Impacts of feeding organic acid-based feed additives on diarrhea, performance, and fecal microbiome characteristics of pigs after weaning challenged with an enterotoxigenic strain of *Escherichia coli*

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ABSTRACT: Post weaning diarrhea (PWD) caused by enterotoxigenic strains of *E. coli* (ETEC) remains a major problem in the industry, causing decreases in performance and survival of weaned pigs. Traditionally, antimicrobials have been used for its mitigation/control. This study tested the hypothesis that a combination of two organic acid (OA)-based commercial feed additives, Presan FX [an OA, medium-chain fatty acid (MCFA) and phenolic compound-based product] and Fysal MP (free and buffered OA based on formic acid), would reduce PWD and improve post-weaning performance in pigs challenged with an F4-ETEC. This combination was assessed against a Negative control diet without any feed additives and a diet containing amoxicillin. Combined with a reduction in temperature during the infection period, inoculation with F4-ETEC resulted in 81% of pigs developing diarrhea, but with no differences between treatments ($P > 0.05$). However, between days 14 to 20 of the study and due to colonization by *Salmonella* serovars, pigs fed the combination of Presan FX and Fysal MP showed less ($P = 0.014$) diarrhea commensurate with a lower ($P = 0.018$) proportion of *Salmonella* numbers relative to total bacterial numbers. This caused less ($P = 0.049$) therapeutic antibiotic administrations relative to the diet with amoxicillin during this time. The diversity of bacteria within amoxicillin-treated pigs was lower ($P = 0.004$) than the diversity in control or Presan FX + Fysal MP-treated pigs ($P = 0.01$). Pair-wise comparisons showed that amoxicillin-treated pigs had altered ($P < 0.001$) fecal microbial communities relative to both Presan FX + Fysal MP-treated pigs and control pigs. Amoxicillin-treated pigs were characterized by an increased abundance of bacterial families generally linked to inflammation and dysbiosis in the gastrointestinal tract (GIT), whereas Presan FX + Fysal MP-treated pigs had an increased abundance of bacterial families considered beneficial commensals for the GIT. Control pigs were characterized by an increased abundance of *Spirochaetaceae* associated with healthy piglets, as well as bacterial families associated with reduced feed intake and appetite. The combination of two OA-based feed additives did not reduce the incidence of F4 ETEC-associated diarrhea nor enhance performance. However, the combination markedly reduced diarrhea caused by *Salmonella* that occurred following the ETEC infection, commensurate with less therapeutic administrations relative to the diet with amoxicillin.

Keywords: diarrhea, enterotoxigenic *Escherichia coli*, medium-chain fatty acids, microbiome, organic acids, pig, *Salmonella*, weaning

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INTRODUCTION

Finding sustainable and cost-effective solutions to post-weaning diarrhea (PWD) is essential to limit economic losses and decreases in the performance and survival of weaned pigs. The disease is caused by strains of enterotoxigenic *E. coli* (ETEC; F4 or F18) that adhere to receptors on the enterocytes and colonize the surface of the small intestine after weaning, releasing enterotoxins that enhance the net secretion of water to the intestinal lumen (Pluske et al., 1997; Pluske et al., 2018; Laird et al., 2021). Antimicrobials, including antibiotics, have traditionally been used for the treatment of PWD, but rising consumer/retailer concerns, the development of antimicrobial-resistant bacteria, and bans or restrictions on the use of selected antimicrobials in various jurisdictions, have collectively compelled the need to use different approaches to this issue (Pluske, 2013).

There is a plethora of nutritional, management, and (or) veterinary strategies that can be used to mitigate or treat PWD (de Lange et al., 2010; Liu et al., 2018; Pluske et al., 2018). Amongst nutritional approaches, commercial feed additives exist, for in-feed and (or) water application, that offer potential solutions to PWD. Products based on organic acids (OA) and medium-chain fatty acids (MCFA) have received much attention and are routinely used in the nursery. Numerous studies over many years have shown their beneficial production effects (Partanen and Mroz, 1999; Suiryanrayna and Ramana, 2015; Mesonero Escuredo et al., 2016) and positive impacts on the gastrointestinal tract (GIT), including changes to the microbiome (Roth and Kirchgessner, 1998; Li et al., 2018; Tugnoli et al., 2020).

It is likely that a combination of OA- and MCFA-based feed additive products will be more efficacious in combatting PWD than an individual product alone since products that differ in their composition can have specific targeted effects against pathogens and different modes of action, either in different segments of the GIT and (or) in the feed and water. In the present study, the efficacy of a combination of two commercial products, Presan FX and Fysal MP (Trouw Nutrition; The Netherlands), was evaluated. The study tested the hypothesis that the use of Presan FX and Fysal MP would reduce diarrhea and improve growth performance of weaned piglets challenged with F4-ETEC, relative to a diet containing an antibiotic. It was also anticipated that favorable shifts in the microbiome would occur with this combination.

MATERIALS AND METHODS

This experiment was approved by the Murdoch University Animal Ethics Committee (R3083/13).

Animals, Housing, Experimental Design, and Diets

A total of 84 male weanling pigs (Large White x Landrace) aged approximately 21 d was weighed and ear-tagged on a commercial farm and then transported approximately 45 min to the research facility at Murdoch University. Upon arrival, pigs were blocked in groups of four pigs per pen in a temperature-controlled and ventilated facility and randomized, on the basis of body weight (BW) and pen location, to one of the three treatments (7 replications of 4 pigs per pen, with a total of 28 pigs per treatment). Before weaning, 170 pre-weaned piglets from the farm of origin were genetically screened for the MUC4 gene before the start of the study to be homozygous susceptible to F4-ETEC infection (Sterndale et al., 2019a).

The three treatments tested were 1) basal diet without antibiotic (Negative control; T1); 2) basal diet with antibiotic (amoxicillin; T2); and 3) basal diet with Presan FX (a blend of a phenolic compound (>0.5%), slow release C12 (>5%), target-release butyrate (>10%) and sorbic acid (>5%), MCFA (C8-C10, >20%), OA (>5%), and silica) plus Fysal MP (formic acid >25%, propionic acid >10%, acetic acid >5%, ammonium formate >15%, and silica; T3) (both from Trouw Nutrition, The Netherlands) (Table 1). A wheat/barley-soybean-based diet was formulated without any additional additives or medication to be isocaloric and

Table 1. A summary of the treatments used in the study

Treatment	ZnO, ppm	Amoxicillin, kg/t	Presan FX, kg/t	Fysal MP, kg/t
T1:Basal diet (Negative control)	1,000	–	–	–
T2: Basal diet + Antibiotic (amoxicillin)	1,000	0.020	–	–
T3: Basal diet + Presan FX + Fysal MP	1,000	–	2	4

isonitrogenous (Table 2). Zinc oxide (ZnO) was added to all diets at a sub-pharmacological inclusion level of 1,000 ppm (mg/kg). A master-batch of the basal diet using the same batch of ingredients was prepared and then the additives were added on top of the basal diet, mixed well, and thereafter put into pre-marked feed bins. After the individual feed was made, a representative sample of 2 kg was taken out of the mixer and subsequently divided into two sub-samples (1 kg/each) for subsequent feed analysis.

Experimental F4 ETEC Challenge

All pigs were orally inoculated with 0.8 mL of F4-ETEC broth (serotype O149; F4; toxins LT, STa, STb, EAST) on days 5 and 6 after weaning. The F4-ETEC was encapsulated in a gelatin capsule and administered to the pigs (Sterndale et al., 2019b). The concentrations of F4-ETEC inoculated were 7.84×10^9 cfu (0.8 mL of 9.8×10^9) and 8.16×10^9 cfu (0.8 mL of 1.02×10^{10}) on days 5 and 6, respectively. Fecal consistency (FC), diarrhea index [DI; (total number of days with score 4 diarrhea/number of days) × 100], and the number of therapeutic antibiotic treatments were recorded. Fecal consistency was visually examined at the same time each morning by the same person on days 1 to 14, and scored on a scale of 1 to 5 as follows: 1, dry and granulated; 2, dry and firm shaped; 3, moist and soft with largely retained shape; 4, pasty diarrhea; and 5, watery diarrhea.

Post-weaning diarrhea was recorded when a pig developed pasty or a watery fecal consistency (FC $\geq$4) accompanied with a stained perineum. All pigs were swabbed upon arrival to determine β-hemolytic *E. coli* shedding by inserting an alginated tipped swab into the anus. This was repeated on days 3, 4, 5, 6, 7, and 11 after weaning. Swabs were cultured onto sheep blood (50 mL/liter) agar plates and incubated overnight at 37 °C. An assessment of the proportion of β-hemolytic *E. coli* recovered on each blood plate was made according to the number of sections (0 to 5) expressing continued streaks of clearing hemolysis around colonies displaying morphology characteristic of β-hemolytic *E. coli*, where 0 = no growth and 5 = confluent growth to the last streak. Pigs that developed a diarrhea score of FC≥4 on two consecutive days were treated with antibiotics [Moxylan IM (amoxicillin 150 mg/

Table 2. Ingredient and nutrient composition of the basal diet (as-fed basis)

Ingredients	Amount, %
Barley	10
Wheat	44.1494
Soybean meal	15
Bloodmeal	1.7247
Fishmeal	8.0369
Whey powder	15.873
Canola Oil	3.157
Lysine	0.2856
Methionine	0.2395
Threonine	0.1347
Tryptophan	0.0724
BJ-Pig Grower Plus[a]	0.15
Limestone	0.4603
Dicalcium phosphate	0.2207
Salt	0.2
Zinc oxide	0.1
Choline chloride 60%	0.0458
Calculated analysis (%)	
ME, MJ/kg	14.2
Dry matter	91.7
Protein	21
Fat	5.0
Crude fiber	2.3
Ash	3.5
Av Lysine	1.35
Lysine	1.36
Av Methionine	0.54
Av Cystine	0.27
Av M+C	0.81
Av Threonine	0.85
Av Tryptophan	0.30
Av Isoleucine	0.77
Av Leucine	1.44
Av Phos	0.45
Calcium	0.9
Phosphorus	0.68

[a]Provided the following nutrients (per kg of air-dried diet): vitamins: A, 7000 IU; D3, 1400 IU; E, 20 mg; K, 1 mg; thiamine, 1 mg; riboflavin, 3 mg; pyridoxine, 1.5 mg; cyanocobalamin, 15 **m**g; calcium pantothenate, 10.7 mg; folic acid, 0.2 mg; niacin, 12 mg; biotin, 30 **m**g. Minerals: Co, 0.2 mg (as cobalt sulfate); Cu, 10 mg (as copper sulfate); iodine, 0.5 mg (as potassium iodine); iron, 60 mg (as ferrous sulfate); Mn, 40 mg (as manganous oxide); Se, 0.3 mg (as sodium selenite); Zn, 100 mg (as zinc oxide); BJ Grower 1, BioJohn Pty Ltd., WA, Australia.

mL, Jurox Pty Ltd., Rutherford NSW, Australia)] as recommended by a veterinarian and in compliance with the directions of the Animal Ethics Committee. The treatment continued until the diarrhea stopped.

Temperature in the rooms upon entry was set at ~28 °C, and during inoculation was lowered to ~23 °C to further induce diarrhea (Wathes et al., 1989). Thereafter and until the end of the second week of the study, temperature was maintained at ~28 °C. In the third week, the temperature was reduced to ~26 °C.

Fecal Sampling and Feed Analysis

A representative pooled sample of fresh feces was collected from the floor of each pen within each treatment at day 21 of the study, and then frozen for subsequent PCR and next generation sequencing (NGS) of fecal microbiota.

Feed samples were analyzed (Agrifood Technology, Bibra Lake, WA, Australia) for dry matter, crude protein, crude fat, crude fiber, ash, Ca, P, and Zn according to standard procedures (Table 2). Analysis of sorbic acid, to confirm the addition of Presan FX, was undertaken at the National Measurement Institute (Melbourne, VIC, Australia).

DNA Extraction and Quantitative Real-Time PCR

The Wizard DNA purification Kit (Promega) was used to extract DNA from pooled pen fecal samples according to the manufacturer's instructions. Briefly, 200 mg of feces were suspended in 700 µL of phosphate-buffered saline, heated to 55 °C for 30 min, and then centrifuged at 12,000*g* for 5 min. The supernatant (approximately 0.3 mL) was mixed with 0.8 mL of the Wizard resin and drawn through a column by vacuum. Each sample was washed with 2 mL of 80% isopropanol and dried before DNA was eluted in 50 µL of 10mM Tris, pH 8.0. Real-time polymerase chain reaction (PCR) assays were performed to quantify *E. coli* with F4 fimbria, *Salmonella* spp., and the total bacterial population. Standard curves for each of the qPCRs were constructed in duplicate from 10-fold serial dilutions of each DNA extracted from known numbers of pure cultures of F4 *E. coli* and *Salmonella* Typhimurium enumerated by ISO 16649-2 and ISO 21528-2, respectively. Published primers and probes for F4 *E. coli* (Franklin et al., 1996), total bacteria (Suzuki et al., 2000), and *Salmonella* spp. (Malorny et al., 2004) were synthesized by Biosearch Technologies (Novato, USA). TaqMan probes were synthesized with 5-carboxyfluorescein (FAM) on the 5′ end and Black Hole Quencher (BHQ-1) on the 3′ end. The compound SYBR green was used to visualize amplicons for the F4 *E. coli* RT PCR.

One-fifth volume (5 µL) of undiluted DNA was added to the F4 *E. coli* and *Salmonella* spp. qPCR assays, but DNA was diluted by one-tenth for total bacteria qPCR. The F4 fimbria *E. coli* qPCR cocktail included 7.5 pmoles of both forward and reverse primers in the SensiMix SYBR Green Low-ROX reaction kit (Bioline, Australia). Product was amplified over 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 60 s, following an initial denaturation at 95 °C for 10 min. The other qPCR assays included 5 pmoles of both forward and reverse primers and 1 pmole of the correct probe in the AgPath-ID RT-PCR buffer (Applied Biosystems, Foster City, USA). After an initial denaturation step of 95 °C for 10 min, the PCR products were amplified over 40 cycles of 95 °C for 15 s, followed by annealing at 58 °C for total bacteria or 60 °C for *Salmonella spp*. for 30 s, on a 7500 Fast PCR machine (Applied Biosystems, Foster City, USA).

Next Generation Sequencing

The concentration of DNA from each sample was quantified with the Qubit dsDNA BR assay on the Qubit fluorometer (Molecular Probes), and 75 ng of each extract was submitted to the commercial Ramaciotti Centre for Genomics (University of New South Wales) for 16S rRNA V4 (515f-806r) amplicon library preparation and sequencing (Illumina MiSeqV2, 2 × 250bp runs). All amplified DNA was sequenced in both directions and paired-end reads were imported into the Qiagen CLC Genomics workbench v21. Sequencing adapters and ambiguous nucleotides were trimmed, chimeras removed, sequence reads below 5 were discarded, and sequences with less than 100 reads were filtered out. Overlapping forward and reverse paired reads were merged to produce one high-quality read.

Operational Taxonomic Unit (OTU) clustering was performed according to the manufacturer's instructions (Qiagen, 2021) using the reference database provided (16S_97_otus_GG.clc). The metadata (treatments and sample numbers) were aggregated with the OTU table produced from clustering analysis. Low abundance OTUs (less than 10) were filtered out of the data. A phylogenetic tree of all OTUs was constructed using a maximum likelihood analysis based on multiple sequence alignment of the OTUs generated

by MUSCLE. Alpha diversity analysis used this phylogenetic tree to provide an estimate of the diversity of bacteria within a sample. Rarefaction analysis set the minimum and maximum depth to sample at 1 and 5,000, with 20 depths to be sampled and 100 replicates at each depth. Rarefaction plots were checked for plateauing of the phylogenetic diversity in all 21 samples, to indicate good coverage of bacterial sequences. Significant differences in the median phylogenetic diversity between treatments were determined by the non-parametric Kruskal–Wallis test.

Beta diversity was measured by UniFrac, an analysis tool used to determine whether communities are significantly different by using phylogenetic data for each sample to cluster similar bacterial communities together and display the results with standard multivariate statistical techniques including principal coordinates analysis (PCoA). Beta diversity analysis estimated differences in species diversity between samples. The treatment and pen replicate data were incorporated into the results to observe differences in relative abundance of bacterial species between treatments.

Differential abundance analysis was used to determine which 25 OTUs had the most significantly different abundances across all treatments using a generalized linear model for each OTU. A Likelihood Ratio test was used to determine significance across the three treatments (ANOVA-like). A heat map for abundance and corresponding dendogram was constructed from the output with the dendogram distance set to Euclidian.

Linear discriminate analysis Effect Size (LEfSe) was performed to determine the combination of OTUs (bacterial groups) that most likely explained the microbiome differences between treatments, by combining biological consistency and effect relevance to traditional statistical tests (Segata et al., 2011). This differs from testing significant differences in the relative abundance of individual bacterial families between treatments by ANOVA.

Statistical Analyses

A test for normality and outliers (Shapiro–Wilk statistic; <0.8 was considered to be not Normally distributed) was used for all variables, and data points were removed as appropriate. A one-way analysis of variance (ANOVA) with post hoc comparisons (Least Significant Difference; LSD) was used for the statistical evaluation of the effect of diet on BW, average daily gain (ADG), average daily feed intake (ADFI), feed conversion efficiency, and qPCR data (bacterial numbers were $\log_{10}$ transformed). Feed conversion efficiency was calculated as gain:feed (G:F; g bodyweight gain: g feed intake) for days 1 to 7, to account for the low feed intake and high incidence of BW loss, and was then calculated as feed conversion ratio (FCR; g feed intake: g bodyweight gain) in subsequent periods. The treatment was the fixed factor and block was included as the random factor in the analysis. The pen was the experimental unit (unless otherwise stated). Statistical significance was accepted at $P < 0.05$, and a trend was considered at $P < 0.1$.

The distribution for the fecal data [*E. coli* score, FC score, DI, and number of days pigs treated for diarrhea] was not normally distributed and therefore was analyzed non-parametrically using a Kruskal–Wallis test with post hoc comparisons (LSD) to determine which groups were significantly different from each other. A Chi-squared analysis was used to examine differences between treatments for the percentage of pigs treated for diarrhea. The individual pig was the experimental unit. Statistical significance was accepted at $P < 0.05$, and a trend was considered at $P < 0.1$.

Significant differences in microbial communities between treatments were determined by permutational multivariate analysis of variance (PERMANOVA; Qiagen CLC Genomics Workbench), a non-parametric multivariate statistical test. The null hypothesis tested whether the clustering (made up of center points and dispersion) of bacterial communities was equivalent for all groups, i.e., a measurement of the effect size and significance of beta diversity between treatments. Differences in the ratios of F4 *E. coli* and *Salmonella* spp. to total bacteria between treatments were compared by one-way ANOVA. Statistical significance was accepted at $P < 0.05$, and a trend was considered at $P < 0.1$. All statistical analyses were conducted with SPSS (version 24, IBM corporation, Armonk, NY).

RESULTS

Analysis of feed samples showed generally good agreement with the calculated analysis. The level of (total) Zn in the control diet was lower than that in the other two diets; however, at this level of dietary Zn, no differences in physiological effects were anticipated. The detected level of sorbic acid in the diet Presan FX + Fysal MP was 130 mg/kg (Table 3).

Post-weaning Diarrhea

The oral dose of F4-ETEC combined with a reduction in the temperature during the inoculation period resulted in 81% of the pigs developing

Table 3. Diet composition and analysis (%, as-fed)

	Diet		
Item	Control	Antibiotic	Presan FX + Fysal MP
DE, MJ/kg[a]	13.5	13.6	13.6
Dry matter	90.8	91.1	91.6
Crude protein	20.8	20.7	21.2
Nitrogen-free extract	58.1	58.4	57.7
Crude fiber	2.4	2.1	2.2
Ash	5.5	5.7	6.2
Crude fat	4.0	4.2	4.3
Calcium, mg/kg	9,500	11,000	11,000
Phosphorus, mg/kg	6,900	7,400	8,000
Zinc, mg/kg	800	915	940
Sorbic acid, mg/kg[b]	<5	<5	130

[a]DE, digestible energy; calculated from ME (derived)/0.96.

[b]ND, not detected.

diarrhea, i.e., a score of 4 or 5 at least once between the first day of inoculation and the end of the study. Of these pigs, 48 required treatments with antibiotics (2 d of score 4 or 5/5 diarrhea or clinically unwell). However, 29/48 pigs that received injectable amoxicillin (Moxylan; Jurox Australia) did not respond to the treatments and had to be changed to a different antibiotic. This was unexpected since previous testing of the inoculated strain confirmed sensitivity to amoxicillin. Further to this, several pigs developed more severe clinical signs (wasting, inappetence, lethargy, and dehydration) than expected.

Consequently, fecal samples were taken from two pigs that had received a full course (5 d) of amoxicillin with no improvement in diarrhea score. Whilst waiting for the culture and sensitivity results, pigs not responding to injectable amoxicillin were changed to oral Scourban (Bayer AG, Leverkusen, Germany; Sulfadimidine 71 mg/mL, Sulfadiazine 71 mg/mL, Streptomycin sulfate 7.6 mg/mL, Neomycin sulfate 1.8 mg/mL; and in each 30 mL contained Sodium chloride 133 mg, Calcium gluconate 5.9 mg, Magnesium sulfate 1.77 mg, Potassium chloride 57 mg, Hyoscine hydrobromide 0.95 mg, Pectin 159.6 mg, Kaolin 3.1 g, and Glycine 627 mg). In addition to receiving antibiotic treatment where necessary, all pens were offered an electrolyte solution (Vet Only, Pig Restart; Portec Pty Ltd.) twice daily after inoculation via extra feeders placed in the pens, and any pigs with a fever or showing signs of discomfort were given an injectable anti-inflammatory compound (Meloxicam; Troy Laboratories, Australia). Furthermore, any pigs showing signs of inappetence (hollow belly), dehydration or general lethargy were offered gruel in the pen to encourage eating, and extra time was spent with these pigs to orally dose electrolytes and gruel during each morning and afternoon. By day 12 of the experiment, 40% of all pigs required additional treatment.

Fecal (culture) testing from the diarrheic pigs (VetPath; Belmont, Western Australia) showed the isolation of *Salmonella* Derby and *Salmonella* Typhimurium. Both species were found to be resistant to amoxicillin, which further exacerbated the diarrhea. Further testing of feces from other diarrheic pigs confirmed the presence of *Salmonella* serovars, indicating that co-infection with F4-ETEC had occurred. Collectively, the current experiment experienced a post-weaning mortality of 10.2%. Most pigs that died (7/9) were from the diet containing the antibiotic.

β-hemolytic E. coli *Shedding and FC Score*

Pigs fed the diet containing antibiotic shed less ($P < 0.05$) *E. coli* on days 6, 7, and 8 of the experiment compared to pigs on the other diets. Pigs fed the antibiotic also had a lower FC score on days 6 and 7, but a higher FC thereafter (days 14–20), than the control- or the Presan FX + Fysal MP-fed pigs (both $P < 0.05$). Before inoculation, pigs on the antibiotic treatment had a lower FC score than the control pigs, and pigs on the Presan FX + Fysal MP treatment had a FC score similar to pigs on the other two treatments ($P < 0.05$) (Table 4).

Diarrhea Measurements and Antibiotic Treatments

There were no significant differences between treatments for the DI, the average number of days pigs were treated for diarrhea, or the percentage of pigs treated for diarrhea over the study (Table 5). However, from days 14 to 20, pigs fed the

Table 4. Effects of feeding a Negative control diet, a diet supplemented with antibiotic, or a diet supplemented with Presan FX and Fysal MP, on fecal *E. coli* scores, and fecal consistency (FC) scores before and after *E. coli* infection

	Treatment			*P* value
Item	Control	Antibiotic	Presan FX + Fysal MP	
n	28 [x]	28 [y]	28	
*E. coli score**				
d 0	0.36 ± 0.201	0.25 ± 0.142	0.39 ± 0.195	0.897
d 5	0.07 ± 0.050	0	0.07 ± 0.071	0.368
d 6	2.25 ± 0.328[a]	0.46 ± 0.189[b]	1.89 ± 0.339[a]	<0.001
d 7	2.21 ± 0.301[a]	0.46 ± 0.209[b]	2.00 ± 0.381[a]	<0.001
d 8	1.61 ± 0.214[a]	0.39 ± 0.165[b]	2.00 ± 0.329[a]	<0.001
d 11	1.43 ± 0.310	1.75 ± 0.370	2.11 ± 0.350	0.511
FC score†				
d 0–5 (before inoculation)	2.3 ± 0.05[a]	2.1 ± 0.05[b]	2.2 ± 0.05[ab]	0.016
d 6 and 7 (inoculation)	3.8 ± 0.22[a]	2.8 ± 0.22[b]	3.6 ± 0.22[a]	0.004
d 8–13 (after inoculation)	3.1 ± 0.14	3.1 ± 0.18	3.0 ± 0.11	0.949
d 14–20 (after inoculation)	2.4 ± 0.12[a]	3.2 ± 0.22[b]	2.4 ± 0.11[a]	0.009

Data are expressed as mean ±SE of the mean.

n= number of pigs per treatment on day 0 of the experiment.

[x]1 pig euthanized on day 17 of the experiment.

[y]2 pigs euthanized on day 11 and day 15 of the experiment respectively and 2 pigs on day 17 of the experiment.

[a]Expressed according to the number of sections (0 to 5) expressing continued streaks of clearing hemolysis around colonies displaying morphology characteristic of *E. coli*, where 0 = no growth and 5 = confluent growth to the last streak. Data analyzed non-parametrically.

[b]FC (fecal consistency) score: scored on a scale of 1 to 5 as follows: 1, dry and granulated; 2, dry and firm shaped; 3, moist and soft with largely retained shape; 4, pasty diarrhea and 5, watery diarrhea. Data were analyzed non-parametrically.

[a,b]Means in a row not having the same superscript are significantly different ($P < 0.05$).

Table 5. Effects of feeding a Negative control diet, a diet supplemented with antibiotic or supplemented with Presan FX and Fysal MP, on diarrhea index (DI), the average number of days that pigs were treated therapeutically for diarrhea over the study with an antibiotic, and the percentage of pigs therapeutically treated for diarrhea after *E. coli* infection

	Treatment			*p* value
Item	Control	Antibiotic	Presan FX + Fysal MP	
n	28[x]	28[y]	28	
DI, %[a]	23.6 ± 3.16	27.0 ± 4.11	20.4 ± 2.75	0.590
Average number of days pigs treated for diarrhea	4.5 ± 0.71	5.0 ± 0.78	3.2 ± 0.60	0.279
Percentage of pigs treated for diarrhea[b]	71 (20/28)	68 (19/28)	61 (17/28)	0.687

Data are expressed as mean ±SE of the mean.

n= number of pigs per treatment on day 0 of the experiment.

[x]1 pig euthanized on day 17 of the experiment.

[y]2 pigs euthanized on day 11 and day 15 of the experiment respectively and 2 pigs on day 17 of the experiment.

[a]DI: diarrhea index; calculated as (total mean number of days with FC score 4 or 5 diarrhea/number of days) x 100. Data were analyzed non-parametrically.

[b]A diarrhea score of FC ≥ 4 on two consecutive days and treated therapeutically with antibiotics. Data were analyzed using a Chi-square test.

antibiotic-supplemented diet had a higher DI and were treated for clinical diarrhea for more days on average than the control or the Presan FX + Fysal MP-fed pigs ($P < 0.05$). The percentage of pigs treated for diarrhea in the antibiotic treatment was also higher than in the Presan FX + Fysal MP treatment ($P = 0.049$), but similar to the control pigs (Table 6).

Production Performance

There were no differences ($P > 0.05$) in BW or ADG between treatments during the 20-d experimental period. There was a trend ($P = 0.098$) for pigs fed the antibiotic diet to gain more weight than pigs on the control diet during the inoculation period (days 6 to 7), whereas the weight gain

Table 6. Effects of feeding a Negative control diet, a diet supplemented with antibiotic, or a diet supplemented with Presan FX and Fysal MP, on the diarrhea index (DI), the average number of days that pigs were treated therapeutically for diarrhea from days 14 to 20 of the study with an antibiotic, and the percentage of pigs therapeutically treated for diarrhea after *E. coli* infection

	Treatment			*P* value
Item	Control	Antibiotic	Presan FX + Fysal MP	
N	28[x]	28[y]	28	
DI, %[a]	18.7 ± 3.53[a]	36.7 ± 6.91[b]	14.3 ± 4.41[a]	0.014
Average number of days pigs treated for diarrhea[b]	1.3 ± 0.35[a]	3.3 ± 0.54[b]	1.1 ± 0.39[a]	0.005
Percentage of pigs treated for diarrhea[c]	43[ab] (12/28)	64[a] (18/28)	32[b] (9/28)	0.049

Data are expressed as mean ±SE of the mean.

[x]1 pig euthanized on day 17 of the experiment.

[y]2 pigs euthanized on day 11 and day 15 of the experiment respectively and 2 pigs on day 17 of the experiment.

[a]DI, diarrhea index; calculated as (total mean number of days with FC score 4 or 5 diarrhea/number of days) x 100. Data were analyzed non-parametrically.

[b]Data were analyzed non-parametrically.

[c]A diarrhea score of FC ≥ 4 on two consecutive days and treated therapeutically with antibiotics. Data analyzed using a Chi-square test.

[a,b]Means in a row not having the same superscript are significantly different ($P < 0.05$).

Table 7. Effects of feeding a Negative control diet, a diet supplemented with antibiotic, or a diet supplemented with Presan FX and Fysal MP, on body weight (BW) and average daily gain (ADG) of weaned pigs during the 20-d experimental period

	Treatment			*P* value
Item	Control	Antibiotic	Presan FX + Fysal MP	
n	7 [x]	7 [y]	7	
BW, kg				
d 0	6.72 ± 0.062	6.72 ± 0.067	6.76 ± 0.071	0.864
d 5	6.82 ± 0.057	6.89 ± 0.058	6.95 ± 0.058	0.317
d 7	6.77 ± 0.086	7.02 ± 0.086	6.98 ± 0.086	0.111
d 13	7.37 ± 0.175	7.65 ± 0.175	7.63 ± 0.176	0.448
d 20	9.35 ± 0.327	9.17 ± 0.327	9.60 ± 0.329	0.656
ADG, g				
d 0–5	17 ± 11.5	31±11.51	43±11.6	0.317
d 6 and 7	−27[a] ± 22.0	64[b] ± 22.0	14[ab] ± 22.1	0.031
d 8–13	99 ± 23.7	91 ± 23.7	110 ± 23.8	0.856
d 14–20	272 ± 43.1	191 ± 43.1	281 ± 43.3	0.293
d 0–20	131 ± 17.9	110 ± 17.9	144 ± 18.0	0.424

Data are expressed as mean ±SE of the mean. The BW at the start was included as a covariate in the analysis. Pigs that died in the study were excluded from the analysis.

n = 7 pens of 4 pigs each.

[x]1 pig euthanized on day 17 of the experiment and performance data adjusted accordingly.

[y]2 pigs euthanized on day 11 and day 15 of the experiment respectively and 2 pigs on day 17 of the experiment. Performance data were adjusted accordingly.

[a,b]Means in a row not having the same superscript are significantly different ($P < 0.05$).

of pigs on the Presan FX + Fysal MP diet was similar ($P > 0.05$) to pigs on the other two diets during the same period (Table 7). The ADFI was similar ($P > 0.05$) between treatments; however, pigs fed amoxicillin showed a trend ($P = 0.098$) to have a poorer feed conversion efficiency for the entire study compared to the other treatments (Table 8).

Real-Time PCR Quantification of Specific Bacterial Species

Standard curves ($\log_{10}$) were linear ($R^2 \geq 0.997$) over the serially diluted standards for each of the three qPCRs: F4 *E. coli* (5.4×10^7 to 5.4×10^3), *Salmonella* Derby (4.5×10^6 to 4.5×10^2), and total bacteria (1.1×10^7 to 1.1×10^3). *Salmonella*

Table 8. Effects of feeding a Negative control diet, a diet supplemented with antibiotic or supplemented with Presan FX and Fysal MP, on average daily feed intake (ADFI) and feed conversion efficiency (FCE) of weaned pigs during the 20-d experimental period

	Treatment			*P* value
Item	Control	Antibiotic	Presan FX + Fysal MP	
n	7 [x]	7 [y]	7	
ADFI, g				
d 0–5	80 ± 6.0	88 ± 6.0	89 ± 6.0	0.557
d 6 and 7	86 ± 12.7	102 ± 15.3	93 ± 13.3	0.715
d 8–13	140 ± 12.5	163 ± 18.2	174 ± 7.5	0.216
d 14–20	398 ± 19.7	343± 36.7	364 ± 25.6	0.439
d 0–20	210 ± 11.5	201 ± 16.9	211 ± 8.9	0.831
FCE, g:g				
d 0–5	0.08 ± 0.212	0.32 ± 0.132	0.49 ± 0.095	0.195
d 6 and 7	-0.19±0.953	-1.53 ± 2.42	2.00 ± 1.04	0.318
d 8–13	1.52 ± 0.234	2.27±1.139	2.31 ± 0.750	0.738
d 14–20	1.49 ± 0.099	2.61±1.832	1.47 ± 0.237	0.803
d 0–20	1.61 ± 0.097	2.52 ± 0.560	1.54 ± 0.120	0.098

Data are expressed as mean ±SE of the mean. Pigs that died in the study were excluded from the analysis.

n= 7 pens of 4 pigs each.

[x]1 pig euthanized on day 17 of the experiment and performance data adjusted accordingly.

[y]2 pigs euthanized on day 11 and day 15 of the experiment respectively and 2 pigs on day 17 of the experiment. Performance data were adjusted accordingly.

Table 9. Effects of feeding a Negative control diet, a diet supplemented with antibiotic, or a diet supplemented with Presan FX and Fysal MP, on selected fecal bacterial ratios 21 d after weaning

Treatment	F4 *E. coli*: Total bacteria	Salmonella: Total bacteria	Total bacterial numbers
T1Basal diet (Negative control)	7.2×10^{-4}	$1.3 \times 10^{-5\,ab}$	$7.6 \times 10^{5\,b}$
T2 Basal diet + Antibiotic	1.6×10^{-3}	$5.7 \times 10^{-5\,a}$	$3.9 \times 10^{5\,a}$
T3 Basal diet + Presan FX + Fysal MP	9.0×10^{-5}	$4.2 \times 10^{-6\,b}$	$1.1 \times 10^{6\,b}$
P value	0.452	0.018	0.005

[a,b]Values in the same column with different superscripts are significantly different.

serovars were not detected by qPCR in any 21-d post-weaning fecal samples from pigs treated with the Presan FX + Fysal MP diet; hence, a random number below the linear detection threshold of the *Salmonella* qPCR was given to all 'negative' samples. The ratio of *Salmonella* to total bacteria was reduced ($P = 0.018$) in pigs fed the Presan FX + Fysal MP diet relative to antibiotic-fed pigs, but diet had no effect ($P > 0.05$) on the proportion of F4 *E. coli*. However, the variance in the ratio of F4 *E. coli* to total bacteria was different between treatments ($P < 0.001$), with the smallest variance in pigs fed the Presan FX + Fysal MP diet (1.5×10^{-8}) relative to control pigs (2.5×10^{-6}) and amoxicillin-treated pigs (1.6×10^{-5}). As identical weights of each pooled fecal sample were not used for DNA extraction and qPCR, it is not possible to accurately compare absolute numbers of pathogens between treatments; however, the antibiotic diet reduced ($P = 0.005$) total bacterial numbers in fecal samples relative to the two other diets (Table 9).

Next-Generation Sequencing of the Microbiota

The phylogenetic diversity was reduced in four of the seven pens of pigs treated with amoxicillin, observed as a reduced plateau in diversity on rarefaction plots (data not shown). In addition, species richness (Chao 1) of bacteria within amoxicillin-fed pens was lower than the richness in control ($P = 0.004$) or Presan FX + Fysal MP-treated pigs ($P = 0.011$). The alpha diversity of fecal microbiomes at 21 d after weaning in control- and Presan FX + Fysal MP-treated pigs were not different ($P > 0.05$; Figure 1).

Analysis of microbial communities by PERMANOVA demonstrated that diet had statistically significant effects on clustering of microbial communities. Pair-wise comparisons showed that amoxicillin-fed pigs had altered fecal microbial communities relative to both Presan FX + Fysal MP-treated pigs ($P < 0.001$) and control pigs ($P = 0.001$). No significant difference existed

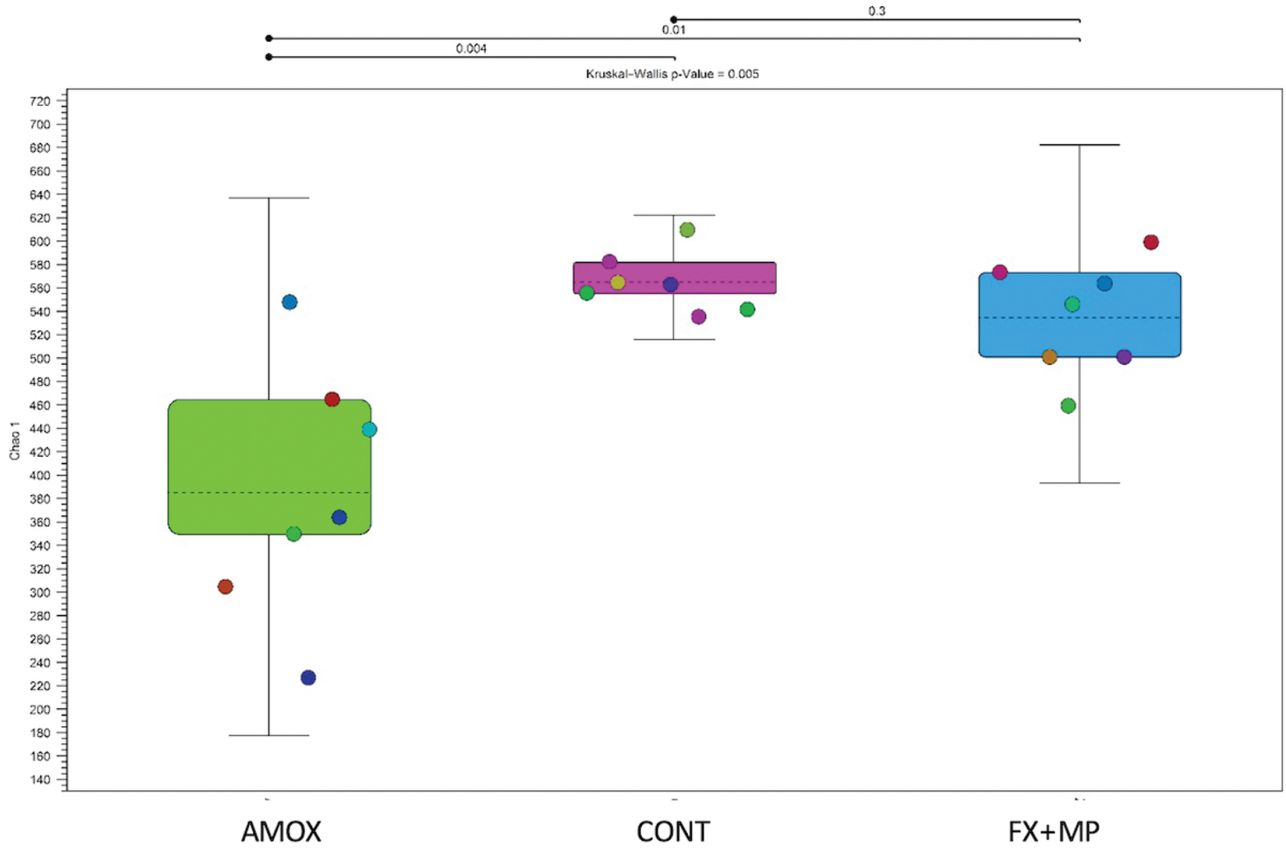

Figure 1. Box plot of bacterial diversity (Chao-1 alpha diversity) within pooled fecal samples of pigs fed with amoxicillin (AMOX), the control diet (CONT), or a diet with Presan FX + Fysal MP (FX+MP) at 21 d after weaning.

Table 10. The PERMANOVA analysis (Bray–Curtis) of pair-wise comparisons of clustering of fecal microbial communities between amoxicillin-, Presan FX + Fysal MP-, and control-fed pigs

Treatment 1	Treatment 2	Pseudo *f*-statistic	*P* value	*P* value (Bonferroni)
Amoxicillin	Presan FX+ Fysal MP	4.051	<0.001	0.002
Amoxicillin	Control	3.083	0.001	0.004
Presan FX + Fysal MP	Control	1.322	0.105	0.315

in clustering of microbial communities between Presan + Fysal MP-treated pigs and control pigs (Table 10).

At the phylum level, diet had no effect on the relative abundance of the two most common phyla in the porcine intestinal microbiome Firmicutes or Bacteroidetes (or the ratio of Firmicutes to Bacteroidetes). However, feeding amoxicillin increased ($P < 0.05$) the abundance of the phyla Proteobacteria, Fusobacteria, and Actinobacteria relative to control-and Presan FX + Fysal MP-fed pigs (Table 11).

Diet had no significant effect on any bacterial class within Proteobacteria, although there was a trend ($P < 0.1$) for elevated Betaproteobacteria (*Alcaligenaceae*) and Gammaproteobacteria (predominantly *Enterobacteriaceae, Pasteurellaceae* and *Succinivibrionaceae*) in amoxicillin-fed pigs. The Presan FX + Fysal MP-fed pigs showed a large reduction in the abundance of *Enterobacteriaceae* ($P = 0.016$) relative to both control- and amoxicillin-fed pigs. *Fusobacteriaceae*, the only abundant family within the phylum Fusobacteria, was more abundant in amoxicillin-treated pigs relative to other treatments (Table 11). Two most abundant families in Actinobacteria are *Coriobacteriaceae* and *Bifidobacteriaceae*, and *Coriobacteriaceae* were more abundant in amoxicillin-fed pigs ($P = 0.016$). In contrast, the phyla Spirochaetes and Tenericutes (predominantly *Mollicutes* order RF39) were more abundant in controls relative to amoxicillin- and Presan FX + Fysal MP-fed pigs.

Diet similarly had no significant effect on the relative abundance of Bacteroidetes, but amoxicillin-fed pigs showed increased abundance of the families *Prevotellaceae* and S24-7 ($P < 0.05$) and a trend toward elevated *Bacteroidaceae* relative to control- and Presan

Table 11. Effects of feeding a Negative control diet, a diet supplemented with amoxicillin, or a diet supplemented with Presan FX and Fysal MP, on the relative abundance of specific bacterial phyla in weaner pig feces

Bacterial Phylum	Control	Amoxicillin	Presan FX + Fysal MP	*P* value
Actinobacteria	0.0024 [a]	0.0092 [b]	0.0013 [a]	0.017
Fusobacteria	0.00001 [a]	0.0013 [b]	0.00001 [a]	0.05
Proteobacteria	0.0033 [a]	0.012 [b]	0.003 [a]	0.018
Spirochaetes	0.0085 [a]	0.002 [b]	0.0042 [b]	0.029
Tenericutes	0.099 [a]	0.016 [b]	0.051 [b]	0.006

[a,b]Means in a row not having the same superscript are significantly different ($P < 0.05$).

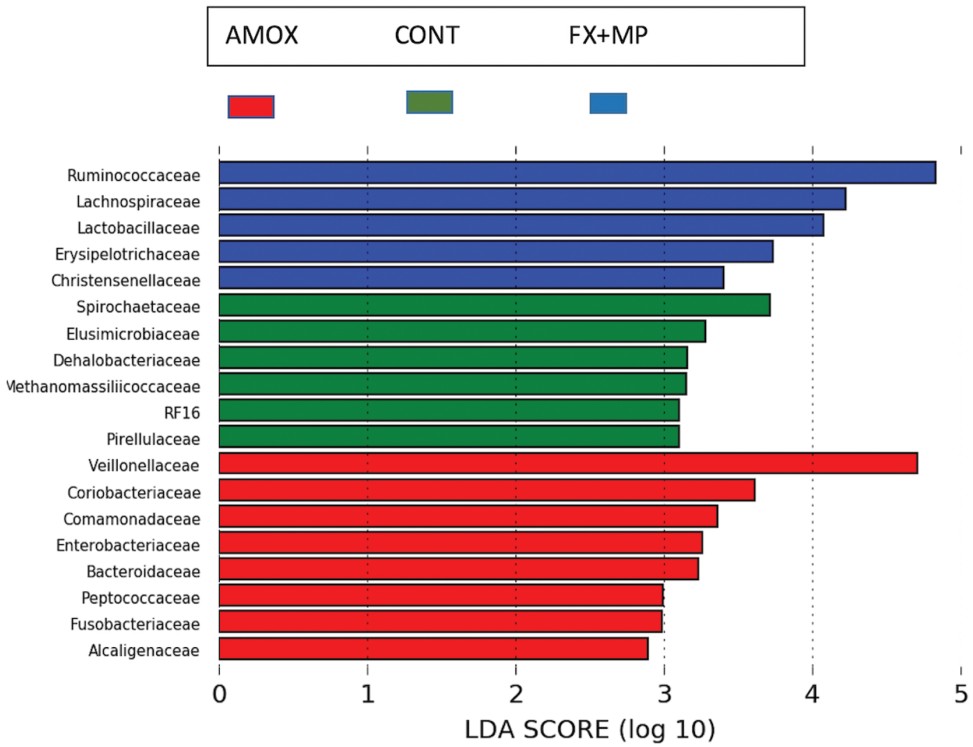

Figure 2. Linear discriminate analysis of the combination of bacterial groups (OTUs) that characterizes the microbiome of treatment groups.

FX + Fysal MP-fed pigs ($P = 0.071$). Within the phylum Firmicutes, amoxicillin-fed pigs showed an increased abundance of *Veillonellaceae* and reduced abundance of *Erysipelotrichaceae* and *Dehalobacteraceae* relative to the other treatments ($P < 0.05$).

Using linear discriminate analysis, amoxicillin-fed pigs were characterized by an increased abundance of *Veillonellacaeae* and *Enterobacteriaceae*, along with the *Coriobacteriaceae, Bacteroidaceae, Peptococcaceae, Fusobacteriaceae*, and *Alcaligenaceae* (Figure 2). In contrast, the Presan FX + Fysal MP-fed pigs were characterized by an increased abundance of *Ruminococcaceae, Lachnospiraceae, Lactobacillaceae, Erysipelotrichaceae*, and *Christensenellaceae* families (Figure 2). Control pigs were characterized by an increased abundance of *Spirochaetaceae*, *Elusimicrobiaceae, Dehalobacteriaceae, Pirellulaceae, Methanomassilicoccaceae*, and RF16 (unidentified family of Bacteroidetes).

Differential abundance of genera and families between treatments was analyzed by a generalized linear model with significance demonstrated with a Likelihood ratio test, and the 25 OTUs with the most significantly different differential abundances between treatments visualized with heat maps and a dendogram. Amoxicillin-fed pigs showed an increased differential abundance of the genera *Parabacteroides, Prevotella* and family S24-7 in the order Bacteroidales, along with the genus *Acidaminococcus* in the family *Veillonellaceae* (phyla *Firmicutes*) and just one of the 400 OTUs in the *Ruminococcaceae* family, relative to control- and Presan FX + Fysal MP-fed pigs. It was harder to show differential differences in abundance of OTUs between control and Presan FX + Fysal MP-fed pigs, suggesting that the microbiome of these two treatments was more similar. Control pigs appeared

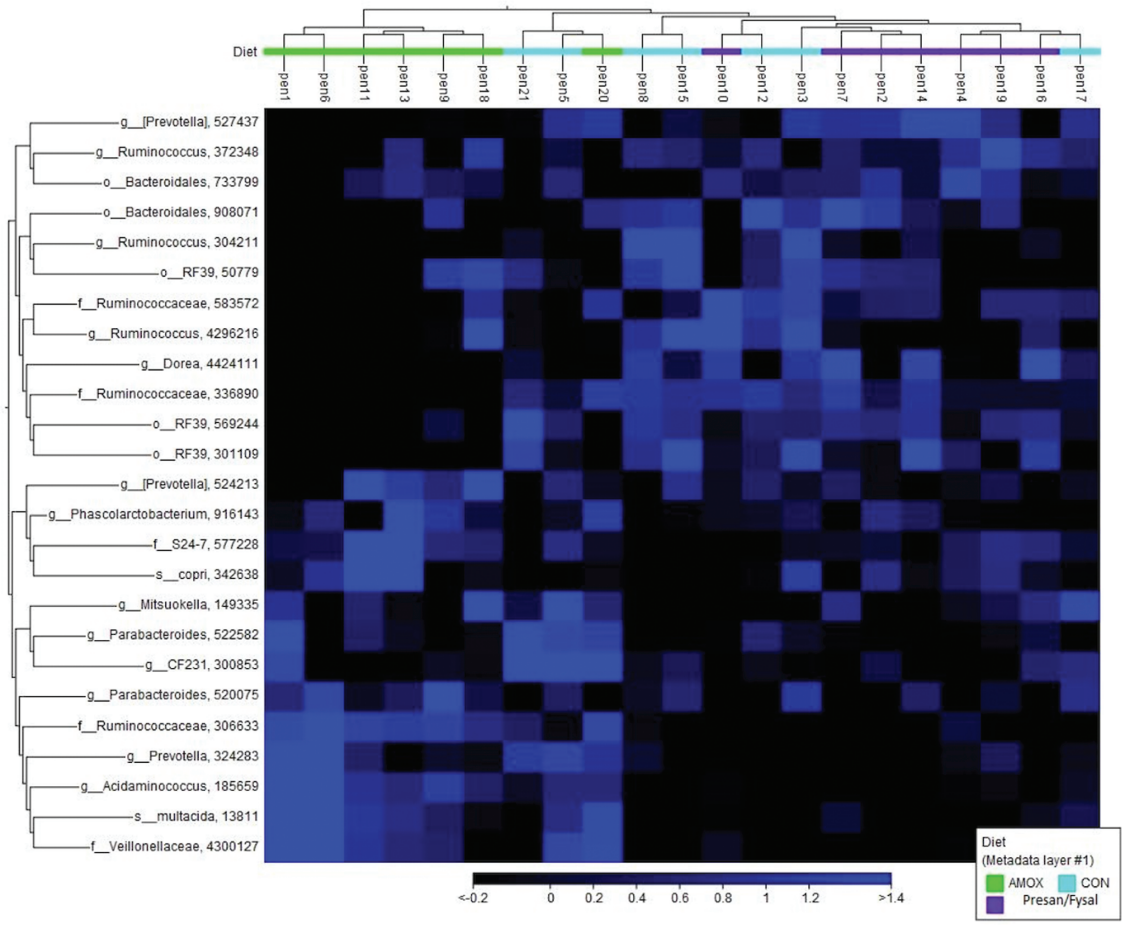

Figure 3. Differential abundance (log_2 fold change) of 25 most different OTUs (bacterial groups) across treatments expressed as a heat map with increased abundance shown in light blue and reduced abundance in black. Treatment and pen identities are outlined on top axis of heat map and OTU identities are outlined on left axis.

to show a differentially increased abundance of the order Bacteriodales and Mollicutes RF39, and two *Ruminococcus* genera (Figure 3).

DISCUSSION

This experiment examined the hypothesis that a combination of Presan FX and Fysal MP would reduce F4-ETEC diarrhea and improve growth performance relative to a diet containing an antibiotic (amoxicillin). There were no positive effects of Presan-FX + Fysal MP on indicators associated with diarrhea and fecal ETEC shedding associated with F4-ETEC inoculation, with pigs fed amoxicillin showing reduced fecal *E. coli* excretion and less diarrhea in the peri-infection period than pigs on both other treatments. However, over the entire study, amoxicillin-fed pigs showed no statistical differences in the average number of days they were treated therapeutically for diarrhea with an antibiotic, and the percentage of pigs therapeutically treated for diarrhea after ETEC infection. Nevertheless, a significant amount of the diarrhea observed in this study occurred on days 14 to 20 due to an unanticipated, opportunistic infection by *Salmonella* serovars including Derby and Typhimurium. Pigs fed Presan FX and Fysal MP showed markedly less diarrhea commensurate with a significantly lower proportion of *Salmonella* numbers relative to total bacterial numbers (as determined by qPCR). The dietary combination of Presan FX and Fysal MP caused significantly fewer therapeutic antibiotic administrations relative to the diet with amoxicillin during this time and demonstrated the potential beneficial effects of this combination under such circumstances.

The combination of F4-ETEC inoculation and amoxicillin treatment is likely to have caused the observed increased abundance of Proteobacteria and specifically *Enterobacteriaceae*, microbial dysbiosis, and loss of commensal bacteria, which are normally able to competitively inhibit or exclude pathogens such as *Salmonella* Derby or Typhimurium from colonizing the GIT (Shin et al., 2015; Anderson and Kendall, 2017). Increased abundance of Proteobacteria is a marker of dysbiosis or unstable intestinal microbial communities and

is associated with colitis in mammals (Shin et al., 2015). In contrast, no *Salmonella* were detected in the feces of pigs treated with Presan-FX + Fysal MP. Comparisons between control- and Presan-FX + Fysal MP-fed pigs showed no significant differences in alpha and beta diversity, and only minimal differences in differential abundance of OTUs.

However, the Presan-FX + Fysal MP-fed pigs were characterized by the increased abundance of short-chain fatty acid-producing bacteria such as *Ruminococcaceae, Lachnospiraceae*, *Lactobacillaceae*, *Erysipelotrichaceae*, and *Christensenellaceae*. Butyric acid (butyrate) production by these bacteria is considered beneficial for the GIT in the post-weaning period (Bedford and Gong, 2018). Moreover, genera affiliated with *Lachnospiraceae* and grouped in the *Clostridium* cluster *XIVa* populate the areas between the mucosal folds in the large intestine and potentially contribute to immune homeostasis and anti-inflammatory effects. They have been shown to induce the accumulation and differentiation of colonic T regulatory cells when colonizing the gut of germ-free mice and have been found to be less abundant, for example, in IBD patients compared with healthy subjects (Ivanov and Honda, 2012; Mancabelli et al., 2017).

The dietary inclusion of Presan-FX + Fysal MP was also associated with a reduction in the abundance of other potential enteric pathogens including *Fusobacteriaceae*, relative to amoxicillin-treated pigs. *Fusobacteriaceae* are associated with diarrhea and increased expression of GIT inflammatory factors (Nosho et al., 2016; Yang et al., 2017). Antimicrobial treatment is known to induce intestinal dysbiosis (Collier et al., 2003; Looft et al., 2014; Yu et al., 2017), which can, in turn, induce inflammation, increase GIT permeability, and ultimately increase translocation of bacteria including *Salmonella* spp. across the intestinal mucosa (Zeineldin et al., 2019). Given ever-changing circumstances occurring worldwide with respect to antimicrobial resistance and the resultant likelihood of a greater incidence of enteric bacterial co-infections (Luppi, 2017), product combinations such as Presan-FX + Fysal MP are likely to become more important in controlling post-weaning diarrheal events in the future.

Pigs are often asymptomatic carriers of *Salmonella*, highlighting the importance of identifying high-prevalence farms and effective treatment methods such as diet and (or) water additives for its control (Schut et al., 2019). Previous research exploring the use of feed additives such as OA and MCFA has tended to concentrate on pigs in the grow-finish period (van der Wolf et al., 2001; Rasschaert et al., 2016) where a higher prevalence occurs (Casanova-Higes et al., 2019). However, relatively little information is known about *Salmonella* infections in younger pigs, even though it is likely that the lactation and nursery periods influence the dynamics of infection during this period. Recent work has shown that newly-weaned pigs can become sub-clinically infected and act as active carriers of *Salmonella*, with there being a close relationship between *Salmonella* infection in piglets and sows as the same serotypes and strains were found in both populations (Casanova-Higes et al., 2019). After weaning, disease caused by colonization of *Salmonella* in the GIT most commonly develops because of the intestinal dysbiosis seen when multiple challenges, including other pathogens such as F4-ETEC, arise (Pluske et al., 2002). In this regard, we found no evidence in the feed or water of the same *Salmonella* serovars that were cultured, and presume that the *Salmonella* serovars were already present in the GIT and clinical infection was induced by the F4-ETEC infection itself.

Nonetheless, several studies have explored the use of some feed additives on *Salmonella* excretion and survival after weaning, but generally with mixed results. In weaned pigs experimentally infected with *Salmonella* Derby or F4 ETEC, addition of 1.2% potassium diformate or 0.9% OA (75% formic and 25% propionic acid) reduced *Salmonella* shedding and resulted in significantly lower counts of *Salmonella* and *E. coli* in the stomach content, indicating an improved efficacy of the stomach barrier (Taube et al., 2009). Another study (Walsh et al., 2012) weaned pigs at 19 ± 2 d of age in a 14-day study to evaluate the effects of water-delivered direct-fed microbials (DFM) or OA on growth, immune status, *Salmonella* infection and shedding, and intestinal microbial populations after intranasal inoculation of *Salmonella* Typhimurium (10^{10} cfu/pig). Treatments were 1) control diet; 2) control diet + DFM (*Enterococcus faecium*, *Bacillus subtilis*, and *Bacillus licheniformis*) in drinking water at 10^9 cfu/L for each strain of bacteria; 3) control diet + an OA-based blend (predominantly propionic, acetic, and benzoic acid) in drinking water at 2.58 mL/liter; and 4) control diet + 55 mg/kg of carbadox. These authors showed that the DFM and OA treatments offered little or no benefits to pigs infected with *Salmonella*. In contrast, a later study (Fabà et al., 2020) showed that feed additive blends including mannan-rich hydrolyzed copra meal and fermented rye combined with OA (88% formic

acid and 12% lactic acid) reduced peak shedding and mean shedding of *Salmonella* Typhimurium in nursery pigs under a 7-d challenge that was evaluated for 21 d. Shedding was not influenced using coated butyrate with the OA blend. More recently, it was shown that feeding a commercial blend of MCFA salts distilled from coconut oil reduced the hindgut colonization by pathogenic populations such as enterobacteria, ETEC and *Salmonella* Typhimurium in orally challenged weaned piglets (López-Colom et al., 2019). Differences in the types of acids and their concentrations, as well as different diet formulations (e.g., use of mineral compounds such as ZnO, fiber types and contents, and other bacterial modifying compounds), weaning age, and (or) environmental conditions, likely account for the disparity seen between studies.

The present experiment showed no major overriding positive effects on any production indices except for the peri-infection period, where pigs fed amoxicillin performed better only than control-fed pigs. Feed conversion efficiencies for diet Presan-FX + Fysal MP and the control diet were improved compared to the pigs fed amoxicillin, a result explained by the greater incidence of diarrhea that occurred in the latter part of the study. In a study comparing the efficacy of a mixture of two commercial OA products like those used in the present study (Li et al., 2018), and relative to a diet with 3,000 ppm ZnO and a diet with 10 mg/kg zinc bacitracin, 5 mg/kg colistin sulfate, and 5 mg/kg olaquindox, no production benefits or reductions in PWD were similarly seen with the OA-based products. However, the study observed that the genus *Prevotella* was increased in the colon and the microbial community structure was significantly altered with various acid combinations, and that the OA blends showed a similar growth-promoting effect as antibiotics in the less digestible diet to which high levels of ZnO had not been added.

Some significant microbial changes in the feces occurred at the end of the study in response to pigs fed Presan FX + Fysal MP. Relative to amoxicillin-fed counterparts, pigs offered Presan FX + Fysal MP showed greater (alpha) diversity. This has similarly been observed in several previous studies exploring similar types of OA- and MCFA-based feed additives (Li et al., 2018; Soler et al., 2018). There is debate related to the importance of a higher GIT microbial diversity in the post-weaning period, with, for example, some authors (Reese and Dunn, 2018) stating that the framework for understanding diversity in host-associated microbial communities should not be as simple as "more diversity is better." Indeed, factors such as genotype, age, and diet profoundly impact diversity of the GIT after weaning as the pig adapts to new challenges (Frese et al., 2015; Mach et al., 2015; Yang et al., 2019). In the present study, the high relative abundance of *Salmonella* spp. in amoxicillin-fed pigs at the end of the study was likely confounded with the lower diversity and species richness observed. Nonetheless, several studies have described a reduction in diversity of the fecal microbiota associated with the administration of antimicrobials (Janczyk et al., 2007; Looft et al., 2012; Ubeda and Pamer, 2012; Gresse et al., 2017; Li et al., 2018; Soler et al., 2018).

In this context, the microbial structure in weaner pigs receiving antimicrobials or a proprietary feed additive product was examined in a recent study (Soler et al., 2018). All animals received a diet with ZnO during the first week after weaning and then pigs were split into three groups: 1) the control group received a standard diet without antibiotics or additives; 2) the antibiotic group received the same diet as the control group but with amoxicillin (15 mg/kg BW/day) and colistin sulphate (5 mg/kg BW/day); and 3) the feed additive group received the same diet as the control group but with a dietary feed additive at 3 kg/tonne that contained 71% coated short-chain fatty acids (butyrate and propionate), 10% MCFA (caprylic, capric and lauric acid), and 19% essential oil components (thymol, cinnamaldehyde and eucalyptus oil). The highest bacterial diversity was observed for the control and the feed additive groups but was very similar in the antibiotic group, which agrees with data found in the present study.

Soler et al. (2018) also reported an increase in abundance of *Bacillus* and *Lactobacillus spp*., part of the Firmicutes phylum, within the feed additive group versus the antibiotic and control groups, as well as a decrease in abundance of Proteobacteria in the antibiotic-treated group and the shift in abundance of the *Prevotellaceae* family occupying their niche. These changes are in harmony with data from the current study. Significantly, a negative correlation between productive performance and the abundance of the family *Prevotellaceae* species has been observed (Unno et al., 2015), although this could not be established in the present study. Furthermore, non-diarrheic pigs after weaning were shown to have a continuous decrease in *Lactobacillus* and *Escherichia* and a gradual increase in *Prevotella* with the transition to solid food after weaning (Janczyk et al., 2007; Frese et al., 2015). These authors proposed that an altered relationship between *Prevotella* and *Escherichia* may

be the main cause of diarrhea in pre-weaned piglets, whereas reduced numbers of *Bacteroides*, *Ruminococcus*, *Bulleidia*, and *Treponema* that are responsible for the digestion and utilization of solid feeds may be related to the onset of post-weaning diarrhea. Indeed, a higher abundance of Prevotella has been reported as a dominant feature of the fecal microbiota in healthy pigs as compared to diarrheic pigs after weaning (Dou et al., 2017; Karasova et al., 2021), and those performing better after weaning (Luise et al., 2021).

CONCLUSIONS

This study showed no overriding positive effects of the combination of Presan FX and Fysal MP on either a reduction in F4 ETEC-induced diarrhea or performance indices relative to the control diet or a diet with amoxicillin. Nonetheless, the co-infection with *Salmonella* serovars that occurred in the latter part of the experiment clearly demonstrated the positive impacts of a mixture of Presan FX and Fysal MP to reduce fecal *Salmonella* spp. counts consistent with reduced diarrhea and improved feed conversion efficiency under such conditions, relative to pigs fed amoxicillin. Changes in the diversity and composition of the fecal microbiome were observed at day 21 of the study. The diversity of bacteria within amoxicillin-treated pigs was lower than the diversity in control- or Presan FX + Fysal MP-fed pigs commensurate with a significantly altered fecal microbial community. Amoxicillin-treated pigs were characterized by an increased abundance of pathogenic families, predominately *Veillonellacaeae* and *Enterobacteriaceae*. In contrast, the Presan FX + Fysal MP-fed pigs were characterized by an increased abundance of *Ruminococcaceae*, *Lachnospiraceae*, *Lactobacillaceae*, *Erysipelotrichaceae*, and *Christensenellaceae*, all of which are considered potential beneficial commensals. Control pigs were characterized by an increased abundance of *Spirochaetaceae*, associated with healthy piglets, as well as *Elusimicrobiaceae* and RF16, associated with reduced feed intake and appetite.

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LITERATURE CITED

Anderson, C. J., and M. M. Kendall. 2017. Salmonella enterica serovar typhimurium strategies for host adaptation. Front. Microbiol. 8:1983. doi:10.3389/fmicb.2017.01983

Bedford, A., and J. Gong. 2018. Implications of butyrate and its derivatives for gut health and animal production. Anim. Nutr. 4:151–159. doi:10.1016/j.aninu.2017.08.010

Casanova-Higes, A., C. M. Marín-Alcalá, S. Andrés-Barranco, A. Cebollada-Solanas, J. Alvarez, and R. C. Mainar-Jaime. 2019. Weaned piglets: another factor to be considered for the control of *Salmonella* infection in breeding pig farms. Vet. Res. 50:45. doi:10.1186/s13567-019-0666-7

Collier, C. T., M. R. Smiricky-Tjardes, D. M. Albin, J. E. Wubben, V. M. Gabert, B. Deplancke, D. Bane, D. B. Anderson, and H. R. Gaskins. 2003. Molecular ecological analysis of porcine ileal microbiota responses to antimicrobial growth promoters. J. Anim. Sci. 81:3035–3045. doi:10.2527/2003.81123035x

de Lange, C. F. M., J. R. Pluske, J. Gong, and M. Nyachoti 2010. Strategic use of feed ingredients and feed additives to stimulate gut health and development in young pigs. Livest. Sci. 134:124–134. doi:10.1016/j.livsci.2010.06.117

Dou, S., P. Gadonna-Widehem, V. Rome, D. Hamoudi, L. Rhazi, L. Lakhal, T. Larcher, N. Bahi-Jaber, A. Pinon-Quintana, A. Guyonvarch, et al. 2017. Characterisation of early-life fecal microbiota in susceptible and healthy pigs to post-weaning diarrhoea. Plos One 12:e0169851. doi:10.1371/journal.pone.0169851

Fabà, L., R. Litjens, J. Allaart, and R. Roubos-van den Hil. 2020. Feed additive blends fed to nursery pigs challenged with Salmonella. J Anim Sci. 98: skz382. doi:10.1093/jas/skz382

Franklin, M. A., D. H. Francis, D. Baker, and A. G. Mathew. 1996. A PCR-based method of detection and differentiation of K88+ adhesive *Escherichia coli*. J. Vet. Diagn. Invest. 8:460–463. doi:10.1177/104063879600800410

Frese, S. A., K. Parker, C. C. Calvert, and D. A. Mills. 2015. Diet shapes the gut microbiome of pigs during nursing and weaning. Microbiome 3:28. doi:10.1186/s40168-015-0091-8

Gresse, R., F. Chaucheyras-Durand, M. A. Fleury, T. Van de Wiele, E. Forano, and S. Blanquet-Diot. 2017. Gut microbiota dysbiosis in postweaning piglets: understanding the keys to health. Trends Microbiol. 25:851–873. doi:10.1016/j.tim.2017.05.004

Ivanov, I. I., and K. Honda. 2012. Intestinal commensal microbes as immune modulators. Cell Host Microbe. 12:496–508. doi:10.1016/j.chom.2012.09.009

Janczyk, P., R. Pieper, W. B. Souffrant, D. Bimczok, H. J. Rothkötter, and H. Smidt. 2007. Parenteral long-acting amoxicillin reduces intestinal bacterial community diversity in piglets even 5 weeks after the administration. Isme J. 1:180–183. doi:10.1038/ismej.2007.29

Karasova D., M. Crhanova, V. Babak, M. Jerabek, L. Brzobohaty, Z. Matesova, and I. Rychlik. 2021. Development of piglet gut microbiota at the time of weaning influences development of postweaning diarrhea

– a field study. Res. Vet. Sci. 135:59–65. doi:10.1016/j.rvsc.2020.12.022

Laird, T. J., S. Abraham, D. Jordan, J. R. Pluske, D. J. Hampson, D. J. Trott, and M. O'Dea. 2021. Porcine enterotoxigenic *Escherichia coli:* antimicrobial resistance and development of microbial-based alternative control strategies. Vet. Microbiol. 258:109117. doi:10.1016/j.vetmic.2021.109117

Li, S., J. Zheng, K. Deng, L. Chen, X. L. Zhao, X. Jiang, Z. Fang, L. Che, S. Xu, B. Feng, et al. 2018. Supplementation with organic acids showing different effects on growth performance, gut morphology, and microbiota of weaned pigs fed with highly or less digestible diets. J. Anim. Sci. 96:3302–3318. doi:10.1093/jas/sky197

Liu, Y., C. D. Espinosa, J. J. Abelilla, G. A. Casas, L. V. Lagos, S. A. Lee, W. B. Kwon, J. K. Mathai, D. M. D. L. Navarro, N. W. Jaworski, et al. 2018. Non-antibiotic feed additives in diets for pigs: a review. Anim. Nutr. 4:113–125. doi:10.1016/j.aninu.2018.01.007

Looft, T., H. K. Allen, T. A. Casey, D. P. Alt, and T. B. Stanton. 2014. Carbadox has both temporary and lasting effects on the swine gut microbiota. Front. Microbiol. 5:276. doi:10.3389/fmicb.2014.00276

Looft, T., T. A. Johnson, H. K. Allen, D. O. Bayles, D. P. Alt, R. D. Stedtfeld, W. J. Sul, T. M. Stedtfeld, B. Chai, J. R. Cole, et al. 2012. In-feed antibiotic effects on the swine intestinal microbiome. Proc. Natl. Acad. Sci. U. S. A. 109:1691–1696. doi:10.1073/pnas.1120238109

López-Colom, P., L. Castillejos, A. Rodríguez-Sorrento, M. Puyalto, J. J. Mallo, and S. M. Martín-Orúe. 2019. Efficacy of medium-chain fatty acid salts distilled from coconut oil against two enteric pathogen challenges in weanling piglets. J. Anim. Sci. Biotechnol. 10:89. doi:10.1186/s40104-019-0393-y

Luise, D., M. Le Sciellour, A. Buchet, R. Resmond, C. Clement, M. N. Rossignol, D. Jardet, O. Zemb, C. Belloc, and E. Merlot. 2021. The fecal microbiota of piglets during weaning transition and its association with piglet growth across various farm environments. Plos One 16:e0250655. doi:10.1371/journal.pone.0250655

Luppi, A. 2017. Swine enteric colibacillosis: diagnosis, therapy and antimicrobial resistance. Porcine Health Manag. 3:16. doi:10.1186/s40813-017-0063-4

Mach, N., M. Berri, J. Estellé, F. Levenez, G. Lemonnier, C. Denis, J. J. Leplat, C. Chevaleyre, Y. Billon, J. Doré, et al. 2015. Early-life establishment of the swine gut microbiome and impact on host phenotypes. Environ. Microbiol. Rep. 7:554–569. doi:10.1111/1758-2229.12285

Malorny, B., E. Paccassoni, P. Fach, C. Bunge, A. Martin, and R. Helmuth. 2004. Diagnostic real-time PCR for detection of Salmonella in food. Appl. Environ. Microbiol. 70:7046–7052. doi:10.1128/AEM.70.12.7046-7052.2004

Mancabelli, L., C. Milani, G. A. Lugli, F. Turroni, D. Cocconi, and D. van Sinderen. 2017. Identification of universal gut microbial biomarkers of common human intestinal diseases by meta-analysis. FEMS Microbiol. Ecol. 93. Doi:10.1093/femsec/fix153

Mesonero Escuredo, J. A., Y. van der Horst, J. Carr, and D. Maes. 2016. Implementing drinking water feed additive strategies in post-weaning piglets, antibiotic reduction and performance impacts: case study. Porcine Health Manag. 2:25. doi:10.1186/s40813-016-0043-0

Nosho, K., Y. Sukawa, Y. Adachi, M. Ito, K. Mitsuhashi, H. Kurihara, S. Kanno, I. Yamamoto, K. Ishigami, H. Igarashi, et al. 2016. Association of *Fusobacterium nucleatum* with immunity and molecular alterations in colorectal cancer. World J. Gastroenterol. 22:557–566. doi:10.3748/wjg.v22.i2.557

Partanen, K. H., and Z. Mroz. 1999. Organic acids for performance enhancement in pig diets. Nutr. Res. Rev. 12:117–145. doi:10.1079/095442299108728884.

Pluske, J. R. 2013. Feed- and feed additives-related aspects of gut health and development in weanling pigs. J. Anim. Sci. Biotechnol. 4:1. doi:10.1186/2049-1891-4-1

Pluske, J. R., D. W. Pethick, D. E. Hopwood, and D. J. Hampson. 2002. Nutritional influences on some major enteric bacterial diseases of pigs. Nutr. Res. Rev. 15:333–71. doi:10.1079/NRR200242

Pluske, J. R., D. L. Turpin, and J. C. Kim. 2018. Gastrointestinal tract (gut) health in the young pig. Anim. Nutr. 4:187–196. doi:10.1016/j.aninu.2017.12.004

Pluske, J. R., I. H. Williams, and D. J. Hampson. 1997. Factors influencing the structure and function of the small intestine in the weaned pig: a review. Livest. Prod. Sci. 51:215–236. doi:10.1016/S0301-6226(97)00057-2

Qiagen. 2021. CLC Microbial genomics module User Manual 21.1. OTU clustering step by step. https://resources.qiagenbioinformatics.com/manuals/clcmgm/current/User_Manual.pdf

Rasschaert, G., J. Michiels, M. Tagliabue, J. Missotten, S. De Smet, and M. Heyndrickx. 2016. Effect of organic acids on salmonella shedding and colonization in pigs on a farm with high salmonella prevalence. J. Food Prot. 79:51–58. doi:10.4315/0362-028X.JFP-15-183

Reese, A. T., and R. R. Dunn. 2018. Drivers of microbiome biodiversity: a review of general rules, feces, and ignorance. mBio 9:e01294–18. doi:10.1128/mBio.01294-18

Roth, F. X., and M. Kirchgessner. 1998. Organic acids as feed additives for young pigs: nutritional and gastrointestinal effects. J. Anim. Feed Sci. 7:25–33. doi:10.22358/jafs/69953/1998

Schut, C. H., A. Farzan, M. H. Ainslie-Garcia, R. M. Friendship, and B. N. Lillie. 2019. Antibody responses to salmonella in pigs from weaning up to marketing and presence of *Salmonella* at slaughter. Foodborne Pathog. Dis. 16:187–194. doi:10.1089/fpd.2018.2454

Segata, N., J. Izard, L. Waldron, D. Gevers, L. Miropolsky, W. S. Garrett, and C. Huttenhower. 2011. Metagenomic biomarker discovery and explanation. Genome Biol. 12:R60. doi:10.1186/gb-2011-12-6-r60

Shin, N. R., T. W. Whon, and J. W. Bae. 2015. Proteobacteria: microbial signature of dysbiosis in gut microbiota. Trends Biotechnol. 33:496–503. doi:10.1016/j.tibtech.2015.06.011

Soler, C., T. Goossens, A. Bermejo, L. Migura-García, A. Cusco, O. Francino, and L. Fraile. 2018. Digestive microbiota is different in pigs receiving antimicrobials or a feed additive during the nursery period. PLoS One. 13:e0197353. doi:10.1371/journal.pone.0197353

Sterndale, S. O., D. J. Evans, J. P. Mansfield, J. Clarke, S. Sahibzada, S. Abraham, M. O'Dea, D. W. Miller, J. C. Kim, and J. R. Pluske. 2019a. Effect of mucin 4 allele on susceptibility to experimental infection with enterotoxigenic F4 *Escherichia coli* in pigs fed experimental diets. J. Anim. Sci. Biotechnol. 10:56. doi:10.1186/s40104-019-0366-1

Sterndale, S. O., D. W. Miller, J. P. Mansfield, J. C. Kim, M. O'Dea, and J. R. Pluske. 2019b. Technical note: novel

delivery methods for an enterotoxigenic *Escherichia coli* infection model in MUC4-locus sequenced weaner pigs. J. Anim. Sci. 97:4503–4508. doi:10.1093/jas/skz303

Suiryanrayna, M. V., and J. V. Ramana. 2015. A review of the effects of dietary organic acids fed to swine. J. Anim. Sci. Biotechnol. 6:45. doi:10.1186/s40104-015-0042-z

Suzuki, M. T., L. T. Taylor, and E. F. DeLong. 2000. Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5'-nuclease assays. Appl. Environ. Microbiol. 66:4605–4614. doi:10.1128/AEM.66.11.4605-4614.2000

Taube, V. A., M. E. Neu, Y. Hassan, J. Verspohl, M. Beyerbach, and J. Kamphues. 2009. Effects of dietary additives (potassium diformate/organic acids) as well as influences of grinding intensity (coarse/fine) of diets for weaned piglets experimentally infected with *Salmonella* Derby or *Escherichia coli*. J. Anim. Physiol. Anim. Nutr. (Berl). 93:350–358. doi:10.1111/j.1439-0396.2008.00894.x

Tugnoli, B., G. Giovagnoni, A. Piva, and E. Grilli. 2020. From acidifiers to intestinal health enhancers: how organic acids can improve growth efficiency of pigs. Animals. 10:134.

Ubeda, C., and E. G. Pamer. 2012. Antibiotics, microbiota, and immune defense. Trends Immunol. 33:459–466. doi:10.1016/j.it.2012.05.003

Unno, T., J. M. Kim, R. B. Guevarra, and S. G. Nguyen. 2015. Effects of antibiotic growth promoter and characterization of ecological succession in Swine gut microbiota. J. Microbiol. Biotechnol. 25:431–438. doi:10.4014/jmb.1408.08063

van der Wolf, P. J., F. W. van Schie, A. R. Elbers, B. Engel, H. M. van der Heijden, W. A. Hunneman, and M. J. Tielen. 2001. Administration of acidified drinking water to finishing pigs in order to prevent *Salmonella* infections. Vet. Q. 23:121–125. doi:10.1080/01652176.2001.9695097

Walsh, M. C., M. H. Rostagno, G. E. Gardiner, A. L. Sutton, B. T. Richert, and J. S. Radcliffe. 2012. Controlling *Salmonella* infection in weanling pigs through water delivery of direct-fed microbials or organic acids. Part I: effects on growth performance, microbial populations, and immune status. J. Anim. Sci. 90:261–271. doi:10.2527/jas.2010-3598

Wathes, C., B. Miller, and F. Bourne. 1989. Cold stress and post-weaning diarrhoea in piglets inoculated orally or by aerosol. Anim. Sci. 49:483–496. doi:10.1017/S0003356100032694

Yang, Q., X. Huang, S. Zhao, W. Sun, Z. Yan, P. Wang, S. Li, W. Huang, S. Zhang, L. Liu, et al. 2017. Structure and function of the fecal microbiota in diarrheic neonatal piglets. Front. Microbiol. 8:502. doi:10.3389/fmicb.2017.00502

Yang, Q., X. Huang, P. Wang, Z. Tan, W. Sun, S. Zhao, and S. Gun. 2019. Longitudinal development of the gut microbiota in healthy and diarrheic piglets induced by age-related dietary changes. Microbiol. Open. 8:e923. doi:10.1002/mbo3.923

Yu, H. T., X. L. Ding, N. Li, X. Y. Zhang, X. F. Zeng, S. Wang, H. B. Liu, Y. M. Wang, H. M. Jia, and S. Y. Qiao. 2017. Dietary supplemented antimicrobial peptide microcin J25 improves the growth performance, apparent total tract digestibility, fecal microbiota, and intestinal barrier function of weaned pigs. J. Anim. Sci. 95:5064–5076. doi:10.2527/jas2017.1494

Zeineldin, M., B. Aldridge, and J. Lowe. 2019. Antimicrobial effects on swine gastrointestinal microbiota and their accompanying antibiotic resistome. Front. Microbiol. 10:1035. doi:10.3389/fmicb.2019.01035