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Non Ruminant Nutrition

Postbiotic effects of *Lactobacillus* fermentate on intestinal health, mucosa-associated microbiota, and growth efficiency of nursery pigs challenged with F18+ *Escherichia coli*

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Abstract

This study determined the supplemental effects of *Lactobacillus* fermentate (**LBF**, Adare Biome, France) on intestinal health and prevention of postweaning diarrhea caused by F18+ *Escherichia coli* in nursery pigs. Sixty-four weaned pigs (6.6 ± 0.7 kg body weight) were allotted in a randomized complete block design to four treatments: **NC**: no challenge/no supplement; **PC**: *E. coli* challenge/no supplement; **AGP**: *E. coli* challenge/bacitracin (30 g/t feed); and **PBT**: *E. coli* challenge/LBF (2 kg/t feed). Bacitracin methylene disalicylate (**BMD**) was used as a source of bacitracin. On day 7, challenged groups were orally inoculated with F18+ *E. coli* (2.4×10^{10} CFU), whereas NC received sterile saline solution. Growth performance was analyzed weekly, and pigs were euthanized at the end of 28 d feeding to analyze intestinal health. Data were analyzed using the Mixed procedure of SAS 9.4. During the post-challenge period, PC tended to decrease ($P = 0.067$) average daily gain (**ADG**) when compared with NC, whereas AGP increased ($P < 0.05$) when compared with PC; PBT tended to increase ($P = 0.081$) ADG when compared with PC. The PC increased fecal score ($P < 0.05$) during day 7 to 14 when compared with NC, whereas AGP decreased it ($P < 0.05$) during day 14 to 21 when compared with PC. The PC increased ($P < 0.05$) protein carbonyl, crypt cell proliferation, and the relative abundance of *Helicobacter rodentium* when compared with NC. However, AGP decreased ($P < 0.05$) crypt cell proliferation and *H. rodentium* and increased ($P < 0.05$) villus height, *Bifidobacterium boum, Pelomonas* spp., and *Microbacterium ginsengisoli* when compared with PC. The PBT reduced ($P < 0.05$) crypt cell proliferation and *H. rodentium* and increased ($P < 0.05$) *Lactobacillus salivarius* and *Propionibacterium acnes* when compared with PC. At the genus level, AGP and PBT increased ($P < 0.05$) the alpha diversity of jejunal mucosa-associated microbiota in pigs estimated with Chao1 richness estimator when compared with PC. Collectively, F18+ *E. coli* reduced growth performance by adversely affecting microbiota and intestinal health. The LBF and BMD improved growth performance, and it was related to the enhanced intestinal health and increased diversity and abundance of beneficial microbiota in pigs challenged with F18+ *E. coli*.

Lay Summary

Newly weaned pigs are susceptible to multiple stressors that may lead to postweaning diarrhea, thereby causing significant economic losses in the swine industry. Enterotoxigenic *Escherichia coli* strains are the major agents causing diarrhea in newly weaned pigs. Subtherapeutic antibiotics have been employed by producers around the world to mitigate this issue. However, the use of antibiotics as growth promoters has become a public health concern because of microbial resistance. This study used *Lactobacillus* fermentate (**LBF**) as a postbiotic to help maintain healthy microbiota on the intestinal mucosa and to prevent postweaning diarrhea caused by *E. coli* F18+. Therefore, the aim of this study was to evaluate the effects of dietary supplementation of LBF on intestinal microbiota, intestinal health, and prevention of postweaning diarrhea caused by a challenge with *E. coli* F18+ in newly weaned pigs. Our model confirmed that the E. coli F18+ reduced growth performance by causing diarrhea, disruption of the microbiota composition, and increased immune response and oxidative stress in the small intestine of newly weaned pigs. *Lactobacillus* fermentate improved growth performance, and it was related to enhanced intestinal health and increased microbiota diversity in *E. coli* F18+-challenged pigs.

Key words: *Escherichia coli*, intestinal health, *Lactobacillus*, newly weaned pigs, postbiotics

Abbreviations: ADFI, average daily feed intake; ADG, average daily gain; AGP, antibiotics as a growth promoter; BMD, bacitracin methylene disalicylate; BW, body weight; CFU, colony-forming unit; CP, crude protein; ELISA, enzyme-linked immunosorbent assay; ETEC, enterotoxigenic *Escherichia coli*; G:F, gain to feed ratio; IgA, immunoglobulin A; IgG, immunoglobulin G; IL-6, interleukin-6; IL-8, interleukin-8; LBF, *Lactobacillus* fermentate; MDA, malondialdehyde; OTU, operational taxonomic unit; PBS, phosphate-buffered saline solution; PBT, postbiotic; PC, protein carbonyl; PWD, postweaning diarrhea; RA, relative abundance; STa, heat-stable toxins A; STb, heat-stable toxins B; TNF-α, tumor necrosis factor-alpha; VH:CD, villus height to crypt depth ratio

Introduction

During the postweaning period, nursery pigs face survival challenges including environmental, psychological, and nutritional that can disrupt the intestinal functions and reduce the resistance to pathogenic microorganisms, such as enterotoxigenic *Escherichia coli* (**ETEC**) causing postweaning diarrhea (**PWD**), impaired intestinal health, and consequently reduced growth performance (Sun and Kim, 2017; Duarte and Kim,

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2021). F4 and F18 are two common fimbria adhesins causing PWD in swine. The fimbria is a virulence factor of ETEC that targets specific receptors on intestinal epithelial cells, enabling the bacteria to colonize the cell surface, followed by secretion of enterotoxins that suppress water absorption and induce water secretion in the small intestine, thereby causing diarrhea (Fairbrother et al., 2005; Nagy and Fekete, 2005). Intestinal health is an important factor to promote the growth of nursery pigs, especially during the weaning period (Willing et al., 2012; Kim and Duarte, 2021; Zheng et al., 2021).

Attempting to improve intestinal health, the addition of antibiotics as a growth promoter (**AGP**) aims to prevent and reduce the severity of PWD and increase the growth performance of pigs (Dibner and Richards, 2005). However, the indiscriminate use of antibiotics has also brought problems such as the enhancement of drug resistance, the residue of antibiotics in animal products, and the pollution of excreta to the environment (Castanon, 2007). It has been reported that there are global concerns about antibiotic resistance that are leading scientists and allied industries to develop alternatives for antibiotics to avoid drug resistance in both animals and humans (WHO, 2014).

Numerous strategies have been investigated to prevent or minimize the negative effect of ETEC invasion and promote the growth of pigs (Liu et al., 2010; Kim et al., 2019; Duarte et al., 2020). Studies have demonstrated that probiotics have beneficial effects to promote intestinal health by enhancing intestinal immune response and preventing pathogen proliferation (Siggers et al., 2008; Videlock and Cremonini, 2012; He et al., 2020a; Sun et al., 2021). Compared with probiotics, postbiotics consist of non-living microorganisms or their components, and in some cases, the metabolites produced by the microorganisms and microorganism cell debris (Salminen et al., 2021); therefore, it shares similar mechanisms as probiotics as well as the beneficial effects to the swine intestine (Taverniti and Guglielmetti, 2011; Arimori et al., 2012; Sun et al., 2021). *Lactobacillus* is a Gram-positive bacterium that has been used as probiotics to enhance intestinal health by reducing inflammation, crypt cell proliferation, and diarrhea symptoms in nursery pigs (Konstantinov et al., 2008; Zhang et al., 2010; Yi et al., 2018). Studies have shown that the use of *Lactobacillus* as a postbiotic had beneficial health effects in humans and mice (Murosaki et al., 1998; Canducci et al., 2000). However, the efficiency of postbiotics made from *Lactobacillus* in a swine challenge model is still under investigation.

A postbiotic consisting of heat-inactivated *Limosilactobacillus fermentum* and *Lactobacillus delbrueckii* dried with their spent culture medium (*Lactobacillus* fermentate, **LBF**) has been shown to reduce diarrhea in humans and to reduce mucosal adherence and virulence of pathogens in vitro and in vivo (Liévin-Le Moal, 2016). Moreover, LBF modulates the intestinal microbiome of mice in vivo (Warda et al., 2019, 2020) and of humans in vitro (Warda et al., 2021).

Therefore, it was hypothesized that LBF, as a postbiotic, could enhance intestinal health and modulate the microbiota in the intestine to promote the growth performance of weaning pigs challenged with F18$^+$ *E. coli*. To test the hypothesis, this study was proposed to evaluate the effects of dietary supplementation of LBF as postbiotics on intestinal health and prevention of postweaning diarrhea caused by F18$^+$ *E. coli* in newly weaned pigs.

Materials and Methods

The experimental protocol was approved by the Institutional Animal Care and Use Committee of North Carolina State University (Raleigh, NC, USA).

Animals, design, diets, and inoculation

Sixty-four newly weaned pigs at 21 d of age (32 barrows and 32 gilts) with initial body weight (**BW**) of 6.55 ± 0.70 kg were allotted to four dietary treatments based on a randomized complete block design with initial BW and sex as blocks. Pigs were housed individually in a pen and had ad libitum access to feeds and water. Sixteen pens were assigned to four BW blocks and two sex blocks to each of four treatments: **NC**: no challenge/no supplement; **PC**: *E. coli* challenge/no supplement; **AGP**: *E. coli* challenge/bacitracin (30 g/t feed); and **PBT**: *E. coli* challenge/LBF (2 kg/t feed). Bacitracin methylene disalicylate (**BMD**) was used as a source of bacitracin. The LBF contained 6×10^{10}/g of powder of heat-inactivated *Lactobacillus* (*L. fermentum* and *L. delbrueckii*) as well as components of the fermented culture medium, including peptides, amino acids, carbohydrates, and minerals (LBiotix, Adare Biome, Houdan, France).

Pigs were fed the assigned experimental diets for 28 d based on 1 phase. The feeds met the nutrient requirements of NRC (2012) (Table 1). All pigs were fed the experimental diets for 7 d (pre-challenge period) until F18$^+$ *E. coli* were orally inoculated to 48 pigs (challenged groups) in PC, AGP, and PBT on day 7 (1.2×10^{10} CFU of strain 2144 (O147: non-motile) and on day 8 (1.2×10^{10} CFU of strain S1191 (O139). Pigs in NC (unchallenged group) received a sterile saline solution. The cultures of the F18$^+$ *E. coli* strains 2144 (O147: non-motile) and S1191 (O139), producing heat-stable toxins A (**STa**) and heat-stable toxins B (**STb**), were prepared following our standard protocol as previously reported (Cutler et al., 2007; Duarte et al., 2020). Briefly, a single colony of each strain was collected and grown for 24 h in Luria Broth medium at 37 °C with shaking at 150 rpm. Both cultures were serial diluted and plated to count the CFU/mL of culture. The cultures were then centrifuged at 4,000 × *g* for 10 min at 4 °C. The pellets were suspended in 5% nonfat dry milk and 20% dextrose. Each challenge dose consisted of 1.2×10^{10} CFU/mL of strain 2144 (O147: non-motile) for day 7 and 1.2×10^{10} CFU of strain S1191 (O139) for day 8. Sows and piglets used in this study were not selected for F18$^+$ *E. coli*, and they were not vaccinated against F18$^+$ *E. coli*.

Growth performance and fecal score

Growth performance was measured by obtaining average daily gain (**ADG**), average daily feed intake (**ADFI**), and gain to feed ratio (**G:F**) on days 0, 7, 14, 21, and 28. Health status was measured by obtaining fecal scores during the entire study. The daily records of fecal score from each pig were averaged for each week (days 0 to 7, 7 to 14, 14 to 21, and21 to 28) prior to the statistical analysis. The fecal score scale was: 1) very hard and dry stool, 2) firm stool, 3) normal stool, 4) loose stool, and 5) watery stool with no shape as previously reported by Duarte et al. (2020).

Sample collection and processing

After 28 d feeding, 12 pigs per treatment, a total of 48, were selected based on the initial BW blocks, excluding sampling the heaviest and the lightest pigs within sex. The 48 selected

Table 1. Composition of basal diet, as-is basis

Item	Basal diet
Feedstuff, %	
Corn, yellow dent	54.4
Soybean meal, 48% crude protein (CP)	23.5
Whey permeate, 80% lactose	10.0
Poultry meal	4.0
Blood plasma	3.0
Poultry fat	2.0
L-Lys HCl	0.47
L-Met	0.18
L-Thr	0.13
Dicalcium phosphate	0.85
Limestone	0.85
Vitamin premix[1]	0.03
Mineral premix[2]	0.15
Salt	0.22
Additive[3]	0.22
Total	100.0
Calculated composition	
ME, Mcal/kg	3.40
CP, %	21.55
SID[4] Lys, %	1.35
SID Met + Cys, %	0.74
SID Trp, %	0.22
SID Thr, %	0.79
Ca, %	0.80
STTD[5] P, %	0.40
Analyzed composition	
Dry matter, %	88.66
CP, %	20.75
Total P, %	0.65
Ca, %	0.80

[1]The vitamin premix provided per kilogram of complete diet: 6,614 IU of vitamin A as vitamin A acetate, 992 IU of vitamin D3, 19.8 IU of vitamin E, 2.64 mg of vitamin K as menadione sodium bisulfate, 0.03 mg of vitamin B12, 4.63 mg of riboflavin, 18.52 mg of D-pantothenic acid as calcium pantothenate, 24.96 mg of niacin, and 0.07 mg of biotin. CJ Bio (Fort Dodge, IA) provided the supplemental amino acids.
[2]The trace mineral premix provided per kilogram of complete diet: 33 mg of Mn as manganous oxide, 110 mg of Fe as ferrous sulfate, 110 mg of Zn as zinc sulfate, 16.5 mg of Cu as copper sulfate, 0.30 mg of I as ethylenediamine dihydroiodide, and 0.30 mg of Se as sodium selenite.
[3]Corn + bacitracin methylene disalicylate (bacitracin: 30 g/t feed), or corn + *Lactobacillus* Fermentate (LBF, LBiotix, Adare Biome, Houdan, France, 2 kg/t feed).
[4]SID, standardized ileal digestibility.
[5]STTD P, standardized total tract digestible phosphorus.

pigs were euthanized by exsanguination after penetration of a captive bolt to the head to remove the gastrointestinal tract for sampling. Tissue from mid-jejunum (3 m after the pyloric duodenal junction) was collected (approximately 5 cm), rinsed with 0.9% saline solution, and placed in 50 mL tubes with 10% buffered formaldehyde solution at room temperature for further histological analysis.

Another mid-jejunum section (15 cm) was longitudinally opened and scraped by microscopy glass slides to collect mucosa. For each selected pig, two tubes of mucosa samples were thus collected and immediately placed in liquid nitrogen for snap-freezing. Samples were then stored at −80 °C. Jejunal mucosa samples (1 g) were suspended in 1 mL of phosphate-buffered saline (**PBS**) and homogenized on ice using a tissue homogenizer. After centrifugation at 13,000 × *g* for 15 min, the supernatant was collected and divided into four sets of 200 µL each and stored at −80 °C for further analysis.

Inflammatory and oxidative stress parameters

The homogenized protein samples were used to measure the concentration of total protein, tumor necrosis factor-alpha (**TNF-α**), interleukin-6 (**IL-6**), interleukin-8 (**IL-8**), immunoglobulin G (**IgG**), immunoglobulin A (**IgA**), protein carbonyl, and malondialdehyde (**MDA**) in the mucosa of the jejunum. Total protein concentration was measured by using The Pierce Bicinchoninic Acid Protein Assay kit (23227, ThermoFisher Scientific, Inc., Wilmington, DE, USA). Samples were diluted (1:50) in PBS to meet the working range of 20 to 2,000 µL. After incubation, the absorbance was measured at 562 nm following Duarte et al. (2020), and the standard curve was fitted in polynomial with fourth order. The concentration of total protein was used to normalize the concentration of protein carbonyl, MDA, TNF-α, IL-6, IL-8, IgG, and IgA.

The concentration of TNF-α was measured using the porcine TNF-α enzyme-linked immunosorbent assay (**ELISA**) Kit (PTA00, R&D Systems, Inc. Minneapolis, MN) with the working range of 23 to 1,500 pg/mL, and the absorbance was measured at 450 and 540 nm following Jang and Kim (2019). The concentration of IL-6 in the mucosa of the jejunum was measured using the porcine ELISA Kit (P6000B, R&D Systems) with the working range of 18 to 1,200 pg/mL, and the absorbance measured at 450 and 540 nm. The concentration of IL-8 in the mucosa of the jejunum was measured using the porcine ELISA Kit (P8000, R&D Systems), with the working range of 62.5 to 4,000 pg/mL, and the absorbance measured at 450 and 540 nm.

Protein carbonyl concentration in the mucosa of the jejunum was measured using the OxiSelect Protein carbonyl ELISA Kit (Cell Biolabs, Inc, San Diego, CA). Before analysis, samples were diluted to reach the protein concentration of 10 µg/mL to fit in the working range of 0.0 to 7.5 nmol/mg. The absorbance was measured at 450 nm following Duarte et al. (2021). The concentration of MDA in the mucosa of the jejunum was measured using the OxiSelect TBARS Assay Kit (Cell Biolabs, Inc), with the working range at 0 to 125 µM, and the absorbance measured at 532 nm.

The concentration of IgG in the mucosa of the jejunum was measured using the Pig IgG ELISA Quantitation Set (E100-104, Bethyl Laboratories Inc, Montgomery, TX). Before analysis, samples were diluted (1:1,200) in PBS to fit in the working range of 8 to 500 ng/mL, and the absorbance was measured at 450 nm. The concentration of IgA in the mucosa of the jejunum was measured using the Pig IgA ELISA Quantitation Set (E100-102, Bethyl Laboratories Inc). Before analysis, samples were diluted (1:1,200) in PBS to fit in the working range of 16 to 1,000 ng/mL, and the absorbance was measured at 450 nm.

Intestinal morphology and crypt cell proliferation

After 48 h in 10% buffered formaldehyde solution, two sections of jejunum per pig were transversely cut, placed into a cassette in 70% ethanol, and sent to the North Carolina State University Histology Laboratory (College of Veterinary Medicine, Raleigh, NC) for Ki67$^+$ staining using

their internal standard protocol as previously described by Jang et al. (2020). The villus height (**VH**), villus width, and crypt depth (**CD**) were measured using a microscope Olympus CX31 with Infinity 2-2 digital CCD camera. Pictures of well-shaped villi and crypts in 40× magnification were taken for measuring the length of villi and crypts. The VH was measured from the top of the selected villi to the villus–crypt junction, the villus width was measured from the middle part of the villi, and the CD was measured from the villus-crypt junction to the bottom of the crypt. The villus height to crypt depth ratio (**VH:CD**) was calculated after measurement.

Pictures of crypts in 100× magnification were taken for $Ki67^+$ cell measurement. The ImageJS software was used for calculating the percentage of dyed $Ki67^+$ cells in the total cells in the crypt. The percentage of $Ki67^+$ cells was used as an indicator of the enterocyte proliferation in the crypt (Jang et al., 2021). All analyses of the histology were executed by the same person, and the average of 10 measurements for each sample was calculated and reported as one number per sample.

Relative abundance and diversity of the mucosa-associated microbiota in the jejunum

Jejunal mucosa samples were used for DNA extraction for microbiome analysis using the QIAamp Fast DNA Stool Mini Kit (51604) following the instructions of the manufacturer. The extracted DNA was sent to Mako Medical Laboratories for microbial sequencing. In the lab, the template was prepared by Chef Instrument, and the Ion S5 system was used to perform sequencing (ThermoFisher Scientific). Using the Ion 16S Metagenomic Kit (ThermoFisher Scientific), the regions V2 to V9 of the 16S rRNA gene were amplified and then sequenced (Hypervariable regions) to the raw unaligned sequence data file by Torrent Suite Software (version 5.2.2; ThermoFisher Scientific) to produce.bam files for further analysis. The taxonomy was assigned against specific primers for microbiota, the GreenGenes (anybody) and MicroSeq (experts) databases. Alpha diversity rare fraction plot generation and the operational taxonomic unit (**OTU**) table generation were performed by the Ion Reporter Software Suite (version 5.2.2) of bioinformatics analysis tools (Thermo Fisher Scientific Inc.) with 98% similarity. The Ion Reporter's Metagenomics 16S workflow powered by Qiime (version w1.1) was used to analyze the samples. The depth of sequencing coverage was >5,000 × sample. OTU data were transformed to relative abundance (**RA**) for further statistical analysis, and the OTU data with less than 0.05% abundance within each level were combined as "other." At the species level, the most abundant bacteria were separated as Gram–, Gram+, aerobic, anaerobic, and facultative anaerobic.

Statistical analysis

Data were analyzed using the Mixed procedure of SAS Software. Treatments and sex were the fixed effects, whereas initial BW blocks were considered as random effects. The LSMEANS statement was used to calculate the least squared mean values as preplanned. Orthogonal contrasts were made between NC vs. PC, PC vs. AGP, and PC vs. PBT. To account for the variation in the BW at day 7 (**BW7**) on the response to $F18^+$ *E. coli* challenge, the growth performance data were analyzed considering the BW7 as a covariate (including BW, ADG, ADFI, and G:F). Statistical differences were considered significant with $P < 0.05$ and tendency with $0.05 < P < 0.10$.

Results

Growth performance

The growth performance of pigs was not affected by the treatments during the pre-challenge period, day 0 to 7 (Table 2). On days 14 and 21, the BW of pigs on NC was greater ($P < 0.05$) than pigs on PC. On day 28, the BW of pigs on NC tended to be greater ($P = 0.068$) than pigs on PC, whereas pigs on AGP tended to have greater BW on day 14 ($P = 0.061$) and had greater ($P < 0.05$) BW on days 21 and 28 when compared with pigs on PC. Pigs on PBT tended to have greater BW on days 14 ($P = 0.067$) and 28 ($P = 0.087$) and had greater ($P < 0.05$) BW on day 21 when compared with pigs on PC.

From day 7 to 14, the ADG of pigs on NC tended to be greater ($P < 0.05$) than pigs on PC, whereas pigs on AGP had greater ($P < 0.05$) ADG than pigs on PC and pigs on PBT tended to have greater ($P = 0.079$) ADG than pigs on PC. From day 21 to 28, the ADG of pigs on NC tended to be greater ($P = 0.077$) than pigs on PC, whereas pigs on AGP had greater ($P < 0.05$) ADG than pigs on PC. From day 7 to 21, the ADG of pigs on NC tended to be higher ($P < 0.05$) than pigs on PC; however, pigs on AGP had greater ($P < 0.05$) ADG than pigs on PC. Pigs on PBT tended to have greater ($P = 0.081$) ADG than pigs on PC. From day 7 to 28, the ADG of pigs on NC tended to be higher ($P = 0.080$) than pigs on PC, whereas pigs on AGP had greater ($P < 0.05$) ADG than pigs on PC. In the overall period, the ADG of pigs on NC tended to be higher ($P = 0.080$) than pigs on PC, whereas pigs on AGP had greater ($P < 0.05$) ADG than pigs on PC. However, the ADG of pigs on PBT did not differ from pigs on PC.

From day 7 to 14, the ADFI of pigs on NC was greater ($P < 0.05$) than pigs on PC, whereas pigs on AGP tended to have greater ($P = 0.061$) ADFI than pigs on PC. The ADFI of pigs on PBT did not differ from pigs on PC. From day 21 to 28, pigs on AGP had greater ($P < 0.05$) ADFI than pigs on PC. From day 7 to 21, the ADFI of pigs on NC was higher ($P < 0.05$) than pigs on PC; however, pigs on AGP and PBT had greater ($P < 0.05$) ADFI than pigs on PC. From day 7 to 28, the ADFI of pigs on NC tended to be higher ($P = 0.077$) than pigs on PC; however, pigs on AGP had greater ($P < 0.05$) ADFI than pigs on PC. Pigs on PBT tended to have greater ($P = 0.078$) ADFI than pigs on PC. In the overall period, the ADFI of pigs on NC tended to be higher ($P = 0.067$) than pigs on PC, whereas pigs on AGP had greater ($P < 0.05$) ADFI than pigs on PC. Pigs on PBT tended to have greater ($P = 0.074$) ADFI than pigs on PC. From day 7 to 14, the G:F of pigs on NC and PBT did not differ from pigs on PC, whereas pigs on AGP tended to have greater ($P = 0.093$) G:F than pigs on PC. The G:F of pigs on NC, AGP, and PBT did not differ from pigs on PC during other experimental periods.

Fecal score

There was no difference in the fecal score from day 0 to 7 (Table 3), confirming that pigs assigned to negative (unchallenged) and positive control (challenged) had similar health status on the day of the challenge. From day 7 to 14, the fecal score of pigs on NC was lower ($P < 0.05$) than pigs on PC; however, the fecal scores of pigs on AGP and PBT were not different from pigs on PC. From day 14 to 21, the fecal score of pigs on PC tended to be greater ($P = 0.082$) than pigs on

Table 2. Growth performance of pigs challenged with F18+ *Escherichia coli* and fed diets supplemented with antibiotic (bacitracin methylene disalicylate) or *Lactobacillus* fermentate (LBF)

Item[2]	Treatment[1]				SEM	*P*-value		
	NC	PC	AGP	PBT		NC vs. PC	PC vs. AGP	PC vs. PBT
BW, kg								
Initial	6.6	6.6	6.6	6.6	0.7	0.981	0.981	0.990
Day 7	7.2	6.9	7.1	7.1	1.0	0.476	0.677	0.640
Day 14	9.1	8.3	9.2	9.0	0.3	0.036	0.020	0.067
Day 21[3]	11.6	10.3	12.1	11.7	0.4	0.048	0.007	0.036
Day 28[4]	14.9	13.1	15.7	14.9	0.7	0.068	0.012	0.087
ADG, g/d								
Day 0 to 7	98	54	80	83	57	0.457	0.660	0.627
Day 7 to 14	288	169	293	269	53	0.037	0.030	0.079
Day 14 to 21	319	310	390	395	46	0.892	0.217	0.201
Day 21 to 28	476	356	514	442	46	0.077	0.032	0.201
Day 7 to 21	321	232	354	328	31	0.048	0.007	0.036
Day 7 to 28	373	284	408	370	35	0.067	0.012	0.081
Overall	300	234	326	295	42	0.080	0.015	0.107
ADFI, g/d								
Day 0 to 7	214	158	161	190	50	0.143	0.928	0.400
Day 7 to 14	393	310	385	368	48	0.039	0.061	0.148
Day 14 to 21	509	437	545	572	58	0.379	0.184	0.104
Day 21 to 28	663	568	731	676	55	0.226	0.041	0.176
Day 7 to 21	469	365	472	461	39	0.028	0.022	0.042
Day 7 to 28	535	438	559	535	39	0.077	0.039	0.078
Overall	451	375	460	450	39	0.067	0.041	0.074
G:F								
Day 0 to 7	0.42	0.34	0.49	0.43	0.24	0.748	0.552	0.723
Day 7 to 14	0.73	0.58	0.74	0.71	0.06	0.118	0.093	0.169
Day 14 to 21	0.60	0.63	0.69	0.71	0.06	0.740	0.480	0.348
Day 21 to 28	0.75	0.66	0.68	0.64	0.05	0.137	0.711	0.798
Day 7 to 21	0.65	0.63	0.73	0.64	0.06	0.828	0.277	0.874
Day 7 to 28	0.71	0.61	0.72	0.66	0.05	0.186	0.130	0.533
Overall	0.64	0.63	0.69	0.69	0.04	0.741	0.257	0.254

[1]Treatments: NC, no-challenge/no-supplement; PC, *E. coli* challenge/no-supplement; AGP, *E. coli* challenge/bacitracin (30 g/t feed); and PBT, *E. coli* challenge/LBF (2 kg/t feed; LBiotix, Adare Biome, Houdan, France).
[2]ADFI, average daily feed intake; ADG, average daily gain; BW, body weight; G:F, gain to feed ratio.
[3]Three pigs died (two from PC only with symptoms of diarrhea—score 5, and one from PBT with mild diarrhea—score 4 and respiratory problem).
[4]Four pigs died (two from PC and two from PBT only with symptoms of diarrhea—score 5).

NC, whereas pigs on AGP had a lower ($P < 0.05$) fecal score than pigs on PC. However, the fecal score of pigs on PBT was no different from pigs on PC. From day 21 to 28, the fecal score of pigs on NC, AGP, and PBT did not differ from pigs on PC.

Inflammatory and oxidative stress parameters

The concentration of MDA, TNF-α, IL-6, and IgG in jejunal mucosa of pigs on NC, AGP, and PBT was not different from pigs on PC (Table 4). The concentration of protein carbonyl in jejunal mucosa of pigs on NC was lower ($P < 0.05$) than pigs on PC, whereas the concentration of protein carbonyl in jejunal mucosa of pigs on AGP and PBT was not different from pigs on PC. The concentration of IgA in jejunal mucosa of pigs on NC tended to be lower ($P = 0.052$) than pigs on PC, whereas pigs on PC tended to have greater ($P = 0.052$) concentration of IgA than pigs on AGP and had no difference from pigs on PBT. The concentration of IL-8 in jejunal mucosa of pigs on NC tends to be lower ($P = 0.073$) than pigs on PC, whereas pigs on PC tended to have greater ($P = 0.078$) concentration of IL-8 than pigs on PBT and had no difference from pigs on AGP.

Intestinal morphology and enterocyte proliferation in crypt

The VH on the jejunum of pigs on NC tended to be higher ($P = 0.068$) than pigs on PC (Table 5), whereas pigs on PC had lower ($P < 0.05$) VH than pigs on AGP but was not different from pigs on PBT. The villus width and CD of pigs on NC, AGP, and PBT were not different from pigs on PC. The VH:CD ratio of pigs on NC was greater ($P < 0.05$) than pigs on PC, whereas pigs on PC had a lower ($P < 0.05$) VH:CD ratio than pigs on AGP but had no difference from pigs on PBT. The enterocyte proliferation in the jejunal crypt of pigs

Table 3. Fecal score of pigs challenged with F18-positive *Escherichia coli* and fed diets supplemented with antibiotic (bacitracin methylene disalicylate) or *Lactobacillus* fermentate (LBF)

Item	Treatment[1]				SEM	*P*-value		
	NC	PC	AGP	PBT		NC vs. PC	PC vs. AGP	PC vs. PBT
Pre-challenge								
Day 0 to 7	3.50	3.38	3.50	3.69	0.21	0.650	0.650	0.259
Post-challenge								
Day 7 to 14	3.41	3.93	3.67	3.72	0.13	0.004	0.140	0.236
Day 14 to 21	3.58	3.93	3.48	3.90	0.15	0.082	0.025	0.854
Day 21 to 28	3.41	3.50	3.28	3.64	0.16	0.572	0.176	0.376

[1]Treatments: NC, no-challenge/no-supplement; PC, *E. coli* challenge/no-supplement; AGP, *E. coli* challenge/bacitracin (30 g/t feed); and PBT, *E. coli* challenge/LBF (2 kg/t feed; LBiotix, Adare Biome, Houdan, France).

Table 4. Oxidative stress and immune status of pigs challenged with F18-positive *Escherichia coli* and fed diets supplemented with antibiotic (bacitracin methylene disalicylate) or *Lactobacillus* fermentate (LBF)

Item[2]	Treatment[1]				SEM	*P*-value		
	NC	PC	AGP	PBT		NC vs. PC	PC vs. AGP	PC vs. PBT
MDA, nmol/mg of protein	0.74	0.73	1.11	1.00	0.16	0.968	0.101	0.244
Protein carbonyl, nmol/mg protein	0.76	1.12	1.20	1.11	0.10	0.016	0.620	0.906
IgG, μg/mg of protein	2.47	3.08	2.41	3.61	0.41	0.302	0.258	0.333
IgA, μg/mg of protein	2.88	4.10	2.69	3.19	0.48	0.080	0.052	0.184
TNF-α, pg/mg of protein	0.88	0.99	0.72	0.67	0.21	0.714	0.372	0.287
IL-6, pg/mg of protein	0.75	0.70	0.65	0.62	0.13	0.818	0.783	0.653
IL-8, pg/mg of protein	0.42	0.77	0.48	0.42	0.14	0.073	0.142	0.078

[1]Treatments: NC, no-challenge/no-supplement; PC, *E. coli* challenge/no-supplement; AGP, *E. coli* challenge/bacitracin (30 g/t feed); and PBT, *E. coli* challenge/LBF (2 kg/t feed; LBiotix, Adare Biome, Houdan, France).
[2]IgG, immunoglobulin G; IgA, immunoglobulin A; IL-6, interleukin-6; IL-8, interleukin-8; MDA, malondialdehyde; TNF-α, tumor necrosis factor-alpha

on NC was lower ($P < 0.05$) than pigs in PC, whereas pigs in PC had greater ($P < 0.05$) enterocyte proliferation in the jejunal crypt than pigs in AGP and PBT.

RA and diversity of the mucosa-associated microbiota in the jejunum

At the phylum level (Table 6), there were no differences in the RA of jejunal mucosa-associated microbiota between NC and PC. The RA of Actinobacteria in pigs on AGP and PBT was greater ($P < 0.05$) than in pigs on PC. The RA of Cyanobacteria in pigs on AGP was greater ($P < 0.05$) than in pigs on PC. The RA of Spirochaetes in pigs on AGP was lower ($P < 0.05$) than in pigs on PC and tended to be lower ($P = 0.051$) in pigs on PBT than in pigs on PC.

At the family level (Table 7), the RA of Campylobacteraceae in pigs on NC tended to be greater ($P = 0.094$) than in pigs on PC. The RA of Bifidobacteriaceae, Burkholderiaceae, Comamonadaceae, Enterobacteriaceae, Microbacteriaceae, Moraxellaceae, Pseudomonadaceae, and Sphingomonadaceae of pigs on AGP was greater ($P < 0.05$) than PC, whereas the RA of Brachyspiraceae of pigs on AGP tends to be lower ($P = 0.056$) than pigs on PC. The RA of Veillonellaceae in pigs on AGP tended to be greater ($P = 0.052$) than in pigs on PC. Also, the RA of Propionibacteriaceae in pigs on PBT was greater ($P < 0.05$) than in pigs on PC, and the RA of Brachyspiraceae of pigs on PBT tended to be lower ($P = 0.062$) than in pigs on PC.

At the genus level (Table 8), the RA of *Selenomonas* in pigs on NC was lower ($P < 0.05$) than in pigs on PC. The RA of *Acinetobacter, Bifidobacterium, Megasphaera, Microbacterium, Mitsuokella, Pelomonas, and Pseudomonas* of pigs on AGP was greater ($P < 0.05$) than pigs on PC, whereas the RA of *Clostridium* and *Propionibacterium* of pigs on PBT was greater ($P < 0.05$) than pigs on PC.

At the species level (Table 9), the RA of *Helicobacter rodentium* in pigs on NC was lower ($P < 0.05$) than in pigs on PC. The RA of *Bifidobacterium boum, Microbacterium ginsengisoli, Pelomonas puraquae,* and *Pelomonas aquatica* in pigs on AGP was greater ($P < 0.05$) than in pigs on PC. The RA of *Helicobacter mastomyrinus* and *H. rodentium* of pigs on AGP was lower ($P < 0.05$) than in pigs on PC. The RA of *Lactobacillus salivarius* and *Propionibacterium acnes* in pigs on PBT was greater ($P < 0.05$) than in pigs on PC. The RA of *H. rodentium* in pigs on PBT was lower than in pigs on PC. The RA of Gram-negative bacteria in the jejunal mucosa of pigs on AGP was lower ($P < 0.05$) than in pigs on PC. The RA of Gram-positive bacteria in the jejunal mucosa of pigs on PBT was greater ($P < 0.05$) than in pigs on PC. The RA of aerobic and anaerobic bacteria in the jejunal mucosa of pigs on NC, AGP, and PBT was not different from pigs on PC, whereas the RA of facultative anaerobic bacteria in the jejunal mucosa of pigs on PBT was greater ($P < 0.05$) than in pigs on PC.

There were no differences between NC and PC on the alpha diversity of jejunal mucosa-associated microbiota estimated

Table 5. Jejunal morphology and enterocyte proliferation in the crypt of pigs challenged with F18-positive *Escherichia coli* and fed diets supplemented with antibiotic (bacitracin methylene disalicylate) or *Lactobacillus* fermentate (LBF)

Item	Treatment[1]				SEM	*P*-value		
	NC	PC	AGP	PBT		NC vs. PC	PC vs. AGP	PC vs. PBT
Villus height, μm	369	309	377	362	22.64	0.068	0.038	0.102
Villus width, μm	118	110	118	116	4.22	0.194	0.204	0.295
Crypt depth, μm	213	230	219	248	10.29	0.259	0.475	0.227
VH:CD[2]	1.74	1.35	1.75	1.48	0.10	0.011	0.009	0.362
$Ki67^{+3}$, %	27.70	36.16	32.42	26.96	1.34	<0.001	0.034	<0.001

[1]Treatments: NC, no-challenge/no-supplement; PC, *E. coli* challenge/no-supplement; AGP, *E. coli* challenge/bacitracin (30 g/t feed); and PBT, *E. coli* challenge/LBF (2 kg/t feed; LBiotix, Adare Biome, Houdan, France).
[2]Villus height to crypt depth ratio.
[3]Enterocyte proliferation in the crypt.

Table 6. Relative abundance of jejunal mucosa-associated microbiota at the phylum level in pigs challenged with F18-positive *Escherichia coli* and fed diets supplemented with antibiotic (bacitracin methylene disalicylate) or *Lactobacillus* fermentate (LBF)

Item, %	Treatment[1]				SEM	*P*-value		
	NC	PC	AGP	PBT		NC vs. PC	PC vs. AGP	PC vs. PBT
Actinobacteria	1.59	1.94	7.50	6.81	1.18	0.833	0.002	0.006
Bacteroidetes	7.55	8.55	9.30	7.33	3.69	0.824	0.870	0.788
Cyanobacteria	0.20	0.34	0.71	0.39	0.13	0.471	0.049	0.770
Firmicutes	10.82	15.63	18.74	25.36	5.11	0.509	0.669	0.186
Proteobacteria	67.04	52.90	53.03	38.60	7.06	0.165	0.990	0.160
Spirochaetes	12.67	12.50	3.73	3.91	3.02	0.969	0.047	0.051
Tenericutes	0.07	7.56	6.61	16.91	7.06	0.457	0.924	0.354
Other	0.06	0.64	0.39	0.67	0.30	0.127	0.510	0.922

[1]Treatments: NC, no-challenge/no-supplement; PC, *E. coli* challenge/no-supplement; AGP, *E. coli* challenge/bacitracin (30 g/t feed); and PBT, *E. coli* challenge/LBF (2 kg/t feed; LBiotix, Adare Biome, Houdan, France).

with the Chao1 richness estimator, Shannon, and Simpson diversity index at family and genus levels (Table 10). Whereas, at the family level, the Chao1 richness in pigs on AGP tended to be greater ($P = 0.095$) than in pigs on PC. The Shannon and Simpson diversity index in pigs on AGP was greater ($P < 0.05$) than in pigs on PC. At the genus level, the Chao1 richness in pigs on AGP and PBT was greater ($P < 0.05$) than in pigs on PC. The Shannon and Simpson diversity index in pigs on AGP was greater ($P < 0.05$) than in pigs on PC, whereas the Shannon diversity index in pigs on PBT tended to be greater ($P = 0.093$) than in pigs on PC. The Simpson in pigs on PBT was not different from pigs on PC.

Correlations with mucosa-associated microbiota

Bacteroidaceae was negatively correlated with the ADG ($r = -0.39$; $P < 0.05$), G:F ($r = -0.79$; $P < 0.05$), and VH ($r = -0.31$; $P < 0.05$), whereas it was positively correlated with TNF-α ($r = 0.46$; $P < 0.05$), IL-8 ($r = 0.70$; $P < 0.05$), and IgG ($r = 0.30$; $P < 0.05$) in jejunal mucosa (Table 11). Similarly, *H. rodentium* was negatively correlated with ADG ($r = -0.29$; $P < 0.05$), G:F ($r = -0.74$; $P < 0.05$), and VH ($r = -0.29$; $P < 0.05$), whereas it was positively correlated with TNF-α ($r = 0.48$; $P < 0.05$), IL-8 ($r = 0.64$; $P < 0.05$), and $Ki67^{+}$ ($r = 0.32$; $P < 0.05$). Enterobacteriaceae was negatively correlated with G:F ($r = -0.41$; $P < 0.05$), whereas it was positively correlated with IL-8 ($r = 0.39$; $P < 0.05$) and MDA ($r = 0.32$; $P < 0.05$) in jejunal mucosa. Comamonadaceae was negatively correlated with TNF-α ($r = -0.30$; $P < 0.05$), whereas it was positively correlated with protein carbonyl ($r = 0.33$; $P < 0.05$) in jejunal mucosa. Similarly, *P. puraquae* and *P. aquatica* were positively correlated with protein carbonyl in jejunal mucosa ($r = 0.37$, $r = 0.38$, respectively; $P < 0.05$). Ruminococcaceae was positively correlated with TNF-α ($r = 0.38$; $P < 0.05$) and IgG ($r = 0.29$; $P < 0.05$). Burkholderiaceae was negatively correlated with TNF-α ($r = -0.31$; $P < 0.05$), whereas it was positively correlated with protein carbonyl ($r = 0.39$; $P < 0.05$) in jejunal mucosa. Moraxellaceae was negatively correlated with IL-6 ($r = -0.29$, $P < 0.05$). Streptococcaceae was positively correlated with IL-6 ($r = 0.32$; $P < 0.05$), IgG ($r = 0.29$; $P < 0.05$), and IgA ($r = 0.31$; $P < 0.05$). Similarly, *Streptococcus alactolyticus* was positively correlated with TNF-α ($r = 0.34$; $P < 0.05$), IL-6 ($r = 0.34$; $P < 0.05$), and IgG ($r = 0.38$; $P < 0.05$). Microbacteriaceae was positively correlated with protein carbonyl ($r = 0.37$; $P < 0.05$). Similarly, *M. ginsengisoli* and *Lactobacillus ruminis* were positively correlated with protein carbonyl ($r = 0.37$, $r = 0.37$, respectively; $P < 0.05$). *Lactobacillus kitasatonis* was positively correlated with IgG ($r = 0.30$; $P < 0.05$), whereas *L. delbrueckii* and *Lactobacillus mucosae* were positively correlated with IgA ($r = 0.34$, $r = 0.30$, respectively; $P < 0.05$). Pseudomonadaceae was positively correlated with the VH ($r = 0.34$; $P < 0.05$) and the VH:CD ($r = 0.40$; $P < 0.05$). Mycoplasmataceae was positively

Table 7. Relative abundance of jejunal mucosa-associated microbiota at the family level in pigs challenged with F18-positive *Escherichia coli* and fed diets supplemented with antibiotic (bacitracin methylene disalicylate) or *Lactobacillus* fermentate (LBF)

Item, %	Treatment[1]				SEM	*P*-value		
	NC	PC	AGP	PBT		NC vs. PC	PC vs. AGP	PC vs. PBT
Bacteroidaceae	0.07	1.66	0.07	0.83	0.80	0.166	0.165	0.463
Bifidobacteriaceae	0.30	0.19	2.13	1.21	0.52	0.883	0.011	0.172
Brachyspiraceae	12.50	12.14	3.66	3.85	3.05	0.934	0.056	0.062
Burkholderiaceae	0.12	0.18	1.54	0.66	0.22	0.842	0.001	0.138
Campylobacteraceae	39.43	21.85	12.15	14.16	7.24	0.094	0.349	0.457
Clostridiaceae	0.73	1.55	1.83	3.19	1.15	0.512	0.817	0.188
Comamonadaceae	0.78	1.13	6.97	2.55	0.93	0.790	<0.001	0.283
Enterobacteriaceae	0.19	0.81	2.29	0.86	0.44	0.323	0.021	0.930
Helicobacteraceae	22.73	24.25	13.84	14.24	6.92	0.877	0.293	0.312
Lachnospiraceae	0.71	0.97	1.50	2.68	0.88	0.821	0.645	0.141
Lactobacillaceae	4.52	5.92	6.26	11.71	2.96	0.740	0.936	0.174
Microbacteriaceae	0.22	0.27	1.96	0.75	0.29	0.909	0.001	0.250
Moraxellaceae	0.38	0.28	5.57	1.72	1.43	0.959	0.012	0.478
Mycoplasmataceae	0.00	7.51	6.55	16.83	7.06	0.456	0.924	0.356
Porphyromonadaceae	0.20	0.56	0.40	1.08	0.54	0.592	0.807	0.455
Prevotellaceae	6.91	6.02	7.96	4.76	3.17	0.816	0.611	0.742
Propionibacteriaceae	0.44	0.16	0.50	3.55	0.46	0.661	0.600	< 0.001
Pseudomonadaceae	0.67	0.36	2.59	1.17	0.49	0.625	0.001	0.196
Ruminococcaceae	0.59	0.79	0.90	0.92	0.44	0.746	0.862	0.828
Sphingomonadaceae	0.21	0.20	1.21	0.49	0.15	0.978	<0.001	0.174
Staphylococcaceae	0.49	0.90	0.67	0.43	0.39	0.453	0.670	0.392
Streptococcaceae	0.22	2.07	1.31	0.68	1.05	0.221	0.615	0.357
Succinivibrionaceae	1.54	0.76	0.66	0.46	0.66	0.361	0.908	0.720
Veillonellaceae	2.22	1.24	4.09	2.93	1.19	0.494	0.052	0.242
Other	3.84	8.23	13.39	8.30	2.25	0.174	0.112	0.982

[1]Treatments: NC, no-challenge/no-supplement; PC, *E. coli* challenge/no-supplement; AGP, *E. coli* challenge/bacitracin (30 g/t feed); and PBT, *E. coli* challenge/LBF (2 kg/t feed; LBiotix, Adare Biome, Houdan, France).

correlated with CD (r = 0.30; P < 0.05). Similarly, *Mycoplasma sualvi* was positively correlated with CD (r = 0.29; P < 0.05). Veillonellaceae, Bifidobacteriaceae, and Sphingomonadaceae were positively correlated with the VH:CD (r = 0.32, r = 0.41, r = 0.32, respectively; P < 0.05). Similarly, *B. boum* was positively correlated with VH:CD (r = 0.51; P < 0.05). Lachnospiraceae and Porphyromonadaceae were negatively correlated with the Ki67$^+$ cells (r = –0.30, r = 0.32; P < 0.05, respectively).

Discussion

This study showed that F18$^+$ *E. coli* is associated with PWD causing negative impacts on intestinal health, changing the mucosa-associated microbiota, and consequently reducing the growth performance of nursery pigs similar to previously reported (Duarte et al., 2020; Sun et al., 2021). Dietary supplementation with PBT and AGP, however, mitigated the negative impacts of F18$^+$ *E. coli* challenge on intestinal health and promoted growth performance. The enterotoxins, including Sta and Stb, produced by ETEC, including F18$^+$ *E. coli*, are the major reasons causing diarrhea in pigs. Interference in the electrolyte fluid would be caused by fimbria receptor interaction, whereas *E. coli* fimbria binds to glycoproteins on the surface of enterocytes (Bijlsma et al., 1982; Fairbrother et al., 2005). There are several contributing factors of ETEC infection to pigs upon weaning, including dietary, environmental change, and behavioral stresses. These factors suppress the immune function and thus increase the possibility of ETEC infection, leading to intestinal inflammation and PWD (Moeser et al., 2007; Sun and Kim, 2017). In this study, the challenge with F18$^+$ *E. coli* reduced BW gain and feed intake similarly to those previously reported (Kim et al., 2019; Duarte et al., 2020; Sun et al., 2021). However, the feed efficiency of pigs was not affected by F18$^+$ *E. coli*. These results suggest that the reduced BW gain was mainly due to the reduced feed intake caused by the F18$^+$ *E. coli* challenge in nursery pigs.

During ETEC invasion, the production of Sta from *E. coli* stimulates the secretion of IL-6 and IL-8 (Wang et al., 2019b), which can reduce the secretion of growth hormone and damage the intestinal epithelial cell in the jejunum impairing barrier function (Moeser et al., 2007). The IL-8 is transcriptionally regulated by mitogen-activating protein kinase (MAPK) and regulates neutrophil chemotaxis toward sites of infection while inducing inflammation (Zhou et al., 2003). ETEC infection in nursery pigs could increase IL-8 production (Godaly et al., 1997; Loos et al., 2013). As the main site of nutrient absorption in the small intestine, the jejunum plays an important role in maintaining metabolism for growth. The *E. coli*

Table 8. Relative abundance of jejunal mucosa-associated microbiota at the genus level in pigs challenged with F18-positive *Escherichia coli* and fed diets supplemented with antibiotic (bacitracin methylene disalicylate) or *Lactobacillus* fermentate (LBF)

Item, %	Treatment[1]				SEM	*P*-value		
	NC	PC	AGP	PBT		NC vs. PC	PC vs. AGP	PC vs. PBT
Acinetobacter	0.69	0.28	6.82	2.01	1.68	0.860	0.007	0.458
Bifidobacterium	0.28	0.21	2.84	1.25	0.71	0.948	0.012	0.306
Campylobacter	32.36	25.21	11.21	13.29	7.58	0.509	0.199	0.273
Clostridium	0.71	0.67	2.23	3.48	1.07	0.974	0.226	0.032
Corynebacterium	0.20	1.53	0.27	0.24	0.66	0.158	0.180	0.172
Helicobacter	36.15	30.27	17.17	20.33	7.74	0.594	0.238	0.369
Lactobacillus	8.02	8.14	8.33	13.39	3.46	0.980	0.969	0.289
Megasphaera	0.98	0.37	1.53	0.76	0.46	0.178	0.012	0.390
Microbacterium	0.47	0.44	2.62	1.04	0.39	0.964	0.001	0.280
Mitsuokella	0.40	0.32	1.29	0.62	0.27	0.827	0.011	0.410
Mycoplasma	0.00	8.50	6.96	17.78	7.37	0.419	0.883	0.378
Pelomonas	1.60	1.63	6.61	2.89	0.93	0.979	0.001	0.344
Prevotella	6.55	5.98	4.98	2.76	3.15	0.892	0.812	0.446
Propionibacterium	0.92	0.31	0.69	4.88	0.64	0.503	0.673	<0.001
Pseudomonas	1.26	0.36	3.39	1.51	0.63	0.257	0.001	0.150
Selenomonas	1.39	0.15	0.45	0.32	0.45	0.038	0.603	0.772
Staphylococcus	0.34	1.16	0.99	0.66	0.42	0.178	0.779	0.408
Streptococcus	0.36	2.30	1.93	0.76	1.25	0.278	0.833	0.387
Succinivibrio	2.13	0.79	0.80	0.66	0.87	0.238	0.995	0.903
Other	5.21	11.38	18.90	11.37	3.25	0.187	0.109	0.998

[1]Treatments: NC, no-challenge/no-supplement; PC, *E. coli* challenge/no-supplement; AGP, *E. coli* challenge/bacitracin (30 g/t feed); and PBT, *E. coli* challenge/LBF (2 kg/t feed; LBiotix, Adare Biome, Houdan, France).

infection in the jejunum could damage the epithelial cells and the villi reducing the absorption of nutrients and, therefore, decreasing the growth performance of pigs (Kim, et al., 2019; Duarte et al., 2020; He et al., 2020b). This study showed that *E. coli* infection increased IL-8 in the jejunal mucosa of pigs potentially indicating increased neutrophil migration in the mucosal tissue by *E. coli* challenge (Godaly et al., 1997).

The LBF tended to reduce IL-8 concentration in the jejunal mucosa of *E. coli*-challenged pigs, which is in line with earlier observations that LBF reduces IL-8 production in cultured epithelial cells challenged with *Salmonella typhimurium* (Coconnier et al., 2000). Reduced IL-8 expression would be associated with reduced inflammation. Indeed, LBF was shown to reduce colitis in mice challenged with *Citrobacter rodentium* (Warda et al., 2020). Feed additives containing microorganisms were shown to improve intestinal health by preventing *E. coli* infection and by reducing the inflammatory response. Sun et al. (2021) found that feeding probiotics for 7 d before the F18$^+$ *E. coli* challenge reduced intestinal inflammation and oxidative stress, thereby consequently increasing the growth of nursery pigs. The LBF effectively reduced the BW loss and feed intake loss after the F18$^+$ *E. coli* challenge as previously observed by Lee et al. (2012) using *Lactobacillus* as a probiotic. These results indicate that dietary supplementation with LBF has a similar effect to the use of *Lactobacillus* as a probiotic. According to Zhang et al. (2005) and Ko et al. (2007), *Lactobacillus* spp. could suppress the expression of IL-8 during the inflammatory response preventing epithelial damage.

Increased immune response by *E. coli* infection can provoke exhaustion of the antioxidant mechanism causing oxidative damages to cellular protein, lipids, and DNA. Protein carbonyl and MDA are effective biomarkers of oxidative stress that indicate protein and lipid oxidation (Dalle-Donne et al., 2003; Zhao et al., 2013; Zhao and Kim, 2020). F18$^+$ *E. coli* challenge also significantly increased the concentration of protein carbonyl. Studies have previously reported increased oxidative stress in the jejunal mucosa of pigs challenged with F18$^+$ *E. coli* (Duarte et al., 2020; Sun et al., 2021). Oxidative stress may induce cell apoptosis (Assimakopoulos et al., 2011). F18$^+$ *E. coli* challenge tended to reduce villi length indicating a potential epithelial damage in the jejunum. As villi length decreases, the enterocyte proliferation in the crypt increases in order to recover the damaged tissue. The increased enterocyte proliferation in the crypt observed in the challenge group is another indicator of intestinal infection caused by F18$^+$ *E. coli*. The Ki67$^+$ is an antigen associated with cellular proliferation (Jang et al., 2021). Although the LBF supplementation did not show differences in MDA and protein carbonyl concentration after the F18$^+$ *E. coli* challenge, it reduced the enterocyte proliferation in crypts of the jejunum. These results indicate the potential benefit of the LBF as a postbiotic reducing the need for crypt cell proliferation by preventing epithelial damage. Similar to LBF, BMD supplementation reduced cell proliferation rate in the jejunum and increased VH compared with the positive control group.

Compared with LBF, supplementation with BMD promoted the growth performance of nursery pigs during the challenge period by increasing ADG and ADFI. During the challenge period, nursery pigs fed diet with BMD tended to have a lower concentration of IgA. The IgA is one of

Table 9. Relative abundance of jejunal mucosa-associated microbiota at the species level in pigs challenged with F18-positive *Escherichia coli* and fed diets supplemented with antibiotic (bacitracin methylene disalicylate) or *Lactobacillus* fermentate (LBF)

Item, %	Treatment[1]				SEM	*P*-value		
	NC	PC	AGP	PBT		NC vs. PC	PC vs. AGP	PC vs. PBT
Gram-negative	82.14	74.73	52.30	61.26	10.01	0.444	0.024	0.167
Campylobacter coli	41.07	29.83	14.98	15.51	10.11	0.367	0.235	0.252
Helicobacter canadensis	0.05	0.05	3.73	0.01	1.84	0.999	0.164	0.988
Helicobacter mastomyrinus	10.26	11.37	1.32	9.14	3.42	0.820	0.044	0.646
Helicobacter rappini	15.73	11.00	7.23	11.72	3.85	0.390	0.493	0.897
Helicobacter rodentium	0.43	3.65	0.12	0.04	1.01	0.030	0.018	0.016
Helicobacter sp.	3.10	2.19	3.18	0.76	1.54	0.677	0.651	0.516
Mycoplasma sualvi	0.00	7.73	6.99	17.13	7.27	0.457	0.943	0.366
Pelomonas puraquae	1.36	1.10	6.26	2.26	0.89	0.837	0.001	0.365
Pelomonas aquatica	0.31	0.39	2.22	0.82	0.29	0.847	< 0.001	0.300
Prevotella copri	5.81	5.05	3.56	2.15	2.90	0.849	0.710	0.470
*Prevotella*sp.	0.82	0.59	1.36	0.35	0.47	0.712	0.208	0.685
Prevotella stercorea	1.81	1.31	0.93	0.79	0.81	0.665	0.737	0.647
Succinivibrio dextrinosolvens	1.38	0.46	0.42	0.59	0.67	0.262	0.965	0.871
Gram-positive	6.97	7.21	14.16	21.11	4.71	0.967	0.227	0.018
Bifidobacterium boum	0.04	0.04	1.97	0.38	0.54	0.995	0.016	0.662
Lactobacillus delbrueckii	0.76	1.58	1.76	2.60	0.87	0.511	0.885	0.411
Lactobacillus kitasatonis	0.82	0.79	0.99	0.62	0.36	0.961	0.693	0.741
Lactobacillus mucosae	1.55	2.97	3.25	4.53	1.55	0.523	0.898	0.481
Lactobacillus ruminis	1.80	0.06	0.29	0.21	0.87	0.167	0.853	0.901
Lactobacillus salivarius	0.03	0.30	0.56	4.11	1.17	0.872	0.874	0.027
Microbacterium ginsengisoli	0.45	0.47	2.97	0.98	0.43	0.982	0.001	0.397
Propionibacterium acnes	1.28	0.40	1.26	7.35	0.99	0.533	0.541	< 0.001
Streptococcus alactolyticus	0.24	0.60	1.10	0.31	0.51	0.619	0.496	0.686
Other	10.89	18.06	33.54	17.63	4.29	0.244	0.015	0.944
Aerobic	72.78	60.05	42.01	41.22	11.1	0.283	0.130	0.115
Anaerobic	11.65	7.52	8.54	4.48	4.49	0.501	0.869	0.620
Facultative anaerobic	4.68	14.36	15.92	36.66	6.89	0.326	0.874	0.027

[1]Treatments: NC, no-challenge/no-supplement; PC, *E. coli* challenge/no-supplement; AGP, *E. coli* challenge/bacitracin (30 g/t feed); and PBT, *E. coli* challenge/LBF (2 kg/t feed; LBiotix, Adare Biome, Houdan, France).

Table 10. Alpha diversity of jejunal mucosa-associated microbiota estimated in pigs challenged with F18-positive *Escherichia coli* and fed diets supplemented with antibiotic (bacitracin methylene disalicylate) or *Lactobacillus* fermentate (LBF)

Item	Treatment[1]				SEM	*P*-value		
	NC	PC	AGP	PBT		NC vs. PC	PC vs. AGP	PC vs. PBT
Family								
Chao1	51.83	56.08	69.84	67.48	5.69	0.600	0.095	0.164
Shannon	2.25	2.25	3.56	2.94	0.33	0.993	0.007	0.141
Simpson	0.62	0.58	0.78	0.68	0.06	0.655	0.036	0.276
Genus								
Chao1	49.11	48.80	76.40	71.55	6.06	0.972	0.003	0.011
Shannon	2.19	2.00	3.53	2.81	0.33	0.690	0.002	0.093
Simpson	0.59	0.52	0.74	0.65	0.07	0.444	0.026	0.188

[1]Treatments: NC, no-challenge/no-supplement; PC, *E. coli* challenge/no-supplement; AGP, *E. coli* challenge/bacitracin (30 g/t feed); and PBT, *E. coli* challenge/LBF (2 kg/t feed; LBiotix, Adare Biome, Houdan, France).

the antibodies that are upregulated during inflammatory responses against *E. coli* (Zhang et al., 2010; Mantis et al., 2011; Wang et al., 2019a). The result observed in the concentration of IgA may reflect the reduced concentration of IL-8 observed in BMD, indicating that the supplementation of BMD could minimize inflammatory response during the

Table 11. Pearson correlation coefficients (*r*) between mucosa-associated microbiota and other variables measured in pigs fed diets with postbiotics preventing postweaning diarrhea caused by F18-positive *Escherichia coli*

Item[1]	Family (*P*-value, *r*)	Species (*P*-value, *r*)
ADG	Bacteroidaceae (0.006, –0.39)	*Helicobacter rodentium* (0.047, –0.29)
G:F	Enterobacteriaceae (0.004, –0.41)	*Helicobacter rodentium* (<0.001, –0.74)
	Bacteroidaceae (<0.001, –0.79)	
TNF-α	Comamonadaceae (0.036, –0.30)	*Helicobacter rodentium* (<0.001, 0.48)
	Ruminococcaceae (0.007, 0.38)	*Streptococcus alactolyticus* (0.018, 0.34)
	Bacteroidaceae (0.001, 0.46)	
	Burkholderiaceae (0.032, –0.31)	
IL-8	Enterobacteriaceae (0.007, 0.39)	*Helicobacter rodentium* (<0.001, 0.64)
	Bacteroidaceae (<0.001, 0.70)	
IL-6	Moraxellaceae (0.044, –0.29)	*Streptococcus alactolyticus* (0.020, 0.34)
	Streptococcaceae (0.031, 0.32)	
IgG	Streptococcaceae (0.044, 0.29)	*Lactobacillus kitasatonis* (0.038, 0.30)
	Ruminococcaceae (0.042, 0.29)	*Streptococcus alactolyticus* (0.008, 0.38)
	Bacteroidaceae (0.037, 0.30)	
IgA	Streptococcaceae (0.036, 0.31)	*Lactobacillus delbrueckii* (0.020, 0.34)
		Lactobacillus mucosae (0.044, 0.30)
MDA	Enterobacteriaceae (0.028, 0.32)	
PC	Comamonadaceae (0.024, 0.33)	*Microbacterium ginsengisoli* (0.010, 0.37)
	Microbacteriaceae (0.010, 0.37)	*Lactobacillus ruminis* (0.011, 0.37)
	Burkholderiaceae (0.006, 0.39)	*Pelomonas puraquae* (0.010, 0.37)
		Pelomonas aquatica (0.009, 0.38)
VH	Pseudomonadaceae (0.020, 0.34)	*Helicobacter rodentium* (0.049, –0.29)
	Bacteroidaceae (0.032, –0.31)	*Bifidobacterium boum* (0.039, 0.30)
CD	Mycoplasmataceae (0.037, 0.30)	*Mycoplasma sualvi* (0.042, 0.29)
VH:CD	Veillonellaceae (0.025, 0.32)	*Bifidobacterium boum* (<0.001, 0.51)
	Pseudomonadaceae (0.005, 0.40)	
	Bifidobacteriaceae (0.004, 0.41)	
	Sphingomonadaceae (0.029, 0.32)	
Ki67$^+$	Lachnospiraceae (0.039, –0.30)	*Helicobacter rodentium* (0.029, 0.32)
	Porphyromonadaceae (0.029, –0.32)	

[1]ADG, average daily gain; G:F, gain to feed ratio; TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6; IL-8, interleukin-8; IgG, immunoglobulin G; IgA, immunoglobulin A; MDA, malondialdehyde; PC, protein carbonyl; VH, villus height; CD, crypt depth; VH:CD, villus height to crypt depth ratio

challenge period by suppressing the *E. coli* attachment to epithelial cells.

The mucosa-associated microbiota has an important role in intestinal epithelial defense preventing the overgrowth of opportunistic pathogens (Shi et al., 2017; Ma et al., 2018; Duarte and Kim, 2021). Although the diarrhea symptoms caused by *E. coli* last 7 to 11 d (Kim et al., 2019; Duarte et al., 2020; Sun et al., 2021), according to Duarte and Kim (2022), the changes in jejunal mucosa-associated microbiota caused by the F18$^+$ *E. coli* challenge affected intestinal health of pigs at least until 21 d post challenge. In the current study, the F18$^+$ *E. coli* challenge imbalanced the jejunal mucosa-associated microbiota by increasing the abundance of *H. rodentium*, a bacteria belonging to Proteobacteria, whereas it decreased the RA of *Selenomonas* that belongs to Bacteroidetes. Multiple studies showed that the RA of *Helicobacter* and *Selenomonas* in the jejunal mucosa of nursery pigs is about 30% and 1.5%, respectively, which was also similar to those in pigs without *E. coli* challenge in this study (Duarte et al., 2020; Cheng et al., 2021; Moita et al., 2021).

Helicobacter spp., a Gram-negative commensal bacteria in the intestine of pigs, are considered opportunistic pathogens that can adhere to the intestinal epithelial surface, and it has been associated with reduced the intestinal barrier properties and VH increasing the inflammatory response and the crypt cells proliferation rate (Shin et al., 2015; Zhang et al., 2017; Duarte et al., 2020). However, the F18$^+$ *E. coli* challenge can stimulate the overgrowth of *Helicobacter* spp. disrupting the balance of mucosa-associated microbiota (Duarte et al., 2020). The disrupted microbiota results in increased inflammatory response and oxidative stress (Duarte et al., 2020; Cheng et al., 2021; Moita et al., 2021). The increased *Helicobacter* spp. in jejunal mucosa of pigs challenged with F18$^+$ *E. coli* competitively suppressed the *Selenomonas* indicating the negative effect of ETEC to host microbiota (Shin et al., 2015; Duarte et al., 2020). Additionally, the reduced *Selenomonas* affects the fermentation of soluble carbohydrates, which may further affect the growth of the animal (Hespell et al., 2006).

The reduction of *H. rodentium* indicates that LBF similar to BMD can enhance intestinal health and reduce the negative

effects of F18+ *E. coli*. Mucosal adhesion of bacterial cell wall compounds in LBF is proposed to be an underlying mechanism for the reduction of diarrhea based on several human studies with LBF (Liévin-Le Moal, 2016). Selected cell components and metabolites produced by Gram-positive bacteria reduce the adherence of pathogenic bacteria in Caco-2 cells (Liévin-Le Moal, 2002; Cheng et al., 2021) by attaching to receptors on the intestinal epithelial cells (Johnson-Henry et al., 2007) and by activating immune response via Toll-like receptors (Hopkins and Sriskandan, 2005). Of note, LBF has direct anti-*Herlicobacter pilori* effects and reduces the adhesion of *H. pilori* to mucosal epithelial cells (Coconnier et al., 1998). The adhesion-preventative effect of *Lactobacillus* has been observed for other species as well. According to Konstantinov et al. (2008), *Lactobacillus sobrius* as a probiotic could reduce Proteobacteria by blocking the receptor on the epithelial cell surface.

Changes in microbiota in the jejunum could promote the growth performance of pigs by enhancing the environment for beneficial microbiota and reducing the abundance of harmful bacteria (Jang and Kim, 2019; Cheng et al., 2021; Jang et al., 2021; Moita et al., 2021). In this study, BMD reduced the abundance of Gram-negative bacteria, whereas LBF increased Gram-positive and facultative anaerobic bacteria. Consequently, both LBF and BMD increased the diversity of the mucosa-associated microbiota in this study. The LBF supplement may have provided support for the growth of *Lactobacillus* spp. indicated by increased abundance of *Lactobacillus salivarius* as an example. The aerobic metabolism of *Lactobacillus* provides the environment for the growth of anaerobic microbiota, therefore, increasing their abundance as well (Zotta et al., 2014), whereas maintaining the intestinal hemostasis. Although antibiotics are generally shown to reduce microbial diversity (Grazul et al., 2016), bacitracin was shown to increase microbial diversity in poultry (Díaz Carrasco et al., 2018; Proctor and Phillips, 2019). According to Schokker et al. (2014), antibiotics could also increase microbiota diversity specifically by improving microbial colonization and improving the abundance of anaerobic bacteria such as *Bifidobacterium* which was also observed in this study. The results of this study indicate that BMD and LBF depleted a few bacterial species and stimulated the establishment of others. The diets supplemented with LBF and BMD increased the abundance of Actinobacteria by increasing Propionibacteriaceae and decreased the abundance of Spirochaetes by reducing Brachyspiraceae. Actinobacteria is one of four major phyla of intestinal microbiota and plays an important role in maintaining intestine homeostasis (Binda et al., 2018). Spirochaetes is known as a potential pathogen in pigs. Colonization of Spirochaetes could increase the inflammatory response, thus suppressing the growth of pigs (Hampson and Ahmed, 2009) as also observed in this study.

The F18+ *E. coli* challenge considerably changed the jejunal mucosa-associated microbiota; increased immune response, oxidative stress, and crypt cell proliferation; and ultimately reduced the growth performance of pigs. However, the LBF, similar to BMD supplementation, increased the diversity of jejunal mucosa-associated microbiota and the abundance of beneficial bacteria, whereas it reduced the potentially harmful bacteria. The LBF alters the gut microbiota in rodents (Warda et al., 2019, 2020) and has a Bifidogenic effect ex vivo, at least with human samples (Warda et al., 2021), whereas the similar effects were not shown in this study. This may be due to the insignificant abundance of Bifidobacteria in the small intestine of pigs compared with humans (Leser et al., 2002).

In this study, the changes in mucosa-associated microbiota led to a reduction of the inflammatory response and also the need for crypt cell proliferation to recover from villus damage, which consequently increased the growth performance of pigs. Bacteroidaceae and *H. rodentium* were negatively correlated with VH and growth performance, whereas they were positively correlated with immune response in the jejunal mucosa. Additionally, *H. rodentium* was positively correlated with crypt cell proliferation. *Lactobacillus* spp. were positively correlated with IgG, IgA, and protein carbonyl. *Bifidobacterium boum* was positively correlated with VH and VH:CD. Duarte and Kim (2021) reported that bacteria including *Lactobacillus* and *Bifidobacterium* associated with the jejunal mucosa are associated with enhanced intestinal health in pigs, whereas the increased abundance of jejunal mucosa-associated Enterobacteriaceae, *Streptococcus*, and *Helicobacter* are generally associated with intestinal dysbiosis.

In conclusion, the challenge with F18+ *E. coli* reduced the growth performance of nursery pigs associated with increased intestinal inflammation, oxidative stress, crypt cell proliferation, and abundance of harmful bacteria in the jejunal mucosa. Dietary supplementation of LBF, as a postbiotic, enhanced the growth performance associated with enhanced diversity and abundance of beneficial microbiota, increased feed intake, prevention of villus damage, and reduced needs of crypt cell proliferation in the jejunum after F18+ *E. coli* challenge. Compared with a diet with antibiotics, supplementation of LBF successfully improved growth performance and intestinal health.

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Conflict of interest statement

The authors declare that they have no conflict of interests.

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