by nNOS is a major noradrenergic noncholinergic neurotransmission (NANC), responsible for relaxation of smooth muscle and regulation of gastrointestinal motility (Boeckxstaens et al., 1993). NOS inhibitors, such as L-NNA and L-NAME, are reported to antagonize the relaxation effect of NANC (Bult et al., 1990; Boeckxstaens et al., 1991; D'Amato et al., 1992). L-NAME (10 mg/kg, ten times the concentration used in this report) administration to rats inhibited descending colon smooth muscle relaxation, delaying colonic transit (Mizuta et al., 1999). The difference in nNOS mRNA expression was only observed in

the cecum and not in the colon. No similar studies were performed comparing the NOS expression in the cecum. Due to the fermentation that occurs in the cecum, we postulated that this process may limited the available amount of compound that could reach the colon. Thus, would explain the absence of difference in colon and cecum. Additionally, the difference was only observed at the nNOS mRNA levels. L-NAME has more affinity for nNOS than for eNOS and iNOS, meaning that the compound bind at a higher level to the nNOS sites than the other isoforms resulting in its strong inactivation (Víteček et al., 2012). While we did not observe any clinical abnormalities, the biological relevance (if any) of the increased levels of nNOS mRNA detected in L-NAME treated healthy animals should be further evaluated, especially at higher doses and in long-term treatments.

The study conducted here shed a light on the effects of oral administration of L-NAME to the large intestine of healthy mice. Our findings suggest no changes in fecal consistency, intestinal microscopical architecture, daily weight gain, feed, and water intake following oral delivery of L-NAME for 7 days. This work is a step towards clarifying the effectiveness of this enzyme blocker in treating the clinical signs of colitis in pigs.

3.6 Acknowledgements

Thanks to Nayara Pestana and RAR staff for assistance with mice handling.

3.7 Figures

		Experimental period (days)						
Group	**Mouse ID**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Control	1	+	+	+	+	+	+	+
	2	+	+	+	+	+	+	+
	3	+	+	+	+	+	+	+
	7	+	+	+	+	+	+	+
L-NAME	5	+	+	+	+	+	+	+
	6	+	+	+	+	+	+	+
	4	+	+	+	+	+	+	+
	8	+	+	+	+	+	+	+

Fecal score		Mice clinical assessment	
	Score 0 = Formed pellet	Score 0 (+)	Active behavior
	Score 1 = Formed pellet with mucus		
	Score 2 = Soft, mucoid feces	Score 1 (-)	Not active behavior
	Score 3 = Feces with blood		

Figure. 3.7.1 Heat map of fecal and clinical behavior scores during the 7-days experimental period for animals in the control and treatment group.

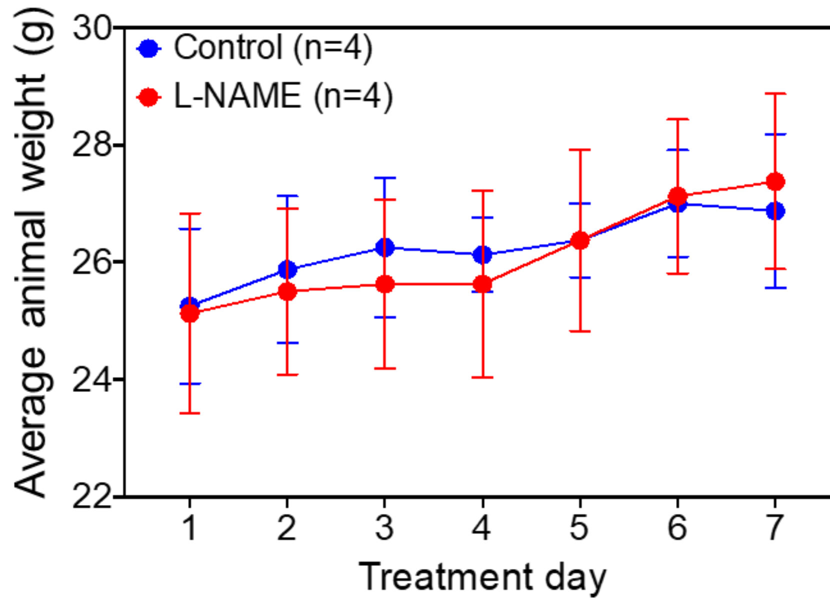

Figure 3.7.2 Mice body weight during the experimental period. Each data point depicts the average (± standard deviation) body weight (in grams) for mice in a given treatment group throughout the seven treatment days.

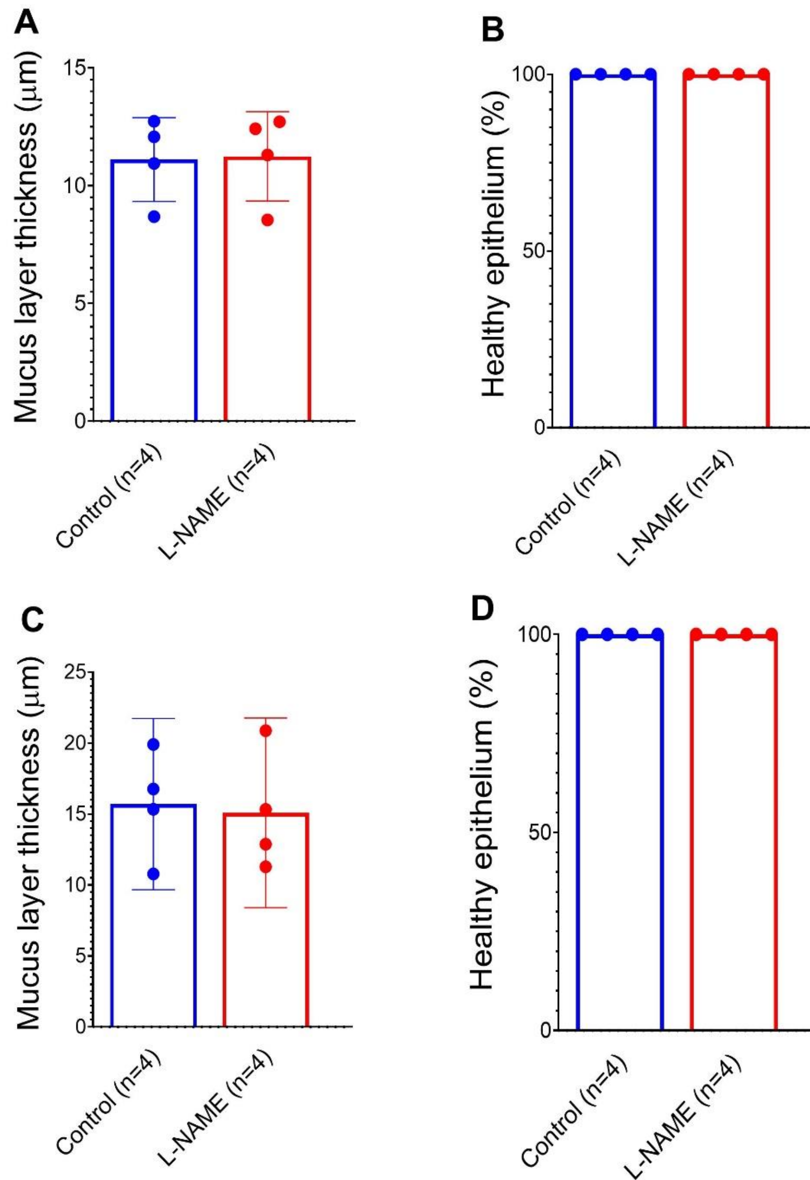

Figure. 3.7.3 Mucus layer thickness (A & C) and epithelial coverage (B & D) from 2 sections of the cecum and two sections of the colon per mouse were averaged and compared between control and treatment (L-NAME) groups during the 7-days experimental period. Bars depict the average and whiskers the standard deviation for tissue sections from a treatment group.

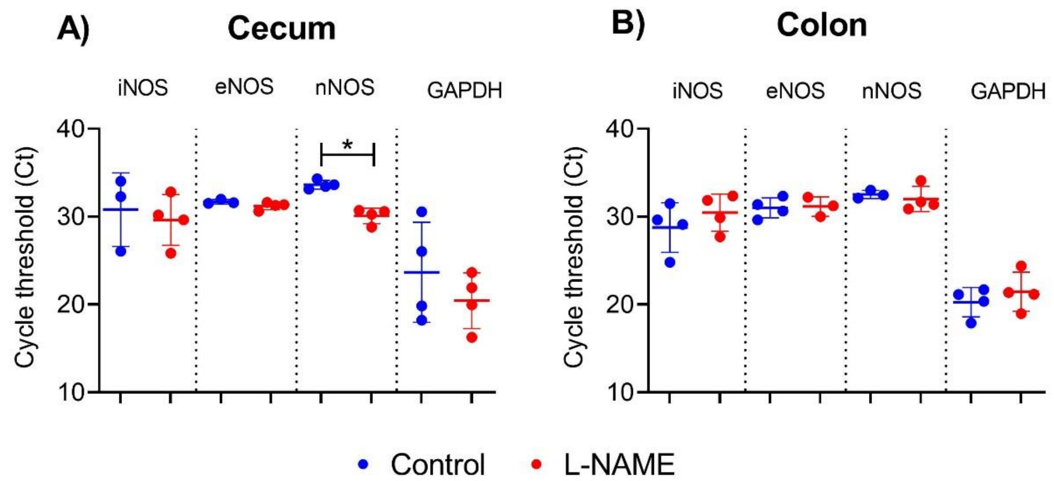

Figure 3.7.4. Gene expression data from cecum (n=4; A) and colon (n=4; B). Star denotes statistical difference between groups. Each data point represents the average from two technical replicates from samples obtained from a given mouse. Missing values are due to undetermined expressions.

3.8 Tables

Table 3.8.1 Cohort data and experimental design summary. Average daily weight gain, total water and feed intake per cage (n=4) for the 7-days experimental period.

Group	Treatment	Total water intake (ml)	Total feed intake (g)	Average daily weight gain (g)
Control (n=4)	None	95	124	0.23 ± 0.09^{a}
Treatment (n=4)	L-NAME (0.1 mg/ml)	90	104.5	0.32 ± 0.10^{a}

*Similar superscripts indicate no statistical difference within a column between lines.

Table 3.8.2 Mice clinical behavior adapted from Arras et al., 2007 and M H Ullman-Culleré and C J Foltz, 1999.

Mice clinical assessment	Score 0 (Active: +)	Score 1 (No active: -)
Spontaneous behavior	Sleeping, resting, digging, running, walking, rearing, climbing, eating, drinking, grooming, sniffing	Sudden movements, backwards movements, transient involuntary muscular contraction of any body part, kicking with hind paws
Posture	Lying, sitting, moving	Hunched, arched back, crouched
Breathing	Undisturbed, regular	Exerted irregular
Coat condition	Clean, smooth, well-groomed	Ruffled, dirty, unkempt, piloerection, hair loss (alopecia)
Eyes	Clear, bright	Discharge
Behavior after weighing	Alert, ready to take flight	Apathetic, sedated, highly aggressive, increased vocalization

Table 3.8.3 Fecal scoring chart based on Ek et al., 2017.

Score	Clinical description
0	Formed fecal pellet
1	Formed pellet with a trace mucous tail
2	Soft, mucoid feces
3	Feces with blood (+/− mucus)

Table 3.8.4 Summary of microscopic lesions colon (n=2/mouse) and cecum (n=2/mouse) H&E stained sections.

Mouse ID	Group	Colon						Cecum					
		Catarrhal inflammation		Neutrophilic infiltration		Epithelial regeneration		Catarrhal inflammation		Neutrophilic infiltration		Epithelial regeneration	
1	Control	0	0	0	0	0	0	0	0	0	0	0	0
2		0	0	0	0	0	0	0	0	0	0	0	0
3		0	0	0	0	0	0	0	0	0	0	0	0
7		0	0	0	0	0	0	0	0	0	0	0	0
5	L-NAME	0	0	0	0	0	0	0	0	0	0	0	0
6		0	0	0	0	0	0	0	0	0	0	0	0
4		1	0	0	0	0	0	0	0	0	0	0	0
8		0	0	0	0	0	0	0	0	0	0	0	0

General conclusion

Swine dysentery (SD), ileitis, and salmonellosis significantly impact pork production worldwide, affecting the performance of grower and finisher pigs. Control and treatment of these infections rely mostly on antimicrobials, which has been associated with an increased number of bacterial strains developing resistance to the drugs used. In parallel, strict measures and legislations to reduce the use of antimicrobials for livestock treatment were adopted in most countries with significant animal protein production. This scenario demanded the development of novel alternatives to antimicrobials to control and treat bacterial diseases, including those mentioned above. Organic acids, prebiotics, and phytobiotics were previously demonstrated to support intestinal function. NOS inhibitors have not yet been implemented as a therapeutic aimed at intestinal infections in swine. The overarching objective of this work was to investigate the efficacy of alternative treatments to antimicrobials in mitigating lesions associated with SD, ileitis, and salmonellosis.

In chapter 2 we evaluated the effectiveness of five non-antimicrobial compounds (D-phytochemical, F - prebiotic, L - enzyme blocker, P - blend of SCFA, and S - blend of OA) in preventing the lesions caused by *B. hyodysenteriae*, *L. intracellularis* or *S.* Typhimurium. *In vitro* porcine colon culture was used to model the infections. This was a responsible and effective approach to minimize the use of animals at this pre-clinical, screening stage. A summary of our findings was presented in Table 2.8.1. The models standardized here were effective in allowing the host-pathogen-compound interactions, as differences between control and treatment groups were observed. We hypothesize that those compounds that were deemed ineffective in this

study, or led to tissue damage, may require a pre-challenge treatment period or lower doses to induce a protective effect. These adaptations should be explored in future studies. Results from compound L, an enzyme blocker (L-NAME), revealed an exciting opportunity for the development of a novel strategy to treat infectious colitis in pigs. As it prevents the activation of NO-dependent inflammatory pathways, it blocks one of the host molecular cascades exploited by *B. hyodysenteriae,* leading to SD. While this approach was relatively successful *in vitro*, further *in vivo* testing is required. In parallel, compound F (prebiotic) was shown to help mitigate *L. intracellularis* associated lesions. Replication of these experiments using a wild-type, virulent strain is strongly recommended. In addition, we believe that the *in vitro* intestinal culture model can be further explored to investigate the early interactions of *L. intracellularis* and the host, fulfilling current knowledge gaps related to disease pathophysiology.

At this point, the pathophysiology of SD is not fully understood. This is a clear impediment to the development of control and treatment methods for the disease. In the third chapter, we investigated if oral delivery of L-NAME (compound L) to mice would induce any detrimental effects to their intestinal tract and overall health. L-NAME inhibits any NOS isoform, not only the immune system associated iNOS. Our results suggested that L-NAME administration in drinking water was safe for mice, not altering intestinal function. No change in feed or water intake was observed. Thus, weight gain was found to be statistically the same between the treatment and control groups. However, this study was limited to a small cohort of mice. In addition, *Mus*

musculus is not the species of interest for SD, and the experimental period was limited to 7 days. Additional investigations using a larger group of animals treated for longer periods are necessary to establish the safety of long-term L-NAME usage. Finally, it would be interesting to investigate differences between treated and untreated animals following challenge with *B. hyodysenteriae*. Particularly, the effect of L-NAME on MUC2 and MUC5AC expression should be investigated. Nevertheless, we were able to provide initial evidence that L-NAME may be a safe treatment option, based on the combination of our *in vivo* and *in vitro* data. This work advanced our knowledge about the gastrointestinal effects of L-NAME in healthy animals, serving as a first step towards the development of L-NAME as a therapeutic approach for SD in pigs.

In conclusion, the work presented in this thesis demonstrated that non-antimicrobial compounds are promising alternatives to mitigate lesions associated with bacterial infections in pigs and may contribute to reducing the use of antimicrobials. Additionally, identification of a novel treatment strategy (NOS blocker) *in vitro*, found to be safe when administered to healthy mice, was explored. Our findings shed a light on the future steps towards the reduction, and potential replacement, of antimicrobials for therapeutic use in swine production.

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