Metabolic response of porcine colon explants to *in vitro* infection by *Brachyspira hyodysenteriae* – a leap into disease pathophysiology

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Contents

Abstract

Introduction

Swine dysentery caused by *Brachyspira hyodysenteriae* is a production limiting disease in pig farming. Currently antimicrobial therapy is the only treatment and control method available.

Objective

The aim of this study was to characterize the metabolic response of porcine colon explants to infection by *B. hyodysenteriae*.

Methods

Porcine colon explants exposed to *B. hyodysenteriae* were analyzed for histopathological, metabolic and pro-inflammatory gene expression changes.

Results

Significant epithelial necrosis, increased levels of L-citrulline and IL-1α were observed on explants infected with *B. hyodysenteriae*

Conclusions

The spirochete induces necrosis *in vitro* likely through an inflammatory process mediated by IL-1α and NO.

Introduction

Swine dysentery is a syndrome characterized by mucohaemorrhagic colitis and diarrhea in pigs, first described in 1921 (Whiting et al., 1921). At that time the etiological agent was unknown and it was not until the 1970's that *Brachyspira hyodysenteriae* (known then as *Treponema hyodysenteriae*) was identified as the cause of disease (Taylor et al., 1971, Harris et al., 1972). *Brachyspira* are Gram-negative aerotolerant spirochetes of which several species can colonize the large intestine of swine. Diarrhea associated with *Brachyspira spp.* is common among growing-finishing pigs, but post-weaning piglets are also susceptible. At the farm level, high morbidity is observed with clinical signs ranging from mild mucoid diarrhea to severe clinical cases with mucohaemorhagic diarrhea, which can be followed by death due to dehydration (Hampson et al., 2006).

Swine dysentery caused by *B. hyodysenteriae* is a worldwide problem causing economical losses due to mortality, sub-optimal performance, poor feed conversion rates and subsequently growth retardation. Based on a limited number of studies available, the prevalence of *B. hyodysenteriae* varies significantly ranging from 0-40% (Carvajal et al., 2006, Fellström et al., 1996, Møller et al., 1998, Stege et al., 2000, Suh et al., 2005). The variations in prevalence may originate from the use of different diagnostic measures, differences in production systems, management and biosecurity or use of antimicrobial feed additives as growth promoters (Alvarez-Ordonez et al., 2013).

Brachyspira pilosicoli is also known to be able to colonize the large intestine of the pig but causes less severe colitis and diarrhea resulting in a poor feed conversion that may persist after clearing the infection and recovering (Duhamel et al., 2001). *Brachyspira murdochii, Brachyspira innocens* and *Brachyspira intermedia* are also associated with mild clinical signs resembling infection with *B. hyodysenteriae*, consisting of mucoid diarrhea (Chander et al., 2012). In Sweden, a novel strain was identified from pigs with dysentery and was provisionally named "*Brachyspira suanatina"* (Råsbäck et al., 2007). More recently, a new strong ß-hemolytic species distinct from every other known *Brachyspira* spp., was associated with pigs showing clinical signs of dysentery. In the USA, Canada and Europe, "*Brachyspira hampsonii"* was isolated from dysenteric pigs (Chander et al., 2012, Rubin et al., 2013, Mahu et al., 2014). Challenge studies using *B. hampsonii and B. suanatina* resulted in mucohaemorhagic diarrhea undistinguishable from *B. hyodysenteriae* (Rubin et al., 2013, Råsbäck et al., 2007).

Knowledge regarding the pathogenesis of mucohaemorrhagic diarrhea in pigs is limited. *B. hyodysenteriae* predominantly colonizes the mucus layer and reaches deep into the colonic crypts of the centripetal part and apex of the spiral colon. Bellgard et al. (2009) clarified some of the adaptions displayed by *B. hyodysenteriae*, making it possible for it to survive in the large intestine. A high proportion of genes involved in amino acid transport and metabolism assist in its survival in the protein rich environment of the large intestine. Genes encoding putative virulence

factors included proteases able to destruct host tissue, ankyrin proteins, known to bind to host chromatin playing a vital role in the interaction with host cells, haemolysins and genes associated with flagella, motility and chemotaxis. Key genes necessary for the biosynthesis of lipooligosaccharides were also identified as possible virulence factors (Alvarez-Ordóez et al., 2013, Bellgard et al., 2009).

Our understanding concerning the disease pathogenesis and the host response to the disease is limited. Argenzio et al. (1980) established that *B. hyodysenteriae* causes a decreased net absorption of the colon. No significant changes were seen in the permeability or net secretion of the colon. In the early stages increases in expelled mucins can be observed together with superficial mucosal necrosis, infiltration of neutrophils of the lamina propria, elongated crypts and haemorrhage is typically observed. 5 days after infection hyperplasia of mucosal goblet cells is observed together with superficial erosion of the mucosa, haemorrhage and fibrinous exudate. Mucus production was described to be 5 times higher in pigs experimentally infected with *B. hyodysenteriae,* compared with healthy pigs. (Burrough et al., 2016)

Kruse et al. (2008) found, after experimentally infecting ten pigs with *B. hyodysenteriae,* elevated blood concentrations of IL-1β, serum amyloid alpha (SAA), neutrophils, monocytes, CD8α+ lymphocytes during dysentery and the increase in IL-10, γδ T cells and *B. hyodysenteriae*-specific antibodies during the recovery period.

Better understanding of the pathogenesis and host response to *B. hyodysenteriae* will enable us to investigate predisposing factors involved in swine dysentery and possibly develop efficient prophylactic methods other than antimicrobial therapy. Furthermore, increased insights in the host response might assist in the development of a vaccine against *B. hyodysenteriae,* which has been unsuccessful up to this day (Alvarez-Ordóez et al., 2013). Based on a previously successful model using metabolomics on tissues inoculated with Hepatitis C virus by Roe et al. (2011), our goal is to profile the metabolites in swine colon explants after *in vitro* exposure to *Brachyspira hyodysenteriae* for 8 hours.

Materials and methods

1.1 *Pathogen isolation and characterization*

Brachyspira hyodysenteriae isolation from fecal samples, propagation and characterization was performed as previously described for other *Brachyspira* spp. (Rubin et al. 2013). Briefly, fresh feces were collected from a naturally infected pig with mucohaemorrhagic diarrhea and transported on ice for isolation. A sterile loop was used to inoculate BJ agar and, after 72 hours of anaerobic incubation at 37ºC, patches of strong β-haemolysis were observed from which motile spirochetes could be seen on phase microscopy. This procedure was repeated two times to obtain an isolated spirochete culture. Areas of clear, strong β-haemolysis were sampled and DNA was extracted using a commercial kit (DNeasy Blood and Tissue Kit; QIAGEN, Hilden, Germany). To speciate the isolate, a genus specific PCR targeting the NADH-oxidase (*nox*) gene (Rohde et al. 2002) was performed and amplicons were submitted for sequencing. Amplicon (939 bp) BLAST analysis revealed 99% similarity to other *Brachyspira hyodysenteriae* isolates (accession number).

1.2 *Colon segment collection and explant preparation*

Three piglets between 5-7 weeks of age, fed commercial diet and free of gastrointestinal clinical signs were euthanized for reasons unrelated to this study. Pigs were sedated with 8mg/kg IM of azaperone (Stresnil, Lifarma BV, Baexem, The Netherlands) and euthanized by pentobarbital overdose (intracardiac, Euthasol 20%, AST farma BV, Oudewater, The Netherlands). Tissue collection and preparation for culture was performed as previously described (Costa et al. 2016). Immediately after euthanasia an 8 cm segment of spiral colon (apex) was surgically excised through a 10 cm incision along the *linea alba*. The colon segment was placed in 30 mL of refrigerated Dulbecco phosphate buffered saline (DPBS without Ca^{2+} and Mg^{2+}, 0.1 M, pH 7.0) supplemented with an antibiotic mix selective for *Brachyspira* spp. (200 µg/ml spectinomycin, 6.25 µg/ml vancomycin, 6.25 µg/ml colistin, 25 µg/ml spiramycin, 12.5 µg/ml rifampicin). Within one hour of euthanasia, colonic serosa was surgically separated from the mucosa on a refrigerated surface embedded in the same solution used for transport. The mucosa from each colon segment was then divided into 28 explants of 1,5 cm x 1,5 cm, which were placed on individual 3 cm x 3 cm x 0,75 cm agar blocks (1% w/v in distilled water) kept in dishes (100 mm x 15 mm) containing 8 mL of culture media. Next, dishes were placed in a modular incubator chamber and it was gassed for 2 minutes at 10 L/min with a 99% O_2 – 1% CO_2 gas mix (Medical Carbogeen, Linde Healthcare Benelux, Eindhoven, The Netherlands). This setup was incubated at 37ºC. Culture media consisted of keratinocyte basal media (KBM-gold Keratinocyte, Lonza Benelux BV, Breda, The Netherlands) supplemented with 1.5mM Ca^{2+} and the above mentioned *Brachyspira* spp. selective antibiotic mix.

1.3 *Inoculum preparation*

Agar pieces from cultures of the isolate described above (1 cm x 1 cm) displaying strong β-haemolysis were used to inoculate glass vials containing 25 ml of JBS broth. Vials were placed on a shaker (160 rpm) and incubated anaerobically at 37°C for 48 hours. Immediately before infecting the explants, culture vials were retrieved from the incubator and an aliquot was collected for verification of bacterial activity and quantification by quantitative PCR (kept frozen at -80ºC until processing). Phase contrast microscopy was used to visually assess spirochete motility as an indicator of viability. 1 ml of culture broth containing active, motile spirochetes was centrifuged for 10 minutes at 2500 × g. The supernatant was discarded and the pellet was resuspended in 1 mL of sterile PBS with above mentioned antibiotic solution. For preparation of control inoculum, 25 ml of sterile JBS broth was incubated, centrifuged and resuspended as the infected inoculum. Before inoculation, lipopolysaccharide (LPS, *E. coli* O:127 B:8, Sigma, Zwijndrecht, The Netherlands) was diluted in sterile PBS (10µg/ml). All inocula were supplemented with the same antibiotic mix selective for *Brachyspira* spp.

1.4 *Infection trial*

In this study, three groups were investigated: PBS group (negative control), where explants were inoculated with the control inoculum; LPS (positive control), where explants received the previously mentioned LPS preparation; and B.hyo, where explants received active, pure cultures of *Brachyspira hyodysenteriae*. Before inoculation, explants were randomly assigned to one of the three groups (n=9/group/pig). Each explant received a polypropylene inoculation ring (0.5 cm diameter x 1 cm height) attached to its mucosal (luminal) aspect by an innocuous, medical-grade silicone adhesive (Kwik-Cast, World Precision Instruments, Sarasota, Fla., USA). The ring was used to prevent inoculum spillage beyond the mucosal side. Each explant receiving a total of 100 µl of inoculum, independent of group. After inoculation, explants were co-incubated with inocula for 8 hours in the conditions described above for explant culture. At this time, 2 explants/group/pig were snap frozen in liquid nitrogen, and stored at -80ºC until qPCR analysis. Other 1 explant/group/pig was fixed in Carnoy's solution (60% absolute ethanol, 30% chloroform and 10% glacial acetic acid v/%.) for 2 hours, and then moved to a 70% ethanol, 30% distilled water solution until processing for histopathology. The remaining 6 explants/group/pig were also snap frozen in liquid nitrogen and stored in -80ºC until further processing for metabolite extraction and analysis. One extra colon section per pig was processed up to inoculation, and then immediately fixed in Carnoy's solution to be used as a technique control (0 hour samples).

1.5 *H&E stained sections*

Carnoy's fixed tissue was routinely processed in paraffin, sectioned and stained using hematoxylin and eosin (H&E). From each explant (n=9), four 4 µm sections were prepared in order to have representative samples of the entire explant length. An examiner blinded to the identity of samples carried out the analysis under optical microscopy at 20× magnification. A semi-

quantitative scoring system was applied, which evaluated epithelial cells in regards to nuclei karyopyknosis or karyorrhexis, loss of cell membrane delimitation and increased cytoplasmic vacuolization. Score 0: 0-20% of crypt cells were necrotic; score 1: 21-50% of crypt cells were necrotic; score 2: 51-70% of crypt cells were necrotic; score 3: 71-100% of crypt cells were necrotic.

1.6 *Quantitative PCR assays*

Analysis of mRNA levels from explants targeted the GAPDH, interleukin-1α (IL-1α), interleukin-8 (IL-8), interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α) genes. Assays were conducted as previously described (Costa et al. 2016). Briefly, total RNA was extracted from RNAlater fixed samples and cDNA was assembled using commercially available kits (RNeasy minikit, Qiagen NV, Venlo, The Netherlands, iScript cDNA synthesis kit, Bio-Rad, Veenendaal, the Netherlands). All quantitative PCR reactions were conducted on a Bio-Rad MyiQ single color Real Time Detection System (Bio-Rad, Veenendaal, the Netherlands). Each 25 µL reaction contained 1× IQ SYBR Green Supermix (Bio-Rad, Veenendaal, the Netherlands), forward and reverse primers (0.8 µM each), and 1 µL of cDNA template. Reactions were incubated at 95°C for 3 min, followed by 40 cycles of [30 secs at 95°C, 30 secs at 64°C and 30 secs at 72°C] and a final extension step of 5 min at 72°C. cDNA template used for all reactions was normalized to the lowest amount detected across all samples. All reactions were run in duplicate, and both extraction negatives and no-template controls were included for each assay. Reaction duplicates that differed by more than 1 Cq value were repeated. Amplification efficiencies for all PCR assays were between 95% and 103%.

Quantification of *B. hyodysenteriae* from pure cultures used a genus-specific quantitative PCR protocol adapted from a previously described technique (Rubin et al. 2013).

1.7 *Metabolite extraction and quantification*

Prior to liquid chromatography-mass spectrometry (LC-MS) analysis, explants were homogenized using a liquid nitrogen cooled mini-mortar (Bel-Art Products). When the tissue was reduced to fine powder, metabolites were extracted by adding 300µl of methanol/acetonitrile/ ULC/MS grade water (Biosolve BV, Valkenswaard, The Netherlands) (2:2:1) lysis buffer and sonicating in a Bioruptor Plus (Diagenode, Liège, Belgium) at high power with 8 sonication cycles (15 sec. ON, 60 sec. OFF). After sonication, samples were shaken for 10 min at 4°C and centrifuged for 15 min at 14,000g and 4°C. Supernatants were collected and transferred into a separated vial and stored at −80 °C awaiting subsequent analysis. Liquid chromatography-mass spectrometry analysis was performed using an Exactive mass spectrometer (Thermo Scientific), coupled to a Dionex Ultimate 3000 auto sampler and pump (Thermo Scientific). The mass spectrometry operated in polarity-switching mode with spray voltages of 4.5 kV and -3.5 kV. Metabolites were separated using a Sequant ZIC-pHILIC column (2.1 x 150 mm, 5 mm, guard column 2.1 x 20 mm, 5 mm; Merck) using a linear gradient of acetonitrile and eluent A (20 mM

$(NH_4)_2CO_3$, 0.1% NH_4OH in ULC/MS grade water (Biosolve BV, Valkenswaard, The Netherlands) and a flow rate of 150 µl/min. Peak intensities were normalized on median ion count using IDEOM software (Creek et al. 2012). Metabolites were identified and quantified using LCquan software (Thermo Scientific) on the basis of exact mass within 5 ppm and further validated by concordance with retention times of commercially available standards.

1.8 *Statistical analysis*

Histopathology and gene expression data analyses were carried out using SPSS version 24 (SPSS Inc., Chicago, USA) and R Studio version 0.99.893 (RStudio Team 2015), respectively. Necrosis scores were compared using Kruskall-Wallis test adjusted by the Bonferroni correction for multiple tests followed by Dunn's *post-hoc* test. this data is presented as median ± absolute deviation. Explant mRNA levels were compared using the MCMC.qpcr (version 1.2.2) R package (Matz et al. 2013). This method applies generalized linear mixed model with Poisson-lognormal errors and a Bayesian Marco Chain Monte Carlo sampling scheme to infer fold changes in mRNA levels in response to fixed factors and random differences attributable to technical replicates. Gene expression data were graphed as mean fold-change (± standard error of the mean) using PBS samples as a reference group.

Metabolomics data were log-transformed, scaled by each variable standard deviation, and analyzed by multivariate PCA, orthogonal PLS-DA, and univariate ANOVA with pairwise comparisons and *post-hoc* correction for multiple hypothesis testing using Fisher's least significant difference method embedded in the MetaboAnalyst 3.0 suite (Xia et al. 2015). The latter was also used for integrated enrichment and pathway topography analysis, which employed the *Homo sapiens* library as a reference since a *Sus scrofa* library is not available.

Results and Discussion

1.9 *Increased epithelial necrosis and mucosal erosion*

Explants from B.hyo group received an average of 4.50×10^6 genome equivalents of *B. hyodysenteriae* upon inoculation. A total of 36 H&E stained explant sections were evaluated and a score difference between groups was observed (P=0.015). *Post-hoc* analysis revealed that *B. hyodysenteriae* infected group had greater scores than both PBS and LPS groups (P=0.037 and 0.04, respectively, Figure 1). All 0-hour samples received a score of 0.

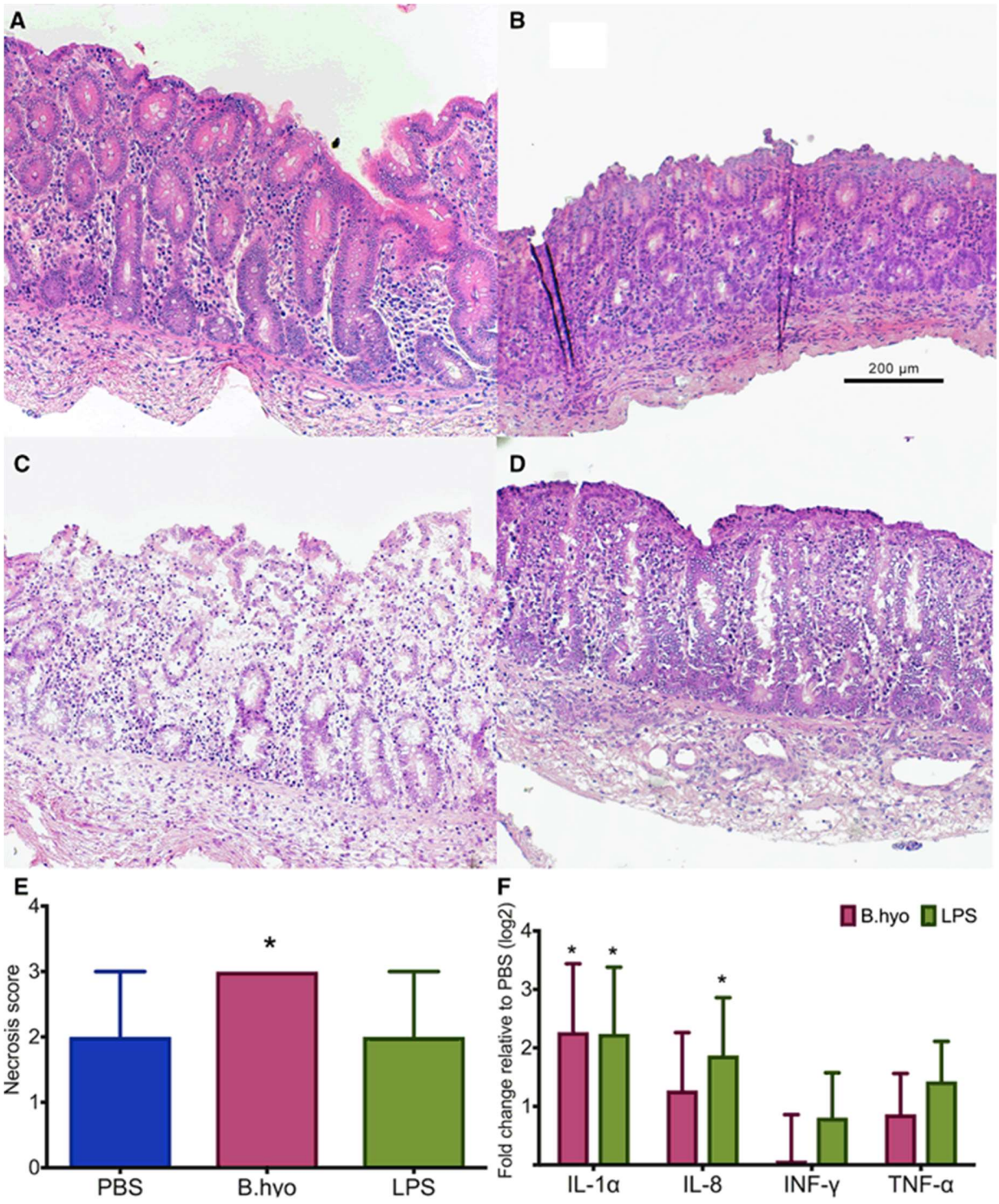

Fig. 1 Histopathology and gene expression data summaries. Explants were fixed in Carnoy's solution. a Typical section of control explant sampled at 0 h. b PBS-inoculated explant (score 1). c Representative B. hyo explant section (score 3), displaying significant epithelial necrosis and sloughing off epithelial cells. d LPS group explant section (score 2). eBars represent median (±interquartile range) epithelial necrosis scores for each inoculation group ($n = 12$ sections/group). Star denotes statistical difference between B. hyo and both other groups ($P < 0.05$). f Each bar shows mean fold-change (±standard error of the mean) in mRNA levels from a given group relative to the PBS group ($n = 6$/group). Star denotes significant mRNA levels difference between a given group and PBS ($P \leq 0.05$).

In this study, exposure of explants to live *B. hyodysenteriae* cells resulted in a greater number of necrotic cells than LPS or PBS. *In* vivo, the earliest lesion detectable in clinical swine dysentery cases is mucosal intercellular edema, described before the onset of diarrhea but days after pigs were inoculated with the spirochete (Albassam et al. 1985). Disease progression leads to epithelial necrosis and erosion of the mucosa, exudation of fluid and necrotic cells from the exposed lamina propria and engorged superficial vessels (Hughes et al. 1977). More recently, inoculation of different *Brachyspira* strains capable of inducing mucohaemorrhagic diarrhea in a pig model revealed neutrophilic inflammation, crypt elongation and mucosal ulceration (Wilberts et al. 2014). We observed similar lesions to what was reported *in vivo*, characterized by crypt cell necrosis and mucosal ulceration. In explants, the lack of physiological features such as peristalsis, for clearance of luminal mucus, and blood circulation, for metabolite removal and immune cell migration, may have prevented the reproduction of typical microscopical findings.

1.10 *Pro-inflammatory cytokine profile after spirochete inoculation*

Analysis of mRNA fold changes between groups revealed that both LPS and *B. hyodysenteriae* inoculated groups had increased expression of IL-1α mRNA (P=0.05) when compared to the PBS group. Concomitantly, LPS exposure led to increased levels of IL-8 and TNF-α mRNA (P=0.05 and P=0.03, respectively). No significant difference between groups was observed for INF, even though LPS and *B. hyodysenteriae* groups had increased mRNA levels when compared to the PBS groups (Figure 1). The GAPDH gene, which was used as a prior in the gene expression statistical model, had an average Cq difference between PBS and both other groups of equal or less than 1 Cq across all samples.

These results show a pro-inflammatory cytokine profile after explant exposure to *B. hyodysenteriae* that resembles the description from clinical cases of swine dysentery: increased IL-1β and TNF-α during peak clinical signs (Kruse et al. 2008). The inflammatory response characterized in this study is a combination of the response built by leukocytes present in the mucosa at the time of euthanasia, the epithelium and stroma cells, and it lacks the enhancement from blood stream migrating immune cells and other immune modulators.

1.11 *Increased citrulline concentration in B. hyodysenteriae explants*

Overall, 110 metabolites were identified from the 54 explants analyzed by LC-MS. PCA analysis revealed greater between-subject variation than between-group variation, leading to no clear group structure when plotting components. A supervised discriminant approach revealed three different clusters, one for each inoculum group. This group difference was further validated by a permutation test (Q^2=0.82, P^2=0.92, 1000 permutations, P<0.0001). Integrated enrichment and pathway topography analysis disclosed a set of metabolic pathways affected by explant exposure to *B. hyodysenteriae*, when compared to PBS inoculated explants (Supplementary Table 1). Among the most deeply impacted pathways are the metabolism of alanine, aspartate and glutamate, synthesis and degradation of ketone bodies, and pyruvate metabolism. Taken

individually, the concentration of 21 metabolites was significantly different across groups (Supplementary Table 2). Out of these, only 3 metabolites were found in greater amounts in the *Brachyspira* group than the others: L-Citrulline, Cytosine and D-Glucose 6-phosphate. This remarkable tissular accumulation of citrulline was unexpected.

Citrulline is a non-essential, non-protein forming amino acid that in higher mammals mainly participates in three metabolic pathways: arginine bio-synthesis, characterized by citrulline exchanges at whole body level; nitric oxide (NO) cycle, involved in local recycling of citrulline; and the complete urea cycle, which occurs in the liver. Under physiological conditions, most of the citrulline is found in the liver and the intestines (Curis et al. 2005). Enzymatic production of NO is catalyzed by nitric oxide synthase (NOS). Out of the three NOS isoforms described, only one is Ca^{2+}-independent and inducible by bacterial toxins and cytokines (iNOS), leading to endothelial hyperpermeability and inflammation (Witthoft et al. 1998; Chavez et al. 1999). Citrulline is the main by-product of enzymatic NO synthesis, which uses D or L-Arginine as substrate (Nagase et al. 1997; Singer et al. 1996). Interestingly, we found that L-Arginine, creatine (for which arginine is a precursor) and glutamic acid (which can fuel the urea cycle through the synthesis of aspartic acid) concentrations were significantly lower in *Brachyspira*-exposed explants than in LPS and PBS groups (Figure 2). NO exerts a range of functions in the gastrointestinal tract, acting as a pro-inflammatory stimulus, stimulating mucus secretion, opening chloride channels in the colon and promoting vasodilation (Brown et al. 1992; Tamai and Gaginella 1993; Fan et al. 1998). In addition, other authors have shown that citrulline alone induces endothelial relaxation as it enhances the production of NO, probably due to its recycling into arginine (Raghavan and Dikshit 2001). Particularly in the explant model described here, this mechanism would explain why *B. hyodysenteriae* exposed explants had a greater degree of necrosis than the LPS group, even though the TNF-α mRNA levels detected were lower. In light of the lack of evidence of toxin and tissue invasion damage by *B. hyodysenteriae,* it is hypothesized that an indirect mechanism leading to overproduction of NO may play a role in the pathogenesis of swine dysentery, especially in regards to tissue inflammation and luminal leakage of red blood cells. By focusing on the metabolic profile during infection, this study lacked further evidence of the hypothesis. The authors warrant further investigations to conclusively associate *B. hyodysenteriae* with the increased expression of iNOS and the production of NO in the swine colon.

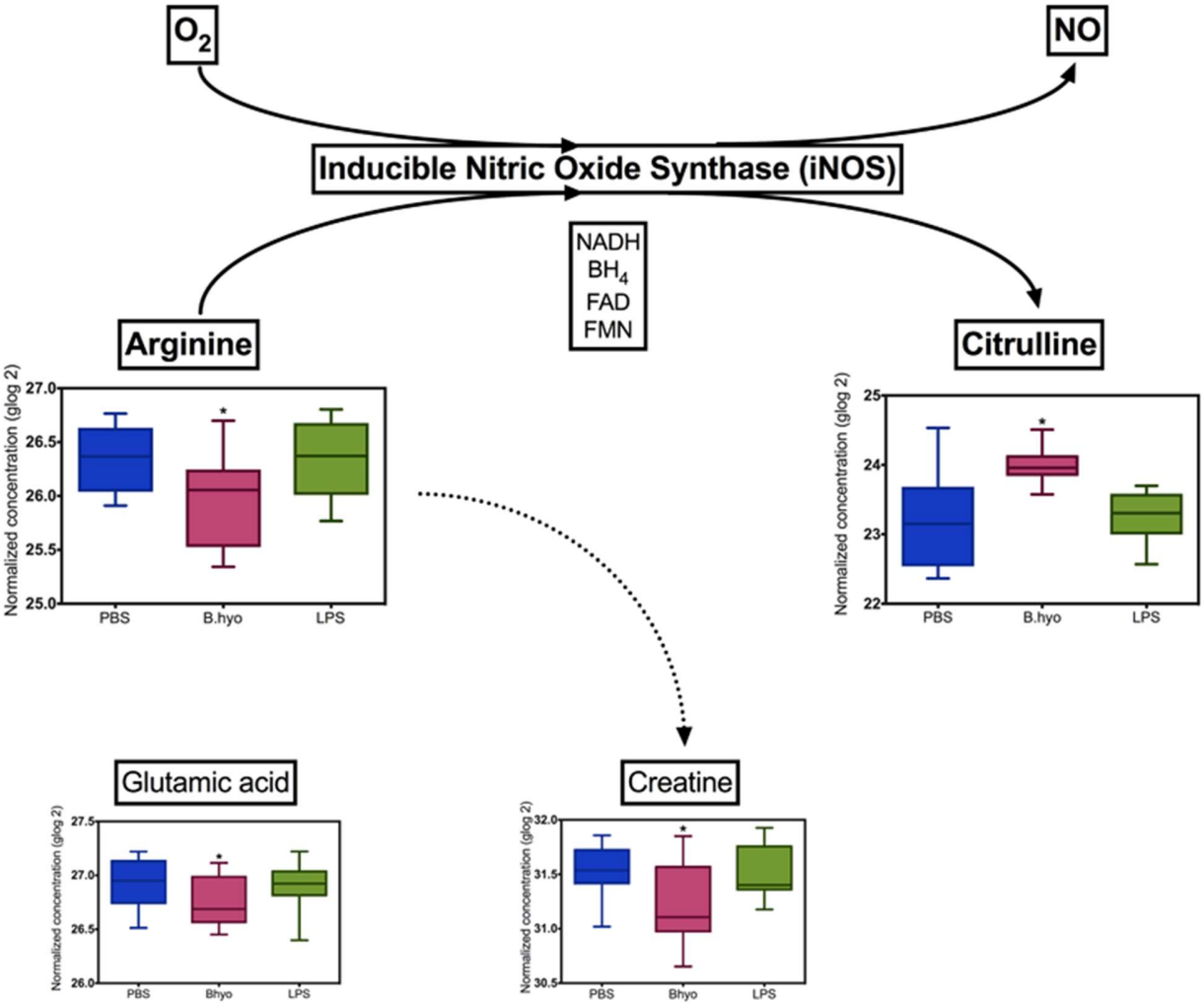

Fig. 2 Inducible nitric oxide synthase (iNOS) catalyzed reaction. Arginine and molecular oxygen are used as substrates by iNOS in a reaction that produces NO, leaving citrulline as a by-product. NADH, BH_4 (sapropterin), FAD and FMN (flavin mononucleotide) are co-factors. Glutamic acid is a precursor of arginine, whereas creatine is synthesized from arginine. *Dotted lines* are pathways not directly related to the synthesis of NO. Charts display transformed concentrations of metabolites obtained from explants exposed to a given inoculum for 8 h. *Bars* represent mean concentrations (5–95 percentile range) for each group (n = 18). *Star* denotes significant differences in the levels of metabolite detected between groups.

Conclusions

Herein, we have presented data demonstrating that *B. hyodysenteriae* induces a pro-inflammatory response in porcine colon explants, affecting different metabolic pathways that culminates with crypt epithelial necrosis. We also observed a tissular *B. hyodysenteriae*-induced accumulation of citrulline that may have resulted from the synthesis of NO, which in turn leads to vasodilation, mucus secretion, opening of chloride channels and inflammation. It is postulated that this mechanism may play a role in the pathophysiology of mucohaemorrhagic diarrhea *in vivo*. Further research to characterize this hypothesis is warranted, which will contribute to our ability to treat and prevent clinical disease and its consequent economic impact to producers.

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Supplementary Table 1 Integrated enrichment and pathway topography analysis results.

Compounds	Total Compounds in the pathway	Hits	Raw p	LOG(p)	Holm adjust	FDR	Impact
Alanine, aspartate and glutamate metabolism	24	10	0.0019067	6.2624	0.10296	0.018749	0.75404
Synthesis and degradation of ketone bodies	6	3	0.80738	0.21396	1	0.8213	0.7
Pyruvate metabolism	32	5	0.45821	0.78043	1	0.55172	0.56886
Cysteine and methionine metabolism	56	10	0.053073	2.9361	1	0.12043	0.54736
Arginine and proline metabolism	77	13	0.0014296	6.5504	0.080058	0.018749	0.46129
Pyrimidine metabolism	60	14	0.019649	3.9297	0.91812	0.082806	0.43845
Citrate cycle (TCA cycle)	20	8	0.033995	3.3815	1	0.098086	0.33318
Glycolysis or Gluconeogenesis	31	7	0.29362	1.2255	1	0.42253	0.29508
beta-Alanine metabolism	28	6	0.34127	1.0751	1	0.44745	0.26813
Fatty acid elongation in mitochondria	27	1	0.48352	0.72665	1	0.55937	0.26765
Purine metabolism	92	12	0.26582	1.325	1	0.39658	0.26548
Butanoate metabolism	40	8	0.0010913	6.8204	0.063295	0.018749	0.26263
Pantothenate and CoA biosynthesis	27	7	0.3122	1.1641	1	0.42962	0.253
Pentose phosphate pathway	32	6	0.33617	1.0901	1	0.44745	0.2475
Galactose metabolism	41	4	0.039593	3.2291	1	0.10156	0.22669
Aminoacyl-tRNA biosynthesis	75	18	0.057098	2.863	1	0.12095	0.22536
Biotin metabolism	11	2	0.31311	1.1612	1	0.42962	0.20325
Glycine, serine and threonine metabolism	48	10	0.028604	3.5542	1	0.098086	0.19432
Glycerolipid metabolism	32	2	0.17907	1.72	1	0.30186	0.18847
Fatty acid metabolism	50	2	0.35851	1.0258	1	0.45983	0.17559
Propanoate metabolism	35	6	0.015839	4.1453	0.77609	0.078189	0.16822
Lysine degradation	47	2	0.60553	0.50165	1	0.68705	0.14675
Riboflavin metabolism	21	1	0.83265	0.18315	1	0.83265	0.14504
Histidine metabolism	44	3	0.14156	1.955	1	0.24565	0.14039
D-Glutamine and D-glutamate metabolism	11	2	0.057401	2.8577	1	0.12095	0.13904
Thiamine metabolism	24	3	0.70785	0.34553	1	0.74577	0.12481
Vitamin B6 metabolism	32	3	0.0013545	6.6043	0.077208	0.018749	0.1229
Phenylalanine metabolism	45	5	0.0052397	5.2515	0.26723	0.034349	0.11906
Tryptophan metabolism	79	2	0.027679	3.5871	1	0.098086	0.10853
Methane metabolism	34	3	0.01001	4.6042	0.5005	0.059059	0.10367
Lysine biosynthesis	32	3	0.74138	0.29925	1	0.76739	0.09993
Valine, leucine and isoleucine degradation	40	5	0.26887	1.3135	1	0.39658	0.0713
Valine, leucine and isoleucine biosynthesis	27	5	0.2159	1.5329	1	0.34428	0.06148
Amino sugar and nucleotide sugar metabolism	88	7	0.0009611	6.9474	0.056705	0.018749	0.05792
Nicotinate and nicotinamide metabolism	44	8	0.037366	3.287	1	0.10021	0.05642
Taurine and hypotaurine metabolism	20	4	0.032694	3.4206	1	0.098086	0.05395
Tyrosine metabolism	76	5	0.0034892	5.6581	0.18493	0.029409	0.04724
Fructose and mannose metabolism	48	1	0.62377	0.47197	1	0.69439	0.04115
Glyoxylate and dicarboxylate metabolism	50	6	0.050386	2.988	1	0.11891	0.03375
Sulfur metabolism	18	2	0.034912	3.3549	1	0.098086	0.03307
Inositol phosphate metabolism	39	3	0.20728	1.5737	1	0.33971	0.02406
Glutathione metabolism	38	6	0.028608	3.5541	1	0.098086	0.02214
Fatty acid biosynthesis	49	1	0.48352	0.72665	1	0.55937	0.0218
Glycerophospholipid metabolism	39	2	0.65109	0.42911	1	0.71138	0.0212
Starch and sucrose metabolism	50	1	0.13931	1.9711	1	0.24565	0.01703
Ascorbate and aldarate metabolism	45	1	0.2454	1.4049	1	0.38102	0.01617
Nitrogen metabolism	39	10	0.12011	2.1193	1	0.22815	0.0083
Phenylalanine, tyrosine and tryptophan biosynthesis	27	5	0.083361	2.4846	1	0.16394	0.008
Selenoamino acid metabolism	22	1	0.0018255	6.3059	0.1004	0.018749	0
Sphingolipid metabolism	25	1	0.0051153	5.2755	0.266	0.034349	0
Porphyrin and chlorophyll metabolism	104	1	0.015903	4.1413	0.77609	0.078189	0
D-Arginine and D-ornithine metabolism	8	2	0.019534	3.9356	0.91812	0.082806	0
One carbon pool by folate	9	2	0.03487	3.3561	1	0.098086	0
Glycosylphosphatidylinositol(GPI)-anchor biosynthesis	14	1	0.042071	3.1684	1	0.10343	0
Cyanoamino acid metabolism	16	3	0.069911	2.6605	1	0.14223	0
Folate biosynthesis	42	1	0.12374	2.0896	1	0.22815	0
Terpenoid backbone biosynthesis	33	2	0.39957	0.91738	1	0.50158	0
Pentose and glucuronate interconversions	53	2	0.45333	0.79113	1	0.55172	0
Ubiquinone and other terpenoid-quinone biosynthesis	36	1	0.68587	0.37707	1	0.73575	0

Supplementary Table 2 ANOVA analysis of individual metabolite concentration differences across groups.

Compound	f.value	p.value	LOG10(p)	FDR	Fisher's LSD
Pyridoxine	100.48	2.04E-18	17.691	2.42E-16	Bhyo - LPS; PBS - Bhyo; PBS - LPS
Citrulline	16.966	2.25E-06	5.6482	0.00013375	Bhyo - LPS; Bhyo - PBS
Cytosine	15.029	7.39E-06	5.1313	0.00029316	Bhyo - LPS; Bhyo - PBS
D-Glucose 6-phosphate	11.981	5.43E-05	4.2654	0.0016145	Bhyo - LPS; PBS - LPS
N-Acetyl-D-Glucosamine 6-Phosphate	10.865	0.00011728	3.9308	0.0024429	LPS - Bhyo; PBS - Bhyo
N-acetylglucosamine/N-acetylmannosamine	10.796	0.00012317	3.9095	0.0024429	LPS - Bhyo; PBS - Bhyo
Succinic acid	10.083	0.00020422	3.6899	0.0034717	PBS - Bhyo; PBS - LPS
5-Methyltetrahydrofolic acid	8.638	0.00058776	3.2308	0.008743	LPS - Bhyo; LPS - PBS
Oxidized glutathione	8.3209	0.00074566	3.1275	0.0098593	LPS - Bhyo; PBS - Bhyo
Creatine	7.8897	0.0010343	2.9854	0.012308	LPS - Bhyo; PBS - Bhyo
L-Alanine	6.8593	0.0023004	2.6382	0.024886	PBS - Bhyo; PBS - LPS
L-Arginine	5.9513	0.0047532	2.323	0.04351	LPS - Bhyo; PBS - Bhyo
Guanosine monophosphate	5.4032	0.0074422	2.1283	0.06242	PBS - Bhyo
D-Glucose 6-phosphate	4.466	0.01632	1.7873	0.12138	Bhyo - LPS; Bhyo - PBS
L-Serine	4.3258	0.018394	1.7353	0.12876	PBS - Bhyo
N-Acetylglutamic acid	4.1465	0.021451	1.6686	0.13101	Bhyo - LPS; PBS - LPS
L-Dihydroorotic acid	4.1417	0.021539	1.6668	0.13101	PBS - LPS
L-Glutamic acid	4.1162	0.022018	1.6572	0.13101	LPS - Bhyo; PBS - Bhyo
D-Malic acid	4.0136	0.024056	1.6188	0.13632	PBS - Bhyo; PBS - LPS
Myo-Inositol	3.6454	0.033132	1.4798	0.17759	LPS - Bhyo
L-Acetylcarnitine	3.605	0.034324	1.4644	0.17759	LPS - Bhyo; PBS - Bhyo