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Non Ruminant Nutrition

Effect of an organic acid blend as an antibiotic alternative on growth performance, antioxidant capacity, intestinal barrier function, and fecal microbiota in weaned piglets

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Abstract

This study was conducted to evaluate the effects of dietary organic acid blend on growth performance, antioxidant capacity, intestinal barrier function, and fecal microbiota in weaned piglets compared with antibiotic growth promoters (AGPs). A total of 90 weaned crossbred barrows (24 ± 1 d of age) with an initial body weight of 7.40 kg were allocated into three experimental treatments. Each treatment consisted of six replicate pens, with five piglets housed in each pen. The dietary treatments included the basal diet (NC), the basal diet supplemented with antibiotics (PC), and the basal diet supplemented with organic acid blend (OA). On day 42, one piglet per pen was randomly selected for plasma and small intestinal sample collection. The results showed that dietary AGP significantly improved growth performance and reduced diarrhea incidence compared to the NC group ($P < 0.05$). Dietary OA tended to increase body weight on day 42 ($P = 0.07$) and average daily gain from days 0 to 42 ($P = 0.06$) and reduce diarrhea incidence ($P = 0.05$). Dietary OA significantly increased plasma catalase (CAT) activity and decreased the plasma concentration of malondialdehyde (MDA), tumor necrosis factor-α (TNF-α), interleukin (IL)-8, and IL-6, which were accompanied by upregulated the relative mRNA abundance of *superoxide dismutase 1 (SOD1)*, *glutathione peroxidase 1 (GPX1)*, and *nuclear factor erythroid 2-related factor 2 (NRF2)* in comparison to that in the NC group ($P < 0.05$). Moreover, pigs fed the OA diet significantly increased the ratio of villus height to crypt depth and upregulated the relative expression of *zonula occludens-1 (ZO-1)* and *Claudin1* gene in the jejunum compared to the NC group ($P < 0.05$). Interestingly, dietary AGP or OA did not affect the fecal microbiota structure or volatile fatty acid content ($P > 0.05$). In conclusion, our results suggested that dietary OA supplementation could improve growth performance and antioxidant capacity and protect the intestinal barrier of weaned piglets, therefore, it has the potential to be considered as an alternative to AGP in the pig industry.

Lay Summary

In the era of antibiotics prohibition, there is an urgent need to develop green and efficient alternatives to antibiotics in the current pig industry to mitigate the economic losses associated with antibiotic bans. Organic acids (OA) are a class of substances that have long been used as feed additives due to their bacteriostatic properties, the ability of reducing feed pH, increasing the activity of digestive enzymes, and other beneficial effects. This study was conducted to evaluate the effects of dietary OA on growth performance, antioxidant capacity, intestinal barrier function, and fecal microbiota structure in weaned piglets. The results showed that OA supplementation can effectively improve the growth performance and intestinal health of weaned piglets. This study provides a reference for the application of OA as an alternative to antibiotics in weaned piglets.

Keywords: organic acid, weaned piglets, growth performance, postweaning diarrhea, antioxidant capacity, barrier function

Abbreviations: ADFI, average daily feed intake; ADG, average daily gain; AGP, antibiotic growth promoters; BW, body weight; CAT, catalase; CD, crypt depth; G:F, the ratio of average daily gain to average daily feed intake; GPX1, glutathione peroxidase 1; H_2O_2, hydrogen peroxide; HO-1, heme oxygenase-1; IL, interleukin; MDA, malondialdehyde; MUC2, mucin 2; NQO1, NAD(P)H: quinone oxidoreductase 1; NRF2, nuclear factor erythroid 2-related factor 2; OA, organic acid; OTUs, operational taxonomic units; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α; VH, villus height; VFA, volatile fatty acid; VH: CD, villus height-to-crypt depth ratio; ZO-1, zonula occludens-1

Introduction

In the swine industry, the problems of excessive drug dependence and sub-health of pigs have become increasingly prominent due to the impact of various multiple stressors, such as weaning, changes in the nature of diet and living environment (Giannenas et al., 2014; Xun et al., 2015). Although the application of antibiotics in animal feeds largely copes with some health problems and exerts a positive impact on animal growth performance. However, long-term misuse of antibiotics not only causes the emergence of resistant bacteria and subsequent issues such as intestinal flora imbalance

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but also leads to drug residues in animal products and the surrounding environment, which directly affect the safety of animal-derived foods and further pose potential harm to human health (Fournier et al., 2006; Pluske, 2013; Muaz et al., 2018). The European Union and China have successively implemented bans on the use of antibiotic growth promoters (AGPs) in animal feed in 2006 and 2020, respectively (Long et al., 2018; Han et al., 2020). Therefore, the identification and development of alternative agents that can improve growth, alleviate diarrhea, and maintain intestinal health similar to AGPs has become a top priority.

The products with the potential to replace antibiotics, such as acidifiers, enzyme preparations, plant extracts, probiotics, and antimicrobial peptides, are widely researched. Organic acids are a class of substances that have long been used as feed preservatives due to their bacteriostatic properties and their ability to reduce feed pH (Ferronato and Prandini, 2020). In recent years, increasing evidence has shown that organic acids (OAs) have the function of increasing the activity of digestive enzymes, improving the digestibility of nutrients, inhibiting the formation of macromolecules inside the bacteria, and destroying the membrane of bacteria (Dai et al., 2021; Wang et al., 2022b). Meanwhile, OA also can improve intestinal health by enhancing intestinal integrity and alleviating inflammatory responses (Sabour et al., 2019; Dai et al., 2022). In addition, previous studies have reported the beneficial effects of OA and their ammonium salts on the growth performance of piglets and their potential as alternatives to antibiotics (Ferronato and Prandini, 2020; Han et al., 2020). However, a gap exists in the understanding of how OA elicits greater growth performance and better intestinal health.

Thus, the present study was conducted to investigate the effect of dietary supplementation of OA on growth performance and diarrhea incidence on weaned piglets, and try to elucidate the beneficial effects from antioxidant capacity, intestinal barrier function, and fecal microbiota community aspects. We assume that the piglets supplemented with OA will exert a better growth performance which may related to the healthier intestine. Based on the results, we found that OA effectively improved growth performance and intestinal health in weaned piglets, which was tightly related to the improvement of the antioxidant capacity and the protection of the intestinal barrier of weaned piglets. Our results may provide a theoretical and practical basis for the application of OA, as replacements for AGPs, in swine production.

Materials and Methods

The animal protocol for this research was approved by the Animal Care and Use Committee of the Institute of Feed Research of the Chinese Academy of Agricultural Sciences.

Experimental design and animal management

The experiment was carried out at the Langfang experimental station of the Institute of Feed Research of the Chinese Academy of Agricultural Sciences. Ninety healthy weaned crossbred barrows (Duroc × Landrace × Yorkshire), with similar initial body weight (BW, 7.40 ± 0.11 kg) and age (24 ± 1 days), were randomly allotted into one of the following dietary treatments: basal weaning diet supplemented with (i) no additive (NC), (ii) 0.04 g/kg Surmax + 0.1 g/kg Olaquindox (PC), (iii) 2 g/kg OA feed additive (Presan-FX, Selko Feed Additives, Nutreco, The Netherlands). The organic acid blend contained phenolic compound, slow-release C12 (mainly a lauric acid), target-release butyrates, medium-chain fatty acids (MCFAs), and free and buffered organic acids. The dosage of OA in the current study was based on the previous reports, which could result in a greater feed intake, lower diarrhea incidence, and greater intestine function in weaned piglets (Kuang et al., 2015; Li et al., 2018; Long et al., 2018). There were six replicate pens per treatment and five piglets per pen. During the experiment, piglets were allowed ad libitum access to water and feed, and the pens were cleaned regularly. A combination of daylight and artificial light was used, and ventilation was achieved by using variable-speed fans. The starting temperature of 28°C was adjusted weekly to reach a final temperature of 25°C. Piglets were housed in pens (5 piglets/pen), located beside an 80-cm walkway with 12 pens (2.00 × 2.00 m^2) on each side, with a slatted floor. Each pen was equipped with two water nipples and a feed trough. The diet for the piglets was formulated according to National Research Council (2012) nutrient requirements (Table 1) (Council, 2012). Prestarter feeds were given to piglets from days 0 to 14 of the trial, and the starter feeds were fed to animals from days 14 to 42 of the trial. The experimental diets were administered as meals (grinding diameter of 1.5 mm). The basal diet was manufactured 1 week before the trial began, without the inclusion of any antibiotic growth promoters.

Sample collection

On day 14, fresh fecal samples were collected from at least 2 piglets in each pen via rectal massage and immediately stored at −80 °C until processing. At the end of the trial (day 42), one piglet with the average pen BW was selected, and they were euthanized by intravenous injection of pentobarbital sodium (6 mg/kg BW) after blood collection. During necropsy, jejunum mucosa samples were collected and then a length of approximately 3 cm of the jejunum (50 cm from the pylorus) of each piglet was fixed in fresh 4% paraformaldehyde at least for 24 h and then embedded in paraffin.

Growth performance and diarrhea incidence measurements

Body weight was recorded on days 0, 14, 28, and 42 of the study, and the average daily gain (ADG), average daily feed intake (ADFI), and gain to feed ratio (G: F) were calculated for each pen. To determine the incidence of diarrhea, fecal scores were monitored daily by visual appraisal of each subject using a five-point fecal consistency scoring system: 1 = hard, dry pellet; 2 = firm, formed stool; 3 = soft, moist stool that retains its shape; 4 = soft, unformed stool; 5 = watery liquid that can be poured. Liquid consistency (score 4-5) was considered indicative of diarrhea (Jiang et al., 2015a, 2015b). Diarrhea incidence (%) = (number of diarrheic piglets × diarrhea days)/(total piglets × experimental days) × 100%.

Assay of plasma antioxidant and immune cytokines indices

The antioxidant-related enzyme activity of catalase (CAT) and superoxide dismutase (SOD), as well as the concentration of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) in the plasma, were measured using a corresponding reagent kit according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). In addition, the determination of plasma tumor necrosis factor (TNF)-α, interleukin (IL)-8, and IL-6 was assayed by

Table 1. Ingredient and nutrient composition of the basal diet (as fed basis)

Items	Prestarter diet[1]	Starter diet[2]
Ingredient, %		
Corn	24.27	37.76
Expanded corn	26.00	21.00
Soybean meal, 46%	15.00	20.50
Extruded soybean	4.00	5.50
SDPP	5.00	-
Fish meal	5.00	3.00
Whey powder	15.00	5.00
Wheat bran	2.60	4.00
Soybean oil	0.50	-
Calcium dihydrogen phosphate	0.50	0.80
Limestone	0.95	1.10
Choline chloride	0.05	0.05
Salt	0.30	0.30
L-Lysine HCl65%	0.38	0.51
DL-Met	0.10	0.09
Threonine	0.06	0.10
Vitamin and mineral premix[3]	0.29	0.29
Analysed nutrition composition		
Crude protein, %	19.67	18.06
Calcium, %	0.97	0.91
Phosphorus, %	0.66	0.58
Calculated nutrition composition		
ME, kcal/kg	3,375	3,300
Crude protein, %	20.00	18.50
Calcium, %	0.85	0.80
Phosphorus, %	0.75	0.68
Available P (STTD), %	0.54	0.43
Lys, %	1.30	1.16
Met, %	0.39	0.37
Met + Cys, %	0.75	0.67
Thr, %	0.79	0.70
Trp, %	0.23	0.20
Val, %	0.87	0.75

[1]Prestarter period: 1st to 2nd week after weaning at 24 d of age.
[2]Starter period: 3rd to 6th week after weaning at 24 d of age.
[3]Premix supplied per kilogram of diet: vitamin A, 35.2 mg; vitamin D3, 7.68 mg; vitamin E, 128 mg; vitamin K3, 8.16 mg; vitamin B1, 4 mg; vitamin B2, 12 mg; vitamin B6, 8.32 mg; vitamin B12, 4.8 mg; niacin, 38.4 mg; calcium pantothenate, 25 mg; folic acid, 1.68 mg; biotin, 0.16 mg; iron ($FeSO_4 \cdot H_2O$), 171 mg; manganese ($MnSO_4 \cdot H_2O$), 42.31 mg; copper ($CuSO_4 \cdot 5H_2O$), 125 mg; selenium (Na_2SeO_3), 0.19 mg; cobalt ($CoCl_2$), 0.19 mg; iodine ($Ca(IO_3)_2$), 0.54 mg.

commercial porcine-specific ELISA kits according to the protocols provided by the manufacturer (Shanghai Meimian Biotechnology Co., Ltd).

RNA extraction and gene quantification

RNA extraction and gene quantification were performed according to the previous study (Cai et al., 2020). Briefly, total RNA was isolated from jejunal mucosa with the Trizol reagent (Thermo Fisher Scientific, Inc., Boston, MA) according to the manufacturer's instructions. Then, reverse transcription was performed using the PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd, Dalian, China) after the detection of the concentration and quality of RNA. The real-time quantitative PCR reaction was performed using the CFX96 Touch real-time PCR instrument (Bio-Rad Laboratories Inc., Berkeley, CA, USA). Table 2 lists the primer sequences used in this study. The quantification of target mRNA relative expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by the $2^{-\Delta\Delta CT}$ method.

Jejunum morphology

The specimens of jejunum were dehydrated in graded ethanol series, cleared with xylene, and embedded in paraffin., Subsequently, three pieces of 5 μm thick sections of intestinal samples were prepared, and then they were stained with hematoxylin-eosin. Under an optical microscope, 10 fields were randomly selected to measure the villus height (VH) and the crypt depth (CD), and then the villus height-to-crypt depth ratio (VH: CD) were calculated.

DNA extraction and 16S rRNA gene sequencing

Genomic DNA was extracted from fecal samples using the E.Z.N.A. stool DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's instructions. The purity and quality of the genomic DNA were checked using 1% agarose gels and a NanoDrop spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) were used to amplify the V3-4 hypervariable region of bacterial 16S rRNA gene. The cycling parameters of PCR were 95 °C for 5 min, followed by 28 cycles of 95 °C for 45 s, 55 °C for 50 s, and 72 °C for 45 s with a final extension at 72 °C for 10 min. The PCR products were purified using an Agencourt AMPure XP Kit. Deep sequencing was performed on the Miseq platform at Allwegene Company (Beijing). After that, image analysis, base calling, and error estimation were performed using Illumina Analysis Pipeline Version 2.6.

Volatile fatty acid

The volatile fatty acid (VFA) concentrations in the fecal sample were determined by a gas chromatographic method following the procedures of the previous report (Alfa et al., 2018). In brief, fecal samples were dissolved in sterile PBS and then were centrifuged at 10,000 × *g* for 10 min at 4 °C. The concentration of SCFAs (acetic acid, propanoic acid, and butyric acid) in supernatant was analyzed using an 883 Ion Chromatograph (IC; Metrohm, Switzerland) after filtration and dilution.

Statistical analysis

Data were analyzed as a completely randomized block design by ANOVA using the GLM procedure of the SAS software (version 9.2, SAS Institute Inc., Cary, NC, USA). The model included the treatment effect, and the pen represented the experimental unit for growth performance, while the individual piglet was the experimental unit for gene expression, intestinal morphology, and VFA concentrations. Treatment comparisons were done using Tukey's honestly significant difference test for multiple testing. Moreover, the Chi-square test was used to analyze diarrhea incidence. Differences were considered statistically significant at $P < 0.05$, whereas a treatment effect trend was noted for $0.05 \leq P < 0.10$.

Table 2. Primer sequences for quantitative real-time PCR in this study

Gene	Accession number	Primer sequence (5′–3′)	Size, bp
GAPDH	NM_001,206,359.1	F: GCTTGTCATCAATGGAAAGG	86
		R: CATACGTAGCACCAGCATCA	
CAT	NM_214,301.2	F: CCTGCAACGTTCTGTAAGGC	72
		R: GCTTCATCTGGTCACTGGCT	
SOD1	NM_001,190,422.1	F: GAAGACAGTGTTAGTAACGG	93
		R: CAGCCTTGTGTATTATCTCC	
GPX1	NM_214,201.1	F: TCTCCAGTGTGTCGCAATGA	104
		R: TCGATGGTCAGAAAGCGACG	
HO-1	NM_001,004,027.1	F: GAGAAGGCTTTAAGCTGGTG	74
		R: GTTGTGCTCAATCTCCTCCT	
NQO1	NM_001,159,613.1	F: GGACATCACAGGTAAACTGA	68
		R: TATAAGCCAGAGCAGTCTCG	
NRF2	XM_005,671,981.3	F: GACCTTGGAGTAAGTCGAGA	103
		R: GGAGTTGTTCTTGTCTTTCC	
ZO-1	XM_021098827.1	F: CGATCACTCCAGCATACAAT	111
		R: CACTTGGCAGAAGATTGTGA	
Occludin	NM_001163647.2	F: TCAGGTGCACCCTCCAGATT	112
		R: TGGACTTTCAAGAGGCCTGG	
Claudin1	NM_001244539.1	F: CCTCAATACAGGAGGGAAGC	76
		R: CTCTCCCCACATTCGAGATGATT	
MUC2	XM_021082584.1	F: CCGCATGGATGGCTGTTTCT	147
		R: CATTGCTCGCAGTTGTTGGT	

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CAT, catalase; SOD1, superoxide dismutase 1; GPX1, glutathione peroxidase 1; HO-1, heme oxygenase-1; NQO1, NAD(P)H: quinone oxidoreductase 1; NRF2, nuclear factor-erythroid2-related factor 2; ZO-1: zonula occludens 1; MUC2, mucin 2.

Results

Growth performance and diarrhea incidence

The effects of dietary OA supplementation on growth performance and diarrhea incidence in weaned piglets were shown in Table 3. Piglets fed the PC diet had increased BW on day 42 ($P < 0.05$), ADG from days 28 to 42 and from days 0 to 42 ($P < 0.05$), and ADFI from days 28 to 42 ($P < 0.05$) compared to those fed the NC diet. In addition, the G: F values of the PC-treated piglets were greater than the NC animals from day 14 to 28 ($P = 0.071$) and day 0 to 42 ($P < 0.05$). Dietary OA tended to increase BW on day 42 ($P = 0.065$), ADG from day 14 to 28, from day 28 to 42 and from day 0 to 42 ($P = 0.079$, 0.096 and 0.062, respectively), and G: F ratio from days 28 to 42 and from days 0 to 42 ($P = 0.059$ and 0.053, respectively) compared to the NC group. Moreover, compared to the NC group, dietary PC significantly decreased the diarrhea incidence ($P < 0.05$), whereas dietary OA tended to reduce the diarrhea incidence from day 0 to 14 ($P = 0.053$). It' is worth noting that in terms of all measured growth parameters and diarrhea incidence, no significant difference was observed between the PC and OA treatments ($P > 0.05$).

Antioxidant and inflammatory cytokines indices in plasma

The effects of dietary OA on plasma antioxidant and immune cytokines in weaned piglets were shown in Figure 1. Compared with the NC group, the activity of CAT was greater in piglets fed with OA, while the concentration of MDA was lower in piglets fed with PC and OA in plasma ($P < 0.05$). In addition, dietary OA significantly decreased the concentration of TNF-α, IL-8, and IL-6, while dietary AGP addition decreased IL-8 content in the plasma, compared with the NC group ($P < 0.05$).

Jejunal NRF2 signaling pathway gene expression

To further explore the effects of dietary OA on intestinal antioxidant capacity, we detected the expression of NRF2 signaling pathway-related genes (Figure 2). We found that dietary OA and PC significantly upregulated the relative mRNA abundance of *GPX1* and *NRF2* compared with the NC group ($P < 0.05$). In addition, dietary OA also augmented the relative mRNA abundance of *SOD1* in comparison to that in the NC group ($P < 0.05$). However, there were no significant differences in relative mRNA expressions of *CAT*, *HO-1*, and *NQO1* between treatments ($P > 0.05$).

Jejunal barrier function gene expression

The effects of dietary OA supplementation on jejunal barrier gene expression in weaned piglets were shown in Figure 3. The OA group showed significantly upregulated ZO-1 and Claudin1 gene levels, while the PC group exhibited significantly upregulated Claudin1 levels when compared with the NC group ($P < 0.05$). However, there were no significant differences in the relative expression of Occludin and MUC2 among all groups ($P > 0.05$).

Jejunal morphology

The effects of dietary OA on the jejunal morphology of weaned piglets were shown in Table 4. Dietary OA significantly increased the villus height-to-crypt depth ratio and

Table 3. Effect of dietary organic acid on growth performance and diarrhea incidence of weaned piglets[1]

Items	NC	PC	OA	SEM	*P*-value
BW, kg					
Day 0	7.40	7.41	7.40	0.36	0.916
Day 14	9.50	9.49	9.34	0.47	0.463
Day 28	12.74	13.35	13.25	0.74	0.130
Day 42	$17.10^{b,y}$	18.92^{a}	$18.38^{ab,x}$	0.98	0.012
ADG, g					
Days 0-14	150	149	138	15	0.435
Days 14-28	222^{y}	266^{xy}	270^{x}	31	0.062
Days 28-42	$335^{b,y}$	429^{a}	$394^{ab,x}$	30	0.013
Days 0-42	$233^{b,y}$	277^{a}	$264^{ab,x}$	20	0.011
ADFI, g					
Days 0-14	239	256	267	14	0.392
Days 14-28	386	420	433	53	0.350
Days 28-42	641^{b}	759^{a}	686^{ab}	48	0.034
Days 0-42	416	470	456	34	0.148
G: F, g/g					
Days 0-14	0.625	0.579	0.516	0.051	0.146
Days 14-28	0.580^{y}	0.650^{x}	0.627^{xy}	0.033	0.079
Days 28-42	0.519^{y}	0.565^{xy}	0.579^{x}	0.020	0.059
Days 0-42	$0.558^{b,y}$	0.591^{a}	$0.580^{ab,x}$	0.011	0.053
Diarrhea incidence[2], %					
Days 0-14	$13.25^{a,x}$	6.67^{b}	$9.05^{ab,y}$	-	0.005

NC, basal diet without additive; PC, basal diet + 0.04 g/kg surmax + 0.1 g/kg olaquindox; OA, basal diet + 2 g/kg organic acid blend.
[a,b]Means listed in the same row with different superscripts are significantly different ($P \leq 0.05$).
[x,y]Means listed in the same row with different superscripts tended to be different ($0.05 < P \leq 0.10$).
[1]Six pens per treatment.
[2]Statistical analysis was conducted by Chi-square test.

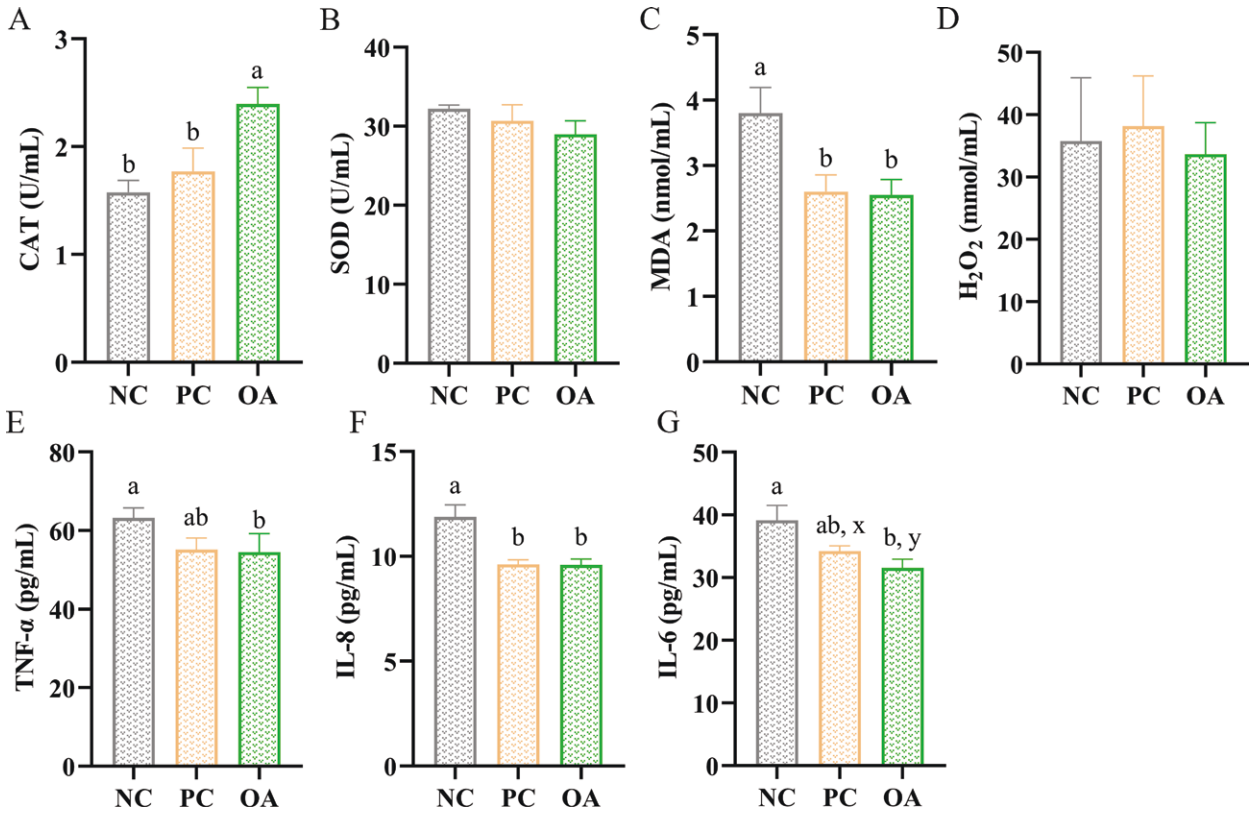

Figure 1. Effects of organic acid on the antioxidant and inflammatory cytokines indices in plasma of weaned piglets. The activity of CAT (A) and SOD (B). The content of MDA (C), H_2O_2 (D), TNF-α (D), IL-8 (D), and IL-6 (D). NC = basal diet without additive; PC = basal diet + 0.04 g/kg surmax + 0.1 g/kg olaquindox; OA = basal diet + 2 g/kg organic acid blend. [a,b] The values with different superscripts are significantly different ($P < 0.05$).

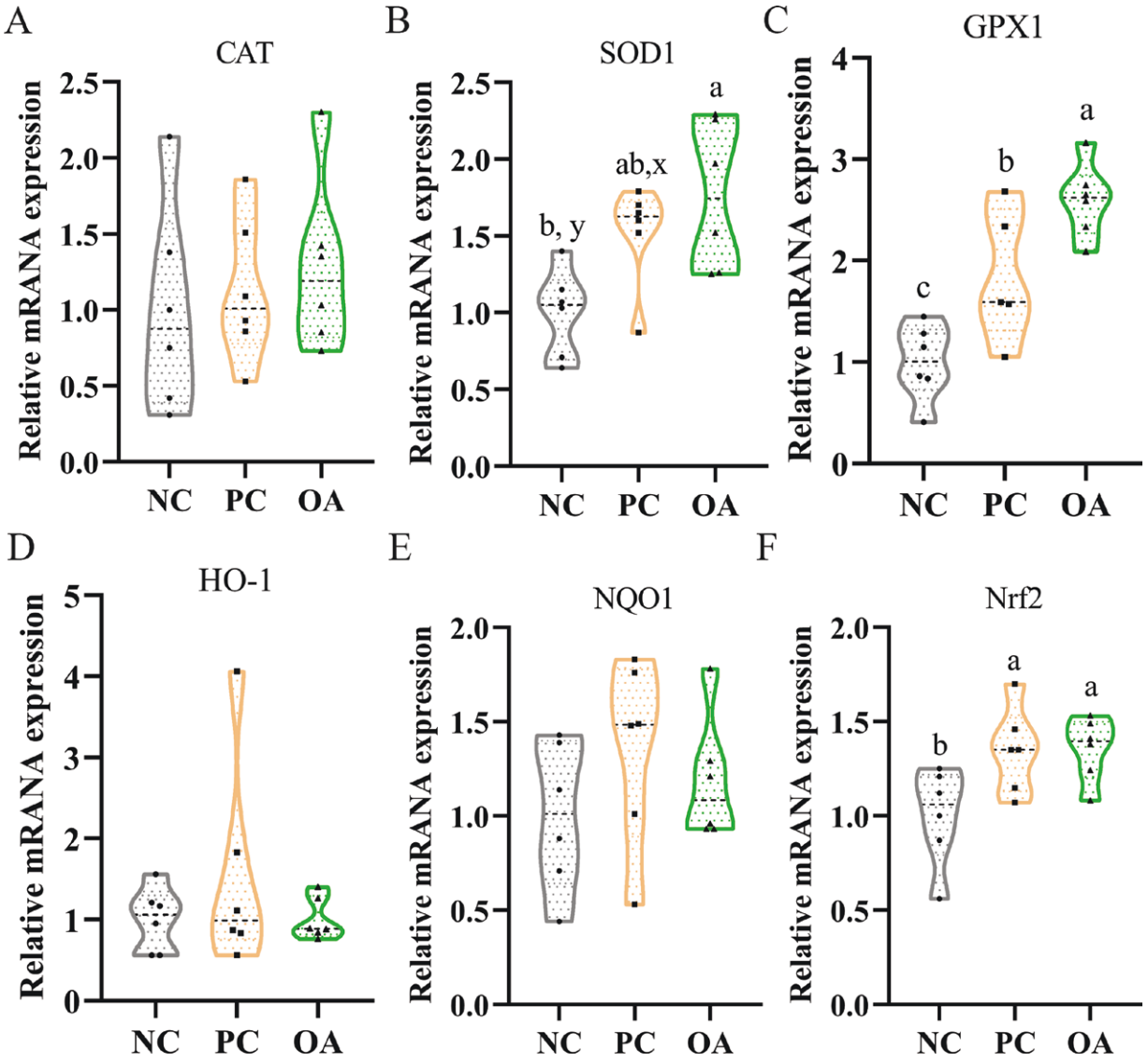

Figure 2. Effects of organic acid on jejunal Nrf2 signaling pathway genes expression in weaned piglets. The relative expression of CAT (A), SOD1 (B), GPX1 (C), HO-1 (D), NQO-1 (E), and Nrf2 (F). NC = basal diet without additive; PC = basal diet + 0.04 g/kg surmax + 0.1 g/kg olaquindox; OA = basal diet + 2 g/kg organic acid blend. [a,b] The values with different superscripts are significantly different ($P < 0.05$).

tended to reduce the crypt depth compared to the NC group ($P = 0.018$, $P = 0.105$, respectively). However, there was no significant diet effect on villus height ($P > 0.05$).

Faecal microbiota analysis by 16S rDNA

The effects of dietary OA on fecal microbiota in weaned piglets was shown in Figure 4. The number of common operational taxonomic units (OTUs) was 614, and the number of unique OTUs in the NC, PC, and OA groups was 26, 5, and 49, respectively (Figure 4D). There were no significant differences in the Chao1, observed_species, and Shannon indices of the fecal microbiota among the treatments ($P > 0.05$) (Figure 4A-C). Principal component analysis was used to evaluate the β-diversity and the results showed a trend of aggregation of fecal microbial communities among all groups ($P > 0.05$) (Figure 4E). The dominant phyla were Bacteroidetes and Firmicutes, which represented 58.3% and 35.8% of the total community, respectively. The abundance of Spirochaetae tended to decrease due to OA treatment ($P = 0.097$). (Figure 4F). In addition, *Unidentified*, *Prevotella*, and *Prevotellaceae_NK3B31_group* were the dominant genus in all groups (Figure 4G). There were no significant differences in the relative abundance of bacterium that relative proportion greater than 1% at the genus level in the genus level among all groups.

Fecal volatile fatty acid

The effects of dietary OA on VFA concentration in the fecal sample was presented in Table 5. No dietary effect on acetic acid, propanoic acid, and butyric acid concentration was observed in the fecal sample of weaned piglets ($P > 0.05$).

Discussion

During the weaning period, piglets face multiple challenges such as underdeveloped gastrointestinal development, changes in nutritional forms, and environmental variations. There has been a continuous effort to explore green and safe growth promoters to reduce the risk of diarrhea and mitigate

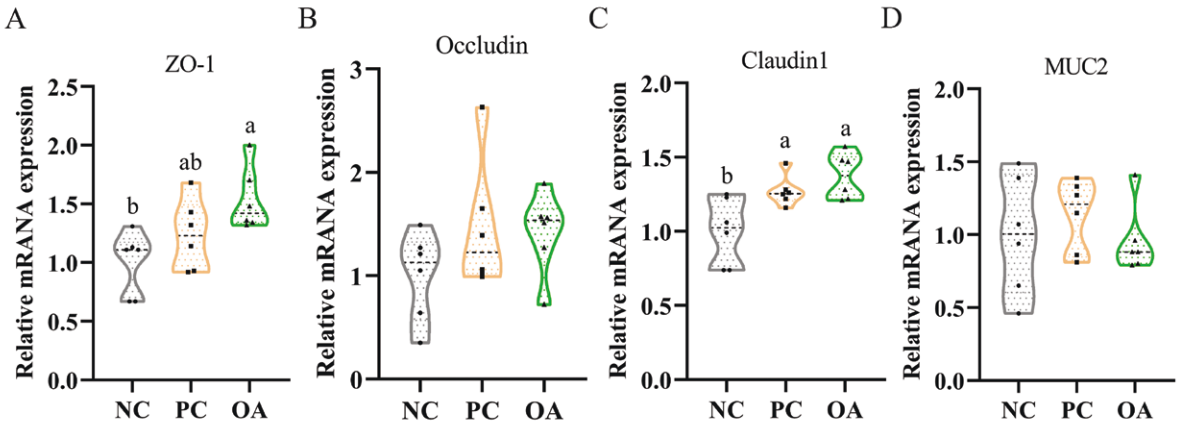

Figure 3. Effects of organic acid on jejunal barrier function genes expression in weaned piglets. The relative expression of ZO-1 (A), Occludin (B), Claudin1 (C), and MUC2 (D) in the jejunum. NC = basal diet without additive; PC = basal diet + 0.04 g/kg surmax + 0.1 g/kg olaquindox; OA = basal diet + 2 g/kg organic acid blend. [a,b] The values with different superscripts are significantly different ($P < 0.05$).

Table 4. Effect of dietary organic acid on jejunum morphology of weaned piglets[1]

Items	NC	PC	OA	SEM	*P*-value
Villus height, μm	402	398	409	22	0.842
Crypt length, μm	303	262	237	17	0.105
VH: CD, μm/μm	1.34[b]	1.54[ab]	1.75[a]	0.08	0.018

NC = basal diet without additive; PC = basal diet + 0.04 g/kg surmax + 0.1 g/kg olaquindox; OA = basal diet + 2 g/kg organic acid blend.
[a,b]Means listed in the same row with different superscripts are significantly different ($P \leq 0.05$).
[1]Six pens per treatment.

economic losses. Previous studies have reported the positive effects of organic acids on pig nutrition (Han et al., 2018; Yang et al., 2018). The current study showed that dietary OA tended to improve the body weight of piglets and increase ADG as well as the G: F ratio, indicating that adding OA to the diet elicits superior growth performance comparable to AGP in weaned piglets, despite no effect of OA supplementation on ADFI was found in the current study at all stages of the experiment. Our results were in agreement with the observations by Xu et al. (2018) that dietary supplementation with fumaric acid improved the ADG of weaning pigs. Additionally, Diao et al. (2016) reported a greater ADG and feed conversion efficiency of weaning pigs with a diet supplemented with benzoic acid. However, there were also reports found that adding organic acids had varying effects on growth performance. Li et al. (2018) reported that adding different levels of OA to a highly digestible basal diet did not significantly affect the growth performance of weaned pigs. Risley et al. (1993) observed no effect of OA treatment on post-weaning performance of pigs challenged with *E. coli*. These inconsistent results might have been caused by the type and dose of OA used in the diet, the dietary-specific composition of basal diets, and different physiological phases of pigs as well as feeding management conditions.

Diarrhea can significantly impact the feed intake and weight gain of weaned piglets. It is quite widespread for piglets to have a higher incidence of diarrhea early in the post-weaning period. In the present study, our results indicate that diarrhea incidence tended to decrease in piglets offered the OA treatment compared to those in the NC treatment, and there is no significant difference between the OA and PC groups. Consistent with results from the present study, Lei et al. (2017) observed that animals fed OA showed significantly reduced post-weaning diarrhea. Tsiloyiannis et al. (2001) also found that supplementation OA positively affected postweaning diarrhea of pigs.

Next, we explored the underlying mechanisms of increased growth performance and decreased diarrhea incidence in response to OA treatment. Piglets face intense oxidative stress and inflammatory response during the weaning period. Excessive reactive oxygen species (ROS) would cause serious redox imbalance, which leads to the oxidation of lipids, proteins, and DNA, ultimately resulting in tissue damage (Minelli et al., 2009; Cao et al., 2018). The complex systems of antioxidant enzymes play a crucial role in protecting the body from oxidative damage (Minelli et al., 2009). In particular, superoxide dismutase can catalyze the generation of superoxide anion radicals into oxygen and hydrogen peroxide, which can be decomposed into H_2O and O_2 by catalase and glutathione peroxidase, thereby reducing the excessive generation of ROS and consequent oxidative stress (Tang et al., 2020). In the current study, we found that dietary OA increased plasma CAT activity and reduced the concentration of MDA, a typical marker of lipid peroxidation. These results indicated that dietary OA could improve antioxidant properties in weaned piglets by enhancing antioxidant enzyme activities and subsequently decreasing lipid peroxidation levels. Consistent with our results, a previous study showed that OA supplementation increased serum total antioxidant capacity and decreased MDA content in weaned piglets (Han et al., 2018). Interestingly, we also found piglets fed OA diets had decreased the content of TNF-α, IL-8, and IL-6 in the plasma. Consistent with our results, Liu et al. (2023) reported that butyric-lauric acid addition reduced serum TNF-α and IL-1β in mice fed a high-fat diet. Sodium butyrate supplementation decreased the concentrations of TNF-α, IFN-γ, IL-6, and IL-1β in Deoxynivalenol-exposed piglets (Zong et al., 2023). The KEAP1/NRF2 cascade signaling pathway plays a critical role in maintaining cellular

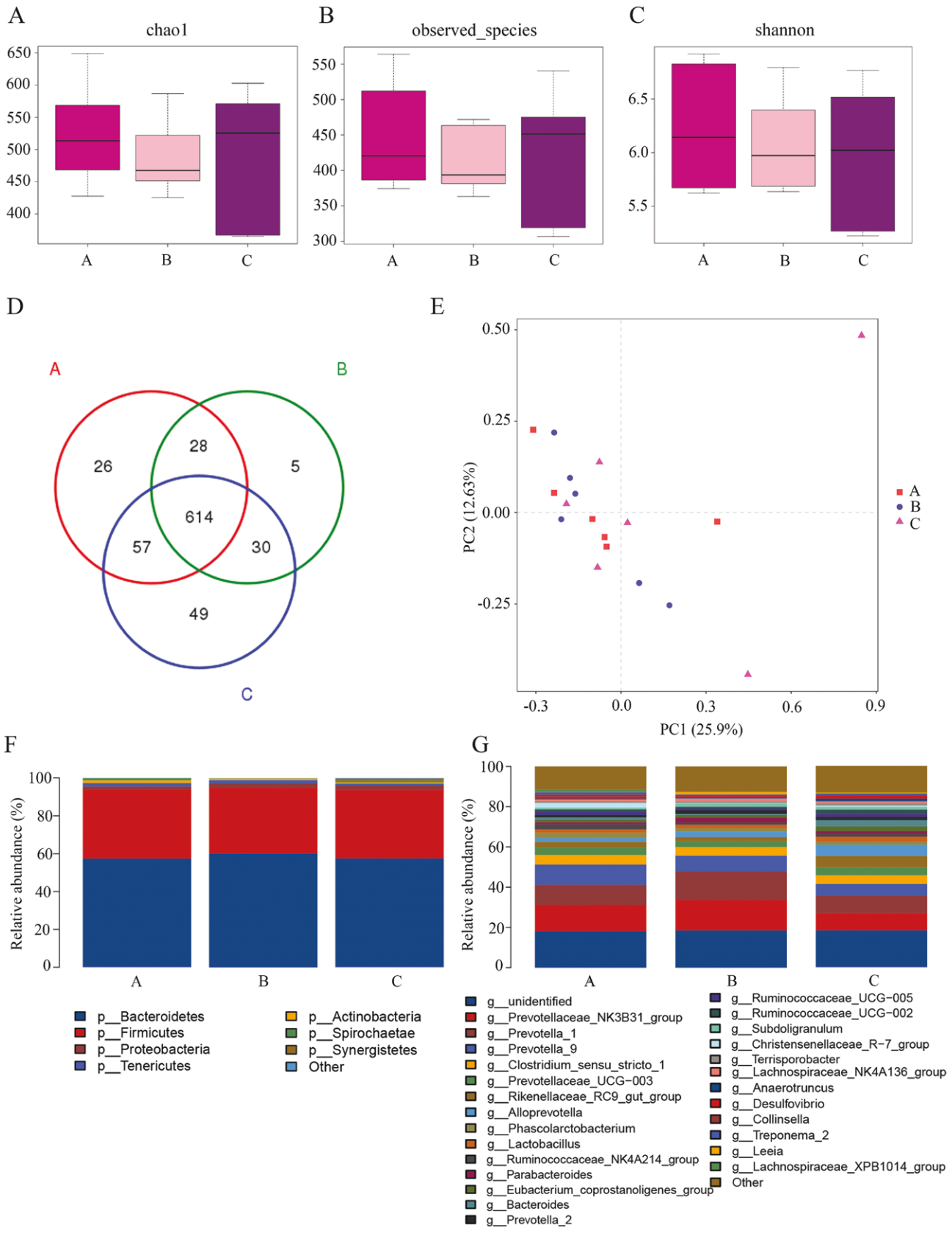

Figure 4. Effects of organic acid on the fecal bacterial community of weaned piglets. (A-C) α-diversity index. (D) Ven diagram of OTU. (E) Principal component analysis. The relative abundance of microbiota at phylum (F) and genus (G) levels. A = basal diet without additive; B = basal diet + 0.04 g/kg surmax + 0.1 g/kg olaquindox; C = basal diet + 2 g/kg organic acid blend.

Table 5. Effect of dietary organic acid on volatile fatty acid concentration in the feces of weaned piglets[1]

Items	NC	PC	OA	SEM	*P*-value
Acetic acid, mg/g	5.36	4.84	4.70	0.35	0.511
Propanoic acid, mg/g	1.22	1.08	1.03	0.14	0.624
Butyric acid, mg/g	1.09	1.00	0.94	0.19	0.521

NC = basal diet without additive; PC = basal diet + 0.04 g/kg surmax + 0.1 g/kg olaquindox; OA = basal diet + 2 g/kg organic acid blend.
[1]Six pens per treatment.

redox balance, which can promote the production and secretion of endogeneity antioxidant enzymes to deal with oxidative damage (Qin and Hou, 2016). In the present study, dietary AGP or OA significantly upregulated the relative mRNA abundance of *NRF2* and its downstream related genes *SOD1* and *GPX1* in the jejunum of weaned piglets. Similarly, some previous studies reported that dietary lauric acid or butyrates could suppress oxidative stress by regulating NRF2 signaling in vivo and in vitro trials (Wang et al., 2022a; Bian et al., 2023). Altogether, these results indicated that OA supplementation could resist weaning-induced oxidative stress and inflammation by enhancing the antioxidant capacity via activating the NRF2 signaling pathway in weaned piglets, which may partly explain why dietary OA can improve growth performance and decreased diarrhea incidence in weaned piglets.

Diarrhea was generally considered to be closely related to intestinal atrophy characterized by reduced villus height and deepening of crypts caused by weaning in piglets. The development of villi and crypts in the small intestine reflects the absorptive capacity of nutrients. A previous study demonstrated that dietary supplementation with OA and its ammonium salts as substitutes for AGP had a beneficial effect on the intestinal morphology and function of weaned piglets (Xu et al., 2020). Here, we found that both PC and OA treatments exerted a positive impact on the gut morphometric properties of assessed piglets. Piglets fed a diet that contained OA significantly increased villus height-to-crypt depth ratio, and numerically decreased crypt depth compared with the NC group. This partially agrees with the results of a previous study, which found the jejunal crypt depth was significantly decreased, and the VH: CD ratio was enhanced in pigs fed OA diets (Diao et al., 2016). Therefore, adding OA may impact the growth performance of weaned piglets by promoting the digestion and absorption of nutrients through the improvement of intestinal development. The intestinal epithelial barrier is the first line of defense against the invasion of pathogens and harmful substances into the circulatory system. Maintaining the expression of intestinal tight junction proteins such as Claudin1 and ZO-1 is crucial for improving intestinal epithelial integrity (Hu et al., 2013; Chen et al., 2016). Claudin1 protein plays an important role in regulating paracellular permeability, barrier formation, and cell polarity (Jia et al., 2022). ZO-1 acts as a scaffolding molecule that is primarily involved in constructing transmembrane tight junctions (Klunker et al., 2013). In the presented study, dietary OA and AGP elevated the expression levels of Claudin1 and ZO-1, Claudin1 in the jejunum, respectively. Consistently, our results were in support of a previous study where dietary OA increased the mRNA expression of Claudin3 and ZO-1 in the jejunum of broilers (Dai et al., 2021). In addition, there were no differences in the relative expression of MUC2 among all groups, indicating that dietary OA did not affect the chemical barrier. The improved intestinal barrier function may play a role in preventing the invasion of pathogenic bacteria, thereby reducing the occurrence of diarrhea.

Intestinal microbiota plays an attractive role in nutrient digestion, enterocyte development, and immune function enhancement of piglets. A superior microbiota community is conducive to intestinal health and improves growth performance (Qi et al., 2021; Mahmud et al., 2023; Xun et al., 2023). Surprisingly, beyond original expectations, dietary OA had no significant effect on the composition of the fecal bacteria, although OA treatment owned more unique OTUs than the NC and PC groups in this study. What's more, there was no significant difference in bacteria composition between NC and PC group, which may be related to the low dosage of antibiotics used in the diet and the antibiotic resistance of pathogenic microorganisms (Hedges and Linton, 1988; Monger et al., 2021; Van Goethem et al., 2024). In addition, another possible reason is that we chose the fecal sample, not the foregut content. There is a study, demonstrating that the foregut microbiota has a more obvious change than the hindgut after antibiotic treatments (Mu et al., 2017). Contrarily, some studies have shown that dietary OA has a positive effect on the intestinal microbial community of piglets. For example, adding OA increased microbial diversity and colonization of more potentially beneficial bacteria, such as *Blautia*, *Turicibacter* as well as reduced the population of some harmful species at the meantime (Wei et al., 2021). Moreover, antibiotic treatment also did not show any effect on bacterial community structure. The VFA plays an important role in maintaining gastrointestinal tract function and integrity, and host immune status (Ferronato and Prandini, 2020). Consistently, no effect of dietary supplementation with antibiotics or OA on VFA concentrations, the microbial metabolites, was observed in the fecal content of weaned piglets. The lack of dietary treatment effect on the fecal microbiota and its metabolite observed in the present study may be closely related to several factors, including the ability of OA to reach the small intestine, the genetic characteristics of pigs, and the superior experimental conditions. It was reported that dietary OA increases *Lactobacillus* and *Bacilli* abundances in the intestine of piglets after enterotoxigenic *Escherichia coli* F4 challenge ($K88^+$) (Xu et al., 2020), and stabilizes the gut bacterial community structure (Han et al., 2020). We speculated that dietary OA may deliver a beneficial effect on the microbial composition of weaning piglets in stress conditions. In addition, the fecal microbiota structure may not fully reflect the composition of microbiota in the gut, and OA treatment may have beneficial effects on the microflora in the small intestine even the colon. Therefore, it is necessary to conduct more specific trials to confirm our conjecture. Above all, the dietary

OA can improve the antioxidant capacities and intestinal barrier, but showed no effect on intestinal microbial composition. A healthy gut and strong antioxidant abilities made more weaned piglets free from diarrhea. Thereby the growth performance of piglets were significantly improved.

Conclusion

In conclusion, our observations demonstrated that dietary supplementation with OA could improve growth performance and attenuate postweaning diarrhea, which may be closely related to the improved the antioxidant capacity and intestinal integrity of weaned piglets. The beneficial effect of OA on weaned piglets was similar to antibiotics, which may provide the theoretical basis for using OA as an alternative to antibiotics in the pig industry.

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Conflicts of Interest Statement

The authors declare no conflict of interest.

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