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Original Research Article

Piglet growth performance improved by dietary supplementation of porous or nano particles of zinc oxide may be related to the gut microbiota

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ABSTRACT

Previous studies on porous or nano particles zinc oxide (ZnO) in the piglets have mainly focused on growth performance and intestinal inflammation, but have scarcely explored the efficacy on gut microbiota. In addition, the efficacy of nano particles ZnO, which is related to its product quality, remains undefined. This study aimed to determine the efficacy of dietary 500 mg/kg porous or nano particles ZnO on the growth performance and gut microbiota of the weaned piglets. A total of 128 weaned piglets were randomly assigned to the dietary groups: NC (basal diet), PC (basal diet + 3,000 mg/kg conventional ZnO), 500HiZ (basal diet + 500 mg/kg porous particles ZnO), and 500ZNP (basal diet + 500 mg/kg nano particles ZnO). Compared with the NC diet group, both 500HiZ and 500ZNP increased ($P < 0.05$) average daily feed intake (1 to 28 d) and average daily gain (1 to 28 d), and the 500ZNP tended to decrease feed to gain ratio (F:G ratio, 1 to 28 d) ($P = 0.09$). Both 500HiZ and 500ZNP decreased crypt depth of the ileum and increased claudin-2 in the duodenum and zonula occludens-1 in the ileum ($P < 0.05$). Moreover, both 500HiZ and 500ZNP decreased IL-1β and tumor necrosis factor-α (TNF-α) in the jejunum and decreased TNF-α and IL-6 in the ileum ($P < 0.05$). Both 500HiZ and 500ZNP increased microbial β-diversity index in the ileum and microbial α-diversity indices in the colon of piglets ($P < 0.05$). The probiotic genera *Coprococcus* (500ZNP) and *Blautia* (500HiZ) were positively correlated with the F:G ratio (1 to 28 d) in colon of piglets ($P < 0.05$). In addition, 500HiZ promoted mitochondrial fusion protein 1 (MFN1) and zinc transporter-1 (ZnT-1) in the jejunum ($P < 0.05$), whilst 500ZNP decreased MFN1 in the jejunum and ZnT-1 in the ileum ($P < 0.05$). In summary, both 500HiZ and 500ZNP improved the growth performance of piglets, which is likely via the genera *Blautia* and *Coprococcus*, respectively. Both 500HiZ and 500ZNP improved barrier function and inflammation of the intestine, and 500HiZ achieved better efficacy than 500ZNP on intestine mitochondrial functions.

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1. Introduction

Weaning is one of the most critical periods in the lifetime of a pig. Pigs are susceptible to multiple stressors, such as nutrition and their environment, which can induce post-weaning stress and even lead to death in extreme cases (Campbell et al., 2013; Fernandes et al., 2020; Laine et al., 2008). In the past, antibiotics were typically adopted as a feed additive to address problems with weaning.

However, issues such as antibiotic resistance and antibiotic residues in meat foods have arisen due to antibiotic abuse, which may adversely affect human health (Bacanli and Basaran, 2019; Simon 2005). As a result, many countries have decided to ban the use of antibiotics as feed additives. Thus, a new alternative to replace in-feed antibiotics is required (Graham et al., 2007).

Zinc is an essential trace element that is involved in numerous biological reactions in the body (Ao et al., 2011). It has been added to feeds for a long time, and the recommended dietary requirement for weaned piglets ranges from 50 to 100 mg/kg (NRC, 2012). Zinc oxide (ZnO) is generally recognized as an antiseptic substance that has been widely used in foods (Xie et al., 2011). In the feed industry, high doses of ZnO have been regularly used as an effective strategy to prevent diarrhea in piglets (Barreto et al., 2017; Carlson et al., 2004). Previous studies reported that high doses of dietary ZnO at 2,500 to 4,000 mg/kg decrease the incidence of diarrhea and at 1,500 to 2,000 mg/kg improve the growth performance of piglets (Hill et al., 2001; Poulsen, 1995). Dietary supplementation of ZnO more than 1,500 mg/kg is necessary to achieve a favorable prevention or treatment effect on diarrhea in piglets (Hill et al., 2001; Pluske et al., 2002). Consequently, a dietary high dose of ZnO, approximately 3,000 mg/kg, has been widely accepted by the pig industry worldwide (Heo et al., 2013; Kim et al., 2012). However, concerns regarding environmental pollution have been raised, and conventional ZnO has been banned or restricted in many countries. As a result, conventional ZnO cannot be used as a viable alternative to antibiotics (Durosoy, 2011; Heo et al., 2013).

However, at present, ZnO remains the only real alternative to antibiotics (Durosoy, 2011), and several highly bioactive or potentiated ZnO additives have emerged to address the problems created by high doses of ZnO in the diet. Potentiated porous or nano particles of ZnO have smaller size (less 100 nm), larger surface area, and higher bioavailability than the conventional ZnO (Cho et al., 2013; Long et al., 2017; Wang et al., 2017; Xie et al., 2011). Furthermore, both porous and nano particles of ZnO have been already applied in animals (Wang et al., 2016). New types of ZnO, such as lipid-coated ZnO (100 or 200 mg/kg) (Byun et al., 2017), porous ZnO particles (500, 750, or 1,500 mg/kg) (Long et al., 2017; Peng et al., 2019, 2020), and nano ZnO particles (200, 500, or 1,200 mg/kg) (Long et al., 2017; Oh et al., 2021; Wang et al., 2017), can achieve the same efficiency at a lower dietary dose compared to the high dietary levels when using conventional ZnO. In addition, according to the above-mentioned studies, it can be concluded that 500 mg/kg of dietary porous or nano particles of ZnO is sufficient to achieve the same efficiency as high doses of conventional ZnO. It is important to note that previous studies have primarily focused on growth performance, barrier function, and intestinal inflammation, but their effects on gut microbes in weaned piglets have not been explored to date. Furthermore, the efficacy of nano ZnO particles primarily depends on the product quality (Durosoy, 2011). Previously, favorable efficacy of nano ZnO particles has been observed in weaned piglets (Long et al., 2017). However, the efficacy of nano ZnO particles remains undefined. Overall, the present study aimed to determine the efficacy of dietary supplementation with porous or nano particles of ZnO at 500 mg/kg on the growth performance and gut microbiota of the weaned piglets.

2. Materials and methods

2.1. Animal ethics statement

This study was conducted according to the guidelines of the Declaration of Helsinki. All animal experiments complied with the ARRIVE guidelines, and the procedures of experimental piglets were approved by the animal welfare committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences (ISA-2016-012).

2.2. Animals and experimental design

A total of 128 piglets (Duroc × Landrace × Yorkshire, average body weight = 10.89 ± 1.16 kg) weaned at 35 d were selected for a 28-day experiment. The piglets were assigned to one of the following four dietary groups: negative control (NC, basal diet), positive control (PC, basal diet + 3,000 mg/kg standard conventional ZnO), 500HiZ diet (basal diet + 500 mg/kg porous ZnO particles), and 500ZNP diet (basal diet + 500 mg/kg nano ZnO particles). Eight replicates were used for each group with four piglets per replicate (half barrows and half gilts). The basal diet (corn-soybean based meal) was designed according to the National Nutrient Requirements of Swine (NRC, 2012). The dietary ingredients and the nutrient levels of the basal diet have been reported previously (Long et al., 2017). The experimental piglets had a 5-d adaption period and had access to feed and water ad libitum during the experiment. Feed intake was determined daily at 08:00, and the body weight of each individual piglet was measured after a period of fasting for at least 12 h. The average daily feed intake (ADFI), average daily gain (ADG), and feed to gain ratio (F:G ratio) of piglets were calculated based on the measured values of body weight and feed intake.

2.3. Samples

Once the trial ended, one piglet (medium weight) per pen was selected and slaughtered as previously described (Yin et al., 2015). Nearly 1 cm was carefully taken out per segment of the intestine (duodenum, jejunum, and ileum), then placed into 4% formalin solution for hematoxylin-eosin analysis. Nearly 5 cm of each segment of the intestine was taken out and rapidly frozen in liquid nitrogen, then stored at −80 °C for further analysis. Lumen digesta samples were collected from the proximal distal parts of the ileum and colon (approximately 10 g per sample), followed by storage at −80 °C for further analysis.

2.4. Analysis of intestine morphology

Segments (nearly 1 cm) of the duodenum, jejunum, and ileum were embedded in paraffin and sliced; then, a 5-μm paraffin section was cut and stained with hematoxylin and eosin for subsequent analysis. Images of intestinal morphology (villus and crypt) were examined (40× magnification) using a microscope (BX51, Olympus, Japan). Approximately 10 unbroken villi-crypts per sample were selected and analyzed using Image-Pro Plus 6.0 (Media Cybernetics, USA); then, the villus height to crypt depth ratio was calculated.

2.5. Western blot analysis

The laboratory Western-blot analysis procedure used in the present study is in line with that described in previous studies (Wen et al., 2020; Guo et al., 2021). Briefly, segments of the intestine (duodenum, jejunum, and ileum) were firstly lysed for 30 min at 4 °C using the RIPA lysis buffer (Beyotime, China). This was followed by centrifugation for 15 min at 10,000 × g (4 °C), and then, the liquid supernatants were collected and the total protein content was extracted using a commercial kit (Beyotime, China). The concentration of total protein was detected using a microplate reader (Infinite M200 PRO, Switzerland) with a protein assay kit (Beyotime, China). Twenty milligrams of protein per sample was denatured and isolated with SDS-PAGE, after which the protein was transferred to a polyvinylidene fluoride membrane (Millipore,

Germany). Polyvinylidene fluoride membranes were blocked with bovine serum albumin at room temperature for 1.5 h, followed by incubation overnight with specific primary antibodies at 4 °C, and then membranes were incubated with the corresponding secondary antibody (R&D, USA).

The primary antibodies specific for zonula occludens-1 (ZO-1; MABT11, Abcam, England), claudin-2 (48120S, Abcam), trefoil factor family 1 (pS2/TFF1, NBP2-16376, Abcam), interleukin-1β (IL-1β; AB10626, Abcam), interleukin-6 (IL-6; AB6672, Abcam), tumor necrosis factor-α (TNF-α, AB6671, Abcam), zinc transporter-1 (ZnT-1, BS-6440R, Abcam), mitochondrial fusion protein 1 (MFN1, NBP1-67515, Abcam), and β-actin (Trans, HC201) were used to determine protein expression. Proteins with polyvinylidene fluoride membranes were visualized using a specific substrate ECL (Bio-Rad, USA) and an imaging system (ChemiDoc MP, Bio-Rad, USA). The protein expression levels were quantified by Photoshop CS6 (Adobe, USA) and normalized to the expression level of β-actin.

2.6. DNA extraction, 16S rRNA sequencing, and bioinformatics analysis

The total DNA of ileal and colonic microbes was extracted using a commercial DNA extraction kit (Omega Bio-Tec, USA). The purity and concentration of DNA was verified on 1% agarose gels. The barcoded primer pairs (341F 5′-CCTAYGGGRBGCASCAG-3′ and 806R 5′-GGACTACNNGGGTATCTAAT-3′) were used to amplify the V3–V4 fragments of bacterial 16S rDNA. The polymerase chain reaction (PCR) cycle reaction conditions included an initial denaturation step at 98 °C for 1 min, 30 cycles of 98 °C for 10 s, 50 °C for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 5 min. All PCR amplicon products were visualized on 2% agarose gels and purified with a DNA gel extraction kit (OXYGEN, China). Then, sequencing libraries were constructed by the TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, USA). Qualified libraries were then pair-end (PE250) sequenced by HiSeq2500 (Illumina, USA).

Ambiguous or mismatched bases in the primer regions were excluded. Sets of sequences aligned with ≥97% identity were defined as an operational taxonomic unit (OTU) using UPARSE software (version 7.0.1001). Rarefaction curves, rank abundance curve, microbial composition, α-diversity indices (Chao1, Goods-coverage, Observed-species, Shannon, and Simpson), and β-diversity evaluation (beta-OTUs index) were assessed using QIIME (version 1.7.0) based on weighted UniFrac distance metrics. The Venn Plot was obtained by R software (3.0.3, R package = "Venn Diagram"). Principal coordinate analysis was performed based on the weighted UniFrac distance metrics of samples. The linear discriminant analysis effect size (LEfSe) was calculated by LEfSe software (version 1.0, LDA score > 2) to analyze ileal and colonic microbial biomarkers. Raw sequencing data have been deposited in the Genome Sequence Archive (accession number: CRA008951) at the China National Center for Bioinformation.

2.7. Statistical analysis

In this study, pen is regarded as the basic statistical unit, and data were all first subjected to normality and homogeneity tests by statistical analysis software (SAS 8.1, Institute, Inc., Cary, NC, USA). Non-parametric Kruskal–Wallis test was performed for data that were not normally distributed or lacked homogeneity. One-way ANOVA analysis was performed for other data and significance was examined by Duncan's multiple comparison. In addition, the relative abundances of microbes in piglets were analyzed using the Kruskal–Wallis test. Significance is declared at a probability (P) of less than 0.05.

3. Results

3.1. Effects on the growth performance parameters of piglets

As shown in Table 1, during the first 1 to 14 d, no difference was observed in ADFI, ADG, and F:G ratio ($P > 0.05$). However, compared with the NC diet group, the F:G ratio in the PC diet group decreased by 11.3% (0.22). During 15 to 28 d, the PC and 500HiZ diet groups had higher ($P < 0.01$) ADFI and ADG than the NC diet group. Even though the 500ZNP diet group had a comparable ADFI with the NC diet group, the ADG in the 500ZNP diet group increased ($P < 0.01$). The lower F:G ratio ($P < 0.01$) in the 500ZNP diet group may be the possible reason for the observed increase. During the entire trial period (1 to 28 d), the ADFI and ADG in the NC diet group were lower ($P < 0.01$) than in other diet groups. In addition, the F:G ratio in 500ZNP diet group showed a tendency to be lower than that of the NC diet group ($P = 0.09$).

3.2. Effects on the intestinal morphology and barrier function in piglets

As shown in Table 2, no significant differences were found in the villus height, crypt depth, and villus height to crypt depth ratio (VH:CD ratio) for the duodenum and jejunum of piglets among all diet groups ($P > 0.05$). Compared with the NC diet group, although different dietary forms of ZnO did not affect the ileal villus height ($P > 0.05$), a lower ileal crypt depth ($P < 0.05$) was observed both in the 500HiZ and 500ZNP diet groups. Moreover, a higher ileal VH:CD ratio ($P < 0.05$) was also observed in the 500ZNP diet group compared to the PC diet group.

Compared to the NC diet group, different dietary forms of ZnO increased ($P < 0.05$) the expression of claudin-2 in the duodenum, while it decreased ($P < 0.05$) its expression in the jejunum (Fig. 1). Moreover, compared with the PC diet group, the 500HiZ and 500ZNP diet groups showed decreased ($P < 0.05$) claudin-2 expression in the ileum, which was comparable with the NC diet group. No significant difference ($P > 0.05$) was observed in the expression of ZO-1 in the duodenum between diet groups. However, the 500ZNP diet group showed increased ($P < 0.05$) expression of ZO-1 in the jejunum compared to the NC and 500HiZ diet groups, but it was not different from the PC diet group. Both 500HiZ and 500ZNP diet groups showed an increase ($P < 0.05$) in ZO-1 expression in the ileum compared to the NC diet group, but it was not different from the PC diet group. The expression of pS2/TFF1 in the duodenum of the PC diet group was higher than in the NC and 500ZNP diet groups ($P < 0.05$), but it was comparable with the expression in the 500HiZ diet group. The ileal protein expression of pS2/TFF1 in the 500HiZ diet group tended to be higher than in the 500ZNP diet group ($P = 0.05$). No significant difference ($P > 0.05$) was observed in the expression of pS2/TFF1 in the jejunum between the diet groups.

3.3. Effects on inflammation and expression of ZnT-1 and MFN1 in the intestine of piglets

Compared with the NC diet group, the PC diet group showed decreased ($P < 0.05$) TNF-α expression in the duodenum, as well as in the jejunum (Fig. 2A and B). Both the 500HiZ and 500ZNP diet groups did not differ from the NC diet group in the expression of TNF-α in the duodenum, while its expression decreased ($P < 0.05$) in both the jejunum and ileum (Fig. 2). The PC diet group showed decreased ($P < 0.05$) IL-6 expression in the duodenum and its expression tended to decrease in the jejunum ($P = 0.09$) compared to the NC diet group. Furthermore, in the 500HiZ diet group, the IL-6 expression decreased ($P < 0.05$) in the duodenum, and the

Table 1
Effects on the growth performance of piglets.

Item	Diet groups				*P*-value
	NC	PC	500HiZ[1]	500ZNP[2]	
1 to 14 d					
ADFI, g	602 ± 37.2	700 ± 48.9	652 ± 32.6	676 ± 30.6	0.32
ADG, g	330 ± 30.1	420 ± 32.6	367 ± 23.4	377 ± 7.1	0.13
F:G ratio	1.91 ± 0.156	1.69 ± 0.082	1.80 ± 0.099	1.87 ± 0.084	0.52
15 to 28 d					
ADFI, g	1062 ± 24.9^C	1216 ± 23.7^A	1192 ± 23.3^{AB}	1132 ± 24.0^{BC}	<0.01
ADG, g	547 ± 23.3^B	638 ± 14.7^A	633 ± 24.4^A	676 ± 11.5^A	<0.01
F:G ratio	1.95 ± 0.047^A	1.91 ± 0.037^A	1.85 ± 0.057^{AB}	1.73 ± 0.036^B	<0.01
1 to 28 d					
ADFI, g	870 ± 28.8^B	1002 ± 34.2^A	978 ± 8.0^A	963 ± 16.0^A	<0.01
ADG, g	439 ± 17.9^B	529 ± 21.8^A	500 ± 17.6^A	520 ± 7.9^A	<0.01
F:G ratio	1.99 ± 0.041^a	1.90 ± 0.048^{ab}	1.94 ± 0.050^{ab}	1.83 ± 0.020^b	0.09

ADFI = average daily feed intake; ADG = average daily gain; F:G ratio = feed to gain ratio; NC = negative control; PC = positive control.
Data are shown as means ± SEM (*n* = 7 or 8). Means labeled with different uppercase letters indicate significant differences (*P* < 0.05), and means labeled with different lowercase letters indicate a trend (0.05 < *P* < 0.10).
[1] 500HiZ, 500 mg/kg porous ZnO particles in the diet.
[2] 500ZNP, 500 mg/kg of nano ZnO particles in the diet.

Table 2
Effects on the intestinal morphology of piglets.

Item	Diet groups				*P*-value
	NC	PC	500HiZ[1]	500ZNP[2]	
Duodenum					
Villus height, μm	408.3 ± 13.95	370.5 ± 24.40	360.3 ± 12.03	373.6 ± 20.15	0.31
Crypt depth, μm	154.5 ± 10.60	130.3 ± 11.04	160.7 ± 5.49	151.1 ± 10.47	0.17
VH:CD ratio	2.72 ± 0.176	2.57 ± 0.124	2.49 ± 0.142	2.41 ± 0.151	0.52
Jejunum					
Villus height, μm	342.0 ± 14.00	360.3 ± 13.46	362.4 ± 26.37	349.9 ± 12.94	0.83
Crypt depth, μm	137.2 ± 12.52	141.5 ± 12.07	154.2 ± 11.22	133.8 ± 7.95	0.59
VH:CD ratio	2.39 ± 0.149	2.65 ± 0.177	2.38 ± 0.136	2.66 ± 0.141	0.39
Ileum					
Villus height, μm	320.4 ± 15.01	336.5 ± 19.88	321.0 ± 16.78	322.5 ± 14.86	0.89
Crypt depth, μm	141.5 ± 10.14^{AB}	166.4 ± 11.79^A	134.9 ± 10.24^B	119.7 ± 6.07^B	0.02
VH:CD ratio	2.3 ± 0.107^{AB}	2.07 ± 0.138^B	2.43 ± 0.129^{AB}	2.65 ± 0.147^A	0.03

NC = negative control; PC = positive control; VH:CD ratio = villus height to crypt depth ratio.
Data are shown as means ± SEM (*n* = 6 to 8). Means labeled with different uppercase letters indicate significant difference (*P* < 0.05).
[1] 500HiZ, 500 mg/kg porous ZnO particles in the diet.
[2] 500ZNP, 500 mg/kg nano ZnO particles in the diet.

500ZNP diet group showed a tendency to decrease (*P* = 0.09) its expression in the jejunum. In addition, the 500HiZ and 500ZNP diet groups both showed decreased (*P* < 0.05) IL-6 expression in the ileum. The protein expression of IL-1β of the PC diet group in the duodenum, jejunum, and ileum did not differ from that in the NC diet group. However, the 500HiZ and 500ZNP diet groups both showed decreased (*P* < 0.05) expression of IL-1β in the jejunum and showed a tendency of decreased expression (*P* = 0.05) in the duodenum when compared to the NC diet group. No significant difference (*P* > 0.05) was found in the expression of IL-1β in the ileum between the diet groups (Fig. 2C).

Compared with other diet groups, in the PC diet group, the expression of ZnT-1 decreased in the duodenum (*P* < 0.05, Fig. 3A), while it increased (*P* < 0.05) in both the jejunum (Fig. 3B) and ileum (Fig. 3C). Compared with the NC diet group, the 500HiZ diet group showed increased ZnT-1 expression in the jejunum (*P* < 0.05, Fig. 3B). Although the expression level of ZnT-1 in the duodenum and jejunum was comparable between the 500ZNP diet group and the NC diet group, the 500ZNP diet group showed decreased ZnT-1 expression in the ileum (*P* < 0.05, Fig. 3C). The MFN1 expression in the duodenum of the 500HiZ and 500ZNP diet groups tended to be higher than that of the PC diet group (*P* = 0.05, Fig. 4A). Moreover, compared with the NC and PC diet groups, the 500HiZ diet group showed increased MFN1 expression in the jejunum (*P* < 0.05,

Fig. 4B). In addition, the jejunal expressions of MFN1 in the PC and 500ZNP diet groups were lower than in the NC diet group (*P* < 0.05). No significant difference in the expression of MFN1 in the ileum was observed between diet groups (*P* > 0.05, Fig. 4C).

3.4. The ileal and colonic microbial α- and β-diversity levels of piglets

A total of 26,447 and 28,448 sequences as well as 1241 and 1036 OTUs were obtained from 16S rDNA sequencing in the ileal and colonic digesta of piglets, respectively. After clustering analysis of the total reads based on sequences with an identity ≥97%, the average valid numbers of OTUs (reads ≥ 1) of ileal microbes obtained in the NC, PC, 500HiZ, and 500ZNP diet groups were 365, 405, 339, and 456, respectively. The average numbers of OTUs of the colonic microbes were 477, 541, 589, and 626, respectively. According to the Venn plot, among the diet groups, 380 OTUs are shared in the ileum (Fig. S1) and 567 OTUs are shared in the colon (Fig. S2). Furthermore, there are 36, 55, 179, and 39 unique sequences in the ileum and 12, 25, 20, and 23 unique sequences in the colon for the NC, PC, 500HiZ, and 500ZNP diet groups, respectively. Although a difference was found between the diet groups in the Goods coverage index of the microbes in the ileum (*P* < 0.05, Fig. S1C), type "S" variation of the samples in the rarefaction curve
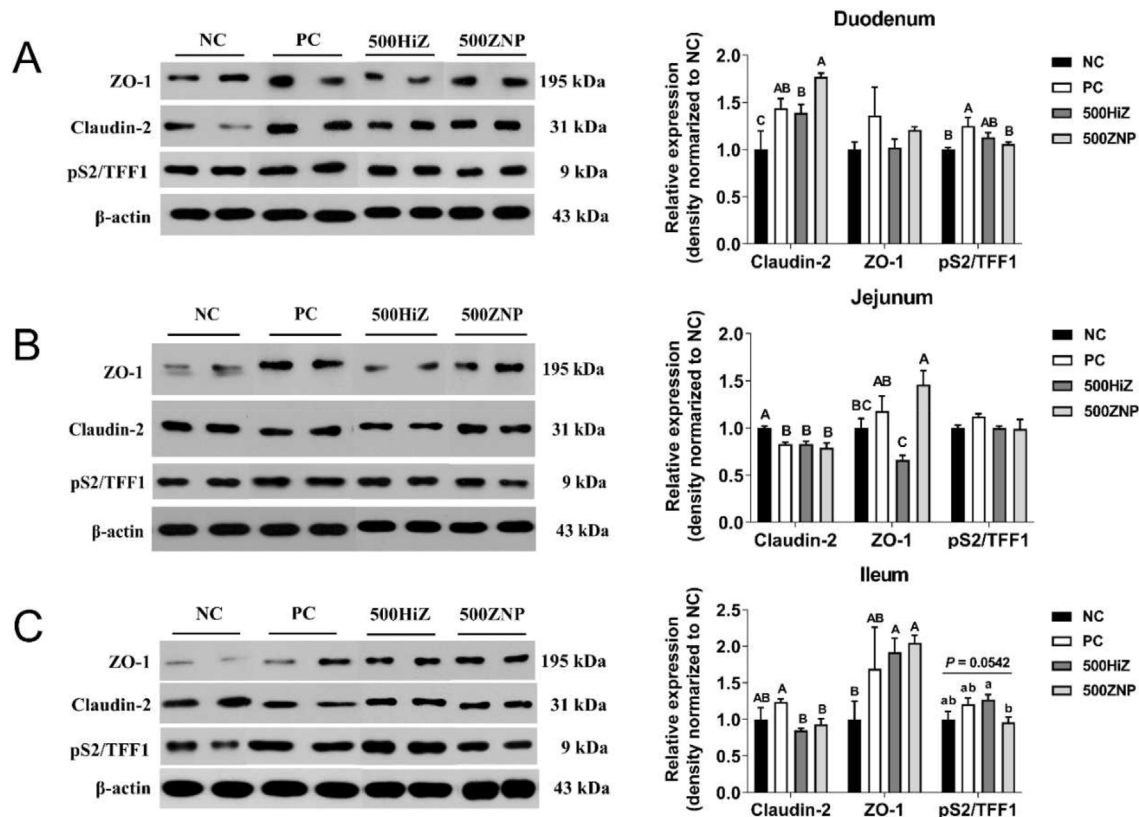
Fig. 1. Effects on the protein expression of claudin-2, ZO-1, and pS2/TFF1 in the duodenum (A), jejunum (B), and ileum (C) of piglets. Data are shown as means ± SEM (n = 4 to 5). [A, B, C]Different uppercase letters on the bars indicate significant differences ($P < 0.05$). [a, b]Different lowercase letters indicate a trend ($0.05 < P < 0.10$). NC, negative control; PC, positive control; 500HiZ, 500 mg/kg porous ZnO particles in the diet; 500ZNP, 500 mg/kg nano ZnO particles in the diet. pS2/TFF1 = trefoil factor family 1; ZO-1 = zonula occludens-1.

indicates that the sequencing depth is sufficient to reflect the microbial diversity in the ileum (Fig. S1A) and colon (Fig. S2A). The non-overlapping rank abundance curve between samples in the ileum (Fig. S1B) and colon (Fig. S2B) suggests that different dietary forms of ZnO may modulate the composition and diversity of microbes.

Regarding microbial α-diversity, in the ileum of piglets, the chao1 index and observed species index were lower in the 500HiZ diet group than in other diet groups ($P < 0.05$, Fig. 5). Moreover, the Simpson index was higher ($P < 0.05$) in the PC diet group than in other diet groups. In the colon of piglets (Fig. 6), compared with the NC diet group, both the 500HiZ and 500ZNP diet groups had increased ($P < 0.05$) observed species index and Shannon index in the colon and the levels were comparable to the PC diet group. In addition, the colonic Simpson index tended to be higher ($P = 0.09$) in the 500HiZ diet group than in the NC diet group. For the microbial β-diversity in the ileum, Fig. S3A shows a separation between the H1 (NC), H2 (PC), and H4 (500ZNP). However, no significant difference was found in the projected distance of samples between the diet groups (Fig. S3B). The microbial β-diversity index in the H2 (PC), H3 (500HiZ), and H4 (500ZNP) groups was higher ($P < 0.05$) than in H1 (NC), and that in H3 (500HiZ) was higher ($P < 0.05$) than in H2 (PC) (Fig. S4). In the colon, a clear separation was found between NC (J1), PC (J2), 500HiZ (J3), and 500ZNP (J4) (Fig. S5A). In addition, the sample projected distance in 500ZNP (J4) was higher than in the other diet groups ($P < 0.05$, Fig. S5B). The colonic β-diversity index was higher ($P < 0.05$) in 500HiZ (J3) than in NC (J1) and 500ZNP (J4), and tended to be higher than in PC (J2) ($P = 0.06$, Fig. S6).

3.5. Ileal and colonic microbial composition in piglets

The microbial composition in the ileum (Fig. S7, top 10) and colon (Fig. S8, top 20) of piglets only show the phylum and genus levels. Regarding the composition of microbes in the ileum, at the phylum level (Fig. 7A), the relative abundance of TM7 was higher ($P < 0.05$) in the NC diet group than in other diet groups. The relative abundance of Synergistetes in the NC diet group tended to be higher than in other diet groups ($P = 0.05$). Compared with the NC diet group, the relative amount of Deferribacteres in the PC and 500ZNP diet groups increased ($P < 0.05$). At the genus level (Fig. 7B), the relative amounts of bacterial genera *Klebsiella*, *Fusobacterium*, *Shewanella*, and *Serratia* in the NC diet group were higher than in other diet groups ($P < 0.05$). In addition, the relative amounts of bacterial genera *CF231* ($P = 0.08$) and *Prevotella* ($P = 0.09$) in the PC and 500ZNP diet groups trended to be higher than in the NC diet group.

Regarding the composition of microbes in the colon, at the phylum level (Fig. 8), compared with other diet groups, the relative abundance of the bacterial phylum Actinobacteria in the NC diet group increased ($P < 0.05$) while that of Bacteroidetes decreased ($P < 0.05$). The 500ZNP diet group had a higher amount of Bacteroidetes than the 500HiZ diet group ($P < 0.05$). Compared with the NC and PC diet groups, the 500HiZ and 500ZNP diet groups both showed increases of the bacteria Fibrobacteres and Spirochaetes ($P < 0.05$), whereas Proteobacteria decreased ($P < 0.05$). Compared with the NC diet group, the PC and 500ZNP diet groups tended to have an increased Cyanobacteria amount ($P = 0.05$). At the genus level (Fig. 9), compared with the NC and PC diet groups, the 500HiZ
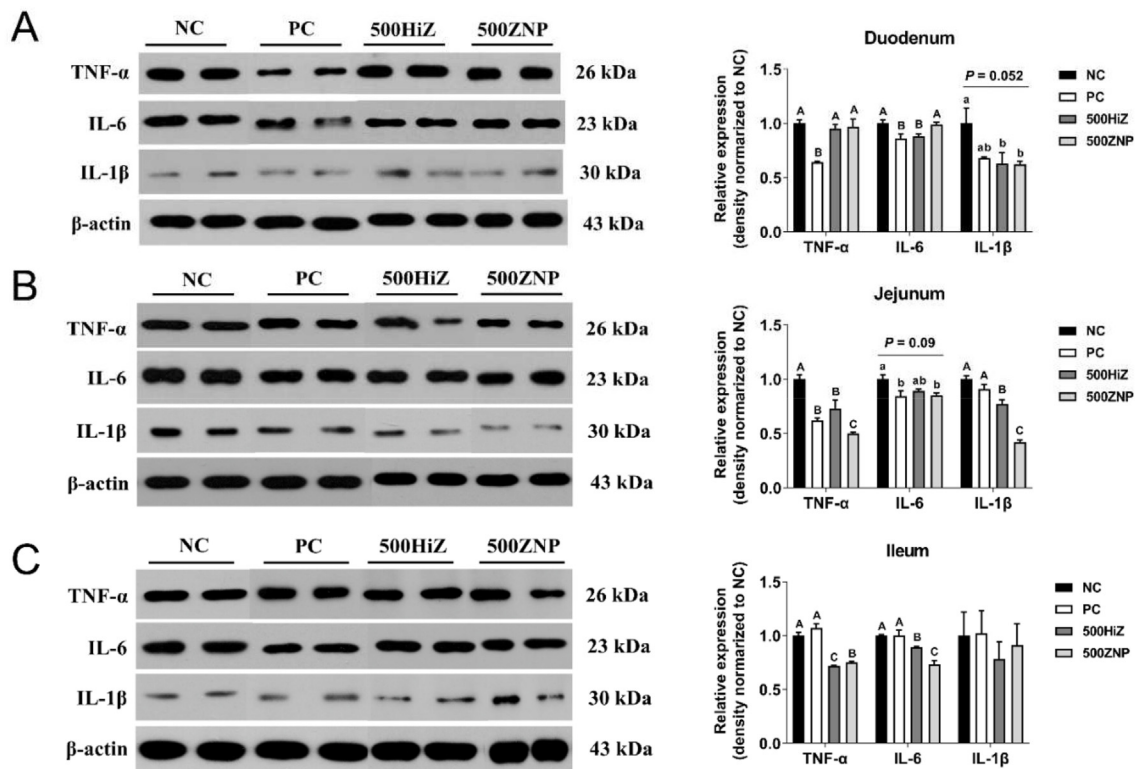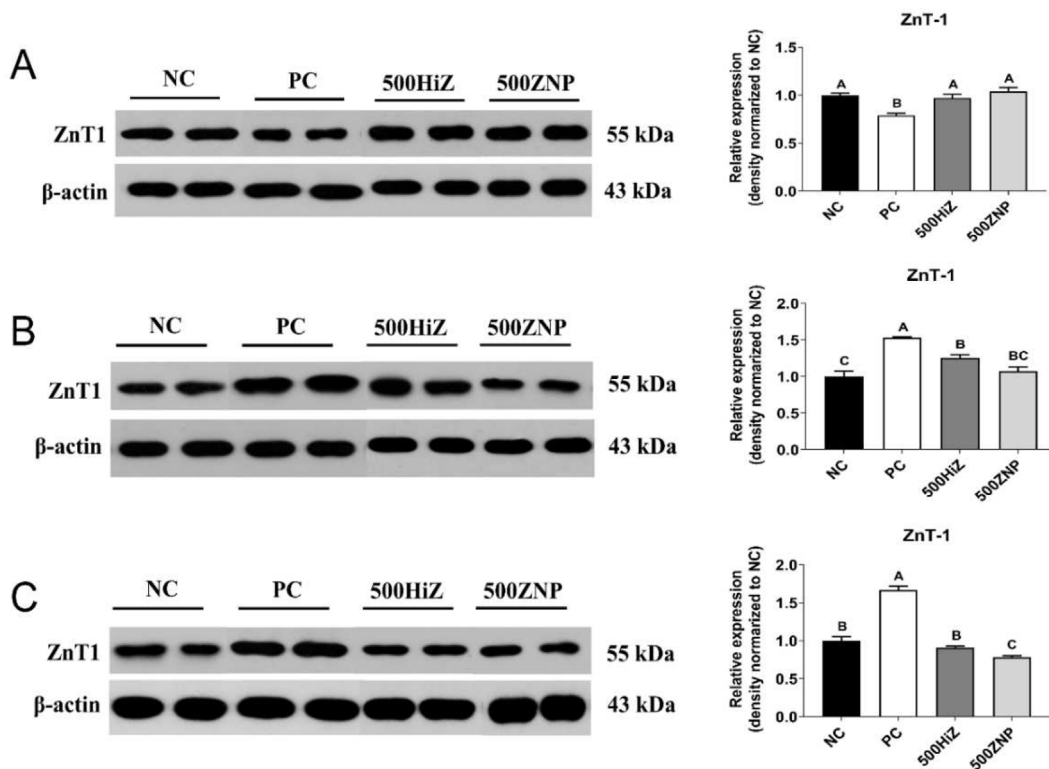
Fig. 2. Effects on the protein expression of TNF-α, IL-6, and IL-1β in the duodenum (A), jejunum (B), and ileum (C) of piglets. Data are shown as means ± SEM (n = 4 to 5). [A, B, C]Different uppercase letters on the bars indicate significant differences ($P < 0.05$). [a, b]Different lowercase letters indicate a trend ($0.05 < P < 0.10$). NC, negative control; PC, positive control; 500HiZ, 500 mg/kg porous ZnO particles in the diet; 500ZNP, 500 mg/kg nano ZnO particles in the diet. IL-1β = interleukin-1β; IL-6 = interleukin-6; TNF-α = tumor necrosis factor-α.

Fig. 3. Effects on the protein expression of ZnT-1 in the duodenum (A), jejunum (B), and ileum (C) of piglets. Data are shown as means ± SEM (n = 4 to 5). [A, B, C]Different uppercase letters on the bars indicate significant differences ($P < 0.05$). NC, negative control; PC, positive control; 500HiZ, 500 mg/kg porous ZnO particles in the diet; 500ZNP, 500 mg/kg nano ZnO particles in the diet. ZnT-1 = zinc transporter-1.
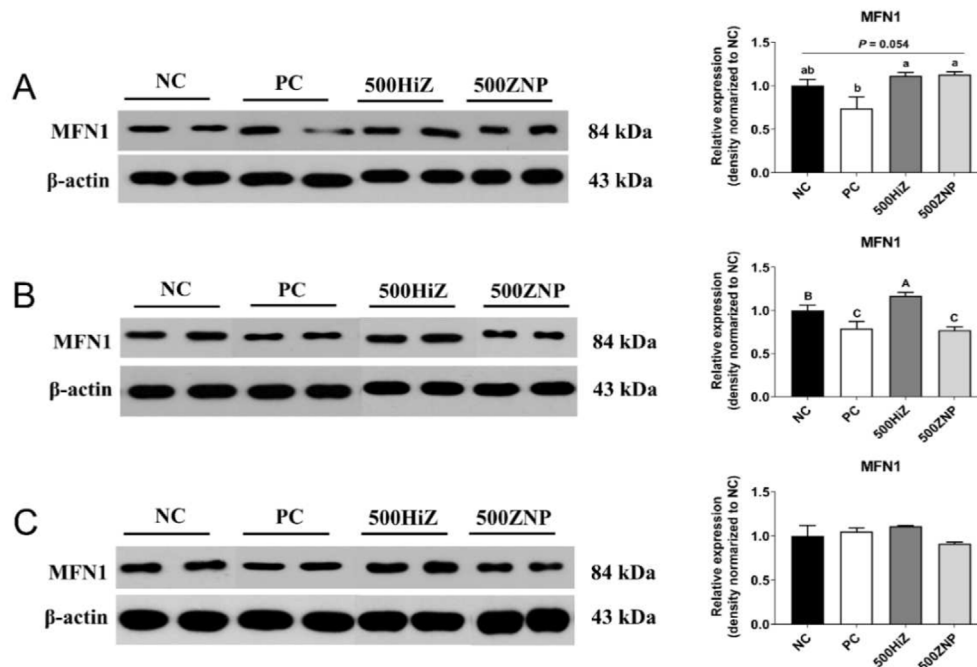
Fig. 4. Effects on the protein expression of MFN1 in the duodenum (A), jejunum (B), and ileum (C) of piglets. Data are shown as means ± SEM ($n = 4$ to 6). [A, B, C]Different uppercase letters on the bars indicate significant differences ($P < 0.05$). [a, b]Different lowercase letters indicate a trend ($0.05 < P < 0.10$). NC, negative control; PC, positive control; 500HiZ, 500 mg/kg porous ZnO particles in the diet; 500ZNP, 500 mg/kg nano ZnO particles in the diet. MFN1 = mitochondrial fusion protein 1.
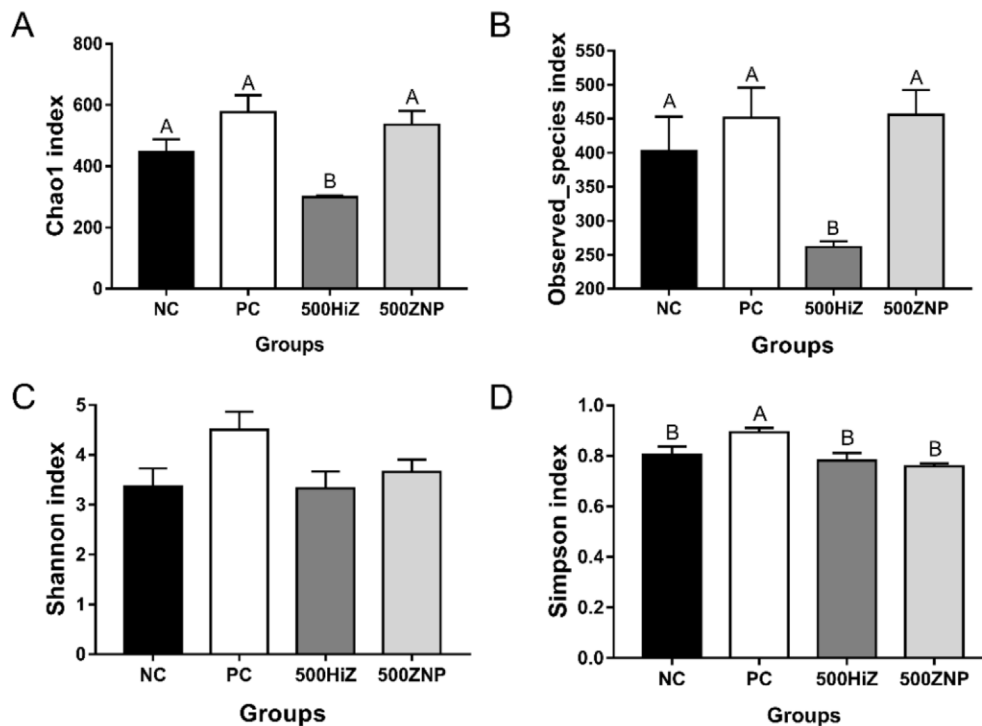

Fig. 5. Effects on the ileal microbial α-diversity indexes in the ileum of piglets. (A) Chao1 index, (B) Observed species index, (C) Shannon index, and (D) Simpson index of microbes. Data are shown as means ± SEM ($n = 4$). [A, B]Different uppercase letters on the bars indicate significant differences ($P < 0.05$). NC, negative control; PC, positive control; 500HiZ, 500 mg/kg porous ZnO particles in the diet; 500ZNP, 500 mg/kg nano ZnO particles in the diet.

and 500ZNP diet groups showed decreased ($P < 0.05$) *Actinobacillus* and *Lactobacillus* amounts. The abundances of the bacterial genera of *Bifidobacterium*, *Dialister*, *Escherichia*, and *Klebsiella* in the NC diet group were higher than in other diet groups ($P < 0.05$). Additionally, *Roseburia* in the NC diet group tended to be lower than in other diet groups ($P = 0.07$). The amounts of bacterial genera *CF231* ($P = 0.07$) and *Sphaerochaeta* ($P = 0.05$) in the 500ZNP diet group tended to be higher than in other diet groups. Moreover, the amount of bacterium *Bacteroides* in the 500ZNP diet group tended to be higher ($P = 0.09$) than in the 500HiZ diet group.
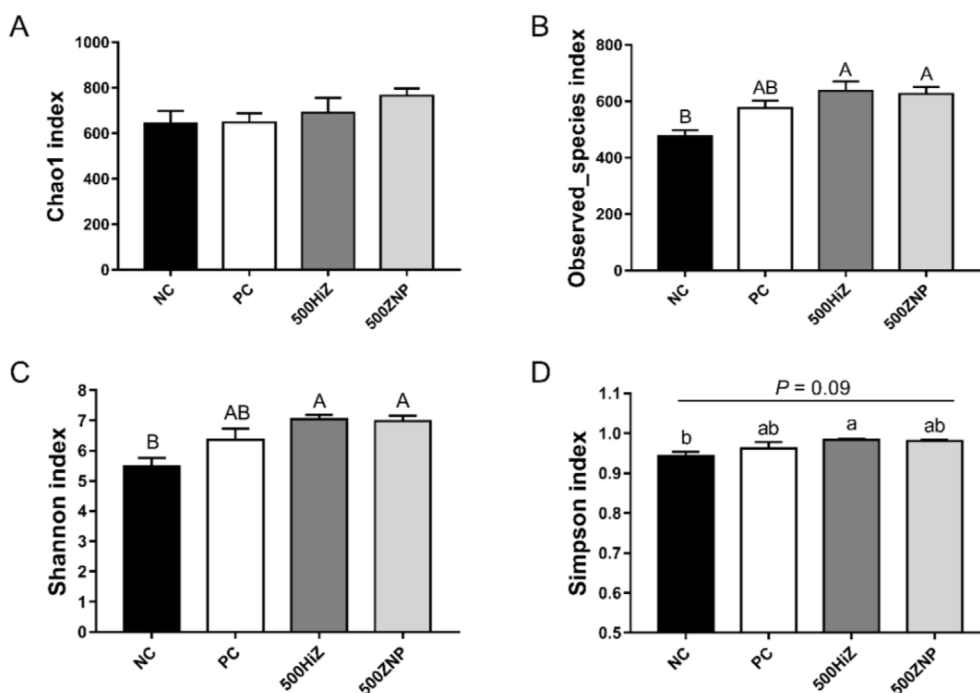
Fig. 6. Effects on the colonic microbial α-diversity indices in the colon of piglets. (A) Chao1 index, (B) Observed species index, (C) Shannon index, and (D) Simpson index of microbes. Data are shown as means ± SEM ($n = 4$). [A, B]Different uppercase letters on the bars indicate significant differences ($P < 0.05$). [a, b]Different lowercase letters indicate a trend ($0.05 < P < 0.10$). NC, negative control; PC, positive control; 500HiZ, 500 mg/kg porous ZnO particles in the diet; 500ZNP, 500 mg/kg nano ZnO particles in the diet.

3.6. Correlation between microbial biomarkers and performance parameters of piglets

Because of the significant difference observed in the microbial composition between diet groups, microbial biomarkers in the ileum (Fig. S9) and colon (Fig. S10) of piglets were examined by LEfSe analysis. In the ileum of piglets, at the phylum level, TM7 in NC (H1) was the only microbial biomarker observed. At the genus level, *Klebsiella*, *Asticcacaulis*, *Finegoldia*, *Paraprevotella*, *TG5*, *Serratia*, and *Suttonella* were biomarkers in NC (H1). In addition, *Gemella* and *Staphylococcus* were microbial biomarkers in PC (H2), and *YRC22* and *Weissella* were microbial biomarkers in 500ZNP (H4). In the colon of piglets, at the phylum level, bacteria Actinobacteria (NC, J1), Fibrobacteres (500HiZ, J3), Spirochaetes and Bacteroidetes (500ZNP, J4) were observed. At the genus level, *Parabacteroides*, *Lactobacillus*, *Dialister*, *Bifidobacterium*, *Klebsiella*, and *Escherichia* were the microbial biomarkers found in NC (J1). *Collinsella*, *Dorea*, and *Actinobacillus* were the microbial biomarkers found in PC (J2). In addition, the microbial biomarkers observed in 500HiZ (J3) were *Blautia*, *Fibrobacter*, *vadinCA11*, and *Treponema*. In 500ZNP (J4), the observed microbial biomarkers included the genera *Butyricicoccus*, *Coprococcus*, and *Sphaerochaeta*.

Pearson correlation analysis was conducted to assess the associations between microbial biomarkers and performance parameters of piglets. As shown in Fig. S11, a significantly positive correlation was found between ADG (1 to 28 d) and genus *Staphylococcus* in the ileum and a significantly negative correlation was found between ADFI (15 to 28 d) and genus *Weissella* in the ileum. In the colon, a significantly negative correlation was observed between the ileal VH:CD ratio and phylum Actinobacteria (Fig. S12). As shown in Fig. 10, a positive ($P < 0.05$) correlation was found between the ADFI (1 to 28 d) and genus *Dorea*. In addition, a positive correlation ($P < 0.05$) was observed between F:G ratio (1 to 28 d) and genera *Coprococcus*, *Collinsella*, and *Blautia*. The F:G ratio (1 to 28 d) was negatively ($P < 0.05$) correlated with the genus

Escherichia. The F:G ratio (15 to 28 d) was also found to be positively ($P < 0.05$) correlated with the genus *Blautia* while negatively ($P < 0.05$) correlated with the genus *Escherichia*. The genera *Dorea*, *Blautia*, and *vadinCA11* were positively ($P < 0.05$) correlated with VH:CD ratio in the ileum of piglets.

4. Discussion

The present study aimed to determine the efficacy of the supplementation of 500 mg/kg porous and nano ZnO particles in the diet of piglets compared to the dietary supplementation with high dose of conventional ZnO (3,000 mg/kg). The growth performance, intestinal morphology, inflammation, and gut microbiota of piglets were evaluated. This study showed that dietary porous and nano particles of ZnO improved the growth performance of piglets during the complete experimental period, which was in accordance with previous literatures (Long et al., 2017; Peng et al., 2020). The porous and nano particles are polymers, which have small size (less 100 nm) and large surface area, thus high absorption rate (Croteau et al., 2010; Wang et al., 2012). Wang et al. (2017) showed that 1,200 mg/kg dietary nano particles ZnO improved the growth performance of weaned piglets, and the content of serum Zn in piglets fed with nano ZnO particles was comparable to that of piglets fed with a high dose of conventional ZnO (3,000 mg/kg). This may be attributed to the higher absorption rate of nano ZnO particles in the gastrointestinal tract of piglets. Peng et al. (2020) reported that porous ZnO particles in the diet increased ADG (750 or 1,500 mg/kg) and decreased F:G ratio (1,500 mg/kg) in piglets. However, the results of the present study demonstrated that the F:G ratio of piglets unaffected by dietary supplementation of 500 mg/kg porous ZnO particles. This inconsistent result may be attributed to the different doses of porous ZnO particles in the piglet diet between studies.

Post-weaning stress induces serious diarrhea or bowel diseases in piglets (Huting et al., 2021; Vente-Spreeuwenberg et al., 2004).
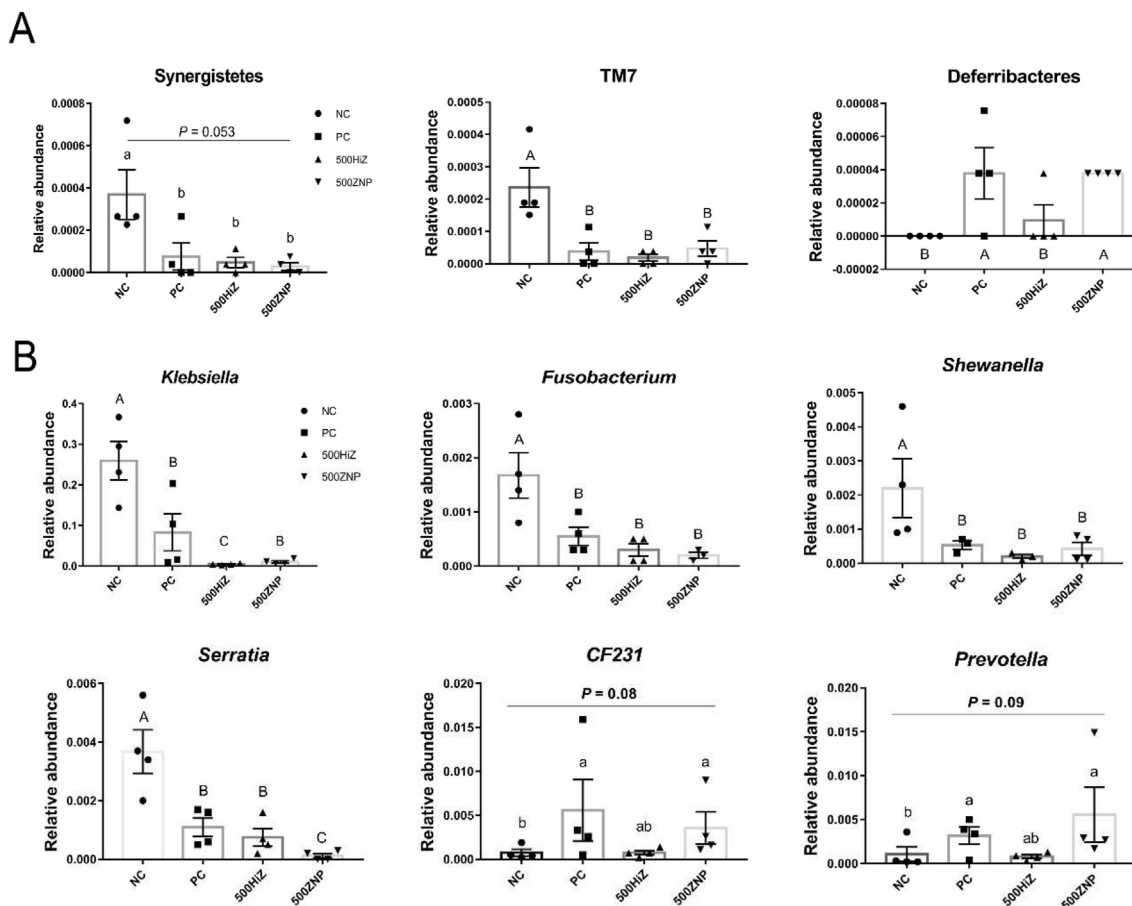
Fig. 7. Effects on the relative abundances of microbes at the phylum (A) and genus (B) levels in the ileum. Data are shown as means ± SEM ($n = 4$). [A, B, C]Different uppercase letters on the bars indicate significant differences ($P < 0.05$). [a, b]Different lowercase letters indicate a trend ($0.05 < P < 0.10$). NC, negative control; PC, positive control; 500HiZ, 500 mg/kg porous ZnO particles in the diet; 500ZNP, 500 mg/kg nano ZnO particles in the diet.

ZnO in the diet is not only beneficial to the growth performance and the diarrhea prevention of piglets, but also improves gut health (Hu et al., 2014). It has been reported that dietary supplementation of porous particles ZnO increased the jejunal villus length (750 mg/kg) and the crypt depth (500 mg/kg) of piglets (Long et al., 2017; Peng et al., 2020). In the present study, however, dietary supplementation of porous and nano particles of ZnO merely decreased crypt depth in the ileum of piglets. In addition, in the studies mentioned above, a high dose of dietary ZnO (3,000 mg/kg) improved morphology of the intestine, which was not observed in the present study. These paradoxical results suggest that differences in dietary dosage and between piglets themselves may be the primary reasons. The intestine plays a critical role in digestion and absorption, and the integrated barrier function of intestine is necessary to counter harmful or toxic substances originating from the lumen (Satheeshkumar et al., 2010; Tanaka et al., 2014). In the present study, 500 mg/kg of porous or nano ZnO particles in the diet increased the expression of claudin-2 and ZO-1 in both the duodenum and ileum. Moreover, nano ZnO particles also upregulated ZO-1 expression in the jejunum. This suggests that dietary supplementation of porous and nano particles of ZnO improves the intestine barrier function of piglet. Similarly, dietary supplementation of nano ZnO particles (1,200 mg/kg) increased ZO-1 expression in the ileum, while dietary supplementation of porous ZnO particles (750 or 1,500 mg/kg) increased it in the jejunum (Peng et al., 2020; Wang et al., 2017). These results suggest that either the form or the dose of dietary ZnO affects the intestine

barrier function of piglets. Piglets that were weaned early commonly suffer from intestinal inflammation because of the immaturity of immune and digestive functions (McCracken et al., 1999; Schulte et al., 2016). In the present study, 500 mg/kg porous and nano particles of ZnO in the diet decreased intestinal proinflammatory cytokines (IL-6, IL-1β, and TNF-α), implying that such ZnO supplementation can improve intestinal inflammation in piglets. Consistently, porous ZnO (750 and 1,500 mg/kg) in the diet improves intestinal inflammation by decreasing the expression of IL-8 and increasing the expression of TGF-β in the jejunum of piglets (Peng et al., 2020). Taken together, 500 mg/kg dietary porous ZnO particles is not only beneficial to alleviate inflammation, but also improves the barrier function of the intestine of piglets.

In the body, ZnO ultimately acts as a zinc ion (Zn^+). Zinc transporters are mainly responsible for the transferal of Zn^+ to the cytoplasm by promoting Zn uptake into the cell or its excretion out of the cell (Brugger et al., 2021). Once physiological conditions change, the specific zinc transporter is modulated to maintain zinc homeostasis in the body. ZnT-1 is thought to be one of the active carrier proteins for the transfer of Zn into the circulation (McMahon and Cousins, 1998), which is primarily expressed in the enterocytes of jejunum (Brugger et al., 2021). In the present study, dietary supplementation of 500 mg/kg porous ZnO particles in the piglet diet increased the expression of jejunal ZnT-1, while 500 mg/kg dietary porous ZnO particles did not. This suggests that porous ZnO particles may be easier than nano ZnO particles to translocate to the
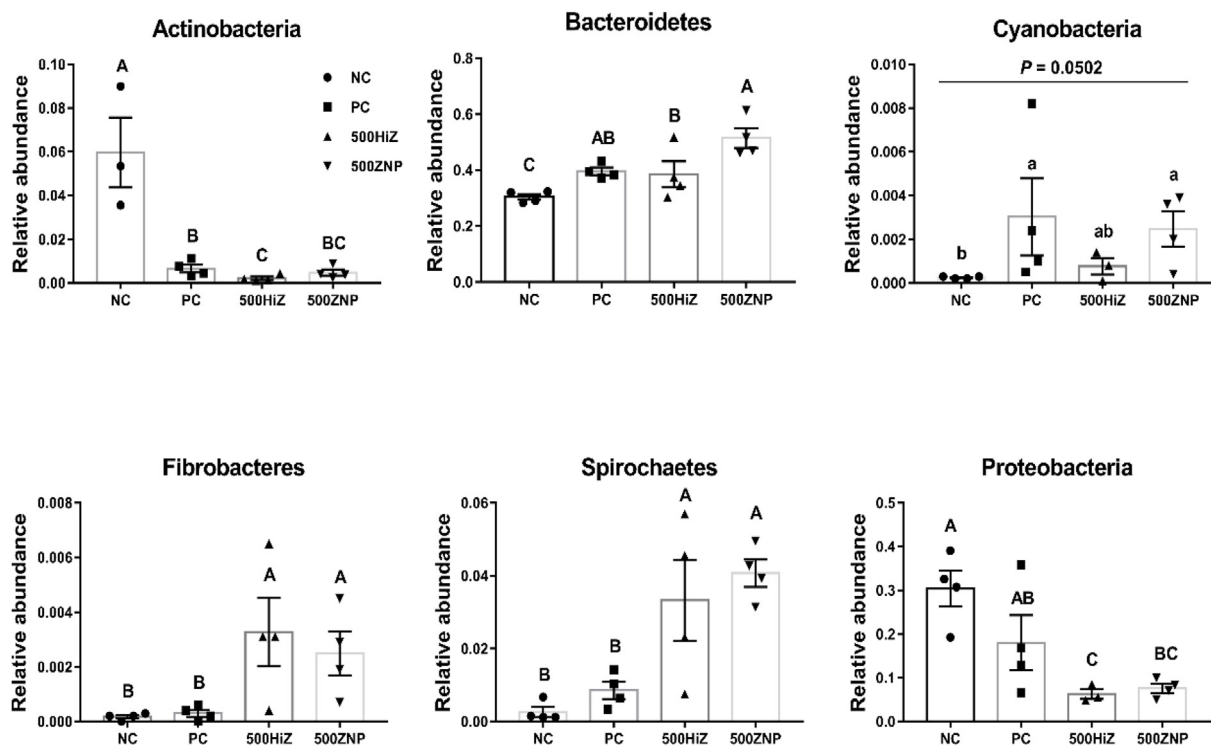
Fig. 8. Effects on the relative abundances of microbes (phylum) in the colon of piglets. Data are shown as means ± SEM (*n* = 4). [A, B, C]Different uppercase letters on the bars indicate significant differences (*P* < 0.05). [a, b]Different lowercase letters indicate a trend (0.05 < *P* < 0.10). NC, negative control; PC, positive control; 500HiZ, 500 mg/kg porous ZnO particles in the diet; 500ZNP, 500 mg/kg nano ZnO particles in the diet.

intestinal epithelium. Zinc is potentially highly toxic when its dosage exceeds toxicity thresholds of a specific tissue or organ (Piao et al., 2003). Notably, a high dose of conventional ZnO (PC diet group) decreased the expression of ZnT-1 in the duodenum, which may be closely related to zinc toxicity. However, this requires further research in the future. Intracellular mitochondria are dynamic organelles that continuously undergo fission and fusion, and the disruption of mitochondrial dynamics causes metabolic disorders and can even cause cancer. MFN1 is one of two mitochondrial fusion proteins in mammals and participates in the fusion of the outer mitochondrial membrane (Gao and Hu, 2021). In the present study, a high dose of dietary ZnO (3,000 mg/kg) was found to reduce MFN1 expression in the duodenum as well as in the jejunum. It has been reported previously that MFN1 is primarily responsible for the docking of the outer membrane in mitochondria (Cipolat et al., 2004). As a result, a high dose of dietary ZnO (e.g., 3,000 mg/kg) may obstruct the fusion of the outer mitochondrial membrane, thus disturbing the normal mitochondrial function. However, additional research is needed to verify this hypothesis. It should be noted that the underlying mechanism of mitochondrial fusion remains unknown. How porous or nano particles of ZnO in the diet influence the mitochondrial function in the jejunum of piglets is an intriguing question.

The gut is home to trillions of commensal microorganisms, which have numerous effects on their hosts (Christian et al., 2017; Tanaka et al., 2014). In addition, the composition and amounts of gut microbes are susceptible to modulation by dietary nutrients (Huting et al., 2021). In the present study, porous ZnO particles in the piglet diet increased microbial β-diversity index in both the ileum and colon. Moreover, it decreased microbial α-diversity in the ileum but increased it in the colon. This result suggests that the dietary supplementation of porous ZnO particles has altered the gut microflora of piglets. Similarly, high doses of dietary ZnO have

previously been reported to affect the diversity of gut microbes (Yu et al., 2017). In addition, Peng et al. (2019) reported that porous ZnO particles in the diet (500 mg/kg) increased the populations of *Lactobacillus* spp. in both the ileum and cecum. However, the results of the present study suggest that porous ZnO particles in the diet decreases the relative abundance of *Lactobacillus* in the colon, which is inconsistent with previous results. In the present study, supplementation with porous and nano ZnO particles in the diet (both at 500 mg/kg) decreased the relative abundances of phylum TM7 and genera *Klebsiella*, *Fusobacterium*, *Shewanella*, and *Serratia* in the ileum of piglets. Inversely, supplementation with conventional ZnO in the diet (at 3,000 mg/kg) increased the relative abundance of phylum TM7 in the ileum (Yu et al., 2017). It has been reported previously that phylum TM7 can induce colitis via the inflammasome pathways (Chow et al., 2011). This induction of colitis suggests that porous or nano ZnO particles in the diet (500 mg/kg) may contribute to alleviating intestinal inflammation by decreasing the abundance of phylum TM7 in the ileum (Fig. 2C). Dietary components, particularly dietary protein, not only influence the gut microflora but also Zn absorption (Adams et al., 2018; Singh et al., 2017). It should be noted that in the present study, a higher dietary protein level (22.84%) was found than that previously reported (19.20%) by Yu et al. (2017). The resulting inconsistent findings can be attributed to differences in dietary protein level. Until now, the associations between dietary nutrients and gut microflora remain undefined. In the present study, a notably positive correlation was observed between ADG (1 to 28 d) and the genus *Staphylococcus* in the ileum (PC diet). Most species of the genus *Staphylococcus* are involved in carbohydrate metabolism (Götz et al., 2006). Hence, increasing the abundance of the genus *Staphylococcus* may be beneficial to providing piglets with additional energy substrates to promote their growth. A notably positive correlation between ADFI (1 to 28 d) and the *Dorea* genus (PC
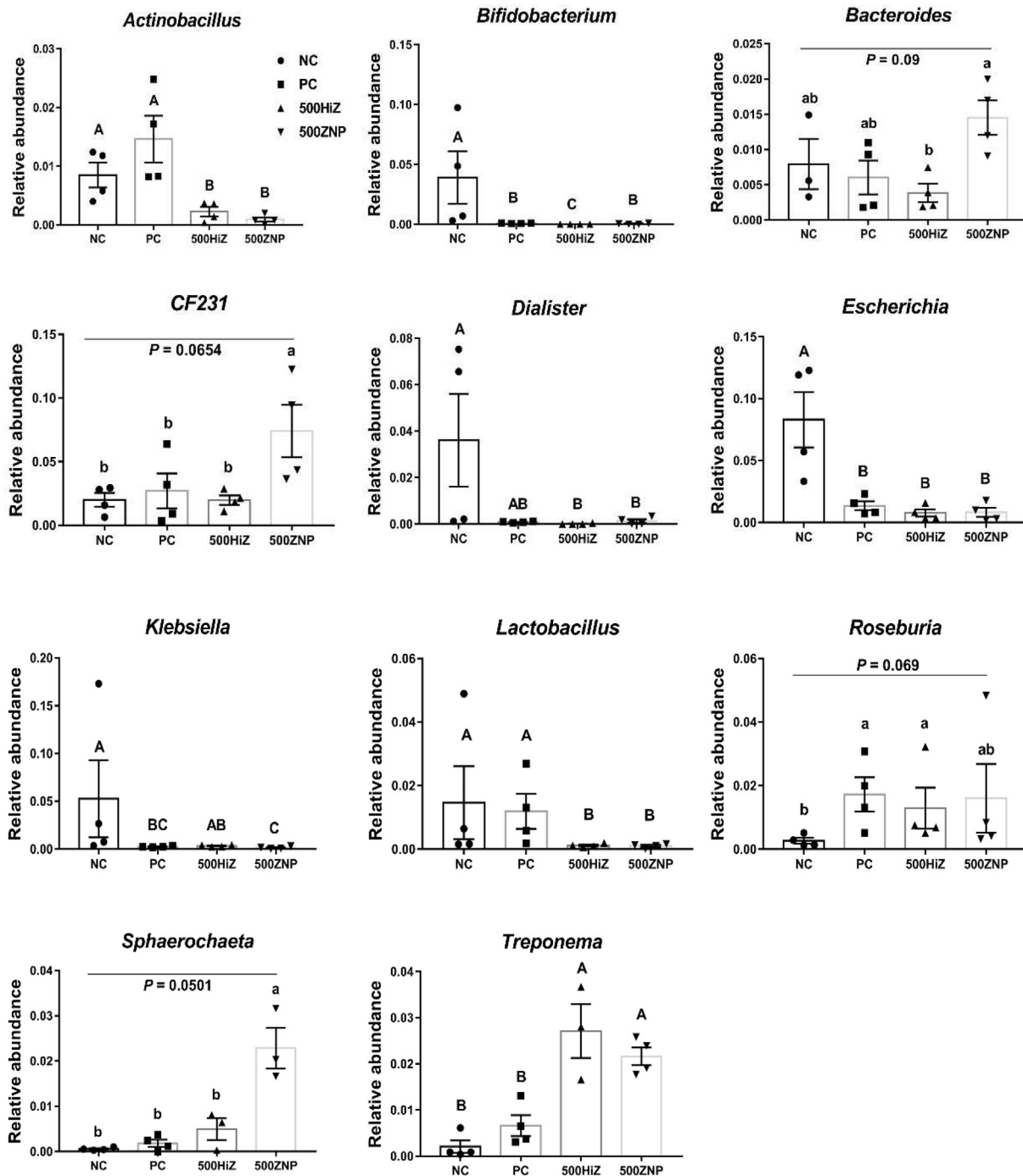
Fig. 9. Effects on the relative abundances of microbes (genus) in the colon. Data are shown as means ± SEM ($n = 4$). [A, B, C]Different uppercase letters on the bars indicate significant differences ($P < 0.05$). [a, b]Different lowercase letters indicate a trend ($0.05 < P < 0.10$). NC, negative control; PC, positive control; 500HiZ, 500 mg/kg porous ZnO particles in the diet; 500ZNP, 500 mg/kg nano ZnO particles in the diet.

diet) in the colon was observed, and the F:G ratio (1 to 28 d) was positively correlated with the genera *Coprococcus* (500ZNP diet), *Collinsella* (PC diet), and *Blautia* (500HiZ diet) in the colon of piglets. The genus *Dorea*, which is efficient in preventing non-alcoholic fatty liver disease (Juárez-Fernández et al., 2023), may thus be closely related to the lipid metabolism in the liver of piglets. However, whether it contributes to the growth or intestinal health of piglets remains unclear. The genus *Collinsella* belongs to the species of phylum Actinobacteria and has the ability to produce butyrate. Moreover, the genus *Collinsella* is closely related to insulin and triglycerides in serum, and thus, may be involved in the lipid metabolism of host (Astbury et al., 2020; Gomez-Arango et al., 2018; Qin et al., 2019). The genus *Coprococcus* is one of the butyrate-producing bacteria and is closely relevant to host glucose homeostasis (Altemani et al., 2021; Guo et al., 2018). It has been reported previously that butyrate-producing bacteria contribute to preventing inflammatory bowel disease and maintaining the intestinal barrier function (Bach Knudsen et al., 2018; Qin et al., 2019).
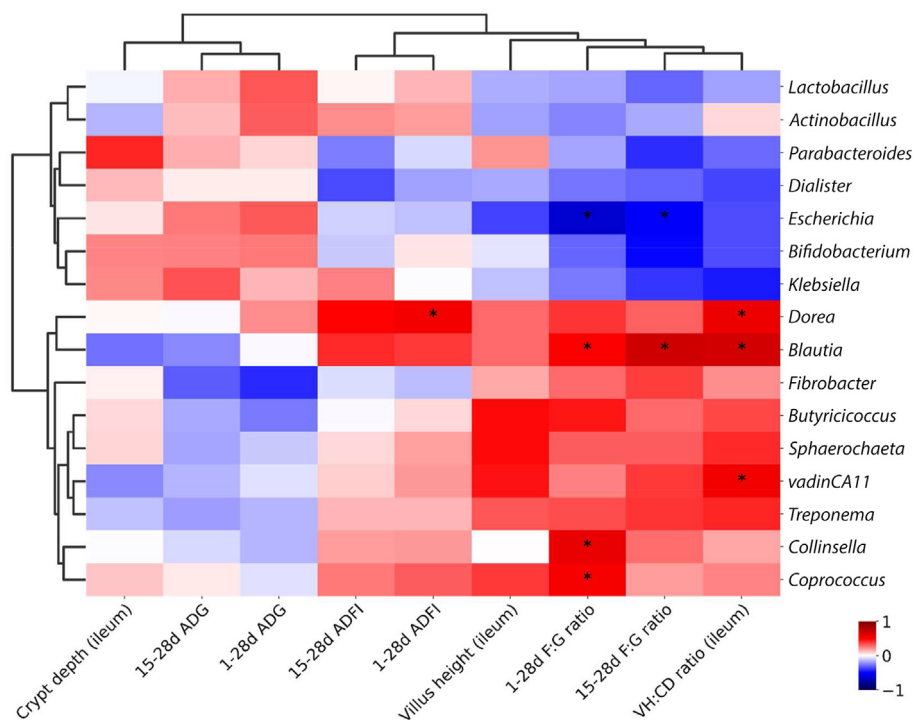
Fig. 10. Pearson correlation between the performance parameters of piglets and colonic microbial biomarkers at the genus level. ADFI = average daily feed intake; ADG = average daily gain; F:G ratio = feed to gain ratio; VH:CD ratio = villus height to crypt depth ratio. Asterisk (*) indicates significant differences ($P < 0.05$).

As a result, the improved growth performance parameters observed in the PC diet group (dietary ZnO at 3,000 mg/kg) may be closely related to the abundance of the genus *Collinsella*. The reduced F:G ratio in the 500ZNP diet group (500 mg/kg nano particles ZnO) may be closely related to the genus *Coprococcus*. Notably, the genus *Coprococcus* participates in the progress of metabolic disorders, such as inflammation and oxidative stress (Di Luccia et al., 2015). However, whether the genus *Coprococcus* adversely affects the gut health of piglets remains uncertain. The genus *Blautia*, which can produce acetic acid or butyric acid, is negatively associated with the visceral fat deposition (Liu et al., 2015, 2021; Ozato et al., 2019). Therefore, the reduced F:G ratio in the 500ZNP diet group may also be relevant to the genus *Blautia*, but this association needs to be confirmed in the future. In the present study, a negative correlation was found between the F:G ratio (1 to 28 d), colonic *Escherichia* (NC diet), VH:CD ratio, and the phylum Actinobacteria. The commensal *E. coli* (Yu et al., 2021) resides in the colonic mucosal layer and most species of this bacterial genus rarely cause disease in animals. However, there are several highly adapted *E. coli* strains that can induce diarrhea or other diseases (Gomes et al., 2016; Kaper et al., 2004). In the present study, it is uncertain whether the identified *Escherichia* species in the NC diet group belongs to the pathogenic *E. coli*. It has been reported previously that the members of the phylum Actinobacteria can produce numerous secondary metabolites, such as antibiotics, and while several species have beneficial effects on livestock, others adversely affect animals (Ranjani et al., 2016). Moreover, the phylum Actinobacteria contains 6 classes and 16 orders (Barka et al., 2016). As a result, identification of the specific *Actinobacteria* species that are negatively correlated with the ileal VH:CD ratio of piglets requires further research. Together, the present study identified several intriguing bacterial species for subsequent research, which are primarily associated with intestinal

inflammation, lipid metabolism, and glucose homeostasis in the weaned piglets.

5. Conclusion

In summary, supplementation of 500 mg/kg porous and nano particles of ZnO in the diet improves the growth performance of piglets and can achieve comparable or even better efficiency compared to that of high dose of conventional ZnO (3,000 mg/kg). In addition, porous and nano particles of ZnO in the diet improve the barrier function and inflammation level of the intestine. Moreover, the colonic bacterial genera *Coprococcus* (500ZNP) and *Blautia* (500HiZ) are positively correlated with improved piglet performance, which may contribute to these changes.

Author contributions

Lina Long: Conceptualization, Methodology, Project administration, Writing-review & editing; **Xichen Zhao:** Visualization, Formal analysis, Data curation, Writing-original draft, Writing-review & editing; **Jie Chen:** Resources, Validation; **Zixi Wang:** Investigation, Formal analysis, Validation; **Yanfang Tang:** Investigation, Validation; **Jian Huang:** Investigation, Validation; **Yulong Yin:** Conceptualization, Funding acquisition, Supervision, Writing-review & editing.

Declaration of competing 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 and/or company that could be construed as influencing the content of this paper.

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Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2023.08.011.

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