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Barbara Ribeiro de Souza Cortez

TISSUE DAMAGE IN SWINE COLONIC EXPLANTS EXPOSED TO *Brachyspira hyodysenteriae* STRAINS WITH DIFFERENT LEVELS OF PATHOGENICITY

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Advisor: Prof. Roberto Mauricio Carvalho Guedes

Co-Advisor: Prof. Matheus de Oliveira Costa

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Dr.(a). Matheus de Oliveira Costa

Dr.(a). Raphael Rocha Wenceslau

Dr.(a). Amanda Gabrielle de Souza Daniel

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"The most important thing we learn at school is the fact that the most important things can't be learned at school."

Haruki Murakami

Acknowledges

In my world from the start, there was only three of us. Thanks mom and dad for being there for me. Then, three little girls stood with me side by side facing all fears we could find. Thanks, sisters, for all the love and strength you gave me. In the meantime, there were a few who have decided to stick around even though I still can't quite see the reason for that. Thanks, my friends, for letting me know that I am important for you. When I started to feel that loneliness was nearly unbearable, you were there. Thank you my everything, for being with me. All of a sudden, an entire family became mine too. Thank you for treasuring my company and teaching more than I could understand. From where I stand now, I see all these people who have made this achievement possible, and I can only be thankful for them: all the professors, graduate students and good souls who have crossed my way. In special, professors Roberto Guedes, Maria Isabel Guedes and Matheus de Oliveira Costa: thank you for giving me the strength to move forward when I thought I couldn't.

ABSTRACT

Swine dysentery (SD) is a major concern for pig farmers worldwide. SD prevention relies solely on biosafety measures and prophylactic use of growth promoters, which contain low dosages of antibiotics. With the global awareness towards the conscious use of antimicrobials this practice is gradually being discouraged, and cases of SD started to re-emerge. In this sense, a robust but underexplored research tool might be employed: the in vitro organ culture (IVOC). Many recent studies were published in the last decades using this approach with impactful results, although Brazilian institutions have not disseminated this practice yet. Amongst the reasons for that are: the high cost of implementation, specialized equipment and refined techniques which require personnel training. Accordingly, the present study had the objective of (1) adapting an already existing IVOC colon culturing method to be accessible for most Brazilian laboratories, and to (2) seek for unique characteristics of pathogenic strains of *Brachyspira hyodysenteriae* which could direct further studies at the pathogenesis of SD. A total of 480 explants were cultured using the adapted IVOC technique and divided into 4 experimental groups: negative control (PBS), *B. hyodysenteriae* highly pathogenic strain (B_High), *B. hyodysenteriae* low pathogenic strain (B_Low) and positive control (LPS). 160 out of 480 explants were submitted for qPCR analysis of the TFF3 gene, a marker of acute intestinal inflammation. The remaining 320 explants were submitted for histopathological processing and morphological analysis of parameters such as crypt necrosis and explant survival. Technique standardization results have shown that the adaptations made to the original protocol caused reduced explants survival, which have not impaired the use of the technique. It was found that the adapted method of IVOC for swine colon explants is suited for reproduction in research laboratories for similar research purposes to this study, although the use of KGM media and conditioned media are recommended if higher epithelial cover or longer time in culture is required. At the SD trial was observed an increased gene expression of TFF3 at both B_High and LPS infected groups (0.7 ±0.19 and 0.6 ±0.23 respectively) in comparison to explants from the negative control and B_Low groups. B_High and LPS treated groups have also shown reduced explant survival (B_High: 0.47 ±0.13; LPS: 0.27 ±0.11) and reduced goblet cell abundance (0.29 ±0.11 and 0,28 ±0.10 respectively) compared with both PBS and B_Low groups. Assembling our data, it was possible to suggest that the increased TFF3 gene expression signaled the activation of the wound-healing mechanism and the release of mucins. This innovative link between TFF and *B. hyodysenteriae* infection can be used as lead in further investigation of SD pathogenesis.

Keywords: *in vitro* colon culture, swine dysentery, trefoil factors, gene expression, histopathology.

RESUMO

A disenteria suína (DS) é uma grande preocupação para os suinocultores em todo o mundo. De forma a estudar medidas profiláticas eficazes, uma ferramenta de pesquisa robusta, mas pouco explorada pode ser empregada: a cultura de órgãos *in vitro* (IVOC). Não são muitas as instituições brasileiras que se utilizam técnica atualmente. Entre as razões para isso estão: o alto custo de implantação, equipamentos especializados e técnicas refinadas que exigem treinamento de pessoal. Assim, o presente estudo teve o objetivo de (1) adaptar um método de cultura de cólon IVOC já existente para ser acessível à maioria dos laboratórios brasileiros e (2) buscar características únicas de cepas patogênicas de Brachyspira hyodysenteriae que possam direcionar novos estudos na patogênese da DS. Um total de 480 explantes foram cultivados usando a técnica IVOC adaptada e divididos em 4 grupos experimentais: controle negativo (PBS), cepa de alta patogenicidade de B. hyodysenteriae (B_High), cepa de baixa patogenicidade de B. hyodysenteriae (B_Low) e controle positivo (LPS). 160 dos 480 explantes foram submetidos à análise de qPCR do gene TFF3, um marcador de inflamação intestinal aguda. Os 320 explantes restantes foram submetidos ao processamento histopatológico e análise morfológica de parâmetros como necrose de criptas e sobrevivência do explante. Os resultados da padronização da técnica mostraram que as adaptações feitas ao protocolo original causaram redução da sobrevida dos explantes, o que não prejudicou o uso da técnica. Verificou-se que o método adaptado de IVOC para explantes de cólon suíno é adequado para reprodução em laboratórios de pesquisa para fins de pesquisa semelhantes a este estudo, embora o uso de meios KGM e meios condicionados sejam recomendados se for necessária maior cobertura epitelial ou maior tempo em cultura. No ensaio acerca da DS foi observada uma expressão gênica aumentada de TFF3 em ambos os grupos infectados B_High e LPS (0,7 ±0,19 e 0,6 ±0,23 respectivamente) em comparação aos explantes dos grupos controle negativo e B_Low. Os grupos tratados com B_High e LPS também mostraram sobrevivência reduzida dos explantes (B_High: 0,47 ±0,13; LPS: 0,27 ±0,11) e abundância reduzida de células caliciformes (0,29 ±0,11 e 0,28 ±0,10 respectivamente) em comparação com os grupos PBS e B_Low. Reunindo nossos dados, foi possível sugerir que o aumento da expressão do gene TFF3 sinalizou a ativação do mecanismo de cicatrização e a liberação de mucinas. Esta ligação inovadora entre TFF e infecção por B. hyodysenteriae pode ser usada como base pare investigações adicionais da patogênese da DS.

Palavras-chave: cultura de cólon *in vitro*, disenteria suína, fatores do trevo, expressão gênica, histopatologia.

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Abbreviations List

σ: standard deviation

µg: micrograms

µl: microliters

µm: micrometers

µM: micromolar

ºC: Celsius

B2M: beta-2-microglobulin

B_High: *Brachyspira hyodysenteriae* high pathogenicity strain

B_Low: *Brachyspira hyodysenteriae* low pathogenicity strain

BHI: brain-heart infusion

CEUA: Ethics Committee on the Use of Animals

CM: conditioned media

CMRL: Connaught Medical Research Laboratories

Ct: cycle threshold

CO_2: carbon dioxide

DDS: dextran sulfate sodium

DMEM: Dulbecco's modified Eagle's medium

DNA: Deoxyribonucleic acid

DON: deoxynivalenol

DPBS: Dulbecco's phosphate buffered saline

EU: European Union

FBS: fetal bovine serum

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GEE: generalized estimating equations

GLP: glucagon-like peptides

H_2: hydrogen

HBSS: Hanks' Balanced Salt Solution

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPRT1: hypoxanthine phosphoribosyl transferase 1

IVOC: *in vitro* organ culture

KBM: keratinocytes basal medium

KRB: Krebs-Ring Bicarbonate

LHC-9: Lechner and LaVeck

LPS: lipopolysaccharide

MHC-I: major histocompatibility complex class I

MIC: Minimum Inhibitory Concentration

mm^2: square millimeters

ml: millilitres

MUC2: mucin type 2

MUC5AC: mucin type 5AC

N_2: nitrogen

NSA: Necrosis Average Score

OIE: World Organisation for Animal Health

qPCR: quantitative polymerase chain reaction

PBS: phosphate buffered saline

PCS: Porcine colonic spirochetosis

PDMS: poly-dimethylsiloxane

ROS: reactive oxygen species

RPMI: Roswell Park Memorial Institute Medium

SD: Swine dysentery

SR: Survival rate

TEER: transepithelial electrical resistance

TIM: TNO Gastro-Intestinal Model

TNO: The Netherlands Organization

TFF: trefoil factors

TFF1: trefoil factor 1

TFF2: trefoil factor 2

TFF3: trefoil factor 3

TSA: tryptone soy agar

Summary

1. INTRODUCTION

Swine dysentery (SD) is a disease caused by pathogenic species of the genus *Brachyspira* sp. (Burrough, 2017), the most isolated species in Brazil being *B. hyodysenteriae* (Daniel et al., 2017). Characterized by muco-hemorrhagic diarrhea, it is responsible for economic losses related with both morbity and reduced growth rate (Alvarez-Ordóñez et al., 2013). Re-emerging in the swine industry, its aggravating factor is the emergence of antimicrobial-resistant strains combined with misuse of the available antimicrobials (Hidalgo et al., 2011) and the incomplete understanding of the pathogenesis mechanism of the bacteria in the host, which makes it difficult to produce preventive methods against the disease (Costa, 2015). Recent studies have verified the application of intestinal *in vitro* organ culture (IVOC) as a tool to obtain information on the pathophysiology of swine dysentery, noting important aspects of it, such as the induction of loss of homeostasis of the microbiota and the mitochondrial dysfunction in enterocytes caused by the bacteria (Costa et al., 2020; Costa & Harding, 2020). Explants are histologically similar to tissue *in vivo*, which gives the advantage of maintaining tissue structure, which cannot be replicated in cell culture, in addition to reducing the number of animals needed for the studies. In the specific case of *Brachyspira* sp. pathogenic of swine, colonic crypts are consistently described as the main site of colonization of these spirochetes in vivo and are usually populated by many bacterial cells (Burrough et al., 2012), being the ideal IVOC model to reproduce such conditions *in vitro*. In addition, this technique provides greater control of environmental factors compared to the *in vivo* model and provides the possibility of studying the initial phases of contact of the infectious agent with the host, a moment that is impractical to be precise in the *in vivo* model (Costa et al., 2016). Despite this, in Brazil this method is still rarely explored in the research of swine dysentery, being used predominantly in studies related to swine nutrition and the effects of mycotoxins at the large and small intestine of this species (Régnier et al., 2017). In order to attest to the reliability of the use of IVOC in future research that seeks to elucidate the genetic, metabolic and molecular aspects of *Brachyspira hyodysenteriae* infection, the present study sought to compare the infection in IVOC from colon by two strains of *B. hyodysenteriae*, one being high pathogenic and the other of low pathogenicity.

2. HYPOTHESIS

IVOC is suitable model to study *B. hyodysenteriae* infection and enables the evaluation of morphological and molecular differences between *B. hyodysenteriae* strains of high and low pathogenicity.

3. OBJECTIVES

3.1. GENERAL OBJECTIVES

Use the swine colonic explant model to compare lesions following infection with a virulent and a non-virulent strain of *B. hyodysenteriae* through morphological and molecular assays.

3.2. SPECIFIC OBJECTIVES

a) Adapt a pre-existing *in vitro* colon culture technique for the available apparatus and culture media.
b) Evaluate differences at TFF3 gene expression and morphological indicators at the early stage of interaction between host and infectious agent using a highly and a low pathogenic *B. hyodysenteriae* strains.

4. LITERATURE REVIEW

4.1. SWINE DYSENTERY

Swine dysentery (SD) is a common enteric disease from growing to finishing pigs (8 to 26 weeks of age). Its presence was first confirmed in the 20's (Whiting et al., 1921), being worldwide spread nowadays and more prevalent in the Americas, Europe and Asia (Burrough, 2017).

Brachyspira hyodysenteriae, *B. hampsonii*, and *B. suanatina* are the main known species of *Brachyspira* which are throughout associated with SD. Other species such as *B. murdochii*, *B. intermedia* and *B. innocens* have diverse hemolytic activity in culture and strains with different levels of pathogenicity demonstrated under field and experimental conditions (Rubin et al., 2013). *Brachyspira pilosicolli* is a weak-hemolytic species of *Brachyspira* which causes porcine colonic spirochetosis (PCS), a mostly subclinical disease characterized by reduction in growth performance, poor feed conversion and loose stool at beginning stages of the growing phase (Dors et al., 2019), and therefore will not be approached at the present review.

Animals clinically affected by SD suffer from mucohaemorrhagic diarrhea, dehydration, mild inappetence, apathy and hyperthermia (Hampson & Burrough, 2019). Economic losses derived from this enteric disease in naïve herds are significant, as the morbidity and mortality rates recorded in such cases are of 90% and 30%, respectively (Burrough, 2017). Moreover, a peculiar characteristic of *B hyodysenteriae* is the subclinical or inapparent clinical presentation of some strains in some herds. This condition has been described on swine farms in Brazil for decades, and a recent study highlighted the importance of this characteristic for maintenance of infection within herds (Sato et al., 2022). Farms with *B. hyodysenteriae* positive animals might be marked by impaired growth, reduced feed efficiency and batch heterogeneity (Nielsen et al.,

2022). In these cases, changes in diet and co-infection with other pathogens can trigger SD, despite the precise mechanisms for which the bacteria cause disease are still numbed (Hampson et al., 2015).

Pathogenic species of *Brachyspira* have the ability to infect the colon of healthy hosts but numerous are the factors which can play a role at resulting a successful colonization. Predisposing factors can be divided into those related with the host, the environment and the bacterial strain. Nevertheless, the complete pathogenesis of SD and the mechanisms which disease-causing *Brachyspira* spp. uses to live in eubiosis or to disturb host's microbiota and cause dysbiosis remains not completely understood (Hampson & Burrough, 2019).

Variations at host's microbiota composition may predispose colonization, as those with more abundant bacteria from *Fusobacterium*, *Campylobacter*, *Mogibacterium* and *Desulfovibrio* genera have been correlated with SD development (Burrough et al., 2017). Moreover, the inclusion of dietary fiber its known to play a role at *B. hyodysenteriae* colonization since soluble fiber fermentation at large intestine results in lactic acid formation and favors acid-lactic bacteria such as *Bifidobacterium* and *Lactobacillus*, which were more abundant in pigs which did not develop SD *post* inoculation in comparison with those who became ill. Convergingly, mucolytic anaerobic bacteria such as *Shuttleworthia*, *Ruminococcus torques*, and *Mogibacterium* were shown to exacerbate the disease in inoculated animals, probably by creating a more favorable environment for *Brachyspira* spp. colonization in pigs feed a diet with restricted levels of non-soluble fibers (Helm et al., 2021).

Brachyspira spp. colonization depends on colonic mucus layer integrity and composition, being a component of great importance to SD pathogenesis. Mucins are the main structures composing the colonic mucus layer of pigs; indeed, the acidic mucins (sialylated and sulfomucins) are the most prevalent type. Pathogenic species of *Brachyspira* have shown to have ability of binding to mucins, invade colonic crypts and protect itself from the oxygen present in the lumen (Je-Han Lin et al., 2021). Corroborating these findings, disorganization of mucus layer structure and increased mucus production were associated with *B. hyodysenteriae* infection in pigs. In addition, the sialylated mucins MUC2 and MUC5AC were shown to be more abundant and to have increased binding sites in animals affected by SD (Quintana-Hayashi et al., 2021).

The prevention of SD relies greatly on management and biosafety measures such as purchase of animals from SD-free farms, diet changes, effective pest control, and eradication programmes which consists of medication and depopulation, since there is no commercial vaccine available (Neirynck et al., 2020). Concomitantly with this circumstance, the use of subtherapeutic doses of infeed antimicrobials was disseminated in the 90's to improve herds' sanitary status and

prevent SD outbreaks (Hampson & Burrough, 2019). This practice has been gradually discontinued due to the collective awareness towards the conscious use of antimicrobials. As for an example, The French Agency for Food, Environmental and Occupational Health & Safety considers the systematic use of antibiotics at pig husbandry for prevention and aiming the reduction of selective pressure an at-risk practice to be abandoned without delay (ANSES Opinion, 2014). In fact, the World Organization for Animal Health considered *Brachyspira spp* a pathogen with high vaccine research priority since this prevention tool would significantly aid the reduction of antimicrobials use in livestock (OIE ad hoc Group, 2015).

In the late 2000's many countries in the EU and North America reported the increase in SD cases (Costa & Harding, 2020). As a consequence of this re-emergence and the lack of non-antimicrobials alternatives for preventing SD, the usage of treatment dosages of antimicrobials such as carbadox, lincomycin, and tiamulin had to be instituted. The above-described scenario contributed to the reduced sensitivity from *Brachyspira* spp. to antimicrobials, mainly pleuromutilins, reported in many regions such as Europe, North America Australia and Japan (Card et al., 2018).

Farmers now struggle with multidrug resistant *B. hyodysenteriae* strains which are being increasingly reported all around the world (García-Martín et al., 2022). A recent review showed that the minimum inhibitory concentration (MIC), a measure for antimicrobial resistance, for *Brachyspira* field strains against the main antimicrobial agents for animal use, have increased with time (D. J. Hampson et al., 2019). In Brazil the situation is aggravating since restrictions concerning in-feed antimicrobials use are less severe compared to North America and Europe. Research made with various field strains from the main pig producing Brazilian states showed a decreased susceptibility to the majority of antimicrobials of choice to treat SD, including tylosin and lincomycin (Daniel et al., 2017).

Unrevealing *Brachyspira* spp. virulence genes is an arduous task which researchers direct their efforts to, with goals including but not limited to develop subunit vaccines, enabling the use of substances which act at the virulence site and inhibit the bacteria more efficiently. Previous studies have encountered hemolysis-associated (*tlyA* and *tly*B) and outer membrane protein genes (*bhlp*16, *bhlp29.7* and *bhmp39f*) being correlated with increased SD severity (Giovagnoni et al., 2022; Hampson & Burrough, 2019) .

Despite the unravelling of such genes, throughout the years researchers suffered several frustrated attempts to develop a vaccine which triggered a protective immunological response. The difficulties encountered were encompassed by flawed protection against a diversity of strains, need of multiple reinforcement applications, the continued shedding of immunized

animals and scarce bacteria obtained from culturing, in the case of bacterin vaccines. Nevertheless, the development of a swine dysentery vaccine is currently acknowledged through patent registration (Nistal et al., 2021) although it is not yet possible either to attest its effectivity under field conditions, especially those encountered in other geographic locations, or to purchase the produced vaccine.

As a result, it is possible to say SD poses a great challenge for swine and global health, mainly due to antimicrobial resistance, lack of well-stablished preventive measures and gaps in knowledge of the complete pathogenesis. Research surrounding SD advances and gradually aid to fill these gaps in order to provide non-antimicrobial alternatives to be used as preventive tools. Most studies require *in vivo* experiments, as SD is an infectious disease well described in clinically affected animals. Recent studies are attempting to correlate the morphological and molecular alterations found *in vitro* cultured colon explants to those experienced *in vivo* (Baydoun et al., 2017; Costa et al., 2016; Fleury et al., 2017), in a manner to reduce the use of animals besides several advantages which shall be discussed in detail at the next section.

4.2. *IN VITRO* INTESTINAL CULTURE TECHNIQUES APPLIED AT VETERINARY RESEARCH

Intestinal mucosa is one of the main surfaces of interaction between individuals and the outside environment. It is responsible for dealing with microorganisms, pathogens, and other potentially harmful agents. Your complex arrangement of specialized tissues interacts in countless ways, therefore a sophisticated model which synthetizes these myriads of processes during pathologic and physiologic states is critical for medical research, either veterinary or human.

Cell models possess a limited clonal population and fail to reproduce organism's *in vivo* architecture. Nevertheless, proper access to intestinal mucosa for *in vivo* sampling is anatomically, technically, and ethically challenging. Despite this, animal gastrointestinal models pose a considerable laborious maintenance, intensive logistics and are often financial unapproachable (Costa et al., 2018). Consequently, intestinal *in vitro* organ culture (IVOC) models have been developed and are increasingly being used to investigate intestinal physiology, pathogenic mechanisms, and pharmacological interactions with the advantage of maintaining cellular variety and differentiation simultaneously with tissue interactions. This intestinal explant-based model offers a more controlled environment for experimental manipulation in comparison with *in vivo* models.

The word explant, when comes to biology, translates to a piece of tissue or organ that has been removed from an organism and cultivated maintaining its cells alive and able to divide. This practice allows researchers to perform interspecies studies in reproducible conditions, to harvest

several explants from a single donor reducing the number of animals needed, and to control and sensibly manipulate the surroundings conditions, being ideal for a detailed exploration of factors controlling migration and cellular differentiation on several intestinal compartments (Randall et al., 2011). New intestinal IVOC techniques thus represent a robust alternative for several demands presented by veterinarian researchers worldly.

There are different types of methods for culturing intestinal explants. The level of complexity of each technique is often related to the type of outcome desired. For example, in most cases, to maintain explants for longer periods, a more specialized equipment will be required. Also, if the response measured is related to living organisms from the gut, the technique will necessarily be more sophisticated since no microbiome suppressors can be used and therefore tissue degradation is exacerbated. In this way, the present review will be divided into categories, beginning with simpler methods and scaling up for methods which requires specialized apparatus for maintenance of intestinal explants.

4.2.1. Methods suited for Embryonic Tissues Culture

Methods that aim to maintain embryonic tissues alive were the firsts to be developed. Beck & Thomson in 1914 and Fischer in 1922 cultured embryonic tissues from chickens and obtained different degrees of differentiation in vitro (Beck & Thomson, 1914; Fischer, 1922). Whereas the first attempt to culture mature organs was accomplished by Trowell in 1954 and then in 1959 with an enhanced apparatus, culture medium and methodology to cultivate several types of tissues up to nine days sustaining a satisfactory morphological state. Intestinal culture was not performed back then, as it was considered too problematic due to bacterial contaminants inherent to this organ.

Hearn and colleagues in 1999 developed a method termed as “catenary culture”. Its name is related to the catenary form acquired by wires when attached in its edges. Intestinal explants are fixed at the ends of a “V” shape cut made in 3 mm^2 pieces of 0.45 µm Millipore paper. This setting is then placed inside a well of a Terasaki plate which is filled with culture media. This method allows cultivation with morphological maintenance for up to 10 days, what is only possible due to the embryonic and thus aseptic origin of the intestine. The purpose of this technique was to access enteric neural crest-derived cells migration in embryos, maintaining proper formation of other structures and functions such as muscle layer, goblet cells and gut motility. The peculiar positioning of explants is required for maintaining organ’s tubular shape with serosal side facing outwards, since eversion and deformation of explants have been observed by previous authors that performed submerged culture.

4.2.2. Methods for culturing explants using simple apparatus

In 1969, Browning & Trier detailed a modified version of Trowell's technique which made possible the cultivation of human small intestinal mucosal biopsies up to 24 hours, maintaining morphological and functional features. Instead of the apparatus previously proposed, these researchers used a circular plastic dish containing a smaller well in its center where the medium was added. A triangular metal mesh was docked on top of the inner dish, so that its bottom touched the surface of medium lying beneath. Intestinal explants were placed above the mesh, with basolateral side facing down and mucosal side facing up. In this arrangement, the medium is constantly absorbed by explants through capillarity. Then dishes were incubated at 37ºC, 95% O_2 and 5% CO_2 from 12 to 24 hours. To study cell proliferation and the ability of enterocytes to absorb micellar fat, researchers exposed explants to thymidine-H and to a micellar solution respectively. It was showed that, after 24 hours of cultivation, crypt cells had evidence of DNA synthesis and enterocytes function of absorbing lipids and synthetizing triglycerides was conserved.

Adaptations for cultivation of human large intestinal mucosal biopsies cultivation was evaluated by Eastwood & Trier (1973). The apparatus used was the same metal grid coupled into the small well centered inside the culture dish described previously, with the addition of a sponge saturated with water around the central well for better atmosphere hydration. The entire setting is placed inside an incubator with a 95% O_2 and 5% CO_2 atmosphere. Authors encountered that explants maintained signs of epithelial cell proliferation and preserved morphological features for at least 24 hours.

In 1978, Dr. Shamsuddin and colleagues proposed a method for cultivation of colonic tissue stripped from serosal layer. Each fragment measured 5 mm^2 and were placed in the edge of large Petri dishes (60 mm diameter) with scarce amount of medium (2 ml). When placed into a rocker platform, this setting allowed explants to be bathed 50% of the time. Plates were placed inside a chamber gassed with 95% O_2 and 5% CO_2 at 37ºC. Medium was renewed after 24 hours, and then every 2 days. Authors claim to have achieved a cultivation time of 20 days. Other authors, such as Rosberg et al. (1991) used the above-suggested medium and compared the submerged explant setting from this author to the metal grid setting proposed by Browning & Trier (1969). They found that the metal grid setting was superior for maintaining a better morphological state and higher viability, but the optimal time of cultivation was of 24 hours, as suggested earlier by Eastwood & Trier (1973). Using the latter method, Rosberg and collaborators (1991) cultivated swine gastric mucosa for 72 hours and evaluated the interaction of *Helicobacter pylori* within explants. They highlighted the reliability of this method for further *H. pylori* studies, which can be relevant to elucidate the mechanisms that aid gastric

ulcer formation in swine. This method was adapted and used by Robertson et al. (2000) for assessing the impacts of *Salmonella* Enteritidis on mice ileum explants. This research highlighted the importance of the bacterial flagella at mucosa invasion and attachment to enterocytes. Nietfeld et al. (1991) achieved better results using RPMI 1640 cell culture medium instead of CMRL1066 as previously recommended by Shamsuddin et al. (1978), obtaining 48 hours of culture.

In 1995, Zhu et al. improved this method by placing explants on top of biopsy sponges that were humidified with culture medium underneath it. Recently, Pérez Gaudio et al. (2018) accomplished a pharmacological study of fosfomycin using swine jejunal explants infected with *Lawsonia intracellularis* aiming to evaluate the concentration of this antibiotic inside enterocytes, using Zhu's technique with modifications. Briefly, jejunal fragments measuring 3 cm of length were collected, opened on mesenteric border, placed on Petri dishes and washed twice with physiologic saline solution (PSS) trough shaking. Then, circular sections of 1.3 cm^2 weighting 0.1 g were placed above sponges with mucosa facing up. Each sponge was submerged into a well of 6-well culture plates containing Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine, high glycose and F-12 nutrient mix from Gibco. The time of preparation of explants was completed in less than one hour after animal's euthanasia. Subsequently, 580 μg/ml of fosfomycin was added to explants with 500 μl of the bacteria, obtained from the commercial vaccine Enterisol Ileitis®, in half of them. Plates were maintained in a shaker at 37 ºC for up to 48 hours. This study concluded that explants were better conserved histologically after 24 hours of cultivation. Moreover, the presence or absence of *L. intracellularis* did not impair intracellular concentrations of fosfomycin. Also using this method, Girard and collaborators (2005) aided the comprehension of the mechanism used by enteropathogenic *Echerichia coli* to cause disease in swine after observing the attaching and effacing phenotype in ileal explants. In addition, they reinforced the finding that *E. coli* subtypes containing the intimin virulence factor present a more effective adherence to enterocytes.

Kolf-Clauw et al. (2009) adapted the method and applied it to study the mycotoxicity on swine intestine. Researchers used the same medium without the ascorbic acid and containing 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 50 μg/mL of gentamycin. Their study sought to validate this explant model for mycotoxin assays trough histological evaluation of exposed explants. They concluded that this approach is sensitive to investigate the effects of food contaminants on intestinal epithelium. Moreover, additional studies concerning the effects of mycotoxins in the intestine aroused. As an example, da Silva et al. (2014, 2019) published two research articles displaying that phytic acid, a natural antioxidant, was able to reduce

histological changes and oxidative stress on swine jejunal explants exposed to fumonisin and deoxynivalenol.

In 2012, Tsilingiri et al. introduced an innovative method to add stimuli to explants. Instead of adding bacteria, food additives or medicines into the culture medium, this technique polarized the stimuli by confining it to a cylinder adhered to the intestinal mucosa. This apparatus is composed by a Petri dish with an inner well in its center. Inside the inner well, culture media is added, and a triangular metal grid is sustained by its edges, similar to the Eastwood & Trier's (1973) setting. Intestinal fragments striped from serosal layer are placed on top of the mesh, with serosal side facing downwards and mucosal side upwards. The main difference is the cylindrical structure placed on top of each explant, in a way that the desired substance can be placed inside it and stimulus remains confined at mucosal surface.

The study performed by Tsilingiri 2012 displayed the interaction of probiotics and a pathogenic species of *Salmonella* comparing both methods: polarized and non-polarized. The results showed that in the polarized model a better representation of *in vivo* contamination occurs, besides a greater conservation of mucus layer. This layer is a natural protective barrier, and several pathogenic agents have their attachment to enterocytes hampered or assisted by alteration on its mucin's composition (Gustafsson et al., 2012).

Few years later, in 2016 Costa et al. developed a technique optimized for porcine colon explants culture. It consists in sectioning a segment of 8 centimeters from spiral colon's apex which is placed into a vial containing refrigerated Dulbecco's phosphate buffered saline (DPBS) without calcium or magnesium and supplemented with antibiotics. Then, the segment is opened in its mesenteric border and serosal layer is mechanically removed. Afterwards, sections of 1.5 cm^2 are obtained and placed into 1% agar blocks, arranged inside Petri dishes and containing 8 ml of keratinocytes basal medium (KBM) supplemented with 1.5 mM of calcium and antibiotics. Polypropylene cylinders are attached to explants using tissue glue for polarization of stimulus (100 µl of inoculum containing Brachyspira hampsonii was added in this study). Subsequently the plates were incubated inside hyperoxic chambers containing 99% of O_2 and 1% of CO_2 for 8 hours at 37ºC.

Researchers aimed to demonstrate the pathological changes found in pigs diseased by swine dysentery, by means of measuring metabolic response of explants exposed to the causing agent, in addition to their histopathological evaluation. The study concluded that this pathogenic species of *Brachyspira* is capable of inducing necrosis *in vitro* probably through a mechanism mediated by the IL-1α cytokine, and reactive oxygen species (ROS). Later in 2018, the same authors published a book chapter displaying the above-described technique with the upgrade of

using a cell strainer to contain the explant, replacing the agar block. As result, the method became much easier and approachable to apply, without any loss at the explant survival and morphological preservation, achieving 5 days under culture.

In 2017, Udden et al. had developed a method for colon culture which lasts for 24 hours and is suitable for studying microbial host defense response. Authors obtained 1 cm^2 colon pieces and then transferred them into 100 μm cell strainers placed inside 6-well plates and added 5 ml of DMEM/F12 medium with antibiotics. In this method, colon segments were throughout submerged. The plates were incubated at 37ºC with 95% air and 5% CO^2 atmosphere. Each two hours for three times, media was renewed with using an antibiotic-free one. Then, colon explants were transferred into a well of a 12-well culture plate, with DMEM/F12 and 5% fetal bovine serum (FBS) and cultivated for another 12 hours. In this study, explants were stimulated with IL-1β, and IL-18 or left untreated, then submitted to RT-PCR for interleukins and antimicrobial peptides gene expression measurement. Their supernatant was collected and incubated with *E. coli* to assay whether explants with induced inflammation are better at releasing antimicrobial peptides at the supernatant. Authors have found that stimulated explants expressed higher levels of antimicrobial peptides and interleukins, producing a supernatant more capable of killing the bacteria compared to control. Thus, this is an additional valuable method for studying intestinal antimicrobial immune response.

Similarly to Uden et al. (2017), which created a submerged culture of intestinal explants instead of maintaining the explant outside the culture media, Bareiss et al. (2008) proposed the three-dimensional culture model. According to the authors, this method allows colon explants to survive for up to two weeks. The technique consists in cleaning colon fragments with HBSS containing penicillin, streptomycin and metronidazole, followed by its opening at the mesenteric border, slicing 2 mm^2 explants and transferring them into Millipore membranes of 0.45 μm pore size. Then, they added 1 ml of HEPES buffered DMEM/F12 medium containing 10% horse adult serum, penicillin, streptomycin, L-glutamine, insulin/transferrin/selenite mix, albumin, hydrocortisone, glucagon, 3,3',5'-triiodo-L- thyronine, ascorbate-2-phosphate, linoleic acid, estradiol and keratinocyte growth factor. The plates containing 6 explants each were placed in humidified incubators with a 5% CO2 and 37ºC atmosphere considering that every two days the medium was renewed. Researchers infected explants with a wild-type and a knockout strain of *Candida albicans*, and they verified that the yeast cause similar effects while infecting explants compared to *in vivo* infection, concluding that this *in vitro* model is suitable for gut infection experiments.

Baydoun et al. (2017) released an adapted enhanced version, which differences consisted in using 12 mm^2 explants and filtering the medium prior use with 0.22 μm filter. Researchers used

severe combined immunodeficient mice what seemingly contributed for extended explant survival since they reached at least 4 weeks of cultivation. This research highlighted the potential use of this technique for *Cryptosporidium parvum* infection assays, as well as further drug screening for this parasite, in addition to a technique that ensures a relative long-term cultivation period.

In 2015, Ripken & Hendriks described a method referred to as segmental model which also uses the submerged approach. It consists at obtaining intestinal fragments and placing them in ice-cold Krebs-Ring Bicarbonate (KRB) buffer gazed with 95% O_2 and 5% CO_2 until processing. Then the segment is cut open in its mesenteric border and the serosal layer is striped out carefully. Circular fragments are sectioned with 8 mm biopsy punches, which are placed inside 24-well culture plates filled with 500 µl of buffer. Plates are displayed inside humidified incubators containing 5% CO_2 v/v and cultivated for 1 hour and 30 minutes.

This method is indicated for studying the effects of nutrients and compounds at gut metabolism and intestinal hormone release, which can be analyzed either from supernatant and explants. Voortman et al. (2012) used this technique to evaluate the effects of long and short-chain fatty acids on the release of gastrointestinal hormones. Authors have found increased levels of glucagon-like peptides 1 and 2 (GLP-1, GLP-2) when explants received both fatty acid types. This research highlighted the relevance of the approach for studying satiety compounds, what might be relevant for animals' heath and production.

In 2016, Barato and colleagues described a method for culturing fish intestinal explants that uses the same principles from the previous submerged techniques. Anterior intestine portions of 4 cm each obtained from tilapias (*Oreochromis* spp.) were placed in 140 mm^2 Petri dishes and 20 ml of high glucose Dulbecco's modified Eagle medium were added and incubated at 28 ºC. Next, intestinal lumen was rinsed with DMEM three times through syringe application, and explants were moved into 24-well plates containing DMEM with 10% of FBS. Plates were incubated inside chambers with controlled atmosphere (5% CO_2 and 80% humidity) at 28ºC under mild agitation for up to 40 minutes. The authors evaluated the ability of adherence from encapsulated and unencapsulated *Streptococcus agalactiae* to intestinal epithelium. The research concluded that the capsule impairs bacterial adhesion during the first steps of infection, and, in addition, that an acidic environment favors the adhesion of encapsulated bacteria. Later in 2019, Vásquez-Machado applied the same technique and unraveled that the bacteria discard its capsule prior attachment to the apical border of intestinal villi. After entering enterocyte's cytoplasm, the bacteria continue to replicate and subsequently achieves basolateral portions, where it invades lamina propria and travels through microcirculation, without evident inflammatory response or caliciform cell's reaction.

4.2.3. Methods Using Specialized Apparatus

4.2.3.1. Ussing Chamber

In 1951, Ussing & Zerahn created the Ussing chamber, a device capable of measuring ongoing ion transport within cultivated tissues. In this study, a fragment of frog skin was cultivated for five hours. The relevant breakthrough achieved here was due to the elucidation of processes such as electrogenic Cl^- secretion, electroneutral NaCl absorption and electrogenic Na^+ coupled glucose absorption in diverse contexts at intestinal mucosa. Concomitantly, it was the first technique which allowed stimulus separation of basolateral from apical surfaces. Ussing chambers can be used for a broad range of purposes on intestinal explants such as micronutrients transportation evaluation, inflammation, toxicological and hormonal studies, pathogenesis of intestinal agents and enzymatic activity measurement. The apparatus is engineered in a way that apical and basolateral side have separated chambers which can receive different stimuli. A supply of oxygen and carbonic gas is constantly pumped into the media, which is recirculated inside each chamber. The device has an underlying electrical current which is used to measure transepithelial electrical resistance (TEER) and allows to detect ionic differences related to Na^+/H^+ and $Cl^-/HCO3^-$ membrane exchangers (Westerhout et al., 2015). As an example of its use and pertinence so far, a study from 2020 aided the understanding of swine dysentery pathogenesis trough this technique. Researchers observed that the bacteria responsible for this disease is capable of inhibit Na^+ absorption in pig's colon, causing an osmotic imbalance which leads to diarrhea (Enns et al., 2020).

In 2012, Gustafsson et al. proposed the use of a perfusion chamber, similar to the Ussing chamber, for culturing colonic and ileal explants. Through this approach researchers could measure mucus layer penetration, thickness and spontaneous mucus growth. Immediately after obtaining intestinal portions, they were washed with cold Krebs' solution oxygenated with 95% O_2 and 5% CO_2, enriched with 116.0 mM NaCl, 1.3 mM $CaCl_2$, 3.6 mM KCl, 1.4 mM KH_2PO_4, 23.0 mM $NaHCO_3$, and 1.2 mM $MgSO_4$ at pH 7.4. The muscular layer was mechanically removed, and explants were mounted into perfusion chambers for mucus thickness measurement, or image chambers for mucus penetrability studies. Their study found that the spontaneous growth of mucus layer is conserved in colonic explants, and the proportions regarding inner and outer mucus layer were similar to that reported *in vivo*.

It is important to mention that due to the short time of cultivation provided by this mechanism on intestinal tissues (up to 150 minutes) animals were required to be exposed to certain stimulus, mainly pathogenic agents, prior euthanasia. Thereby, the advantage of reducing animals within an experimental group and diminishing costs regarding facilities and animal care is not feasible in cases where researchers seek a late response from a given stimulus. Another

limitation is the low throughput of the technique, since it requires one chamber setting for each explant.

New devices are arising to overcome this limitation. The so-called horizontal chambers come as an alternative for the classical Ussing chamber, where tissues are cultivated in a vertical orientation. As an example, two main devices have been designed for this purpose. First, the NaviCyte Horizontal Ussing System that supports 6 chambers at a time, maintaining inlets and outlets pumps for liquids recirculation. Second, the inTESTine^{TM} system which allows simultaneous cultivation of 24 to 96 explants by using simplified smaller chambers. The later system does not support recirculation - instead the apparatus must be placed in a rocker platform so liquids can be agitated inside chambers (Westerhout et al., 2015).

4.2.3.2. An Intestinal Gut Organ Culture System for Analyzing Host-Microbiota Interactions

In 2017, a polarized adapted model developed by Yissachar and collaborators was better suited at preserving intestinal architecture, and allowed to assess interactions between microbiome, nervous system and intestinal immune response using mice intestinal explants. Due to these characteristics, this system currently is the most sophisticated and accurate method to evaluate interactions between enteropathogens and substances in different species *in vitro* (Etienne-Mesmin et al., 2019). Briefly describing it, a fragment of intestine is plugged into an input and an output port which is responsible for allowing flow transit inside the lumen. The serosal side is also equipped with an input and output plugs, which circulates the culture media at the intestine surroundings. Each setting contains six of the above-described chambers, which are enbibed with a controlled O_2/CO_2 atmosphere.

The method developed by Costa et al. (2018) – previously described here – was also able to provide insights into the microbiota of antibiotic treated and *Salmonella* Typhimurium infected explants. Their work have showed that the microbiota between animals' intestine and explants is not different, besides observing that the modulated caused at the microbiota by amoxicillin treatment might've contributed to reducing intestinal lesions from the *Salmonella* Typhi challenge (Costa et al., 2020).

4.2.3.3. PigutIVM

A method for intestinal explants cultivation named PigutIVM was published by Fleury et al. (2017). The authors optimized a bioreactor for the culture of intestinal explants, prioritizing the maintenance of intestinal microbiota. As the result, this method allows explants survival for at least 28 days and is suitable for studying probiotics, antibiotics, feed ingredients, and other

subjects related to the microbiota. The biggest disadvantage here is the requirement of a specific bioreactor and the time-consuming task of learning how to operate it.

4.2.3.4. Interphase Microfluidic Culture System

Another mechanism engineered for intestinal explant cultivation is the interphase microfluidic culture system proposed by Baydoun and collaborators (2020). It consists of a central well that relays on top of a porous membrane, which is supported by a micro-channel apparatus. Three explants are placed on top of the culture chamber, which is permeated by microchannels conducting the culture media from the inlets to the outlets and provides nutrients trough the circular porous membrane. The individual culture apparatus is involved with poly-dimethylsiloxane (PDMS), a silicon-like material which holds all elements in place. This system allows to culture 3 explants at the same time, with no morphological abnormalities for up to 192 hours. Authors claim that improvements have to be made to increase the rate of surviving explants at the end of the trial.

4.2.3.5. Organ-on-a-chip

The organ-on-a-chip model is in early steps of development, but already represents a robust tool. It allows applying desirable cellular, chemical, and mechanical parameters in many types of organs. This unique characteristic can be used in numberless analysis of molecular processes concomitant to enteropathies and assist the development of new therapies. The setting is composed of a layer of intestinal epithelium which is organized inside the central compartment of the chip. The surface and the basolateral side of the epithelium are separated and each one receives a different substance in a constant flow. The flow maintenance in intestinal explants is a difficult feature to implement and is of great value for clearance of epithelium exfoliation and transit of the resident microbiota (Bein et al., 2018).

4.2.3.6. Peristalsis Recording Methods

In 1958, Bülbring et al. described a method for recording peristalsis in isolated intestinal portions. The technique relies in a device which produces electric waves induced through the intestine and registered in a volume recorder. Neither this or later published studies evaluated the viability of the tested intestinal segment by means of histopathology or any other method, since the goal concerned the muscle layer electrical responsiveness. As explants for definition must maintain cells alive and able to divide, the method for recording intestinal peristalsis is unfit for the present review. Although it is a valuable method for studying drugs and evaluating its effects on longitudinal muscle contractions, intraluminal pressure and volume of fluid expelled through the intestinal segment.

It is relevant to stand out a mighty, although expensive option developed by Minekus et al. (1999) in association with The Netherlands Organization for applied scientific research (TNO). The device referred to nowadays as TNO Gastro-Intestinal Model (TIM) was optimized for controlling peristalsis and physiological fluids, as to measure absorption of nutrient and drugs (Brodkorb et al., 2019). This device uses synthetic tissues instead of explants, not being a suitable subject for great detailing at the present review either.

4.2.3.7. Organoids

Organoids, or enteroids when steam cells originate enterocytes, have the advantage of using a single animal to obtain an enteroid culture which can be used for undefined time, increasing statistical representativity. The main impairment is the spherical format that they assume, which occludes the lumen and makes difficult to expose the mucosa to pathogens or substances, besides being a technique of poor yield (Daoud & Múnera, 2019). A method to invert polarity was developed allowing the mucosa surface to face outside, but its use is still insipient (Co et al., 2019). In addition, a technique of microinjection which poses high output and allow to add microbiota to enteroids have been developed recently (Williamson et al., 2018). They are a more suitable model to evaluate regulation and renew of epithelium but is still underdeveloped for characterization of its interaction with enteropathogens (Zhang & Sun, 2019).

4.2.4. Consideration about intestinal explants

Intestinal explants cultivated in vitro represents a powerful and underexplored tool in veterinary research. The methodology is already consolidated at global academia. Its performance is feasible and less costly than in vivo experiments and innovative *in vitro* techniques. Following world's tendency of reducing animal use and the great representativity of intestinal functions offered by this model, it would be reasonable to point that intestinal IVOC dissemination is the next step for animal science to advance at testing pharmaceutics, microorganisms, and feed additives.

4.3. SUMMARY OF PHYSIOLOGICAL AND PATHOLOGICAL RELEVANCE OF INTESTINAL TREFOIL FACTORS

The mucus layer is composed of water, mucins, lipids and peptides. Trefoil factors are types of peptides composing the mucus layer, and similarly to the mucins, they are secreted by the goblet cells from healthy intestinal epithelium. The trefoil factor 3 (TFF3) is one of the three isoforms of trefoil factors, and is also called the intestinal trefoil factor, while the other two (TFF1 and TFF2) are classified as spasmolytic peptides. The peptide TFF3 plays an important roll at maintaining epithelial restitution and cellular migration, being more expressed in the colon and small intestine rather than the upper intestinal tract, where spasmolytic peptides are more

present. Its expression is basal in the healthy gut and is exacerbated in the face of epithelial injury (Liu et al., 2018).

The insertion of the TFF3 peptide at intestinal wound-healing mechanism is incompletely understood. Previous studies have linked the hyperplasic stimulating activity of interleukin 33 (IL-33) to be activated by TFF3 peptide, trough IL-1 receptor (ST2). Increased levels of IL-33 as well as Th2 cytokines is found in ulcerative colitis, a disease which have the propriety of arising "ulcer-associated cell linage" at intestinal epithelium, cells with notably production of TFF3 peptides. Similarly, oral administration of TFF3 was shown to prevent colitis induced by dextran sulfate sodium (DSS) and TFF3 knockout mouse exhibited a much more severe colitis compared with regular mouse in face of DSS challenge. In addition, the intestinal protection mechanism regulated by commensal bacteria recognition of the toll like receptor 2 (TLR2) has the TFF3 as a key effector molecule (Brent Polk & Frey, 2012).

Trefoil factors production is intrinsically related to mucus production throughout the digestive tract. In the intestine, TFF3 peptide can play a role at MUC2 polymerization, a mucin present at the colonic mucus layer and, therefore, aiding the stabilization of this physiological barrier (Wong et al., 1999).

Despite the existence of lacunes surrounding the precise role of TFF3 at epithelial repairing, there are well-known characteristics of this peptide. All trefoil factors are motogen substances, what means that they stimulate cellular motility and aid the resolution of intestinal damaged areas. In response to injury, enterocytes expand its cytoplasm and perform a spatial rearrangement, avoiding leakage of electrocytes and entrance of luminal antigens, what is triggered trough substances secreted locally, trefoil peptides being a part of them, as shown in *in vitro* studies using organoids and cellular culture. Interestingly, some injury patterns of intestinal tract produce different types of TFF expression profiles. As for an example, in mice with colitis induced by DSS the expression of TFF3 was throughout increased, while in acetic acid-induced damage an initial reduction at TFF3 gene expression followed by an upregulation in the healing phase was noticed (Aihara et al., 2017).

Studies analysing TFF gene expression in pigs are scarce. Scholven et al. (2009) performed a characterization of the expression profile in weaning piglets compared with younger and older piglets and detected increased expression of TFF2 at the distal jejunum and a 2-fold increase of TFF3 at distal jejunum and ileum in this age group, which is marked by physiological alterations at the mucosal composition. A more recent study characterized the effects of deoxynivalenol (DON) at trefoil factors expression in pigs' jejunal explants and simultaneously the effects at wound-healing in Caco-2 cells and found that TFFs expression is initially

increased after a 3-hour exposure, then suppressed after 48 hours, and that tissue repair was throughout impaired under cellular culture using low doses of DON (Graziani et al., 2019).

Through these findings, it is possible to infer that the analysis of TFF factors has great predictive value in injuries of the gastrointestinal tract, specially TFF3 at the large intestine. Nonetheless, TFF3 factor is intrinsically connected to the MUC2 release form goblet cells, what enriches the search of its gene in diseases which disturb the colonic mucus layer. Previous research was done using the gene expression analysis of TFF3 at pig's intestinal explants with successful results. In this way, the present work attempted to measure and compare TFF3 gene expression in colonic explants from pigs challenged with different strains of *B. hyodysenteriae*.

5. MATERIALS AND METHODS

5.1. ANIMALS AND EXPERIMENTAL DESIGN

For this experiment, ten healthy 4-week-old barrows acquired from the Veterinary School's Experimental Farm herd and housed into the University's facilities with access to food and water *ad libitum* were euthanized - by electro stunning followed by exsanguination – throughout a period of six days (approved under the protocol number 23/2021, Ethics Committee on the Use of Animals - CEUA). From each animal 48 explants were obtained, which were submitted to an equal processing method and divided into all experimental groups in every animal.

In total, 480 explants were divided equally into four experimental groups: negative control (PBS) which received PBS pH=7.4 (n=120), inoculated with *B. hyodysenteriae* highly pathogenic strain GB2 (B_High) (n=120), inoculated with *B. hyodysenteriae* low pathogenic strain AM35017 (B_Low) (n=120), and positive control (LPS) which received 100 µg of lipopolysaccharide (LPS from E. coli O:127 (Sigma, St. Louis, USA)) (n=120). After culture, 160 explants were submerged in homogenization solution and snap frozen in liquid nitrogen for qPCR of the trefoil factor 3 gene (TFF3) and 320 explants were fixed in formalin and processed for histopathological evaluation of necrosis score and survival rate. An extra tissue sample was collected from each animal at the moment of euthanasia and submitted to all analysis (time zero control).

5.2. BACTERIAL STRAINS CULTIVATION AND INOCULUM

The high and low pathogenic strains used in this study were obtained respectively from a clinically SD ill animal from a SD herd, and from a healthy pig from a herd with no history of SD and no clinical signs of the disease. Both isolates were obtained from commercial farms in Minas Gerais State, Brazil (Sato et al., 2022).

Highly and low pathogenicity strains were cultured in tryptone soy agar (TSA) inside anaerobic chambers gazed with N_2 (80%), CO_2 (10%) and H_2 (10%) at 42ºC for 72 hours. At the time of inoculation, plates were washed with 2 ml of brain-heart infusion (BHI) broth supplemented with 5% fetal bovine serum (FBS). The bacterial suspension was transferred into transparent vials and broth was added until the desired concentration of 10^8 bacteria/ml was reached according to suspension's turbidity compared to the 2.0 McFarland turbidity standard. A sample was removed and evaluated by optic microscopy for purity confirmation. Bacterial inoculation was performed using 100 µl of the above-described solution. Negative control received 100 µl of BHI broth and positive control received 100 µg of LPS from *E. coli* O:127 (Sigma, St. Louis, USA).

5.3. SWINE COLON EXPLANTS *IN VITRO* CULTURE

The intestinal explant culture method utilized was an adaptation from the method proposed by Costa et al. (2018). Animals were desensitized with electrical stunning and then euthanized by exsanguination at the necropsy room from the Veterinary College (UFMG). Next, the abdominal cavity was cut open and a 20 centimetres fragment from the apex of the spiral colon was removed, opened longitudinally at the mesenteric border and washed with ice cold sterile cleaning solution (KCl 0.4 g/L, KH_2PO_4 0.06 g/L, $NaHCO_3$ 0.35 g/L, NaCl 8.0 g/L, Na_2HPO_4 0.04788 g/L, D-glucose 1.0 g/L; pH 7.4) added with 1.5 mM $CaCl_2$. Clean fragments were transferred to a laminar flow cabinet and refrigerated on ice. Smaller fragments were cut with scissors and placed into 90 x 15 mm Petri dishes displayed on top of refrigerated surfaces and added with 1 ml of culture media (DMEM + $CaCl_2$ 1.5 mM, fetal bovine serum 100 µL/ml and L-glutamine 200 mM 0,4 µL/ml (Life Technologies Co., Carlsbad, CA)). The serosal layer was mechanically removed by pinching each layer of the tissue with a toothed forceps and pulling it gently. After serosal removal, fragments were positioned with mucosal side facing up for the obtainment of sections of 2 cm^2 using two scalpels. Then explants were transferred into 6-well culture plates mounted with 1ml of culture media and one cell strainer (100 µm) per well. Subsequently, inoculation rings (0,5 ml Eppendorf tubes without lid and bottom) were positioned at the center of each explant and glued with cyanoacrylate (TEKBOND, Embu das Artes, BR). After adding the correspondent inoculum to each explant, plates were placed on a metal tray revested with a polypropylene vacuum bag measuring 54 cm x 85 cm (Original Space Bag, Storage Packs, San Diego, CA). A vacuum pump was used to remove the bag's air and then 99% oxygen was used to re-inflate the vacuum bag. Less than one hour after animal's euthanasia, trays were placed inside 37ºC incubators where explants underwent cultured for 2 hours. A schematic representation of explants is shown in Figure 1.

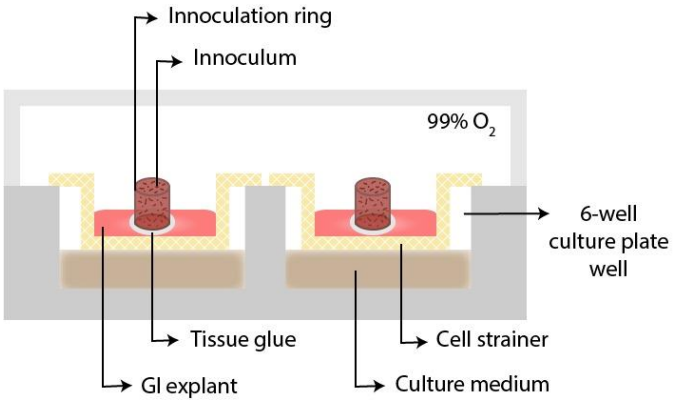

Figure 1 – *In vitro* colon culture setting based on what proposed by Costa et al. (2018). Each plate contains 6 explants independently cultured with polarized stimulus. The setting is composed of a 6-well culture plate, each with a layer of culture media underneath the cell strainer touching its bottom. One explant per well was placed with mucosal side facing upwards and serosal side facing downwards. Then an inoculation ring is attached with cyanoacrylate glue, and the inoculum is placed inside.

5.4. IVOC METHOD STANDARZATION

Following the method stablished by Costa et al. (2018), a series of tests were conducted in order to adapt this protocol to our conditions. Changes included culture media, chamber's gas composition, tissue glue, wash and transport media, conditioned media and time under culture, which were tested in explants obtained from intestine taken from a nearby slaughterhouse. In total, 4 protocols were performed evaluating the necrosis score (NS) of explants, with a final trial prior the experiment in a pilot 4-week-old freshly euthanized animal, as can be seen at Table 1 bellow.

Table 1 - Summary of the protocols used at the IVOC method standardization.

Protocol	Nature of Alteration	Alteration	Protocol	Nature of Alteration	Alteration
1	Culture media	DMEM	3	Culture media	KGM
		KGM		Environmental condition	Oxygen
	Environmental condition	CO2		Time of culture	2 hours
		Tri-gas			8 hours
	Time of culture	2 hours		Cleaning and transport solution	Manufactured HBSS
		8 hours			Commercial HBSS
2	Culture media	DMEM	4	Culture media	DMEM
		KGM			KGM
	Environmental condition	Oxygen		Incubation methods	Anaerobic jar
		CO2			Vacuum bag
	Time of culture	2 hours		Cleaning and transport solution	Manufactured HBSS
		8 hours			Commercial HBSS

Protocols are listed from 1 to 4 in the first column, followed by the nature of alteration and the alteration made at the original protocol from Costa et al., 2018.

The culture media originally used by Costa et al (2018) was the KGM™ Keratinocyte Growth Medium BulletKit™ (KGM) which costs approximately BRL R$ 1500,00 (500 ml), which was above our budget. The culture media used in this present study was DMEM with the addition of $CaCl_2$ 1.5 mM, fetal bovine serum 100 µL/ml and L-glutamine 200 mM 0,4 µL/ml, which is routinely available in our laboratory.

The environmental condition inside the culture chamber preconized at the original protocol was 99% O_2. Due to the pandemic's scenario where our tests were conducted, oxygen supply was unavailable and initially we tested the culture inside a 5% CO_2 incubator and compared to the tri-gas composed of N_2 (80%), CO_2 (10%) and H_2 (10%) gassed in vacuum bags. After the oxygen became available it was used as the method of choice for the technique, as stablished by Costa et al. (2018) as it provides pH stability for the media and oxygen supply for the tissue.

Two different approaches for explants incubation were attempted. The first one used the anaerobic jar with 6 cycles of vacuum followed by addition of pure oxygen. This procedure led to a delay of 30 minutes in the preparation of explants, what is undesirable since it predisposes explant degradation. In this way, we tried the second approach which consisted in aspirating the air from a vacuum bag with a vacuum pump and inflating once with pure oxygen.

The conditioned media used to enhance crypts proliferation and enterocyte maintenance described at the original protocol needed to be produced through the supernatant from the culture of L-WRN cells. A sample of this particular cell line worth BRL R$20.000,00, approximately, which was above our budget. For this reason, we tried to perform this study without the use of this supplement which would be added to the culture media. According to previous experiences by Dr. Costa (personal communication), a severe reduced survival rate would be expected (≤50%).

The tissue glue used to fixate the inoculation ring into the intestinal mucosa of explants at the original protocol is tissue adhesive for veterinary use which base is a purified n-Butyl Cyanoacrylate. In Brazil, the use of these tissue glue in veterinarian clinics is incipient, what makes the acquisition of such glue difficult and expensive – around BRL R$ 500,00 for 0,8 ml. For this reason, we chose to use TEKBOND 793 (cyanoacrylate), a much more interesting option at a financial perspective and largely available in Brazil.

The transport media used in the original protocol is Hanks' Balanced Salt Solution (HBSS 6648, Sigma, St. Louis, USA) added with 1.5 mM $CaCl_2$ which currently costs BRL R$ 500,00 (500 ml). To properly rinse an intestinal fragment one bottle per animal is needed, which would cost BRL R$5.000,00 for the present experiment. In an attempt to make the method more viable for

most Brazilian research facilities, we used the formulation of HBSS and produced a similar formula at our laboratory and tested it in comparison to the commercial formula.

Morphological score data were obtained and compared as the mean score value for each group following the necrosis score raging from 0 to 3 as detailed at the section "5.6. Histopathology and morphological analysis". In each variable analysed, explants showing reduced score of survival rate at the pilot trials were correlated with an inferior approach for explant culturing.

5.5. RNA EXTRACTION AND QUANTITATIVE PCR OF TFF3 GENE

Harvested explants were submerged on 200 µl of Homogenization Solution (Promega Co., Madison, USA) and snap frozen in liquid nitrogen until processing. For tissue disruption samples were tawed on ice, each received one 3 mm tungsten carbide bead and were placed into the TissueLyser LT device, using two cycles of 2 minutes at 50 Hz oscillation frequency (QUIAGEN, Germantown, USA). Then extraction was performed according to Maxwell® RSC simplyRNA Tissue Kit manual (Promega Co., Madison, USA). Briefly, 200 µl of Lysis Buffer was added to the tissue homogenate and the mixture was added to the well #1 from Maxwell® RSC's cartridge. Then cartridges were prepared with the addition of 10 µl of DNAse I Solution on well #4 and the addition of 50 µl of RNAse free water to elution tubes. Maxprep™ Software was used to set up automated extraction under simplyRNA Tissue settings. Then, cartridge tray was placed on Maxwell® RSC Instrument and samples underwent RNA extraction. As extraction was completed, elution tubes were removed and frozen at -80ºC.

Sample RNA content was assessed trough Qubit® Fluorometer using Qubit® RNA BR Assay Kit following the user guide (Life Technologies Co., Carlsbad, USA). In summary, 199 µl of working solution and 1 µl of sample was mixed by vortexing, incubated for 2 minutes in room temperature and then read at the Qubit® 2.0 Fluorometer. Then, normalization of samples was performed trough dilution at final concentration of 250 ng/µl. Quantitative PCR carried out using SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invritogen Co., Carlsbad, USA) according to manufacturer specifications. Firstly, the reaction mix was prepared aiming the final volume of 25 µl per reaction, adding: 12,5 µl of 2X SYBR® Green Reaction Mix; 0,75 µl of each forward and reverse primers (concentration of 10 µM); 0,1 µl of ROX Reference Dye; 0,5 µl of SuperScript® III RT/Platinum® Taq Mix; 1 µl of RNA template (concentration of 250 ng/µl) and 9,4 µl of RNAse/DNAse free water. Reactions were carried out on a QuantStudio™ 7 Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, USA) under a cycling program of: 50 ºC for 3 minutes hold; 95 ºC for 5 minutes hold; 40 cycles of 95ºC for 15 seconds and 60ºC for 30 seconds; and 40ºC for 1 minute, followed by melting curve analysis. The following forward and reverse primers sequences were used for the trefoil factor 3 (TFF3) gene (forward CAG GAT GTT CTG GCT GCT AGT G; reverse GCA GTC CAC CCT

GTC CTT G) (Graziani et al., 2019).For the housekeeping gene it was chosen the beta-2-microglobulin (B2M) gene, which has the following sequence: forward CAA GAT AGT TAA GTG GGA TCG; reverse TGG TAA CAT CAA TAC GAT TTC (Wang et al., 2018).

5.6. HISTOPATHOLOGY AND MORPHOLOGICAL ANALYSIS

Cultured explants were harvested and submitted for histopathology tissue processing according to Rieger et al., 2021 with some modifications. Firstly, fragments were placed on filter paper pieces and inserted inside embedding cassettes, which were submerged in neutral buffered formalin immediately and fixed for at least 24 hours. Then samples were transferred into the automatic tissue processor Leica TP1020 (Leica Biosystems, Nussloch, DE) for tissue processing. Following tissues were positioned longitudinally inside embedding molds and embedded with fresh paraffin. After paraffin hardened, paraffin blocks were frozen overnight at -20ºC for slide preparation. Sections of 4 μm were cut, placed on glass slides and dried overnight at room temperature. Haematoxylin and Eosin (H&E) staining was performed next by incubating the tissue slides at 70ºC for 30 minutes, then proceeding to the following hydration battery: xylene for 15 minutes (twice), ethanol 100% for 10 minutes (twice), ethanol 90% for 3 minutes, ethanol 80% for 3 minutes, ethanol 70% for 3 minutes and distilled water for 3 minutes. Next, the staining was performed by submerging slides into hematoxylin solution for 3–7 minutes, rising with running tap water for 10 minutes, submerging in eosin solution for 5–10 minutes, and rinse by dipping in distilled water for 5 seconds. Slides subsequently underwent dehydration and were mounted with Canada Balsam (Dinamica, Indaiatuba, BR).

From the original sample size of 320 explants for histopathological analyses, 266 were evaluated - with hidden group identifications - by optical microscopy using high power objective (40x) for the following variables: survival rate; necrosis score; goblet cell abundance; cellularity of lamina propria; cellular crypt content; and distention of crypts. 54 samples were lost while cutting the paraffin embedded blocks, since the sample was too small (6 mm in diameter and 1 mm in thickness) and difficult to visualize.

The survival rate was evaluated by the average value of epithelium cover (0 to 100% of epithelium covering the lamina propria), epithelium necrosis (0 to 100%; from the epithelium covering the lamina propria, which percentage of cells were necrotic) and crypt necrosis (0 to 100% as described below). An average bellow 25% was given a score 0; an average between 25 and 50% was given a score 1; an average between 50 and 75% was given a score 2; and a average between 75 and 100% was given a score 3.

The necrosis score was performed according to what was previously described by Costa et al., 2017. It consists on the evaluation of 25 crypts at 5 equally distant points of the explant and

ranges from 0 to 3 according to the following criteria: 0 – necrosis absence; 1 – from 0 to 20% of necrotic crypts; 2 – from 20 to 50% of necrotic crypts; 3 – more than 50% necrotic crypts.

Goblet cell abundance was given by considering a normal ratio of 1:1 goblet cell: enterocytes under a score from 0 to 3 as described: 0 – 0 to 25% of crypts with an increased goblet cells-: enterocytes ratio; 1 – 25 to 50% of crypts with an increased goblet cells: enterocytes ratio; 2 – 50 to 75% of crypts with an increased goblet cells : enterocytes ratio; 3 – 75 to 100% of crypts with an increased goblet cells : enterocytes ratio.

Cellular crypt content and distention of crypts were given respectively by the score of crypts containing any necrotic cellular debris (0 – 0 to 25% of crypts with cellular debris; 1 – 25 to 50% of crypts with cellular debris; 2 – 50 to 75% of crypts with cellular debris; 3 – 75 to 100% of crypts with cellular debris) and by the score of crypts with an increase of the luminal volume (0 – 0 to 25% of crypts with increased luminal volume; 1 – 25 to 50% of crypts with increased luminal volume; 2 – 50 to 75% of crypts with increased luminal volume; 3 – 75 to 100% of crypts with increased luminal volume).

Lamina propria cellularity score was given by the reduction or increase in the cellular population contained at the lamina propria compared with normal histological slide of intestine as follows: 0 – significant reduced cellular population, with more than 75% of clear space showing at the lamina propria; 1 – slightly reduced cellular population, with 50% of clear space showing at the lamina propria; 2 – normal cellular population, with similar fulfillment of the lamina propria as the normal histological cut; 3 – increased cellular population with less than 25% of clear space at the lamina propria.

5.7. STATISTICAL ANALYSIS

qPCR statistical analysis was performed using the MCMC.qpcr package in R according to Matz et al. (2013), a method suited for qPCR analysis without the use of a reference gene. Firstly, the data from qPCR containing CT values were formatted as genes and experimental groups displayed in columns (one column per gene) without averaging of replicates. Then generalized linear model was applied by converting counts using the formula "Count = $E^{(Cq1 - Cq)}$" where E = 2 (considering a 100% efficiency) and Cq1 is the number of qPCR cycles required to detect a single target molecule. Following application of data trough MCMC.qpcr package, a validation step was made through diagnostic plots to access this linear modeling was appropriate for this experiment dataset. Next, results were extracted and visualized by plotting relative abundance graphs. Comparisons were made first by removing the data from the animal control (intestinal fragment collected upon euthanasia) and then analysing groups by the difference of expression between PBS control group and challenged groups.

Histopathology score analysis was carried out using generalized estimating equations (GEE), where the outcomes were the treatments (which could have an unknow correlation) and the subjects the individuals (pigs). The analysis was undertaken by performing pairwise comparisons from estimated marginal means based on the original scale of each treatment (dependent variable). A 95% Wald confidence interval test was conducted to assess the significance of the independent variables analysed. It was considered a statistically significant result those with *P value* inferior to 0.05.

6. RESULTS

6.1. IVOC METHOD STANDARZIDATION

6.1.1. Protocol #1 – Culture Media vs. Environmental Culturing Conditions

A slight improve in CO_2 incubator compared to the tri-gas was noticed (CO_2: NSA =1,2 and 1,1 at 2 hours and NSA = 2,6 and 2,8 at 8 hours; Tri-gas: NSA = 2,8 and 1,2 at 2 hours and NSA = 3,0 and 3,0 at 8 hours) and an improved condition of explants in the KGM media compared to the DMEM media (KGM: NSA = 1,1 and 1,2 at 2 hours and NSA = 2,8 and 3 at 8 hours; DMEM: NSA = 1,2 and 2,8 at 2 hours and NSA = 2,6 and 3,0 at 8 hours), although these results were preliminary and more tests were needed to ensure our decision.

The average necrosis score from 3 explants *per* group under 2 and 8 hours of culture upon histopathology for the culture media and gas used. The histological conservation of crypts was scored from 0 to 3 for each explant (0 – necrosis absence; 1 – from 0 to 20% of necrotic crypts; 2 – from 21 to 50% of necrotic crypts; 3 – more than 51% necrotic crypts) and an average necrosis score per group was summarized in Table 1.

Table 2 – Necrosis Score Average (NSA).

Protocol	Culture Media	Environmental Condition	Culture Time (hours)	Necrosis Score Average
1	DMEM	CO2	2	1,2
	KGM	CO2		1,1
	DMEM	Tri-gas		2,8
	KGM	Tri-gas		1,2
1	DMEM	CO2	8	2,6
	KGM	CO2		2,8
	DMEM	Tri-gas		3
	KGM	Tri-gas		3

DMEM or KGM culture media, and CO_2 incubator or tri-gas were tested as culturing conditions. A necrosis score average (NSA) was calculated with the necrosis score (NS) from 3 explants per group, scored as follows: 0 – no necrosis; 1 – from 0 to 20% of necrotic crypts; 2 – from 21 to 50% of necrotic crypts; 3 – more than 51% necrotic crypts.

6.1.2. Protocol #2 – Culture Media vs. Different Environmental Culturing Conditions

Improved explant conditions were noticed at the 2-hour culture time using KGM culturing media compared with DMEM (KGM: NSA = 1,2 and 1,5; DMEM: NSA = 2,4 and 1,9) while explants who underwent culture for 8 hours had decreased necrosis score using DMEM media (KGM: NSA = 2,6 and 2,9; DMEM: NSA = 2,2 and 2,5), making it difficult to pinpoint a decision.

Due to the high necrosis score from the 2-hour cultured explants at the first protocol, we concluded that the oxygen was primarily important for survival. And as tri-gas showed the worst results compared to CO_2, we decided to remove it and perform a test using a vacuum bag inflated with pure oxygen ceded by the Veterinary Hospital from the Veterinary School (Universidade Federal de Minas Gerais) and compare with the CO_2 incubator for 2 and 8 hours under culture. Results are as shown in Table 2.

Table 3 – Necrosis Score Average (NSA).

Protocol	Culture Media	Environmental Condition	Culture Time (hours)	Necrosis Score Average
2	DMEM	Oxygen	2	2,4
	KGM	Oxygen		1,2
	DMEM	CO2		1,9
	KGM	CO2		1,5
2	DMEM	Oxygen	8	2,2
	KGM	Oxygen		2,6
	DMEM	CO2		2,5
	KGM	CO2		2,9

DMEM or KGM culture media and CO_2 incubator or pure oxygen were tested as culturing conditions. A necrosis score average (NSA) was calculated with the necrosis score (NS) from 3 explants per group, scored as follows: 0 – necrosis absence; 1 – from 0 to 20% of necrotic crypts; 2 – from 21 to 50% of necrotic crypts; 3 – more than 51% necrotic crypts.

6.1.3. Protocol #3 – Commercial HBSS vs. Manufactured HBSS

Manufactured HBSS showed increased NSA compared with commercial HBSS for both culture times (Man. HBSS: NSA = 1,3 and Comm HBSS: NSA = 0,3 at 2 hours; Man. HBSS: NSA = 2,8 and Comm HBSS: NSA = 2,1 at 8 hours, as shown in Table 3. Although the pH from the manufactured HBSS was not adjusted before use. So, we inferred that this could have led to an erroneous decision. In this way we have decided to repeat the test with both cleaning solutions and both culture media at Protocol #4. Nevertheless, improved explant conditions were noticed in general at this protocol at the 2-hour time point compared with previous protocols. Since no visible reduction at the 8-hour time point was noticed so far, we have decided to continue tests and to perform the experiment using only the 2-hour time point.

Table 4 – Necrosis Score Average (NSA).

Protocol	Culture Media	Environmental Condition	Culture Time (hours)	Cleaning and Transport Solution	Necrosis Score Average
3	KGM	Oxygen	2	Man. HBSS	1,3
				Comm. HBSS	0,3
3	KGM	Oxygen	8	Man. HBSS	2,8
				Comm. HBSS	2,1

Manufactured HBSS or commercial HBSS tested as cleaning and transport solutions, with the culturing conditions of pure oxygen and KGM as culture media. A necrosis score average was calculated with the NS from 6 explants per group, scored as follows: 0 – necrosis absence; 1 – from 0 to 20% of necrotic crypts; 2 – from 21 to 50% of necrotic crypts; 3 – more than 51% necrotic crypts.

6.1.4. Protocol #4 – Anaerobic Jar vs. Vacuum Bags for Explants Incubation with Pure Oxygen

In this protocol we compared both incubation methods (anaerobic jar and vacuum bag), besides both culturing media (DMEM and KGM) and cleaning and transport solutions (manufactured and commercial HBSS). For the isolated comparison of DMEM vs. KBM, an average value of each culture media was made, resulting in an NSA of 1,4 and 1,2 respectively. Based on this and previous results, it was decided to perform the experiment using supplemented DMEM as culture media, since the NSA was similar using either of them. Comparing the anaerobic jar with the vacuum bag, an average value of NSA for each variable was 0,7 and 1,9, respectively. Due to the unviability of continuing to consume large amounts of oxygen to fill the anaerobic jar for 6 cycles, it was decided to perform the experiment using the vacuum bag, despite the results shown at this protocol. Regarding the cleaning and transport solution tested (manufactured and commercial HBSS), the results shown an NSA of 1,1 and 1,8, respectively in average (Table 4). Based in this result, it was decided to use the manufactured HBSS instead of the commercial in our experiment.

Table 5 – Necrosis Score Average (NSA).

Protocol	Culture Media	Incubation Methods	Culture Time (hours)	Cleaning and Transport Solution	Necrosis Score Average
4	DMEM	Anaerobic Jar	2	Man. HBSS	1,3
				Comm. HBSS	0,3
	DMEM	Vacuum Bag		Man. HBSS	1,0
				Comm. HBSS	3,0
4	KGM	Anaerobic Jar	2	Man. HBSS	0,3
				Comm. HBSS	0,6
	KGM	Vacuum Bag		Man. HBSS	0,6
				Comm. HBSS	3,0

Tests consisted of comparing DMEM and KGM as culture media, anaerobic jar and vacuum bag as incubation method, and manufactured HBSS and commercial HBSS as cleaning and transport solution. A necrosis score average was calculated with the necrosis score (NS) from 3 explants per group, scored as follows: 0 – necrosis absence; 1 – from 0 to 20% of necrotic crypts; 2 – from 21 to 50% of necrotic crypts; 3 – more than 51% necrotic crypts.

6.2. QUANTITATIVE PCR

The qPCR results for both TFF3 and B2M genes were analysed using average per treatment from raw Ct values, which are displayed in Figure 2.

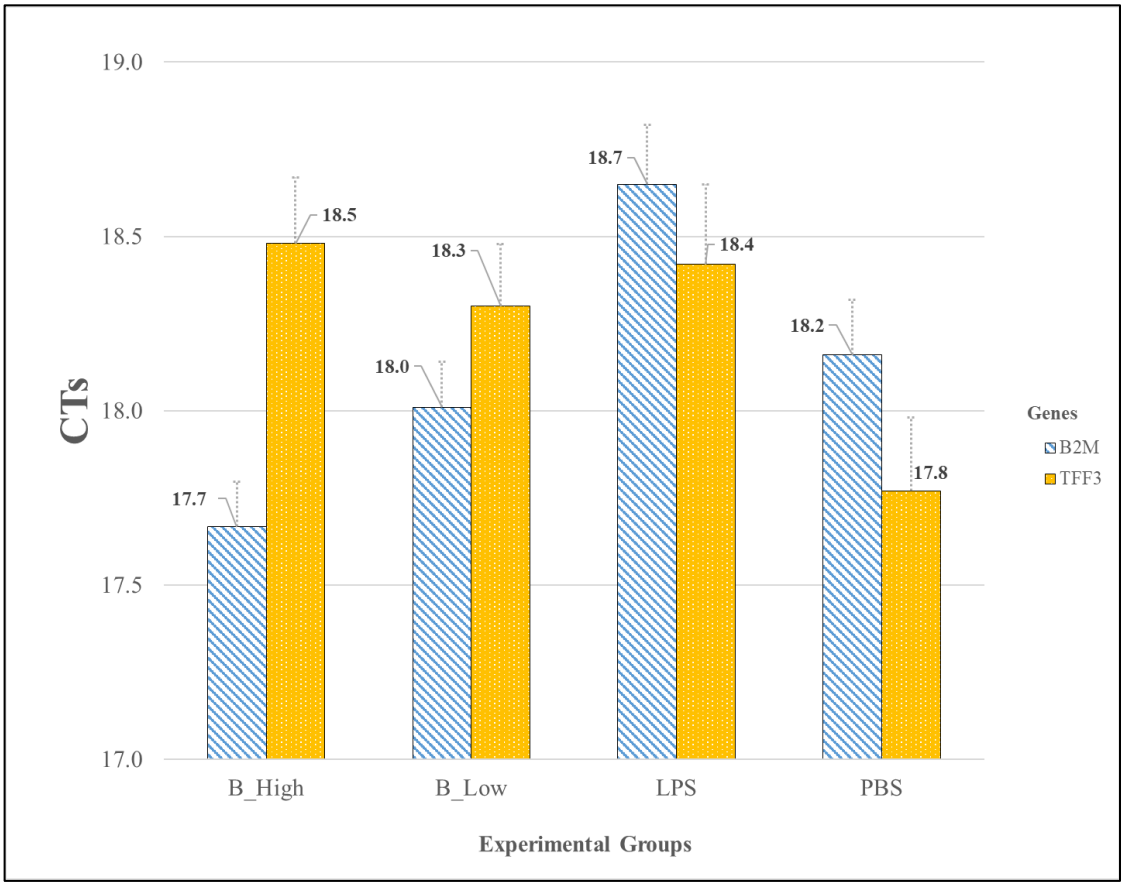

Figure 2 – B2M and TFF3 gene expression from experimental groups. B_High: explants inoculated with *B. hyodysenteriae* highly virulent strain; B_Low: explants inoculated with *B. hyodysenteriae* low virulent strain; LPS: positive control group - explants inoculated LPS; PBS: negative control group - explants inoculated PBS. Standard deviations are shown as vertical bars in colored in gray.

For the B2M gene (housekeeping), the experimental group infected with *B. hyodysenteriae* highly virulent strain showed a mean Ct value of 17.7 ±0.1; while the one infected with *B. hyodysenteriae* low virulent strain showed a mean Ct value of 18.0 ±0.1; and the positive control group showed a mean Ct value of 18.7 ±0.2; and the negative control group a mean Ct value of 18.2 ±0.2.

For the TFF3 gene the experimental group infected with *B. hyodysenteriae* highly virulent strain showed a mean Ct value of 18.5 ±0.2; while the one infected with *B. hyodysenteriae* low virulent strain showed a mean Ct value of 18.3 ±0.2; and the positive control group showed a mean Ct value of 18.4 ±0.2; and the negative control group a mean Ct value of 17.8 ±0.2. Comparisons between groups were made by subtracting the CTs of the PBS control group from the CTs of the other experimental groups (Figure 3).

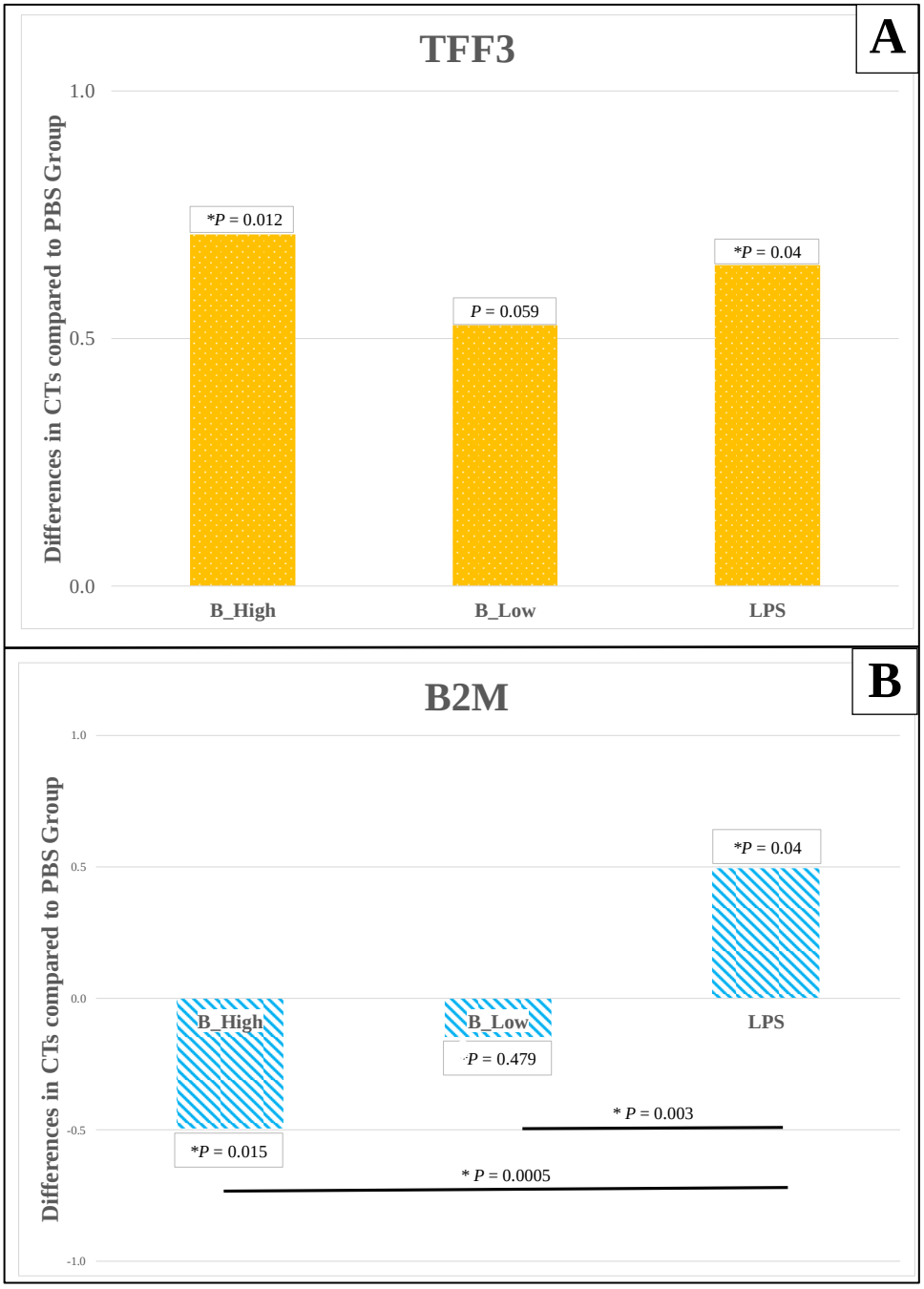

Figure 3 – Relative gene expression between negative control group (PBS) and B_High, B_Low, and LPS for TFF3 (Fig. 3A) and B2M (Fig. 3B) genes. *P* <0.05 was considered as statistical difference (*P* values preceded by asterisks). Statistical differences between groups are indicated by black bars, and for differences between PBS and other treatments the box containing the *P* value is displayed. Treatments: B_High - explants inoculated with *B. hyodysenteriae* highly virulent strain; B_Low - explants inoculated with *B. hyodysenteriae* low virulent strain; LPS - positive control group infected with LPS.

Compared with the negative control group, gene B2M showed a reduction at gene expression for explants exposed to *B. hyodysenteriae* highly virulent strain of -0.5 ±0.13 with *P* = 0.015; an increase in gene expression for explants exposed to LPS group of 0.5 ±0.17 (*P* = 0.04); and had no statistical difference compared with explants exposed to *B. hyodysenteriae* low virulent strain (-0.1 ±0.13; *P* = 0.479).

A reduction of B2M gene expression was noted in the *B. hyodysenteriae* low virulent strain compared to the LPS positive control group ($P = 0.003$) and also in the *B. hyodysenteriae* highly virulent compared to the LPS positive control group ($P = 0.0005$).

Compared with the PBS group, TFF3 gene showed a increase in the gene expression for explants exposed to *B. hyodysenteriae* highly virulent strain of 0.7 ±0.19 ($P = 0.012$); in explants exposed to LPS positive control group of 0,6 ±0,23 ($P = 0.04$); and a trend to in explants exposed to *B. hyodysenteriae* low virulent strain of 0.5 ±0.18 ($P = 0.059$).

6.3. HISTOPATHOLOGY AND MORPHOLOGICAL ANALYSIS

The results of histopathology and morphological evaluations are described in Table 5.

Table 6– Histological scores from the PBS, B_Low, B_High, LPS groups and uncultured tissues.

	Survival Rate	**Crypt Necrosis Score**	**Lamina Propria Celullarity**	**Goblet Cells Abundance**	**Cellular Crypt Content**	**Distention of Crypts**
PBS	1.4 ±0.9	2.0 ±0.9	1.0 ±0.8	1.5 ±0.8	0.5 ±0.9	0.8 ±0.9
B_Low	1.1 ±0.8	2.2 ±0.8	1.0 ±0.9	1.4 ±0.7	0.5 ±0.71	0.5 ±0.7
B_High	0.9 ±0.9	2.5 ±0.7	1.1 ±0.8	1.3 ±0.9	0.5 ±0.8	0.9 ±1.0
LPS	1.1 ±0.7	2.2 ±0.7	1.2 ±0.9	1.3 ±0.8	0.5 ±0.9	0.7 ±0.8
Uncultured Tissue	3.0 ±0.0	0.6 ±0.9	0.9 ±0.8	2.1 ±0.6	0.2 ±0.5	0.5 ±0.9

A score ranging from 0 to 3 score was assigned for suvival rate, crypt necrosis, lamina propria cellularity, goblet cell abundance, cellular crypt content and distetnion of crypts. Each explant was subjected to one of the treatments: PBS (negative control), B_low (*B. hyodysenteriae* low pathogenicity strain), B_high (*B. hyodysenteriae* high pathogenicity strain), LPS (positive control). Intestinal tissue sampled at euthanazia is displayied as unculutred tissue. Standard deviation values are included after the ± sign for each score.

The results from the histopatological variables were analysed by performing a mean value of each treatment and then plotting a bloxpot graph with the averages and variances. Then a Wald test (binomial confidence interval) was performed for statistical differences, assuming a 95% confidence interval ($P < 0.05$) as shown in Figure 4.

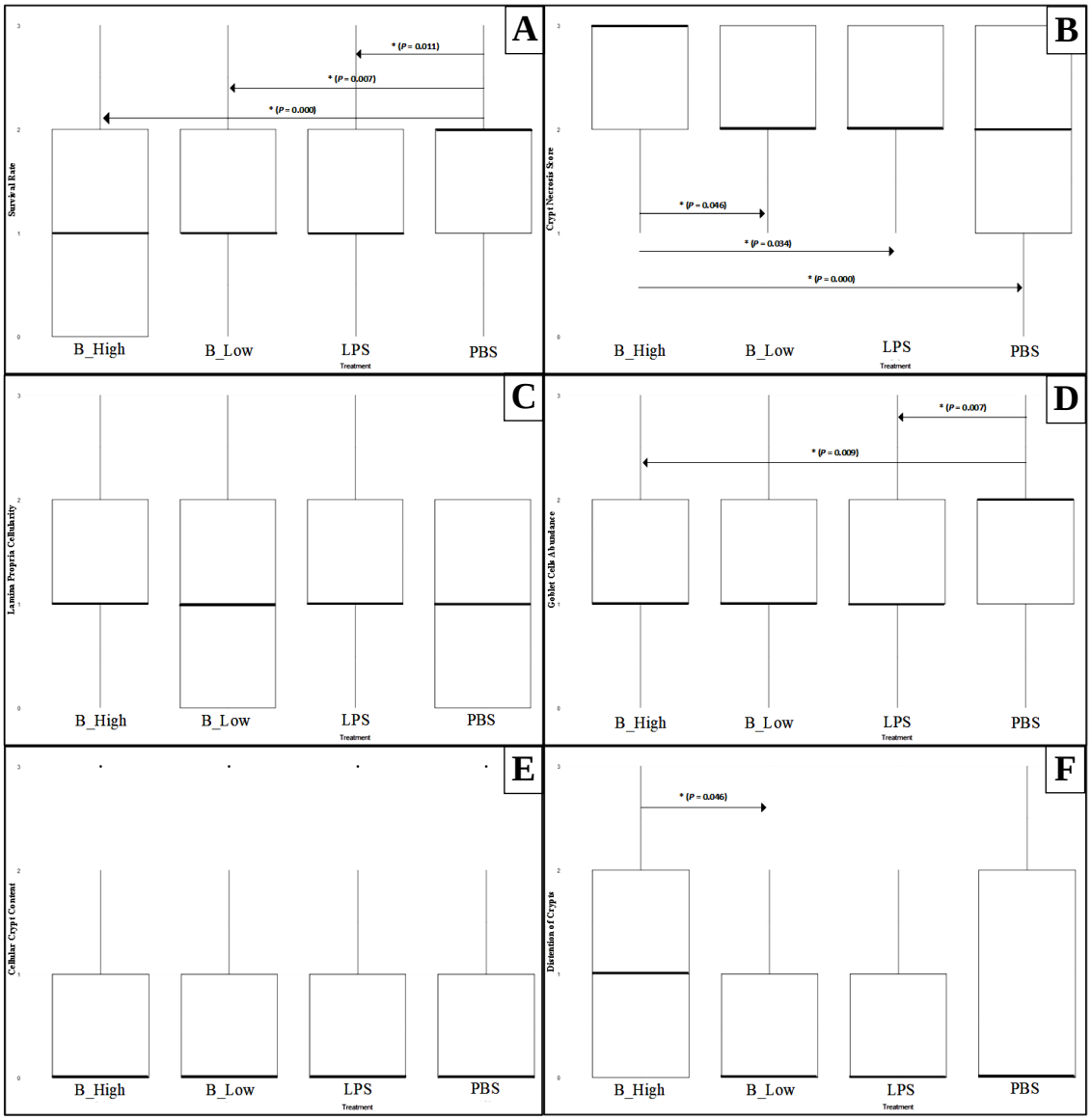

Figure 4 – Boxplots of GEE distribution for histopathological scores *versus* treatments from each variable as follows: (A) Survival rate; (B) Crypt necrosis score; (C) Lamina propria cellularity; (D) Goblet cell abundance; (E) Cellular crypt content and (F) Distension of crypts. Treatments: B_High - explants inoculated with *B hyodysenteriae* high pathogenic strain; B_Low - explants inoculated with *B hyodysenteriae* low pathogenic strain; LPS - explants inoculated with LPS (positive control); PBS - explants inoculated with PBS (negative control). One-sided arrows denotates statistical differences from one group to another, as indicated by the arrow direction. Asterisks represents statistical difference (P <0.05).

PBS group had higher survival rate than the other three groups, with a mean difference of 0.47 ±0.13 (P = 0.000) in relation to B_High; of 0.32 ±0.12 (P = 0.007) in relation to B_Low; and of 0.27 ±0.11 (P = 0.019) in relation to LPS.

B_High group had more crypt necrosis than the other three groups, with a mean difference of 0.21 ±0.10 (P = 0.046) in relation to B_Low, of 0.23 ±0.11 (P = 0.034) in relation to LPS, and of 0.43 ±0.11 (P = 0.000) in relation to PBS.

PBS group showed higher goblet cell abundance than the B_High and the LPS groups, with a mean difference of 0.29 ±0.11 (P = 0.009) in relation to B_High and of 0,28 ±0.10 (P = 0.007) in relation to LPS. There was no difference between this variable for PBS and the B_Low groups (0.12 ±0.10; P = 0.22).

B_High group showed higher distention of crypts than B_Low group, with a mean difference of 0.33 ±0.13 (P = 0.012). For the lamina propria cellularity and cellular crypt content parameters there were no statistical differences among treatments.

7. DISCUSSION

Explants culturing techniques are well consolidated and largely used in veterinary research globally. In Brazil, the use of such methodology in published articles is still incipient. In the present work, an *in vitro* colon culture technique already stablished in other countries underwent a series of adaptations so it could be more accessible for using in most research groups in Brazil.

The adapted protocol did result in good crypt conservation but failed to provide a mucosal layer with more than 90% of epithelial cover as preconized at the original protocol. Considering that, explants have the major advantage of providing all morphological features while maintaining similar structure to the origin organ. As a result, the mucosal layer has great importance at host-pathogen interaction, and the loss of epithelium represents an important negative outcome.

One of the factors which could have contributed to these results is the absence of conditioned media (CM) which would have been added to the culture media following the original protocol. CM is the supernatant obtained from the culture of L-WRN cells which contains three factors: Wnt, R-spondin and noggin. The first two are important at promoting cellular renewal trough stimulus of canonical Wnt signaling, being indispensable at intestinal stem cells isolation. The noggin factor aids the maintenance of enteroids cultured *in vitro*, being largely used in intestinal organoids culture. The creators of this cell line have reported success in culturing mature enteroids extracted from adult mice intestine and emphasize the importance of these factors for enteric cellular culture (Miyoshi & Stappenbeck, 2013).

In the present work, the addition of CM might have improved survival rate and favored cellular renewal, what would had improved epithelial layer maintenance. Complete replacement of cells at mucosal surface occurs within 4 to 5 days in pigs (Rao & Wang, 2010),what perhaps makes the use of CM more relevant when performing experiments with longer culturing periods.

Another factor which might have interfered with explants' SR was the culturing media used. Unfortunately, to the best of my knowledge, there are few studies comparing culturing media

efficacy in order to enrich and guide an argumentation. One study noticed the reduced effects of antigenic molecules in bronchiolar cells grown *in vitro* when cultured with KGM media, in comparison with the other media used (LHC-9), which has a less nutritious composition (Veranth et al., 2008).

The original protocol is restricted when comes to alternatives for changing the culture media used for colonic explant culture. Previous attempts were made with other culturing media, but KGM was the media which consistently provided a healthy epithelium (personal communication). In sight of the results found in the present study, although it is possible to cultivate explants with inferior media, if a higher morphological quality and longer cultivation periods are needed the use of KGM media is recommended.

As for the method used for explant incubation, the environment containing pure oxygen was shown to be required for the successful performance of the study. It was observed that the vacuum bag could be used as a more approachable option for maintaining the gas atmosphere without losses in explant survival.

For the TFF3 gene expression, it was found that all infected groups (either with *Brachyspira* or with LPS) showed higher levels of TFF3 when compared to the PBS group. It is noteworthy to say that *B. hyodysenteriae* low virulent strain had no statistical difference compared to the control group ($P = 0.059$); while the other two groups (B_High and LPS) were higher than the control ($P < 0.05$); suggesting that the low virulent strain is not as pathogenic as the high *B. hyodysenteriae*. This finding corroborates what was demonstrated by Sato (2017) who performed an experimental inoculation study using this low virulence *B. hyodysenteriae* strain. Sato (2017) detected very mild and isolated clinical signs in pigs inoculated with the same low pathogenic strain used in this experiment, despite the presence of macroscopic lesions noted at necropsied animals (18 days post inoculation), which were milder than those found at the highly virulent *B. hyodysenteriae* infected pigs.

It is a characteristic of bacteria from the genus *Brachyspira* to have its pathogenicity modulated by factors such as host's microbiota, bacterial strain and environmental challenge. It is not rare to find reports of mildly pathogenic species of *Brachyspira* colonizing the gut of healthy pigs. In Brazil there is a study showing that commensal spirochaetes, such as *B. innocens, B. intermedia* and *B. murdochii* are found throughout the territory without association with clinical disease (Barcellos et al., 2000).

The extent of the damage caused by these species of *Brachyspira* is still not clear. There are a few studies demonstrating the change in pathogenicity of these Brachyspira species. Jensen et al. (2010) reported colitis caused *B. murdochii,* a weakly beta-hemolytic spirochete. Komarek et

al. (2009) found that chronically diseased pig herds with individuals showing decreased weight gain and diarrhea were infected by non-pathogenic species of *Brachyspira* such as *B. murdochii*, *B. innocens* and *B. intermedia*, indicating that these species could have subclinical impacts at animal's health and intestinal physiology.

Previous studies have characterized atypical strains of *Brachyspira hyodysenteriae* that showed week hemolysis in agar culture and were isolated from clinically healthy herds (La et al., 2016). This phenomenon has been studied extensively. A recent study by Mahu et al. (2016) showed promising results, where an avirulent strain of *B. hyodysenteriae* induced an IgA intestinal response which led to a late onset of the disease on vaccinated animals. In addition, La et al. (2019) demonstrated the potential of an avirulent strain of *B. hyodysenteriae* in reducing swine dysentery in inoculated pigs. This highlights the importance of uncovering aspects of atypical *B. hyodysenteriae* strains, to look for pertinent solutions for the swine industry.

The significantly increased gene expression of TFF3 at B_High and LPS infected groups in comparison to the PBS group are in agreement with previous knowledge which shows that the expression of trefoil factors is consistently exacerbated in the face of acute epithelial injury (Liu et al., 2018). TFF3 is a MUC2 associated protein which aids the stability of the mucus layer, since it is a peptide which participates at the polymerization of this mucin (Wong et al., 1999). *B. hyodysenteriae* have shown the ability to trigger the secretion of MUC2, favoring its colonization (more ligation sites available for spirochetes) and penetration trough the mucus layer, which becomes disorganized and less cohesive (Quintana-Hayashi et al., 2015).

Assembling this information with our data, it is reasonable to suggest that the mechanism involved at increased mucus production unleashed by *B. hyodysenteriae* infection relates to the activation of the TFF3 pathway, although more studies are necessary to confirm this link. Thus, the increased TFF3 gene expression at the two experimental groups which undergone more severe damage is likely to be attributed to the activation of the wound-healing mechanism and the exacerbation of MUC2 production associated with swine dysentery, which was also found by Costa & Harding (2020).

To the best of our knowledge there are no other studies associating swine spirochaete infection with TFF3 gene expression, although there are few studies that are tangential to the subject. Smith et al. (2014) uncovered that the colonization of *Lawsonia intracellularis*, an obligate intracellular bacterium which causes disease in swine, is facilitated trough the MUC2 and TFF3 inhibition. Convergently, Graziani et al. (2019) found that the expression of all trefoil factors is reduced in porcine goblet cells exposed to the mycotoxin deoxynivalenol. Lastly, Raja et al. (2012) analysed the effects of the infection by *Shigella dysenteriae* (the most common agent of

epidemic human dysentery) at the expression of trefoil factors and mucins, revealing that both MUC2 and TFF3 genes were upregulated in the face of acute infection.

Beta-2-microblulin (B2M) is commonly used as a housekeeping gene and its physiological role is to participate in the translation of the major histocompatibility complex class I (MHC-I) (Sake et al., 2019). Its expression in the colon of piglets during the weaning period (26 to 46 days of age) was characterized by Wang et al. (2018) and compared with other commonly used housekeeping genes such as GAPDH. The results showed that in the colon of swine from this age-group, B2M and HPRT1 were the most stable ones, while GAPDH was the least stable.

Previous studies have found that B2M was not stable enough for being used as a housekeeping gene in qPCR analysis. Nazari et al. (2015) found that B2M was not reliable for gene expression studies in equine adipose and marrow derived mesenchymal stem cells, Nihon-Yanagi et al. (2013) also found that B2M is unsuitable to be used as reference gene at the analysis of mRNA in colorectal cancer from humans. In fact, a recent Scientific Reports study from Ho & Patrizi (2021) demonstrated that specific experimental conditions might interfere with the housekeeping gene expression, making necessary to perform a validation for a candidate housekeeping gene inside each trial's scenario. With that and our results, we postulate that B2M apparently is not a highly stable gene which could be used as reference gene under the experimental conditions provided in this study.

The use of B2M as the housekeeping gene in the present study was established according to Wang et al (2018) results and for the advantage of minimizing differences among starting materials when using a housekeeping gene. Surprisingly, our qPCR data from the chosen housekeeping gene displayed statistical differences among groups and, therefore, it was not possible to use B2M as a reference gene. Despite that our comparisons were not affected by the absence of a housekeeping gene. It is interesting finding that B2M had its expression increased in explants exposed to LPS.

Morphological analysis demonstrated that survival rate was improved in PBS group compared to all other groups. This suggests *B. hyodysenteriae* and the positive control treatment (LPS) had a destructive effect in explants. In agreement with this postulate, the B_high group showed a worst crypt necrosis score in comparison with the other three groups, indicating that the disease-causing mechanism of the bacteria was responsible for the lesions and TFF3 expression levels findings.

Previous research has described the acting mechanism of pathogenic species of *Brachyspira* in explants cultured at similar swine colon IVOC setting. Costa & Harding (2020) showed that a pathogenic strain of *B. hampsonii*, a SD-causing spirochaete, can more efficiently impair the

host's immune response and decrease mechanisms of epithelial wound repair compared with a non-pathogenic strain. Hemolysins and lipooligosaccharides are recognized as putative tissue damage virulence factors and are present in most highly pathogenic strains of *Brachyspira* sp. (Hampson & Burrough, 2019), which aids the explanation of the crypt necrosis found at B_High group.

The goblet cell abundance and the distention of crypts parameters were in accordance with each other, insofar as B_High had increased-sized crypts with clear luminal content indicating the presence of mucus and presented smaller goblet cell abundance compared to the negative control. What is likely to have happened is that in the face of stimuli, goblet cells rapidly discharge their stored intracellular mucin together with the plasma membrane, occasioning in the deflation and shrinking of cells, what makes them seem absent at the microscopical slide (Specian & Oliver 1991). This finding is in accordance with the increased crypt damage caused by the *B. hyodysenteriae* high pathogenic strain, which caused more stimuli to the goblet cells and occasioned the larger crypts found at morphological analysis.

As expected, lamina propria cellularity and cellular crypt content parameters were not altered by the treatments used in this study. Explants are disconnected from blood supply; therefore, the cellular content of lamina propria and crypt's lumen should not change since there is no immune cell recruitment.

8. CONCLUSION

The adapted IVOC for swine colon method is suited for reproduction under similar research purposes to this study. Although, if higher epithelial cover or longer time in culture is required for other researcher purposes, the use of KGM media and the conditioned media are recommended. The model could successfully depict the initial pathogen-host interaction, demonstrating histological lesions and changes in gene expression levels compared to what previous studies have shown.

Assessing the gene expression of TFF3 at *B. hyodysenteriae* infection of swine intestine enabled to suggest an existing link between the induction of TFF3 production, activation of the wound-healing mechanism and stimulus to the release of mucins in the swine colon. This ground-breaking correlation can bring insights into the activation of the TFF3 pathway at SD pathogenesis, yielding a lead for further investigation.

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