Original Research Article

Mixed organic acids as an alternative to antibiotics improve serum biochemical parameters and intestinal health of weaned piglets

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A R T I C L E   I N F O
Article history:
Received 4 August 2020
Received in revised form 9 September 2020
Accepted 21 November 2020
Available online 23 June 2021

Keywords:
Weaned piglet
Organic acid
Growth performance
Intestinal health

A B S T R A C T
The primary aim of this experiment was to critically explore the relationship between the different levels of mixed organic acids (MOA) and growth performance, serum antioxidant status and intestinal health of weaned piglets, as well as to investigate the potential possibility of MOA alternative to antibiotics growth promoters (AGP). A total of 180 healthy piglets (Duroc × [Landrace × Yorkshire]; weighing 7.81 ± 1.51 kg each, weaned at d 28) were randomly divided into 5 treatments: 1) basal diet (CON); 2) CON + chloromycine (75 mg/kg) + virginiamycin (15 mg/kg) +吉他霉素 (50 mg/kg) (AGP); 3) CON + MOA (3,000 mg/kg) (OA1); 4) CON + MOA (5,000 mg/kg) (OA2); 5) CON + MOA (7,000 mg/kg) (OA3). This study design included 6 replicates per treatment with 6 piglets per pen (barrow:gilt = 1:1) and the experiment was separated into phase 1 (d 1 to 14) and phase 2 (d 15 to 28). In phases 1, 2 and overall, compared with the CON, the feed conversion ratio (FCR) was reduced (P < 0.01) and the average daily gain (ADG) was increased (P < 0.05) in piglets supplemented with AGP, OA1 and OA2. The concentration of serum immunoglobulins G (IgG) was improved (P < 0.05) in piglets supplemented with OA1 and OA2. The villus height:crypt depth ratio was significantly increased (P < 0.01) in piglets fed AGP and OA1. The mRNA expression level of claudin-1 and zonula occludens-1 (ZO-1) (P < 0.01) was up-regulated in piglets supplemented with OA1 and OA2. The piglets fed AGP, OA1 and OA2 showed an increase (P < 0.05) in the content of acetate acid and total volatile fatty acids (TVFA) in the cecum, and butyric acid and TVFA in the colon compared with CON. Also, OA1 lowered (P < 0.05) the content of Lachnospiraceae in piglets. These results demonstrated that MOA at 3,000 or 5,000 mg/kg could be an alternative to antibiotics due to the positive effects on performance, immune parameters, and intestinal health of weaned piglets. However, from the results of the quadratic fitting curve, it is inferred that MOA at a dose of 4,000 mg/kg may produce a better effect.

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1. Introduction

With new rearing regimes being practiced in modern swine production, the weaning period of piglets has changed from the original 7–8 wk to 4 wk. The piglets are faced with stress from nutrition, environment, physiology and other aspects in advance, which has led to increased diarrhea, decreased digestive enzyme ability, and stunted growth of piglets (Heo et al., 2013). Therefore, antibiotics have often been added to the feed of weaned piglets to prevent diarrhea and improve growth performance (Liu et al., 2018). However, the overuse of antibiotics has increased resistance to bacteria, and resulted in residual accumulation of drugs in the body (Suiryanrayna and Ramana 2015). In 2006, the European Union banned the addition of antibiotics to feed, and the United States and China have gradually begun to ban the use of antibiotics. Consequently, safe and green feed additives replacing antibiotics were found to solve the negative effect on performance, immune parameters, and intestinal health of weaned piglets. However, from the results of the quadratic fitting curve, it is inferred that MOA at a dose of 4,000 mg/kg may produce a better effect.

At present, the commonly used alternative feed additives are plant essential oils, organic acids, antibacterial peptides, prebiotics,
enzyme preparations, etc. Organic acids are widely used in livestock production because of their advantages such as lowering the pH value of the gastrointestinal tract, improving the structure of the intestinal microbial flora, immune function and performance (Ahmed et al., 2014; Liu et al., 2014; Yang et al., 2019). Several researchers have demonstrated that the application of a small amount of mixed organic acids (MOA) has had a wide range of effects compared with that of a single organic acid in pigs (Kim et al., 2005; Partanen et al., 2007a).

Currently, there are still differences concerning the optimum concentration of MOA as a substitute to antibiotics on improving performance and intestinal health of weaned piglets, such as blood biochemistry, the abundance of microbiota, the relative mRNA expression of tight junction protein and so on. To better explore the optimum concentration of MOA, we investigated 3 levels of MOA to explore the potential possibility of MOA as an antibiotic substitute for improving the performance and health status of weaned piglets as well as to identify the optimum concentration for MOA in weaned piglets.

2. Materials and methods

All the procedures used in the animal experiment were authorized and approved by the Institutional Animal Care and Use Committee of China Agricultural University (Beijing, China).

2.1. Mixed organic acid product and antibiotic mixture

The commercial products of MOA used in this experiment were sourced from the Netherlands (Nutreco, Amsterdam, Netherlands). The MOA product mainly included formic acid (11%), ammonium formate (13%), propionic acid (10%), acetic acid (5.1%) and citric acid (3.7%) and the carrier was silica. The antibiotic mixture (chloromycin, virginiamycin and guitaromycin) was provided by Beijing Tongli XingKe Agricultural Technology Company Limited (Beijing, China).

2.2. Animals, diets and experimental design

A total of 180 healthy piglets (Duroc × [Landrace × Yorkshire]; weighing 7.81 ± 1.51 kg each, weaned at d 28) were randomly divided into 5 treatments: 1) basal diet (CON); 2) CON + chloromycin (75 mg/kg) + virginiamycin (15 mg/kg) + guitaromycin (50 mg/kg) (antibiotics growth promoters [AGP]); 3) CON + MOA (3,000 mg/kg) (OA1); 4) CON + MOA (5,000 mg/kg) (OA2); and 5) CON + MOA (7,000 mg/kg) (OA3). This study designed 6 replicates per treatment with 6 piglets per pen (barrow:gilt = 1:1) and the experiment was separated into phase 1 (d 1 to 14) and phase 2 (d 15 to 28). The diet formula and the composition of nutrition are presented in Table 1, which refer to the recommendations of the NRC (2012).

The piglets were housed at the piglet facility which has 1.5 m × 1.5 m experimental pens with a leaky plastic floor, stainless steel adjustable trough, and duckbill waterer in each pen. The temperature was maintained at 25 ± 1 °C with 60% to 70% humidity. Pigsties were disinfected once a month to maintain health and hygiene and to prevent the spread of certain diseases in all pigs. Moreover, to decrease the stress, the feed was transferred step by step for the first 3 d post-weaning based on below procedure: the first day, 75% creep feed + 25% nursery feed (trial feed); the second day, 50% creep feed + 50% nursery feed; the third day, 25% creep feed + 75% nursery feed. All experimental piglets had free access to water and feed overall. Feed consumption and diarrheas were recorded daily.

2.3. Experimental procedures and sampling

On d 1, 14 and 28 of the experiment, the piglets and feed were weighed and recorded, respectively before calculating the average daily gain (ADG), average daily feed intake (ADFI), and feed conversion rate (FCR).

Blood samples of 10 ml were collected from 1 piglet of each replicate from all groups through the jugular vein to vacuumainer (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) on the morning of d 14 and 28. The samples were centrifuged at 3,000 × g at 4 °C for 10 min, and stored at −20 °C until analysis. The immunoglobulins (IgG, IgM and IgA) and tumor necrosis factor-α (TNF-α) were determined using an ELISA kit (IgG, IgM, IgA and TNF-α quantitation kit; Bethyl Laboratories, Texa). Serum total antioxidant capacity (T-AOC), malondialdehyde (MDA), hydroxyl radical (OH), glutathione peroxidase (GSH-PX) and superoxide dismutase (SOD) were detected by Spectrophotometry (Leng GuangSFZ1606017568, Shanghai, China) following the kit manufacturer’s operating instructions. (Jiancheng Institute of Bioengineering, Nanjing, China).

Digestive juices from the cecum and colon were collected, immersed in liquid nitrogen immediately, and stored at −80 °C for subsequent analysis of volatile fatty acid (VFA). Gently scraping with a sterile plate scalpel, the mucosal tissue of jejunum, ileum and pancreas was collected, and liquid nitrogen was used to freeze the mucous membrane mixture, which was preserved at −80 °C. The digestive enzymes were analyzed via spectrophotometry (PU 8720 UV/VIS Scanning Spectrophotometer, Pye Unicam, UK) according to the methods of Lee et al. (2003).

At the 28th day of the experiment, one piglet with average BW was selected in each pen to collect aseptic intestinal samples for detection of morphology. The histological specimens were immobilized with 10% neutral buffer formalin. The small intestine was sliced at a 6-μm thickness, dehydrated, paraffin-embedded, and cut into 4 cross-sections, stained with hematoxylin and eosin. Then random measurements were taken of the heights of 10 oriented villi and their crypts on small covers by optical microscope using an eyepiece scale.

On the 28th day of the experiment, the cm jejunal of the three piglets with the medium weight of each treatment was taken, frozen and washed with cold phosphate-buffered saline (PBS), persevered at −80 °C till protein mRNA extraction, and the mRNA expressions of tight junction protein were detected by RT-PCR. Sample RNA was extracted with Trizol, and 20-μL RNase-free water was added to dissolve the RNA according to the protocol of the supplier. The cDNA was synthesized by 1 μg total RNA treated with RNase-free DNase I, in which the main reaction contained 1-μL oligo (DT) 18, 1-μL reverse transcriptase M-MLV. This was quantified by measuring the absorbance at 260 and 280 nm. The comparative cycle threshold was compared with the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to compute the relative mRNA expression of tight junction protein. Table 2 shows the primer sequences of GAPDH and the tight junction protein.

On the 28th day of the experiment, VFA analysis was performed on fresh chyme samples collected from the cecum, colon, and rectum. Approximately 1.5-g sample was mixed with 1.5-ml sterile water and placed in the centrifuge tube before centrifuge at 15,000 × g at 4 °C for 10 min. This was later transferred to a sample bottle of a gas chromatograph, and 200 μL metaphosphoric acid was added. The bottle was immersed in ice for 30 min, centrifuge at 4 °C at 15,000 × g for 10 min. The contents of VFA were tested using Hewlett Packard 5890 gas chromatography (HP, Pennsylvania) (Pan et al., 2016).
GAPDH were purified by a QIAquick PCR purification kit. The quality of the extracted DNA was quantified by a Qubit 2.0 fluorometer (Thermo Fisher Scientific, USA) to achieve a uniform concentration. Then the paired-end reads of 250 bp were obtained by sequencing on the Illumina HiSeq PE250 platform (Illumina, San Diego, USA) according to the standard protocol. The paired-end reads were assembled into longer tags, and the quality was filtered to remove the tags with length <220 nucleotide (nt) and average quality score <20, as well as the tags containing >3 ambiguous bases used by PANDAseq software (version 2.9) to clean the reads. All clean reads were compared with the operational taxonomy unit (OTU) to acquire the full mapped reads (Edgar 2013). After discarding the singleton, the high-quality tags were clustered into OTU for species classification by using the Usearch (version 7.0.1090) in Quantitative Insights Into Microbial Ecology (QIIME) pipeline software (version 1.8.0) with a similarity threshold of 97% and the OTU were further taxonomically analyzed using the Ribosomal Database Project (RDP) database, using the RDP classifier at an 0.80 confidence level. Alpha diversity and beta diversity were tested by QIIME (University of California, San Diego, USA). Linear discriminant analysis (LDA) and effect size (LEfSe) analyses were operated via the LEfSe tool (Segata et al., 2011).

### 2.4. Statistical analysis

SAS statistical software (SAS version 9.2, SAS Institute Inc., Cary, NC, USA) was used for data analysis with each pen of pigs considered as an experimental unit, and measurement data were expressed as standard errors of the least squares mean (SEM). The treatments were considered as fixed effects and the piglets as a random effect. The optimal requirement of MOA was evaluated for performance of piglets by using the quadratic curve model in nonlinear regression (NLIN): \( y = a \times x^2 + b \times x + c \), maximum \( = -b / (2 \times a) \), where \( y \) is ADG or FCR; \( x \) is concentration of MOA (Robbins et al., 2006). The differences and composition of bacterial abundance in cecal samples were analyzed by Kruskal–Wallis rank-sum test. \( P \leq 0.05 \) was considered statistically significant different; when \( 0.05 < P < 0.10 \), the trends were noted.

### 3. Results

#### 3.1. Growth performance, diarrhea rate and the optimum dosage of MOA

In phase 1 (d 1 to 14), compared with the CON, the piglets supplemented with AGP could improve \( P < 0.05 \) the ADG (Table 3), piglets fed OA1, AGP had decreased \( P < 0.05 \) the FCR, and...
the diarrhea rate of piglets fed OA2 was lower ($P < 0.05$). The fitted quadratic indicated that the supplementation level of MOA for the maximal ADG of weaned piglets in phase 1 was 3,675 mg/kg (Fig. 1).

In phase 2 (d 15 to 28), compared with the CON, the piglets supplemented with OA1 and AGP increased ($P < 0.05$) the ADG, and FCR was reduced ($P < 0.05$) in piglets fed AGP, OA1 and OA2. In the entire experimental period, piglets fed AGP, OA1 and OA2 increased the ADG and reduced ($P < 0.05$) the FCR. Additionally, no piglets developed diarrhea in phase 2.

In the entire experimental period, compared with the CON, piglets fed AGP, OA1 and OA2 increased ($P < 0.05$) ADG by 12.7%, 8.8%, and 5.7%, respectively. Further, piglets supplemented with AGP, OA1 and OA2 lowered FCR by 14.5%, 12.3%, and 10.6% ($P < 0.05$) respectively. The fitted quadratic indicated that supplementation level of MOA for the optimum FCR of weaned piglets was 3,931 mg/kg during the entire study period (Fig. 2).

### 3.2. Digestive enzyme activity

There was no effect of dietary supplementation of organic acids or AGP on digestive enzyme activity compared with the CON (Table 4), but the piglets fed OA1 tended to promote the digestive enzyme activity of pancreas in weaned piglets.

### 3.3. Immune parameters in serum

In phase 1, compared with the CON, the content of GSH-Px was increased ($P < 0.05$) in piglets fed OA2 and OA3 (Table 5). In phase 2, the dietary supplementation of OA2 showed higher concentrations of IgG compared with the CON.

### 3.4. Intestinal morphology

In the duodenum, the crypt depth of the piglets supplemented with AGP and OA1 was decreased ($P < 0.01$) compared with those fed OA2 and OA3 (Table 6). Also, the villus height: crypt depth ratios in piglets fed AGP, OA1 and OA2 were improved ($P < 0.01$).

In the ileum, compared with the CON, the villus height: crypt depth ratio in piglets supplemented with AGP and OA2 was improved ($P < 0.05$).

### 3.5. Relative mRNA expression of tight junction protein

Compared with the CON, the relative mRNA expression of claudin-1 and ZO-1 were up-regulated ($P < 0.05$) in the jejunum of piglets supplemented with OA1 and OA2 (Fig. 3).

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**Table 3**

| Item                      | Dietary treatments 1 | SEM | $P$-value |
|---------------------------|----------------------|-----|------------|
|                          | CON                  | AGP | OA1        | OA2        | OA3        |
|                           | d 1 to 14            |     |            |            |            |
| ADG, g                    | 350 a, b             | 391 a | 378 ab     | 378 ab     | 362 ab     |
| ADFI, g                   | 631                  | 612  | 612        | 613        | 595        |
| FCR, g/g                  | 1.80 a, b            | 1.56 b | 1.61 b     | 1.63 b     | 1.64 b     |
| Diarrhea rate, %          | 5.36                 | 3.21 b | 2.50 b     | 1.07        | 2.86 b     |
|                           | d 15 to 28           |     |            |            |            |
| ADG, g                    | 532                  | 602 a | 583 ab     | 555 b      | 528 c      |
| ADFI, g                   | 954                  | 910  | 901        | 873        | 924        |
| FCR, g/g                  | 1.79                 | 1.51 a | 1.53 b     | 1.58 a      | 1.75 b     |
|                           | d 1 to 28            |     |            |            |            |
| ADG, g                    | 441                  | 497 a | 480 ab     | 466 b      | 445 c      |
| ADFI, g                   | 792                  | 761  | 756        | 743        | 760        |
| FCR, g/g                  | 1.79                 | 1.53 a | 1.57 b     | 1.60 ab     | 1.70 ab     |

ADG = average daily gain; ADFI = average daily feed intake; FCR = feed conversion ratio.

1 Different letters within a row mean a statistical difference ($P < 0.05$).

2 No piglets developed diarrhea during d 15 to 28.

CON: control; AGP: chlorhinomycin at 75 mg/kg + virginiamycin at 15 mg/kg + guitaromycin at 50 mg/kg; OA1: MOA at 3,000 mg/kg; OA2: MOA at 5,000 mg/kg; OA3: MOA at 7,000 mg/kg.

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Fig. 1. Fitted quadratic of ADG of piglets given diets with different supplemental levels of mixed organic acids (MOA) during phase 1. The formula of forecasting broken line: $y = -2 \times 10^{-6} x^2 + 0.0147 x + 351.9$, $R^2 = 0.49$. The fitted quadratic showed the breakpoint as 3,675 mg/kg, indicating that the maximal ADG (378.9 g/d) could be obtained by supplementing MOA at 3,675 mg/kg ($P < 0.05$).
3.6. Volatile fatty acids

In the cecum, compared with the CON, the content of acetate acid and total volatile fatty acids (TVFA) were enhanced ($P < 0.01$) in piglets fed AGP, OA1 and OA2 (Table 7). Also, the piglets supplemented with OA1 and OA2 showed an enhanced ($P < 0.05$) content of propionic acid.

In the colon, compared with the CON, OA1 enhanced ($P < 0.05$) the content of butyrate acid and TVFA of piglets.

3.7. Cecal microbiota

There was no significant effect on $\alpha$-diversity (Fig. 4). At the phylum level, the cecum contents of the piglets were mainly composed of Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria, which accounted for more than 98.5% of the total cecal flora. The proportions of Firmicutes in the OA1 group and the AGP group were 87.38% and 81.96%, respectively, which were lower than the CON (87.73%) (Fig. 5A, Fig. 6A).

At the family level (Figs. 5B and 6B), Lactobacillaceae, Ruminococcaceae, Lachnospiraceae and Streptococcaceae were the major Firmicutes in the cecum contents of weaned piglets. Bacteroidetes were mainly composed of Prevotellaceae. Most of the cecum contents of the CON were Lactobacillaceae (42.03%), Ruminococcaceae (12.89%) and Lachnospiraceae (17.49%). Most of the cecum contents in piglets supplemented with AGP were Lactobacillaceae (22.12%), Ruminococcaceae (17.99%) Lachnospiraceae (19.65%) and Prevotellaceae (10.05%). The relative abundances of the cecum of the OA1 group were mainly composed of Lactobacillaceae (41.32%), Ruminococcaceae (15.51%) and Streptococcaceae (11.43%). The relative abundances of main flora in the cecum of piglets supplemented with OA2 were Lactobacillaceae (53.72%), Ruminococcaceae (12.75%) and Lachnospiraceae (14.37%). The relative abundances of the cecum of the OA3 group were mainly composed of Lactobacillaceae (15.22%), Ruminococcaceae (24.40%), Lachnospiraceae (18.02%) and Streptococcaceae (21.19%). The content of Lactobacillaceae in the cecum of piglets fed OA2 was increased ($P < 0.05$) compared with those fed AGP and OA3. Compared with the CON, the content of Lachnospiraceae in the cecum of piglets fed OA1 was declined ($P < 0.05$). Furthermore, piglets fed OA1 and OA2 had a tendency to lower ($P = 0.07$) the relative abundance of Escherichia-Shigella (Fig. 7).

### Table 4

| Item       | Dietary treatments | SEM | P-value |
|------------|--------------------|-----|---------|
| Jejunum    | CON                |     |         |
| Trypsin    | 21.10              |     |         |
| Lipase     | 36.55              |     |         |
| Amylase    | 67.26              |     |         |
| Ileum      | 20.44              |     |         |
| Trypsin    | 19.05              |     |         |
| Lipase     | 38.53              |     |         |
| Amylase    | 71.54              |     |         |
| Pancreas   | Trypsin            |     |         |
| Lipase     | 32.31              |     |         |
| Amylase    | 52.44              |     |         |

a, b Different letters within a row mean a statistical difference ($P < 0.05$).

1 CON: control; AGP: chloramphenicol at 75 mg/kg + virginiamycin at 15 mg/kg + gatifloxacin at 50 mg/kg; OA1: MOA at 3,000 mg/kg; OA2: MOA at 5,000 mg/kg; OA3: MOA at 7,000 mg/kg.

### 4. Discussion

The application of organic acids in swine production was suggested in a review article published by Sirivanya and Ramana (2015). Many studies have proved the utility of MOA as a potential alternative to antibiotics in developing performance and enhancing microbial flora in pigs (Grecco et al., 2018; Li et al., 2019). However, no systematic research has been dedicated to the optimum concentration of MOA in the feed. In this research, MOA was supplemented at 3 levels based on the previous findings of Long et al. (2017), who demonstrated that piglets supplemented with MOA at 3,000 mg/kg could show improved the growth group were 87.38% and 81.96%, respectively, which were lower than the CON (87.73%) (Fig. 5A, Fig. 6A).

At the family level (Figs. 5B and 6B), Lactobacillaceae, Ruminococcaceae, Lachnospiraceae and Streptococcaceae were the major Firmicutes in the cecum contents of weaned piglets. Bacteroidetes were mainly composed of Prevotellaceae. Most of the cecum contents of the CON were Lactobacillaceae (42.03%), Ruminococcaceae (12.89%) and Lachnospiraceae (17.49%). Most of the cecum contents in piglets supplemented with AGP were Lactobacillaceae (22.12%), Ruminococcaceae (17.99%) Lachnospiraceae (19.65%) and Prevotellaceae (10.05%). The relative abundances of the cecum of the OA1 group were mainly composed of Lactobacillaceae (41.32%), Ruminococcaceae (15.51%) and Streptococcaceae (11.43%). The relative abundances of main flora in the cecum of piglets supplemented with OA2 were Lactobacillaceae (53.72%), Ruminococcaceae (12.75%) and Lachnospiraceae (14.37%). The relative abundances of the cecum of the OA3 group were mainly composed of Lactobacillaceae (15.22%), Ruminococcaceae (24.40%), Lachnospiraceae (18.02%) and Streptococcaceae (21.19%). The content of Lactobacillaceae in the cecum of piglets fed OA2 was increased ($P < 0.05$) compared with those fed AGP and OA3. Compared with the CON, the content of Lachnospiraceae in the cecum of piglets fed OA1 was declined ($P < 0.05$). Furthermore, piglets fed OA1 and OA2 had a tendency to lower ($P = 0.07$) the relative abundance of Escherichia-Shigella (Fig. 7).
serum biochemical index of weaned piglets as affected by dietary mixed organic acids (MOA) and antibiotic growth promoter (AGP) supplementation.

| Item                              | Dietary treatments¹ | SEM  | P-value |
|-----------------------------------|---------------------|------|---------|
|                                  | CON  | AGP  | OA1  | OA2  | OA3  |
| d 1 to 14                         |      |      |      |      |      |
| IgG, mg/mL                        | 13.18 | 12.49 | 11.59 | 8.95 | 12.73 | 2.11 | 0.64 |
| IgA, mg/mL                        | 28.11 | 34.25 | 27.90 | 23.20 | 32.04 | 4.01 | 0.38 |
| IgM, mg/mL                        | 14.00 | 17.00 | 13.93 | 10.78 | 14.95 | 2.18 | 0.41 |
| IgG, mg/mL                        | 2.87  | 2.30  | 2.36  | 2.23  | 2.16  | 0.33 | 0.36 |
| IgA, mg/mL                        | 13.10 | 14.23 | 13.57 | 13.26 | 13.73 | 0.97 | 0.93 |
| T-AOC, U/mL                       | 13.00 | 13.25 | 13.15 | 15.48 | 15.62  | 0.52 | <0.01 |
| MDA, nmol/mL                      | 1.16  | 1.10  | 1.21  | 1.28  | 1.17  | 0.08 | 0.61 |
| SOD, U/mL                         | 45.71 | 47.53 | 44.10 | 45.27 | 45.76 | 0.91 | 0.17 |
| TNF-α, ng/mL                      | 97.37 | 90.04 | 90.90 | 75.84 | 101.83 | 13.31 | 0.70 |
| d 15 to 28                        |      |      |      |      |      |
| IgG, mg/mL                        | 5.26  | 8.00  | 8.14 | 10.07  | 8.69  | 0.93 | 0.03 |
| IgA, mg/mL                        | 19.88 | 24.05 | 19.64 | 26.27 | 21.16 | 2.99 | 0.47 |
| IgM, mg/mL                        | 9.47  | 11.94 | 10.17 | 11.52 | 12.18 | 1.21 | 0.60 |
| OH, mg/mL                         | 2.32  | 2.73  | 2.17  | 2.19  | 2.43  | 0.20 | 0.31 |
| T-AOC, U/mL                       | 13.35 | 14.28 | 13.63 | 14.82 | 13.73 | 0.64 | 0.53 |
| GSH-Px, μmol/L                    | 16.44 | 16.70 | 15.91 | 15.16 | 16.79 | 0.83 | 0.23 |
| MDA, nmol/mL                      | 1.06  | 1.18  | 1.09  | 1.11  | 1.11  | 0.05 | 0.44 |
| SOD, U/mL                         | 44.64 | 45.64 | 44.92 | 44.44 | 43.85 | 0.83 | 0.65 |
| TNF-α, ng/mL                      | 66.96 | 82.66 | 85.98 | 88.88 | 82.39 | 8.13 | 0.20 |

¹-Con: control; AGP: chloromycin at 75 mg/kg + virginiamycin at 15 mg/kg +吉他霉素 at 50 mg/kg; OA1: MOA at 3,000 mg/kg; OA2: MOA at 5,000 mg/kg; OA3: MOA at 7,000 mg/kg.

Table 6
Intestinal morphology of weaned piglets as affected by dietary mixed organic acids (MOA) and antibiotic growth promoter (AGP) supplementation.

| Item                              | Dietary treatments¹ | SEM  | P-value |
|-----------------------------------|---------------------|------|---------|
|                                  | CON  | AGP  | OA1  | OA2  | OA3  |
| Duodenum                          |      |      |      |      |      |
| Villus height, μm                 | 302.56 | 301.65 | 300.41 | 306.56 | 292.20 | 3.25 | 0.11 |
| Crypt depth, μm                   | 204.89 | 194.61 | 187.90 | 222.59  | 216.83  | 4.33 | <0.01 |
| villus height: crypt depth ratio  | 1.48  | 1.55  | 1.60  | 1.38  | 1.35  | 0.03 | <0.01 |
| Jejunum                           |      |      |      |      |      |
| Villus height, μm                 | 283.01b | 311.15b | 347.42a | 326.06a | 275.20  | 12.07 | 0.01 |
| Crypt depth, μm                   | 198.69 | 191.48 | 183.32 | 198.89 | 189.21 | 7.89 | 0.72 |
| villus height: crypt depth ratio  | 1.46  | 1.62  | 1.90  | 1.64  | 1.45  | 0.03 | <0.01 |
| Ileum                             |      |      |      |      |      |
| Villus height, μm                 | 249.02 | 223.40 | 259.09 | 242.16 | 218.26 | 15.08 | 0.35 |
| Crypt depth, μm                   | 183.69 | 149.69 | 160.65 | 165.81 | 163.30 | 10.25 | 0.31 |
| villus height: crypt depth ratio  | 1.30  | 1.49  | 1.61  | 1.46  | 1.34  | 0.02 | <0.01 |

²-Con: control; AGP: chloromycin at 75 mg/kg + virginiamycin at 15 mg/kg +吉他霉素 at 50 mg/kg; OA1: MOA at 3,000 mg/kg; OA2: MOA at 5,000 mg/kg; OA3: MOA at 7,000 mg/kg.
production of α-ketoglutarate, and converted into glutamic acid. Therefore, MOA can effectively prevent the GSH synthesis obstacle caused by the lack of precursor materials, improve the feedback regulation of GSH-Px synthesis, promote the increase of GSH-Px enzyme activity, and accelerate to remove free radicals, which was demonstrated by Tezcan et al. (2009), who found that citric acid has strong antioxidant activities. Also, the activities of GSH-Px decreased at 15 to 28 d indicated that there were more free radicals and active antioxidant system in the early stage of weaning stress. Still, the adaptability of piglets to stress became stronger with the increase in age, resulting in a decrease of stress intensity and the activity of antioxidant enzymes. IgA, IgG and IgM can specifically bind to corresponding antigens and are the main substances of humoral immunity. The content of immunoglobulin in serum can decreased at 15 to 28 d indicated that there were more free radicals and active antioxidant system in the early stage of weaning stress. Still, the adaptability of piglets to stress became stronger with the increase in age, resulting in a decrease of stress intensity and the activity of antioxidant enzymes. IgA, IgG and IgM can specifically bind to corresponding antigens and are the main substances of humoral immunity. The content of immunoglobulin in serum can

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**Fig. 3.** The relative mRNA expression of claudin-1 (A), occludin (B), and ZO-1 (C) in the jejunum of piglets as affected by dietary mixed organic acids (MOA) and antibiotic growth promoter (AGP) supplementation (n = 3). CON: control; AGP: chlorinomycin at 75 mg/kg + virginiamycin at 15 mg/kg + guitaromycin at 50 mg/kg; OA1: MOA at 3,000 mg/kg; OA2: MOA at 5,000 mg/kg; OA3: MOA at 7,000 mg/kg. Data are expressed as means ± SEM. a, b Different letters mean a statistical difference (P < 0.05).

**Table 7**

Volatile fatty acids in the cecum, colon and rectum of piglets as affected by dietary mixed organic acids (MOA) and antibiotic growth promoter (AGP) supplementation (mg/g).

| Item                  | Dietary treatments | SEM | P-value |
|-----------------------|--------------------|-----|---------|
|                       | CON                | AGP | OA1     | OA2     | OA3     |     |
| Cecum Acetic acid     | 4.37b              | 4.84b| 5.62a   | 5.51a   | 4.28c   | 0.09| <0.01 |
| Propionic acid        | 2.66b              | 3.00ab| 3.58a   | 3.44a   | 3.01ab  | 0.14| 0.01  |
| Butyric acid          | 0.81               | 0.96 | 1.31    | 1.46    | 1.08    | 0.14| 0.07  |
| Valeric acid          | 0.10               | 0.13 | 0.19    | 0.21    | 0.16    | 0.05| 0.58  |
| Total volatile fatty acid | 7.91b             | 8.95b| 10.71a  | 10.63a  | 8.54bc  | 0.20| <0.01 |
| Colon Acetic acid     | 5.60               | 5.68 | 6.46    | 6.19    | 5.68    | 0.19| 0.05  |
| Propionic acid        | 2.83               | 3.00 | 3.67    | 2.96    | 2.87    | 0.23| 0.15  |
| Butyric acid          | 1.00b              | 1.13b| 1.65a   | 1.45ab  | 1.37ab  | 0.10| 0.01  |
| Valeric acid          | 0.20               | 0.25 | 0.39    | 0.32    | 0.21    | 0.04| 0.05  |
| Total volatile fatty acid | 9.70b              | 10.11b| 12.28a  | 11.00ab | 10.15ab | 0.44| 0.02  |
| Rectum Acetic acid    | 4.62               | 4.64 | 5.16    | 4.77    | 4.61    | 0.31| 0.69  |
| Propionic acid        | 1.62               | 1.79 | 2.01    | 1.77    | 1.87    | 0.15| 0.50  |
| Butyric acid          | 0.62               | 0.67 | 0.70    | 0.70    | 0.68    | 0.08| 0.95  |
| Valeric acid          | 0.08               | 0.09 | 0.16    | 0.13    | 0.14    | 0.03| 0.36  |
| Total volatile fatty acid | 7.52               | 7.80 | 8.85    | 8.04    | 7.92    | 0.37| 0.22  |

a, b Different letters within a row mean a statistical difference (P < 0.05).

1 CON: control; AGP: chlorinomycin at 75 mg/kg + virginiamycin at 15 mg/kg + guitaromycin at 50 mg/kg; OA1: MOA at 3,000 mg/kg; OA2: MOA at 5,000 mg/kg; OA3: MOA at 7,000 mg/kg.
measure the immune function of the body. In a previous study, organic acids were shown to improve the immune system of piglets, especially by increasing serum concentration of IgG (Ahmed et al., 2014). In our research, the piglets fed MOA tended to increase the content of IgA, IgM and IgG compared with the CON in phase 2, which is in agreement with Yang et al. (2019), who revealed that piglets which received diets supplemented with MOA increased the concentration of IgM and IgG. Ren et al. (2020) indicated that piglets fed 1% mixture of formic acid and propionic acid reduced the inflammatory response in ETEC K88 attack piglets, improved immune response and reduced fever and diarrhea. Allaire et al. (2018) also reported that there is a correlation between immunity and the number of intestinal epithelial cells. Therefore, the improvement of immune performance by MOA in piglets was probably related to the improvement of intestinal flora and intestinal morphology.

Intestinal morphology is one of the indexes used to evaluate intestinal digestive performance (Jha et al., 2019). The villus length represents the absorption capacity of nutrients in livestock and poultry. The longer the villi, the more epithelial cells and lymphocytes, and the stronger the immune response. The depth of the crypts represents the rate of cell formation. The shallower the crypt, the better the cell maturity and the higher the secretory function. The higher the villus height is, the greater the villus height:crypt depth ratio, also the larger the total villus area, the more sufficient the digestive enzyme development is, resulting in higher digestibility (Van, Leeuwen et al., 1996). Studies have shown that weaning stress can reduce the height of villi, while villi atrophy is the result of an increase in programmed cell death and a decrease in cell renewal rate (Hampson and Kidder, 1986). In our research, the piglets fed MOA at 3,000 mg/kg showed a positive effect on villus height and villus height:crypt depth ratio in the duodenum, jejunum and ileum. Diao et al. (2014) revealed that piglets fed benzoic acid at 5,000 mg/kg demonstrated improved villus height:crypt depth ratio in the duodenum, jejunum and ileum on day 14 and 42, and thus enhanced the nutrient absorption of piglets. Long et al. (2017) indicated that piglets fed MOA could significantly lower the crypt depth in the jejunum, improve the ratio of villus height and crypt depth in jejunum and ileum of piglets. Previous research has revealed that the improvement of the acidic environment (Lupton and Jacobs, 1987) and the increase of volatile fatty acid concentration both contribute to the increase of intestinal morphology (Karlsson et al., 2013). Therefore, MOA may help to improve the morphology of the intestinal tract and can alleviate intestinal stress in piglets.

Tight junction (TJ) proteins play a vital role in maintaining the difference of substances on both sides of epithelial cells and maintaining cell polarity which mainly contains claudin, occludin, and zonula occludens (ZO) proteins, that take a systematic way to modulate TJ function (Yin et al., 2015; Roxas et al., 2010). Bacteria, viruses, and endotoxins can regulate the expression of transmembrane protein occludin, claudin and ZO-1 via regulating or affecting some cytokines and protein kinase C, and reduce the barrier function of intestinal epithelial cells (Weiler et al., 2005;
Zhang et al., 2010). Hu et al. (2013) indicated that early-weaning could hamper intestinal barrier function by destroying the intestinal structure, altering the expression of TJ proteins, and increasing the intestinal permeability, activating mitogen-activated protein kinases in pigs, which may be attributed to altering expression of TJ proteins in intestinal epithelial cells. The results in the present research indicated that piglets fed MOA at 3,000 and 5,000 mg/kg increased the relative mRNA expression of claudin-1 and ZO-1 in jejunal mucosal epithelial cells, which was consistent with Feng et al. (2018), who revealed that piglets fed sodium butyrate at 2,000 mg/kg facilitated expression of the TJ protein in the jejunum by acting on the protein kinase B (PKB) signaling pathway in a GPR109A dependent manner, which possibly activated or triggered a series of signaling pathways. But MOA at 7,000 mg/kg declined the relative mRNA expression of claudin-1 and ZO-1, which may be due to the high concentration of MOA shutting down some signal pathway, and then inhibiting the expression of TJ protein. The detailed mechanism of action, therefore, needs further research.

Microorganisms in the digestive tract of piglets (especially colon and cecum) can ferment carbohydrates (Canh et al., 1998) and produce a variety of VFA (propionic acid, butyric acid, etc.). These short-chain fatty acids can provide energy for piglets, resist pathogenic microorganisms and maintain animal intestinal health, among which butyric acid is particularly important. It is easily absorbed by colon epithelial cells and used as energy, and it can also reduce the incidence of colon disease (Corrier et al., 1990; Howard et al., 1995). Kathrin (2009) revealed that piglets fed organic acids showed an increase in the content of butyric acid, which was similar to our research in that dietary supplementation of MOA at 5,000 mg/kg (OA2) increased acetic acid and propionic acid in the cecum and increased the butyric acid in the colon. Likewise, the total VFA concentration was increased in the cecum and colon when piglets were fed OA2 and OA3, which is in agreement with Diao et al. (2014) who noted that piglets fed organic acids showed an enhanced content of total VFA in the cecum. Long et al. (2017) and Li et al. (2019) have demonstrated that MOA increased the content of total VFA in feces in piglets and growing-finish pigs, respectively. This study did not find any difference between the CON and OA3 on VFA concentration, which may be because the high concentration of organic acids can inhibit the secretion of gastric acid by gastric bottom gland cells, hinder the development of gastric secretion function, and make organic acids exhausted in the stomach so that the organic acid could not continue to play a role in intestinal tract. The full effects of high concentration of MOA on VFA

Fig. 5. Microbial community in cecal samples of piglets as affected by dietary mixed organic acids (MOA) and antibiotic growth promoter (AGP) supplementation. Microbial community barplot at (A) the phylum level and (B) the family level (n = 3). CON: control; AGP: chloromycine at 75 mg/kg + virginiamycin at 15 mg/kg + guitaromycin at 50 mg/kg; n = 3. OA1: MOA at 3,000 mg/kg; OA2: MOA at 5,000 mg/kg; OA3: MOA at 7,000 mg/kg. Results were expressed as mean values of different bacteria. Different colors indicated the bacteria abundance of each treatment.
in piglets are still unknown. Therefore, the detailed mode of MOA in the intestinal tract needs to be further researched.

Symbiotic microbiota in the gastrointestinal tract acts in a vital role on the growth and health of the host by performing metabolic, immunologic and protective functions (Duan et al., 2019). In the current research, the relative abundances of Firmicutes, Bacteroides and Proteobacteria were more than 95%, which belong to the predominant microbiota, and the results were consistent with the previous study of Chen et al. (2017) in suckling piglets. Lactobacillaceae family belongs to the Firmicutes phylum, which is a valuable microorganism for improving intestinal barrier function and immune regulation of intestinal health (Yang et al., 2018). Many studies have documented that piglet supplementation with MOA could increase the relative abundance of Lactobacillaceae and improve the microflora in the intestinal tract (Grecco et al., 2018; Sotira et al., 2020), which is in line with our findings. Xu et al. (2018) also showed that MOA modulated the microbial populations by enhancing the Lactobacillus in faeces of weaned piglets, which may be related to the promotion of proliferation of epithelial cells. Lachnospiraceae are one of the most abundant families from the order Clostridiales, and play a role in maintaining gut health (Biddle et al., 2013). Studies have confirmed that Lachnospiraceae is more abundant in NOD (non-obese diabetic) mice that develop diabetes and less abundant in mice that do not develop diabetes (Krych et al., 2015). In our research, the piglets supplemented with MOA at 3,000 mg/kg lowered the relative abundance of Lachnospiraceae, which implies that the addition of MOA inhibits the growth of some potentially harmful bacteria and increases the health of intestinal microorganisms. Moreover, we also observed that the piglets fed MOA group tended to decrease the relative abundance of Escherichia-Shigella, which was also similar to previous research (De Busser et al., 2011; Silva et al., 2020). This is mainly because the MOA provides an acidic environment and lowers the pH of the gastrointestinal tract, which inhibits the reproduction of Escherichia-Shigella (the optimal pH value was 6.0 to 8.0). Additionally, a higher relative abundance of Firmicutes and a lower population of...
Bacteroides was found in piglets fed OA1, which enhanced the ratio of Firmicutes to Bacteroidetes. Ley et al. (2006) have proven that the proportion of Firmicute to Bacteroidetes is positively correlated with body weight. Therefore, MOA may enhance the performance of piglets by increasing the proportion of Firmicutes/Bacteroidetes and improving the intestinal microflora. Still, a high concentration of MOA did not result in optimal performance in piglets, which probably related to the inhibition of gastric fundus gland cells due to high concentration of organic acids. In short, these findings revealed that the relative abundance and composition of the intestinal microbiota could differ in piglets fed MOA, so further research is now warranted to clarify the underlying mechanisms of MOA for intestinal microbiota, and clarify the potential prospect of replacing antibiotics with optimal concentrations of MOA.

The effect of AGP on piglets is mainly achieved by improving the ADG and FCR. However, it has a negative impact, to some extent, on the intestinal health of piglets (mainly affecting the structure of intestinal microflora and the expression of intestinal epithelial tight junction protein) (Francois, 1961; Visek, 1978; Thomas, 2015). In our study, dietary supplementation of MOA at 3,000 and 5,000 mg/kg had beneficial effects on performance, immune function, and intestinal health in piglets. These findings revealed that MOA could replace AGP to some extent to achieve a growth-promoting effect, and may be even better than antibiotics in improving digestive enzyme activity, intestinal morphology, immune capacity and intestinal microflora in weaned piglets.

5. Conclusions

In summary, the evidence from this research suggested that MOA at dosages of 3,000 and 5,000 mg/kg could potentially serve as AGP substitutes based on their beneficial effect on performance, immune function, the relative mRNA expression of TJ protein, and the gut microbiota community in weaned piglets. Moreover, our study found that the optimal content of MOA (3,675 mg/kg) in piglets can be obtained from the maximum ADG (378.9 g/d) in phase 1, and the optimal content of MOA (3,931 mg/kg) can be obtained from the optimum FCR (1.554) during the entire experimental period.

Author contributions

Jiayu Ma: Conceptualization, Software, Data Curation, Writing-Original Draft Preparation, and Visualization; Jiayu Ma and Shenfei Long: Methodology; Qinghui Shang and Sujie Liu: Investigation; Xiangshu Piao: Supervision; Validation; Xiangshu Piao, Shenfei Long and Shad Mahfuz: Writing- Reviewing and Editing.

Conflict of interest

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

Acknowledgements

This research was supported by the National Natural Science Foundation of China (31772612) and Beijing Municipal Natural Science Foundation (6202019). We acknowledge Nutreco Company (Amsfoort Netherlands) for providing the new organic acid commercial products and Beijing Tongli XingKe Agricultural Technology Company Limited for providing the antibiotic mixture.

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