Effects of paraformic acid supplementation, as an antibiotic replacement, on growth performance, intestinal morphology and gut microbiota of nursery pigs

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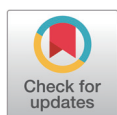
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Abstract

A total of 150 crossbred male pigs (21 ± 1 days old; 8.85 ± 0.15 kg body weight [BW]) were randomly assigned to five dietary treatments with five replicates per treatment and six pigs per pen to evaluate the effect of paraformic acid (PFA), as a substitute to antibiotics, on growth performance, intestinal morphology, and gut microbiota of nursery pigs. The treatments were: 1) Negative control (NC): nutrient adequate control diet; 2) PFA1: similar to NC plus 0.30% PFA; 3) PFA2: similar to NC plus 0.60% PFA; 4) PFA3: similar to NC plus 1.0% PFA; and 5) Positive control (PC): similar to NC plus 0.15% of chlortetracycline. Pigs were fed the same nutritional profile during the two-phase feeding regime (phase 1 [P1; d 0–14], and phase 2 [P2; d 15–30]). Initial BW, and BW and feed disappearance at the end of each phase were recorded to calculate average daily feed intake (ADFI), average daily gain (ADG), and feed to gain ratio (F: G). The Fecal score was determined at the end of P1, while the intestinal morphology and microbiota analysis were performed at the end of P2. Pigs fed PFA2 had higher ADG than those fed NC in P1. A quadratic response was found in the overall phase 1 and phase 2 (P1&2) with the highest ADG in pigs fed PFA2 (*p* < 0.05). Pigs fed PC had the highest ADFI during P2 and overall P1&2 (*p* < 0.05). The PFA2 group had the lowest F:G ratio among treatments in P1 and P2, with a quadratic response in the overall P1&2 (*p* < 0.05). Pigs fed PFA1, PFA2, PFA3, and PC showed better fecal consistency than NC (*p* < 0.05). No differences were found in intestinal morphology among treatments. PFA groups supplementation modulated the relative abundance of *Lactobacillus* and *Streptococcus* in the jejunum. In the cecum, PFA2 had a higher relative abundance of *Prevotella* when compared to NC, but lower than PC. In addition, pigs fed the NC diet

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Availability of data and material
Upon reasonable request, the datasets of this study can be available from the corresponding author.

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Ethics approval and consent to participate
The protocol was reviewed and approved by the Animal Care and Use Committee of the South China Agricultural University, Guangzhou, China (approval number 2021f082).

had higher abundance of *Treponema* and *Methanobrevibacter* than other treatments. In conclusion, the supplementation of 0.6% PFA improved growth performance and modulated gut microbiota in nursery pigs.

Keywords: Paraformic acid, Nursery pigs, Microbiota, Intestinal morphology, Antibiotics

INTRODUCTION

In the modern swine industry, suckling pigs face early weaning stress [1,2], involving dietary and social changes such as switching from sow´s milk to a solid and less palatable plant-based feed, adaptations to a new facility, and establishment of hierarchy between pigs from other litters [3,4]. These sudden events affect normal feed consumption behavior [5]. A reduced feed intake generates morpho-functional modifications of intestinal villus, hyperplasia of crypt depth (CD) [6], reduction in digestive enzyme secretions [7], as well as increased permeability to antigens and toxins [8]. Besides these, the inefficient gastric enzyme activity of pigs during the weaning period, due to a low capacity of hydrochloric acid secretion, allows the flow of a high amount of undigested and contaminated feed to the hindgut [2,9]. As a consequence, it provides ideal conditions for the proliferation of pathogenic bacteria and the onset of post-weaning diarrhea (PWD) [10].

For decades, PWD; one of the most economically relevant diseases in pigs [11], has been efficiently controlled by the therapeutic use of antibiotics [12,13]. However, the continued overuse of antibiotics to combat diseases in both livestock and humans has resulted in the development of bacterial resistance to therapeutic treatments [14,15]. Given the necessity of reducing the use of antibiotics, because of public health concern, it is crucial to develop new feed additive-based nutritional strategies to control gastrointestinal infections related to the weaning transition without adverse effects on human health and the environment [10].

The organic acids, based on their acidifying property and their capacity to control the growth of fungal and enteropathogenic bacteria [16], have been efficiently used for decades as feed hygiene enhancers in animal diets [17,18]. Nursery studies have evidenced that organic acids could be used as a powerful tool in maintaining gut health by suppressing the proliferation of pathogenic bacteria such as *Escherichia coli* [19,20] *Clostridium perfringens* [21], and *Salmonella* [22].

Formic acid has especially been demonstrated to enhance gastric activity [23], gut health [24], immune status [25], and modulate the microbiota [25], leading to improvement of growth performance in nursery pigs. However, formic acid is corrosive [26,27], thus affecting equipment life, creating handling difficulties, and also causing general irritation to workers [28,29]. These disadvantages limit its usage in animal husbandry [16]. Interestingly, formic acid derivatives have been receiving more attention regarding animal feed formulations due to their non-corrosive and non-irritating characteristics [16], without loss of their antimicrobial properties and improvements in growth performance [19,30].

Paraformic acid (PFA), a new formic acid derivative, is a dimer formed from two formic acid molecules and obtained through a polymerization process [22]. Up to now, there is no evidence of whether PFA exhibits beneficial effects on the performance of nursery pigs. Therefore, this study aimed to evaluate the effect of PFA supplementation at different concentrations on growth performance, intestinal morphology, and gut microbiota of nursery pigs.

MATERIAL AND METHODS

Animal care

The protocol was reviewed and approved by the Animal Care and Use Committee of the South

China Agricultural University, Guangzhou, China (approval number 2021f082). The animal experiment was conducted according to the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China). The maximum dosage of formic acid allowed in all species is 10,000 mg/kg according to European Union (EU) regulations 2017/940. The highest level of PFA used in this study was 10 kg/Ton of formulated feed to follow the regulations established by the EU [31]. PFA is a new molecular ingredient made from formic acid and is broken into formic acid molecules in low pH solutions. The dosages used in this experiment did not show any sign of toxicity in the pigs.

Animals and experimental diets

A total of 150 crossbred male pigs (21 ± 1 day old; 8.85 ± 0.15 kg of body weight [BW]) were transferred to the conventional nursery facility of Numega Livestock Research Center, Foshan, China, for a 30-day nursery study. Pigs were randomly assigned to five dietary treatments with five replicates (pen) per treatment and six pigs per replicate. The pigs were raised in a naturally ventilated house and had ad libitum access to feed and water during the entire experiment.

There were five dietary treatments: 1) Negative control (NC): nutrient-adequate control diet, formulated to meet or exceed the nutritional requirement according to the NRC [32]; 2) PFA1: similar to NC plus the addition of 0.30% of PFA (paraformic acid®, Numega Nutrition); 3) PFA2: similar to NC plus the addition of 0.60% of PFA; 4) PFA3: similar to NC plus the addition of 1.0% of PFA; 5) Positive control (PC): similar to NC plus the addition of 0.15% of chlortetracycline (Citifac 20% chlortetracycline; CP BIO). Pigs were fed the same nutritional profile during the two-phase feeding regime (phase 1 [P1; d 0–14], and phase 2 [P2; d 15–30]; Table 1).

Chemical analysis of diets

The percentage of crude protein, crude fat, crude fiber, calcium and phosphorous were determined following the method AOAC 976.05, AOAC 920.39, AOAC 962.09, AOAC 927.02, AOAC 964.06, respectively (Table 2) [33].

Data recording and sample collection

Performance

Individual BW on d 0, and BW and feed disappearance at the end of each phase were recorded to calculate average daily gain (ADG), average daily feed intake (ADFI), and feed to gain ratio (F:G) per phase.

Fecal consistency

At the end of P1, rectal stimulation was performed with sterile swabs to obtain fresh feces. Fecal samples were used to evaluate the fecal consistency following the scoring index described by Sherman et al. [34]: 0, normal (feces firm and well-formed); 1, soft consistency (feces soft and formed); 2, mild diarrhea (fluid feces, usually yellowish); and 3, severe diarrhea (feces watery and projectile).

Intestinal morphology

One pig per pen was sacrificed at the end of P2, following the method described by Hu et al. [35]. Per treatment, a total of six subsamples of middle sections of jejunum tissue were collected and used for measuring intestinal morphology according to the procedure described by Núñez et al. [36]. After sampling, tissues were immediately fixed in 10% neutral buffer formalin, dehydrated with normal saline, carefully embedded in paraffin, and then sliced into 6 μm thick sections. Finally,

Table 1. Diet formulation and calculated composition of basal diet (as-fed basis)

Ingredients	NC	PFA1	PFA2	PFA3	PC
Corn	31.77	31.47	31.17	30.77	31.62
Broken rice	20.00	20.00	20.00	20.00	20.00
Fermented soybean meal	12.50	12.50	12.50	12.50	12.50
Whey power	10.00	10.00	10.00	10.00	10.00
Powercookies	5.00	5.00	5.00	5.00	5.00
Fish meal (Peru)	5.00	5.00	5.00	5.00	5.00
Concentrate soybean meal	5.00	5.00	5.00	5.00	5.00
Extruded soybean	4.77	4.77	4.77	4.77	4.77
Glucose	2.50	2.50	2.50	2.50	2.50
Di-Calcium phosphate	0.53	0.53	0.53	0.53	0.53
Vitamin premix[1]	0.50	0.50	0.50	0.50	0.50
Mineral premix[2]	0.50	0.50	0.50	0.50	0.50
L-lysine HCL	0.63	0.63	0.63	0.63	0.63
DL-Methionine	0.33	0.33	0.33	0.33	0.33
Salt	0.29	0.29	0.29	0.29	0.29
L-threonine	0.28	0.28	0.28	0.28	0.28
ZnO	0.25	0.25	0.25	0.25	0.25
Choline chloride (50%)	0.10	0.10	0.10	0.10	0.10
L-tryptophan	0.05	0.05	0.05	0.05	0.05
Paraformic acid	0.00	0.30	0.60	1.00	0.00
Antibiotic (chlortetracycline)	0.00	0.00	0.00	0.00	0.15
Total	100.00	100.00	100.00	100.00	100.00
Calculated composition					
Metabolizable energy (kcal)	3,258.00	3,235.00	3,209.81	3,177.70	3,246.52
Crude protein (%)	20.00	19.74	19.48	19.13	19.87
Crude fat (%)	6.48	6.37	6.26	6.12	6.43
Crude fiber (%)	2.86	2.81	2.76	2.70	2.84
Ash (%)	4.67	4.63	4.59	4.53	4.65
Calcium (%)	0.75	0.75	0.75	0.75	0.75
Phosphorus (%)	0.81	0.73	0.65	0.54	0.77
Available phosphorus (%)	0.39	0.39	0.39	0.39	0.39
Lysine (%)	1.35	1.28	1.21	1.11	1.31
Methionine + cysteine (%)	0.74	0.63	0.51	0.36	0.68
Threonine (%)	0.87	0.78	0.69	0.57	0.83
Tryptophan (%)	0.22	0.22	0.22	0.22	0.22

[1]The vitamin premix provided per kilogram diet contain: 11375 IU of vitamin A, 3500 IU of vitamin D3, 26.3 IU of vitamin E, 3.5 mg of vitamin K3, 3.5 mg of vitamin B1, 8.8 mg of riboflavin, 5.4 mg of vitamin B6, 0.03 mg of vitamin B12, 17.5 mg of pantothenic acid, 35.0 mg of niacin; 1.75 mg of folacin, 0.14 mg of biotin.

[2]The mineral premix provided per kilogram of diet: 64.4 mg of Cu (cupric glycinate), 165.4 mg of Fe (iron glycine), 47.8 mg of Mn (manganese glycinate), 47.8 mg of Zn (zinc glycinate), 0.54 mg of Se (yeast selenium), 0.68 mg of I (calcium iodate), 0.1 mg of Co (cobaltous sulfate).

NC, negative control; PFA, paraformic acid; PC, positive control.

tissues were stained with haematoxylineosin for histological evaluation. The villus height (VH), villus width (VW), CD, and the villus height to crypt depth ratio (VH:CD) conformed to the morphological analysis and were addressed by a computer-assisted system (image-analysis system;

Table 2. Chemical composition of experimental diets

Nutrients	NC	PFA1	PFA2	PFA3	PC
Crude protein (%)	20.03	19.96	19.89	19.92	20.12
Crude fat (%)	6.45	6.39	6.35	6.24	6.51
Crude fiber (%)	2.87	2.90	2.81	2.76	2.86
Calcium (%)	0.73	0.72	0.70	0.71	0.72
Phosphorous (%)	0.82	0.81	0.80	0.82	0.81

NC, nutrient adequate control diet; PFA1, similar to NC plus 0.3% of PFA; PFA2, similar to NC plus 0.6% of PFA; PFA3, similar to NC plus 1.0 % of PFA; PC, similar to NC plus 0.15% of chlortetracycline.

NC, negative control; PFA, paraformic acid; PC, positive control.

Biowizard, Thaitec). The VH was measured from the tip of the villus to the base between individual villi. The VW was determined as the distance of the base width of the duodenal villi, while the CD measurements were taken from the valley between individual villi to the basal membrane. The CH:CD was calculated as the VH divided by CD.

Sampling, DNA extraction, and sequencing

Sterile swabs were used to collect jejunum and cecum digesta samples. Samples were preserved in Puritan® Liquid Amies and transported to lab on ice, then stored at –80℃ until DNA extraction. The genomic DNA was extracted using the Omega Bio-tek E.Z.N.A. TM stool DNA kit, followed by agarose gel electrophoresis and Nanodrop to detect the purity and concentration of the DNA. The V4 region of 16S rRNA was amplified using the 515F and 806R primer. The TIANSeq Rapid DNA Library kit (TIANGEN Biotech) was used to build a sequencing library, and then sequencing was performed through the Illumina Miseq System (illumine).

Data analysis

Data were analyzed using the PROC GLM procedure of SAS (SAS Institute) as a Randomized Complete Design. Pen was the experimental unit for ANOVA. Orthogonal contrasts were used to determine the linear and quadratic effect of increased levels of PFA in diets (PFA1, PFA2, and PFA3). Probability (p) value < 0.05 were considered significant, and p values between 0.05 and 0.10 as trends. Raw sequencing data were analyzed via QIIME2 (2019. 10 release). Alpha diversity and beta diversity were used to analyze the complexity of species diversity based on different indexes (Shannon index, and Chao1 index).

RESULTS

All piglets were healthy throughout the experimental period. In P1, there were statistical differences in ADG, with the highest gain in pigs fed PFA2 (p < 0.05), while there were no differences in ADG during P2 (Table 3). However, a quadratic response was observed (p < 0.05) in the overall phase 1 and phase 2 (P1&2) with the highest ADG in pigs fed PFA2. The results of ADG were consistent with the BW per phase, where there was a significant difference in the BW at the end of P2 (p < 0.05) and a quadratic tendency on the final BW, being those fed PFA2 the heaviest pigs. No differences were observed in the ADFI during P1. Pigs fed PC showed the highest ADFI (p < 0.05) in P2 and P1&2, with NC, PFA2, and PFA3 as intermediate, and the PFA1 group with the lowest ADFI. Furthermore, there was a positive linear response (p < 0.05) in ADFI in pigs fed increasing levels of PFA (PFA1, PFA2, PFA3) in P2 and P1&2. Regarding to F: G ratio, pigs fed any PFA level showed lower F: G than NC and PC treatments in P1, P2, and P1&2 (p < 0.05). Additionally, a quadratic response was observed in the P1&2 (p < 0.05) with the lowest ratio in pigs

Table 3. Effects of PFA on growth performance of nursery pigs

Performance Parameters[3]	Treatments[1]					SEM	*p*-value[2]		
	NC	PFA1	PFA2	PFA3	PC		Treatment	Linear[1]	Quad[1]
BW (kg)									
d 0	8.39[a]	8.69[a]	8.33[a]	8.73[a]	8.62[a]	0.23	0.62	0.88	0.14
d 14	13.05[a]	13.92[b]	13.92[b]	14.06[b]	13.95[b]	0.30	0.05	0.70	0.83
d 30	20.42[a]	21.36[a]	22.31[a]	21.33[a]	21.46[a]	0.58	0.29	0.95	0.10
ADG (g)									
P1 (d 0–14)	332.5[a]	373.14[b]	399.13[b]	380.28[b]	380.67[b]	12.50	0.02	0.66	0.13
P2 (d 15–30)	468.98[a]	471.62[a]	533.30[a]	459.26[a]	475.59[a]	31.37	0.49	0.79	0.11
P1&2 (d 1–30)	400.73[a]	422.38[a]	466.21[a]	419.77[a]	428.13[a]	16.07	0.10	0.90	0.03
ADFI (g)									
P1 (d 0–14)	431.35[a]	442.02[a]	423.00[a]	434.21[a]	462.03[a]	12.41	0.26	0.64	0.30
P2 (d 15–30)	736.13[ab]	661.98[a]	721.0[ab]	789.85[b]	790.75[b]	32.00	0.05	0.03	0.92
P1&2 (d 1–30)	583.74[ab]	552[a]	572[ab]	612.03[bc]	626.38[c]	16.67	0.03	0.03	0.64
F: G									
P1 (d 0–14)	1.30[c]	1.18[abc]	1.06[a]	1.15[ab]	1.22[bc]	0.04	0.01	0.55	0.07
P2 (d 15–30)	1.62[abc]	1.46[abc]	1.39[a]	1.77[c]	1.72[c]	0.10	0.05	0.06	0.10
P1&2 (d 1–30)	1.46[bc]	1.32[ab]	1.22[a]	1.46[bc]	1.47[c]	0.045	0.01	0.07	0.02

[1]NC: nutrient adequate control diet; PFA1, similar to NC plus 0.3% of PFA; PFA2, similar to NC plus 0.6% of PFA; PFA3, similar to NC plus 1.0 % of PFA; PC, similar to NC plus 0.15% of chlortetracycline.

[2]Ortogonal contrast to determine linear and quadratic response effects of increased levels of PFA in diets (PFA1, PFA2, and PFA3).

[3]Experiment was carried out after weaning during two nursery phases: P1, from day 0 to day 14; P2, from day 15 to day 30; P1&2, from day 0 to 30.

[a–c]Superscripts indicate statistically significant differences (*p* < 0.05).

PFA, paraformic acid; NC, negative control; PC, positive control; BW, body weight; ADG, average daily gain; p, phases; ADFI, average daily feed intake; F:G, feed to gain ratio.

fed PFA2.

Pigs fed any level of PFA (0.3%, 0.6%, and 1.0%) or PC had better fecal scores than pigs fed the NC diet (*p* < 0.05; Table 4). Furthermore, increasing the level of PFA led to a linear reduction in the fecal score at the end of P1 (*p* < 0.05).

There were no statistical differences in the VH, VW, CD, and VH:CD. Pigs fed PFA2 had the best numerical response regarding the morphological parameters evaluated in this study (Table 5; Fig. 1).

The bacterial diversity and richness were not significantly influenced by the different dietary treatments in the jejunum (Shannon index and Chao1 index: Figs. 2A and 2B, respectively), while the weighted and unweighted Unifrac based on principal coordinate analysis show differences in community structures based on treatment groups (Figs. 2C and 2D). In the cecum, no differences were observed in the Shannon index between treatments (Fig. 2E), while a tendency to differ was observed in the Chao1 index, with higher diversity in the PFA3 group (Fig. 2F). In addition, differences in community structures among treatments were observed in the weighted and

Table 4. Effects of PFA on fecal score of nursery pigs

Parameter	Treatments[1]					SEM	*p*-value[2]		
	NC	PFA1	PFA2	PFA3	PC		Treatment	Linear	Quad
Fecal score	2.0[a]	1.05[b]	1.03[b]	0.95[b]	0.92[b]	0.14	0.05	0.02	0.93

[1]NC, nutrient adequate control diet; PFA1, similar to NC plus 0.3% of PFA; PFA2, similar to NC plus 0.6% of PFA; PFA3, similar to NC plus 1.0 % of PFA; PC, similar to NC plus 0.15% of chlortetracycline.

[2]Ortogonal contrast to determine linear and quadratic response effects of increased levels of PFA in diets (PFA1, PFA2, and PFA3).

[a,b]Superscripts indicate statistically significant differences (*p* < 0.05).

PFA, paraformic acid; NC, negative control; PC, positive control.

Table 5. Effects of PFA on intestinal morphology of nursery pigs

Performance Parameters	Treatments[1]					SEM	p-value[2]		
	NC	PFA1	PFA2	PFA3	PC		Treatment	Linear	Quad
VH (mm)	0.39[a]	0.42[a]	0.43[a]	0.4[a]	0.4[a]	0.04	0.95	0.72	0.71
VW (mm)	0.18[a]	0.15[a]	0.19[a]	0.17[a]	0.16[a]	0.01	0.24	0.34	0.07
CD (mm)	0.052[a]	0.051[a]	0.045[a]	0.058[a]	0.05[a]	0.01	0.65	0.34	0.18
VH:CD	7.68[a]	8.54[a]	9.48[a]	7.31[a]	8.48[a]	0.80	0.38	0.33	0.16

[1]NC, nutrient adequate control diet; PFA1, similar to NC plus 0.3% of PFA; PFA2, similar to NC plus 0.6% of PFA; PFA3, similar to NC plus 1.0 % of PFA; PC, similar to NC plus 0.15% of chlortetracycline.

[2]Ortogonal contrast to determine linear and quadratic response effects of increased levels of PFA in diets (PFA1, PFA2, and PFA3).

[a]Superscripts indicate statistically significant differences ($p < 0.05$).

PFA, paraformic acid; NC, negative control; PC, positive control; VH, villus height; VW, villus width; CD, crypt depth; VH:CD, villus height to crypt depth ratio.
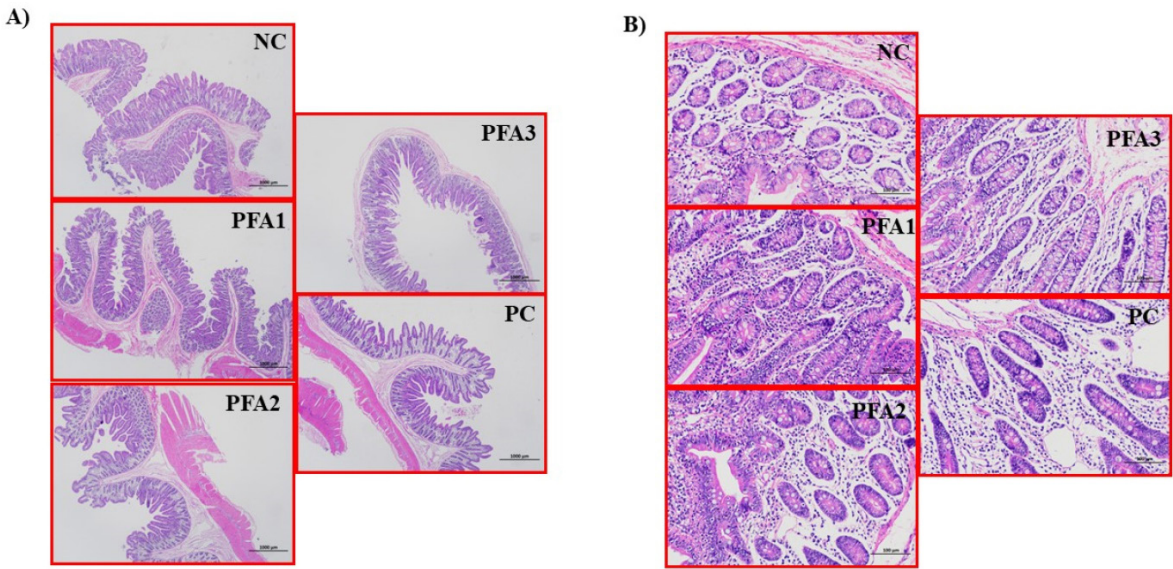

Fig. 1. Histological representation of jejunal A) villi and B) crypt depth of nursey pigs at the end of phase 2 (d 30) under different experimental diets. NC, nutrient adequate control diet; PFA1, similar to NC plus 0.3% of PFA; PFA2, similar to NC plus 0.6% of PFA; PFA3, similar to NC plus 1.0 % of PFA; PC, similar to NC plus 0.15% of chlortetracycline. NC, negative control; PFA, paraformic acid; PC, positive control.

unweighted Unifrac based on principal coordinate analysis (Fig. 2G and 2H, respectively).

The relative abundance of the most dominant jejunal and cecal microbiota is shown in Fig. 3. *Lactobacillus* and *Streptococcus* showed higher relative abundance in pigs fed PFA3 and PFA2, respectively, (Fig. 4A and 4B). On the other hand, the most notable changes in the relative abundance, at the genus level, in cecum samples were *Prevotella*, *Treponema*, and *Methanobrevibacter* (Fig. 4C, 4D, and 4E, respectively). Pigs fed PC had the highest relative abundance of *Prevotella* among treatments, followed by PFA groups (PFA1, PFA2, and PFA3) as intermediate, and NC as the lowest group. Furthermore, PFA1, PFA2, PFA3, and PC treatment had a lower relative abundance of *Treponema* and *Methanobrevibacter* than the NC group.

DISCUSSION

Organic acids have gained attention in the last few years due to their antimicrobial effects on
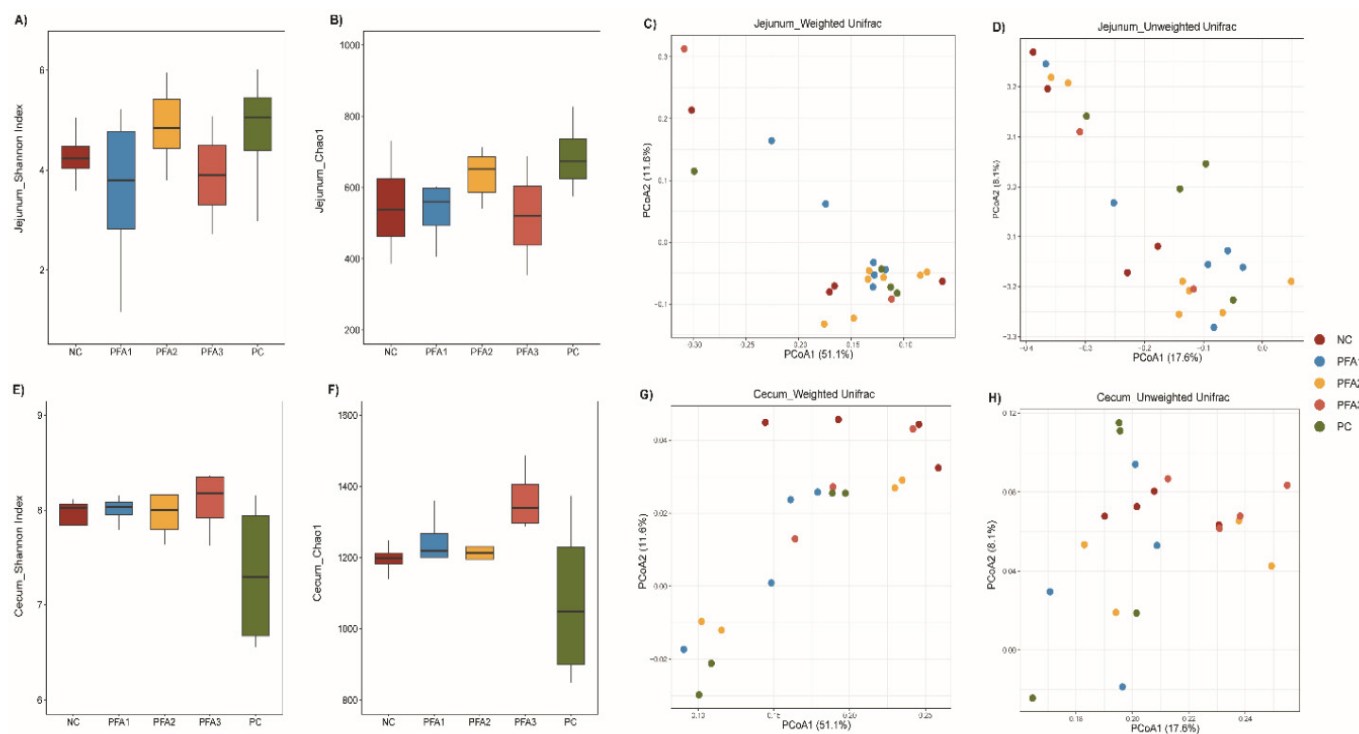
Fig. 2. Effects of paraformic acid (PFA), as antibiotic replacement, on community richness of the gut microbiota in A) jejunum Shannon index; B) jejunum Chao1 index; C) jejunum weighted unifrac; D) jejunum unweighted unifrac; E) cecum Shannon index; F) cecum Chao1 index; G) cecum weighted unifrac; and H) cecum unweighted unifrac. NC, nutrient adequate control diet; PFA1, similar to NC plus 0.3% of PFA; PFA2, similar to NC plus 0.6% of PFA; PFA3, similar to NC plus 1.0 % of PFA; PC, similar to NC plus 0.15% of chlortetracycline. NC, negative control; PC, positive control.
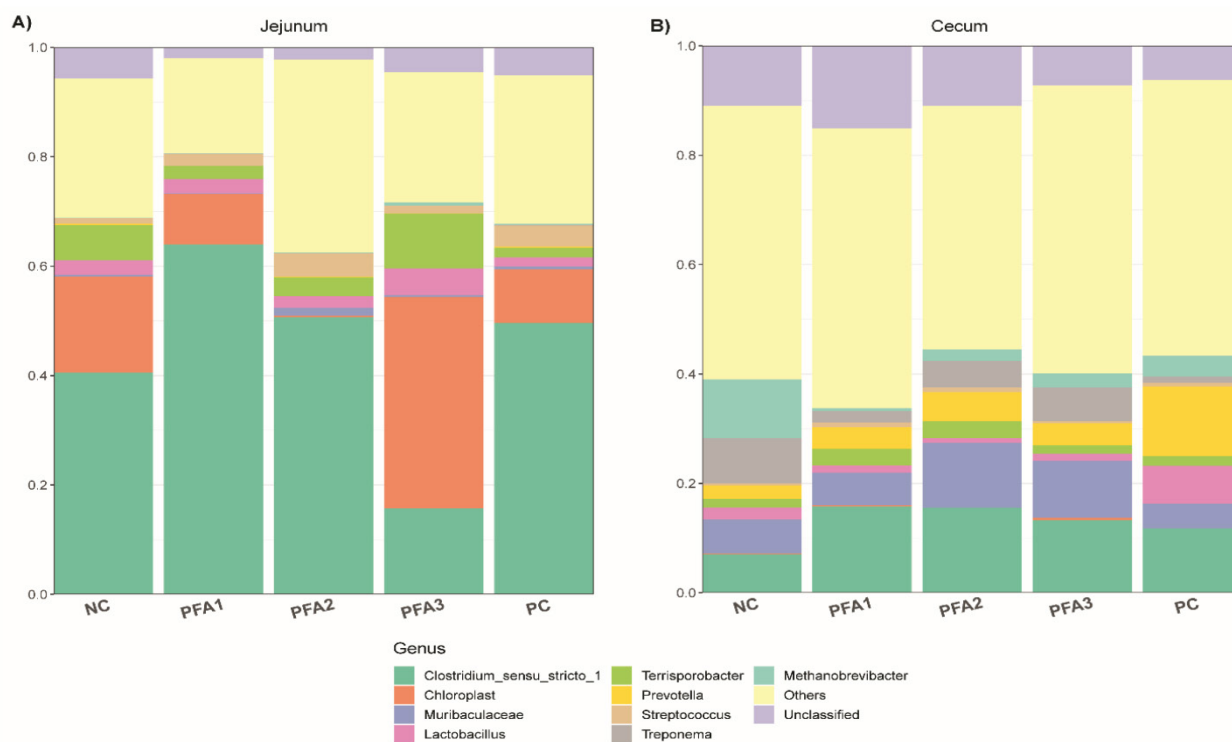
Fig. 3. Relative bacterial abundance of top 10 genus in A) jejunum and B) cecum. NC, nutrient adequate control diet; PFA1, similar to NC plus 0.3% of PFA; PFA2, similar to NC plus 0.6% of PFA; PFA3, similar to NC plus 1.0 % of PFA; PC, similar to NC plus 0.15% of chlortetracycline. NC, negative control; PFA, paraformic acid; PC, positive control.

Fig. 4. Relative abundance of cecal microbiota (at the genus level) of A) *Lactobacillus* **and B)** *Streptococcus* **in the jejunum, while C)** *Prevotella*; **D)** *Treponema* **and E)** *Methanobrevibacter* **in the cecum.** NC, nutrient adequate control diet; PFA1, similar to NC plus 0.3% PFA; PFA2, similar to NC plus 0.6% of PFA; PFA3, similar to NC plus 1.0 % of PFA; PC, similar to NC plus 0.15% of chlortetracycline. NC, negative control; PFA, paraformic acid; PC, positive control.
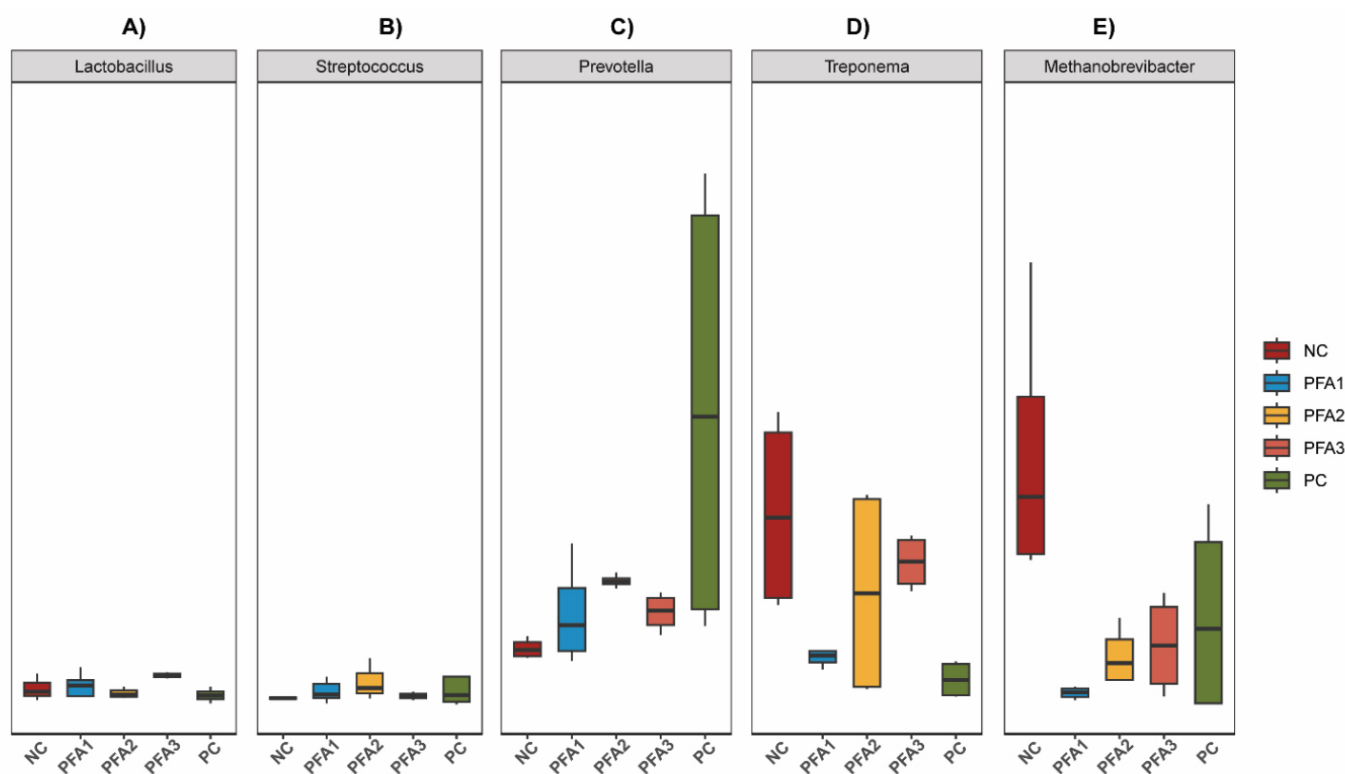
gut microbiota and improvements in the general performance of pigs [24,37,38]. Several studies summarized by Luise et al. [16] suggested that incorporating formic acid as a feed supplement might improve the general performance of nursery pigs. Among them, the main evidence indicates that formic acid modifies the acidic condition of the feed, hindering the growth of pathogenic bacteria and improving the hygiene of the feed [39]. Furthermore, formic acid reduces stomach pH, offering the ideal condition for more efficient activity of digestive enzymes [23] as well as acting as an antimicrobial agent, suppressing the survival and colonization of low pH intolerant pathogenic bacteria [40].

In the current study, the ADG of pigs that received PFA-supplemented nursery feed highlighted the health benefits that eased weaning transition stress. The supplementation of PFA2 evidenced a better daily gain of 66.63 g and 65.48 g over pigs fed NC in P1 and P1&2, respectively, and 18.46 g and 38.08 g over pigs fed PC diet in P1 and P1&2, respectively. Similar results were reported by Dahmer et al. [25] where nursery pigs fed 0.70% of formic acid showed higher ADG than those supplemented with the basal diet. Interestingly, pigs had an ADG of 470 g, similar to the ADG found in this study (466 g) with 0.60% of PFA inclusion. Additionally, Luise et al. [41] reported overall improvements in ADG with nursery pigs supplemented with 0.64% of formic acid on day 21 after weaning. The growth performance improvements found in this study with pigs fed PFA might be due to the reduction of pathogenic bacteria in the feed attributed to the acid´s presence before consumption, as well as the enhancement of pepsin enzyme activity by lowering the stomach pH, which in turn improved the nutrient utilization, and a lower amount of undigested feed available in the gut for pathogenic bacteria growth. This assumption might

be supported by the results of the fecal score, where the pigs under PFA supplementation or PC had a similar fecal consistency, classified between normal and soft and well-formed feces, while those fed NC showed an incidence of mild diarrhea. The incidence of diarrhea in nursery pigs is a consequence of a complex interaction of several infectious agents that colonize the intestines and secrete their endotoxins [11], which in turn generate a cascade of inflammatory responses, intestinal tissue damage as well as secretion of fluids [1]. As a result of these complex interactions, PWD is generated leading to a reduction in nutrient utilization, and reductions on the general growth performance of nursery pigs.

Some studies have reported no positive effects on ADFI and F:G ratio in nursery pigs fed 0.2 % [42] or 0.5% [43] of formic acid. Such results are contradictory to the findings of this study, where increasing the level of PFA stimulated the ADFI and showed a lower F:G ratio, mainly in those fed intermediate levels of PFA (0.6%), when compared to those fed NC or PC diets. Based on the physicochemical properties of organic acids, a normal formic acid molecule has a pungent odor plus irritating and corrosive characteristics [28,44]. Eisemann and van Heugten [45] evaluated three different levels of formic acid (0.8%, 1.0% y 1.2%) in combination with ammonium formate, and reported a reduction in feed intake as the inclusion level of formic acid was increased during the nursery phase 2 and grower phases. However, feed intake tended to increase in those pigs fed diets devoid of formic acid plus ammonium formate. Furthermore, Ettle et al. [46] studied the self-selection of feed with or without acidifier and its impact on feed intake behavior. Pigs under the feed self-selection study had preferences for unacidified diets versus acidified diets with 1.2% or 2.4% of K-diformate. However, in the second part of the experiment, pigs were given the choice between a 1.2% formic acid diet or 1.2% sorbic acid diet, and they showed a preference for the sorbic acid-based diet over the formic acid-based diet, reducing feed intake due to possible low palatability. Based on the above-mentioned, it is possible to speculate that the supplementation of PFA might not exert negative effects on feed palatability, allowing the supplementation with a higher inclusion level of formic acid without reductions on ADFI as evidenced by the positive linear response as increased the PFA inclusion on the overall ADFI. Additionally, the supplementation of PFA2 showed to exert the highest benefit on feed efficiency, supported by the reduction in the F:G ratio as well as the obtained quadratic response.

Overall, pigs fed NC and PC consumed 11.74 g and 54.36 g, respectively, more than pigs fed PFA2. Interestingly, pigs fed PFA2 gained 66.21 g and 38.08 g more than the NC and PC groups, respectively. The highest daily gain obtained in the PFA2 group supports the BW of PFA2 pigs with 1.89 kg over NC group and 0.85 kg over PC group. These results show that the PFA practical inclusion of 0.6 % in nursery diets is feasible as a potential substitute for antibiotics, during the early nursery period. Further studies should be conducted to evaluate PFA supplementation from the nursery and follow-up on pig performance through the finisher period to determine the potential impact of PFA supplementation compared with antibiotics at the end of the fattening period.

It has been well evidenced that weaning is a stressful period that affects intestinal morphology and health through a reduction in intestinal cell renewal and increments of apoptosis or cell death [47,48]. However, healthy intestinal morphological structures such as VH, and CD are important morpho-functional characteristics for nutrient digestion and absorption that exert pronounced effects on performance [49]. In the current study, the supplementation of PFA at different concentrations, or PC did not show differences in VH, CD, VW, and VH:CD ratio. However, the PFA2 group showed a remarkable numerical increase in VH, VW, VH:CD ratio, and lower CD than pigs under the PC diet or NC. Long et al. [50] evaluated a synergistic blend of free and buffered short-chain fatty acids composed of formic acid, acetic acid, and propionic acid at a 0.30% inclusion level in nursery pigs. They found a lack of notable changes in VH and CD

in the duodenum, jejunum, or ileum compared to the antibiotic or control group. Furthermore, Manzanilla et al. [43] reported no differences in VH and CD with pigs fed 0.5% formic acid versus 0.30% of a plant extract containing carvacrol, cinnamaldehyde, and capsicum oleoresin. Similarly, a chicken study reported no changes in morphological structures of the intestine when the birds were fed 0.05% or 0.10% of formic acid, plant extract mixture, or antibiotic as growth promoters [51]. VH reflects a balance between the mitotic activity of the crypt enteric cells and the desquamation produced principally by external aggressors [43]. Additionally, antimicrobial compounds such as organic acids have been evidenced to control the pathogenic load in the intestines, which in turn decreases the presence of toxins and reduces the damage on intestinal morphology, mainly on the VH, thus offering conditions for nutrient utilization [52]. PFA at a concentration of 0.6% might potentially maintain better gut health based on the slight increase in VH reported in this study. Furthermore, the positive effects on F:G ratio of pigs fed PFA2 might be due to the slight improvements in VH, VW, and VH:CD ratio, offering a better absorptive area for nutrient utilization.

A balanced microbiota has been correlated with gut health and is responsible for different functions in the host such as nutrient absorption, metabolism, gastrointestinal development, and immune function [53]. Additionally, a good healthy condition has been linked with a high alpha diversity in humans [54,55] and pigs [56,57]. The Chao1 index is an indicator of microbial richness [58]. In this study, pigs fed PFA3 showed to stimulate the cecal microbial diversity, as reported by the Chao index. An organic acid-based study by Wei et al. [59] reported a higher diversity of microbial species in nursery pigs fed 0.10% of a blend of organic acids than those fed the control diet. Likewise, Li et al. [22] evaluated the supplementation of 0.1% of PFA in 42-day broiler chickens and evidenced a greater microbial richness. Nursery pigs are predisposed to face gut dysbiosis during the first weeks of weaning, and this imbalance of microbiota dramatically affects the microbial richness and predisposes the pigs to gastrointestinal disorders [10]. Based on these results, the use of PFA might help minimize dysbiosis and maximize the proliferation of beneficial bacteria, leading to improved bacterial richness.

It has been well reported that the genera *Lactobacillus* [59] and *Streptococcus* are two of the most dominant groups of lactic acid bacteria in the proximal small intestine [60]. *Lactobacillus* and *Streptococcus* produce lactic acid, which benefits the control of some harmful bacteria in the gut. However, some potential pathogenic bacteria can multiply and colonize the main site of nutrient absorption and generate significant damage to intestinal morphology [61]. Because organic acids have demonstrated to reduce pH of stomach and small intestine due to their acidifying properties, the supplementation with PFA2 and PFA3 seems to modulate the proliferation of these bacteria, possibly, by adequations of the intestinal pH, thus offering the ideal condition for their proliferation. The improvements in growth performance might also be influenced by the proliferation of healthy microbiota and reduction of the development of potential pathogenic bacteria in the site of nutrient utilization.

Mathanobrevibacter, a genus belonging to the order Methanobacteriales, is H2-oxidizing methanogens [62]. Approximately, 1.2% of ingested energy is lost by methane production in pigs, thus contributing to the greenhouse effect [63]. Recently, Li et al. [22] evaluated the feed supplementation of 0.1 % PFA for broiler chickens and reported a significant reduction in the relative abundance of methanogenic bacteria. Our results are similar to those evidenced by Li, where the supplementation of PFA reduced the abundance of *Methanobrevibacter*. Together, these results suggest that the supplementation of PFA reduces methane emissions, thus providing for a more environmentally friendly swine industry.

Several species of treponemes are swine pathogens [64]. The genus *Treponema* causes ear

necrosis and ulcers in pigs [65]. Interestingly, organic acids have been shown to efficiently reduce the *Treponema* abundance, specifically, the *Brachyspira hyodysenteriae* isolated from pigs [66]. The supplementation of PFA might help to maintain a healthier microbial population one month post-weaning by reducing the *Treponema* abundance in the gut. In addition, the abundance of *Prevotella*, a group of fiber-fermenting bacteria, gradually increases during the transition period from a milk-based diet to a solid plant-based diet [67], and has been positively correlated with the growth performance of nursery pigs [68]. The supplementation of PFA groups or PC increases the relative abundance of *Prevotella*. Similar results were reported by Pluske et al. [69] where a blend of organic acids, including formic acid, modulates the prevotella abundance similarly to an amoxicillin-supplemented diet, demonstrating that organic acid derivatives can help to maintain healthy gut microbiota.

CONCLUSION

This study demonstrated that the supplementation of 0.6% PFA in nursery pig diets can efficiently replace the use of antibiotics, as a growth promoter, through beneficial modulation of the gut microbiota, enhancement of intestinal morphology, control of diarrhea incidence, and improvements in growth performance. This finding supports the benefits of using PFA as a feed additive in nursery pig diets. Further studies have to be conducted to evaluate PFA supplementation during nursery and follow-up on pig performance through the fattening period to determine the potential practical implication of PFA supplementation compared to antibiotics.

REFERENCES

1. Campbell JM, Crenshaw JD, Polo J. The biological stress of early weaned piglets. J Anim Sci Biotechnol. 2013;4:19. https://doi.org/10.1186/2049-1891-4-19

2. Heo JM, Opapeju FO, Pluske JR, Kim JC, Hampson DJ, Nyachoti CM. Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. J Anim Physiol Anim Nutr. 2013;97:207-37. https://doi.org/10.1111/j.1439-0396.2012.01284.x

3. Zheng L, Duarte ME, Sevarolli Loftus A, Kim SW. Intestinal health of pigs upon weaning: challenges and nutritional intervention. Front Vet Sci. 2021;8:628258. https://doi.org/10.3389/fvets.2021.628258

4. Weary DM, Jasper J, Hötzel MJ. Understanding weaning distress. Appl Anim Behav Sci. 2008;110:24-41. https://doi.org/10.1016/j.applanim.2007.03.025

5. Jayaraman B, Nyachoti CM. Husbandry practices and gut health outcomes in weaned piglets: a review. Anim Nutr. 2017;3:205-11. https://doi.org/10.1016/j.aninu.2017.06.002

6. Spreeuwenberg MAM, Verdonk JMAJ, Gaskins HR, Verstegen MWA. Small intestine epithelial barrier function is compromised in pigs with low feed intake at weaning. J Nutr. 2001;131:1520-7. https://doi.org/10.1093/jn/131.5.1520

7. Xiong X, Tan B, Song M, Ji P, Kim K, Yin Y, et al. Nutritional intervention for the intestinal development and health of weaned pigs. Front Vet Sci. 2019;6:46. https://doi.org/10.3389/fvets.2019.00046

8. Brown DC, Maxwell CV, Erf GF, Davis ME, Singh S, Johnson ZB. The influence of different management systems and age on intestinal morphology, immune cell numbers and mucin production from goblet cells in post-weaning pigs. Vet Immunol Immunopathol. 2006;111:187-98. https//doi.org/10.1016/j.vetimm.2005.12.006

9. Castillo M, Martín-Orúe SM, Nofrarías M, Manzanilla EG, Gasa J. Changes in caecal microbiota and mucosal morphology of weaned pigs. Vet Microbiol. 2007;124:239-47. https://doi.org/10.1016/j.vetmic.2007.04.026

10. Gresse R, Chaucheyras-Durand F, Fleury MA, Van de Wiele T, Forano E, Blanquet-Diot S. Gut microbiota dysbiosis in postweaning piglets: understanding the keys to health. Trends Microbiol. 2017;25:851-73. https://doi.org/10.1016/j.tim.2017.05.004

11. Rhouma M, Fairbrother JM, Beaudry F, Letellier A. Post weaning diarrhea in pigs: risk factors and non-colistin-based control strategies. Acta Vet Scand. 2017;59:31. https://doi.org/10.1186/s13028-017-0299-7

12. Yang H, Paruch L, Chen X, van Eerde A, Skomedal H, Wang Y, et al. Antibiotic application and resistance in swine production in China: current situation and future perspectives. Front Vet Sci. 2019;6:136. https://doi.org/10.3389/fvets.2019.00136

13. Cao R, Ben W, Qiang Z, Zhang J. Removal of antibiotic resistance genes in pig manure composting influenced by inoculation of compound microbial agents. Bioresour Technol. 2020;317:123966. https://doi.org/10.1016/j.biortech.2020.123966

14. Marshall BM, Levy SB. Food animals and antimicrobials: impacts on human health. Clin Microbiol Rev. 2011;24:718-33. https://doi.org/10.1128/CMR.00002-11

15. Du L, Liu W. Occurrence, fate, and ecotoxicity of antibiotics in agro-ecosystems. A review. Agron Sustain Dev. 2012;32:309-27. https://doi.org/10.1007/s13593-011-0062-9

16. Luise D, Correa F, Bosi P, Trevisi P. A review of the effect of formic acid and its salts on the gastrointestinal microbiota and performance of pigs. Animals. 2020;10:887. https://doi.org/10.3390/ani10050887

17. Berge AC, Wierup M. Nutritional strategies to combat Salmonella in mono-gastric food animal production. Animal. 2012;6:557-64. https://doi.org/10.1017/S1751731111002217

18. El-hack MEA, El-Saadony MT, Salem HM, El-Tahan AM, Soliman MM, Youssef GBA, et al. Alternatives to antibiotics for organic poultry production: types, modes of action and impacts on bird's health and production. Poult Sci. 2022;101:101696. https://doi.org/10.1016/j.psj.2022.101696

19. Upadhaya SD, Lee KY, Kim IH. Effect of protected organic acid blends on growth performance, nutrient digestibility and faecal micro flora in growing pigs. J Appl Anim Res. 2016;44:238-42. https://doi.org/10.1080/09712119.2015.1031775

20. Li S, Zheng J, Deng K, Chen L, Zhao XL, Jiang X, et al. 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. 2018;96:3302-18. https://doi.org/10.1093/jas/sky197

21. Gómez-García M, Sol C, de Nova PJG, Puyalto M, Mesas L, Puente H, et al. Antimicrobial activity of a selection of organic acids, their salts and essential oils against swine enteropathogenic bacteria. Porcine Health Manag. 2019;5:32. https://doi.org/10.1186/s40813-019-0139-4

22. Li J, Liu Y, Niu J, Jing C, Jiao N, Huang L, et al. Supplementation with paraformic acid in the diet improved intestinal development through modulating intestinal inflammation and microbiota in broiler chickens. Front Microbiol. 2022;13:975056. https://doi.org/10.3389/fmicb.2022.975056

23. Kristoffersen S, Gjefsen T, Svihus B, Kjos NP. The effect of reduced feed pH, phytase addition and their interaction on mineral utilization in pigs. Livest Sci. 2021;248:104498. https://doi.org/10.1016/j.livsci.2021.104498

24. Lee J, Kim JW, Hall H, Nyachoti CM. Effect of dietary organic acids supplementation on

growth performance, nutrient digestibility, and gut morphology in weaned pigs. Can J Anim Sci. 2022;102:255-65. https://doi.org/10.1139/cjas-2021-0080

25. Dahmer PL, Harrison OL, Jones CK. Effects of formic acid and glycerol monolaurate on weanling pig growth performance, fecal consistency, fecal microbiota, and serum immunity. Transl Anim Sci. 2022;6:txac145. https://doi.org/10.1093/tas/txac145

26. Badea GE, Ionita D, Cret P. Corrosion and passivation of the 304 stainless steel in formic acid solutions. Mater Corros. 2014;65:1103-10. https://doi.org/10.1002/maco.201307491

27. Yan Y, Zou C, Zhang L, Zhu Y, Wu L, Zhou H, et al. A study on corrosion products and processes of patinated tin bronze in formic acid. Res Chem Intermed. 2020;46:5087-99. https://doi.org/10.1007/s11164-020-04252-2

28. Balagué N, Vostrel P, Beaulieu JY, van Aaken J. Third degree formic acid chemical burn in the treatment of a hand wart: a case report and review of the literature. Springerplus. 2014;3:408. https://doi.org/10.1186/2193-1801-3-408

29. Nielsen GD. Sensory irritation of vapours of formic, acetic, propionic and butyric acid. Regul Toxicol Pharmacol. 2018;99:89-97. https://doi.org/10.1016/j.yrtph.2018.09.012

30. Bosi P, Sarli G, Casini L, de Filippi S, Trevisi P, Mazzoni M, et al. The influence of fat protection of calcium formate on growth and intestinal defence in Escherichia coli K88-challenged weanling pigs. Anim Feed Sci Technol. 2007;139:170-85. https://doi.org/10.1016/j.anifeedsci.2006.12.006

31. European Union. Commission implementing regulation (EU) 2017/940 of 1 June 2017 concerning the autorisation of formic acid as a feed additive for all animal species. Off J Eur Union. 2017;66:40-2.

32. National Research Council. Nutritional requirements of swine. 11th ed. The National Academies Press; 2012.

33. AOAC (Association of Official Analytical Chemists). Official methods of analysis of the AOAC. 15th ed. AOAC; 1990.

34. Sherman DM, Acres SD, Sadowski PL, Springer JA, Bray B, Raybould TJG, et al. Protection of calves against fatal enteric colibacillosis by orally administered Escherichia coli K99-specific monoclonal antibody. Infect Immun. 1983;42:653-8. https://doi.org/10.1128/iai.42.2.653-658.1983

35. Hu Y, Zhang Y, Liu C, Qin R, Gong D, Wang R, et al. Multi-omics profiling highlights lipid metabolism alterations in pigs fed low-dose antibiotics. BMC Genet. 2020;21:112. https://doi.org/10.1186/s12863-020-00918-3

36. Núñez MC, Bueno JD, Ayudarte MV, Almendros A, Ríos A, Suárez MD, et al. Dietary restriction induces biochemical and morphometric changes in the small intestine of nursing piglets. J Nutr. 1996;126:933-44. https://doi.org/10.1093/jn/126.4.933

37. Papatsiros VG, Tassis PD, Tzika ED, Papaioannou DS, Petridou E, Alexopoulos C, et al. Effect of benzoic acid and combination of benzoic acid with a probiotic containing Bacillus cereus var. toyoi in weaned pig nutrition. Pol J Vet Sci. 2011;14:117-25. https://doi.org/10.2478/v10181-011-0017-8

38. Kuang Y, Wang Y, Zhang Y, Song Y, Zhang X, Lin Y, et al. Effects of dietary combinations of organic acids and medium chain fatty acids as a replacement of zinc oxide on growth, digestibility and immunity of weaned pigs. Anim Feed Sci Technol. 2015;208:145-57. https://doi.org/10.1016/j.anifeedsci.2015.07.010

39. Partanen KH, Mroz Z. Organic acids for performance enhancement in pig diets. Nutr Res Rev. 1999;12:117-45. https://doi.org/10.1079/095442299108728884

40. Diebold G, Eidelsburger U. Acidification of diets as an alternative to antibiotic growth

promoters. In: Barug D, de Jong J, Kies AK, Verstegen MWA, editors. Antimicrobial growth promoters, where do we go from here?. Wageningen Academic Publishers; 2006. p. 311-27.

41. Luise D, Motta V, Salvarani C, Chiappelli M, Fusco L, Bertocchi M, et al. Long-term administration of formic acid to weaners: influence on intestinal microbiota, immunity parameters and growth performance. Anim Feed Sci Technol. 2017;232:160-8. https://doi.org/10.1016/j.anifeedsci.2017.06.015

42. Kil DY, Piao L, Long HF, Lim JS, Yun MS, Kong CS, et al. Effects of organic or inorganic acid supplementation on growth performance, nutrient digestibility and white blood cell counts in weanling pigs. Asian-Australas J Anim Sci. 2006;19:252-61. https://doi.org/10.3390/ani10050887

43. Manzanilla EG, Perez JF, Martin M, Kamel C, Baucells F, Gasa J. Effect of plant extracts and formic acid on the intestinal equilibrium of early-weaned pigs. J Anim Sci. 2004;82:3210-8. https://doi.org/10.2527/2004.82113210x

44. Liu X, Li H, Zhao X, Chen Y, Wang S. Comparison of the corrosion behavior of copper tubes in formic acid and acetic acid environment. Mater Corros. 2021;72:1919-27. https://doi.org/10.1002/maco.202112568

45. Eisemann JH, van Heugten E. Response of pigs to dietary inclusion of formic acid and ammonium formate. J Anim Sci. 2007;85:1530-9. https://doi.org/10.2527/jas.2006-464

46. Ettle T, Mentschel K, Roth FX. Dietary self-selection for organic acids by the piglet. Arch Anim Nutr. 2004;58:379-88. https://doi.org/10.1080/00039420400005067

47. van der Peet-Schwering CMC, Jansman AJM, Smidt H, Yoon I. Effects of yeast culture on performance, gut integrity, and blood cell composition of weanling pigs. J Anim Sci. 2007;85:3099-109. https://doi.org/10.2527/jas.2007-0110

48. Wang D, Piao XS, Zeng ZK, Lu T, Zhang Q, Li PF, et al. Effects of keratinase on performance, nutrient utilization, intestinal morphology, intestinal ecology and inflammatory response of weaned piglets fed diets with different levels of crude protein. Asian-Australas J Anim Sci. 2011;24:1718-28. https://doi.org/10.5713/ajas.2011.11132

49. Sekirov I, Russell SL, Antunes LCM, Finlay BB. Gut microbiota in health and disease. Physiol Rev. 2010;90:859-904. https://doi.org/10.1152/physrev.00045.2009

50. Long SF, Xu YT, Pan L, Wang QQ, Wang CL, Wu JY, et al. Mixed organic acids as antibiotic substitutes improve performance, serum immunity, intestinal morphology and microbiota for weaned piglets. Anim Feed Sci Technol. 2018;235:23-32. https://doi.org/10.1016/j.anifeedsci.2017.08.018

51. García V, Catalá-Gregori P, Hernández F, Megías MD, Madrid J. Effect of formic acid and plant extracts on growth, nutrient digestibility, intestine mucosa morphology, and meat yield of broilers. J Appl Poult Res. 2007;16:555-62. https://doi.org/10.3382/japr.2006-00116

52. Xu ZR, Hu CH, Xia MS, Zhan XA, Wang MQ. Effects of dietary fructooligosaccharide on digestive enzyme activities, intestinal microflora and morphology of male broilers. Poult Sci. 2003;82:1030-6. https://doi.org/10.1093/ps/82.6.1030

53. Jandhyala SM, Talukdar R, Subramanyam C, Vuyyuru H, Sasikala M, Reddy DN. Role of the normal gut microbiota. World J Gastroenterol. 2015;21:8787-803. https://doi.org/10.3748/wjg.v21.i29.8787

54. Le Chatelier E, Nielsen T, Qin J, Prifti E, Hildebrand F, Falony G, et al. Richness of human gut microbiome correlates with metabolic markers. Nature. 2013;500:541-6. https://doi.org/10.1038/nature12506

55. Kong F, Hua Y, Zeng B, Ning R, Li Y, Zhao J. Gut microbiota signatures of longevity. Curr Biol. 2016;26:R832-3. https://doi.org/10.1016/j.cub.2016.08.015

56. Dou S, Gadonna-Widehem P, Rome V, Hamoudi D, Rhazi L, Lakhal L, et al. Characterisation of early-life fecal microbiota in susceptible and healthy pigs to post-weaning diarrhoea. PLoS One. 2017;12:e0169851. https://doi.org/10.1371/journal.pone.0169851

57. Correa-Fiz F, dos Santos JMG, Illas F, Aragon V. Antimicrobial removal on piglets promotes health and higher bacterial diversity in the nasal microbiota. Sci Rep. 2019;9:6545. https://doi.org/10.1038/s41598-019-43022-y

58. Liu W, Zhang R, Shu R, Yu J, Li H, Long H, et al. Study of the relationship between microbiome and colorectal cancer susceptibility using 16SrRNA sequencing. BioMed Res Int. 2020;2020:7828392. https://doi.org/10.1155/2020/7828392

59. Wei X, Bottoms KA, Stein HH, Blavi L, Bradley CL, Bergstrom J, et al. Dietary organic acids modulate gut microbiota and improve growth performance of nursery pigs. Microorganisms. 2021;9:110. https://doi.org/10.3390/microorganisms9010110

60. Richards JD, Gong J, de Lange CFM. The gastrointestinal microbiota and its role in monogastric nutrition and health with an emphasis on pigs: current understanding, possible modulations, and new technologies for ecological studies. Can J Anim Sci. 2005;85:421-35. https://doi.org/10.4141/A05-049

61. Dowarah R, Verma AK, Agarwal N. The use of Lactobacillus as an alternative of antibiotic growth promoters in pigs: a review. Anim Nutr. 2017;3:1-6. https://doi.org/10.1016/j.aninu.2016.11.002

62. Zhang H, DiBaise JK, Zuccolo A, Kudrna D, Braidotti M, Yu Y, et al. Human gut microbiota in obesity and after gastric bypass. Proc Natl Acad Sci USA. 2009;106:2365-70. https://doi.org/10.1073/pnas.0812600106

63. Monteny GJ, Groenestein CM, Hilhorst MA. Interactions and coupling between emissions of methane and nitrous oxide from animal husbandry. Nutr Cycl Agroecosyst. 2001;60:123-32. https://doi.org/10.1023/A:1012602911339

64. Stamm LV, Walker RL, Read DH. Genetic diversity of bovine ulcerative mammary dermatitis-associated Treponema. Vet Microbiol. 2009;136:192-6. https://doi.org/10.1016/j.vetmic.2008.10.022

65. Karlsson F, Svartström O, Belák K, Fellström C, Pringle M. Occurrence of Treponema spp. in porcine skin ulcers and gingiva. Vet Microbiol. 2013;165:402-9. https://doi.org/10.1016/j.vetmic.2013.03.031

66. Vande Maele L, Heyndrickx M, Maes D, De Pauw N, Mahu M, Verlinden M, et al. In vitro susceptibility of Brachyspira hyodysenteriae to organic acids and essential oil components. J Vet Med Sci. 2016;78:325-8. https://doi.org/10.1292/jvms.15-0341

67. Frese SA, Parker K, Calvert CC, Mills DA. Diet shapes the gut microbiome of pigs during nursing and weaning. Microbiome. 2015;3:28. https://doi.org/10.1186/s40168-015-0091-8

68. Tang S, Xin Y, Ma Y, Xu X, Zhao S, Cao J. Screening of microbes associated with swine growth and fat deposition traits across the intestinal tract. Front Microbiol. 2020;11:586776. https://doi.org/10.3389/fmicb.2020.586776

69. Pluske JR, Turpin DL, Sahibzada S, Pineda L, Han Y, Collins A. 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. Transl Anim Sci. 2021;5:txab212. https://doi.org/10.1093/tas/txab212