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Original Research Article

Dietary inclusion of multispecies probiotics to reduce the severity of post-weaning diarrhea caused by *Escherichia coli* F18$^+$ in pigs

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ABSTRACT

This study was aimed to determine the efficacy of multispecies probiotics in reducing the severity of post-weaning diarrhea caused by enterotoxigenic *Escherichia coli* (ETEC) F18$^+$ on newly weaned pigs. Thirty-two pigs (16 barrows and 16 gilts, BW = 6.99 ± 0.33 kg) at 21 d of age were individually allotted in a randomized complete block design with 2 × 2 factorial arrangement of treatments. Pigs were selected from sows not infected previously and not vaccinated against ETEC. Pigs were fed experimental diets for 25 d based on 10 d phase 1 and 15 d phase 2. The factors were ETEC challenge (oral inoculation of saline solution or *E. coli* F18$^+$ at 2 × 10^9 CFU) and probiotics (none or multispecies probiotics 0.15% and 0.10% for phase 1 and 2, respectively). Body weight and feed intake were measured on d 5, 9, 13, 19, and 25. Fecal scores were measured daily. Blood samples were taken on d 19 and 24. On d 25, all pigs were euthanized to obtain samples of digesta, intestinal tissues, and spleen. The tumor necrosis factor alpha (TNFα), malondialdehyde (MDA), peptide YY (PYY), and neuropeptide Y (NPY) were measured in serum and intestinal tissue. Data were analyzed using the MIXED procedure of SAS. The fecal score of pigs was increased ($P < 0.05$) by ETEC challenge at the post−challenge period. The ETEC challenge decreased ($P < 0.05$) jejunal villus height and crypt depth, tended to increase ($P = 0.056$) jejunal TNFα, increased ($P < 0.05$) ileal crypt depth, and decreased ($P < 0.05$) serum NPY. The probiotics decreased ($P < 0.05$) serum TNFα, tended to reduce ($P = 0.064$) jejunal MDA, tended to increase ($P = 0.092$) serum PYY, and increased ($P < 0.05$) jejunal villus height, and especially villus height-to-crypt depth ratio in challenged pigs. Growth performance of pigs were not affected by ETEC challenge, whereas the probiotics increased ($P < 0.05$) ADG and ADFI and tended to increase ($P = 0.069$) G:F ratio. In conclusion, ETEC F18$^+$ challenge caused diarrhea, intestinal inflammation and morphological damages without affecting the growth performance. The multispecies probiotics enhanced growth performance by reducing intestinal inflammation, oxidative stress, morphological damages.

1. Introduction

Post-weaning diarrhea (PWD) is the primary concerns for the success of pig production because of the economic losses as a result of mortalities and reduced growth rate of surviving pigs. Enterotoxigenic *Escherichia coli* (ETEC) is one of the major factors causing PWD (Fairbrother et al., 2005; Luppi et al., 2016). The most common adhesins associated with PWD are F4 and F18 fimbria type (Li et al., 2020; Luise et al., 2019b; Rhouma et al., 2017). The virulence of ETEC can be characterized by the adhesion mediated by a fimbria receptor interaction followed by colonization of the intestinal epithelium and the production of heat-liable and heat-stable enterotoxins that cause interferences in the electrolytes fluid increasing the fluid secretion to the lumen leading to diarrhea (Nagy and Fekete, 2005; Dubreuil et al., 2016).

As an attempt to promote health and growth performance, prophylactic antibiotics have been included in the diets of pigs for many years (Kirchhelle, 2018). However, repeated use of

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prophylactic antimicrobials increases antibiotic resistance to ETEC strains causing inefficacy of PWD prevention (Burow et al., 2019; Diana et al., 2019). Alternative feed additives have been studied to maintain the health of pigs and consequently promote growth performance (Barba-vidal et al., 2019; Xiong et al., 2019; Zimmermann et al., 2016). Bacteria from the genus *Lactobacillus, Bifidobacterium,* and *Enterococcus* can be good alternatives to conventional antibiotic growth promoter (Gadde et al., 2017; Liu et al., 2018) due to their antagonistic activities against harmful bacteria modulating the gut microbiome balance, their effects on the digestive processes, and on the immunity of the host. These benefits may lead to a protective effect against intestinal diseases, such as PWD (Barba-Vidal et al., 2017; Klingspor et al., 2013; Liao and Nyachoti, 2017; Qiu et al., 2012).

Therefore, it is hypothesized that dietary inclusion of multispecies probiotics of *Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium thermophilum* and *Enterococcus faecium* enhances the health by reducing the severity of PWD and increasing growth performance of newly-weaned pig. To test the hypothesis, the objective of this study was to investigate the efficacy of dietary supplementation of multispecies probiotics to enhance gut health by reducing the severity of PWD, and to increase growth performance of newly-weaned pigs challenged with *E. coli* F18$^+$.

2. Materials and methods

The protocol for the use of animals in this study was approved by the North Carolina State University Animal Care and Use Committee.

2.1. Animals, experimental design, and diets

Thirty-two newly weaned crossbred pigs (21 d of age, 16 barrows and 16 gilts) with an initial body weight (BW) of 6.99 ± 0.33 kg were randomly allotted to 32 pens based on a 2 × 2 factorial arrangement. Pigs used in this study were selected from sows not infected previously and were not vaccinated against ETEC. The first factor was the ETEC challenge (oral inoculation of saline solution or *E. coli* F18$^+$ at 2 × 10^9 colony-forming unit [CFU] on d 13 post-weaning), and the second factor was the multispecies probiotics (none and probiotics at 0.15% on phase 1 [P1] and at 0.10% on phase 2 [P2]). Each factor and their interaction had 8 pens ($n = 8$; 4 pens with barrows and 4 pens with gilts; and 2 BW blocks within sex) and pigs were housed individually in a pen. From d 0 to 9 post-weaning, pigs were fed P1 diet, and from d 10 to 25 post-weaning, pigs were fed P2 diet.

The multispecies probiotics contained *L. acidophilus, L. casei, B. thermophilum* and *E. faecium* with the concentration of 0.25 × 10^8 CFU/g for each strain (PrimaLac, Star Labs/Forage Research, Inc). Body weight and feed intake were measured on d 0, 5, 9, 13, 19, and 25 post-weaning to calculate the average daily gain (ADG), and the average daily feed intake (ADFI). The probiotics were mixed with control diet prior to feeding. During the 25 d of feeding period, all pigs had free access to feed and water. Concentrations of nutrients met the requirements suggested by NRC (1998). The calculated and analyzed nutrient compositions are shown in Table 1.

2.2. ETEC challenge strains

Two strains of *E. coli* F18$^+$-producting were used as challenge. The strain S1191 (0139) was isolated from a pig with gut edema and produced heat-stable toxin A (STa), heat-stable toxin B (STb), and Shiga toxin 2e (Stx2e), and the strain 2144 (0147) was isolated from piglets with PWD and produced toxins STa and STb. The inoculum of *E. coli* F18$^+$ was prepared following our standard protocol as previously described by Cutler et al. (2007). The final concentration of was 2 × 10^9 CFU/mL comprising 1 × 10^9 CFU/mL of each strain orally inoculated in a single dose (Duarte et al., 2020).

Table 1
Ingredients and composition of basal diets (as-fed basis, %).

Item	Phase 1	Phase 2
Ingredients		
Corn	37.11	54.41
Soybean meal	25.00	30.00
Whey permeate[1]	25.00	8.00
Fish meal	4.00	2.00
Blood plasma	3.00	1.00
L-Lys HCl	0.23	0.12
DL-Met	0.16	0.06
L-Thr	0.11	0.02
Poultry fat	3.40	2.20
Salt	0.22	0.22
Vitamin premix[2]	0.03	0.03
Mineral premix[3]	0.15	0.15
Dicalcium phosphate	1.00	1.15
Limestone	0.60	0.65
Total	100.00	100.00
Calculated composition		
DM	91.8	90.4
ME, kcal/kg	3,503	3,438
CP	21.0	21.2
SID Lys	1.35	1.20
SID Met + Cys	0.78	0.68
SID Trp	0.24	0.23
SID Thr	0.85	0.73
Ca	0.92	0.81
STTD P	0.56	0.40
Analyzed composition		
DM	92.83	90.93
CP	19.71	19.75
ADF	2.20	2.42
Ca	0.75	0.71
Total P	0.73	0.69

SID = standardized ileal digestible; STTD = standardized total tract digestible.
[1] Dairy Lac80 (International Ingredient Corporation) was used as a source of whey permeate containing (79.3 ± 0.8)% lactose.
[2] Vitamin premix provided the following per kilogram of complete diet: 22,045,000 IU of vitamin A; 3,306,900 IU of vitamin D_3; 66,138 IU of vitamin K; 88 mg of vitamin B_{12}; 15,432 mg of riboflavin; 88,184 mg of niacin; 61,729 mg of d-pantothenic acid; 8,818 mg of menadione; 220 mg of biotin.
[3] Mineral premix provided the following composition: 1.10% of Cu; 198.0 mg/kg of I; 11.02% of Fe; 2.64% of Mn; 198.4 mg/kg of Se; 11.02% of Zn.

2.3. Sampling

In the morning of d 19 and 24 post-weaning, after the meal, blood samples of all pigs were collected from jugular vein to obtain serum. Blood was collected in vacutainers without anticoagulant (BD, Franklin Lakes, NJ). Serum samples were collected after centrifuging (3,000 × *g* for 15 min at 4 °C) and stored at −80 °C until they were analyzed for concentration of malondialdehyde (MDA) and tumor necrosis factor α (TNFα) as biological indicators of systemic oxidative stress and inflammatory responses, respectively (Duarte et al., 2019). On d 25 post-weaning, all pigs were stunned by an electric device and euthanized by exsanguination. Then the gastric intestinal tract was quickly removed and the small intestine was dissected. The middle sections of jejunum and ileum were isolated and flushed with distilled water. Half of the sections were fixed in 10% formaldehyde-phosphate buffer and kept for microscopic assessment of mucosal morphology. The other half of the sections were then opened for scraping the mucosal layer of the intestine. The mucosa of the jejunum and ileum was scraped into a 2-mL tube and frozen in liquid nitrogen. Mucosa samples were then stored in −80 °C until analyzing for MDA and TNFα concentrations.

One tube of digesta samples (50 mL) from jejunum, ileum and colon was also collected, and digesta pH was measured using a pH meter immediately. Digesta were directly put on ice, and then stored in −20 °C until analyzing. Spleen weight was also measured as an indicator of the expression of pro-inflammatory cytokines such as TNFα and interleukin-β (Touchette et al., 2002).

2.4. Fecal scores and diarrhea frequency

Fecal Scores were measured on d 2, 3, 5, 9, 12, and daily from d 13 post-weaning using a 0 to 3 scale: 0, normal feces; 1, soft feces; 2, mild diarrhea; 3, severe diarrhea, whereas the fecal scores greater than 1 were considered as diarrhea (Kim et al., 2019). The fecal scores of each pig were averaged within each phase and pre- and post−challenge periods.

2.5. Morphological evaluation of small intestine

Sections of the jejunum and ileum were sent to the North Carolina State University Histopathology Laboratory (College of Veterinary Medicine, Raleigh, NC, USA) to prepare polylysine-coated slides with hematoxylin and eosin (H&E) staining. Then the slides were observed using a color video camera (CCD, Sony Electronics, San Diego, CA) attached to a microscope (Olympus Van-ox S, Opelco, Washington, DC). Villus height, villus width (in the middle of the villus), and crypt depth were determined (Shen et al., 2009). Lengths of 10 well-oriented intact villi and their associated crypts were measured in each slide. One person completed all the analysis of small intestinal histomorphology.

2.6. Cytokine measurement

The concentration of TNFα in serum and in the mucosa of the ileum and jejunum was measured. Mucosa samples were homogenized in phosphate-buffered saline (PBS) containing protease inhibitors and the supernatant was collected and analyzed for protein content using a bicinchoninic acid (BCA) assay (Jang and Kim, 2019). Then the supernatant and serum were used to measure the concentration of TNFα using a Porcine TNFα Colorimetric ELISA Kit (Pierce Biotechnology, Inc., Rockford, IL) as an indicator of inflammation and acute phase reaction (Duarte et al., 2019). Briefly, 50 μL of assay diluent plus 50 μL of standard or sample were added to pre-coated microplate wells with capture antibody in conjunction with antibody reagent. Measurement was done by the use of horseradish peroxidase, 3,3′,5,5′-tetramethylbenzidine (TMB) substrate and a stop solution of 0.18 mol/L H_2SO_4. Absorbance was detected at 450 and 540 nm by an ELISA plate reader and the KC4 data analysis software. Detection limit for TNFα was 5 pg/mL. Concentration of TNFα in serum was expressed as picograms per milliliter, and that in mucosa was expressed as picograms per milliliter.

2.7. Oxidative stress status

Malondialdehyde content in serum and mucosa was measured using an OxiSelect thiobarbituric acid reactive substance (TBARS) Assay Kit (Cell Biolabs, Inc., San Diego, CA) as an index of lipid peroxidation (Kim et al., 2019). All the procedures followed the instruction of the manufacturer. Concentration of MDA in serum was expressed as micromoles per milliliter, and concentration of MDA in mucosal tissue was expressed as micromoles per milliliter of protein.

2.8. Gut hormones measurement

The concentration of peptide YY (PYY) and the neuropeptide Y (NPY) were measured in the serum using ELISA kits following Chen et al. (2020). The blood samples were collected in the morning of d 19 post-weaning. The concentration of PYY and NPY was expressed as picograms per milliliter.

2.9. Statistical analysis

Two factors (ETEC challenge and probiotics) and their interaction were fixed effects. Blocks, sex and initial BW, were random effects. The experimental unit was the pig as pigs were individually housed and fed. Data, except the diarrhea data, were analyzed using the Mixed procedure in SAS version 9.3 (SAS Inc., Cary, NC, USA). The means were separated using the LSMEANS statement in SAS. When an interaction between 2 factors was significant or tended to be significant, a pairwise comparison was made using the PDIFF option in SAS. The diarrhea frequency was analyzed using the Proc Freq of SAS. Statistical differences were considered significant with $P < 0.05$, whereas $0.05 \leq P < 0.10$ was considered as a tendency.

3. Results

3.1. Growth performance

Initial BW of pigs did not differ among factors (Table 2). During the entire feeding period, the growth performance was not affected by the ETEC challenge. Whereas, regardless the ETEC challenge, the probiotics tended to increase ($P = 0.099$) the BW of pigs at d 13 and increased it ($P < 0.05$) on d 19 and 25. The probiotics tended to increase ($P = 0.099$) the ADG of pigs during the pre-challenge period (d 0 to 13) and increased ($P < 0.05$) it during the post−challenge period (d 13 to 25), regardless the ETEC challenge. Analyzing the data by phase, the probiotics did not affect the ADG of pigs during P1, whereas, during P2 and overall, the probiotics increased ($P < 0.05$) the ADG of pigs, regardless the ETEC challenge.

The probiotics did not affect the ADFI during the pre-challenge period. However, during the post−challenge period, the supplementation of probiotics increased ($P < 0.05$) the ADFI, regardless the ETEC challenge. During P1 the probiotics did not affect the ADFI, whereas during P2 and overall period, the probiotics increased ($P < 0.05$) the ADFI of pigs, regardless the ETEC challenge. The probiotics increased ($P < 0.05$) the G:F ratio during the pre-challenge period and tended to increase it during P2 ($P = 0.066$) and overall ($P = 0.069$).

3.2. Fecal scores and occurrence of diarrhea

The fecal score was not affected by the factors during the pre-challenge period and P1 (Table 3). In the post−challenge period, the ETEC challenge increased ($P < 0.05$) the fecal score, whereas the probiotics did not affect it. During P2 and overall period the ETEC challenge increased ($P < 0.05$) the fecal score, whereas the probiotics did not affect it. In the pre-challenge period and P1, the factors did not affect the frequency of diarrhea. In the post−challenge period, P2 and overall, the ETEC challenge increased ($P < 0.05$) the number of pigs with diarrhea, whereas the probiotics did not affect it.

Table 2
Growth performance of pigs challenged with *E. coli* F18$^+$ (CH) on d 13 postweaning and fed diets supplemented with multispecies probiotics (PRO).

Item	CH-		CH+		SEM	*P*-value		
	PRO-	PRO+	PRO-	PRO+		CH	PRO	CH × PRO
BW, kg								
Initial	6.99	6.97	6.98	7.03	0.19	0.517	0.735	0.453
d 9	7.35	7.92	7.57	7.70	0.52	0.992	0.337	0.543
d 13	7.83	8.91	7.98	8.65	0.79	0.915	0.099	0.691
d 19	9.45	11.43	9.32	10.96	1.42	0.708	0.032	0.831
d 25	11.79	15.19	11.82	14.20	2.19	0.708	0.031	0.689
ADG, g/d								
Pre-challenge	65	149	77	124	65	0.864	0.099	0.633
Post-challenge	330	523	320	462	118	0.604	0.019	0.708
P1 (d 0 to 9)	40	106	66	74	63	0.937	0.342	0.463
P2 (d 9 to 25)	278	454	266	406	106	0.617	0.013	0.764
Overall	192	329	194	287	89	0.688	0.031	0.666
ADFI, g/d								
Pre-challenge	180	273	234	249	60	0.707	0.203	0.361
Post-challenge	512	756	514	722	159	0.860	0.018	0.839
P1 (d 0 to 9)	145	207	185	190	51	0.716	0.320	0.400
P2 (d 9 to 25)	448	672	472	637	139	0.942	0.023	0.718
Overall	339	505	369	476	105	0.992	0.038	0.646
G:F ratio								
Pre-challenge	0.36	0.54	0.32	0.49	0.14	0.585	0.044	0.967
Post−challenge	0.65	0.70	0.62	0.64	0.05	0.207	0.347	0.655
P1 (d 0 to 9)	0.29	0.52	0.35	0.38	0.18	0.808	0.362	0.468
P2 (d 9 to 25)	0.63	0.68	0.56	0.64	0.05	0.161	0.066	0.683
Overall	0.57	0.65	0.53	0.60	0.07	0.211	0.069	0.922

G:F ratio = gain-to-feed ratio.

Table 3
Fecal score of pigs challenged with *E. coli* F18$^+$ (CH) on d 13 postweaning and fed diets supplemented with multispecies probiotics (PRO).

Item	CH-		CH+		SEM	*P*-value		
	PRO-	PRO+	PRO-	PRO+		CH	PRO	CH × PRO
Pigs with diarrhea/Pigs per treatment (fecal score)[1]								
Pre-challenge	0/8 (0.67)	0/8 (0.77)	0/8 (0.67)	0/8 (0.69)	0.15	0.780	0.691	0.780
Post−challenge	1/8 (0.47)	0/8 (0.38)	5/8 (1.26)	5/8 (1.06)	0.19	0.001	0.467	0.790
P1 (d 0 to 9)	0/8 (0.71)	0/8 (0.88)	0/8 (0.63)	0/8 (0.86)	0.17	0.777	0.272	0.856
P2 (d 9 to 25)	1/8 (0.48)	0/8 (0.37)	5/8 (1.17)	5/8 (0.94)	0.17	0.002	0.338	0.737
Overall	1/8 (0.53)	0/8 (0.51)	5/8 (1.06)	5/8 (0.94)	0.16	0.001	0.934	0.497

[1] Fecal scores > 1 were considered as diarrhea.

Table 4
pH of digesta of pigs challenged with *E. coli* F18$^+$ (CH) on d 13 postweaning and fed diets supplemented with multispecies probiotics (PRO).

Item	CH-		CH+		SEM	*P*-value		
	PRO-	PRO+	PRO-	PRO+		CH	PRO	CH × PRO
pH								
Jejunum	6.85^a	6.31^b	6.71^{ab}	6.84^a	0.21	0.192	0.171	0.028
Ileum	7.00	6.39	6.74	6.50	0.29	0.740	0.065	0.413
Colon	6.67	6.30	6.42	6.22	0.28	0.459	0.192	0.685

[a, b] Within a row, means without a common superscript letter differ ($P < 0.05$).

3.3. Digesta pH

The challenge did not affect the pH in jejunal and ileal digesta (Table 4). The probiotics decreased ($P < 0.05$) the pH of the jejunal digesta in unchallenged pigs, whereas it tended ($P = 0.065$) to reduce the pH in ileal digesta regardless the challenge.

3.4. Histomorphology evaluation

In the jejunum, the ETEC challenge decreased the villus height (Table 5). Whereas, there was an interaction ($P < 0.05$), and the probiotics increased the villus height in pigs challenged with ETEC. The villus width was not affected by the factors. The ETEC challenge reduced ($P < 0.05$) the crypt depth, whereas the probiotics increased ($P < 0.05$) it, regardless the challenge. The ETEC challenge did not affect the villus height-to-crypt depth (VH:CD) ratio, whereas the probiotics tended ($P = 0.087$) to reduce the VH:CD ratio, regardless the challenge. However, there was an interaction ($P < 0.05$), and the prebiotic reduced the VH:CD ratio in unchallenged pigs.

In the ileum, the factors did not affect the villus height, villus width, and VH:CD ratio. However, the ETEC challenge increased ($P < 0.05$) the crypt depth, whereas the probiotics did not affect it.

3.5. Spleen weight, inflammatory cytokine, and oxidative stress status

The ETEC challenge did not affect the spleen weight, whereas the probiotics tended ($P = 0.081$) to increase it. The ETEC challenge increased ($P < 0.05$) the concentration of jejunal TNFα, whereas the probiotics did not affect it (Table 6). In the ileum, the concentration of jejunal TNFα was not affected by the factors. In the serum collected at d 19 after weaning, there was an interaction ($P < 0.05$),

Table 5
Intestinal histomorphology of pigs challenged with *E. coli* F18$^+$ (CH) on d 13 postweaning and fed diets supplemented with multispecies probiotics (PRO).

Item	CH-		CH+		SEM	*P*-value		
	PRO-	PRO+	PRO-	PRO+		CH	PRO	CH × PRO
Jejunum								
Villus height, μm	452.6^a	411.6^{ab}	376.5^b	416.5^{ab}	19.9	0.048	0.978	0.027
Villus width, μm	120.9	111.8	110.5	111.3	4.1	0.139	0.261	0.176
Crypt depth, μm	232.5	248.3	213.8	228.8	8.6	0.010	0.032	0.954
VH:CD ratio	1.95^a	1.66^b	1.77^{ab}	1.82^{ab}	0.07	0.908	0.087	0.015
Ileum								
Villus height, μm	285.2	319.4	318.6	331.2	28.9	0.316	0.299	0.630
Villus width, μm	91.7	97.2	91.4	103.3	7.91	0.600	0.123	0.562
Crypt depth, μm	237.9	244.6	258.6	290.2	18.25	0.019	0.160	0.353
VH:CD ratio	1.18	1.31	1.22	1.15	0.06	0.257	0.611	0.091

VH:CD ratio = villus height-to-crypt depth ratio.
$^{a, b}$ Within a row, means without a common superscript letter differ ($P < 0.05$).

Table 6
Immune status and oxidative stress of pigs challenged with *E. coli* F18$^+$ (CH) on d 13 postweaning and fed diets supplemented with multispecies probiotics (PRO).

Item	CH-		CH+		SEM	*P*-value		
	PRO-	PRO+	PRO-	PRO+		CH	PRO	CH × PRO
Spleen weight, g	21.11	26.95	21.18	28.62	3.59	0.813	0.081	0.827
TNFα								
Jejunum, pg/mg	0.48	0.40	0.56	0.89	0.23	0.039	0.346	0.126
Ileum, pg/mg	0.86	0.77	1.16	0.71	0.33	0.698	0.401	0.567
Serum[1], pg/mL	57.84^{ab}	66.47^a	70.01^a	44.92^b	6.06	0.446	0.187	0.010
Serum[2], pg/mL	34.61	35.77	32.50	46.17	6.67	0.516	0.251	0.330
MDA								
Jejunum, μmol/mg	0.58	0.65	0.64	0.63	0.12	0.830	0.809	0.717
Ileum, μmol/mg	0.84	0.73	0.89	0.64	0.09	0.854	0.064	0.438
Serum[1], μmol/mL	10.19	6.30	14.37	9.88	4.37	0.184	0.153	0.917
Serum[2], μmol/mL	11.42	6.21	12.91	10.12	4.92	0.435	0.252	0.725

TNFα = tumor necrosis factor alpha; MDA = malondialdehyde.
$^{a, b}$ Within a row, means without a common superscript letter differ ($P < 0.05$).
[1] Blood samples were collected at d 19.
[2] Blood samples were collected at d 25.

and the probiotics reduced the concentration of TNFα in pigs challenged with ETEC. In the serum collected at d 25 after weaning, the factors did not affect the concentration of TNFα.

The concentration of MDA was not affected by the factors in the jejunal mucosa or in the serum collected on d 19 and 25 after weaning. Whereas, in the ileal mucosa the probiotics tended ($P = 0.064$) to reduce the concentration of MDA.

3.6. Gut hormones

The ETEC challenge did not affect the concentration of PYY in the serum on d 19 or 24 (Table 7). The probiotics tended to increase ($P = 0.092$) the concentration of PYY in the serum at d 19 after weaning. The ETEC challenge reduced ($P < 0.05$) the concentration of NPY in the serum of pigs on d 19 after weaning, whereas the probiotics did not affect it.

Table 7
Serum peptide YY (PYY) and neuropeptide Y (NPY) of pigs challenged with *E. coli* F18$^+$ (CH) on d 13 postweaning and fed diets supplemented with multispecies probiotics (PRO) (pg/mL).

Item	CH-		CH+		SEM	*P*-value		
	PRO-	PRO+	PRO-	PRO+		CH	PRO	CH × PRO
PYY								
d 19	76.7	103.9	70.2	103.0	17.3	0.829	0.092	0.872
NPY								
d 19	20.8	16.6	3.4	8.7	5.3	0.015	0.914	0.314

4. Discussion

Enterotoxigenic *E. coli* is related to important economic losses in the swine production around the world (Fairbrother et al., 2005; Rhouma et al., 2017; Sun and Kim, 2017). In agreement with the previous reports, this study confirmed *E. coli* F18$^+$ as a pathogen causing PWD, affecting the gut histomorphology, the immune response and the oxidative stress status (Duarte et al., 2020; Gresse et al., 2017; Luise et al., 2019b), however without affecting the growth performance of the pigs (McLamb et al., 2013).

The oral inoculation of *E. coli* F18$^+$ caused diarrhea in 63% of the pigs in this study. The greater fecal score in response to the ETEC challenge is a clinical indicator that the pigs had ETEC infection after the challenge (Luise et al., 2019b; Luppi et al., 2016). The weaning stress factors cause a disturbance on the immune system, increasing the susceptibility of newly-weaned pigs to ETEC infection. The increasing in the fecal score is related to a disruption on the electrolytes fluid system in the intestinal epithelium caused mainly by the enterotoxins (including STa, and STb) from ETEC (Dubreuil et al., 2016; Kaper et al., 2004; Nagy et al., 1997; Nagy and Fekete, 2005).

Although the fecal score of the pigs has increased, the growth performance was not affected by the ETEC challenge. Enterotoxigenic *E. coli* infection can be considered a multifactorial process that, beside the virulence of the strains, requires a combination of many factors to promote a more severe infection (Bin et al., 2018; Luise et al, 2019a, 2019b; Moredo et al., 2015; Opapeju et al., 2010; Wellock et al., 2008). Furthermore, the pigs seem to be recovering from the ETEC infection as demonstrated in the reduction in the

concentration of TNFα from d 6 to 12 post−challenge. McLamb et al. (2013) demonstrated that weaning age has a great impact on the response of the pigs to the *E. coli* F18$^+$ challenge. In oral inoculated challenge models, the dosage and the response of the pigs to the challenge varied widely among studies (Luise et al., 2019b). The results in this study indicated that the *E. coli* F18$^+$ challenge at 2×10^9 CFU leads to a less severe infection in pigs weaned at 21 d of age with BW 6.99 ± 0.33 kg. The pigs used in this study were not tested for ETEC F18 susceptibility, however the pigs were selected from sows not infected previously and not vaccinated against ETEC as suggested by Luise et al. (2019a).

The use of probiotics enhanced the growth performance of the pigs by increasing the ADG, ADFI, and feed efficiency. This results are in agreement with previous studies using the same probiotics mixture for chickens (Chichlowski et al., 2007; Grimes et al., 2008; Rahimi et al., 2011). Rahimi et al. (2009) reported that the improved growth performance and feed efficiency were associated with greater villus density in the duodenum, jejunum, and ileum in birds. Dietary supplementation with *L. acidophilus* or multi-strains of Lactobacilli have been related to improvement of growth performance of pigs after weaning (Huang et al., 2004). The increase in the feed intake and feed efficiency of pigs fed diets supplemented with probiotics reported in the present study can be related to the gut health of pigs further increasing the ADG.

The role of probiotics is to improve gut health and stimulating effect on the digestive processes and the immunity of the host by positively influence the colonization and composition of gut microflora including modulate gut pH (Barba-vidal et al., 2019). It has been proposed that multi-strain/species probiotics can be more effective due to the combination of different modes of action of genus, species and strain (Chapman et al., 2011; Sanders and Veld, 1999; Timmerman et al., 2004). The probiotics used in this study is a mixture of *L. acidophilus, L. casei, B. thermophilum* and *E. faecium*. These microbials are lactic acid-producing bacteria (Pringsulaka et al., 2015; Yang et al., 2015) that can decrease the pH, improve the immune system, increase the intestinal function, and modulate the microbiome, which consequently, can enhance the growth performance of weaning pigs (Chichlowski et al., 2007; Valeriano et al., 2017).

The ETEC challenge did not affected the concentration of TNFα in serum, whereas, the probiotics reduced it on d 6 post−challenge. On d 12 post−challenge, the levels of TNFα in the serum were lower than those on d 6 post−challenge indicating that pigs was recovering from the weaning stress and the *E. coli* F18$^+$ infection. According to Luise et al. (2019a) the peak of *E. coli* F18$^+$ infection in newly-weaned pigs is around 3 to 5 d post−challenge. Roselli et al. (2006) reported that probiotics containing *Bifidobacterium animalis* and *Lactobacillus rhamnosus* can reduce adhesion of ETEC to the intestinal cells, which can reduce the immune response. Barba-Vidal et al. (2017) reported that probiotics with *Bifidobacterium longum* improve immune responses in challenged pigs. The ETEC challenge increased the immune response of the pigs by increasing the jejunal mucosal concentration of the TNFα at the end of the study. These outcomes may indicate that the ETEC challenge have different roles on the immune system in the blood and in the intestinal mucosa. Pigs infected with *E. coli* F18$^+$ have the immune system activated mainly by the fimbriae F18 (Sarrazin and Bertschinger, 1997; Smeds et al., 2011) as well as the lipopolysaccharide (LPS) present in the bacteria wall (Ji et al., 2020). According Liu et al. (2013) the expression of TNFα is stimulated by the LPS on the membrane of the ETEC. Tumor necrosis factor alpha is produced mainly by macrophages and T lymphocytes in different tissues in response to infection (Bradley, 2008). Spleen tissues upregulate the expression of pro-inflammatory cytokines such as TNFα (Touchette et al., 2002). In response to the increasing TNFα, the weight of the spleen was increased by the *E. coli* F18$^+$ challenge in this study. A similar result was found by Kiarie et al. (2009) in pigs challenged with *E. coli* F4$^+$. Liu et al. (2016) related that the spleen tissues showed high expression of TNFα when stimulated by *E. coli* F18$^+$.

Besides playing important roles in the immune system, the TNFα can activate the apoptosis of epithelial cells in the intestine (Schmitz et al., 1999) reducing the villus height, as shown in this study. Becker et al. (2020) also reported that *E. coli* F18$^+$ challenge reduced the villus height and reduced the VH:CD ratio. The cell death in the intestinal epithelium of challenged pigs can also be caused by the enterotoxins from ETEC. The great fluid loss caused by the enterotoxins in infected pigs damages the epithelial cells causing villus atrophy (Berberov et al., 2004). The structure of the intestinal mucosa is an indicator of the gut health status (Pluske et al., 1997). The villus height is usually associate with mucosal surface area and the enterocyte absorption of nutrients (Caspary, 1992).

As an attempt to repair the intestinal epithelium in the challenged pigs, the cell proliferation rate increases (Pluske et al., 2018). The enterocyte proliferation occur on the villus crypt, and a deeper crypt indicates fast tissue turnover in response to normal sloughing or inflammation from pathogens or their toxins and high demands for tissues (Awad et al., 2010; Xiong et al., 2019). Decreased villus height and greater crypt depth can be reasons for poor nutrient absorption, increased gastric secretions, diarrhea, and lower performance (Xu et al., 2003).

Besides the ETEC challenge, the weaning stressors can trigger the production of reactive oxygen species (ROS) (Nakagawa and Miyazaki, 2017). Increasing levels of ROS can damage structural molecules such as proteins, DNA and lipids (Celi and Gabai, 2015; Sido et al., 2017). The final product of the lipid oxidation is MDA, which has been considered an indicator of the oxidative (Mateos and Bravo, 2007). The *E. coli* F18$^+$ challenge did not affect the concentration of MDA in mucosa or serum in this study, although the ETEC challenge has been related to increase the oxidative stress in pigs (Humphrey et al., 2019). The probiotics slightly reduced the concentration of MDA in the ileal mucosa of the pigs. Lactic acid bacteria has been related to exert several biological activities such as antioxidant functions (Nakagawa and Miyazaki, 2017). The antioxidant capacity of the lactic acid bacteria may be related to the exopolysaccharides present in the cell wall of these microbials (Guo et al., 2013; Moscovici, 2015).

The PYY are expressed and secreted by the enteroendocrine L-cells in the ileum and colon (Greiner and Bäckhed, 2016). The plasma concentration of PYY increases in response to feeding and the expression and secretion is stimulated by the lipids, proteins, and carbohydrates content in the diet, as well as the microbiota and their metabolites (Covasa et al., 2019; Steinert et al., 2013). Intestinal microbials, including those added as probiotics, produce short chain fatty acids (SCFA) that stimulate the secretion PYY (Chang et al., 2019). Probiotics has been related to increase the concentration of PYY in the plasma of rats (Lesniewska et al., 2006). The greater concentration of PYY reported in this study can be a consequence of the greater feed intake, considering that the blood was collected after the meal and that the postprandial increases the concentration of PYY (Ueno et al., 2008). The NPY is secreted mainly by the hypothalamic arcuate nucleus (Loh et al., 2015). Beyond others functions, the NPY increase the intestinal absorption of water and electrolytes (Holzer-Petsche et al., 1991). Therefore, the greater fecal score and the low concentration of NPY in challenged pigs reported in this study can indicate that NPY play a role during diarrhea due to the secretion of water to the intestinal lumen.

5. Conclusion

In conclusion, ETEC challenge increased the fecal score of newly weaned pigs, increasing the intestinal immune response and crypt depth, whereas reducing the villus height in the jejunum and the serum concentration of NPY without affecting the growth performance. Dietary supplementation of multispecies probiotics enhanced growth performance by reducing the pH of digesta, systemic immune response, and intestinal oxidative stress, and by increasing the villus height in the small intestine and the concentration of PYY in serum, regardless the ETEC challenge. Therefore, the *E. coli* F18$^+$ challenge affected the intestinal heath of the pigs, whereas the multispecies probiotics seems to be effective in reducing or impairing the effects of *E. coli* F18$^+$ infection.

Author contributions

Y. Sun: data curation; formal analysis; investigation; methodology; resources; software; validation; visualization; roles/writing – original draft; writing – review & editing. **M. E. Duarte**: methodology; resources; software; validation; visualization; roles/writing – review & editing. **S. W. Kim**: conceptualization; data curation; funding acquisition; investigation; methodology; project administration; resources; software; supervision; validation; roles/writing – original draft; writing – review & editing.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that might inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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