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Non Ruminant Nutrition

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The use of an alternative feed additive, containing benzoic acid, thymol, eugenol, and piperine, improved growth performance, nutrient and energy digestibility, and gut health in weaned piglets

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Abstract

This research evaluated a feed additive (benzoic acid, eugenol, thymol, and piperine), associated or not with colistin, in weaned piglets feeding. The parameters evaluated were growth performance, apparent total tract digestibility (**ATTD**) of nutrients, diarrhea incidence, intestinal morphology, relative weights of digestive organs, microbial diversity, and the percentages of operational taxonomic units of microorganisms in the cecum content of pigs. One-hundred and eight crossbred piglets (5.3 ± 0.5 kg) were used in a three-phase feeding program (21 to 35, 36 to 50, 51 to 65 d of age) and fed a control diet with no inclusion of growth promoter feed additive, a diet with 40 ppm of colistin, a diet with 0.3% of alternative additive, and a diet with 0.3% of alternative additive and 40 ppm of colistin. The diets were based on corn, soybean meal, dairy products, and spray-dried blood plasma and formulated to provide 3.40, 3.38, and 3.20 Mcal of ME/kg and 14.5, 13.3, and 10.9 g/kg of digestible lysine, in phases 1, 2, and 3, respectively. The piglets were housed three per pen, with nine replicates per diet, in a complete randomized block design based on initial BW. The data were submitted to ANOVA and means were separated by Tukey test (5%), using SAS. Pigs fed diets with the alternative feed additive had greater ($P < 0.05$) ADG (114.3 vs. 91.8 g) and ADFI (190.1 vs. 163.3 g) in phase 1 than pigs fed diets without the product. The alternative additive improved ($P < 0.05$) ATTD of crude protein (**CP**) in phase 1 (71.0% vs. 68.6%), gross energy in phases 1 (77.4% vs. 75.2%) and 3 (79.0% vs. 77.1%), and dry matter in phase 3 (79.1% vs. 77.1%). The antibiotic inclusion in the diets increased ($P < 0.05$) ATTD of CP in phase 1 (71.5% vs. 68.2%). The alternative feed additive tended ($P = 0.06$) to increase (46%) normal feces frequency, decreased ($P < 0.05$) goblet cells count (104.3 vs. 118.1) in the jejunum, and decreased ($P < 0.05$) small intestine (4.60% vs. 4.93%) and colon (1.41% vs. 1.65%) relative weights, compared with pigs not fed with the alternative additive. There was a tendency ($P = 0.09$) for a lower concentration of *Escherichia–Shigella* (1.46% vs. 3.5%) and lower ($P < 0.05$) percentage of Campylobacter (0.52% vs. 10.21%) in the cecum content of piglets fed diets containing essential oils and

benzoic acid compared with pigs fed diets without the alternative feed additive. The alternative feed additive was effective in improving growth performance, diets digestibility, and gut health in piglets soon after weaning.

Key words: diarrhea, gut health, microbiota, phytogenic additive

Abbreviations

ADFI	average daily feed intake
ADG	average daily gain
AIA	acid insoluble ash
ATTD	apparent total tract digestibility
CP	crude protein
DM	dry matter
GE	gross energy
OM	organic matter
OTU	operational taxonomic unit
PCR	polymerase chain reaction
QIIME	Quantitative Insights Into Microbial Ecology
SID	standardized ileal digestible

Introduction

The post-weaning period is critical in intensive swine production, due to the frequent decrease in piglets' health status and growth performance (de Lange et al., 2010). One of the main strategies to avoid these problems is the use of antibiotics in animals' diets at subtherapeutic levels. Antibiotics are used to promote intestinal health and the optimum expression of animals' genetic potential, reducing mortality caused by subclinical enteric diseases (Brenes and Roura, 2010).

However, there is evidence that the use of antibiotics at subtherapeutic levels can cause bacterial resistance, which could be transferred to human pathogenic bacteria (Teillant et al., 2015). Therefore, addition of antibiotics at subtherapeutic levels for growth promotion in animal feeds has been prohibited in the European Union since 2006 and reduced in many other countries (Cheng et al., 2014).

Phytogenic additives are derived from plants and contain a great diversity of active attributes, with the potentially positive use in animal nutrition. The possible mechanisms of action of these products are antioxidant and antimicrobial effects, resulting in the improvement of intestinal health (Jacela et al., 2010). However, the results from research evaluating phytogenic additives as growth performance enhancers for newly weaned piglets are variable and contradictory (Franz and Novak, 2009; Lallès et al., 2009; de Lange et al., 2010).

Acidifiers present antimicrobial activity, can increase gut and pancreas enzyme secretion, and positively influence intestinal villi by serving as an energetic substrate for epithelial cells (Lallès et al., 2009; de Lange et al., 2010). All of these points together can improve pigs' intestinal health and growth performance and can potentially be an effective alternative to the use of antibiotics.

The objective of the present research was to evaluate an alternative feed additive containing the combination of benzoic acid and the essential oils of eugenol, thymol, and piperine, associated or not with colistin, in the feeding of newly weaned piglets. The parameters evaluated were growth performance, nutrient diet digestibility, diarrhea incidence, digesta transit time, intestinal morphology, relative weights of digestive organs, microbial diversity, and the percentages of operational taxonomic unit (**OTU**) of *Lactobacillus*, *Bifidobacterium*, *Clostridium*, *Escherichia–Shigella*, and *Campylobacter* in the cecum content.

Materials and Methods

The study was conducted at Institute of Animal Science and Pastures, in Nova Odessa, São Paulo, Brazil, and all animal procedures followed the guidelines established by the Brazilian Council of Animal Experimentation and were reviewed and approved by the "Instituto de Zootecnia" Committee of Ethics and Animal Welfare (protocol number 219-15).

Animals, housing, and diets

One-hundred and eight crossbred barrows weaned at 21-d old (5.3 ± 0.5 kg of body weight) were housed in groups of three, in pens (1.0 × 2.0 m) with a slatted plastic floor equipped with 4-feeding space dry feeder and nipple drinkers. The animals had free access to feed and water during all experimental period. The trial was divided into the following phases: phase I—from 21 to 35 d (5.3 ± 0.5 to 6.5 ± 1.2 kg), phase II—from 36 to 50 d (6.5 ± 1.2 to 11.7 ± 2.6 kg), and phase III—from 51 to 65 d (11.7 ± 2.6 to 19.4 ± 3.8 kg).

The animals were submitted to four experimental diets, as follows: a control diet (**CON**) with no inclusion of any type of growth promoter feed additive; a diet with 40 ppm of the antibiotic colistin (diet **COL**); a diet with 0.3 % inclusion of an alternative additive, composed of benzoic acid and the essential oils of eugenol, thymol, and piperine (diet **AA**); and a diet with 0.3 % inclusion of alternative additive and 40 ppm of colistin (diet **AACOL**). The contents of the active components of the alternative additive were: 89.3 % benzoic acid, 1.8 % thymol, and 3.2 % eugenol and piperine. The diets were composed of corn, soybean meal, spray-dried blood plasma, and dairy products, supplemented with vitamins, minerals, and amino acids, and formulated according to the method described by Rostagno et al. (2011) (Table 1).

The animals were submitted to a sanitary challenge (adapted from Kahindi et al., 2014) to properly evaluate the feed additives, which consisted of spraying 2 liters of diluted pig manure at each nursery pen 2 d before the beginning of the trial. The manure was collected from the manure pond of the pig farm where the piglets were born and diluted with water (60% of manure to 40% of water). Additionally, the pens were not washed before and during phase I of the trial.

Experimental procedures and sample collection

Animals and feed were weighed at the beginning and at the end of each phase (days 1, 14, 29, and 44) to determine the average daily gain (**ADG**) and average daily feed intake (**ADFI**) and to calculate feed efficiency (ADG:ADFI). All wasted feed by the pigs was collected from the floor nearby the feeders daily, weighed, and discounted from the amount provided to the pigs.

In the first 14 d of the trial, the presence of diarrhea was monitored, daily, by visual observation of animal feces consistency and signs of stool adhered to pigs' rear. Feces were

Table 1. Ingredients and calculated chemical compositions of experimental diets in phases I (21 to 35 d), II (36 to 50 d), and III (51 to 65 d)

Ingredients, g/kg	Phase I	Phase II	Phase III
Corn	392.55	481.76	631.35
Soybean meal	293.74	324.70	291.99
Dairy product[1]	162.99	30.40	—
Dairy product[2]	41.95	86.14	—
Dried blood plasma	39.54	20.00	—
Dicalcium phosphate	15.84	16.07	16.00
Limestone	7.92	8.11	7.60
Salt	—	1.87	4.43
AIA	10.00	10.00	10.00
Inert[3]	11.33	11.33	9.39
Mineral supplement[4]	1.00	1.00	1.00
Vitamin supplement[5]	3.00	3.00	3.00
Soybean oil	13.46	—	20.75
L-Lysine HCl, 78%	3.16	3.07	2.90
L-Threonine, 98%	1.02	1.03	0.77
DL-Methionine, 99%	1.69	1.32	0.62
L-tryptophan, 98.5%	0.06	—	—
L-valine, 96.5%	0.55	—	—
Antioxidant	0.00	0.20	0.20
Total	1,000.00	1,000.00	1,000.00
Calculated nutrient levels, as-fed			
Metabolizable energy, kcal/kg	3,400	3,375	3,230
CP, g/kg	220.0	214.2	189.1
Available phosphorus, g/kg	4.5	4.1	3.6
Calcium, g/kg	8.5	8.2	7.6
SID lysine, g/kg	14.5	13.3	10.9
SID methionine + cysteine, g/kg	8.1	7.4	6.1
SID threonine, g/kg	9.1	8.3	6.8
SID tryptophan, g/kg	2.6	2.3	1.9
SID valine, g/kg	10.0	9.1	7.9

[1]Nuklospray L70-5—70 % Lactose, Trouw Nutrition Sloten, The Netherlands.
[2]Nuklospray E50—38 % Lactose, Trouw Nutrition Sloten, The Netherlands.
[3]Alternative additive and/or colistin were added to diets substituting part of the inert material (Kaolin).
[4]Mineral supplement (with no growth promoter) provided per kg of diet: Co, 0.168 mg; Cu, 0.015 g; Fe, 0.025 g; I, 1.42 mg; Mn, 0.04 g; and Zn, 0.075 g.
[5]Vitamin supplement (with no growth promoter) provided per kg of diet: Vit. A, 8,000 UI; Vit. D3, 3,000 UI; Vit. K, 8 mg; Vit. B2, 6 mg; Vit. B12, 33 mcg; Vit. B6, 2 mg; Vit. B1, 2 mg; Vit. E, 30 UI; Calcium pantothenate, 21 mg; Niacin, 0.04 g; Folic acid, 1.20 mg; Biotin, 0.05 mg; Selenium, 0.39 mg; and Choline, 0.36 g.

classified according to the following scores: 1—normal feces, 2—doughy feces, and 3—diarrheal feces. These evaluations were always performed by the same observer. Feces assigned with scores 1 and 2 were considered normal, and feces assigned score 3 were considered diarrheal feces. The frequency of feces scores 1, 2, and 3 were the percentage of days pigs presented feces scores 1 or 2 or 3 in each pen, calculated as follows: frequency of feces score 1 or 2 or 3 (%) = {[(P1 × D) + (P2 × D) + (Pn × D)]/*n*/TD} × 100, where P (1, 2…*n*) represents each pig within a pen (*n*); D is the number of days each pig showed feces scores 1 or 2 or 3 within a pen; TD is the total number of days in which diarrhea scores were monitored (Milani et al., 2017). The frequencies of feces scores 1, 2, and 3 were transformed [y = arcsine√(p/100)] for statistical evaluation.

The apparent total tract digestibility (**ATTD**) of dry matter (**DM**), organic matter (**OM**), crude protein (**CP**), and gross energy (**GE**) of the diets were determined by the index method (Adeola, 2001), using acid insoluble ash (**AIA**) as an index compound. The pigs were fed diets with 1% of AIA throughout the trial. Freshly voided feces were collected from 0800 to 1600 hours, by hand grab-sampling from pen floors on days 7, 8, and 9 (phase 1); 22, 23, and 24 (phase 2); and 36, 37, and 38 (phase 3). Feces were pooled by pen and frozen at –20 °C.

At the end of phases I and III, nine animals per diet (one pig per experimental unit) were euthanized for weighing the digestive organs and for the collection of small intestine tissues and cecum content. The pigs were fasted for 12 h, stunned by electronarcosis, euthanized by bleeding, and immediately had their gastrointestinal tracts removed.

The weights of the liver, pancreas, empty stomach, empty small intestine, empty cecum, and colon were recorded, as described by Pond et al. (1988). Organs' weights were expressed as a percentage of the body weight.

Sample processing and laboratory analyses

Feces samples were thawed, homogenized per pen and collection phase, dried at 55 °C for 72 h, ground in a knife mill (1 mm), and analyzed for DM (method 930.15), ash (method 942.05), CP (Nitrogen × 6.25; method 988.05), as described by AOAC (2006), and GE in an adiabatic calorimeter (model 5003; Ika-Werke GmbH & Co., Staufen, Germany). The OM contents of samples were calculated using the determined DM and ash values. AIA determinations were performed according to van Keulen and Young (1977). The ATTD of DM, OM, CP, and GE, and digestible energy values, were calculated according to Adeola (2001).

Tissues samples from the proximal duodenum and jejunum were harvested (approximately 1.0 cm long) to determine the villi height and width, crypt depth, and goblet cell count using light microscopy techniques. Samples were opened in the mesenteric border, washed, extended by serosa, and placed in a fixative Bouin solution for 24 h. Subsequently, samples were washed in running water and in 70% ethanol to remove the fixative solution and then dehydrated in an ascending series of alcohols, from 70% to 100%, rinsed in xylene, and embedded in paraffin. Samples were microtomized over a width of 5 µm, and 15 semi serial cuts were made for each segment of each animal for mounting on a histological slide, which was stained with hematoxylin and eosin (Tolosa et al., 2003).

The sections were examined under a light microscope (model BX41, Olympus, Tokyo, Japan) coupled to a system to capture images (Olympus DP11-N). The images were transferred to a computer, and morphometric evaluations were performed using the software Image-Pro Plus (Media Cybernetics, Inc., Washington, USA). All measurements, villi height and width, crypt depth, and goblet cell count, were performed in 15 well-oriented villi and crypts per sample.

The cecum of each animal was immediately separated after euthanasia in phase I and its content was collected through an incision made with a set of disinfected instruments for each pig, homogenized, and stored into individual sterile containers, which were frozen and maintained at –20 °C until analysis.

Samples of cecum contents were processed for DNA extraction according to Lu et al. (2003), using a DNA isolation kit (MO BIO, Inc., Qiagen, Hilden, Germany). Before DNA amplification, samples were screened for DNA concentration and purity using nanodrop microvolume spectrophotometers (NanoDrop 2000/c ThermoFisher Scientific, Waltham, Massachusetts, USA), and its

integrity was verified using agarose gel (1.5%) electrophoresis. Then, DNA was stored at –20 °C prior to following procedures.

The gene-specific sequences targeting the 16S V3-V4 region were amplified in all samples using the primers Forward (5′-CCT ACG GGN GGC WGC AG-3′) and Reverse (5′-GAC TAC HVG GGT ATC TAA TCC-3′) (Klindworth et al., 2013). The first polymerase chain reaction (**PCR**) was performed for locus-specific amplification. Sequentially, AMPure XP beads (Beckman Coulter Inc., Brea, USA) were used for PCR purification. The size of the fragments generated in the PCR was evaluated by 1.5% agarose gel electrophoresis. The second PCR was performed to ligate the Illumina Nextera XT barcodes (Illumina, Inc., San Diego, USA) and other steps to purify the PCR and to validate the libraries were performed. Subsequently, libraries were quantified and equimolarly joined in a single pool. The libraries were quantified using the KAPA library quantification kit (Roche Sequencing and Life Science Kapa Biosystems, Wilmington, MA, USA). To introduce complexity to sequencing, a control heterogeneous, the 20% of PhiX phage, was combined with the amplicon pool. Finally, denaturation of libraries and PhiX was performed to allow sequencing.

Sequencing was performed using the MiSeq Reagent Kit V2 (500 cycles) 2 × 250 pb (Illumina, Inc., San Diego, USA). The data were analyzed by the Real-Time Analysis program (provided by Illumina), which made the base call of the sequencing images, converting them into FASTQ format sequences, with each base accompanied by a quality Phred score (Ewing et al., 1998). After conversion to FASTQ, initial evaluation of sequencing readings was done with the FastQC version 0.11.4 program (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Initially, reads forward and reverse were merged into a FASTA file using a script implemented in the Quantitative Insights Into Microbial Ecology (**QIIME**) pipeline (Caporaso et al., 2010). Quality control steps included filtering for low quality and truncated size readings, and removing singleton sequences, using the VSEARCH program (Edgar, 2010) (https://github.com/torognes/ vsearch). The analysis was performed following the pipeline recommended by the Brazilian Microbiome Project, with some modifications (Rognes et al., 2016).

Sequence clustering into 97% similarity OTUs was performed using the UPARSE method of UCLUST program (http://drive5.com/usearch/manual/uclust_algo.html) (Pyrlo et al., 2014). Possible chimeras have been removed. Readings were then mapped against OTU, and taxonomies were assigned using the Ribossomal Database Project, version 2.2 method implemented in the QIIME pipeline (Caporaso et al., 2010), using the Silva database, version 128 (Quast et al., 2013).

For OTU-based diversity analysis, the original OTU table was rarified to sequencing depth (minimum sample depth) to minimize sampling effects. The rarefaction α was performed using the phylogenetic diversity, Chao1, and observed species metrics. The β diversity was estimated by computing weighted and unweighted UniFrac distances between samples using the QIIME pipeline.

Calculation and statistical analysis

A randomized block design, based on the animal's initial weight, in a 2 × 2 factorial arrangement (with or without alternative additive; with or without antibiotic) was used. The experimental unit was the pen, with three animals in phase I and two animals in phases II and III, with nine repetitions per treatment. The normality of the errors was tested by the Cramer–von Misses method, and data were examined for outliers. The percentages of OTU of the genus *Lactobacillus*, *Bifidobacterium*, *Clostridium*, *Escherichia–Shigella*, and *Campylobacter* and the results of fecal score were transformed [y = arcseno√(p/100)]. All data were submitted to ANOVA by the PROC MIXED of SAS (SAS 9.1, SAS Institute, Cary, NC, USA); means comparisons were performed using Tukey's test; and statistical significance was set at $P < 0.05$ and tendency between $P > 0.05$ to $P < 0.10$. The pigs were not redistributed to blocks at the end of each phase; therefore, the statistical analyses of growth performance data in phases II and III were performed using the initial body weight of pigs in each phase as a covariate.

Results

The inclusion of an alternative feed additive in newly weaned piglets' diet in phase I, independent of antibiotic usage, increased ($P < 0.05$) pigs ADG by 25%, ADFI by 16%, and final body weight (Table 2), compared with those that did not receive the alternative feed additive in their diets. The ADFI of control pigs was greater ($P < 0.05$) than of pigs fed AA diets, and both groups of pigs did not differ ($P > 0.05$) from COL and AACOL animals, in phase II. There were no effects ($P > 0.05$) of any of the additives on the growth performance of pigs in phase III and in the overall period.

The fecal score evaluation (Table 2) showed a tendency ($P = 0.06$) for a greater normal feces (score 1) frequency as a consequence of alternative additive use. The animals fed the diet AACOL showed 41% of feces score 1 and 17% of feces score 3 (diarrheal feces), while piglets submitted to the control diet presented 28% of normal feces and 23% of diarrheal feces. Although these results were only a tendency, the normal feces frequency was 46% greater, and the diarrheal feces frequency was 26% lower in pigs fed diets with both feed additives compared with pigs fed the control diet.

Regarding ATTD of nutrients and energy (Table 3), the alternative additive use improved ($P < 0.05$) ATTD of CP (71.0% vs. 68.6%) and the digestible energy in phase I (3,207.6 vs. 3,093.4 kcal/kg), ATTD of GE in phases I (77.4% vs. 75.2%) and III (79.0% vs. 77.1%), and ATTD of DM in phase III (79.1% vs. 77.1%). The antibiotic inclusion in the diets increased ($P < 0.05$) ATTD of CP in phase I (71.5% vs. 68.2%). The dietary inclusion of the alternative additive and of the antibiotic interacted for ATTD of DM, OM, CP, GE, and digestible energy in phase II. Piglets submitted to diets AA, COL, and AACOL presented similar means ($P > 0.05$) for all of these variables, but greater ($P < 0.05$) than the ones showed by control piglets.

Concerning the histological evaluations in phase I (Table 4), piglets fed diets with the alternative additive had fewer ($P < 0.05$) goblet cells in the jejunum than the other piglets (104.3 vs. 118.1). The use of colistin promoted higher ($P < 0.05$) villi width and more ($P < 0.05$) goblet cells in the jejunum. Piglets fed diets CON, AA, and AACOL presented similar ($P > 0.05$) crypt depth in jejunum, but greater ($P < 0.05$) than COL pigs.

In phase III (Table 4), pigs fed the diets with antibiotics presented higher ($P < 0.05$) villi height in the jejunum and more goblet cells in the duodenum than piglets that did not receive antibiotics in their diets. Interactions between colistin and the alternative additive use were found for some variables in this phase. Villi height in the duodenum of piglets fed diet AA was lower ($P < 0.05$) than for piglets fed diets AACOL, AA, and CON. With regard to villi width in the duodenum, CON animals showed narrower villi ($P < 0.05$) than pigs fed diets COL, AA, and AACOL. All groups of pigs presented different ($P < 0.05$) villi width in the jejunum; CON animals showed the lowest value; and AACOL pigs had the greatest mean. In addition, in phase III,

Table 2. The effects of diets with alternative additive (A) and colistin (C) on the growth performance of piglets in phase I (21 to 35 d of age), phase II (36 to 50 d of age), phase III (51 to 65 d of age), overall (21 to 65 d of age), and on fecal scores from 21 to 35 d of age[1]

	Diet[2]					P-value		
Item	CON	COL	AA	AACOL	SEM	A	C	A × C
Body weight, kg								
Day 1	5.34	5.31	5.29	5.25	0.05	—	—	—
Day 14	6.64	6.60	6.71	7.13	0.13	0.028	0.125	0.198
Day 29	12.01	11.51	11.73	12.49	0.29	0.49	0.81	0.23
Day 44	19.87	18.45	19.29	20.64	0.80	0.32	0.96	0.09
ADG, g								
Phase 1	93.5	90.1	97.5	131.1	5.8	0.033	0.137	0.074
Phase 2	397.5	392.7	393.5	365.8	12.8	0.427	0.431	0.541
Phase 3	564.0	549.3	513.0	571.0	13.0	0.201	0.378	0.087
Overall	346.5	312.2	332.0	366.1	15.7	0.30	0.99	0.08
ADFI, g								
Phase 1	161.3	165.3	175.2	205.0	6.30	0.016	0.116	0.226
Phase 2	493.4	509.6	479.3	477.7	18.4	0.757	0.335	0.708
Phase 3	949.2[a]	905.0[ab]	848.7[b]	927.5[ab]	27.0	0.466	0.107	0.014
Overall	536.2	478.3	520.8	550.2	24.0	0.339	0.618	0.137
Feed efficiency								
Phase 1	0.568	0.518	0.571	0.591	0.189	0.337	0.690	0.374
Phase 2	0.722	0.668	0.717	0.679	0.019	0.220	0.938	0.821
Phase 3	0.673	0.632	0.649	0.640	0.009	0.180	0.654	0.397
Overall	0.649	0.645	0.621	0.637	0.910	0.191	0.615	0.373
Percentage of fecal score								
Simple means, %								
1	27.8	30.5	36.9	40.9	—	—	—	—
2	48.8	42.5	42.1	42.0	—	—	—	—
3	23.4	27.0	21.0	17.1	—	—	—	—
Transformed means								
1	0.523	0.583	0.627	0.658	0.05	0.056	0.309	0.753
2	0.773	0.618	0.696	0.718	0.05	0.816	0.187	0.082
3	0.485	0.470	0.429	0.342	0.04	0.212	0.488	0.624

[1]Least-squares means based on nine pen observations per diet.
[2]CON, control diet; COL, diet containing 40 ppm colistin; AA, diet with 0.3% inclusion of alternative additive composed of benzoic acid and essential oils of eugenol, thymol and piperine; AACOL, diet with 0.3% AA and 40 ppm colistin.
[a,b]Means within a row with different superscripts differ ($P < 0.05$).

Table 3. The effects of alternative additive (A) and colistin (C) on ATTD of DM and OM, CP, GE, and the digestible energy of diets fed to pigs in phases I (21 to 35 d), II (36 to 50 d), and III (51 to 65 d)[1]

	Diet[2]					P-value		
Item	CON	COL	AA	AACOL	SEM	A	C	A × C
ATTD of DM, %								
Phase I	76.3[b]	78.7[ab]	79.3[a]	78.9[a]	2.3	0.021	0.148	0.048
Phase II	68.3[b]	75.0[a]	75.5[a]	76.8[a]	2.2	<0.001	<0.001	0.002
Phase III	77.2	76.9	78.7	79.5	3.1	0.009	0.703	0.467
ATTD of OM, %								
Phase I	78.8	81.1	81.3	81.2	2.3	0.090	0.136	0.061
Phase II	72.3[b]	78.2[a]	78.6[a]	79.6[a]	2.3	<0.001	<0.001	0.002
Phase III	80.6	79.8	81.3	82.2	3.2	0.086	0.967	0.342
ATTD of CP, %								
Phase I	66.0	71.2	70.3	71.7	2.9	0.038	0.006	0.100
Phase II	59.7[b]	67.8[a]	69.5[a]	70.8[a]	2.2	<0.001	0.006	0.037
Phase III	74.0	74.4	76.6	76.5	3.0	0.068	0.888	0.844
ATTD of GE, %								
Phase I	73.9	76.4	77.5	77.2	2.2	0.011	0.177	0.089
Phase II	65.9[b]	73.0[a]	73.9[a]	74.9[a]	2.2	<0.001	<0.001	0.003
Phase III	77.0	77.1	78.5	79.4	3.1	0.028	0.548	0.656
Digestible energy, kcal/kg								
Phase I	3,049.1	3,137.6	3,207.1	3,208.1	92.5	0.004	0.180	0.190
Phase II	2,639.1[b]	2,906.8[a]	2,950.7[a]	3,002.7[a]	87.8	<0.001	<0.001	0.008
Phase III	3,020.8	3,061.5	3,059.5	3,133.7	123.0	0.100	0.089	0.610

[1]Least-squares means based on nine pen observations per diet.
[2]CON, control diet; COL, diet containing 40 ppm colistin; AA, diet with 0.3% inclusion of alternative additive composed of benzoic acid and essential oils of eugenol, thymol and piperine; AACOL, diet with 0.3% AA and 40 ppm colistin.
[a,b]Means within a row with different superscripts differ ($P < 0.05$).

piglets fed diet AACOL presented greater crypt depth ($P < 0.05$) in the duodenum than animals fed diet COL, whose crypt was deeper ($P < 0.05$) than that of CON and AA pigs. AACOL piglets showed greater ($P < 0.05$) crypt depth on the jejunum than CON, COL, and AA pigs. The number of goblet cells in piglets fed diets CON, AA, and AACOL did not differ ($P > 0.05$) but was lower ($P < 0.05$) than that of COL pigs.

Concerning organs' weights as a percentage of body weight (Table 4), the use of the alternative additive in phase I decreased ($P < 0.05$) small intestine (4.60% vs. 4.93%) and colon (1.41% vs. 1.65%) relative weights compared with swine not fed with the alternative additive. There was an interaction between alternative additive and antibiotic for liver relative weight in phase I and for pancreas relative weight in phase III. Animals receiving diet AACOL presented lower relative liver weights ($P < 0.05$) than pigs receiving diet AA, but there were no differences ($P > 0.05$) between animals that were not fed diets with the alternative additive. The pancreas relative weight of animals submitted to the AACOL diet was greater ($P < 0.05$) than that observed in pigs fed diets COL and AA. CON animals presented similar pancreas relative weight ($P > 0.05$) when compared with pigs fed diets COL, AA, and AACOL.

The alpha diversity measured using Chao 1 index (Figure 1) and beta bacterial diversity examined using principal component analysis (Figure 2), as well as the percentages of *Lactobacillus*, *Bifidobacterium*, and *Clostridium* in the cecum content (Table 5) of pigs, were not affected ($P > 0.05$) by the experimental diets. On the other hand, there was a tendency ($P = 0.09$) for a lower concentration of *Escherichia–Shigella* (1.46% vs. 3.5%) and a lower ($P < 0.05$) percentage of *Campylobacter* (0.52% vs. 10.21%) in the cecum content of piglets fed diets containing essential oils and benzoic acid compared with pigs fed diets without the alternative feed additive.

Table 4. The effects of diets with alternative additive (A) and colistin (C) on villus height and width, crypt depth, number of goblet cells in duodenum and jejunum, and relative empty organ weights of piglets slaughtered at 35 and 65 d of age[1]

	Diet[2]					P-value		
Item	CON	COL	AA	AACOL	SEM	A	C	A × C
Slaughter at 35 d								
Villus height, µm								
Duodenum	614.4	618.4	616.5	618.5	1.7	0.736	0.367	0.754
Jejunum	651.3	652.7	651.2	654.3	1.1	0.724	0.287	0.660
Villus width, µm								
Duodenum	330.8	332.4	332.1	332.8	1.4	0.623	0.474	0.776
Jejunum	351.4	352.5	351.3	359.6	1.2	0.087	0.023	0.080
Crypt depth, µm								
Duodenum	247.3	249.5	251.1	250.1	1.4	0.427	0.836	0.576
Jejunum	266.0[a]	260.8[b]	263.1[a]	265.4[a]	0.7	0.516	0.269	0.006
Goblet cells								
Duodenum	102.2	104.8	105.1	107.0	1.3	0.333	0.399	0.899
Jejunum	113.0	123.1	101.4	107.1	2.1	<0.001	0.004	0.373
Relative weights, % of body weight								
Small intestine	4.99	4.87	4.58	4.62	0.16	0.039	0.781	0.593
Stomach	0.95	0.90	0.94	0.90	0.03	0.899	0.316	0.878
Liver	2.71[ab]	2.99[ab]	3.06[a]	2.60[b]	0.11	0.922	0.524	0.015
Pancreas	0.20	0.22	0.19	0.18	0.01	0.215	0.627	0.371
Cecum	0.29	0.31	0.35	0.29	0.02	0.602	0.613	0.266
Colon	1.60	1.70	1.43	1.39	0.06	0.001	0.695	0.271
Slaughter at 65 d								
Villus height, µm								
Duodenum	720.8[a]	719.0[a]	698.1[b]	715.5[a]	1.9	<0.001	<0.001	<0.001
Jejunum	740.5	758.0	749.7	759.6	2.3	0.118	<0.001	0.257
Villus width, µm								
Duodenum	433.6[b]	444.8[a]	447.3[a]	447.9[a]	1.5	0.002	0.022	0.038
Jejunum	333.0[d]	343.1[c]	352.9[b]	373.6[a]	2.7	<0.001	<0.001	0.043
Crypt depth, µm								
Duodenum	326.1[c]	343.2[b]	331.0[c]	357.3[a]	2.3	<0.001	<0.001	0.028
Jejunum	345.8[b]	353.0[b]	351.3[b]	373.4[a]	2.0	<0.001	<0.001	<0.001
Goblet cells count								
Duodenum	152.3	159.7	152.0	155.8	1.0	0.328	0.014	0.409
Jejunum	99.8[b]	117.3[a]	104.9[b]	97.4[b]	1.9	0.024	0.114	<0.001
Relative weights, % of body weight								
Small intestine	4.50	4.16	3.82	4.21	0.19	0.096	0.891	0.059
Stomach	0.91	0.88	0.86	0.85	0.04	0.134	0.424	0.695
Liver	2.77	2.65	2.80	2.63	0.13	0.993	0.325	0.860
Pancreas	0.22[ab]	0.21[b]	0.20[b]	0.25[a]	0.01	0.495	0.047	0.002
Cecum	0.22	0.24	0.22	0.21	0.06	0.794	0.196	0.380
Colon	1.70	1.60	1.64	1.89	0.09	0.276	0.485	0.115

[1]Least-squares means based on nine observations per diet.
[2]CON, control diet; COL, diet containing 40 ppm colistin; AA, diet with 0.3% inclusion of alternative additive composed of benzoic acid and essential oils of eugenol, thymol and piperine; AACOL, diet with 0.3% AA and 40 ppm colistin.
[a,b]Means within a row with different superscripts differ ($P < 0.05$).

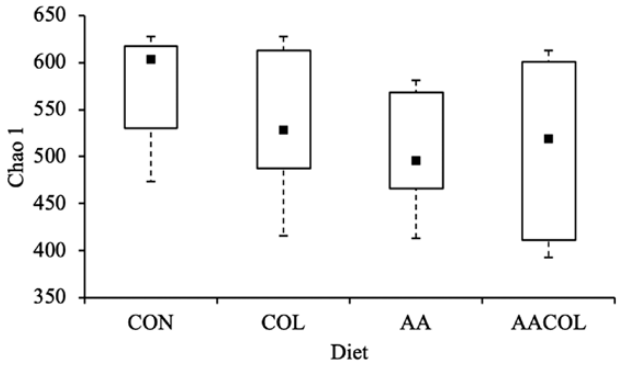

Figure 1. Microbiota alpha-diversity comparison among four dietary treatments. Chao 1 index of four dietary treatments—CON, COL, AA, and AACOL. Pigs were regarded as the experimental unit, diets AA and COL $n = 7$, diet AACOL $n = 6$, and diet CON $n = 5$, due to samples eliminated.

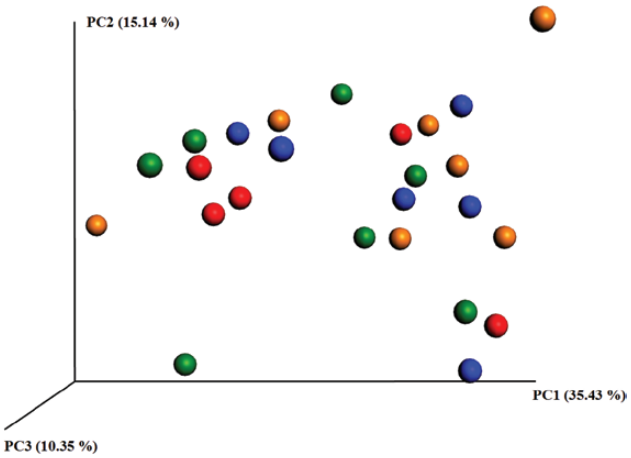

Figure 2. Microbiota beta diversity comparison among four dietary treatments. Principal coordinate analysis to visualize the weighted UniFrac distances of caecum digesta samples from individual pig. Pigs were regarded as the experimental unit, diets AA (orange circles) and COL (green circles) $n = 7$, diet AACOL (blue circles) $n = 6$, and diet CON (red circles) $n = 5$, due to samples eliminated.

Discussion

In general, the use of the alternative additive, solely or associated with an antibiotic, was effective in improving piglets growth performance only in period I. The antibiotic used at 40 ppm, which was the maximum allowed dose for colistin use as a feed additive according to Brazilian legislation, did not show positive results. It is worth highlighting that a sanitary challenge was effectively promoted since the growth performance of control pigs was significantly reduced compared with AA or AACOL piglets.

Initially, effects of the diets on the animals' growth performance were only found in period I. This was possibly related to the fact that the majority of issues that can impair the growth performance of weaned piglets are concentrated in the first 15 d after weaning. Briefly, changes in diet composition and physical form (from sow's milk to solid feed), separation from the sow, mixing with unknown animals, moving to an unknown place (from farrowing to nursery room), and immature immunological and digestive systems of pigs are factors that, taken together, make newly weaned piglets very sensitive, which can frequently be reflected in poor weight gain, high diarrhea incidence, and susceptibility to illness (Boudry et al., 2004; Lallès et al., 2009).

The greater ADG and ADFI of the animals fed diets containing the alternative additive compared with piglets that did not receive these diets in phase I were probably related to the effects of the alternative feed additive on improving diet nutrient digestibility and gut health. The trend for a greater normal feces frequency, the numerical lower diarrhea occurrence, the fewer goblet cells count, and the lower relative weights of small intestine and colon of piglets fed diets containing the alternative additive, compared with pigs fed CON diet, may have occurred through the reduced pathogenic bacteria found in the cecum content of piglets, which may have promoted a better intestinal health.

Some of the possible mechanisms of action of phytogenic additives and organic acids are stimulation of digestion, improvement of digestibility, absorption of nutrients, and stimulation of feed intake (Mellor, 2000; Lallès et al., 2009; de Lange et al., 2010). Essential oils can present diversified actions, such as an increase in feed intake by improvement in feed palatability, stimuli to digestive enzyme secretion, and increases in gastric and intestinal motility (Basmacioğlu Malayoğlu et al., 2010). Organic acids can lower digesta pH, specifically in the stomach, aiding protein digestion, lowering the stomach emptying rate, and stimulating pancreatic enzyme production in the small intestine (de Lange et al., 2010; Diao et al., 2016). All of these processes can improve ATTD of nutrients and energy of diets in pigs and support the results found in this research, because greater feed consumption and improvement in ATTD of nutrients and energy were observed in pigs submitted to diets containing the alternative additive, compared with animals not fed with the product.

Some studies clearly showed the positive effects of organic acids and essential oils on ATTD of nutrients in pigs. The supplementation of weaned piglets diets with fenugreek, clove, and cinnamon (Cho et al., 2006); or a mixture of buckwheat, thyme, *Curcuma*, black pepper, and ginger (Yan et al., 2012); or benzoic acid, thymol, eugenol, and piperine (Zhang et al., 2015); or benzoic acid solely (Diao et al., 2016; Kiarie et al., 2018) improved ATTD of DM and CP compared with pigs submitted to diets with no addition of growth promoter feed additive.

Regarding diarrhea occurrence, one of its major causes is related with intestinal mucosa colonization by pathogenic microorganisms, which produce toxins that impair intestinal health and initiated diarrhea (Pluske et al., 1997; Lallès et al., 2007). Herbal extracts and organic acids can present antimicrobial actions (Lallès et al., 2009; de Lange et al., 2010) by exerting direct effects over pathogenic microorganisms or by favoring the growth of beneficial bacteria for the animal (Kluge et al., 2006; Jacela et al., 2010; Li et al., 2012; Diao et al., 2015). In addition, benzoic acid is considered more effective in killing coliforms and *Salmonella typhimurium* than fumaric, lactic, propionic, formic, and acetic acids (Lallès et al., 2009), and in the present study, the additives were effective in killing *Escherichia–Shigella* and *Campylobacter* in the piglets' cecum.

The organic acids' antimicrobial effects occur by a pH decrease in the stomach, assisting digestibility of nutrients and inhibiting the proliferation of pathogenic microorganisms, and/or by the capacity to penetrate by passive diffusion in their undissociated form through the bacterial wall, inducing internal changes in pH, which are incompatible with certain categories of bacteria which do not tolerate high amplitude of pH and cannot reestablish their homeostasis, leading to its destruction (Kluge et al., 2006; Gräber et al., 2012).

The reduction in *Campylobacter* concentration, the trend for lower *Escherichia–Shigella* content in cecum digesta and the

Table 5. The effects of diets with or without alternative additive (A) and colistin (C) on cecum microbial composition of piglets euthanized at 35 d of age

	Diet[1]					*P*-value		
Item	CON	COL	AA	AACOL	SEM	A	C	A × C
OTU								
Simple means, %								
Bifidobacterium	0.59	0.37	1.18	1.25	0.21	—	—	—
Lactobacillus	19.55	20.73	17.99	24.40	4.20	—	—	—
Clostridium 1	2.09	0.40	2.17	0.90	0.41	—	—	—
Clostridium 2	0.23	1.13	0.19	0.08	0.23	—	—	—
Campylobacter	12.74	7.67	0.09	0.94	1.81	—	—	—
Escherichia–Shigella	4.16	2.84	0.55	2.36	0.66	—	—	—
Transformed means								
Bifidobacterium	0.06	0.06	0.08	0.10	0.01	0.185	0.634	0.587
Lactobacillus	0.40	0.42	0.41	0.45	0.06	0.845	0.780	0.905
Clostridium 1	0.12	0.06	0.11	0.09	0.02	0.717	0.142	0.497
Clostridium 2	0.04	0.06	0.03	0.02	0.01	0.285	0.754	0.456
Campylobacter	0.28	0.18	0.03	0.07	0.04	0.029	0.692	0.360
Escherichia–Shigella	0.18	0.13	0.06	0.10	0.03	0.090	0.921	0.349

[1]CON, control diet; COL, diet containing 40 ppm colistin; AA, diet with 0.3% inclusion of alternative additive composed of benzoic acid and essential oils of eugenol, thymol and piperine; AACOL, diet with 0.3% AA and 40 ppm colistin.
[a,b]Means within a row with different superscripts differ ($P < 0.05$).

absence of effects on bacterial diversity as a result of feeding the alternative additive to pigs found in this study, indicate the specific effects of the additive on pathogenic microorganism and not on beneficial bacteria. These findings are in agreement with other researches that evaluated the effects of organic acids and/or essential oils on intestinal microorganisms. Several studies showed reduced counts of *Escherichia coli* in pigs digesta or feces by the use of benzoic acid (Guggenbuhl et al., 2007); or a mixture of buckwheat, thyme, *Curcuma*, black pepper, and ginger extracts (Yan et al., 2012); or a product containing thymol and cinnamaldehyde (Li et al., 2012); or a feed additive composed of 0.3% benzoic acid plus 0.1% essential oils (thymol, eugenol, and piperine) (Zhang et al., 2015). Similarly, some researchers found not only a reduction in digesta or fecal *E. coli* but also an increase in the concentration of *Bacillus* and *Bifidobacterium* in intestinal content by feeding pigs diets with benzoic acid (Diao et al., 2014) or benzoic acid and thymol (Diao et al., 2015). In addition, Li et al. (2018) did not verify changes in alpha bacterial diversity in the colon of pigs fed organic acids, measured by the Chao 1 index.

Even though *E. coli* may acquire tolerance to acid (Richard and Foster, 2003; Kiarie et al., 2008), testing the perfusion of organic acids in piglets' small intestine infected with enterotoxigenic *E. coli* observed less net fluid and electrolyte losses, indicating the efficacy of these additives in controlling enterotoxigenic *E. coli*-secretory diarrhea. These findings support the results found in the present research, in which piglets fed diets with alternative additive presented a tendency for a lower load of *Escherichia–Shigella* and also a lower load of *Campylobacter* at cecum.

After intestinal mucosa colonization by pathogenic bacteria, the organism of the pig reacts by increasing production of mucus and antimicrobial peptides in goblet cells and by gut wall thickening (Pluske et al., 1997; Maloy and Powrie, 2011). Thus, it was possible to infer that the lower number of goblet cells verified in pigs fed diets AA and AACOL was the result of alternative additive action, solely or combined with colistin, in controlling pathogenic bacteria in piglets' intestines, reducing the stimulus for mucus and antimicrobial peptides production. The lower small intestine and colon relative weights can be related to a reduced, or nonexistent, gut wall thickening in animals fed diets with the alternative additive.

Concerning piglets' growth performance, positive results from organic acids and/or essential oils in piglets feeding were observed in some studies, supporting the results of the present research. The inclusion of benzoic acid in piglets feeding improved feed intake, body weight gain, and feed efficiency by 9%, 15%, and 6%, respectively, compared with piglets fed a control diet (Kluge et al., 2006) or increased the ADG of pigs (Guggenbuhl et al., 2007; Halas et al., 2010; Diao et al., 2016; Kiarie et al., 2018). In addition, piglets fed diets containing a commercial product based on the essential oils of thymol and cinnamaldehyde (Li et al., 2012) or submitted to diets supplemented with 0.5% benzoic acid and 0.3% of a mixture of thymol, eugenol, and piperine (Zhang et al., 2015) presented improvements in ADG, ADFI, and feed efficiency, compared with control pigs that did not receive growth enhancer feed additives.

Regarding the effects of antibiotics on piglets' growth performance, some research showed improvements in ADFI and ADG of pigs fed diets with antibiotics compared with animals submitted to control diets or to diets supplemented with phytogenic additives (Oetting et al., 2006), in contrast to the findings of the present study. However, it is important to highlight that in the research of Oetting et al. (2006), three antibiotics were used (zinc bacitracin, olaquindox, and colistin) at 50 ppm for each one, and these doses were higher than the one used in the present study (40 ppm of colistin).

Feed efficiency was not affected by experimental diets in this study because animals that presented the greatest ADG also demonstrated the highest ADFI. These results are in accordance with those presented by Oetting et al. (2006), who evaluated the use of herbal extracts in piglets feeding. However, Jansons et al. (2011) studied organic acids and phytogenic additives in piglet feed and verified improvements in feed efficiency as a result of feed additives use. In this case, the inclusion of organic acids and phytogenic additives may have stimulated the growth of beneficial microorganisms and also aided in physiological process that resulted in a better feed efficiency (Jacela et al., 2010). Moreover, Kiarie et al. (2018) feeding piglets after weaning

with diets containing 0.5% of benzoic acid, chlortetracycline hydrochloride, and tiamulin or a control diet observed better feed efficiency of the treated animals in relation to the pigs which consumed the diets without the organic acid or the antibiotics.

Intestinal mucosa morphological parameters were not affected by alternative additive use. Similarly, some other researches evaluating herbal extracts (Manzanilla et al., 2006), essential oils and organic acids (Bhandari et al., 2008), and Brazilian red pepper essential oil (Gois et al., 2016) in pigs diets also did not find significant effects of these feed additives over intestinal mucosa morphology in swine.

The results found in the present research corroborate the positive effects of benzoic acid and essential oils on pigs growth performance, ATTD of nutrients, and gut health. New technologies have emerged trying to potentialize its effects, as the combination of organic acids and medium-chain fatty acids to improve the performance of pigs in different ages (Upadhaya et al., 2014, 2016; Devi et al., 2016), suggesting further studies in this area.In the current study, the utilization of the alternative feed additive, containing benzoic acid and the essential oils of thymol, eugenol, and piperine, was positive in piglets feeding, because of the effects on improving ADG, ADFI, ATTD of nutrients and energy, and by reducing pathogenic bacteria of the genus *Escherichia* and *Campylobacter* in the cecum content.

Conflict of interest statement

The authors declare no real or perceived conflicts of interest.

Literature Cited

Adeola, O. 2001. Digestion and balance techniques in pigs. In: Lewis, A. J., and L. L. Southern, editors. *Swine nutrition*. New York (NY): CRC Press; p. 903–916.

AOAC. 2006. *Official methods of analysis*. 18th ed. Arlington (VA): Association of Official Analytical Chemists.

Basmacioğlu Malayoğlu, H., S. Baysal, Z. Misirlioğlu, M. Polat, H. Yilmaz, and N. Turan. 2010. Effects of oregano essential oil with or without feed enzymes on growth performance, digestive enzyme, nutrient digestibility, lipid metabolism and immune response of broilers fed on wheat-soybean meal diets. *Br. Poult. Sci.* **51**:67–80. doi:10.1080/00071660903573702

Bhandari, S. K., B. Xu, C. M. Nyachoti, D. W. Giesting, and D. O. Krause. 2008. Evaluation of alternatives to antibiotics using an *Escherichia coli* K88$^+$ model of piglet diarrhea: effects on gut microbial ecology. *J. Anim. Sci.* **86**:836–847. doi:10.2527/jas.2006-822

Boudry, G., V. Péron, I. Le Huërou-Luron, J. P. Lallès, and B. Sève. 2004. Weaning induces both transient and long-lasting modifications of absorptive, secretory, and barrier properties of piglet intestine. *J. Nutr.* **134**:2256–2262. doi:10.1093/jn/134.9.2256

Brenes, A., and E. Roura. 2010. Essential oils in poultry nutrition: main effects and modes of action. *Anim. Feed. Sci. Tech.* **158**:1–14. doi:10.1016/j.anifeedsci.2010.03.007

Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Peña, J. K. Goodrich, J. I. Gordon, *et al.* 2010. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* **7**:335–33. doi:10.1038/nmeth.f.303

Cheng, G., H. Hao, S. Xie, X. Wang, M. Dai, L. Huang, and Z. Yuan. 2014. Antibiotic alternatives: the substitution of antibiotics in animal husbandry? *Front. Microbiol.* **5**:217. doi:10.3389/fmicb.2014.00217

Cho, J. H., Y. J. Chen, B. J. Min, H. J. Kim, O. S. Kwon, K. S. Shon, I. H. Kim, S. J. Kim, and A. Asamer. 2006. Effects of essential oils supplementation on growth performance, IgG concentration and fecal noxious gas concentration of weaned pigs. *Asian-Australas. J. Anim. Sci.* **19**:80–85. doi:10.5713/ajas.2006.80

Devi, S. M., K. Y. Lee, and, I. H. Kim. 2016. Analysis of the effect of dietary protected organic acid blend on lactating sows and their piglets. *Rev. Bras. Zootecn.* **45**:39–47. doi:10.1590/S1806-92902016000200001

Diao, H., Z. Gao, B. Yu, P. Zheng, J. He, J. Yu, Z. Huang, D. Chen, and X. Mao. 2016. Effects of benzoic acid (VevoVitall®) on the performance and jejunal digestive physiology in young pigs. *J. Anim. Sci. Biotechnol.* **7**:32. doi:10.1186/s40104-016-0091-y

Diao, H., P. Zheng, B. Yu, J. He, X. B. Mao, J. Yu, and D.W. Chen. 2014. Effects of dietary supplementation with benzoic acid on intestinal morphological structure and microflora in weaned piglets. *Livest. Sci.* **167**:249–256. doi:10.1016/j.livsci.2014.05.029

Diao, H., P. Zheng, B. Yu, J. He, X. Mao, J. Yu, and D. Chen. 2015. Effects of benzoic acid and thymol on growth performance and gut characteristics of weaned piglets. *Asian-Australas. J. Anim. Sci.* **28**:827–839. doi:10.5713/ajas.14.0704

Edgar, R. C. 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**:2460–2461. doi:10.1093/bioinformatics/btq461

Ewing, B., L. Hillier, M. C. Wendl, and P. Green. 1998. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res.* **8**:175–185. doi:10.1101/gr.8.3.175

Franz, C., and J. Novak. 2009. Sources of essential oils. In: Baser, K. H. C., and G. Buchbauer, editors. *Handbook of essential oils: science, technology, and applications*. New York (NY): CRC Press; p. 39–81.

Gois, F. D., P. L. G. Cairo, V. D. Cantarelli, L. C. D. Costa, R. Fontana, I. B. Allaman, M. Sbardella, F. M. Carvalho, and L. B. Costa. 2016. Effect of Brazilian red pepper (*Schinus terebinthifolius* Raddi) essential oil on performance, diarrhea and gut health of weanling pigs. *Livest. Sci.* **183**:24–27. doi:10.1016/j.livsci.2015.11.009

Gräber, T., H. Kluge, F. Hirche, J. Brož, and G. I. Stangl. 2012. Effects of dietary benzoic acid and sodium-benzoate on performance, nitrogen and mineral balance and hippuric acid excretion of piglets. *Arch. Anim. Nutr.* **66**:227–236. doi:10.1080/1745039X.2012.676812

Guggenbuhl, P., A. Seon, A. P. Quintana, and C. S. Nunes. 2007. Effects of dietary supplementation with benzoic acid (VevoVitall®) on the zootechnical performance, the gastrointestinal microflora and the ileal digestibility of the young pig. *Livest. Sci.* **108**:218–221. doi:10.1016/j.livsci.2007.01.068

Halas, D., C. F. Hansen, D. J. Hampson, B. P. Mullan, J. C. Kim, R. H. Wilson, and J. R. Pluske. 2010. Dietary supplementation with benzoic acid improves apparent ileal digestibility of total nitrogen and increases villous height and caecal microbial diversity in weaner pigs. *Anim. Feed. Sci. Tech.* **160**:137–147. doi:10.1016/j.anifeedsci.2010.07.001

Jacela, J. Y., J. M. Derouchey, M. D. Tokach, R. D. Goodband, J. L. Nelssen, D. G. Renter, and S. S. Dritz. 2010. Feed additives for swine: fact sheets – prebiotics and probiotics, and phytogenics. *J. Swine Health Prod.* **18**:132–136.

Jansons, I., A. Jemeljanovs, I. H. Konosonoka, V. Sterna, and B. Lujane. 2011. The influence of organic acid additive, phytoadditive and complex of organic acid additive phytoadditive on pig productivity, meat quality. *Agron. Res.* **9**:389–394.

Kahindi, R. K., J. K. Htoo, and C. M. Nyachoti. 2014. Effect of dietary lysine content and sanitation conditions on performance of weaned pigs fed antibiotic-free diets. *Can. J. Anim. Sci.* **94**:115–118. doi:10.4141/CJAS2013-016

van Keulen, J., and B. A. Young. 1977. Evaluation of acid-insoluble ash as natural marker in ruminant digestibility studies. *J. Anim. Sci.* **44**:282–287. doi:10.2527/jas1977.442282x

Kiarie, E., D. O. Krause, and C. M. Nyachoti. 2008. Net fluid and electrolyte losses in enterotoxigenic *Escherichia coli* infected

piglet small intestine upon perfusion with fumaric acid, zinc oxide, egg yolk antibodies or carbadox. *Can. J. Anim. Sci.* **88**:485–488. doi:10.4141/CJAS08011

Kiarie, E., C. Voth, D. Wey, C. Zhu, P. Vingerhoeds, S. Borucki, and E. J. Squires. 2018. Comparative efficacy of antibiotic growth promoter and benzoic acid on growth performance, nutrient utilization, and indices of gut health in nursery pigs fed corn–soybean meal diet. *Can. J. Anim. Sci.* **98**:868–874. doi:10.1139/cjas-2018-0056

Klindworth, A., E. Pruesse, T. Schweer, J. Peplies, C. Quast, M. Horn, and F. O. Glöckner. 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* **41**:e1. doi:10.1093/nar/gks808

Kluge, H., J. Broz, and K. Eder. 2006. Effect of benzoic acid on growth performance, nutrient digestibility, nitrogen balance, gastrointestinal microflora and parameters of microbial metabolism in piglets. *J. Anim. Physiol. Anim. Nutr. (Berl).* **90**:316–324. doi:10.1111/j.1439-0396.2005.00604.x

Lallès, J. P., P. Bosi, P. Janczyk, S. J. Koopmans, and D. Torrallardona. 2009. Impact of bioactive substances on the gastrointestinal tract and performance of weaned piglets: a review. *Animal.* **3**:1625–1643. doi:10.1017/S175173110900398X

Lallès, J. P., P. Bosi, H. Smidt, and C. R. Stokes. 2007. Weaning – a challenge to gut physiologists. *Livest. Sci.* **108**:82–93. doi:10.1016/j.livsci.2007.01.091

de Lange, C. F. M., J. Pluske, J. Gong, and C. 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

Li, S. Y., Y. J. Ru, M. Liu, B. Xu, A. Peron, and X. G. Shi. 2012. The effect of essential oils on performance, immunity and gut microbial population in weaner pigs. *Livest. Sci.* **145**:119–123. doi:10.1016/j.livsci.2012.01.005

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

Lu, J., U. Idris, B. Harmon, C. Hofacre, J. J. Maurer, and M. D. Lee. 2003. Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. *Appl. Environ. Microbiol.* **69**:6816–6824. doi:10.1128/aem.69.11.6816-6824.2003

Maloy, K. J., and F. Powrie. 2011. Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature* **474**:298–306. doi:10.1038/nature10208

Manzanilla, E. G., M. Nofrarías, M. Anguita, M. Castillo, J. F. Perez, S. M. Martín-Orúe, C. Kamel, and J. Gasa. 2006. Effects of butyrate, avilamycin, and a plant extract combination on the intestinal equilibrium of early-weaned pigs. *J. Anim. Sci.* **84**:2743–2751. doi:10.2527/jas.2005-509

Mellor, S. 2000. Herbs and spices promote health and growth. *Pig Progress* **16**:27–30.

Milani, N. C., M. Sbardella, N. Y. Ikeda, A. Arno, B. C. Mascarenhas, and V. S. Miyada. 2017. Dietary zinc oxide nanoparticles as growth promoter for weanling pigs. *Anim. Feed. Sci. Tech.* **227**:13–23. doi:10.1016/j.anifeedsci.2017.03.001

Oetting, L. L., C. E. Utiyama, P. A. Giani, U. S. Ruiz, and V. S. Miyada. 2006. Effects of antimicrobials and herbal extracts on intestinal microbiology and diarrhea incidence in weanling pigs. *Rev. Bras. Zootecn.* **35**:2013–2017. doi:10.1590/S1516-35982006000700019

Pluske, J. R., D. J. Hampson, and I. H. Williams. 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

Pond, W. G., H. G. Jung, and V. H. Varel. 1988. Effect of dietary fiber on young adult genetically lean, obese and contemporary pigs: body weight, carcass measurements, organ weights and digesta content. *J. Anim. Sci.* **66**:699–706. doi:10.2527/jas1988.663699x

Pyrlo, V. S., L. F. Roesch, D. K. Morais, I. M. Clark, P. R. Hirsch, and M. R. Tótola. 2014. Data analysis for 16S microbial profiling from different benchtop sequencing platforms. *J. Microbiol. Methods* **107**:30–37. doi:10.1016/j.mimet.2014.08.018

Quast, C., E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, J. Peplies, and F. O. Glöckner. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* **41**(Database issue):D590–D596. doi:10.1093/nar/gks1219

Richard, H. T., and J. W. Foster. 2003. Acid resistance in *Escherichia coli*. *Adv. Appl. Microbiol.* **52**:167–186. doi:10.1016/s0065-2164(03)01007-4

Rognes, T., T. Flouri, B. Nichols, C. Quince, and F. Mahé. 2016. VSEARCH: a versatile open source tool for metagenomics. *PeerJ.* **4**:e2584. doi:10.7717/peerj.2584

Rostagno, H. S., L. F. T. Albino, J. L. Donzele, P. C. Gomes, R. F. Oliveira, D. C. Lopes, A. S. Ferreira, S. L. T. Barreto, and R. F. Euclides. 2011. *Tabelas brasileiras para aves e suíno: composição de alimentos e exigências nutricionais*. 3rd ed. Viçosa (MG): UFV; p. 252.

Teillant, A., C. H. Brower, and R. Laxminarayan. 2015. Economics of antibiotic growth promoters in livestock. *Annu. Rev. Resour. Econ.* **7**:349–374. doi:10.1146/annurev-resource-100814–125015

Tolosa, E. M. C., C. J. Rodrigues, A. O. Behmer, and A. G. Freitas Neto. 2003. *Manual de técnicas para histologia normal e patológica [Manual techniques to normal histology and pathology]*. 2nd ed. São Paulo (SP): Manole. (in Portuguese).

Upadhaya, S. D., K. Y. Lee, and I. H. Kim. 2014. Protected organic acid blends as an alternative to antibiotics in finishing pigs. *Asian-Australas. J. Anim. Sci.* **27**:1600–1607. doi:10.5713/ajas.2014.14356

Upadhaya, S. D., K. Y. Lee, and I. H. Kim. 2016. Effect of protected organic acid blends on growth performance, nutrient digestibility and faecal micro flora in growing pigs. *J. Appl. Anim. Res.* **44**:238–242. doi:10.1080/09712119.2015.1031775

Yan, L., Q. W. Meng, and I. H. Kim. 2012. Effect of an herb extract mixture on growth performance, nutrient digestibility, blood characteristics, and fecal microbial shedding in weanling pigs. *Livest. Sci.* **145**:189–195. doi:10.1016/j.livsci.2012.02.001

Zhang, Z. F., A. V. Rolando, and I. H. Kim. 2015. Effects of benzoic acid, essential oils and *Enterococcus faecium* SF68 on growth performance, nutrient digestibility, blood profiles, faecal microbiota and faecal noxious gas emission in weanling pigs. *J. Appl. Anim. Res.* **44**:173–179. doi:10.1080/09712119.2015.1031765