JÉSSICA APARECIDA BARBOSA

BRACHYSPIRA-ASSOCIATED COLITIS AND SALMONELLOSIS IN PIGS: NOVEL APPROACHES AND HOST-PATHOGEN INTERACTIONS

LAVRAS - MG
2023

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Zootecnia, área de concentração em Produção e Nutrição de Não Ruminantes, para obtenção do título de Doutor.

Orientador
Prof. Dr. Vinícius de Souza Cantarelli

Coorientadores
Prof. Dr. Matheus de Oliveira Costa
Prof. Dr. Márvio Lobão Teixeira de Abreu

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2023

Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Barbosa, Jéssica Aparecida.

Brachyspira-associated colitis and salmonellosis in pigs: novel approaches and host-pathogen interactions / Jéssica Aparecida Barbosa. - 2023.

140 p.

Orientador(a): Vinícius de Souza Cantarelli.

Coorientador(a): Matheus de Oliveira Costa, Márvio Lobão Teixeira de Abreu.

Tese (doutorado) - Universidade Federal de Lavras, 2023.

Bibliografia.

1. Swine. 2. Enteric diseases. 3. Intestinal health. I. Cantarelli, Vinícius de Souza. II. Costa, Matheus de Oliveira. III. de Abreu, Márvio Lobão Teixeira. IV. Título.

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BRACHYSPIRA E COLITE ASSOCIADAS E SALMONELOSE EM SUÍNOS: NOVAS ABORDAGES E INTERAÇÕES HOSPEDEIRO-PATÓGENO

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Zootecnia, área de concentração em Produção e Nutrição de Não Ruminantes, para obtenção do título de Doutor.

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LAVRAS - MG

2023

À minha família, em especial, à minha mãe,

Nely, pelo apoio, incentivo, compreensão e

por ser o meu maior exemplo de vida!

Dedico!

AGRADECIMENTOS

À Deus, por ser meu guia. “Deus é bom o tempo todo. O tempo todo Deus é bom”.

À minha mãe Nely, pelo amor incondicional, dedicação, respeito, e ensinamentos, e a minha família pelo apoio nessa caminhada.

Ao meu orientador, professor Vinícius Cantarelli, por todo conhecimento compartilhado, oportunidades oferecidas, orientação pessoal e profissional, por acreditar no meu potencial e me fazer pensar “fora da caixa”.

Ao meu co-orientador, professor Matheus Costa, por toda oportunidade concedida e por me fazer enxergar que posso ir além.

Aos professores Cesar Garbossa, Bruno Silva e John Harding pela disponibilidade em participar da minha defesa.

Aos colegas do NESUI e meus companheiros do ASIH (*Animal Science and Intestinal Health*) pela parceria, trabalho e amizades aqui construídas.

Ao Costa Lab (Arthur, Bárbara, Sulove, Ruwini, Maitê, Fernanda, Carolina e Lucie) e o time Harding (Juan e Roman) por todo companheirismo, desespero e risadas.

Ao Brazilian rolês e minha gangue latina por deixarem os dias mais leves no frio canadense.

À minha família canadense, Marlene e Dennis Hoehn, por todo acolhimento.

Ao meu trio, Carol e Ygor, por me segurarem todas as vezes que eu queria cair.

Aos meus companheiros, Diana, Joana e Rodrigo, por toda parceria nessa jornada.

As minhas irmãs, Angel, Iarita e Mari, que Lavras me deu quase na reta final.

À Universidade de Saskatchewan e ao Western College of Veterinary Medicine pelas portas abertas.

À Universidade Federal de Lavras pela oportunidade concedida.

Ao Departamento de Zootecnia pelo aprendizado.

Por último, mas não menos importante, a mim mesma, por ter colocado meus medos e inseguranças no “banco de trás do carro” e ter assumido o controle na direção desse desafio. Mandei bem demais!

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001.

O presente trabalho foi realizado com apoio do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

“Tenha coragem e seja gentil.”

Filme Cinderela

RESUMO

Objetivou-se verificar a associação da microbiota intestinal antes dos sinais clínicos de disenteria suína (DS); a resposta das células B a *Brachyspira* spp. e *Salmonella enterica* sorovar Typhimurium; e a utilidade da calprotectina fecal (FC) como um biomarcador de inflamação intestinal em suínos. No primeiro experimento, 60 amostras fecais foram coletadas de 15 suínos um dia após o contato com suínos inoculados via intragástrica com *B. hyodysenteriae* (d0); dois dias (d-2SD) e 1 dia (d-1SD) antes de se observar diarreia muco-hemorrágica, e no dia em que os animais desenvolveram diarreia muco-hemorrágica (MHD). Os perfis da microbiota fecal foram gerados com base na amplificação e sequenciamento do gene universal *cpn*60. Apenas um aumento no índice Chao1 em d-1SD e MHD em comparação com amostras d0 foi observado ao nível de gênero. A análise diferencial de abundância revelou que variantes de sequência única (ASV) foram modulados nos dias anteriores à observação de diarreia. Em resumo, existe alteração na microbiota fecal nos dias anteriores ao desenvolvimento da DS clínica. No segundo ensaio, células B suínas foram co-incubadas por oito (8) horas com inóculo controle negativo (n=6), *E. coli* LPS (n=6), *B. hyodysenteriae* (n=6), *B. hampsonii* (cepas virulentas e avirulentas, n = 3 em cada), *B. pilosicoli* (n=6), *B. pilosicoli* inativada (n=3), e *S.* Typhimurium (n =6). A mortalidade de células B foi avaliada usando a coloração Azul de Tripan, e a expressão de genes relacionados à ativação de células B foram avaliados através de RT-PCR. Após o período de exposição, apenas a *S.* Typhimurium e o LPS induziram aumento na mortalidade de células B. *B. pilosicoli* reduziu a expressão gênica de CD19, syk, lyn e TNF-α, quando comparado ao grupo controle negativo. Nossos achados sugerem que *B. pilosicoli* não provoca uma resposta imediata de células B independentes de células T, nem desencadeia mecanismos de apresentação de antígenos. Por outro lado, as outras bactérias são capazes de ativar diferentes genes dentro da via de sinalização de células B. No terceiro experimento, amostras fecais de suínos com colite foram coletadas de animais inoculados experimentalmente com *B. hyodysenteriae* ou controles (n = 18). Amostras fecais de suínos com enterite foram coletadas de animais inoculados com *S.* Typhimurium ou de controles (n = 14). As amostras fecais foram classificadas como: 0 = normal; 1 = fezes pastosas; 2 = fezes aquosas; 3 = diarreia mucoide; e 4 = diarreia sanguinolenta. Os níveis de CF foram analisados usando ELISA sanduíche, ensaio imunoturbidimétrico e um teste de fita rápido. A concentração de CF foi maior em amostras de leitões com colite de escore fecal 4 em comparação com escores fecais ≤ 4 usando ELISA, e em fezes com escores 3 e 4 do que ≤ 1 usando imunoturbidimetria. Independentemente do ensaio utilizado, não foram encontradas diferenças nos níveis de FC entre os escores fecais para amostras de enterite. A FC só atinge níveis detectáveis após colite, mas não enterite, indicando seu potencial papel como biomarcador na colite infecciosa em suínos e possível suporte em intervenções terapêuticas mais criteriosas.

Palavras-chave: Doenças entéricas, Saúde intestinal, Células B, Microbioma, Suínos.

ABSTRACT

This thesis aimed to verify the association of the intestinal microbiota preceding the clinical signs of swine dysentery (SD) on disease onset; to investigate the porcine T-independent B-cell response to *Brachyspira* spp. and *Salmonella enterica* serovar Typhimurium; and to assess the usefulness of fecal calprotectin (FC) as a biomarker of intestinal inflammation in *B. hyodysenteriae* and *S.* Typhimurium infected pigs. In the first trial, 60 fecal samples were collected from 15 contact pigs at: one day after contact with seeder pigs inoculated intra-gastrically with *B. hyodysenteriae* (d0); 2 days (d-2SD) and 1 day (d-1SD) before mucohaemorrhagic diarrhea was observed, and at the day when pigs developed mucohemorragic diarrhea (MHD). Fecal microbiota profiles were generated based on amplification and sequencing of the *cpn*60 universal target. Only an increase in the Chao1 index in d-1SD and MHD compared to d0 samples was observed at the genus level for the alpha diversity index. Differential abundance analysis revealed that amplicon sequence variants (ASV) were modulated in the days prior to diarrhea observation. An increase in *Alistipes dispar* and *Parabacteroides gordonii* was detected in MHD. In summary, there was an alteration in the fecal microbiota in the days prior to the development of clinical SD. In the second trial, immortalized porcine B-cells were co-incubated for 8 hours with sham-inoculum (n=6), *E. coli* LPS (n=6), *B. hyodysenteriae* (n=6), *B. hampsonii* (virulent and avirulent strains, n = 3 for each), *B. pilosicoli* (n=6), *B. pilosicoli* dead (n=3), *S.* Typhimurium (n =6). B-cell mortality was evaluated using Trypan blue, and the expression levels of B-cell activation-related genes were assessed using RT-PCR. Only *S.* Typhimurium and LPS led to increased B-cell mortality follow the exposure period. *B. pilosicoli* downregulated CD19, syk, lyn, and TNF-α, when compared to the negative control group. Our findings suggest that *B. pilosicoli* does not elicit a B-cell response, , nor does it trigger antigen presentation mechanisms. All other bacteria could activate different triggers within the T-independent B-cell pathway. In the third trial, fecal samples from pigs with colitis were collected from animals experimentally inoculated with *B. hyodysenteriae* or from sham-inoculated controls (n =18). Fecal samples from pigs with enteritis were collected from animals inoculated with *S.* Typhimurium or from sham-inoculated controls (n = 14). For both groups, fecal samples were scored as: 0 = normal; 1 = soft, wet cement; 2 = watery feces; 3 = mucoid diarrhea; and 4 = bloody diarrhea. FC levels were analyzed using a sandwich ELISA, a turbidimetric immunoassay and a point-of-care dipstick test. FC concentration were higher in colitis samples scoring 4 compared with ≤ 4 fecal scores using ELISA, and in feces scoring 3 and 4 than ≤ 1 using immunoturbidimetry. Regardless of the assay used, no differences in FC levels were found among fecal scores for enteritis samples. This initial data suggest that FC only peaks at detectable levels following colitis but not enteritis. Hence, this indicates its potential role as a biomarker of infectious colitis in pigs and possible support in judicious therapeutic interventions.

Keywords: Enteric diseases, Intestinal health, B-cell pathways, Microbiome, Swine.

RESUMO INTERPRETATIVO

Colite associada a *Brachyspira* spp. e Salmonelose em suínos : novas abordagens e interações patógeno-hospedeiro

Elaborado por **Jéssica Barbosa** e orientado por **Vinícius de Souza Cantarelli**

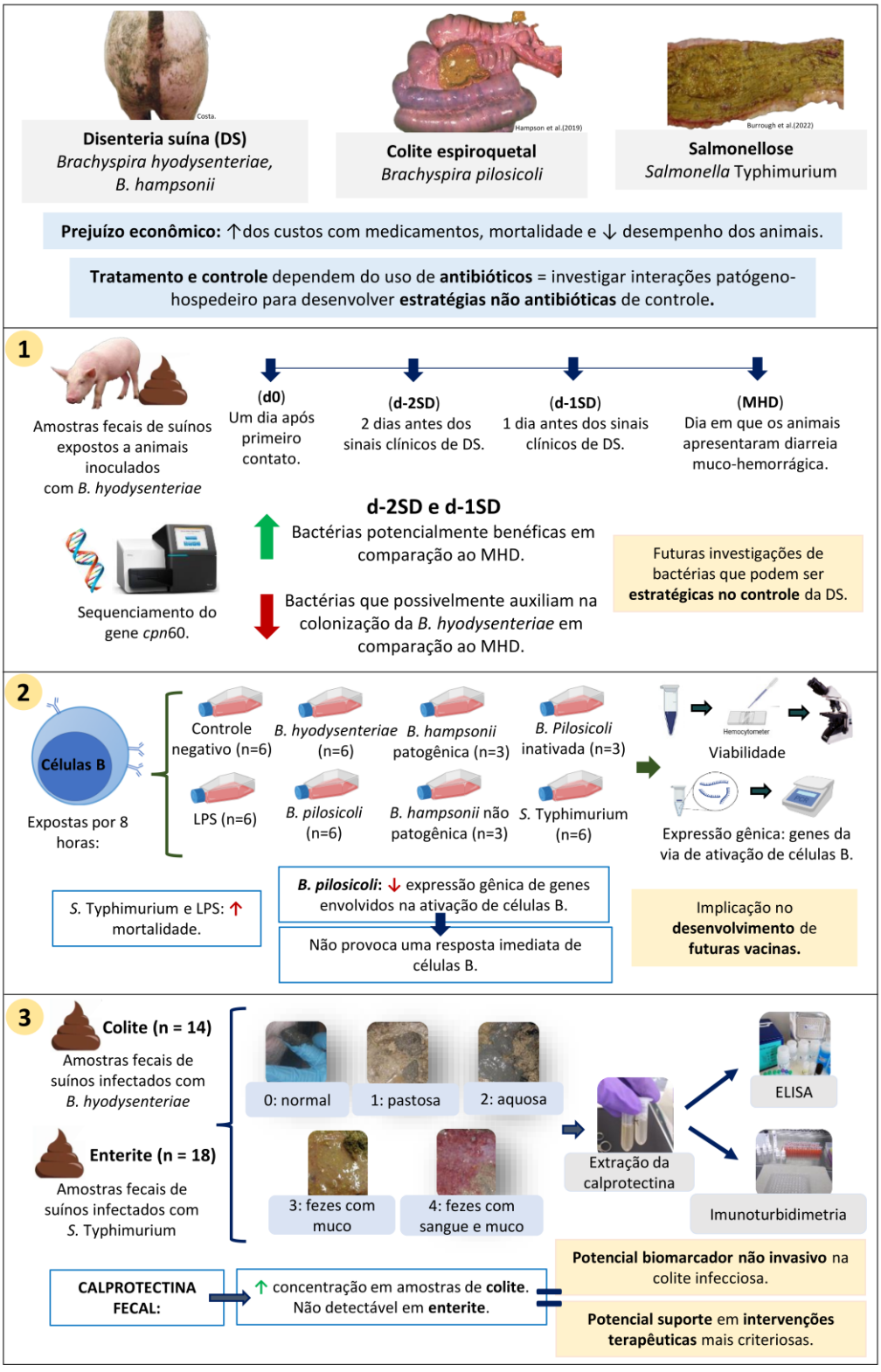
A disenteria suína, a colite espiroquetal e a salmonelose suína são doenças entéricas importantes na suinocultura. Os prejuízos econômicos decorrem do aumento nos gastos com medicamentos, mortalidade e redução no desempenho dos animais. Embora vacinas vem sendo desenvolvidas, a mitigação dessas doenças ainda depende do uso de antibióticos. Visando desenvolver futuras ferramentas não antibióticas, neste trabalho buscamos compreender a interação que ocorre entre os patógenos causadores dessas enfermidades com a microbiota intestinal e a resposta de células B. Além disso avaliamos a utilidade de um biomarcador de inflamação intestinal nas fezes de suínos com disenteria suína e salmonelose.

Experimento 1: Avaliamos se há diferença na microbiota fecal de suínos dois dias antes dos animais apresentarem diarreia com sangue, e no dia que os animais apresentam os sinais clínicos da disenteria suína. Dois dias antes da observação da doença, foram identificadas bactérias potencialmente associadas com proteção da mucosa intestinal contra a colonização da *Brachyspira hyosydenteriae*. Por outro lado, no dia em que os animais apresentaram diarreia com sangue, bactérias que provavelmente auxiliam na alteração do ambiente intestinal favorecendo a colonização e infecção pela *B. hyosydenteriae* foram identificadas. Em conclusão, existe diferença na composição da microbiota imediatamente antes e depois dos sinais clínicos da disenteria suína. Dessa forma, alguns grupos bacterianos podem ser investigados futuramente como possíveis biomarcadores para o tratamento e controle da doença na suinocultura.

Experimento 2: Foi avaliada a resposta das células B expostas a *B. hyodysenteriae, B. hampsonii* (patogênica e não patogênica), *B. pilosicoli,* e *S.* Typhimurium. Apenas *S.* Typhimurium e LPS aumentaram a mortalidade de células B. *B. pilosicoli* diminuiu a expressão de genes relacionados ao desenvolvimento e ativação das células B comparado ao controle. Todos os outros patógenos aumentaram a expressão gênica de pelo menos um desses genes. Esses achados sugerem que, a *B. pilosicoli* não provoca uma resposta imediata de células B. Isso pode implicar no desenvolvimento de vacinas para seu controle.

Experimento 3: Avaliamos a utilidade da calprotectina fecal (CF) como biomarcador de inflamação intestinal em amostras fecais de suínos infectados com *B. hyodysenteriae* (colite) ou *S.* Typhimurium (enterite). Na colite, os níveis de CF foram elevados em amostras fecais de escore fecal 4 (diarreia com sangue) em comparação com escore fecal 0 (normal), 1 (fezes macias), 2 (fezes com água) e 3 (fezes com muco). Não foram encontradas diferenças nos níveis de CF entre os escores fecais nas amostras de enterite. Diante desses resultados, podemos concluir que a CF fecal é um potencial biomarcador não invasivo útil para diagnosticar colite infecciosa em amostras fecais de suínos. Essa potencial ferramenta pode contribuir para uma abordagem antimicrobiana mais prudente e assertiva no cenário de redução e uso prudente de antimicrobianos.

RESUMO GRÁFICO



Três experimentos avaliaram a interação entre os patógenos mencionados acima, aos quais os antibióticos são amplamente aplicados em suínos, com a microbiota, a resposta suína de células B, e um biomarcador não invasivo de inflamação intestinal, respectivamente. Diante dos resultados, futuras estratégias poderão ser desenvolvidas para controlar as doenças e minimizar o uso de antibióticos.

Tese de doutorado em Zootecnia na UFLA, defendida em 25/05/2023.

INTERPRETIVE SUMMARY

Brachyspira-associated colitis and salmonellosis in pigs: novel approaches and host-pathogen interactions

Designed by **Jéssica Barbosa** and supervised by **Vinícius de Souza Cantarelli**

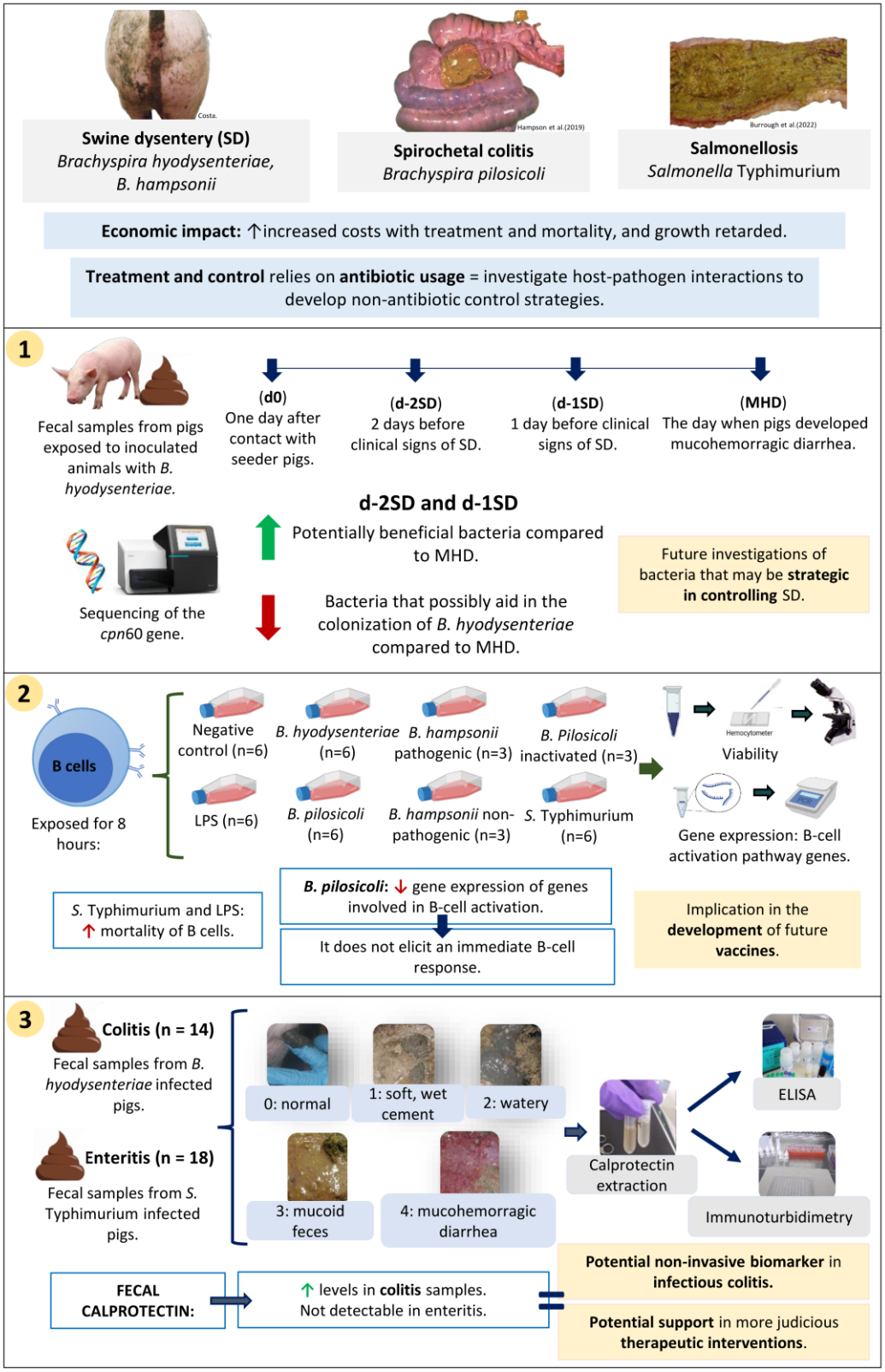
Swine dysentery, spirochetal colitis, and porcine salmonellosis are enteric diseases with global importance in the swine industry. The financial impact arises from increased production costs associated with treatment and mortality and retarded growth. Although vaccines have been developed, the mitigation of these diseases relies on antibiotics. Aiming to develop future non-antibiotic approaches to control these infections, we aimed to understand the interaction between the pathogens that cause these diseases with the intestinal microbiota, and B-cell response. Additionally, we evaluated the usefulness of a non-invasive biomarker in the feces of swine dysentery and salmonellosis affected pigs.

Trial 1: We evaluated whether there is a difference in the fecal microbiota of swine two days before the animals have bloody diarrhea and on the day the animals show the clinical signs of swine dysentery (SD). Two days before disease observation, bacteria potentially associated with the protection of the intestinal mucosa against colonization by *Brachyspira hyosydenteriae* were identified. Moreover, on the day the pigs had bloody diarrhea, bacteria that probably help alter the intestinal environment, favoring colonization and infection by *B. hyosydenteriae*, were observed. There is a difference in the microbiota composition immediately before the clinical signs of SD. Thus, specific bacterial groups can be investigated as possible biomarkers to control the disease in swine herds.

Trial 2: We investigated the response of B cells exposed to *B. hyodysenteriae*, *B. hampsonii* (pathogenic and non-pathogenic), *B. pilosicoli*, and *S.* Typhimurium. Only *S.* Typhimurium and LPS increased B-cell mortality after the exposure period. *B. pilosicoli* decreased expression of genes related to B-cell development and activation compared to control. On the other hand, all other pathogens increased the gene expression of these genes. Our findings suggest that, unlike other bacteria, *B. pilosicoli* does not elicit a B-cell response. This may imply the development of vaccines for its control.

Trial 3: We assessed the usefulness of fecal calprotectin (FC) as a biomarker of intestinal inflammation in fecal samples from swine infected with *B. hyodysenteriae* (colitis) or *S.* Typhimurium (enteritis). In colitis, FC levels were greater in score 4 (bloody diarrhea) compared to fecal score 0 (normal), 1 (soft, wet cement), 2 (watery feces), and 3 (mucoid diarrhea). No differences in FC levels were found between fecal scores for enteritis samples. Given these results, we can conclude that FC may be a potentially useful non-invasive biomarker for diagnosing infectious colitis in swine fecal samples. This potential tool may direct a more assertive antimicrobial approach in the scenario of prudent and suppressing antibiotic usage.

GRAPHICAL ABSTRACT



Three trials evaluated the interaction among the above-mentioned pathogens, to which antibiotics are widely applied in pigs, with the microbiota, the porcine B-cell response, and a non-invasive biomarker of intestinal inflammation, respectively. Given the results, future strategies can be developed to control diseases and minimize antibiotic usage.

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SUMMARY

FIRST SECTION

1 INTRODUCTION

Grower-finisher infectious diarrhea is one of the main economic challenges in the swine industry, since it affects the optimal functioning of the gastrointestinal tract, compromising the performance, health, and welfare of the pigs (JACOBSON et al., 2003; PANAH et al., 2021). The financial impact arises from retarded growth, worse feed conversion ratio, and increased production costs associated with treatment and mortality (SJÖLUND; ZORIC; WALLGREN, 2014).

Swine dysentery (SD), spirochetal colitis (SC), and swine salmonellosis (SS) are among the most common diarrheic diseases affecting growing-finishing pigs worldwide, especially in the major pork producer countries (FUNK; GEBREYES, 2004; ALVAREZ-ORDÓÑEZ et al., 2013). The aero-tolerant spirochetes *Brachyspira hyodysenteriae*, *B. hampsonii* and *B. suanatina* are associated with SD, leading to mucohemorragic diarrhea and colitis (TAYLOR; ALEXANDER, 1971; RÅSBÄCK et al., 2007; RUBIN et al., 2013) SC is caused by *B. pilosicoli*, characterized by mucoid, watery diarrhea and mild colitis (TAYLOR; SIMMONS; LAIRD, 1980). *Salmonella enterica* serovar Typhimurium leads to watery diarrhea, enteritis and dehydration in pigs, and its also a One Health concern associated with food-borne gastroenteritis in humans (HURD et al., 2001; EFSA, 2022). Despite the efforts for vaccine development against SD and SC (LA et al., 2019; CHRISTODOULIDES et al., 2022), and the improvement of available vaccines for salmonellosis (WALES; DAVIES, 2017), in practice, antibiotic therapy remains the major appliance for their mitigations (VAN DUIJKEREN et al., 2014; TENG et al., 2022).

It is well known that colonization of the intestine and development of disease by *Brachyspira* is influenced by complex interactions between the spirochaetes and the indigenous microbiota (WHIPP et al., 1979; COSTA et al., 2014; BURROUGH; ARRUDA; PLUMMER, 2017). However, there is a gap in knowledge regarding what changes the intestinal microbiota undergoes immediately preceding the appearance of clinical signs due to *B. hyodysenteriae* colonization. The T-cell independent B-cell activation takes part in the early response against pathogens and vaccine efficacy (HAVENAR-DAUGHTON et al., 2018). Studies in humans and mice models have demonstrated that some pathogens can interact with B-cell, thus impairing the humoral response (CASTRO-EGUILUZ et al., 2009; GOENKA et al., 2012). Nevertheless, how the swine enteric pathogens *Brachyspira* spp. and *Salmonella* Typhimurium can interact with B-cell impairing its response is still unknown.

Understanding the pathogen-host interactions will provide further insights to clarify pathogenesis mechanisms, as well to support improvement and development of reliable preventative and control tools to mitigate enteric disease in swine operations. The knowledge provided in this work will be supported in the immediate future, especially in the effort to direct judicious therapeutic interventions. Thus, the work described in this thesis aimed to evaluate the host-pathogen-microbiome interactions of agents of grower-finisher diarrhea to which antibiotics are the main control tool used in commercial farms.

2 LITERATURE REVIEW

2.1 Swine dysentery and spirochetal colitis in pigs

The *Brachyspira* genus comprises intestinal spirochaetes, belonging to the phylum Spirochaetes, class Spirochaetes, order Spirochaetales, being the only genus accounting for the family *Brachyspiraceae* (PASTER; DEWHIRST, 2000). In the large intestine, *Brachyspira* spp. colonizes specifically the cecum and colon, near the intestinal epithelium (STANTON, 1997), and can belong to the indigenous microbiota in healthy pigs (HAAKE, 2009; PATTERSON et al., 2013). These species have been isolated from a broad spectrum of hosts, including pigs, poultry, rodents, dog, horse, and humans (STANTON, 2006).

Brachyspira spp. are gram-negative bacteria, obligate anaerobe but oxygen tolerant, thin, helically coiled (spiral-shape), and motile (HOLT, 1978; PASTER; DEWHIRST, 2000). The intensity of β-hemolysis on blood-agar blood has been applied for its differentiation and to assess the development of disease (BURROUGH et al., 2012). *B. hyodysenteriae, B. suanatina*, and *B. hampsonii* develop strongly β-hemolysis (CHANDER et al., 2012; MUSHTAQ et al., 2015; MAHU et al., 2016). Whereas *B. innocens, B. intermedia, B. murdochii*, and *B. pilosicoli* are known as weak β-hemolytic spirochaetes (SAHEB; DAIGNEAULT-SYLVESTRE; PICARD, 1981; JENSEN; CHRISTENSEN; BOYE, 2010; PHILLIPS et al., 2010). Except for *B. pilosicoli*, all those weakly hemolytic *Brachyspira* spp. are indeed regarded mildly or non-pathogenic (HAMPSON, 2018). In pigs, the main pathogenic *Brachyspira* species causing diarrhea are *B. hyodysenteriae, B. hampsonii*, and *B. pilosicoli* (HARRIS et al., 1972; TAYLOR; SIMMONS; LAIRD, 1980; CHANDER et al., 2012). Other species, including *B. intermedia, B. murdochii, B. suaginata* and non-pathogenic *B. innocens* have been also isolated, but are less relevant causing disease in pigs (RÅSBÄCK et al., 2007; PHILLIPS et al., 2010; MUSHTAQ et al., 2015).

2.1.1 Swine dysentery

SD is one of the most production-limiting enteric disease affecting growing-finishing pigs worldwide. The financial impact concern is regarding poor feed conversion, medications costs, mortality, and animal welfare (WOOD; LYSONS, 1988; ÓZSVÁRI, 2017). It is characterized by mucohemorrhagic diarrhea and fibrinonecrotic colitis (TAYLOR; ALEXANDER, 1971; HARRIS et al., 1972). The first report was described in 1921, when SD was experimentally reproduced (WHITING; SPRAY; DOYLE, 1921). Despite that, the causative agent of SD was confirmed years later, and first named *Treponema hyodysenteriae* (TAYLOR; ALEXANDER, 1971; HARRIS et al., 1972). Thereafter, the spirochetes were renamed *Serpula* and *Serpulina*, respectively (STANTON et al., 1991; STANTON, 1992). Since then, the causative agent has been classified as *Brachyspira* spp. (OCHIAI; ADACHI; MORI, 1997).

The primary agent of SD, *B. hyodysenteriae,* is a gram-negative spirochete, with 8-10 μm in length, 0.3–0.4 μm in diameter, strongly β-haemolytic, aerotolerant and positive indole production (OCHIAI; ADACHI; MORI, 1997). Lately, a novel strongly β-haemolytic characterized as *B. hampsonii* was found in outbreaks in Canada, United States, and Europe causing clinical disease indistinguishable from SD (HARDING et al., 2010; CHANDER et al., 2012; MAHU et al., 2014). Experimental inoculations have been confirmed the similarity by reproducing mucohemorrhagic diarrhea and colitis in pigs challenged with *B. hampsonii* isolates (BURROUGH et al., 2012a; COSTA et al., 2014; RUBIN et al., 2013).

2.1.1.1 Epidemiology

SD has a worldwide distribution, occurring mainly in countries with a higher density of pig production. Around the 90s, its prevalence drastically decreased due to biosecurity improvements and global swine industry technification (MIRAJKAR; GEBHART, 2014). Recently, SD has re-emerged in many countries, including Canada, United States, Brazil, European, and Asian countries (HARDING et al., 2010; BURROUGH, 2013a; KAJIWARA et al., 2016; LÖBERT et al., 2016; SATO et al., 2022). SD can affect all ages, but the main target is growing and finishing pigs at approximately 8–26 weeks (BURROUGH, 2017). The prevalence ranging from 0% to 40%, morbidity reaching 90%, and a mortality rate of 50-90% has been reported. Onset has been reported mainly after removing antibiotics, occurring cyclically in the herd (ALVAREZ-ORDÓÑEZ et al., 2013).

The transmission occurs via the fecal-oral route by ingesting feces from infected animals. Moreover, asymptomatic animals are an important source of the organism into the environment. Contaminated clothes, footwear, trucks, and other fomites are also good sources of spirochaetes (HAMPSON; BURROUGH, 2019). Apart from the etiological agent, risk factors such as stress, diet, vectors, the dosage of the agent, and virulence of strains have been associated with disease condition (ZEEH; VIDONDO; NATHUES, 2020). Poor husbandry practice and intestinal microbiota may influence the outcome of infection (WHIPP et al., 1979; ALVAREZ-ORDÓÑEZ et al., 2013; COSTA et al., 2014b).

2.1.1.2 Pathogenesis and clinical signs

As a multifactorial disease, the pathogenesis of SD is complex and has not yet been fully elucidated. *B. hyodysenteriae* colonize the mucosal surface of the cecum and colon, and subsequently settles between the epithelial and goblet cells (GLOCK; HARRIS; KLUGE, 1974; JOENS et al., 1981). Also, the pathogen can establish within crypts and on the luminal surface (KENNEDY; STRAFUSS, 1976). Whether its attachment and penetration are essential for pathogenesis has not yet been fully clarified (GLOCK; HARRIS; KLUGE, 1974; TEIGE et al., 1981). Virulence factors, including mucus chemotaxis, motility, adherence, hemolysin, and endotoxic activity of lipooligosaccharides (LOS), might be the main actors for disease development (MILNER; SELLWOOD, 1994; KENNEDY; YANCEY, 1996; BELLGARD et al., 2009).

It has been shown that NADH activity contributes to pathogen colonization and pathogenic ability, as it consumes oxygen and protects *B. hyodysenteriae* cells from oxygen toxicity (STANTON et al., 1999). The periplasmic flagellar activity assists *B. hyodysenteriae* corkscrew-like motility for penetration and colonization of the mucus layer. The flaA and flaB genes were shown to be involved in this function, since lowered mobility and colonization were observed in mutants lacking flaA and flaB (KENNEDY; ROSEY; YANCEY, 1997). LOS are present on the outer envelope of the *Brachyspira* spp. and has also been implicated as a potential virulence factor (NUESSEN; JOENS; GLOCK, 1983; GREER; WANNEMUEHLER, 1989), mainly inducing local inflammation in the colon (JOENS et al., 1985; HALTER; JOENS, 1988).

The production of hemolysins has been considered the main virulence factor of *B. hyodysenteriae*. As previously mentioned, the intensity of hemolysis has been used to differentiate pathogenic from non-pathogenic *Brachyspira* spp., and the greater colonic inflammation generally is associated with strongly β-hemolytic strains (BURROUGH et al.,

2012). To date, seven potential hemolysin-encoding genes have been identified: tlyA, tlyB, tlyC, and the acyl carrier protein containing β-hemolysin (hylA) (TER HUURNE et al., 1994; HSU et al., 2001), genes encoding putative hemolysin III (BHWA1_00446 and BHWA1_01870) and a putative hemolysin CBS domain (BHWA1_00587) (BELLGARD et al., 2009). Although *in vivo* assays have confirmed the involvement of the identified hemolysins in the occurrence of SD, the pathogenic activity of those remains unclear (TER HUURNE et al., 1992; HYATT et al., 1994).

The strict association with the mucus present on the surface of the epithelium is important for *Brachyspira* infection (KENNEDY et al., 1988). *B. hyodysenteriae* were found to regulate the mucin composition in the colon, altering the mucus layer organization with lacks striations, which may increase binding sites for the spirochetes (QUINTANA-HAYASHI et al., 2015, 2019; VENKATAKRISHNAN et al., 2017; LIN et al., 2023). This structural disorganization at the early infection leads to reduced expression of sulfated mucins, sialomucins, and mucigen within globet cells at the base of crypts. Hence, further excessive and remarkable mucin accumulation is observed at the apex of the spiral colon (WILCOCK; OLANDER, 1979; WILBERTS et al., 2014a).

Investigation in inoculated pigs with *B. hyodysenteriae* or *B. hampsonii* has shown that these changes are associated with enhanced gene expression encoding the gel-forming mucin MUC5AC via *de novo* synthesis (WILBERTS et al., 2014a; QUINTANA-HAYASHI et al., 2015; LIN et al., 2023). Curiously, unlike MUC2, the expression of MUC5AC is not constitutively detected in the colon of healthy pigs (QUINTANA-HAYASHI et al., 2015; USHIO et al., 2020). The presence of neutrophilic infiltration and the expression of pro-inflammatory cytokine IL-1β and IL-17 has been implicated in MUC5AC stimulation (KIM et al., 2002; WILBERTS et al., 2014a; LIN; ARRUDA; BURROUGH, 2021).

At the early stage of the disease, superficial mucosal necrosis, vascular congestion and dilatation, abundant mucus accumulation, edema, and neutrophils in the lamina propria and crypts can be observed (TEIGE; NORDSTOGA, 1979; WILCOCK; OLANDER, 1979). As the disease progresses, exfoliation of the epithelium and the blood cells are remarkable into the lumen, leading to fibrinohemorrhagic colitis (GLOCK; HARRIS; KLUGE, 1974; WILBERTS et al., 2014a). The gross lesions can be multifocal and are limited to the cecum and colon. Thin, fluid-filled colon with serous hyperemia and mesocolon edema, associated with mucus, hemorrhage, fibrinous exudate, and mucohemorragic feces, are typical lesions at the necropsy (BURROUGH, 2017).

As a result of this injury, diarrhea is driven by the complete abolition of water, sodium, and chloride electrolytes absorption due to failures in membrane transport processes and epithelial destruction (ARGENZIO; WHIPP; GLOCK, 1980). Excessive mucin production may also be involved in increased sodium and potassium (WILCOCK; OLANDER, 1979). Diarrhea usually starts with moderate mucus, watery feces and progress to mucohemorragic diarrhea (HAMPSON; BURROUGH, 2019).

At experimental conditions, the onset of clinical signs is observed at 7 – 10 days post-inoculation with *B. hyodysenteriae* (WILCOCK; OLANDER, 1979), and between 4-5 days post-challenge for *B. hampsonii* (RUBIN et al., 2013b; WILBERTS et al., 2014b). Spirochetes may be found in the feces 1- 4 days before the diarrhea onset (KINYON; HARRIS; GLOCK, 1977; COSTA et al., 2014a). The disease usually spreads gradually; the incubation period ranges from 4 days to 3 months. The onset between 10 to 14 days in naturally exposed pigs is reported (HAMPSON; BURROUGH, 2019). The clinical signs are associated with dehydration, including weakness, depression, sunken eyes, weight loss, and hollow flanks. Metabolic acidosis and hyperkalemia increase dehydration, and mortality may occur in severely affected pigs (HAMPSON; BURROUGH, 2019).

2.1.1.3 Influence of microbiota on swine dysentery onset

The synergistic association between intestinal microbiota and spirochetes has been extensively investigated. The first studies dated from the 70s have shown that *B. hyodysenteriae* requires the presence at least of one microorganism for the disease expression (MEYER; SIMON; BYERLY, 1975; HARRIS et al., 1978; WHIPP et al., 1979; WANNEMUEHLER; DORN, 2003). Using the gnotobiotic pigs model, colonic lesions characteristic of SD were reported when the animals were co-inoculated with *B. hyodysenteriae*, *Fusobacterium necrophorum,* and *Bacteroides vulgatus* (HARRIS et al., 1978). On the other hand, gnotobiotic pigs inoculated with B. hyodysenteriae alone failed to reproduce the disease (MEYER; SIMON; BYERLY, 1974; HARRIS et al., 1978). The same was noticed in pigs co-inoculated with *B. hyodysenteriae*, *F. necrophorum*, *B. vulgatus*, a *Clostridium* species, or *Listeria denitrificans*, but not with the spirochete alone (WHIPP et al., 1979). Indeed, this initial discovery opened the door for recent investigations into the relationship between the indigenous microbiota and *Brachyspira* spp. (PALLERONI, 1997; NOCKER; BURR; CAMPER, 2007). Nevertheless, the development of culture-independent methods in the 1980s provided a new perspective on the composition, distribution, diversity, and host-related functions of the microbiota (PACE et al., 1986).

Recent studies applying high throughput sequencing methods have demonstrated a lower Bacteroidetes:Firmicutes ratio linked with mucohaemorrhagic diarrhea clinical signs after experimental inoculation (COSTA et al., 2014b). Further, differences in microbial profile in pigs inoculated with *B. hyodysenteriae* or *B. hampsonii* that develop SD, compared with non-developed SD were assessed. *Campylobacter*, *Mogibacterium*, *Brachyspira*, and *Desulfovibrio* spp. were differential features in SD-pigs, while *Bifidobacterium* spp. and *Lactobacillus* were predominant in pigs without colitis (BURROUGH; ARRUDA; PLUMMER, 2017). Interestingly, the genera found in SD-pigs have been associated with PEDv diarrhea (KOH et al., 2015), colitis in humans (ROWAN et al., 2010), and the degradation of sulfate mucins (COUTINHO et al., 2017). Thus, it is evident that specific microorganisms are able to interact with spirochaetes, reflecting intestinal dysbiosis and diarrhea onset.

Diet is another component explored in the occurrence of SD (SIBA; PETHICK; HAMPSON, 1996; PLUSKE et al., 1998; HANSEN et al., 2010, 2011; HELM et al., 2020). Nevertheless, contradictory results regarding its influence have been reported (KIRKWOOD et al., 2000; LINDECRONA et al., 2003; LEE et al., 2022). On the influence of diet on the intestinal microbiota and *Brachyspira* spp. interaction, lowly fermentable fiber (20% corn distillers dried grain with solubles, DDGS), induced an increased in *Shuttleworthia*, *Ruminococcus torques*, and *Mogibacterium*, which potentially benefit *B. hyodysenteriae* colonization (HELM; GABLER; BURROUGH, 2021). Contrariwise, highly fermentable fiber, specifically promoting acid-lactic and butyric acid-producing bacteria, has been associated with protection against SD. For example, pigs fed a fructan-rich diet had higher proportions of *Bifidobacteria* and *Megasphaera* that inhibit colonization of *B. hyodysenteriae* compared to a resistant carbohydrate diet (MØLBAK et al., 2007).

2.1.2 Spirochetal colitis

In pigs, the disease caused by *B. pilosicoli* is called porcine colonic spirochetosis or porcine intestinal spirochetosis (PIS). The first description of *B. pilosicoli* occurred in the 1980s after a weakly hemolytic strain (P43/6/78) was isolated in feces from pigs (TAYLOR; SIMMONS; LAIRD, 1980). Since then, the spirochete has been isolated from humans (HARLAND; LEE, 1967; HOVIND-HOUGEN et al., 1982), and animals, including chickens, ducks, turkeys, rodents, dogs, and birds (DAVELAAR et al., 1986; OXBERRY; TROTT; INFECTION, 1998; SHIVAPRASAD; DISEASES, 2005; BACKHANS; JOHANSSON, 2010). *B. pilosicoli* has the same phenotypic characteristics as other *Brachyspira* spp. It has two

sets of periplasmic flagella conferring corkscrew-like motility, with 5–11 μm in length and 0.2–0.4 μm in width (HAMPSON; BURROUGH, 2019). The fastidious slow growth in anaerobic conditions justifies the 3 to 5 days to form a zone of weak hemolysis on the surface of blood agar (BROOKE et al., 2000).

PIS has been reported in many countries, but epidemiological studies about its prevalence are sparse. In Danish finishing herds, its prevalence ranged from 5-10%, isolated in 15 of 79 herds (19.0%) (STEGE et al., 2000). In Poland, *B. pilosicoli* was detected in 13.7% (13/95) of herds, considering a low level of occurrence (DORS; CZYŻEWSKA-DORS; WOŹNIAKOWSKI, 2019). Herds with higher PIS cases also have been associated with mixed diarrhea infections, compared with farms without co-diarrhea occurrence (MØLLER et al., 1998; STEGE et al., 2001). In German, *B. pilosicoli* were found positive in 73.4% of farms with *L. intracellularis* and *B. hyodysenteriae* mixed infections (REINER et al., 2011).

As for SD, PIS also affects growing and finishing pigs. However, its clinical manifestation is different from that observed in SD (HAMPSON; BURROUGH, 2019). The spirochete is transmitted orally. Carriers, feral pigs, and other animals are sources of the introduction of infection (OXBERRY; TROTT; INFECTION, 1998; PHILLIPS et al., 2009). Moreover, contaminated fomites and effluent pond water were assigned as an infection source (OXBERRY; HAMPSON, 2003). Changes in diet, antimicrobials removal, or other stress are critical for the appearance of clinical cases in weaned, growing and finishing pigs (NARESH; HAMPSON, 2011; HAMPSON, 2018).

2.1.2.1 Pathogenesis

The complete pathogenesis of spirochetal colitis remains unclear, and few disease outcome-related features have been identified. *Brachyspira* species are actively mobile due to periplasmic flagella that drive the “corkscrew-like” twisting motility to penetrate the mucus layer (CHARON et al., 1992). The swimming speed and wave frequency showed in a two-directional-illuminated dark-field microscopy is one of the factors that sustain *B. pilosicoli* in a viscous environment (NAKAMURA et al., 2006). The interaction between membrane lipoproteins, such as variable surface proteins (Vsp) and specific receptors, contributes to its attachment to the luminal surface of mature apical enterocytes, forming the “false brush border” (TROTT; HUXTABLE; HAMPSON, 1996; TROTT et al., 2001). The production of enzymes from the sialidase family-like proteins (*nanA*, BP951000_2021, BP951000_2022, and BP951000_2023) (WANCHANTHUEK et al., 2010) and, among others, subtilisin-like serine

protease (DASSANAYAKE et al., 2004), trigger the hydrolyze of the colonic mucus layer allowing *B. pilosicoli* penetration.

Once attached to the surface of the enterocytes, the pathogen triggers colitis and diarrhea. The diarrhea onset has been associated with physical disruption of the normal water and electrolyte absorption process caused by massive colonization of the spirochete on the colonic epithelial surface (RODGERS et al., 1986; GAD et al., 2010). Under experimental conditions, the incubation period can reach 20 days, and diarrhea may last from 2 to 7 days, with pathogen shedding occurring within 2 to 7 days (HAMPSON, 2018). Pigs have been shown to have moderate colitis and watery to mucoid diarrhea. The presence of mucus, rare flecks of blood, and color alterations of feces to green or brown have been frequently observed (HAMPSON; BURROUGH, 2019). The disease will reflect a significant loss of performance that leads to poor feed conversion rate and lower slaughter weight (THOMSON; SMITH; MURRAY, 1998).

In naturally or inoculated pigs, macroscopic lesions are attributed to the cecum and colon. The presence of fluid and edema on the serous surface is observed, accompanied by increased mesenteric lymph nodes. Likewise, mild congestion and hyperemia of the mucosa, mucoid content, and necrotic erosions may appear (JENSEN; BOYE; MØLLER, 2004; HAMPSON, 2018). Overall at the microscopy, thickening, hyperemia, and edema of the mucosa, dilation, and elongation of the crypts with neutrophilic infiltrates and lymphocytes in the lamina propria are observed mainly in the mucosa and submucosa layer (THOMSON et al., 1997; DUHAMEL, 2001).

2.1.3 Swine dysentery and spirochetal diagnosis

The observation of clinical signs associated with macroscopic changes and histological findings will determine the success of the final diagnosis. Importantly, *Lawsonia intracellularis, Salmonella* spp., *Escherichia coli, Clostridium perfringens* type C, and *Trichuris suis* are differential diagnoses for *Brachyspira* spp. infections (HAMPSON; BURROUGH, 2019).

Traditional bacterial culture is the gold standard for diagnosing of SD and PIS. *Brachyspira* spp. has a fastidious growth, and are isolated on a selective blood agar medium, requiring an anaerobic environment at 42ºC for two - four days (STANTON; LEBO, 1988). In histological sections, Warthin-Starry silver staining and immunohistochemistry (IHC) techniques are often used to assess the presence of *Brachyspira* spp. (PAULOVICH et al., 2004; BURROUGH et al., 2012). Fluorescent *in situ* hybridization (ISH) using molecular probes

targeting DNA or RNA on formalin-fixed tissue can achieve higher sensitivity and provide rapid identification than culture (BURROUGH et al., 2013).

PCR-based methods have been developed and are routinely used to confirm diagnostic culture isolates. The multiplex or duplex PCR detecting different spirochetes has been an advantageous method for quantification in pigs (BORGSTRÖM et al., 2017). The common targets for *Brachyspira* spp. are the 16S rRNA, 23S rRNA and *nox* gene encoding NADH oxidase (ROHDE; HABIGHORST-BLOME, 2012; BORGSTRÖM et al., 2017; ROJAS et al., 2017). Despite that, the *cpn60* gene has been shown to be more phylogenetically informative than 16S rRNA and *nox* gene (LINKS et al., 2012; ROHDE et al., 2019). Recently, a novel TaqMan 5-plex real-time PCR targeting *cpn60* and *nox* gene was developed to identify *B. hyodysenteriae*, *B. pilosicoli*, *B. suanatina*, and *B. hampsonii* (SCHERRER; STEPHAN, 2021). Although serological tests for monitoring herds exposed to *B. hyodysenteriae* or *B. pilosicoli* has been described (SONG; FREY; HAMPSON, 2012; SONG et al., 2015; HAMPSON et al., 2016), the vast serodiversity and reactivity among *Brachyspira* species is the major gap of serology with high sensitivity and specificity for SD diagnosing (HERBST et al., 2017).

2.1.4 Treatment and control measures

Improvements in management and biosecurity are the main key factor in reducing the exposure risks for SD and PIS in swine herds. When adequately implemented, it can control and prevent the disease from spreading within and between farms (ALVAREZ-ORDÓÑEZ et al., 2013; BURROUGH, 2013b; FABLET, 2018). Elimination by total or partial depopulation associated with biosecurity measures has been implemented (NEIRYNCK et al., 2020).

Treatments relies on antimicrobials. Pleuromutilins (tiamulin and valnemulin), lincomycin, and macrolides (tylosin and tylvalosin) has been used (VAN DUIJKEREN et al., 2014; HAMPSON; BURROUGH, 2019). Tiamulin, valnemulin, carbadox, dimetridazole, and lincomycin are particularly important and used in different countries (PRINGLE; LANDÉN; FRANKLIN, 2006; PRINGLE et al., 2012). However, the resistance of *B. hyodysenteriae* has increased for tiamulin, lincomycin and tylosin (HAMPSON et al., 2019; STUBBERFIELD et al., 2020). Decreased susceptibility to lincosamides and macrolides has been documented for *B. pilosicoli* (ARNOLD et al., 2022).

Multiple vaccines has been evaluated to control SD. Among them, attenuated bacterins (HUDSON et al., 1976; HAMPSON; ROBERTSON; MHOMA, 1993; DIEGO et al., 1995), avirulent vaccines (MAHU et al., 2017; LA et al., 2019), subunits and recombinant vaccines (LA et al., 2004; HOLDEN; COLOE; SMOOKER, 2008), and a reverse method approach

(SONG et al., 2009; CHRISTODOULIDES et al., 2022) has been assessed. To control PIS infection, recombinant vaccines (MOVAHEDI; HAMPSON, 2007, 2010), inactivated bacterins (HAMPSON et al., 2000), and subunit vaccines (CASAS et al., 2017) has been explored. Despite extensive efforts, only partial cross-protection against strains of different serogroups has been reported (HAMPSON; BURROUGH, 2019), and to date, commercial vaccines are not available yet.

2.2 Salmonellosis associated with *Salmonella* Typhimurium in pigs

Given the significant number of foodborne cases and outbreaks yearly, Salmonellosis is a critical topic in world public health. In this perspective, *Salmonella* enterica serovar Typhimurium is highlighted due to its wide distribution in swine production and zoonotic importance (DEANE et al., 2022; EFSA, 2022). Moreover, the One-Health concern has been increasingly associated with the emergence of multidrug-resistant *Salmonella* spp. strains in the pork chain to the highest priority clinical human antimicrobials (JIANG et al., 2019; LAUTERI et al., 2022).

Salmonella enterica belongs to the family *Enterobacteriaceae*. It is a gram-negative rod-shaped bacteria (2–5 µm long by 0.5–1.5 µm wide), non-lactose fermenting, and facultative anaerobic intracellular, with a motile drive by peritrichous flagella (PENNER, 1988; FINIAY; FALKOW, 1989). More than 2600 serovars have been registered, and *Salmonella* can be isolated from the intestinal tract of a wide range of farm and wild animals and humans (CHAUDHURI et al., 2013; HORTON et al., 2013; CAMPOS et al., 2019).

S. Typhimurium has become endemic in swine herds and isolated from pigs worldwide, especially in the major swine-producing countries (MORNINGSTAR-SHAW et al., 2016; DOS et al., 2019; FERRARI et al., 2019). The fecal-oral route is the main route of transmission. Low biosecurity/hygiene (BELOEIL et al., 2007), contaminated feed, equipment, fomites, rodents, pets, and wild birds infected are risk factors for infection in swine herds. Nevertheless, subclinical carriers persistently infected are the most significant source of infection and dissemination (BOYEN et al., 2008), shedding *Salmonella* under stressful situations and being a contamination risk at slaughter (HURD et al., 2001; MASSACCI et al., 2020).

2.2.1 Pathogenesis

The pathogenesis of *Salmonella* Typhimurium infections is complex. The infection process involves establishment in the intestinal lumen, invasion of epithelial and other cells, and subsequent dissemination to lymph nodes and organs (MARTINS et al., 2013). To establish

infection, *S.* Typhimurium has clever strategies to survive at low stomach pH levels and reach the intestine. Its acid tolerance response (ATR) mechanism given by a pH homeostasis system and acid shock proteins (FOSTER, 1993; AUDIA; WEBB; FOSTER, 2001), along with stress, may ensure its successful invasion into the intestinal mucosa of the distal parts of the intestine (MIKKELSEN et al., 2004; RYCHLIK; BARROW, 2005).

Virulence genes located at *Salmonella* pathogenicity islands (SPI) play a significant role in cell invasion and intracellular pathogenesis (MARCUS et al., 2000). At least 12 SPI have already been characterized, encoding genes involved in invasion and survival in epithelial cells, triggering fluid secretion, invasion and survival in macrophages, and protection against oxidative explosion (HENSEL, 2004; EHRBAR; HARDT, 2005; GERLACH; TIERARZTLICHE, 2007; RUSHING; MICROBIOLOGY, 2011). One of the most important virulent factors is the expression of many fimbrial adhesins, which allow *Salmonella* attachment to the apical membrane of enterocytes and other cells (BÄUMLER; TSOLIS; HEFFRON, 1997; VAN DER VELDEN et al., 1998; REHMAN et al., 2019). The presence of lipopolysaccharide (LPS), the expression of invasins, and the Vi antigen are determinants of virulence that help in its survival and intracellular replication (VIRLOGEUX-PAYANT; POPOFF, 1996; HUANG; DUPONT, 2005).

There are several routes for the dissemination of *Salmonella* typhimurium, including microfold cells (M cells), epithelial cells, macrophages, and dendritic cells (LI, 2022). The Peyer's patches have been reported as the main portal of the bacteria entry into the submucosa (JONES; GHORI; FALKOW, 1994; SCHAUSER; OLSEN; LARSSON, 2004). The pathogen migration will be driven by monocyte-derived cells in the gut-associated lymphoid tissue. This allows its dissemination through the Peyer's patches (PP) and mesenteric lymph nodes (MLN) (TAM et al., 2008). Producing cytokines by innate immune cells into the invasion site will be essential (ECKMANN; KAGNOFF, 2001). However, *Salmonella* has strategies to evade the innate and adaptive immune response of the host, allowing its survival and spread (BROZ; OHLSON; MONACK, 2012). Thus, the pathogen can also reach systemic organs such as the liver, spleen, and hematogenous routes exploiting migratory dendritic cells (CHEMINAY et al., 2002).

It has been shown that within a few hours of oral inoculation, *S.* Typhimurium and other serotypes can be observed and detected in high concentrations within enterocytes, cytoplasm, lymph nodes, liver, spleen, intestinal contents, and feces (HURD et al., 2001; LOYNACHAN et al., 2004). Epithelial damage and inflammation at the ileum mucosa can be observed a few hours after ingestion (COLLADO-ROMERO et al., 2012). The interaction between SPI-1 type

three secretion system (T3SS), IL-8 cytokine, and *Salmonella* B external proteins (SopB) is crucial for the diarrhea onset. During the epithelial invasion, the SIP-1 effector SipA protein triggers neutrophil recruitment following IL-8 release, enhancing epithelial inflammation and tissue damage (MCCORMICK et al., 1995; UTHE et al., 2007; VOLF et al., 2007). Through the intracellular release of SopB and consequent increase in intracellular concentrations of inositol 1,4,5,6-tetrakisphosphate (Ins(1,4,5,6)-P4), chloride secretion increases contributing to the development of diarrhea (NORRIS et al., 1998; MARCUS et al., 2002; DRECKTRAH et al., 2005).

Clinical symptomatology of *S.* Typhimurium course with the development of enterocolitis associated with watery diarrhea, without blood or mucus. Pigs can be febrile, prostrated, dehydrated with anorexia transient and decreased feed intake. Furthermore, an increase in *S.* Typhimurium shedding in feces is observed (RODRIGUES et al., 2021). Diarrhea usually lasts three to seven days for individual pigs, and the disease may spread within a few days. The low mortality is given by the rapid clinical recovery of most pigs, and intermittent shedding for at least five months has been reported for remaining carriers (GRIFFITH; CARLSON; KRULL, 2019). Classical macroscopic lesions are thickened turgid and hyperemic intestine walls, with pseudomembranes and adherent yellow-tan fibrinonecrotic exudate. The chronicity of the disease leads to button ulcers in the spiral colon. Mesenteric lymph nodes are dilated and inflamed (GRIFFITH; CARLSON; KRULL, 2019).

2.2.2 Diagnosis, treatment and control

Despite the availability of rapid molecular approaches, bacteriological methods associated with macroscopic lesions are still the main diagnostic tool for Salmonellosis. A pool of ileocecal lymphnodes, ileum, and colon may be satisfactory for isolation in active cases (GRIFFITH; CARLSON; KRULL, 2019). Recently a new multiplex PCR assay was validated to differentiate *S.* Typhimurium from *Salmonella* 1,4,[5],12:I:, targeting the fljB-hin region and *mdh* gene (HONG et al., 2023). Serology based on ELISA test using LPS antigen and/or meat juice to detect antibodies has been described as a surveillance approach (PROUX et al., 2000; SZABÓ et al., 2008).

Controlling *Salmonella* generally requires multiple efforts. Preventing *Salmonella* from entering the farm and reducing the number of infected animals by following basic biosecurity and management practices, results in the low spread of the disease (VIDIC et al., 2015). Prophylactic use of antimicrobials is necessary for controlling *Salmonella* outbreaks and spreading among herds. As previously mentioned, the emergence of swine multidrug resistance

for mainly clinical antimicrobials for humans has become a worldwide concern (MARCHELLO; CARR; CRUMP, 2020; ROASTO et al., 2023). *Salmonella* serotypes resistant to quinolones and beta-lactams was isolated from slaughtered pigs (JIANG et al., 2019). *Salmonella* resistant to cephalosporin without previous use was isolated from piglets (CAMERON-VEAS et al., 2018). Moreover, isolates from pigs with diarrhea were resistant to ampicillin, sulfamethoxazole, and tetracycline (SU et al., 2018).

So far, commercial vaccines against *Salmonella* targeting sows (SMITH et al., 2018; PEETERS et al., 2020; VAN DER WOLF et al., 2021), piglets (HUSA et al., 2009; FARZAN; FRIENDSHIP, 2010; MOURA et al., 2021; SCHMIDT et al., 2021), or fattening pigs (DENAGAMAGE et al., 2007; PEETERS et al., 2020) are available in many countries, focusing not only in preventing clinical salmonellosis but also in decrease shedding and contamination of carcasses at slaughter. Despite the efforts, cross-protection induction between different serovars is still challenging in vaccine development.

2.3 Activation of T-cell-independent B-cell response against pathogens

The art of differentiating into long-lived memory or antigen-specific immunoglobulin (Ig)-producing plasma cells makes B-cell lymphocytes at the centre of the adaptive humoral immune response (HOFFMAN; LAKKIS; CHALASANI, 2016). These functions highlight the role of B cells in protective immunity against reinfection and in vaccine response (HAVENAR-DAUGHTON et al., 2018). Apart from their primary role, mature B cells are professional antigen-presenting cells (APCs) (CLARK et al., 2004; HUA; HOU, 2020). Moreover, through cytokine production, B-cells can regulate the route of immune response in antibody-independent mechanisms (LUND, 2008). B-cells can express a vast number of intra and extracellular receptors, including B-cell receptor (BCR), Toll-like receptors (TLRs), dectin-1, and complement receptors (CR1 [CD35], CR2 [CD21]), capable of recognizing and uptake bacterial antigens (CASTAÑEDA-SÁNCHEZ et al., 2017). These receptors operate as a cell-intrinsic bridge between innate and adaptive immune mechanisms (RAWLINGS et al., 2012; BUCHTA; BISHOP, 2014).

T cell-independent (TI) B-cell response is critical for early protection against pathogen invasion (FAGARASAN; HONJO, 2000). Non-proteinaceous antigens, including bacterial polysaccharides and lipopolysaccharide structures or epitopes of the viral particle, mediate extensive cross-linking of antigen receptors (VOS et al., 2000). Consequently, there is stimulation and intense production of low-affinity antibodies by B-1 and marginal zone B cells, mostly belonging to the IgM and no class-switched (BOES et al., 2000; EHRENSTEIN;

NOTLEY, 2010). Signal transduction via BCR is critical for TI immune response. The cross-linking of BCR by multivalent antigens induces a variety of complex signaling cascades, resulting in the regulation of gene expression and cytoskeleton reorganization. Therefore, influencing the B-cell fate within germinal centers (Figure 1) (PIERCE; LIU, 2010; KIM et al., 2019).

The BCR comprises a membrane-bound immunoglobulin (Ig) with two heavy (H) and two light (L) chains, and the heterodimeric signaling subunit Ig-α/Ig-β (CD79a/CD79b) (RETH; WIENANDS, 1997). Each CD79a and CD79b contains, at the basis of their aminoacids sequence in the cytoplasmic tails, an immunoreceptor tyrosine-based activation motif (ITAM), which is crucial for the B-cell development and maturation process (RETH, 1989; KRAUS et al., 2004). Upon BCR-antigen crosslinking, several signaling cascades are initiated by the proximal kinase Lyn, which belongs to the Src-family tyrosine kinase (SFK). Lyn phosphorylates CD79a/CD79b ITAMs, thereby creating docking sites for the recruitment and activation of splenic tyrosine kinase (Syk) (YAMANASHI et al., 1991; FÜTTERER et al., 1998; XU et al., 2005; GEAHLEN, 2009). Syk interacts with the ITAM motifs amplifying the BCR signal (ROLLI et al., 2002). The ITAM/Syk complex leads to the phosphorylation and activation of downstream molecular pathways, including SH2 domain-containing leukocyte protein (SLP-65/BLNK), Bruton's tyrosine kinase (Btk), phospholipase C gamma 2 (PLCγ2), and phosphoinositide 3-kinase (PI3K) (NIIRO; CLARK, 2002; DEANE; FRUMAN, 2004).

Many of these downstream signaling molecules function as calcium (Ca^{2+}) signals, influencing Ca^{2+} cytosolic alterations. Ca^{2+} is crucial for the development and function of B cells, working as a universal cytosolic messenger in a range of intracellular processes, including transcription factors, protein kinase, mitochondrial physiology, apoptosis, cell adhesion, and migration (SCHARENBERG; HUMPHRIES; RAWLINGS, 2007). The SLP65/BLNK is required for Ca^{2+} mobilization in activated B cells. This adaptor protein organizes the B-cell calcium signalosome, thus promoting Ca^{2+} release and B-cell differentiation (CHIU et al., 2002; KULATHU et al., 2008). The PLCγ2 and other important surface receptors influence Ca^{2+} signaling alterations through the production and accumulation of the second messenger inositol 3,4,5-trisphosphate (IP3) (CAHALAN; WULFF; CHANDY, 2001; KIM et al., 2019). PLCγ2 phosphorylation results in hydrolysis of PI(4,5)P2 to IP3 and diacylglycerol (DAG), which trigger Ca^{2+} influx from endoplasmic reticulum and protein kinase C (PKC) activation, respectively (LI et al., 1997; KUROSAKI et al., 2000; WERNER; HOBEIKA; JUMAA, 2010). PLCγ2 phosphorylated also stimulates MAPK and NF-κB pathways, resulting in B-cell proliferation, differentiation or quiescence (PETRO; KHAN, 2001; ANTONY et al., 2004).

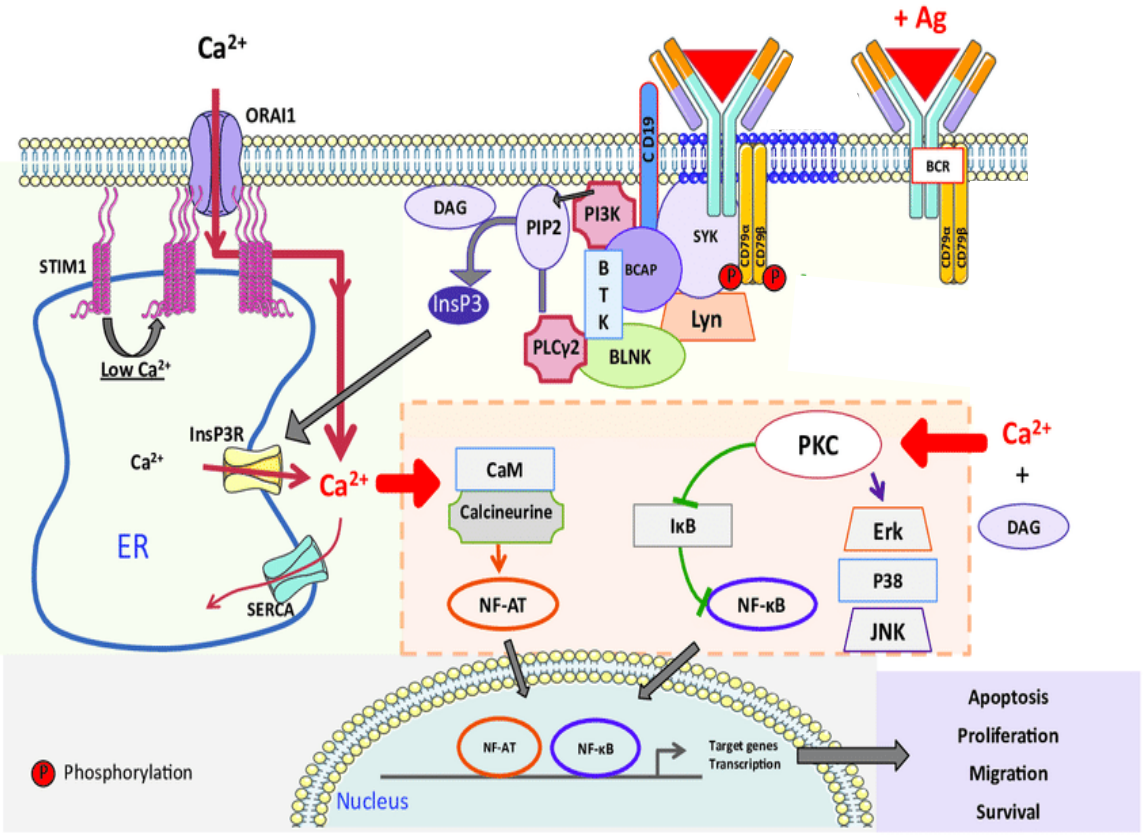

Figure 1. B-cell receptor (BCR) signaling and downstream pathways upon antigen BCR crosslink. Adapted from Debant et al. (2015).

Another important transmembrane molecule essential for B-cell activation is CD19, a co-receptor molecule that forms a complex with CD21, CD81, and CD225. CD19 has been described as a critical co-receptor for amplifying BCR responses, setting intrinsic thresholds of B-cell signaling through modulation of BCR-dependent and independent response (DEL NAGRO et al., 2005; ISHIURA et al., 2010). Its phosphorylation leads to membrane recruitment and activation of PI3K and Akt, thereby enhancing BCR-induced signaling of pro-survival response (FEARON; CARROLL, 2000). Moreover, CD19 can serves as an adaptor protein, influencing the recruitment and amplification of transduction molecules, including Lyn, Btk, PI3K, Ras family, adapter molecules (Vav, Grb2) and also SH2 domain (FUJIMOTO et al., 2000; ISHIURA et al., 2010).

Through tyrosine phosphorylation and Akt kinase, CD19 is required for MHC-II-mediated downstream signaling (BOBBITT; JUSTEMENT, 2000; MILLS; STOLPA; CAMBIER, 2007), which in addition to their role as antigen presentation, is involved in B-cell fate during B-cell and T-cell interaction (SCHOLL; GEHA, 1994; AL-DACCAK; MOONEY;

CHARRON, 2004). In human B cells, CD19/PI3K/Akt/Btk complex is an essential axis integrating BCR and Toll-like receptor 9 (TLR9) signaling, a pattern recognition receptor that recognizes bacterial and viral DNA containing the cytosine–phosphate–guanine (CpG) dideoxynucleotide motif (DALPKE et al., 2006; MORBACH et al., 2016).

Interestingly, some intracellular pathogens have strategies to evade protective humoral immunity, infecting and employing B cells as a host reservoir. Through this mechanism, known as the "Trojan horse," bacteria can multiply and disseminate in the organism (NOTHELFER; SANSONETTI; PHALIPON, 2015). *Salmonella* Typhimurium is one of the examples of pathogens capable of infecting B cells at different developmental stages and exploiting them as an excellent host for its persistence, dissemination and long-term infection niche (CASTRO-EGUILUZ et al., 2009; LOPEZ-MEDINA et al., 2014). Thus, comprehending the molecular mechanisms involved in T cell-independent B-cell activation and the pathogen interaction with them, will provide insights into efficient control strategies.

2.4 Fecal calprotectin as a non-invasive intestinal inflammation biomarker

Calprotectin (CP) is a 24 kDa calcium-binding protein, a member of the S100 family (KLIGMAN; HILT, 1988; CLOHESSY; GOLDEN, 1995), composed of the heterodimeric complex S100A8 and S100A9 (KORNDÖRFER; BRUECKNER; SKERRA, 2007). The protein was first described in the 1980s in inflammatory conditions in human tissues, receiving different names (FAGERHOL; DALE; ANDERSON, 1980; DALE; FAGERHOL; NAESGAARD, 1983; ODINK et al., 1987), but its similar identity was recognized in 1988 (ANDERSSON et al., 1988). Subsequently, it was renamed calprotectin, highlighting its properties of inhibitory activity against *Candida albicans* and Ca^{2+} binding characteristics (MCNAMARA et al., 1988; STEINBAKK et al., 1990). CP comprises approximately 40 - 60% of the total cytosolic protein in neutrophils (EDGEWORTH et al., 1991; HESSIAN; EDGEWORTH; HOGG, 1993) and is also expressed in monocytes, activated macrophages, dendritic cells, and endothelial and epithelial cells (BRANDTZAEG; DALE; FAGERHOL, 1987; ŠPLÍCHAL et al., 2002; KUMAR; STEINKASSERER; BERCHTOLD, 2003).

Many pathological conditions can cause infection and inflammatory response in the intestinal environment, disrupting the intestinal barrier (GERMAN; HALL; DAY, 2003; ROHR et al., 2018). In response to inflammation, recruitment of neutrophil and monocytes leads to massive release of CP by dead granulocytes into the lumen (JOHNE et al., 1997; BERSTAD; ARSLAN; FOLVIK, 2009). As CP is resistant to intestinal proteases, pancreatic secretions, and bacterial degradation in the lumen (RØSETH et al., 1992), its presence in feces

has been widely applied as a biomarker of intestinal inflammation (LEHMANN; BURRI; BEGLINGER, 2015).

2.4.1 The pleiotropic role of calprotectin

Once released into the lumen, pleiotropic functions including antimicrobial, antiproliferative, and apoptotic properties, have been described for CP (STŘÍŽ; TREBICHAVSKÝ, 2004). Recognized triggers account for lipopolysaccharides (LPS), monosodium urate, and pro-inflammatory mediators, such as TNF-α, IL-1β, and eosinophils (SURYONO et al., 2005; REICHMAN et al., 2017). Furthermore, glucocorticoids and non-steroidal anti-inflammatory drugs positively drive CP release (TIBBLE et al., 1999; RYCKMAN et al., 2004; HSU et al., 2005).

As an antimicrobial player, CP allows the chelation of essential divalent metal ions in the extracellular space, such as iron, zinc, calcium, or manganese. This micronutrient-capturing mechanism prevents their uptake and metabolic utilization by pathogens, thus inhibiting bacterial growth (LOOMANS et al., 1998; KEHL-FIE; SKAAR, 2009). The S100A8 and S100A9 protein complexes from CP are essential. CP also contains zinc-binding domains (LOOMANS et al., 1998), and both S100A8 and S100A9 have histidine-based zinc-binding sequences. The heterodimerization of this complex exposes the high-affinity Zn^{2+} sites, which are considered functional sites for the antimicrobial role of CP (DONATO et al., 2013). Antimicrobial properties, particularly against *Staphylococcus aureus* (KEHL-FIE et al., 2011; HAMMER; SKAAR, 2012), *Listeria monocytogenes* (ZAIA et al., 2008), *Klebsiella* (HO et al., 2018), *Borrelia burgdorferi* (LUSITANI; MALAWISTA; MONTGOMERY, 2003), *Salmonella enterica* serovar Typhimurium (NAUGHTON; GRANT; GOLDEN, 1996), *Escherichia coli* (LIPCSEY et al., 2019), and *Candida albicans* (URBAN et al., 2009) has been described. Interestingly, *S.* Typhimurium can express a high-affinity zinc transporter (ZnuABC), which drives its capacity to overcome CP (LIU et al., 2012; DIAZ-OCHOA et al., 2016).

The regulation of inflammation, cell proliferation, differentiation, and tumorigenesis are among the immunomodulatory mechanisms of CP (GHAVAMI et al., 2010; LAOUEDJ et al., 2017). Its interaction with receptor for advanced glycation end products (RAGE) and Toll-like receptor 4 (TLR4) receptors (VOGL et al., 2007; TUROVSKAYA et al., 2008) through NF-κB pathway (RIVA et al., 2012), subsequently induces the expression of pro-inflammatory and anti-inflammatory mediators (SUNAHORI et al., 2006). This was observed in monocytes secreting IL-1β, IL-6, and TNF-α (CESARO et al., 2012), neutrophils promoting IL-8

expression (SIMARD et al., 2014), and overexpression of macrophages increasing IL-10 and extracellular reactive oxygen species (ROS) (YANG et al., 2018). CP secretion has also been reported to induce granulocyte adhesion and migration (I et al., 2000; RYCKMAN et al., 2003).

The apoptotic activity in tumor cells (YUI et al., 2002; NAKATANI et al., 2005; QIN et al., 2010), epidermal keratinocytes, and other cells (SAKAGUCHI et al., 2014; ZHENG et al., 2014) has been registered. CP-induced apoptosis can occur through increased ROS production, which causes mitochondrial dysfunction and damage via BNIP3, the protein associated with mitochondrial dysfunction and cell death (GHAVAMI et al., 2010; LEE et al., 2011). By sequestering zinc, CP promotes the inhibition of matrix metalloproteinases (MMPs) (ISAKSEN; FAGERHOL, 2001), suppressing metastasis of CasKi human cervical cancer cells, thus inducing apoptosis (QIN et al., 2010).

2.4.2 Fecal calprotectin and its role as a biomarker in intestinal disease

Fecal calprotectin (FC) has become a widely used biomarker in human medicine, accurately assessing intestinal inflammatory conditions. Its stability for three to seven days and homogenous distribution in feces, make FC a useful non-invasive biomarker (RØSETH et al., 1992; FOELL; WITTKOWSKI; ROTH, 2009; LASSON et al., 2015). In humans, the applicability of FC has been explored mainly in diagnosing inflammatory bowel disease (IBD) (D'HAENS et al., 2012; LEHMANN; BURRI; BEGLINGER, 2015).

IBD is an organic condition due to inflammation, consisting of Crohn's disease (CD) and ulcerative colitis (UC), and it is characterized by periods of symptomatic relapse and remission (HENDRICKSON; GOKHALE; CHO, 2002). Here, FC has been applied to screening patients with suspected IBD before invasive endoscopy (VAN RHEENEN; VAN DE VIJVER; FIDLER, 2010). Moreover, FC can be used to monitor the recurrence of IBD during postoperative and detect relapses in adults and pediatric patients (WRIGHT et al., 2015; HUKKINEN et al., 2016). Patients with IBD and irritable bowel syndrome (IBS) have similar symptoms, including abdominal pain and diarrhea (WALSHAM; SHERWOOD, 2016). FC is one of the most sensitive approaches in distinguishing organic IBD from functional IBS (SCHOEPFER et al., 2008; CHANG et al., 2014; MARI et al., 2019). Likewise can be applied to distinguish inflammatory from non-inflammatory gastrointestinal disease (VON ROON et al., 2007; BANERJEE et al., 2015; BRESSLER et al., 2015).

Besides IBD, another common gastrointestinal disorder is necrotizing enterocolitis in newborns, and FC has been suggesting a promising biomarker in the diagnosis of premature infants (QU et al., 2020). CF has also been explored in veterinary medicine. Several studies

have highlighted the clinical usefulness of FC as a sensitive biomarker of chronic inflammatory enteropathies (CIEs) in dogs (HEILMANN; SUCHODOLSKI; STEINER, 2008; GRELLET et al., 2013; HEILMANN et al., 2018a, 2019), and potentially biomarker in cats (HEILMANN et al., 2018b; ENDERLE; KÖLLER; HEILMANN, 2022).

It is noteworthy that many conditions are associated with an elevated CF level, such as bacterial infections, NSAID-induced enteropathy, neoplasms, and other infections (ALIBRAHIM; ALJASSER; SALH, 2015; JUKIC et al., 2021). Hence, although FC correlates with the number of neutrophils in the intestinal lumen during intestinal inflammation (KONIKOFF; DENSON, 2006), it is not possible to discriminate distinct potential triggers. Therefore, careful interpretation of FC should be warranted by clinicians.

2.4.3 The usefulness of FC as a non-invasive biomarker in the swine industry

In pigs, studies investigating FC were first evaluated in healthy animals to determine reference values at different ages (LALLES et al., 2005). The authors observed that the mean FC concentrations in adult pigs were in a range similar to that of healthy human adults (13 ± 38 mg/kg feces and 2–47 mg/kg, respectively). On the other hand, the average concentration for newborn piglets was lower (24 ± 60 mg/kg) than for humans newborns (145 ± 78.5 mg/kg). The low FC values were attributed to the higher sanitary conditions that pigs were exposed (LALLES et al., 2005). Lately, FC has been applied to assesses the effects of dietary alternatives to mitigate intestinal inflammation in pigs (XIAO et al., 2014; SLINGER et al., 2019; SÁNCHEZ-URIBE et al., 2022).

Limited information regarding the effectiveness of FC as a biomarker of inflammation caused by enteric pathogens in pigs is available. Previous studies have shown the correlation between CP in plasma and intestinal lumen (ŠPLÍCHAL et al., 2005), and jejunal mucosa (XIAO et al., 2014) following *Escherichia coli* infection. Recently, *Escherichia coli* infection did not elicit a significant increase in FC concentration in challenged weaned piglets, indicating FC may not be a valuable biomarker against *E. coli* enteropathy (BOECKMAN et al., 2022). Hence, given its applicability in human medicine and the lack of knowledge about its application during infectious diarrhea in pigs, it is interesting to investigate its potential as a non-invasive biomarker in swine diarrheal disease.

3 FINAL CONSIDERATIONS

Swine dysentery, spirochetal colitis and salmonelosis, as mentioned above, are a concern to producers, veterinarians and animal science professionals. However, many aspects

of the pathogen-host interaction are unclear and require further scientific investigation to aid clarification on pathogenesis mechanisms, in order to develop preventative tools. While antibiotics have been effective for decades, concerns raised on the pandemic of antimicrobial resistance, animal welfare, and food safety have turned the lights on making better decisions and policies to reduce its usage. Combining analysis with insights into strategies used by pathogens would substantially increase our knowledge of how diarrhea is elicited during infection, which may lead to novel therapeutic and vaccination approaches in the future.

Therefore, the objectives of the studies presented in this thesis were to evaluate the host-pathogen-microbiome interactions of agents of grower-finisher diarrhea to which antibiotics are the main control tool used in commercial farms.

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SECOND SECTION

ARTICLE 1 - CHARACTERIZATION OF THE BACTERIAL FECAL MICROBIOTA COMPOSITION OF PIGS PRECEDING THE CLINICAL SIGNS OF SWINE DYSENTERY

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Characterization of the bacterial fecal microbiota composition of pigs preceding the clinical signs of swine dysentery

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Abstract

Swine dysentery (SD) is a worldwide production-limiting disease of growing-finishing pigs in commercial farms. The importance of the large intestinal microbiota in the swine dysentery pathogenesis has been established, but not well characterized. The objective of this study was to characterize the fecal bacterial microbiota of pigs immediately prior to developing clinical signs of swine dysentery. A total of 60 fecal samples were collected from 15 contact pigs one day after contact with seeder pigs (d0, n=15), 2 days before mucohaemorrhagic diarrhea was observed (d-2SD, n=15), 1 day before mucohaemorrhagic diarrhea was observed (d-1SD, n=15), and the day when pigs developed mucohemorragic diarrhea (MHD, n=15). Sequencing of *cpn*60 amplicons was used to profile the microbiome, and analyses were performed on QIIME2. Increased Chao1 index in d-1SD and MHD samples when compared to the d0 group was the only change observed in alpha diversity. No differences between groups on beta diversity (Bray-Curtis dissimilarity) were found. Differential abundance analysis revealed that

Alistipes dispar and *Parabacteroides gordonii* were increased in MHD fecal samples when compared to d-2SD and d-1SD. Future investigations to verify the specific role of these taxa on the pathogenesis of SD is warranted.

Background

Swine dysentery (SD) is a production limiting enteric disease affecting grower-finisher pigs worldwide. The anaerobic tolerant spirochete *Brachyspira hyodysenteriae* was the initial etiological agent of SD [1], which is characterized by mucohaemorrhagic diarrhea and fibrinonecrotic colitis [1,2]. Recently, *B. suanatina* [3] and *B. hampsonii* [4] were also recognized as agents of SD.

A complex interaction between the large intestine microbiota and *B. hyodysenteriae* has been demonstrated. Studies based on culture have shown that *B. hyodysenteriae* requires the presence of other bacteria for severe SD expression [5–9]. Gnotobiotic pigs inoculated with *B. hyodysenteriae* alone did not develop mucohaemorrhagic diarrhea [5,10]. Colitis and mucohaemorrhagic diarrhea was reported in gnotobiotic pigs co-inoculated with *B. hyodysenteriae* and *Fusobacterium necrophorum* or *Bacteroides vulgatus* [5]. Mucoid diarrhea was found in pigs co-inoculated with *B. hyodysenteriae*, *F. necrophorum*, *B. vulgatus*, a *Clostridium* species, or *Listeria denitrificans,* but not when the spirochete was the only inoculum [9]. Recently, high-throughput sequencing of microbial barcode genes, such as 16S rRNA or *cpn*60, has been applied to study SD. Colonic contents and mucosal scrapings from pigs inoculated with *B. hyodysenteriae* or *B. hampsonii* had decreased species richness than their uninoculated counterparts [11]. Lower Bacteroidetes:Firmicutes ratio was linked to mucohaemorrhagic diarrhea following inoculation of pigs with *B. hampsonii* compared with sham-inoculated control or inoculated pigs that did not develop clinical disease [12]. *Campylobacter* spp., *Mogibacterium* spp., *Brachyspira* spp., and *Desulfovibrio* spp. were found in higher numbers in mucosal scrapings of pigs that developed SD, whereas *Bifidobacterium* spp. and *Lactobacillus* spp. were significantly more abundant in pigs without SD [11].

It has also been suggested that the diet may influence the incidence of SD and the colonic microbiome. However, results have been contradictory [13–16]. Highly fermentable soluble fiber has been associated with a protective effect against SD [17–19]. These diets promoted the growth of lactic acid and butyric acid-producing bacteria, such as *Bifidobacterium* spp., *Megasphaera* spp., and *Faecalibacterium* spp. [16,20,21]. Conversely, poorly fermentable insoluble fiber has been linked to increased odds of pigs developing SD [22]. This observation was combined with augmented loads of anaerobes such as *Shuttleworthia* spp., *Ruminococcus*

torques, and *Mogibacterium* spp., which may play a synergistic role with *B. hyodysenteriae* in inducing SD [21].

It is well established that the colonic microbial community changes following the development of SD [9,11,12,16]. However, little is known regarding the changes preceding the clinical signs of SD. We hypothesize that the fecal microbiome is disturbed immediately before clinical SD is observed. Thus, the goal of this study was to characterize the bacterial fecal microbiota composition of pigs immediately prior to developing clinical signs of swine dysentery.

Methods

Ethics statement

Samples used in this study were collected during a trial conducted in accordance with the Canadian Council for Animal Care and approved by the University of Saskatchewan Animal Research Ethics Board (Protocol #20180046).

Animal trial and samples

Barrows (n=15), age 9 to 10 weeks-old, were obtained from a PRRSV, *Mycoplasma hyopneumoniae* negative, high-health herd farm with no gastrointestinal clinical signs and no history or previous laboratory diagnosis of SD. Animals were housed and allowed to acclimate in a BSL-2 animal care facility for 7 days prior to inoculation. A commercial starter diet, unmedicated, fed *ad libitum* was used. Fecal samples were collected from contact pigs that were exposed to seeder pigs experimentally inoculated intra-gastrically thrice over 72 hours with 100 mL of a 24h broth culture of *B. hyodysenteriae* strain G44 (total dose 3.72×10^{11} genome equivalents/mL). Feces from contact pigs were collected at four different times: one day after contact with seeder pigs (d0, n=15); two (2) days before mucohaemorrhagic diarrhea was observed (d-2SD, n=15); one (1) day before mucohaemorrhagic diarrhea was observed (d-1SD, n=15); and at the day when pigs developed mucohemorragic diarrhea (MHD, n=15), totaling 60 samples. A summary of the samples used from this trial is shown on Table S1. All fecal samples were collected by digital stimulation and stored at -80ºC until processing for analysis. The development of swine dysentery was confirmed by associating clinical signs, a positive fecal *B. hyodysenteriae* culture and gross necropsy lesions.

DNA extraction, cpn60 amplification and sequencing

For each sample, total DNA was extracted from 200 mg of feces using a commercial kit (MagMax DNA Ultra v2.0; Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) on a KingFisher Flex platform (Thermo Fisher Scientific, Waltham, MA, USA). Amplification and indexing of the *cpn*60 universal target barcode region were performed as previously described [23]. Briefly, the *cpn*60 gene was amplified using a primer mix comprised of 100 µM from each of the following primers: M279 Forward (5' – GAIIIIGCIGGIGAYGGIACIACIAC – 3'), M280 Reverse (5' – YKIYKITCICCRAAICCIGGIGC– 3'), M1612 Forward (5' – GAIIIIGCIGGYGACGGYACSACSAC– 3'), and M1613 Reverse (5' – CGRCGRTCRCCGAAGCCSGGIGCCTT– 3'). Primers were mixed in a 1:3 molar ratio of M279:M280 (3 µL each), and M1612:M1613 (9 µL each) and diluted in 276 µL of ultrapure water for a total volume of 300 µL. PCR reactions had a total reaction volume of 50 µL, for 2 µL of DNA template. The master mix was prepared using 38.1 µL ultrapure water, 5 µL of 10x PCR buffer, 2.5 µL of MgCI2 (50mM), 0.4 µL of Platinum Taq Polymerase (Invitrogen, Thermo Fisher Scientific, USA), 1 µL of dNTP mix (10 mM; Invitrogen, Thermo Fisher Scientific, USA) and 1 µL of the primer cocktail described above. Reactions were incubated at 95°C for initial denaturation for 5 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 2 minutes. *Cpn*60 amplicons were purified using NucleoMag NGS beads (Macherey-Nagel Inc., Germany). Indexing PCR for library preparation was performed using a Nextera XT primers library preparation kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's protocol. Indexed amplicons were size-selected using NucleoMag NGS beads. Indexed amplicons were quantified, normalized, and diluted to 10pM libraries containing 5% PhiX DNA, Sequencing was carried out on an Illumina MiSeq (Illumina Inc., San Diego, CA, USA) platform using a 500-cycle reagent kit v2 (401 R1, 101 R2, Illumina Inc., San Diego, CA, USA).

Sequencing data analysis

Following sequencing, on-rig quality control procedures were executed. Raw data was processed by initially removing sequencing and amplification primers using Cutadapt [24], and low quality or short length and technical sequences were trimmed with Trimmomatic [25]. Filtered sequence reads were imported to Quantitative Insights Into Microbial Ecology 2

(QIIME2) [26], and variant calling was carried out using DADA2, truncating at 150 bp from the 5′ end [27]. Reads were mapped to the nonredundant version of cpnDB (cpnDB_nr) using watered BLAST [28]. Downstream analysis used only amplicon sequence variants (ASV) with >55% sequence similarity to a cpnDB_nr match. An feature table was generated and analyzed using a web-based platform for high-throughput sequencing data statistical analyses [29]. For further statistical analysis and visualization, the ASV table with taxa and metadata file were uploaded to the MicrobiomeAnalyst tool (Xia Lab, McGill University, Quebec, Canada; available at: https://www.microbiomeanalyst.ca) [30]. At the data filtering step, a low count filter was used to filter all ASV features with <4 counts in at least 20% prevalence, and 10% minimum variance among samples, leaving 169 ASV [29]. The alpha diversity indices (Chao1 and Shannon's index) were calculated on raw data and comparisons were performed using ANOVA followed by post-hoc Tukey test. Beta-diversity differences between groups were analyzed by permutational multivariate analysis of variance (PERMANOVA) using Bray-Curtis dissimilarity index. Principal coordinate analysis (PCoA) was used to visualize the beta diversity data (MicrobiomeAnalyst). Differential abundance analysis at the phylum and ASV levels between sampling days was performed using DESeq2, and the adjusted *p*-value<0.05 was used to report the significance (R version 4.2.1, RStudio, Boston, MA, USA, Love et al., 2014).

Results

Following quality control steps, sequencing resulted in 2,639,963 high-quality reads (average 44,745 per sample, ranging from 4,755 to 86,947). One sample, 387_MHD (rep 8), was removed from the analysis due to an extremely low number of reads generated (159). A total of 589 ASV were detected, 420 had ≥ 2 reads total and were kept for downstream analyses.

Fecal microbial community composition

Proportional taxa abundance data at the phylum and family level are shown in Figures 1A and 1B, respectively. The top 3 most abundant phyla were firmicutes, terrabacteria-group and bacteroidetes. The top 3 most abundant family were *Bacteroidales*, *Clostridiales* and *Ruminococcaceae.*

No significant differences in alpha diversity indexes (Chao1, $P = 0.056$, Shannon's index, $P = 0.248$) were observed between groups at the phylum level (Figure 2A, 2B). At the genus level, Chao1 index was increased at d-1SD ($P = 0.042$) and MHD ($P = 0.001$) when

compared to d0 samples (Figure 2C), and no significant differences in Shannon's index were observed among groups (Figure 2D; *P* =0.270). No changes in beta diversity were observed at the phylum level. At the genus level, four samples clustered separately from the others significantly affecting the data distribution, but an even distribution of high and low ranks within and between groups was observed as evidenced by the small R^2 value identified (R^2 = 0.091684, *P* = 0.003, Figure 3).

Differential abundance analysis

Reads associated with actinobacteria and spirochetes significantly differed between groups (Figure 4A and 4B). ASVs with more than 10.000 total read counts and differentially abundant between d-2SD vs. MHD, d-1SD vs. MHD and d0 vs. all other groups are presented in Table 1, Table 2, and Supplementary Table 2, respectively.

Discussion

This study characterized the fecal microbiota of pigs on the two days immediately prior to the development of swine dysentery clinical signs. Alpha and beta diversity were not significantly affected on the two (2) days prior the observation of mucohaemorrhagic diarrhea. However, differential abundance analysis revealed ASVs significantly affected prior to the observation of clinical SD. This work is a stepping-stone towards the complete understanding of SD pathogenesis, and the role of the microbiome in this mechanism.

A high abundance of *Eubacterium brachy* and a low abundance of *Parabacteroides gordonii* was observed in d-2SD, when compared to MHD samples. The *Eubacterium* genera is known to be present in the healthy mammalian intestinal microbiota, as demonstrated in mice, humans and pigs [32–34]. *Eubacterium* spp. is suggested to benefit the host largely due to its production of butyrate [35–37]. *Eubacterium brachy*, a Gram-positive strict anaerobe, was frequently isolated from patients with periodontitis and pleuropulmonary infection [38,39]. Mouse models of colitis using dextran sodium sulfate revealed that *E. limosum* and its metabolites were associated with reduced clinical scores through increased butyrate levels. In T84 colonocyte cells, this effect is mediated by reduced IL-6 and TLR4 expression [40]. Additionally, lower abundance of *Eubacterium* spp. has been reported in patients with ulcerative colitis or Crohn's disease, when compared to healthy patients [41,42]. Previous work showing the association between a higher abundance of *Eubacterium* spp. and attenuated colitis corroborates our findings, as this genera was found depleted in MHD samples. In contrast, *Parabacteroides* spp. is found in low amounts in the human gut microbiota [43]. In pigs, it is

suggested to be part of the intestinal microbiota of healthy animals [44–46]. Humans with IBD have decreased abundance of *Parabacteroides*, when compared to healthy patients [47,48]. Bacteria of this genera were found to produce SCFAs [49], regulate immunity in multiple sclerosis through IL-10 induction [50], and relieve intestinal inflammation in mice with acute and chronic colitis by reducing the levels of pro-inflammatory cytokines [51]. *Alistipes dispar* was also found depleted on d-1SD and d-2SD, when compared to MHD. *Alistipes* spp. are enriched in human fecal samples from patients with colorectal cancer [52,53], and other non-intestinal disorders, such as depression and atherosclerotic cardiovascular disease [54,55]. It is suggested to thrive in the inflamed colon of IL-$10^{-/-}$ knocked out mice, being sufficient to induce colitis and tumorigenesis through IL-6–STAT3 signaling [56]. In contrast, decreased abundance of *Alistipes* spp. has been associated with protective effects in IBD patients and ulcerative colitis in mice [57,58]. Interestingly, when *A. finegoldii* was administered together with *Bacteroides eggerthii*, a colitis-predisposing bacterium, it attenuated the severity of dextran sulfate sodium (DSS)-induced colitis in mice depleted of intestinal microbiota [57]. We speculate both anaerobes, *Parabacteroides* spp. and *Alistipes dispar*, increased abundance at MHD is either linked to their opportunistic profile or they truly are part of the ancillary microbiota required for the severe expression of SD, as previously shown to be required [5–7,9,11].

Actinobacteria load decreased from d0 samples to MHD. This has not been reported before, although differences in analytical methods between this study and previous research may explain such finding [11,12]. Interestingly, a significant increase in actinobacteria abundance and decreased incidence of clinical SD was observed in *B. hyodysenteriae*-inoculated pigs consuming a highly fermentable fiber, when compared to pigs fed a low fermentable fiber [21]. Several studies have found a decline in actinobacteria abundance during different gastrointestinal disorders, such as acute hemorrhagic diarrhea in dogs [59], new neonatal porcine diarrhea [60], and post-weaning diarrhea in piglets [61]. Although this phylum has been found in higher abundance in healthy intestinal samples when compared to diseased ones, to clarify the exact role of actinobacteria in SD requires further studies.

Higher Chao1 index was identified on d-1SD, when compared to the control d0 samples. Differing from our findings, Burrough et al. (2017). found a low Chao1 diversity index in colonic contents and mucosal scrapings of *B. hyodysenteriae* or *B. hampsonii* inoculated pigs, when compared to uninoculated controls. No changes in alpha diversity were observed in the fecal microbiota of pigs inoculated with *B. hampsonii* [12], *E. coli* $F18^{+}$ [62,63], or *S.* Typhimurium [64], when compared to matching controls. When compared to other alpha

diversity indices, Chao1 index is considered sensitive to rare taxa [65,66]. Given the inherent limitations of high-throughput sequencing, changes in ASVs with low abundance could be a simple result of the technique used to generate the data, or the bioinformatic algorithms used.

Beta-diversity analysis at the genus level revealed a single cluster with all samples intertwined. A previous study found no significant differences in beta-diversity fecal samples of pigs prior to inoculation and at the onset of mucohaemorrhagic diarrhea [12]. However, differences in beta-diversity between the luminal content and mucosal scrapings were observed in pigs with and without SD [11]. It is known that the mucosal, luminal and fecal microbiomes are compositionally different [67]. As a limitation of our study, the design used relied on ante-mortem samples. Unless surgical intervention was performed, which could co-found the development of clinical signs, sampling the luminal or mucosal microbiome of pigs on the days prior to SD is rather challenging.

While we recognize that a small sample size was used in this study, our investigation revealed that the fecal microbiota changed in the days prior to the development of clinical SD. We suggest that the anaerobes *A. dispar* and *P. gordonii* may play a role in contributing to the development of SD. Further investigation on their specific role may help clarify the importance of other microbes in SD. In addition, studies associating the fecal metabolome to the microbiota taxonomic composition may shed a light on the microbiota role in SD pathogenesis.

Acknowledgements

The authors are grateful to Champika Fernando and Scott do Santos for technical assistance and helpful discussions on data analysis.

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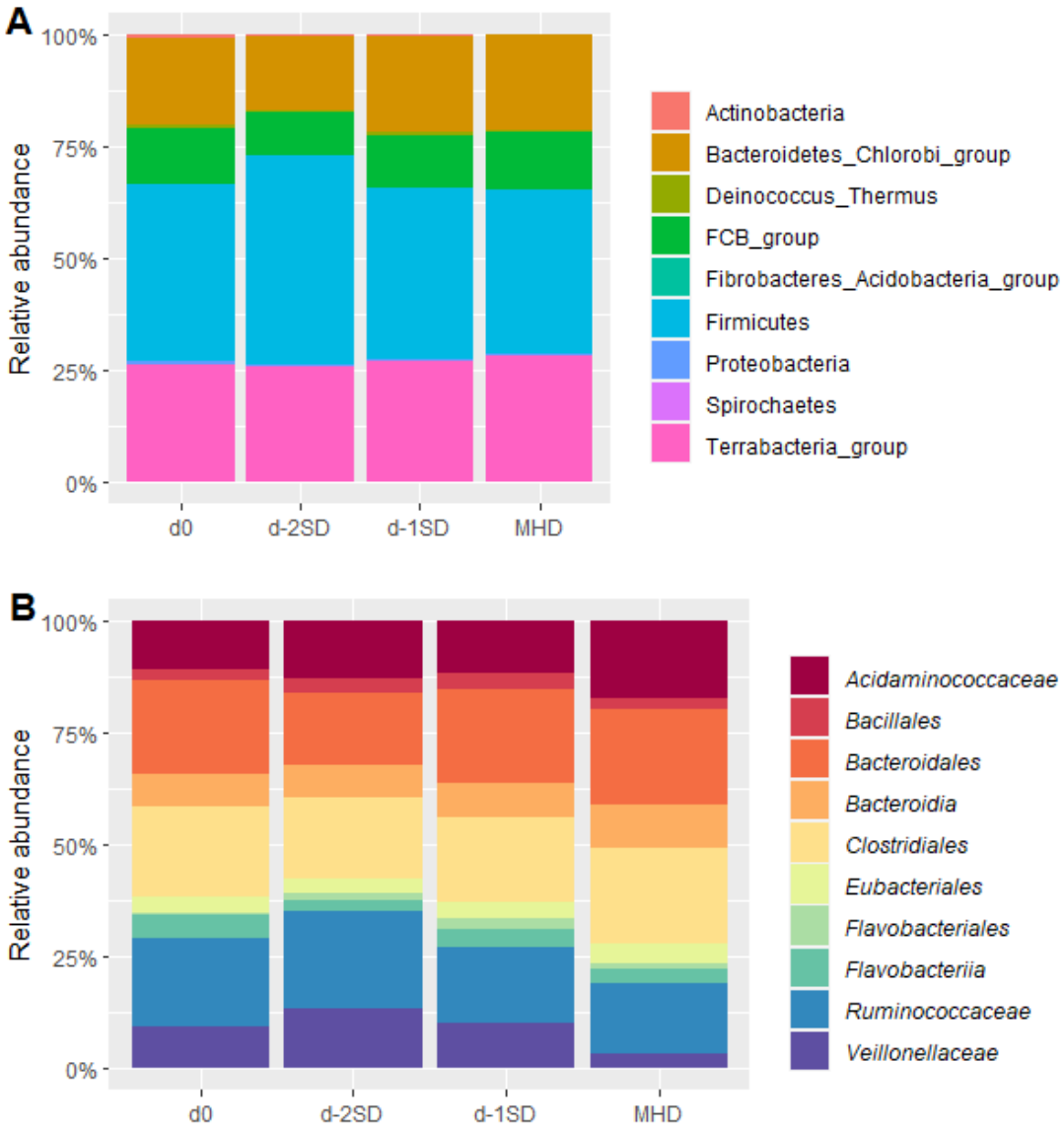

Figure 1. Stacked bar charts representing proportional abundance of bacterial taxa one day after contact with seeder pigs (d0, n=15); 2 days pre-SD (d-2SD, n=15); 1 day pre-SD (d-1SD, n=15); and the day mucohaemorrhagic diarrhea was observed for the first time (MHD, n =14). (A) Depicts data at the phylum level, and (B) at the family level.

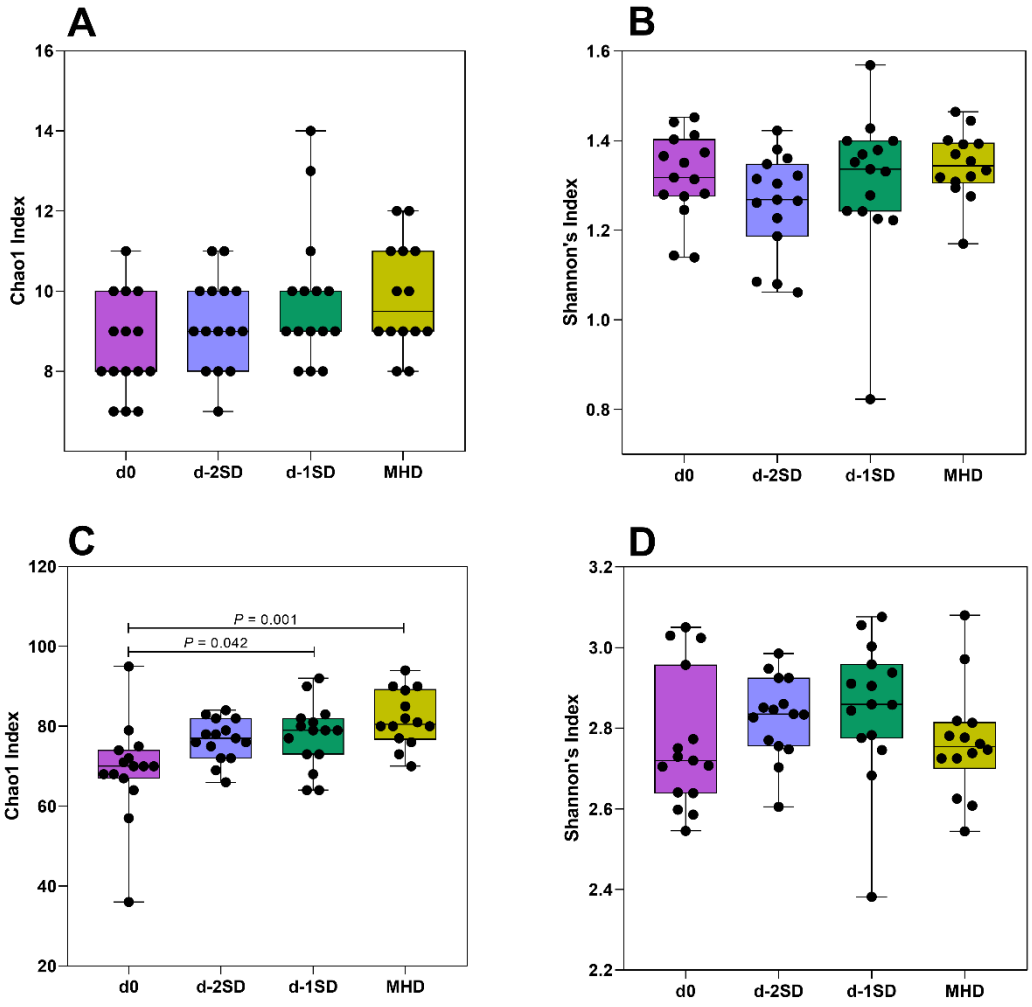

Figure 2. Alpha-diversity metrics from fecal samples collected after contact with seeder pigs (d0, n=15); 2 days pre-SD (d-2SD, n=15); 1 day pre-SD (d-1SD, n=15); and the day mucohaemorrhagic diarrhea was observed for the first time (MHD, n =14). A: Chao1 richness at the phylum level; B: Shannon's diversity index at the phylum level; 2C: Chao1 richness index at the genus level; D: Shannon's diversity index at the genus level. Boxes shows interquartile ranges, whiskers depict the minimum and maximum values.

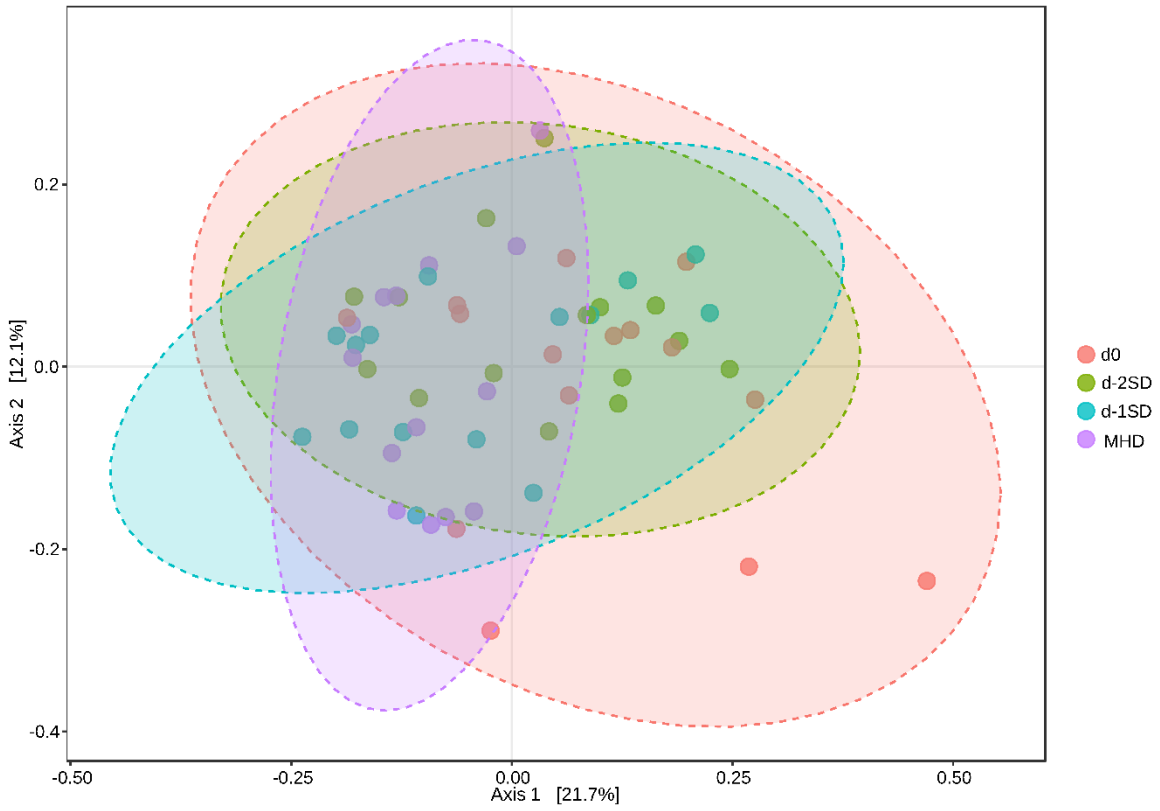

Figure 3. Beta diversity (Bray-Curtis dissimilarity data) at genus level based on principal coordinates analysis (PCoA) of fecal samples from pigs after contact with seeder pigs (d0, n=15); 2 days pre-SD (d-2SD, n=15); 1 day pre-SD (d-1SD, n=15); and the day mucohaemorrhagic diarrhea was observed for the first time (MHD, n =14). (R-squared: 0.091684; *p*-value: 0.003).

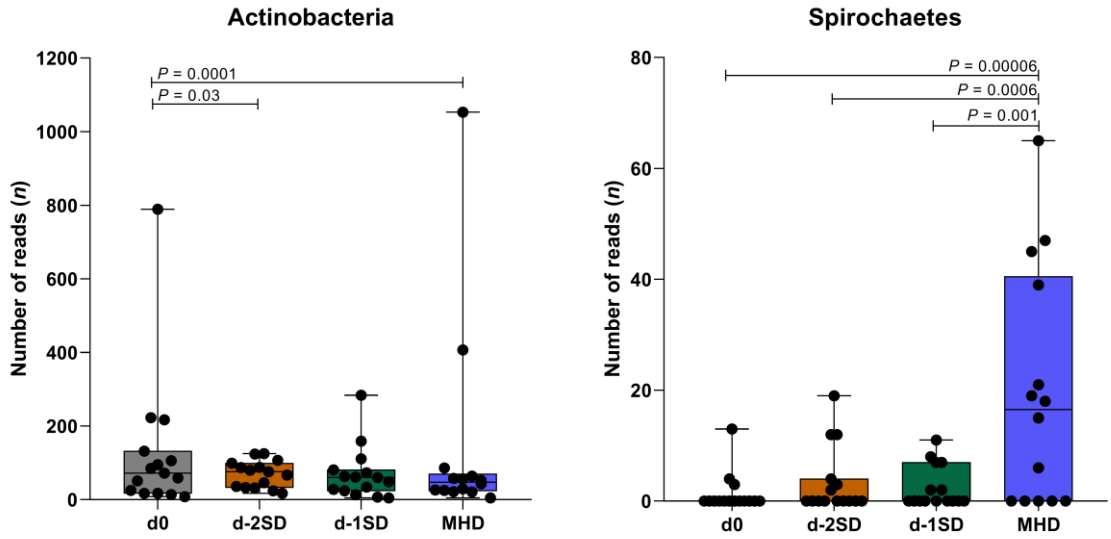

Figure 4. Phyla significantly enriched or depleted in fecal samples after contact with seeder pigs (d0, n=14); 2 days pre-SD (d-2SD, n=15); 1 day pre-SD (d-1SD, n=15); and the day mucohaemorrhagic diarrhea was observed for the first time (MHD, n =14). Boxplot represents the sum of reads associated with a given phylum at a given sampling time, and whiskers depict the minimum and maximum number of reads.

Table 1. Amplicon sequence variants (ASV) significantly different abundance between d-2SD and MHD.

ASV	Total read counts		logFC[3]	adjusted *P*-value
	d-2SD[1]	MHD[2]		
Parabacteroides gordonii	6228	17377	-1.524	0.002
Eubacterium brachy	7112	1546	2.141	0.002
Prevotella buccae	1967	6224	-2.283	0.018
Alistipes dispar	1789	8254	-3.373	0.002

[1]d-2SD: 2 days before mucohaemorrhagic diarrhea was observed (n=15); [2]MHD: day mucohaemorrhagic diarrhea was observed for the first time (n=14); [3]The degree of differential abundance is represented by $\log_2$ fold change (logFC) between d-2SD and MHD samples.

Table 2. Amplicon sequence variant (ASV) with significantly different abundance between d-1SD and MHD.

ASV	Total read counts		logFC[3]	adjusted *P*-value
	d-1SD[1]	MHD[2]		
Alistipes dispar	3089	8254	-3.652	0.002

[1]d-1SD: one day before mucohaemorrhagic diarrhea was observed (n=15); [2]MHD: day mucohaemorrhagic diarrhea was observed for the first time (n=14); [3]The degree of differential abundance is represented by $\log_2$ fold change (logFC) between d-1SD and MHD samples.

Table S1. Description of fecal samples used in this study.

Pig	Pen	Sampling day (DPI)[1]			
		d0[2]	**d-2SD**[3]	**d-1SD**[4]	**d-MHD**[5]
358	Pen 1	0	7	8	9
385	Pen 1	0	7	8	9
484	Pen 1	0	10	11	12
359	Pen 1	0	13	14	15
368	Pen 2	0	9	10	15
573	Pen 3	0	16	17	18
370	Pen 3	0	12	13	14
374	Pen 3	0	7	8	9
410	Pen 3	0	12	13	14
387	Pen 8	0	7	8	9
381	Pen 8	0	7	8	9
392	Pen 8	0	13	14	15
435	Pen 9	0	5	6	7
576	Pen 9	0	7	8	9
593	Pen 10	0	12	13	14

[1]DPI: days post introduction of seeder pigs, [2]d0: day after contact with seeder pigs (n=15), [3]d-2SD: 2 days before mucohaemorrhagic diarrhea was observed (n=15), [4]d-1SD: one day before mucohaemorrhagic diarrhea was observed (n=15), [5]MHD: day mucohaemorrhagic diarrhea was observed for the first time (n=14).

Table S2. Amplicon sequence variants (ASV) with significant differential abundance between d0 and all other groups.

	ASV	Total read counts				logFC[5]	adjusted *P*-value
		d0[1]	d-2SD[2]	d-1SD[3]	MHD[4]		
d0 vs. d-2SD	*Alistipes sp. JC136*	8154	4867	10578	9037	1.914	0.022
	Acetanaerobacterium elongatum	7961	4030	7571	4520	2.386	0.000
	Limosilactobacillus frumenti	592	5606	3661	4042	-2.276	0.033
d0 vs. d-1SD	*Prevotella baroniae*	8918	8306	8530	11078	1.341	0.024
	Dialister succinatiphilus	13411	4355	4583	1866	1.968	0,015
	Limosilactobacillus frumenti	592	5606	3661	4042	-2.463	0.025
d0 vs. d-MHD	*Phascolarctobacterium sp.*	51619	79540	83848	105144	-1.037	0.024
	Cohnella fermenti	11475	19674	25019	14247	0,610	0.030
	Barnesiella intestinihominis	11117	10148	17669	9807	0.957	0.030
	Parabacteroides gordonii	4891	6228	10456	17377	-1.266	0.008
	Eubacterium brachy	10230	7112	7813	1546	3.324	0.000
	Selenomonas bovis	5056	7415	9064	5154	1.072	0.018
	Dialister succinatiphilus	13411	4355	4583	1866	2.705	0.000
	Acetanaerobacterium elongatum	7961	4030	7571	4520	1.758	0.007
	Flavonifractor sp.	2203	4221	5639	8325	-1.583	0.002
	Ruminococcus callidus	6150	3611	5500	1977	1.953	0.033

Prevotella buccae	2454	1967	3943	6224	-2.096	0.030
Alistipes dispar	1321	1789	3089	8254	-2.532	0.024
Limosilactobacillus frumenti	592	5606	3661	4042	-2.279	0.014
Pygmaiobacter massiliensis	4084	2929	3860	2889	1.323	0.005
Ammonifex degensii	1024	3047	4653	4723	-1.865	0.007

[1]d0: day after contact with seeder pigs (n=15), [2]d-2SD: 2 days before mucohaemorrhagic diarrhea was observed (n=15), [3]d-1SD: one day before mucohaemorrhagic diarrhea was observed (n=15), [4]MHD: day mucohaemorrhagic diarrhea was observed for the first time (n=14).[5]The degree of differential abundance is represented by $\log_2$ fold change (logFC) between d0 and all other sample groups.

ARTICLE 2 - T-INDEPENDENT B-CELL EFFECT OF AGENTS ASSOCIATED WITH SWINE GROWER-FINISHER DIARRHEA

ARTICLE FORMATTED ACCORDING TO THE MICROBIAL PATHOGENESIS JOURNAL

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T-independent B-cell response to agents associated with infectious swine grower-finisher diarrhea

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ABSTRACT

Swine dysentery, spirochetal colitis, and salmonellosis are production-limiting enteric diseases of global importance to the swine industry. Despite decades of efforts, mitigation of these diseases still relies on antibiotic therapy. A common knowledge gap among the three agents is the early B-cell response to infection in pigs. Thus, this study aimed to characterize the porcine B-cell response to *Brachyspira hyodysenteriae*, *Brachyspira hampsonii* (virulent and avirulent strains), *Brachyspira pilosicoli*, and *Salmonella* Typhimurium, the agents of the syndromes mentioned above. Immortalized porcine B-cells were co-incubated for 8 hours with each pathogen, as well as *E. coli* LPS and a sham-inoculum (n=3/treatment). B-cell viability following exposure to treatments was evaluated using trypan blue, and the expression levels of

B-cell activation-related genes was profiled using RT-PCR. Only *S.* Typhimurium and LPS led to increased B-cell mortality. *B. pilosicoli* downregulated CD19, syk, lyn, and TNF-α, and elicited no change in CD79b and SLA-DRA expression levels, when compared to the sham-inoculated group. In contrast, all other treatments significantly upregulated CD79b and stimulated responses in other B-cell downstream genes. These findings suggest that *B. pilosicoli* does not elicit an immediate T-independent B-cell response, nor it triggers antigen-presenting mechanisms. All other agents activated at least one trigger within the T-independent pathways, as well as peptide antigen presenting mechanisms. Future research is warranted to verify these findings *in vivo*.

Keyworks: swine dysentery, colitis, salmonellosis, humoral immunomodulation, gene pathways.

1. Introduction

Swine dysentery (SD), spirochetal colitis (SC), and swine salmonellosis (SS) are diarrheic diseases affecting swine in the grower-finisher stage, and are associated with decreased growth performance and increased production costs [1–3]. SD is characterized by mucohaemorrhagic diarrhea and colitis. It is caused by *Brachyspira hyodysenteriae* [4], *B. suanatina* [5] or *B. hampsonii* [6]. *Brachyspira pilosicoli* is the causative agent of SC, clinically described as mucoid, watery diarrhea linked to mild colitis when compared to SD [7]. *Salmonella enterica* serovar Typhimurium causes watery diarrhea and enterocolitis in growing pigs [8]. In practice, these three diseases are often controlled and treated using antimicrobial therapy in commercial operations.

Several different vaccine development strategies have been explored for SD [9–11], and SC [12]. Despite these efforts, only partial protection has been induced and no effective vaccine for SD or SC is commercially available in the major pork producing countries. In contrast, commercial *Salmonella* vaccines are available in many countries [13–15]. However, cross-protection between serovars is questionable, impacting vaccine uptake in commercial farms [16–18]. Thus, the reliance on antimicrobials for disease mitigation. Consequently, the emergence of antibiotic resistant strain is a concern as it is linked to animal welfare, food safety, and security [19,20].

B-cells express multiple intra and extracellular receptors capable of recognizing antigens, including bacterial, that trigger signals to modulate the innate and adaptive immune responses [21]. T-cell independent B-cell activation takes part in the early response against

pathogens through the production of IgM, and serves as a gateway to immunotolerance or immune activation [22]. The B-cell receptor (BCR) is an important player in this mechanism. It is formed by a membrane-bound immunoglobulin (Ig) and a heterodimeric signaling subunit (CD79a/CD79b) [23]. Upon BCR crosslinking by antigens, the proximal kinase lyn initiates the signaling cascade phosphorylating tyrosines in the CD79a/CD79b BCR subunits, which results in recruitment and activation of the spleen tyrosine kinase (syk) [24–26]. Syk leads to the phosphorylation and activation of downstream molecular pathways that lead to B-cell activation, proliferation, and differentiation or quiescence [27,28].

We hypothesized that B-cell exposure to *Brachyspira hyodysenteriae*, *Brachyspira pilosicoli*, *B. hampsonii*, and *Salmonella* Typhimurium activates different triggers within the B-cell intrinsic activation pathways. The goal of this study was to investigate the initial mRNA B-cell response to swine enteric pathogens, independently of T-cells.

2. Materials and Methods

2.1 B-cell culture: A immortalized porcine B-cell line derived from a crossbred pig with lymphoma was used [29]. Cells were cultured at 37°C with 5% CO_2 in a standard bench-top CO_2 incubator (Thermo Fisher Scientific, Waltham, MA, USA) using high quality polystyrene flasks (Sarstedt, Numbest, Germany). Complete RPMI 1640 media with L-glutamine (Gibco Life Technologies, Co., Grand Island, NY, USA), supplemented with 10 mM HEPES buffer (Gibco Life Technologies, Co., Grand Island, NY, USA), 1X non-essential amino acids (Gibco Life Technologies, Co., Grand Island, NY, USA), 1 mM sodium pyruvate (Gibco Life Technologies, Co., Grand Island, NY, USA), 50 μg/mL gentamycin (Gibco Life Technologies, Co., Grand Island, NY, USA), 5,000 U/mL penicillin-streptomycin (Gibco Life Technologies, Co., Grand Island, NY, USA), and 5% fetal bovine serum (Gibco Life Technologies, Co., Grand Island, NY, USA) [29]. Once cells reached 90-100% confluency, they were passaged at a concentration of 1×10^5 cells/mL for inoculation.

2.2 Bacterial inocula culture: Salmonella enterica serovar Typhimurium strain X4232 was cultured at 37°C in Luria-Bertani broth (LB, BD Canada, Oakville, ON, Canada). *Brachyspira hyodysenteriae* strain G44 (*B. hyo*), the virulent *Brachyspira hampsonii* clade II strain 30446 (*B. hampsonii*), the non-pathogenic *Brachyspira hampsonii* clade 2 strain KL-180 (*B.* KL180), and *Brachyspira pilosicoli (B. pilosicoli)* were cultured in brain heart infusion (BHI) broth (Becton and Dickinson Company, Sparks, MD, USA) supplemented with 10% of fetal bovine serum, and incubated under anaerobiosis (Anaerogen, Oxoid Limited, Basingstoke, United Kingdom) at 39ºC. A *B. pilosicoli* aliquot was sonicated (Vibracell Sonicator, Sonics &

Materials Inc., Danbury, Connecticut, USA) for 2 minutes at 20 kHz to inactivate the bacteria (*B. pilo* dead).

2.3 Inoculation procedure: 25 mL flasks containing B-cells at 1×10^5 cells/mL were exposed to one of the following inocula: negative control (sham inoculated, n=6); positive control (100 µg/flask of *E.coli* O111:B4 lipopolysaccharide, LPS, n=6); *B. hyo* (1.69×10^7 genome equivalents (GE)/mL, n=6); *B. hampsonii* (1.49×10^9 GE/mL, n=3), *B. pilosicoli* (3.35×10^{10} GE/mL, n=6), *B.* KL180 (4.79×10^9 GE/mL, n=3), *B. pilo* dead (1.26×10^{11} GE/mL, n=3), and *S.* Typhimurium (4.32×10^9 CFU/mL, n =6). Inocula were prepared by centrifuging bacterial culture broth at 10,000 rpm for 10 minutes. Next, cell pellets were resuspended in 6 mL of cRPMI and inoculated into the flasks containing B-cells. Co-incubation followed for 8 hours at 37°C in 5% CO_2.

2.4 B-cell viability assay: Following the exposure period, B-cell viability was measured using trypan blue (Lonza, Walkersville, MD, USA). Briefly, 0.1 mL of 0.4% trypan blue was added to a 0.4 mL aliquot from each flask, incubated for 2 minutes at room temperature and counted using a hemocytometer chamber (Hausser Scientific, Horsham, PA, USA) and a light microscope at 40x magnification. Results are reported as total dead cells/total cell count x 100.

2.5 Bacterial viability: Before the co-incubation period, 100 µL of each *Brachyspira* inocula were plated on blood agar plates and incubated anaerobically using a commercial system (Anaerogen, Oxoid Limited, Basingstoke, United Kingdom) at 42°C for 48 hours. Similarly, 100 µL of *Salmonella* Typhimurium were plated on LB agar plates (Bectron, Dickinson and Company BD, Sparks, MD, USA) and incubated at 39ºC for 24 hours. After the co-incubation period, 100 µL of cRPMI containing any of the *Brachyspira* inocula or cRPMI inoculated with *Salmonella* Typhimurium were plated on their respective medium plates and environmental conditions described above.

2.6 Relative mRNA expression levels: Expression of CD19 (B-lymphocyte antigen), CD79b (immunoglobulin-associated beta), lyn (tyrosine-protein kinase), syk (spleen associated tyrosine Kinase), SLA-DRA (swine leukocyte antigen class II), tumor necrosis factor alpha (TNF-α), interferon alpha (IFN-α), interferon beta (IFN-β), and interleukin 10 (IL-10) was evaluated by reverse transcriptase, real time PCR. Primers used for amplification were: CD19: forward 5´- GAAATTGCTGAGCCTGAACC-3´, reverse 5´- AGCAACAGAACAGCCTTTCC-3´; CD79b: forward 5´- TGATTTGGAGGAGGGAGTTC-3´, reverse 5´- CATGGGAGAATGGGTTTGAG-3´; LYN: forward 5´- TTGTTGACAAGAGGCTGTGC-3´, reverse 5´ TGGGAAAGACACCAAAGCTC-3´; SYK: forward 5´- CACTTGCCCTTCTTCTTTGG-3´, reverse 5´- CGGTTGAAAGGGTTCTTGAG-

3′; SLA-DRA: forward 5′- ATCTCCCCTTCATGCCCTCA-3′, reverse 5′-AGCTTCAAACTCCCAGTGCT-3′; TNF-α: forward 5′- CCAATGGCAGAGTGGGTATG-3′, reverse 5′- TGAAGAGGACCTGGGAGTAG-3′; IFN-α: forward 5′-GGCTCTGGTGCATGAGATGC-3′, reverse 5′-CAGCCAGGATGGAGTCCTCC-3′; IFN-β: forward 5′-TGCAACCACCACAATTCCAGAAGG-3′, reverse 5′-TCTGCCCATCAAGTTCCACAAGGA-3′, and IL-10: forward 5′-GGTTGCCAAGCCTTGTCAG-3′, reverse 5′-AGGCACTCTTCACCTCCTC-3′. The cytokines primers were previously published and validated [30]. All other primers were validated initially *in silico* by verifying primer nucleotide homology with the target template, followed by amplicon size verification and melt-curve analysis using the PCR conditions described below. Following the co-incubation period, flasks containing B-cells and a given inocula were centrifuged at 500 g for 5 minutes. The supernatant was discarded and 1 mL of RNAlater (Sigma-Aldrich Co., St. Louis, MO, USA) was added to the pellet and vortexed. Samples were stored at -80°C until processing. RNA extraction was performed using a commercial kit (Qiagen RNeasy, Qiagen, Hilden, Germany) and cDNA was synthesized using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. PCR reactions were conducted in a Bio-Rad CFX instrument (Bio-Rad Laboratories Ltd., Mississauga, ON). Each 25 μL reaction contained 12.5 mL of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories Ltd, Hercules, CA, USA), forward and reverse primers (20 μM each), and 2 mL of cDNA template. Reactions were incubated at 94◦C for 3 minutes, followed by 40 cycles of 10 seconds at 95ºC, 10 seconds at 59ºC for SLA-DRA and IFNβ; 63.3ºC for IL-10 and CD19; and 65ºC for IFNα, TNF-α, SYK, LYN and CD79b, and 30 seconds at 72ºC. Negative and no-template controls were included in each plate ran. All reactions were run in duplicates. Reaction duplicates that differed by more than 1 Ct were repeated.

2.7 Statistical analysis: Shapiro-Wilk test was used to evaluate the normality of data. Differences in B-cell mortality levels among the groups were analyzed using one-way ANOVA followed by post-hoc Tukey test. Real-time PCR data were analyzed using generalized linear mixed models based on lognormal-Poisson error distribution, fitted using Marcov chain Monte Carlo sampling (mcmc.qPCR package on R version 4.2.0, RStudio, Boston, MA, USA).

3. Results

B-cell exposure to LPS (P <0.001) or *S.* Typhimurium (P = 0.001) significantly increased mortality when compared to the negative control group for all pairwise comparison.

None of the other treatments led to a significant impact on B-cell viability. A summary of the data is presented in Figure 1.

B-cell exposure to *B. pilosicoli* led to no change in the expression of the BCR signaling component CD79b. In contrast, all other treatments significantly increased CD79b mRNA levels. Other components of the BCR activation pathway (CD19, syk, and lyn) were significantly downregulated only following *B. pilosicoli* exposure (-2.8 fold, $P = 0.0001$; -2.1 fold, $P < 0.0001$; and -1.5 fold, $P = 0.03$, respectively). In contrast, syk mRNA levels was only increased when B-cells were exposed to *B. hampsonii* (2.2 fold, $P = 0.02$) or *B. hyo* (1.7 fold, $P = 0.02$), relative to the negative control group. None of the other treatments significantly altered the expression of lyn.

B. pilosicoli decreased B-cell expression of TNF-α (-2.4 fold, $P = 0.03$), but increased IFN-β (5.5 fold, $P = 0.01$) mRNA production relative to the negative control group. *B. hyo* exposure increased the mRNA levels of IFN-α (2.0 fold, $P = 0.02$) and IFN-β (5.9, $P = 0.01$). *S.* Typhimurium upregulated the expression of IFN-α (1.9 fold, $P = 0.03$) and IL-10 (2.1 fold, $P = 0.03$), in relation to the negative control samples. SLA-DRA was upregulated following *B. hampsonii (*2.5 fold, $P = 0.01$), *B. pilo* dead (2.4 fold, $P = 0.01$), *B. hyo* (1.61 fold, $P = 0.05$), *B. KL180* (1.90 fold, $P = 0.05$), and *S.* Typhimurium (1.7 fold increased compared to control, $P = 0.03$), but remained unaffected in the presence of *B. pilosicoli*. A summary of the RT-PCR data is presented in Figure 2.

Post-inoculation evaluation of the viability of bacterial inocula resulted in no growth of the *Brachyspira* spp. and *S.* Typhimurium in their respective culture medium.

4. Discussion

Here we investigated the T-cell independent B-cell response to enteric pathogens associated with grower-finisher diarrhea in pigs. Surprisingly, B. *pilosicoli* downregulated genes involved in B-cell activation and differentiation, and did not trigger the expression of the major histocompatibility complex type II (MHC-II, SLA-DRA gene). *B. hyodysenteriae*, different strains of *B. hampsonii*, *S.* Typhimurium and killed *B. pilosicoli* triggered activating responses by the host cells. Grower-finisher infectious diarrhea directly impacts profit in commercial swine operations [31,32]. Understanding B-cell response to pathogens to which antibiotics are largely used in pigs may aid in the development of preventative tools.

Our data showed that B-cell exposure to all treatments other than *B. pilosicoli* upregulated CD79b expression. After antigen binding to BCR, CD79b is the initial signaling trigger involved in B-cell maturation and activation [33,34]. Phosphorylation of the tyrosine-

based activation motif (ITAM) on CD79b by Src-family kinases activates syk, followed by downstream signaling molecules, such as phospholipase C gamma 2 (PLC-γ2) and phosphoinositide 3-kinase (PI3K) [27,37]. These molecules form the main BCR signaling cascade involved in B lymphocyte cell-cycle progression and survival pathways [38,39]. CD79b expression is up-regulated in mice kidneys infected with *Staphylococcus aureus* [40], and in sheep mammary tissue infected with *Mycoplasma agalactiae* [41]. *B. pilosicoli* likely did not lead to crosslinking of BCR, as no changes in CD79b expression was identified. CD19 is a co-receptor of the B-cell cell-surface signal-transduction complex (including CD21, CD81, and CD225) that is phosphorylated by the src-family kinase lyn upon BCR cross-linking [42]. CD19 plays an important role on B-cell activation by reducing the BCR activation threshold, and by promoting BCR-independent B-cell expansion through c-MYC protein stability [43,44]. CD19 deficient mice and humans respond poorly to transmembrane signals, leading to impaired humoral response [45–47]. In contrast, overexpression of CD19 leads to increased humoral response and disruption of tolerance mechanisms [48–50]. Its phosphorylation leads to membrane recruitment of PI3K and activation of (serine/threonine-protein kinase) Akt and cell survival pathway [51]. Moreover, through Akt kinase signaling and tyrosines phosphorylation, CD19 is required for MHC-II-mediated downstream signaling [52]. CD19 recruitment of downstream signaling intermediates play a role in immunoglobulin-induced activation of B-cell or their antigen-independent development [53,54]. CD19 also plays a role in TLR9 signaling pathways in human B cells [55], which is activated by bacterial DNA [56]. We found that *B. pilosicoli* exposure to B-cells downregulated CD19 expression. Although other molecules and receptors from CD19-activated pathways were not evaluated in the present study, our findings suggest that *B. pilosicoli* may increase the BCR activation threshold, repress B-cell expansion and impair pathogen recognition via MHC-II or TRL-9, thus crippling the early B-cell response to infection and potentially inducing tolerance to *B. pilosicoli* antigens.

One of the earliest events following BCR activation is phosphorylation of lyn and syk protein kinases [57]. Lyn plays a crucial role in activating or inhibiting BCR signaling [24]. It can enhance B-cell downstream signaling, phosphorylating ITAMs on B-cell receptor Igα/Igβ (CD79a/CD79b) chains triggering the activation of the spleen tyrosine kinase (syk) [58,59]. Lyn also phosphorylates tyrosine-based inhibitory motifs (ITIMs) on inhibitory receptors (CD22 and FcγRIIB) that suppress BCR signaling [60,61]. Syk binds to the BCR [62], phosphorylating not only ITAM tyrosines at CD79a/CD79b but also other proteins, including CD19 and BCAP, activating the PI3K pathway, and the SH2 domain-containing leukocyte protein of 65 kDa (SLP-65) [63,64]. These signals support further development of B-cells from

pro-B to pre-B-cell. Here we showed that the expression of lyn and syk were downregulated after B-cell exposure to live *B. pilosicoli*. Lyn-deficient mice shown reduced numbers of mature follicular B-cells, absence of marginal zone and higher proportion of immature B-cells [65,66]. Lyn deficiency is also involved in decreased phagocytosis and autophagy upon *Pseudomonas aeruginosa* infection of mice alveolar macrophages [67]. Syk deficiency also impaired the differentiation and maturation of B-lineage cells [68–70]. Taken together, the decrease in CD19, lyn, and syk expression following *B. pilosicoli* suggest that the B-cell response to this pathogen is weakened from a BCR-dependent or independent activation perspective, potentially leading to tolerance.

SLA-DRA are expressed mainly in antigen presenting cells, and it is a key player in extracellular peptide antigen processing and presentation, T-cell dependent response and vaccine efficacy [71,72]. In our study, SLA-DRA was upregulated by B-cell treatment with all inocula, except for live *B. pilosicoli* and LPS. Replication of porcine epidemic diarrhea virus (PEDV) in bone marrow-derived dendritic cells inhibited expression of SLA-DRA, showing PEDV has mechanisms to evade the host immune response [73]. Our results suggest that the cell line used recognized all the treatments as foreign antigens, except for live *B. pilosicoli*. The mechanism through which *B. pilosicoli* escapes antigen processing and presentation may be a key feature to enable vaccine development in the future.

We found increased expression of IFN-α upon B-cell exposure to *B. hyodysenteriae*, *S.* Typhimurium, and IFN-β following *B. hyodysenteriae* and *B. pilosicoli* exposure. Type I interferons (IFN-α/IFN-β, T1IFN) are early innate immunity cytokines and have pleiotropic effects on the immune response modulation, with direct and indirect effects on B-cells [74]. Multiple studies have demonstrated the role of IFN-α/IFN-β as immunoregulatory B-cell stimulators during viral infections [74–77]. T1IFN were found to enhance B-cell response and activation during the inflammatory process, increasing BCR sensitivity, which is suggested as a link between the innate and acquired immune responses [78,79]. In contrast, exacerbated exposure to T1IFN has been shown to be harmful to the host, promoting proliferation of self-reactive B-cells in autoimmune diseases in humans [80]. Thus, the role of T1IFN in response to bacterial infection remains to be clarified [81]. Here we found that *B. hyodysenteriae* and *S.* Typhimurium led to increased levels of IFN-α, when compared to the control group. Exogenous or endogenous IFN-α was found to modulate B-cell proliferation and their differentiation into antibody-secreting cells [82]. Interestingly, Domeier et al. (2018) found evidence that intrinsic B-cell T1IFN signaling causes loss of tolerance in germinal center cells. Also, IFN-α amplifies naïve B-cell activation and immunoglobulin production through TLR-9/MyD88-dependent

signaling after stimulation with CpG motifs of bacterial DNA [84]. In parallel, IFN-β was upregulated by *B. hyodysenteriae* and *B. pilosicoli*. IFN-β exposure reduces B-cell capacity to respond to antigen mediated signals, focusing its response on immediate innate system measures [85]. We postulate that *S.* Typhimurium and *B. hyodysenteriae* triggered the observed B-cell responses due to, in part, the increased production of IFN-α. Oppositely, *B. pilosicoli* effect on IFN-β only may explain the lack of antigen-based B-cell response.

TNF-α is one of the earliest responses by B-cells following crosslinking of surface immunoglobulins [86]. This molecule is a required autocrine factor for B-cell growth, promoting cell differentiation [87]. Our results revealed that TNF-α was significantly downregulated following B-cell exposure to *B. pilosicoli*, but not killed *B. pilosicoli* or any other treatment. In contrast, a previous study using Caco-2 cells found that inactivated *B. pilosicoli* led to the upregulation of TNF-α, while live *B. pilosicoli* did not significantly change its expression levels [88]. Caco-2 are epithelial cells derived from human samples, not pigs. This may explain the differences observed here. Multiple bacterial pathogens have evolved to directly or indirectly suppress the production of TNF-α, thus facilitating parasitism [89,90]. It is plausible that *B. pilosicoli* suppresses lymphocyte TNF-α production to support its periplasmatic lifestyle through a mechanism that remains to be clarified.

IL-10 plays a role enhancing B-cell proliferation and differentiation, and regulates MHC-II antigen presentation [91–93]. We found that *S.* Typhimurium was the only pathogen evaluated in this study to increase IL-10 expression after co-exposure with B-cells. It has been shown that *Salmonella* can induce IL-10 production in mice B-cells *in vitro* via stimulation of TLR2, TLR4, and the myeloid differentiation primary response gene 88 (MyD88) [94]. In lymphoblastoid cell lines, *S.* Typhimurium also induced robust production of IL-10 through induction of the signal transducer and activator of transcription 3 (STAT3)-dependent anti-inflammatory pathway [95]. Among its functions, it has been reported that IL-10 acts inhibiting autophagy, through signaling activation of IL10R-STAT3 and AKT-mTOR pathway [96,97]. Although we did not investigate those pathways genes, our results corroborate previous findings that *S.* Typhimurium may hijack IL-10-signalling to favor its intracellular lifestyle.

S. Typhimurium significantly increased B-cell mortality when compared to the negative control group. Previous research *in vivo* and *in vitro* indicated that *Salmonella* is able to infect and survive in B-cell endosomal-lysosomal compartments [98,99]. These cells act as a reservoir for persistence, dissemination and evasion of $CD8^+$ T-cell-mediated responses [100]. This mechanism is linked to a negative regulation in NLRC4, inhibiting the secretion of IL-1β and its cytotoxic effects, preventing B-cell death by pyroptosis [101,102]. A second study showed

that *Salmonella* could also inhibit B-cell autophagy by activating mTORC1 by secreting its virulence protein SopB [103]. This may be linked to the overwhelmingly high amount of bacteria to which B-cells were exposed in our study.

We recognize that there are multiple steps involved in T-independent B-cell activation, and the work presented here focused only on a few key players of these complex mechanisms. Further work dissecting the downstream effects of the pathways found affected in this study is warranted, especially regarding *B. pilosicoli* interaction with the host.

Our findings revealed that *B. pilosicoli* has a profound impact on B-cell activation, both in a T-dependent and T-independent manners. An antigenicity spectrum among the other *Brachyspira* tested was also identified, helping explain their varied virulence. *S.* Typhimurium was the only agent to induce B-cell death, among those tested. Further studies on the consequences of the pathogen-B-cell interactions identified here are suggested to help clarify pathogenesis mechanisms, and may fill in gaps leading to vaccine development.

Abbreviations

CD21: B-lymphocyte antigen 21; CD22: B-lymphocyte antigen 22; CD225: B-lymphocyte antigen 225; CD79a: immunoglobulin-associated alpha; CD79b: immunoglobulin-associated beta; CD81: B-lymphocyte antigen 81; Cells T CD8+: cytotoxic T lymphocytes; CFU: colony-forming unit; FcγRIIB: Fcγ receptor; GE: genome equivalents; I IFNs: Type I interferons; IFN-α: interferon alpha; IFN-β: interferon beta; Ig: immunoglobulin; IgM: Immunoglobulin M; Igα/Igβ: Immunoglobulin alpha/beta; IL-10: interleukin 10; IL10R: interleukin-10 receptor; IL-18: interleukin-18; IL-1β: Interleukin 1-beta; ITAM: tyrosine-based activation motif; ITIMs: tyrosine-based inhibitory motifs; LB: Luria-Bertani broth; LPS: lipopolysaccharide; Lyn: tyrosine-protein kinase; MHC-II: major histocompatibility complex type II; mTOR: mammalian target of rapamycin; mTORC1: mechanistic/mammalian target of rapamycin complex 1; MyD88: myeloid differentiation primary response gene 88; NLR: Nod-like receptor; NLRC4: Family CARD Domain Containing 4; PI3K: phosphoinositide 3-kinase; PLC-γ2: phospholipase C gamma 2; SC: spirochetal colitis; SD: swine dysentery; SFK: src-family-kinase; SLA-DRA: swine leukocyte antigen class II; SLP-65: SH2 domain-containing leukocyte protein of 65 kDa ; SS: swine salmonellosis; STAT: Signal transducer and activator of transcription 3; Syk: spleen tyrosine kinase; TLR2: Toll-like receptor 2; TLR4: Toll-like receptor 4; TLR7: Toll-like receptor 7; TLR9: Toll-like receptor 9; TNF-α: tumor necrosis factor alpha.

Author statement

Jéssica Barbosa: Conceptualization, methodology, validation, formal analysis, investigation, data curation, writing – original draft, writing – review & editing, visualization. **Christine Yang:** Conceptualization, methodology, validation, formal analysis, investigation, writing – review & editing. **Arthur Silva:** Methodology, validation, investigation, writing – review & editing. **Vinícius Cantarelli:** Funding acquisition, writing – review & editing. **Matheus Costa**: Conceptualization, methodology, validation, formal analysis, investigation, data curation, writing – original draft, writing – review & editing, visualization, project administration, supervision, funding acquisition.

Funding

This work was supported by Coordination of Superior Level Staff Improvement – Brazil (CAPES, Barbosa), and a Natural Sciences and Engineering Research Council – Discovery Grant (Costa).

Data availability

Not applicable.

Declaration of competing interest

No competing interests declared.

Ethics approval and consent to participate

Not applicable.

Acknowledgements

The authors would like to thanks Arely Hernandez Davila, Champika Fernando, Ruwini Dharmasiri Gamage and Noreen Rapin for technical assistance.

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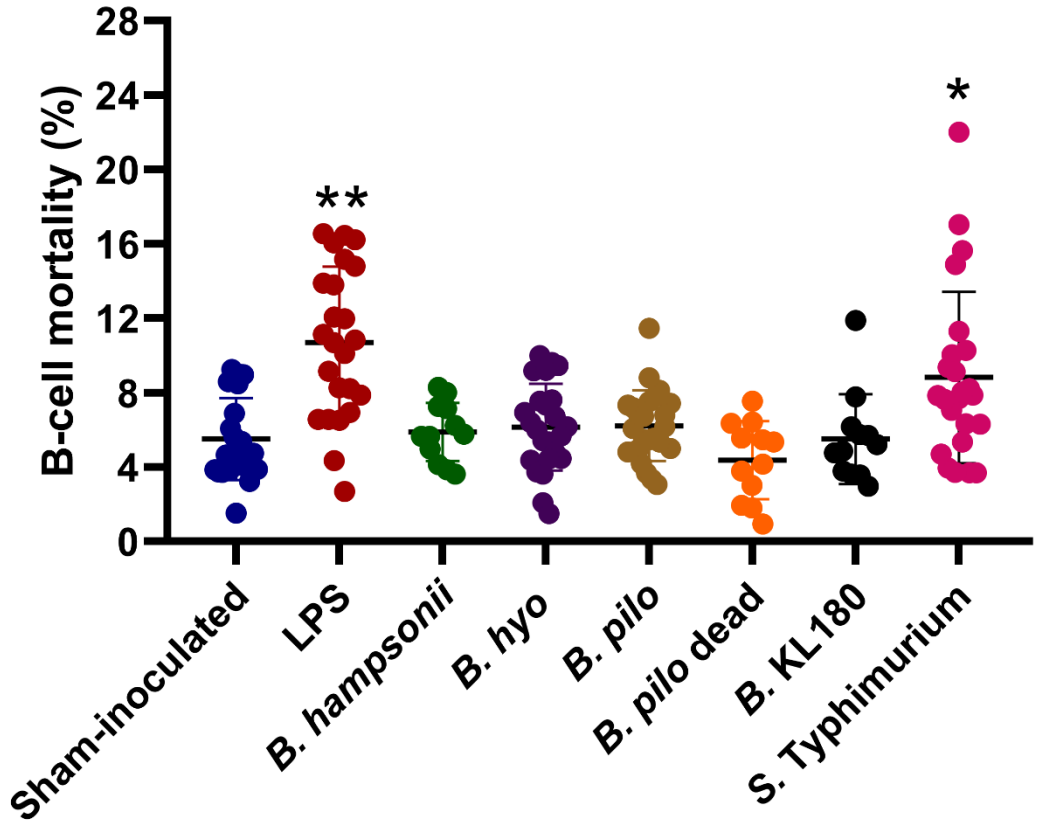

Figure 1. B-cell mortality after 8 hours of exposure to sham-inoculated control (n=6), LPS (n=6), *B. hampsonii* clade II 30466 (*B.hampsonii,* n =3), *B. hyodysenteriae* G44 strain (*B. hyo*, n =6), *B. pilosicoli* (*B. pilo,* n=6), *B. pilosicoli* sonified dead (*B. pilo* dead, n=3), non-pathogenic *B. hampsonii* clade 2 KL180 (*B.* KL180, n=3), and *Salmonella* Typhimurium (*S.* Typhimurium, n=6). *-Denotes statistical significance between *S.* Typhimurium and all groups, except LPS ($P = 0.001$). **-Denotes statistical significance between LPS and all groups, except *S.* Typhimurium ($P < 0.001$).

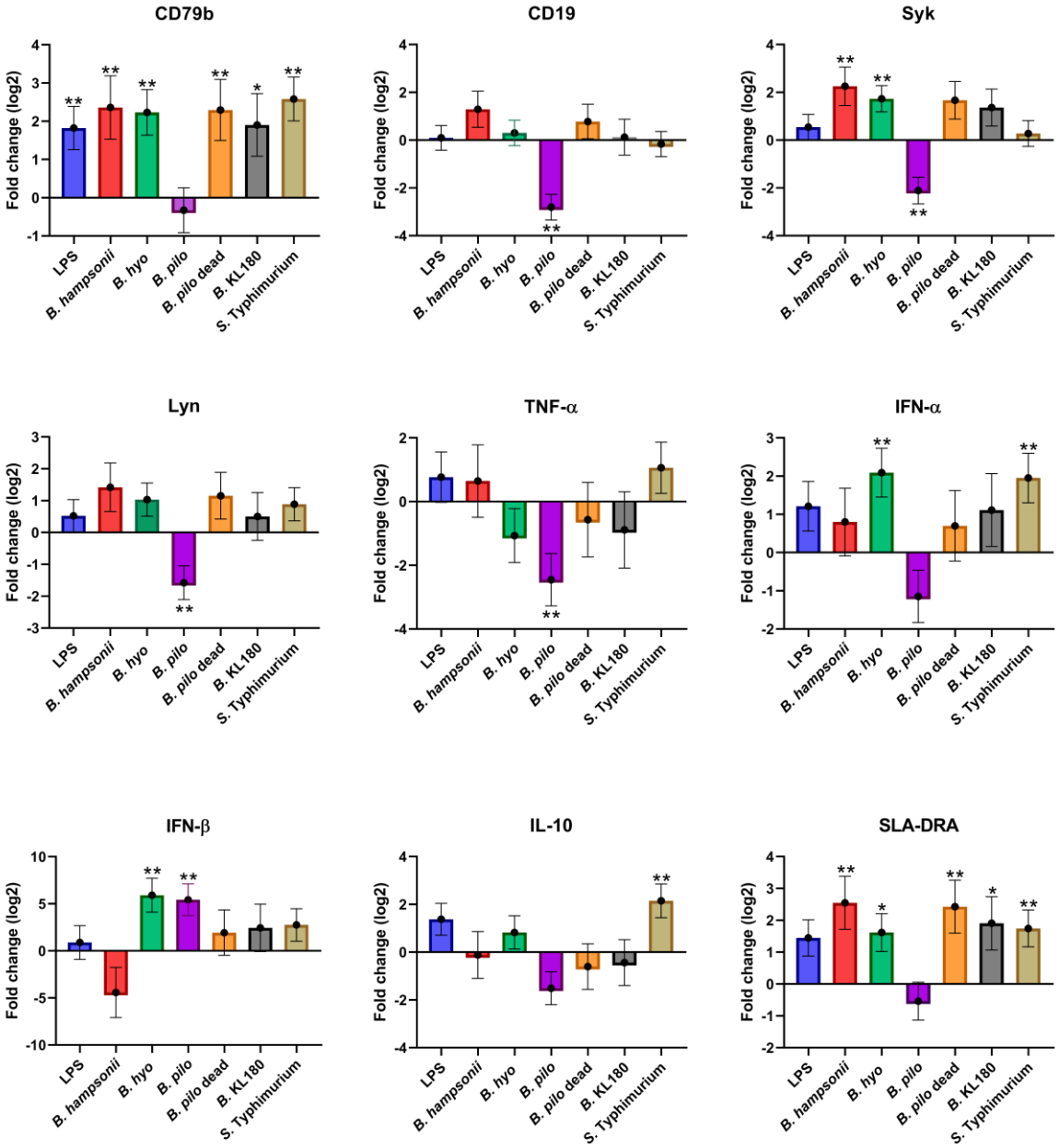

Figure 2. Expression of B-cell activation and proliferation marker genes after 8 hours of exposure to sham-inoculated control (n=6), LPS (n=6), *B. hampsonii* clade II 30466 (*B.hampsonii,* n =3), *B. hyodysenteriae* G44 strain (*B. hyo,* n =6), *B. pilosicoli* (*B. pilo,* n=6), *B. pilosicoli* sonified dead (*B. pilo* dead, n=3), non-pathogenic *B. hampsonii* clade 2 KL180 (*B.* KL180, n=3), and *Salmonella* Typhimurium (*S.* Typhimurium, n=6). measured by quantitative real-time RT-PCR. Bars depict mean fold change (log2) values from eight treatments, relative to the negative control group, and error bars represent 95% confidence intervals. **- Denotes statistical significance (P <0.05). *- Denotes statistical significance (P = 0.05).

ARTICLE 3 - EXPERIMENTAL INFECTIOUS CHALLENGE IN PIGS LEADS TO ELEVATED FECAL CALPROTECTIN LEVELS FOLLOWING COLITIS, BUT NOT ENTERITIS

ARTICLE ACCEPTED FOR PUBLICATION ON PORCINE HEALTH MANAGEMENT

Published: 24 August 2021

https://doi.org/10.1186/s40813-021-00228-9

Experimental infectious challenge in pigs leads to elevated fecal calprotectin levels following colitis, but not enteritis

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Abstract

Background: Fecal calprotectin is largely applied as a non-invasive intestinal inflammation biomarker in human medicine. Previous studies in pigs investigated the levels of fecal calprotectin in healthy animals only. Thus, there is a knowledge gap regarding its application during infectious diarrhea.

This study investigated the usefulness of fecal calprotectin as a biomarker of intestinal inflammation in *Brachyspira hyodysenteriae* and *Salmonella* Typhimurium infected pigs.

Results: Fecal samples from pigs with colitis ($n = 18$) were collected from animals experimentally inoculated with *B. hyodysenteriae* ($n = 8$) or from sham-inoculated controls ($n = 3$). Fecal samples from pigs with enteritis ($n = 14$) were collected from animals inoculated with *Salmonella enterica* serovar Typhimurium ($n = 8$) or from sham-inoculated controls ($n = 4$). For both groups, fecal samples were scored as: 0 = normal; 1 = soft, wet cement; 2 = watery feces; 3 = mucoid diarrhea; and 4 = bloody diarrhea. Fecal calprotectin levels were assayed using a sandwich ELISA, a turbidimetric immunoassay and a point-of-care dipstick test. Fecal calprotectin levels were greater in colitis samples scoring 4 versus ≤ 4 using ELISA, and in feces scoring 3 and 4 versus ≤ 1 using immunoturbidimetry ($P < 0.05$). No differences were found in calprotectin concentration among fecal scores for enteritis samples, regardless of the assay used. All samples were found below detection limits using the dipstick method.

Conclusions: Fecal calprotectin levels are increased following the development of colitis, but do not significantly change due to enteritis. While practical, the use of commercially available human kits present sensitivity limitations. Further studies are needed to validate the field application of calprotectin as a marker of intestinal inflammation.

Keywords: biological markers, intestinal inflammation, enteric disease, swine

Background

The use of antimicrobials as growth promoters (APG) in pork production has been globally discouraged due to the emergence of multi-drug resistant bacterial strains which can impose risks to human and animal health [1, 2]. In most countries with significant pork production, the current policies on the use of antimicrobial agents have resulted in a need for improved on-farm biosecurity, nutritional, husbandry, and welfare practices, as well as the development of tools to guide the use of antimicrobials [3]. A non-invasive biomarker for intestinal inflammation would result in more judicious therapeutic and nutritional interventions during episodes of enteric diseases in commercial operations.

Swine dysentery (SD) and porcine salmonellosis are intestinal disorders of global relevance in grower-finisher pigs. Both diseases are associated with significant economic losses due to increased production costs and poor animal performance [4, 5]. Mucohemorrhagic diarrhea and colitis (inflammation of the large intestine) are the main clinical signs of SD caused

by *Brachyspira hyodysenteriae*, *B. hampsonii* and *B. suanatina* [6]. Currently, the use of antimicrobials is the only strategy to prevent and treat this disease [7]. *Salmonella enterica* serovar Typhimurium causes enteritis (inflammation of the small intestine) and watery diarrhea in pigs [5, 8]. Even though studies have evaluated vaccination to control salmonellosis in pigs, protection is variable due to poor cross-protection across serovars [9, 10], and antimicrobials are still used metaphylactically.

Calprotectin is a 24 kDa calcium binding protein of the S100 family. It accounts for approximately 60% of the cytosolic protein in neutrophils and is also found in monocytes [11, 12]. It is released upon neutrophil activation and displays antimicrobial, antiproliferative and apoptotic properties [12, 13]. Interestingly, calprotectin is resistant to intestinal bacteria proteases [14]. In human medicine, calprotectin has been used to assess the extent of intestinal inflammation [15]. Its concentration in feces is correlated with inflammatory bowel disease (IBD) [16, 17], and necrotic enterocolitis in infants [18]. Fecal calprotectin is used to identify and aids in distinguishing IBD from irritable bowel syndrome (IBS) [19, 20], and is specifically useful to predict disease activity and relapse during treatment [21, 22]. Increased fecal calprotectin levels were associated with endoscopic and histological lesions during episodes of IBD [23, 24] and can be used to distinguish between inflammatory and non-inflammatory colitis in humans [25]. Physicians often apply this concept to distinguish IBD relapses from true infectious colitis and diarrhea [21, 25]. Thus, there is a plethora of commercially available kits aimed at detecting human calprotectin in feces, ranging from laboratory-intensive ELISAs to point-of-care dipsticks.

Studies focused on swine have investigated calprotectin levels in the feces of healthy animals only, suggesting it may be involved in intestinal homeostasis [26, 27]. However, there are no reports on the use of calprotectin as a biomarker of intestinal inflammation in disease-challenged pigs. We hypothesized that, similar to what is observed in humans, pigs with intestinal inflammation have increased levels of fecal calprotectin. The swine calprotectin S100-A8 subunit amino-acid sequence is 72% similar to the human protein, and the S100-A9 subunit is 66% similar. Thus, we also hypothesized that commercial kits aimed at human calprotectin should also detect the swine protein. Therefore, the objective of this study was to evaluate the usefulness of fecal calprotectin as a biomarker of colitis or enteritis in swine using commercially available human kits.

Results

Colitis samples assessment

Using ELISA, fecal samples that scored 4 (bloody diarrhea) had higher calprotectin levels than those that scored 0, 1, or 3 ($P = 0.037$, Fig. 1A). Using immunoturbidimetry, fecal samples that scored 3 and 4 had higher calprotectin levels than those that scored 1 (score 3 $P = 0.039$, score 4, $P = 0.044$ respectively, Fig. 1B). Fecal calprotectin level was positively correlated with fecal consistency scores using ELISA ($\rho = 0.728$; $P = 0.001$, Fig. 1A) and immunoturbidimetry ($\rho = 0.80$; $P = 0.001$, Fig. 1B). ELISA was positively correlated with the immunoturbidimetry assay ($\rho = 0.55$; $P = 0.017$). ROC curve analysis (Fig. 1C) revealed that both ELISA ($P = 0.002$) and immunoturbidimetry ($P < 0.001$) could reliably diagnose a diseased state. Immunochromatographic dipstick tested negative for all samples.

Enteritis samples assessment

No differences were found in calprotectin concentration among fecal score groups when measured using ELISA ($P = 0.098$; Fig. 2A) or immunoturbidimetry ($P = 0.579$; Fig. 2B). However, fecal scores 1 and 2 did have numerically higher fecal calprotectin concentrations than score 0 using either method. Fecal calprotectin concentration was not correlated with fecal consistency scores when analyzed by either ELISA ($\rho = 0.536$; $P = 0.590$; Fig. 2A) or immunoturbidimetry ($\rho = 0.268$; $P = 0.376$; Fig. 2B). The same correlation pattern was observed between ELISA and Immunoturbidimetry assays ($\rho = 0.464$; $P = 0.095$). ROC curve analysis (Fig. 2C) revealed no statistical significance regarding the ability of either ELISA ($P = 0.56$) or immunoturbidimetry ($P = 0.51$) assays in diagnosing a diseased state. Additionally, all samples tested negative when the immunochromatographic dipstick test was used.

Discussion

Grower-finisher infectious diarrhea in commercial swine operations leads to decreased performance and increased production costs associated with treatment and mortality, directly impacting profits [4, 5]. To help direct immediate therapeutic and nutritional interventions following observation of diarrhea, a non-invasive intestinal inflammation biomarker test to differentiate inflammatory from non-inflammatory causes of diarrhea would be beneficial for practitioners. In this study, we observed that fecal calprotectin levels, measured by ELISA or

immunoturbidimetry, increases following the development of colitis and mucoid or bloody diarrhea in pigs challenged with *B. hyodysenteriae.* However, we did not find any changes in fecal calprotectin levels due to enteritis caused by *S.* Typhimurium. Both methodologies were ineffective in discerning between mild, watery diarrhea, and normal feces during colitis or enteritis.

Calprotectin is a calcium binding protein secreted by neutrophilic granulocytes and has a role controlling bacterial growth during inflammation [12, 28]. Recruitment of neutrophils to the intestinal mucosa leads to neutrophil cell shedding and active secretion of calprotectin to the intestinal lumen [12]. Currently, recognized triggers of calprotectin secretion are lipopolysaccharide and monosodium ureate [1, 2]. This is in line with findings showing that, in humans, bacterial agents lead to higher fecal calprotectin levels than viral [41, 56, 57]. Once secreted, calprotectin sequesters essential micronutrients such as iron, zinc, and manganese, inhibiting bacterial growth [29, 30]. Fecal calprotectin concentration has been shown to be correlated with the number of neutrophils released in the intestinal lumen during inflammation, which in humans can be associated with the severity of inflammation [19]. Previous studies investigating calprotectin levels in the feces of healthy pigs suggested it may play a role in intestinal homeostasis [26]. Lallès et al. [27] observed that the average fecal calprotectin concentration from sow samples (13 ± 38 mg/kg of feces) was close to the range described in healthy human adults (range 2–47 mg/kg), but the concentrations found from piglet samples at birth were lower (24 ± 60 mg/kg) than human newborns (145 ± 78.5 mg/kg). The same authors also found very low fecal calprotectin levels in healthy pigs under high sanitary conditions. Elevated fecal calprotectin is a common finding in humans with IBD [16, 22]. In humans, patients with IBD and IBS have similar clinical signs. Calprotectin is already extensively used in human medicine as a biomarker of IBD, as it can help distinguish IBS from IBD, and detect recurrent IBD during treatment [20, 25]. Fecal calprotectin levels reported from dog samples can be used to discern between animals with different causes of chronic inflammatory enteropathies such as steroid-responsive/refractory enteropathy and immunosuppressant-responsive/-refractory enteropathy, and animals with food-responsive enteropathy or antibiotic-responsive enteropathy before treatment [31, 32].

Here elevated fecal calprotectin levels in pigs were associated with mucoid or haemorrhagic colitis, but not enteritis. While further studies using larger populations are needed to validate these results, our data suggests that fecal calprotectin could be a potential tool used to diagnose severe inflammatory colitis, particularly by untrained observers who may, for example, miss blood staining in feces when pigs are housed in large groups. It may also help

distinguish bacterial colitis from other causes of diarrhea in pigs, thus, contributing to a more judicious use of antimicrobials for pork production. We found that mucoid or mucohemorragic feces from pigs with colitis contained the highest calprotectin concentration. Multiple previous reports have characterized the accumulation of neutrophils, a source of antimicrobial peptides such as calprotectin, on the surface of the colonic mucosa during *B. hyodysenteriae* and *B. hampsonii* infection in pigs [33,34,35,36]. Here we found evidence that severe SD clinical signs are associated with increased fecal calprotectin levels, providing further evidence of the importance of neutrophils in the pathogenesis of swine dysentery.

In swine, *S.* Typhimurium invades epithelial cells of the small intestine. It can invade colonocytes as well, leading to inflammatory diarrhea with a marked increase in mucosal neutrophil infiltration [5, 8, 37]. Despite this, we did not observe a significant increase in fecal calprotectin levels following inoculation with *S.* Typhimurium, regardless of the assay used. Our findings differ from previous studies that found increased fecal calprotectin concentration during *S.* Typhymurium infection in rats [38, 39], and *Salmonella* spp. infection in humans [40]. Human patients with severe or moderate bacterial gastroenteritis and fecal mucus have increased fecal calprotectin, but those with mild diarrhea do not [41]. Mucoid feces is not a feature of swine salmonellosis, but it is associated with *Brachyspira* spp. [5]. Moreover, it has been shown that *S.* Typhimurium overcomes the antimicrobial effect of calprotectin by expressing a high affinity zinc transporter (ZnuABC) [39, 42]. We recognize that the lack of histopathology data from either sample cohort is a limitation here and suggest the collection of such samples in future studies.

The literature is contradictory regarding the association between high fecal calprotectin levels and lesion site. There are reports that either ileal or colonic lesions can both be monitored using fecal calprotectin as an indicator of endoscopically active Crohn's disease (CD) [16, 43,44,45]. In contrast, other studies have found that the discriminatory power of fecal calprotectin is greater in ileocolonic and colonic CD, than in jejunal or ileal CD [46,47,48]. Zittan et al. [47] postulated that the slow intestinal transit in the colon could increase calprotectin degradation through intestinal proteases, thereby reducing its concentration in feces. We believe that the lack of difference in calprotectin levels in enteritis samples was due to the proximal location of the lesions, which were most likely associated with the small intestine [49]. Differently from humans, pigs have a functional cecum that may contribute to this disappearance effect by luminal proteases. Furthermore, age may as well impact luminal calprotectin clearance. The gastrointestinal tract length of pigs used in this study were a portion of the size of a finisher pig, together with the functional changes that take place following

weaning these could be factors that influence the disappearance of calprotectin released in the small intestine.

Interestingly, higher concentrations of fecal calprotectin were found when measured using the immunoturbidimetry assay compared to ELISA in both sample cohorts. For human samples, ELISA based on monoclonal antibodies is the gold standard used to quantify fecal calprotectin levels. It is specific to calprotectin heterodimeric and polymeric complexes. However, ELISA is laborious and time-consuming [13, 50] when compared to the a particle enhanced turbidimetric immunoassays (PETIA), based on polystyrene nanoparticles coated with calprotectin-specific antibodies binding to their specific target within the extracted samples. Subsequent quantification of the agglutinated calprotectin-nanoparticle complex detected by light absorbance (turbidity) can be adapted to several commercially available clinical chemistry analyzers and has been proposed as a rapid response test [51]. Labaere et al. [52] compared different calprotectin detection methods (three rapid quantitative immunochromatografic tests, two enzyme-linked immunosorbent assays, and one automated fluoroimmunoassay), and reported significant variations in the calprotectin levels detected. Juricic et al. [53] reported fecal calprotectin concentrations using a commercial ELISA kit to be significantly lower than a turbidimetric immunoassay. Oyaert et al. [54] observed satisfactory diagnostic performance between six different fecal calprotectin immunoassays (two ELISA, two chemiluminescent immunoassays (CLIA), one fluoroenzyme immunoassay (FEIA), and one PETIA), even though there were discrepancies in calprotectin values detected between these kits. These reports are consistent with our findings that different assays resulted in different values for fecal calprotectin. It is worth mentioning that the kits evaluated in this study used monoclonal antibodies specific for human calprotectin, therefore, the low calprotectin levels found by ELISA may be due to the lack of cross reactivity with the swine protein, as previously reported [26]. While we understand the limitation of this approach, commercial kits for fecal calprotectin detection are only available for humans. In addition, there are multiple point-of-care kits commercially available that could be translated into farm-friendly tools. Nevertheless, we still found evidence that human tests can be used in veterinary medicine, taking advantage of this previously developed infrastructure. However, test sensitivity must be further evaluated and optimized for swine, if deemed necessary by future investigations.

We recognize that there are multiple other causes of enteritis and colitis in pigs; we believe that *B. hyodysenteriae* and *S.* Typhimurium are also representative of these syndromes.

We also appreciate that a limited number of samples were utilized in both COL and ENT groups. This likely limited some of our findings related to the less severe fecal scores.

Conclusions

This initial data suggests that fecal calprotectin only peaks to detectable levels following colitis, but not enteritis. The approach used was unable to discern between mild-diarrhea and healthy feces, or when pigs only developed enteritis. Further investigations are suggested as this approach has the potential to support the judicious use of antimicrobials for pork production through the differentiation of infectious from non-infectious causes of colitis.

Methods

Animal trials and fecal samples

Two independent trials (one for each pathogen) were performed where pigs were obtained from the same PRRSV negative, high-health herd with no gastrointestinal clinical signs and historically free from swine dysentery and salmonellosis. Animals were housed and allowed to acclimate in a BSL-2 animal care facility for 7 days prior to inoculation. Colitis samples (COL, $n = 18$) were obtained from 9-to-10-week-old barrow pigs (housed in pens with 6 pigs/pen) experimentally inoculated ($n = 8$) thrice over 72 h with *Brachyspira hyodysenteriae* G44 (obtained from a clinical case), the etiologic agent of swine dysentery, or from sham-inoculated controls ($n = 3$). A commercial starter diet, unmedicated, fed ad libitum was used. Pigs were intragastrically inoculated with 50 mL liquid media averaging 1.69×10^9 genome equivalents/mL as previously described [35]. A summary of the samples used from this trial is shown on Table 1. The development of swine dysentery was confirmed by associating clinical signs, positive fecal *B. hyodysenteriae* culture and gross necropsy lesions (data not shown). Enteritis samples (ENT, $n = 14$) were collected from pigs experimentally inoculated with *Salmonella enterica* serovar Typhimurium var Copenhagen ($n = 9$, isolated from a clinical case), or from non-infected controls ($n = 4$). After a 7-days acclimation period, pigs were orally inoculated twice within 4 h with 1 mL containing 3.3×10^9 CFU/mL/pig of *S.* Typhimurium, as previously described [55], or 1 mL of sterile saline solution (non-infected controls). Pigs were fed a diet that met the minimum requirements for this age, and were group housed in pens with 8 animals [55]. Sample summary is also shown in Table 1. All animals tested negative by culture for their inoculation agent upon arrival at the BSL-2 facility [35, 55]. Daily monitoring for pathogen of interest shedding was also performed

as previously described [35, 55], and only positive samples were used in this study. Briefly, *Brachyspira* spp. culture was performed using BJ agar in anaerobic chambers at 42 °C for up to 10 days. *Salmonella* samples were cultured in brilliant green agar and verified by broth culture using enriched selenite-cysteine broth. As expected, fecal scores for this trial ranged from 0–2. The development of salmonellosis was confirmed by fecal culture, gross necropsy lesions (no signs of typhlitis or colitis were observed), clinical signs, intestinal levels of antioxidant enzymes and performance parameters (data not shown). Feces from both trials were collected following digital stimulation, and only one sample per pig per score was included. Scoring followed a previously developed fecal consistency rubric [35]: 0 = normal; 1 = soft, wet cement; 2 = watery feces; 3 = mucoid diarrhea; and 4 = bloody diarrhea. All fecal samples were obtained from individual pigs and stored at -80ºC until processing for analysis.

Fecal sample extraction

Fecal samples were processed according to the kit manufacturers' instructions, with minor changes as described below (Bühlmann Calprotectin ELISA EK-CAL, Bühlmann Laboratories AG, Switzerland). For each sample, between 50 and 100 mg of feces were weighed into a sterile polypropylene tube (15 mL, VWR Scientific Products, Suwanee, GA, USA). Extraction buffer was added, adjusting the reaction volume to each sample weight to obtain a final 1:10 ratio. Extraction tubes were individually vortexed for 30 s (Fisher Vortex Genie 2, Fisher Scientific, Pittsburgh, PA, USA) at maximum speed and incubated for 30 min at room temperature on a shaker at 400 rpm (G-25 Incubator Shaker, New Brunswick Scientific Co., Inc., Edison, NJ, USA). Samples were vortexed again for 30 s, a 1.5 mL aliquot was transferred to a 2 mL sterile microfuge tube and centrifuged at 3000 g for 5 min. Finally, the supernatant was transferred to a 1.5 mL microfuge tube and stored at -20 °C until analysed.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was carried out following the manufacturer's instructions (Bühlmann Calprotectin ELISA EK-CAL, Bühlmann Laboratories AG, Switzerland). Fecal extracts were thawed and homogenized prior to analysis. Initially, 100 µL of incubation buffer (blank, negative control), five calibrator samples (100 µL/well, ranging from 30 to 1800 µg/g; Additional file 1: Table 1), and low and high control samples (100 µL/well) were included on each microtiter plate precoated with anti-calprotectin monoclonal antibodies (mAb). Finally, 100 µL of fecal extract per sample was analyzed. All samples were analyzed in duplicates, including extraction controls. Following dispensing of samples and controls, reaction plates were incubated for

35 min using an orbital plate shaker at 450 rpm, at room temperature. After incubation, plates were washed three times for 30 s with 300 µL of wash buffer per well. Next, each sample was incubated and mixed for 35 min with 100 µL of enzyme label anti- mAb conjugated with horseradish peroxidase (HRP). The wash step was repeated 5 times as described above and immediately after; the color reaction was induced using 100 µL of tetramethylbenzidine (TBM). The plate was covered with a plate sealer (Bühlmann Laboratories AG, Switzerland) to prevent TBM degradation due to exposure to light, and incubated for 15 min on a plate shaker at 400 rpm at room temperature. The reaction was stopped by adding 100 µL of 0.25 M sulfuric acid to each well and absorbance assessed at 450 nm using a microplate reader (Biotek Epoch, Biotek Instruments, Winooski, Vermont, USA). Calprotectin level was expressed as micrograms per gram (µg/g) of feces and values are reported as the mean value for both duplicates.

Immunoturbidimetry assay

Fecal extracts were thawed and analyzed using the fCal Turbo assay (BÜHLMANN, Laboratories AG, Switzerland). This assay was adapted to be performed on a plate reader. Reaction buffer (150 µL) and immunoparticles (30 µL) were pipetted into all wells of a test plate. Six calibrator samples (10 µL/well, ranging from 0 to 2207.6 µg/g; Additional file 1: Table 2) were included in each plate. Ten µL of fecal extract per sample was tested in duplicate. Absorbance was measured at 546–580 nm using a microplate reader (Biotek Epoch, Biotek Instruments, Winooski, Vermont, USA) using the Gen5 Data Analysis software interface (Biotek Instruments, Winooski, Vermont, EUA).

Immunochromatographic assay

Samples were also analyzed using a point-of-care dipstick test for detection of calprotectin in feces (Actim calprotectin rapid test, Medix biochemica, Espoo, Finland) following the manufacture's instructions. This is a semi-quantitative test with a detection range of 12.5 to 10,000 µg of calprotectin/g of human feces. Briefly, 1 g from each fecal sample was brought to room temperature and added to the dilution buffer container. The container was manually shaken, and the detection stick was inserted in the container once the sample was diluted. Results were read after 10 min contact between the test strip and the sample.

Statistical analysis

One fecal sample from the COL group (the only score 2) was removed from the analyses but is still shown in the plots for visual comparison only. Analyses were performed using SPSS (IBM-SPSS, Chicago, IL, USA). Differences in calprotectin levels among fecal score groups were analyzed using the Kruskal–Wallis test. When there was a significant overall group difference, the Dunn's post-hoc test was used to assess pairwise differences. The association between calprotectin concentration and fecal consistency score, as well as between ELISA and Immunoturbidimetry assays, was assessed by determining the Spearman's correlation coefficient (ρ). Alpha level for determination of significance was 0.05. A receiver operator characteristic (ROC) curve analysis was performed to assess the diagnostic efficiency of each diagnostic method. Fecal scores ≥ 2 were used as the clinical threshold for diarrhea (positive sample).

List of abbreviations

CD: Crohn's disease; CLIA: Chemiluminescent immunoassay; CS: Colitis samples; ELISA: Enzyme-linked immunosorbent assay; ES: Enteritis samples; FEIA: fluoroenzyme immunoassay; IBD: Inflammatory bowel disease; IBS: Irritable bowel syndrome; HRP: Horseradish peroxidase; mAb: Anti-calprotectin monoclonal antibodies; PETIA: particle enhanced turbidimetric immunoassay; PRRSV: Porcine reproductive and respiratory syndrome virus; SD: Swine dysentery; TBM: tetramethylbenzidine; ZnuABC: high affinity zinc transporter.

Declarations

Bühlmann Laboratories AG had no role in study design, data analyses and interpretation.

Ethics approval and consent to participate

Colitis and enteritis samples used in this study were collected during two independent experiments designed and conducted in accordance with the Canadian Council for Animal Care and approved by the University of Saskatchewan Animal Research Ethics Board (AUP #20180046 and AUP #20190003).

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by Coordination of Superior Level Staff Improvement – Brazil (CAPES, Barbosa), and the Natural Sciences and Engineering Research Council of Canada (Costa).

Authors' contributions

All authors contributed to reviewing the topics and writing the present article, and agree with the contents of the review. All authors read and approved the final manuscript.

Acknowledgements

The authors would like to express their gratitude to Collin-Shaw (Bühlmann Laboratories AG, Switzerland), and Dr. Cheryl Dvorak for knowledge exchange with ELISA assays. Bühlmann Laboratories AG had no role in study design, data analyses and interpretation.

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Table 1. Summary of fecal samples used in this study

Fecal Score	Inoculation group	Calprotectin group	Collection day (dpi)
0	Control-SD	Colitis	0
0	Control-SD	Colitis	0
0	Control-SD	Colitis	5
1	SD	Colitis	9
1	SD	Colitis	6
1	SD	Colitis	8
1	SD	Colitis	8
1	SD	Colitis	5
2	SD	Colitis	5
3	SD	Colitis	5
3	SD	Colitis	5
3	SD	Colitis	15
3	SD	Colitis	8
3	SD	Colitis	9
4	SD	Colitis	7
4	SD	Colitis	10
4	SD	Colitis	5
4	SD	Colitis	8
0	Control-ST	Enteritis	2
0	Control-ST	Enteritis	3
0	Control-ST	Enteritis	5
0	Control-ST	Enteritis	5
1	ST	Enteritis	2
1	ST	Enteritis	1
1	ST	Enteritis	5
1	ST	Enteritis	5
1	ST	Enteritis	4
2	ST	Enteritis	2
2	ST	Enteritis	2
2	ST	Enteritis	3
2	ST	Enteritis	2
2	ST	Enteritis	2

SD Swine dysentery, samples from pigs inoculated with *B. hyodysenteriae, ST Salmonella* Typhimurium, samples from a pigs inoculated with *S.* Typhimurium, *Dpi* Days post-inoculation.

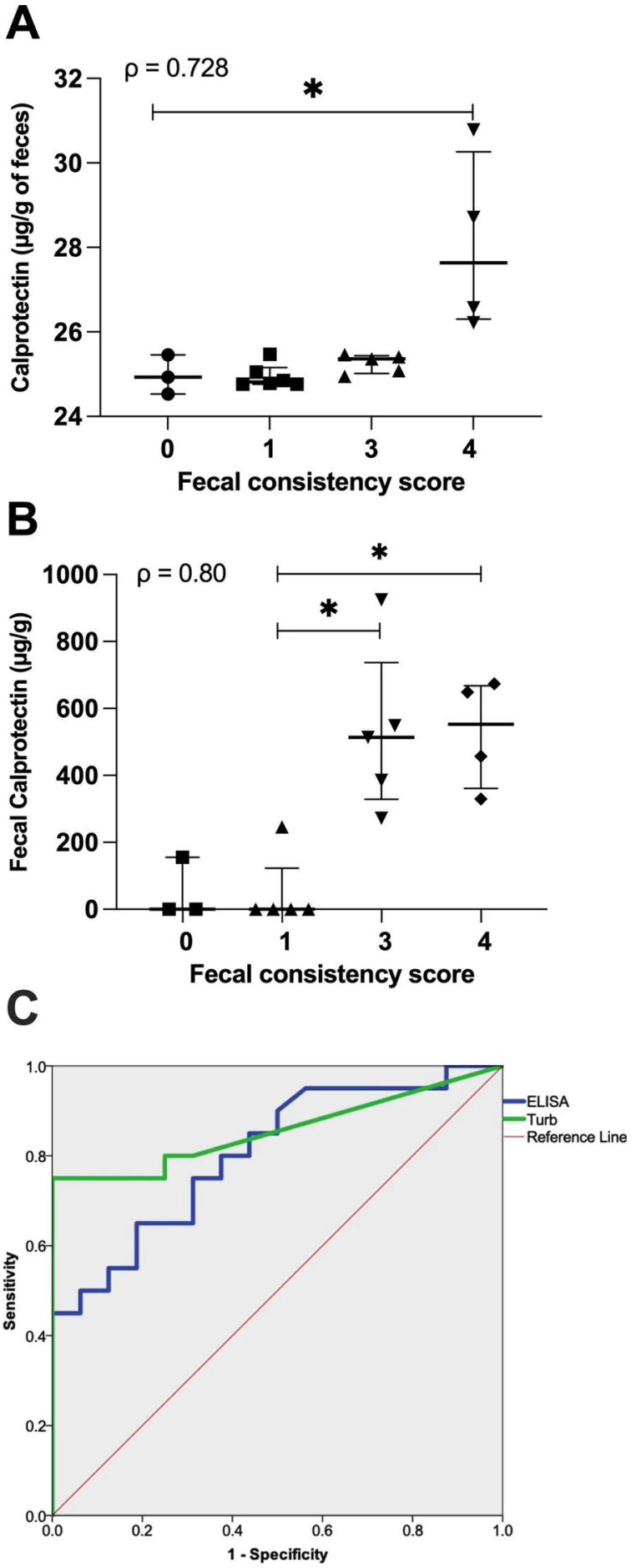

A
ρ = 0.728
*
Calprotectin (µg/g of feces)
32
30
28
26
24
0
1
3
4
Fecal consistency score
B
ρ = 0.80
*
*
Fecal Calprotectin (µg/g)
1000
800
600
400
200
0
0
1
3
4
Fecal consistency score
C
1.0
0.8
0.6
0.4
0.2
0.0
Sensitivity
0.0
0.2
0.4
0.6
0.8
1.0
1 - Specificity
ELISA
Turb
Reference Line

Figure 1. Calprotectin concentration in colitis fecal samples (COL, µg/g) from pigs challenged with *B. hyodysenteriae.* **A** ELISA assay; **B** Immunoturbidimetry assay; **C** ROC curve analysis plot (Turb – immunoturbidimetry assay). Stars denote a significant difference ($P < 0.05$) between fecal scores. Bars denote median, with interquartile range shown error bars. (ρ = Spearman's correlation coefficient).

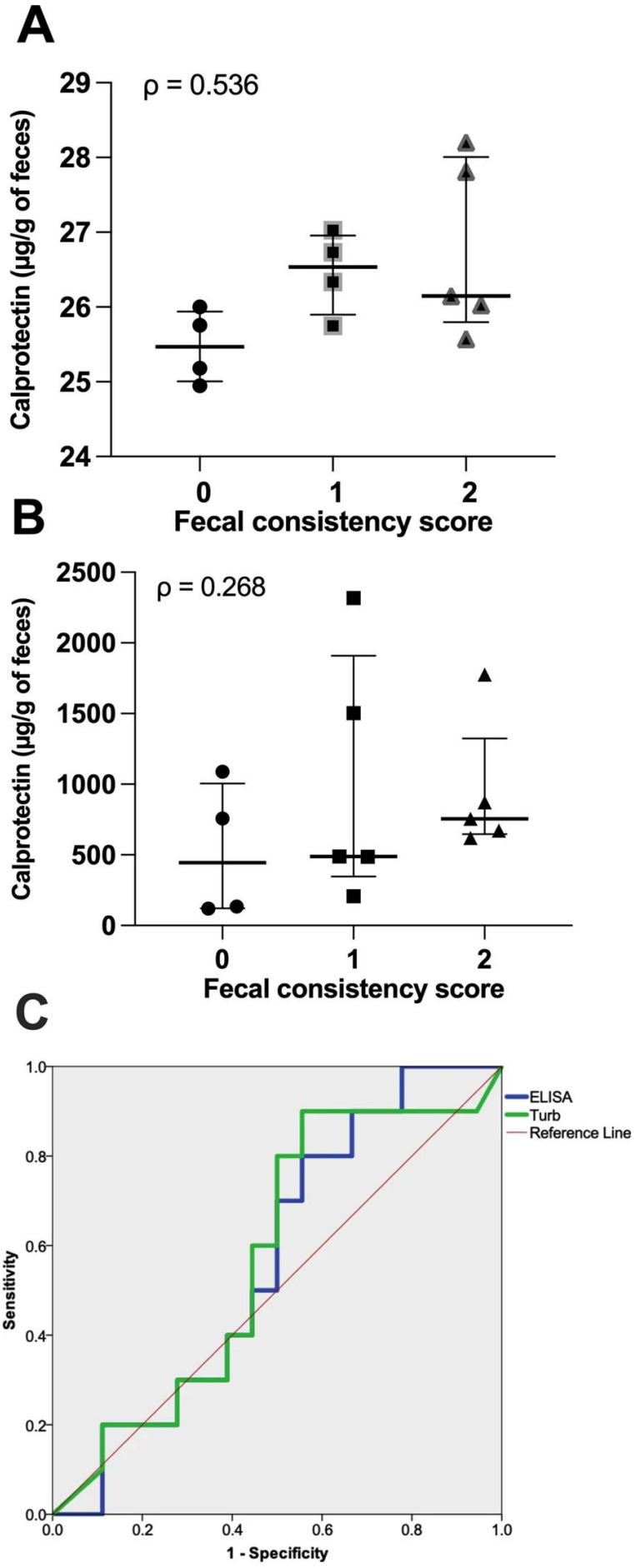

A
ρ = 0.536
Calprotectin (µg/g of feces)
29
28
27
26
25
24
0
1
2
Fecal consistency score
B
ρ = 0.268
Calprotectin (µg/g of feces)
2500
2000
1500
1000
500
0
0
1
2
Fecal consistency score
C
Sensitivity
1 - Specificity
0.0
0.2
0.4
0.6
0.8
1.0
ELISA
Turb
Reference Line

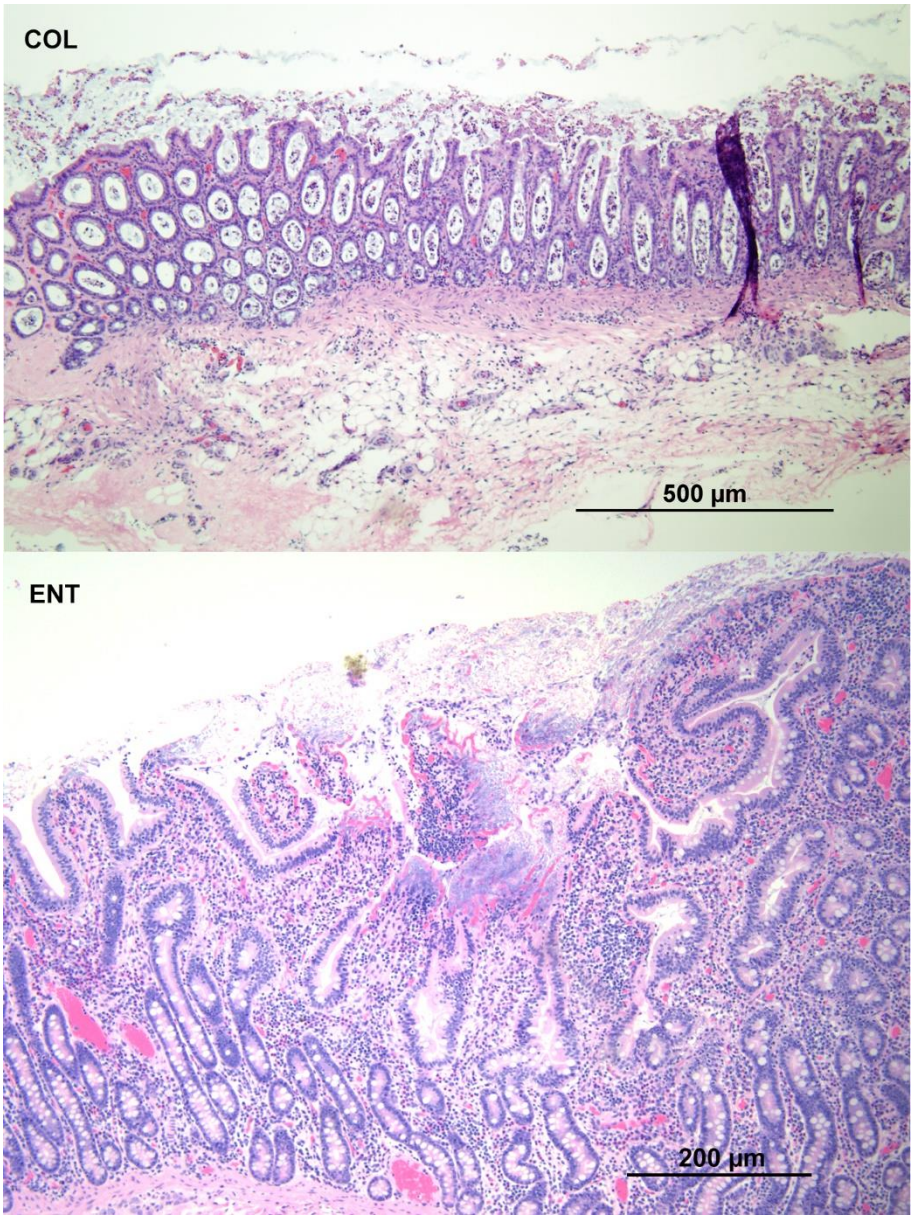
Figure 2. Calprotectin concentration in enteritis fecal samples (ENT, µg/g) from pigs challenged with *S.* Typhimurium. **A** ELISA assay; **B** Immunoturbidimetry assay; **C** ROC curve analysis plot (Turb – immunoturbidimetry assay). Stars denote a significant difference ($P < 0.05$) between fecal scores. Bars denote median, with interquartile range shown error bars. (ρ = Spearman's correlation coefficient).

Supplementary Table 1. Summary of calibrator and reaction control results for ELISA assays

Reagents	Manufacturer predicted value (µg/g)	Measured value (n=2, µg/g)
Blank	N/A	24.601
Calibrator A	30	30
Calibrator B	90	89.999
Calibrator C	300	300
Calibrator D	900	900.001
Calibrator E	1800	1800.031
Low control	66-171	127.436
High control	303-561	408.966

Supplementary Table 2. Summary of calibrator and reaction control results for Immunoturbidimetry assay

Reagents	Manufacturer predicted value (µg/g)	Measured value (n=2, µg/g)
Calibrator 1	0	0
Calibrator 2	55.3	0
Calibrator 3	220.6	536.119
Calibrator 4	552.3	1.127.235
Calibrator 5	1104.1	1.399.032
Calibrator 6	2207.6	1.892.208
Low control	68-104	0
High control	228-342	602.765



Supplementary Figure 1. Representative H&E stained sections of formalin fixed colon (COL group) and ileum (ENT group) samples.