RESEARCH ARTICLE

J Anim Sci Technol 2025;67(5):1050-1066
https://doi.org/10.5187/jast.2024.e70

JAST
Journal of Animal Science and Technology
pISSN 2672-0191 eISSN 2055-0391

Dietary coated copper and zinc improved growth performance by modulating immune responses and fecal microbiota of weaned pigs

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Abstract

The objective of this study was to investigate effects of a low dietary dose of coated $CuSO_4$ and ZnO on growth performance, frequency of diarrhea, nutrient digestibility, immune responses, and fecal microbiota of weaned pigs. In a randomized complete block design (block: initial body weight), a total of 96 weaned pigs [Landrace × Yorkshire) × Duroc; 7.29 ± 0.69 kg of average initial body weight (BW)] were allotted into four dietary treatments (4 pigs/pen, 6replicates/treatment): (1) a basal weaner diet based on corn and soybean meal (CON), (2) CON supplemented with 2,500 ppm standard ZnO (T1), (3) CON supplemented with 100 mg/kg dietary coated $CuSO_4$ and 100 mg/kg dietary coated ZnO (T2), and (4) CON supplemented with 200 mg/kg dietary coated $CuSO_4$ and 200 mg/kg dietary coated ZnO (T3). Dietary T2 and T3 increased ($p < 0.05$) the average daily gain for the first two weeks and the overall experimental period compared to that with CON. In addition, the groups supplemented with $CuSO_4$ and Zno tended to have a decreased ($p < 0.10$) frequency of diarrhea. Pigs fed dietary T2 and/or T3 had lower ($p < 0.10$) the number of white blood cells on day 7 and hematocrit on day 14 compared to those fed CON. However, no difference was observed in the number of red blood cells among the dietary treatments. Regarding immune responses, dietary T2 decreased ($p < 0.10$) serum tumor necrosis factor-α level on day 7 and increased ($p < 0.10$) immunoglobulin G level on day 14 compared with CON. Moreover, pigs fed dietary T2 tended to have increase bacterial abundance of *Limosilacatobacilus* ($p < 0.10$) compared with those fed dietary T1. Dietary T3 had higher ($p < 0.05$) relative abundance of the genus *Agathobacter* compared to those fed CON and dietary T1 and decreased ($p < 0.05$) genus *Terrisporobacter* compared to those fed dietary T1. These results suggested the supplementation of dietary coated ZnO and $CuSO_4$ enhanced growth performance and modulated immune responses associated with changes in the fecal microbiota composition.

Keywords: Copper, Fecal Microbiota, Growth performance, Immune response, Weaned pig, Zinc

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Received: Jun 4, 2024
Revised: Jul 4, 2024
Accepted: Jul 4, 2024

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Competing interests
No potential conflict of interest relevant to this article was reported.

Funding sources
This work was supported by research fund of Chungnam National University.

Acknowledgements
Not applicable.

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

Authors' contributions
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Writing - review & editing: Nam J, Cho JH, Kim HB, Ahn J, Kang Y, Kim S, Kim JT, Jung SW, Song M, Kyoung H.

Ethics approval and consent to participate
The experimental protocol for this study was reviewed and approved by the Institutional Animal Care and Use Committee of Chungnam National University, Daejeon, Korea (approval# 202103A-CNU-080).

INTRODUCTION

Post-weaning diarrhea (PWD) is a potentially fatal disease in swine production worldwide, causing dehydration, growth delay, and death in severe cases causes of negative impacts on the industry [1]. During the post-weaning period, various stress factors, including dietary, environmental, social, and physiological stressors, affect weaning pigs. These factors reduce feed intake and growth, thus resulting in intestinal dysfunction, and an increased susceptibility to inflammation [2,3]. Furthermore, disruption of the intestinal barrier increases intestinal permeability leading to PWD due to infections by intestinal pathogens such as *Escherichia coli* [4]. To solve these issues, in-feed antibiotics have been widely utilized in the livestock industry for growth promotion and disease treatment including PWD. However, the incidence of antimicrobial resistance and residual issues remain concerning for animal and public health due to the overuse of in-feed antibiotics [5]. Therefore, various nutritive alternatives such as probiotics, enzymes, minerals, and others, have been utilized to enhance animal health and performance [6].

It is known that copper (Cu) and zinc (Zn) are vital trace elements that act as components of metabolic enzymes and perform biological functions [7]. Both support immunity, reproduction, and growth of animals [8,9]. The most commercial forms in the swine industry are zinc oxide (ZnO) and copper sulfate ($CuSO_4$) because of their relatively low cost compared with other forms [9,10]. These act as alternatives to in-feed antibiotics for promoting growth and have antimicrobial effects such as reducing diarrhea incidence in weaned pigs at concentration levels exceeding normal nutritional requirements according to the National Research Council [11]. Specifically, pharmacological levels (2,000 to 4,000 mg/kg) of ZnO have been commonly supplemented in weanling diets to promote growth performance and reduce diarrhea frequency by enhancing morphological structure and maintaining gut integrity [12,13] . In addition, previous studies reported that a dose of 150 to 250 mg/kg supplementation of $CuSO_4$ stimulates growth rate and feed intake, and sustains fecal consistency via modulating gut microbiota homeostasis in the intestine [14,15].

However, supplementation of high doses of $CuSO_4$ (200 to 250 mg/kg) and ZnO (2,000 to 4,000 mg/kg) in the nursery diet can be excreted through manure without being normally absorbed into the intestine, resulting in environmental pollution [16–18]. Based on these issues, the European Union has legislated new maximum levels to limit Zn and Cu supplementation to 150 mg/kg up to 4 weeks after weaning [19,20]. Thus, new forms of Cu and Zn at lower doses have been proposed to reduce excretions and improve growth performance during the weaning phase. Lipid-coated and concentrated forms of ZnO and $CuSO_4$, protect against the formation of insoluble complexes with other minerals and dissociation in the stomach. They are dissociated by pancreatic lipase, and efficiently absorbed in the small intestine of monogastric animals [21–23]. A previous *in vitro* study presented the dissociation percentage of coated and uncoated ZnO in the stomach. The results showed that the percentages were 25.03% for coated and 85.26% in uncoated ZnO [24]. Consequently, this reduced the quantity released into the soil through the manure. Despite the growing interest in coated ZnO, there are still limited studies on dietary mixture of coated ZnO and CuSO4 in weaned pigs. Therefore, the objective of this study was to evaluate the effects of low doses of lipid-coated $CuSO_4$ and ZnO on the growth performance, diarrhea, nutrient digestibility, immune responses, and fecal microbiota of weaned pigs.

MATERIALS AND METHODS

All the experimental protocol for this study was reviewed and approved by the Institutional

Animal Care and Use Committee of Chungnam National University, Daejeon, Korea (approval# 202103A-CNU-080).

Source of tested products

The lipid-coated ZnO and $CuSO_4$ practiced in this study were provided by a commercial company (ACC, Seongnam, Korea). These products are concentrated forms of Zn and Cu from ZnO and copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) which are microencapsulated in a lipid matrix by fatty acids and hydrogenated palm oil according to the manufacturer's information.

Experimental design, animals, and diets

A total of 96 weaned piglets ([Landrace × Yorkshire] × Duroc; 7.29 ± 0.69 kg of average initial body weight BW]) were assigned to four dietary treatments (4 pigs/pen; 6 replicates/treatment) in a randomized complete block design (block = initial BW). The dietary treatments were (1) a basal weaner diet based on corn-soybean meal (CON), (2) CON supplemented with 2,500 ppm standard ZnO (T1), (3) CON supplemented with 100 mg/kg dietary coated $CuSO_4$ and 100 mg/kg dietary coated ZnO (T2), and (4) CON supplemented with 200 mg/kg dietary coated $CuSO_4$ and 200 mg/kg dietary coated ZnO (T3). The basal diet was mixed to meet or exceed the

Table 1. Composition of basal diet for weaned pigs (as-fed basis)

Item	Basal diet
Ingredient (%)	100.00
Corn	49.86
Soybean meal (44%)	25.00
Whey powder	12.50
Soy protein concentrate	6.25
Soybean oil	3.00
Limestone	1.14
Monocalcium phosphate	1.05
Vitamin premix[1)]	0.20
Mineral premix[2)]	0.20
L-Lysine·HCl	0.45
DL-Methionine	0.16
L-Threonine	0.13
L-Valine	0.06
Calculated energy and nutrient	
Metabolizable energy (kcal/kg)	3,465
Crude protein (%)	21.26
Calcium (%)	0.81
Phosphorous (%)	0.65
Lysine (%)	1.53
Methionine (%)	0.47
Threonine (%)	0.95
Tryptophan (%)	0.25

[1)]Vitamin premix provided the following quantities of vitamin per kilogram of complete diet: vitamin A, 12,000 IU; vitamin D_3, 2,500 IU; vitamin E, 30 IU; vitamin K_3, 3 mg; D-pantothenic acid, 15 mg; nicotinic acid, 40 mg; choline, 400 mg; and vitamin B_{12}, 12 µg.

[2)]Mineral premix provided the following quantities of mineral per kilogram of complete diet: Fe, 90 mg from iron sulfate; Cu, 8.8 mg from copper sulfate; Zn, 100 mg from zinc oxide; Mn, 54 mg from manganese oxide; I, 0.35 mg from potassium iodide; Se, 0.30 mg from sodium selenite.

 https://doi.org/10.5187/jast.2024.e70

nutritional requirements of the National Research Council [11] for weaned pigs (Table 1). The study was conducted for 6 weeks, and the pigs were allowed *ad libitum* access to feeders and water and were housed in the same-sized pen (2 m × 2 m) throughout the experimental period.

Data and sample collection

In each pen, pigs' BW and remaining feed were weighed on days 1, 14, and 42 to figure out the average daily gain (ADG), average daily feed intake (ADFI), and gain to feed ratio (G:F). The fecal score of the pigs was visually monitored in each pen by two independent observers during the first 2 weeks. The score ranged from 1 to 5 (1 = hard and dry feces, 2 = soft feces, 3 = moist feces, 4 = mild diarrhea, and 5 = mild and severe diarrhea) and were calculated by counting the number of pen days with a diarrhea score of 4 or higher as a percentage. Blood samples were collected from the jugular vein of one randomly selected pig per pen using 10 mL tubes with or without ethylenediaminetetraacetic acid (EDTA) on days 1, 7, and 14. Blood samples from tubes without EDTA were left to clot at room temperature for 2 hours and then centrifuged for 15 min at 3,000×g at 4°C to obtain serum. These samples were stored at −80°C for immune response analysis. In the final week of the experiment, 0.2 % chromic oxide (Cr_2O_3) was fed to all pigs as an indigestible marker. Fecal samples were collected from a randomly one selected pig per pen using rectal palpation for three days after the adaption period and stored at −20°C to measure nutrient digestibility [25,26]. Fecal samples were obtained from three randomly chosen pigs in each dietary treatment on the last day of the experiment and stored at −80°C until metagenomic and fecal microbial analysis

Nutrient digestibility analysis

Diets and fecal samples were dried using a forced-air drying oven at 65°C for 72 h and then ground through a grinder (80350, Hamilton Beach) for apparent total tract digestibility (ATTD) analysis. All ground samples were examined for dry matter (DM), crude protein (CP) by Kjeldahl method, and energy using a bomb calorimeter (Parr 1281 Bomb Calorimeter, Parr Instrument) following the procedures of the Association of Official Analytical Chemists [27]. The concentration of chromium in the samples was determined using an absorption spectrophotometer (Hitachi Z-5000 Absorption Spectrophotometer, Hitachi High-Technologies). The ATTD of the DM, CP, and energy for each dietary treatment were calculated according to the previous study [28].

Blood samples analysis

Whole blood samples were collected in EDTA tubes using an automated hematology analyzer (scil Vet abc hematology analyzer; scil animal care company, F-67120 Altorf, France) [29] . The measurements included the numbers of white blood cell (WBC), red blood cell (RBC), and hematocrit (HCT). The serum samples were applied to determine immune responses including tumor necrosis factor-α (TNF-α) and cortisol using porcine enzyme-linked immunosorbent assay kits (ELISA) kits (R&D Systems). Additionally, levels of serum immunoglobulin A (IgA), serum immunoglobulin G (IgG), and serum immunoglobulin M (IgM) were determined using ELSA kits (Bethyl Laboratories). All assays were performed according to the manufacturer's instructions.

Fecal microbiota analysis

DNA was extracted from fecal samples (200 mg of feces per sample) using the QIAamp Fast DNA Stool Mini Kit (QIAGEN) based on the manufacturer's protocol. The concentration of DNA was measured using the Colibri Microvolume Spectrometer (Titertek Berthold), and the samples with 260/280 ratios between 1.80 and 2.15 were used to additional analysis [30]. The V5 to V6 regions of the 16S rRNA genes were amplified using sets of polymerase chain reaction (PCR) primers consisted of 799F-mod6 and 114R [31]. After PCR amplification, the products were refined using a Wizard® SV Gel and PCR Clean Up System purification kit (Promega). The sequencing of purified 16S rRNA gene was performed using the Illumina MiSeq platform at BRD Inc following the manufacturer's protocols. Quality control of all raw sequence data was checked utilizing FastQC [32], and then the 16S rRNA gene sequences were analyzed using the Deblur algorithm, which is executed in both QIIME2 software and the Microbiome Helper pipeline [33]. After applying the algorithm, sequences were grouped into operational taxonomic units (OTUs), determined at a similarity cutoff of 97% [34]. Alpha diversity indices such as the observed OTUs, Shannon, Simpson, and Chao1 were measured to compare the diversity of microbial communities within each dietary treatment. In addition, principal coordinated analysis (PCoA) based on unweighted and weighted UniFrac distances was used to visualize differences in microbial communities among the dietary treatments. Taxonomic composition of the dietary treatments was expressed as a percentage at the phylum and genus levels based on their relative abundance.

Statistical analyses

Data were subjected to the GLM procedure of SAS (SAS Institute) using a randomized complete block design (block = initial BW). The experimental unit was the pen. The statistical models for growth performance, nutrient digestibility, blood profiles, and immune responses of weaned pigs included dietary treatment as the main effect and initial BW as a covariate. The frequency of diarrhea was analyzed using the Chi-square test. The MicrobiomeAnalyst webtool (https://www.microbiomeanalyst.ca/) was used to analyze alpha and beta diversity. STAMP software v. 2.1.3 [35] was used for taxonomic classification using a two-sided Welch's t-test. Alpha diversity indices were measured using ANOVA and beta diversity based on unweighted and weighted UniFrac distances was estimated using ANOSIM to determine the differences in microbial diversity among the dietary treatments. Statistical difference and tendency for dietary treatment effects were set at $p < 0.05$ and $0.05 \le p < 0.10$, respectively.

RESULTS

Growth performance, frequency of diarrhea, and nutrient digestibility

Pigs fed dietary T2 and T3 had higher ($p < 0.05$) ADG and G:F on day 1 to 14 than those fed CON (Table 2). Additionally, the groups supplemented with Cu and Zn tended to have a lower ($p < 0.10$) frequency of diarrhea than those fed CON. However, no differences were found in overall average ADFI for the first 14 days after weaning among the dietary treatments. In addition, dietary T2 and T3 increased ($p < 0.05$) ADG over the entire experimental period compared with the CON. As shown in Table 3, no differences were found ATTD of DM, CP and energy among dietary treatments.

Blood profiles and immune responses

Pigs fed dietary T2 tended to have lower ($p < 0.10$) the number of WBC on day 7 and HCT on day 14 than those fed CON (Table 4). However, there was no difference in RBC among dietary treatments. Regarding immune responses (Table 4), dietary T1 tended to have a lower ($p < 0.10$) concentration of serum cortisol on day 7 and a higher ($p < 0.10$) serum immunoglobulin M (IgM) than CON. In addition, dietary T2 tended to have decreased ($p < 0.10$) serum concentrations of TNF-α on day 7 and increased ($p < 0.10$) serum IgG on day 14 compared with CON. However, no differences were found in serum IgA levels among the dietary treatments.

 https://doi.org/10.5187/jast.2024.e70

Table 2. Effects of dietary coated CuSO4 and ZnO on growth performance of weaned pigs[1)]

Item[2)]	CON	T1	T2	T3	SEM	*p*-value
Day 1 to 14						
Initial BW (kg)	7.30	7.31	7.29	7.27	0.31	1.000
Final BW (kg)	10.73	11.27	11.75	11.87	0.44	0.503
ADG (g/d)	245[b]	283[ab]	319[a]	328[a]	20.72	0.040
ADFI (g/d)	437	429	415	429	26.52	0.994
G:F (g/g)	0.561[b]	0.659[ab]	0.768[a]	0.766[a]	0.042	0.021
Day 15 to 42						
Initial BW (kg)	10.73	11.27	11.75	11.87	0.44	0.503
Final BW (kg)	21.49[b]	22.23[ab]	22.85[a]	22.98[a]	0.31	0.030
ADG (g/d)	384	391	396	397	13.02	0.925
ADFI (g/d)	956	951	963	965	29.55	0.999
G:F (g/g)	0.402	0.412	0.412	0.411	0.020	0.993
Day 1 to 42						
Initial BW (kg)	7.30	7.31	7.29	7.27	0.31	1.000
Final BW (kg)	21.49[b]	22.23[ab]	22.85[a]	22.98[a]	0.31	0.030
ADG (g/d)	338[b]	355[ab]	371[a]	374[a]	7.28	0.020
ADFI (g/d)	783	777	780	786	24.78	0.999
G:F (g/g)	0.431	0.457	0.475	0.476	0.016	0.419
Frequency of diarrhea[3)] (%)	13.87	9.28	10.14	9.92		0.051

[1)]Each value is the mean of 6 replicates (4 pigs/pen).

[2)]CON, a basal weaner diet based on corn and soybean meal; T1, CON + 2,500 ppm standard ZnO; T2, CON + 100 mg/kg dietary coated $CuSO_4$ and 100 mg/kg dietary coated ZnO; T3, CON + 200 mg/kg dietary coated $CuSO_4$ and 200 mg/kg dietary coated ZnO; BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; G:F, gain to feed ratio.

[3)]Frequency of diarrhea for the first 2 weeks after weaning (%) = (number of diarrhea score of 4 or higher / number of pen days) × 100. Data was analyzed using the Chi-square test.

[a,b]Means in the same row with different superscripts are different ($p < 0.05$).

Table 3. Effects of dietary coated $CuSO_4$ and ZnO on apparent total tract digestibility of weaned pigs[1)]

Item[2)]	CON	T1	T2	T3	SEM	*p*-value
DM (%)	70.68	75.27	80.17	81.15	5.67	0.324
Energy (%)	75.34	77.83	79.74	84.33	5.21	0.414
CP (%)	69.84	73.33	76.40	82.59	6.48	0.542

[1)]Each value is the mean of 6 replicates (1 pig/pen).

[2)]CON, a basal weaner diet based on corn and soybean meal; T1, CON + 2,500 ppm standard ZnO; T2, CON + 100 mg/kg dietary coated $CuSO_4$ and 100 mg/kg dietary coated ZnO; T3, CON + 200 mg/kg dietary coated $CuSO_4$ and 200 mg/kg dietary coated ZnO; DM, dry matter; CP, crude protein.

Fecal microbiota

The microbial alpha diversity indices are shown in Fig. 1. Dietary T1 tended to have lower ($p < 0.10$) Chao1, Simpson, and Shannon indices than CON and T3. The beta diversity of the fecal microbiota determined using PCoA plots is presented in Fig. 2. PCoA plots based on the unweighted UniFrac distance, confirmed that T1 had distinct clustering from other groups, but overlapped clustering with CON, T2, and T3 ($r = 0.463$, $p < 0.05$). However, there was some distinct separation of fecal microbial communities based on the weighted UniFrac distance among the dietary treatments ($r = 0.201$, $p < 0.10$). The relative abundances of the fecal microbes at the phylum and genus level among the dietary treatments are shown in Figs. 3 and 4, respectively. At the phylum level (Fig. 3), Firmicutes were the most predominant bacteria in all dietary treatments

Table 4. Effects of dietary coated $CuSO_4$ and ZnO on blood profiles and immune responses of weaned pigs[1)]

Item[2)]	CON	T1	T2	T3	SEM	*p*-value
WBC (×10^3/μL)						
Day 1	13.88	13.56	12.18	14.88	2.48	0.474
Day 7	23.08	19.76	18.70	20.42	1.31	0.074
Day 14	24.70	23.32	19.20	19.14	2.53	0.459
RBC (×10^6/μL)						
Day 1	4.70	4.94	4.54	4.65	0.18	0.355
Day 7	6.00	6.33	6.19	6.10	0.41	0.984
Day 14	6.45	6.50	6.70	6.74	0.32	0.325
HCT (%)						
Day 1	25.08	26.02	25.02	24.92	0.87	0.885
Day 7	30.66	29.74	29.66	29.54	2.05	0.985
Day 14	35.56	30.30	29.26	29.56	1.85	0.084
TNF-α (pg/mL)						
Day 1	141.21	131.53	125.35	121.35	30.25	0.506
Day 7	108.81	78.57	77.79	78.97	10.08	0.095
Day 14	104.99	84.44	77.27	81.43	38.91	0.550
Cortisol (ng/mL)						
Day 1	115.05	118.74	107.32	105.83	18.64	0.691
Day 7	122.98	81.18	83.77	90.17	13.84	0.063
Day 14	105.50	98.64	92.11	94.88	17.39	0.330
IgG (mg/mL)						
Day 1	5.37	4.86	4.91	5.24	0.71	0.721
Day 7	3.72	3.84	3.77	3.94	1.01	0.650
Day 14	3.86	4.55	4.79	4.75	0.31	0.076
IgM (mg/mL)						
Day 1	1.36	1.57	1.15	1.43	0.27	0.413
Day 7	1.35	1.37	1.44	1.56	0.17	0.361
Day 14	1.23	1.62	1.60	1.51	0.13	0.067
IgA (mg/mL)						
Day 1	0.23	0.36	0.25	0.26	0.08	0.385
Day 7	0.32	0.26	0.28	0.30	0.11	0.857
Day 14	0.40	0.65	0.46	0.55	0.14	0.354

[1)]Each value is the mean of 6 replicates (1 pig/pen).

[2)]CON, basal weaner diet based on corn and soybean meal; T1,= CON + 2,500 ppm standard ZnO; T2,= CON + 100 mg/kg dietary coated $CuSO_4$ and 100 mg/kg dietary coated ZnO; T3, CON + 200 mg/kg dietary coated $CuSO_4$ and 200 mg/kg dietary coated ZnO; WBC, white blood cell; RBC, red blood cell; HCT, hematocrit; TNF-α, tumor necrosis factor-alpha; IgG, immunoglobulin G; IgM, immunoglobulin M; IgA, immunoglobulin A.

(CON, 83.82%; T1, 82.98%; T2, 91.44%; T3, 80.19%), followed by Bacteroidetes in CON (5.83%) and T1 (14.67%), and Actinobacteria and Proteobacteria in T2 (2.9%) and T3 (7.16%). At the genus level (Fig. 4) and based on statistical differences among dietary treatments (Fig. 5), dietary T2 tended to increase ($p < 0.10$) relative abundance of *Limosilactobacilus* (24.77%) compared to those fed dietary T1 (0.33%). In addition, pigs fed dietary T3 had higher ($p < 0.05$) relative abundance of *Agathobacter* (3.39%) than those fed CON (1.26%) and dietary T1 (0.04%). Additionally, dietary T3 had a lower relative abundance of *Terrisporobacter* (1.74%) compared to those fed dietary T1 (12.77%).

 https://doi.org/10.5187/jast.2024.e70

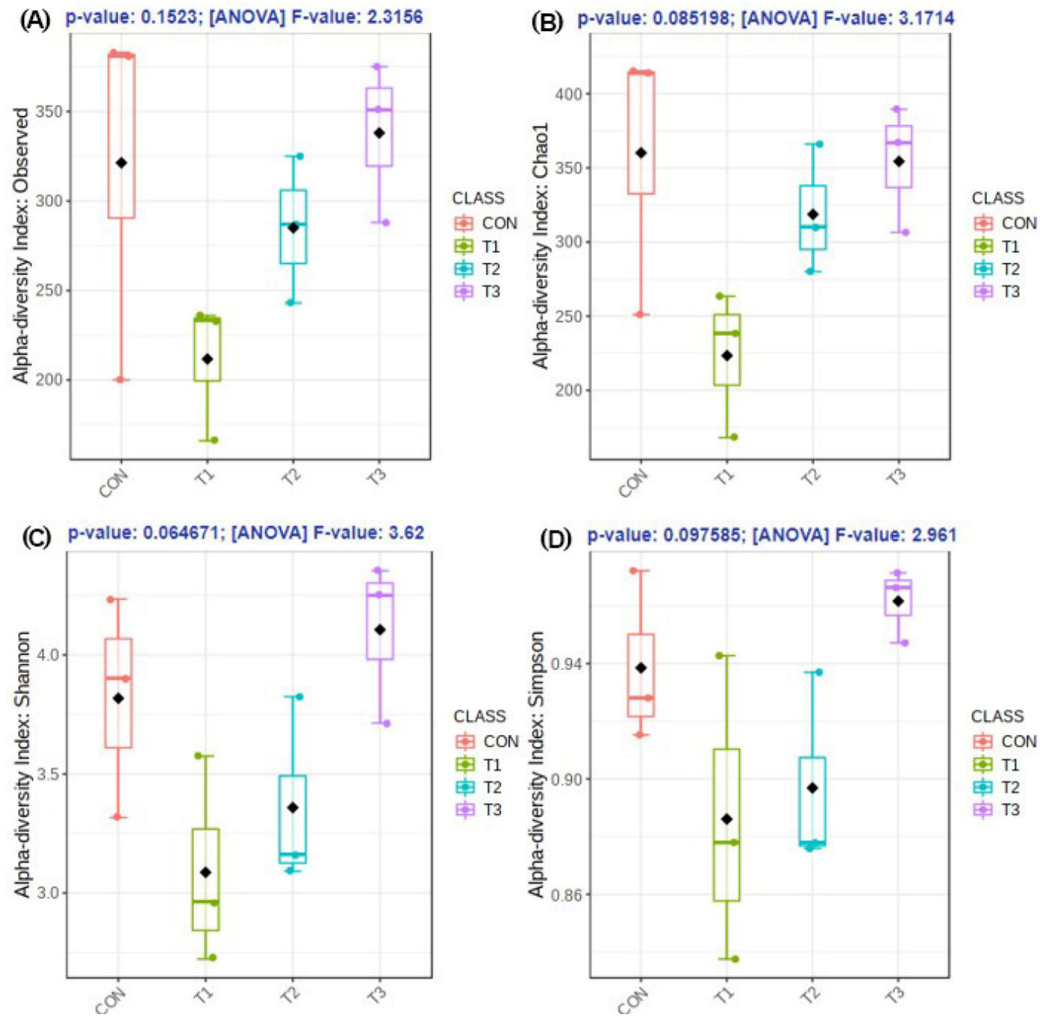

Fig. 1. Effects of dietary coated $CuSO_4$ and ZnO on alpha diversity of fecal microbiota of weaned pigs (n = 3). Alpha diversity indices were (A) observed OTUs, (B) Chao1, (C) Shannon, and (D) Simpson. Statistical difference was performed using the analysis of ANOVA. CON, a basal weaner diet based on corn and soybean meal; T1, CON + 2,500 ppm standard ZnO; T2, CON + 100 mg/kg dietary coated $CuSO_4$ and 100 mg/kg dietary coated ZnO; T3, CON + 200 mg/kg dietary coated $CuSO_4$ and 200 mg/kg dietary coated ZnO.

DISCUSSION

Minerals are inorganic elements that improve growth and reproduction in pigs [11]. In the livestock industry, micro minerals such as Cu and Zn, typically in the form of $CuSO_4$ and ZnO are widely supplemented into the diet in amounts that exceed the nutritional requirements of weanling pigs [36–38]. However, when these compounds reach the stomach of piglets, large amounts of ZnO and $CuSO_4$ are dissociated into Zn and Cu ions, respectively, and great quantities are lost in the digestive tract. Because only a small amount of these complexes can reach the intestinal tract, high doses are required. However, the inclusion level in feed must be lowered owing to global regulations on environmental pollution through excretion and/or malabsorption by overnutrition. Therefore, modified forms such as organic, nanoparticles and lipid-coated forms have been researched and utilized into swine feed [38–41]. Results from this study demonstrated that the dietary T2 and T3 groups enhanced the ADG and G:F for the first two weeks and the overall period compared with that of the CON group, which is consistent with previous studies where supplementation in coated or nano-sized forms [42,43]. An improvement in growth performance was observed with the supplementation of a lower dosage of dietary-coated $CuSO_4$ and ZnO in the present study due

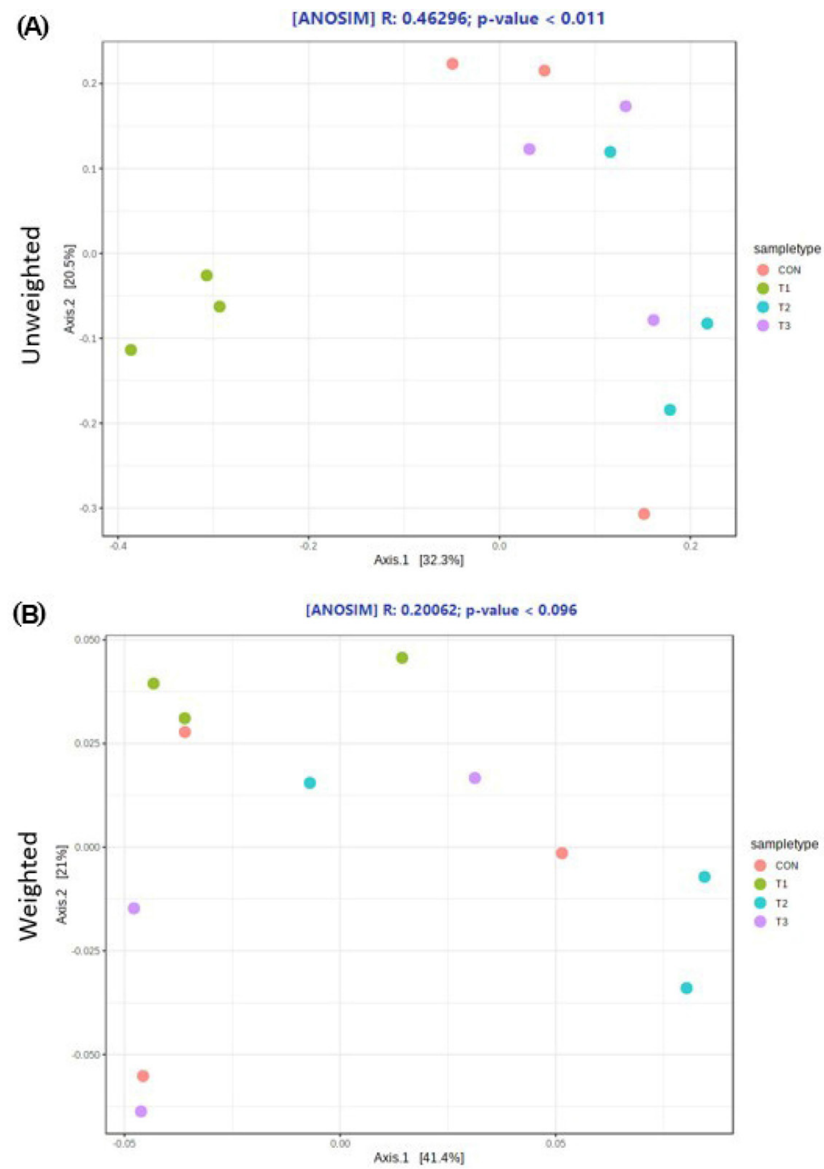

Fig. 2. Effects of dietary coated $CuSO_4$ and ZnO on beta diversity of fecal microbiota of weaned pigs (n = 3). Principal coordinated analysis based on (A) unweighted and (B) weighted Unifrac distances. The ANOSIM test was used for statistically significant distances. CON, a basal weaner diet based on corn and soybean meal; T1, CON + 2,500 ppm standard ZnO; T2, CON + 100 mg/kg dietary coated $CuSO_4$ and 100 mg/kg dietary coated ZnO; T3, CON + 200 mg/kg dietary coated $CuSO_4$ and 200 mg/kg dietary coated ZnO.

to the relatively high bioavailability and absorption of Cu and Zn in the small intestine compared with standard forms of $CuSO_4$ and ZnO. In general, no additive effects were observed when excess Zn was added to Cu [44]. Specifically, metallothionein in the intestinal mucosa is induced by high concentrations of Zn, resulting in Cu binding, and causing Cu deficiency by disturbing its absorption [45,46]. Therefore, the results suggested that balanced doses of coated $CuSO_4$ and ZnO were better absorbed and enhanced growth rate compare with CON as same as supplementation of pharmacological levels of ZnO.

In this study, supplementation with dietary coated $CuSO_4$ and ZnO tended to have decreased the frequency of diarrhea. In addition, HCT in the blood profiles, which increases with dehydration and is used as an indicator of increases in diarrhea [47], did not differ among the dietary treatments

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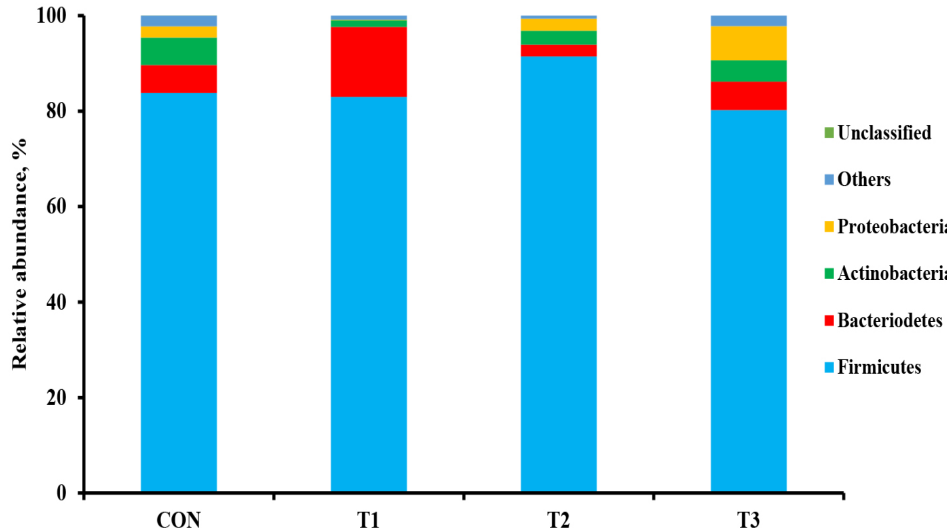

Fig. 3. Taxonomic relative abundance of fecal microbiota at the phylum level among dietary treatments (n = 3). CON, a basal weaner diet based on corn and soybean meal; T1, CON + 2,500 ppm standard ZnO; T2, CON + 100 mg/kg dietary coated $CuSO_4$ and 100 mg/kg dietary coated ZnO; T3, CON + 200 mg/kg dietary coated $CuSO_4$ and 200 mg/kg dietary coated ZnO.

in the first week after weaning. However, supplementation with low dose of dietary coated microminerals tended to decrease HCT on day 14, and we demonstrated that this supplementation positively affected the fecal score. Furthermore, no differences in the ATTD of DM, CP, and energy among the dietary treatments. However, several previous studies have reported that ZnO and $CuSO_4$ supplementation in the form of lipid-coated or nano-type positively affected nutrient and energy digestibility, which were attributed to digestive enzymes and morphological changes in the small intestine [42,43,48]. Therefore, additional research on digestive enzyme activity and nutrient digestibility should be conducted because they may differ depending on the processing type, method, or concentrations of the microminerals.

As intestinal permeability increases due to weaning stress, potentially pathogenic bacteria can penetrate and cause not only intestinal inflammation but also systemic inflammatory responses [47]. Changes in the WBC count, indicative of systemic inflammation, are associated with alterations in the levels of cytokines involved in maintaining immunity and homeostasis [49,50]. Serum TNF-α is one of the pro-inflammatory cytokines, used as a potential indicator of inflammatory reactions and damaging the mucosal barrier system [51]. Changes in these parameters regulate the systemic immune responses against infections or diseases caused by weaning stress. Previous studies reported that supplementation of $CuSO_4$ and ZnO reduced and downregulated the concentrations and mRNA levels of inflammatory cytokines including TNF-α in the intestinal mucosa of weaned pigs [24,37]. The current study further demonstrated that dietary coated $CuSO_4$ and ZnO alleviated systemic immune responses caused by weaning stress through the reduction of WBC and serum TNF-α levels. Serum IgA, IgM, and IgG levels, which are the major components of humoral immunity, are reduced by weaning stress and immature immunity of piglets [52]. In this study, dietary coated $CuSO_4$ and ZnO improved serum IgG levels of weaned pigs, consistent with the results of previous studies [17,53]. IgG is a type of antibody that leads to control the infection via binding many pathogens such as bacteria and viruses by immune cells such as macrophages. Furthermore, it has been reported that free Cu and Zn ions possess antimicrobial properties against *E.coli* in the small intestine [54]. Although the specific mechanisms of microminerals activities are not entirely elucidated yet, it is widely accepted that these metallic ions degrade bacterial cell

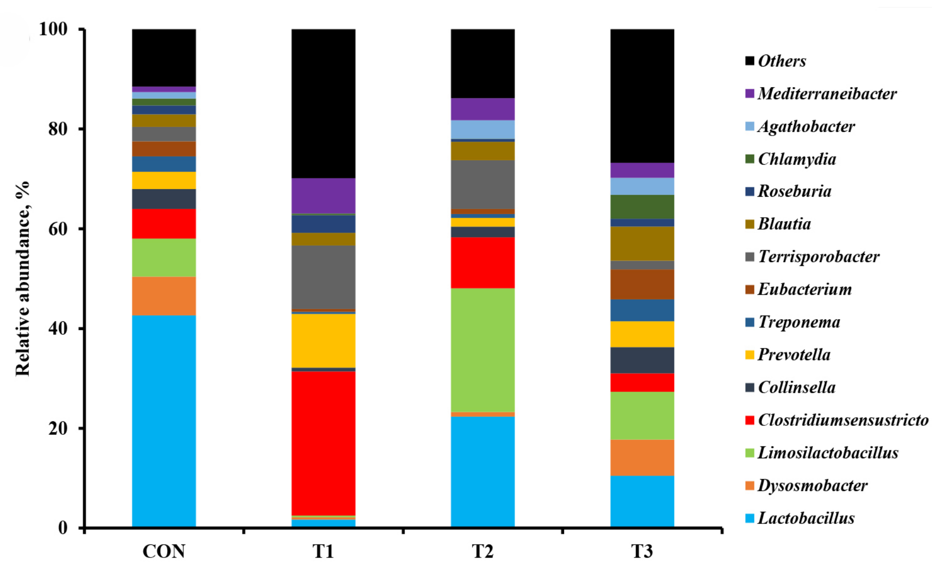

Fig. 4. Taxonomic relative abundance of fecal microbiota at the genus level among dietary treatments (n = 3). CON, a basal weaner diet based on corn and soybean meal; T1, CON + 2,500 ppm standard ZnO; T2, CON + 100 mg/kg dietary coated $CuSO_4$ and 100 mg/kg dietary coated ZnO; T3, CON + 200 mg/kg dietary coated $CuSO_4$ and 200 mg/kg dietary coated ZnO.

membranes by disrupting the integrity of bacterial cell membranes, inducing cell death. In addition, ions increase the release of reactive oxygen species within microorganisms, leading to pathogen destruction [55]. Collectively, dietary supplementation with coated Cu and Zn modulates immune responses by preventing the breakdown of the intestinal barrier and overproduction of pro-inflammatory cytokines.

Many studies have reported that dietary supplementation of pharmacological concentration of Cu and Zn improves intestinal microorganisms by increasing the number of beneficial bacteria and reducing pathogenic microbial composition [56,57]. The diversity and composition of the intestinal microbiota in pigs are considerably affected by health condition and the digestion of nutrients compositions through physiological functions [58]. In our study, the Shannon index tended to increase with coated $CuSO_4$ and ZnO compared to that with the standard dosage of ZnO, indicating greater diversity in the fecal microbiota of piglets. Shen et al. [24] showed that a pharmacological dosage of ZnO reduced the richness of microbial populations in the jejunum and feces of weaning pigs, which was consistent with the results of our study. In general, diversity is often associated with the presence of beneficial bacteria that can counteract pathogens [59]. However, the relative abundance of bacteria among dietary treatments was compared through taxonomic classification for a more precise interpretation. At the phylum level, the Firmicutes and Bacteroidetes accounted for approximately 90% of all treatments in the fecal microbiomes of weaned pigs. Among the dietary treatments, dietary T2 had the highest proportion of Firmicutes. Since *Lactobacillus* and *Clostridium* were dominant genera within Firmicutes, we determined that the overall portion of *Lactobacillus* (22.4%), *Limosilactobacilus* (24.78%), and *Clostridium sensu stricto* (10.23%) in the T2 was higher than in the other treatments. At the genus level, the present study showed that pigs fed dietary T2 had a higher relative abundance of genus *Limosilactobacilus* compared with T1. *Limosilactobacilus* is a genus of lactic acid bacteria that recently split from *Lactobacillus* and includes the species *Limosilactobacilus reuteri*, which is a microorganism with properties that promotes intestinal health and is widely used as a probiotic strain [57,60].

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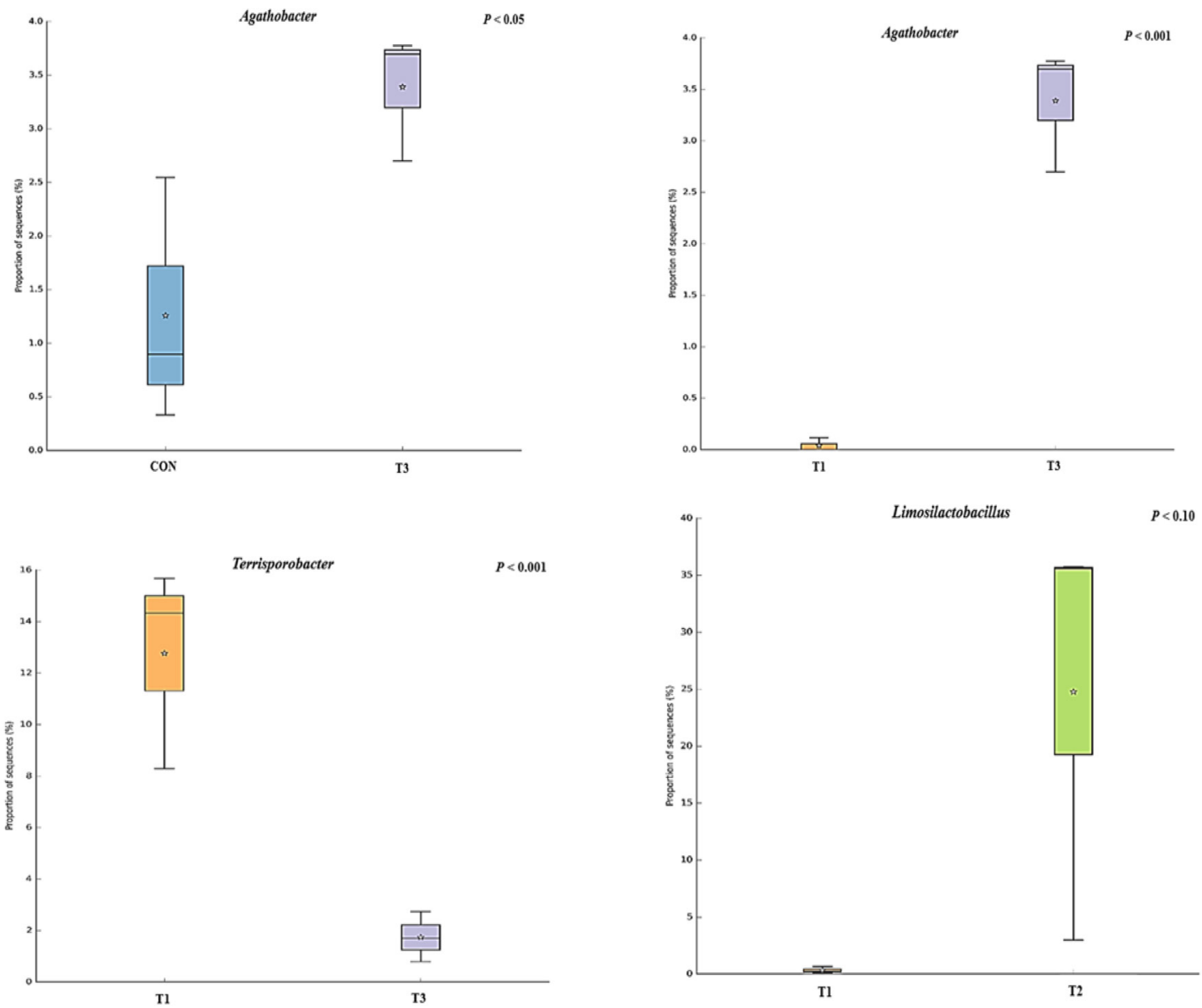

Fig. 5. STAMP analysis identifying the significant different fecal taxa at the genus level in weaned pigs among dietary treatments. Statistical difference was measured using a two-sided Welch's t-test ($p < 0.05$). CON, a basal weaner diet based on corn and soybean meal; T1, CON + 2,500 ppm standard ZnO; T2, CON + 100 mg/kg dietary coated $CuSO_4$ and 100 mg/kg dietary coated ZnO; T3, CON + 200 mg/kg dietary coated $CuSO_4$ and 200 mg/kg dietary coated ZnO.

Furthermore, the relative abundance of fecal microbiota increased *Agathobacter* and decreased Terrisporobacter in dietary T3 compared with that in CON and dietary T1. *Agathobacter* is a beneficial bacterium that contributes to short-chain fatty acid production, particularly butyrate, and is positively correlated with overall gut health in humans through metabolic interactions of the gut microbiota [61]. Furthermore, *Terrisporobacter* shows a positive correlation with increased serum markers such as endotoxin and TNF-α, which promote oxidative stress, inflammation, and malnutrition of gut microbiota in weaned pigs [62,63]. Therefore, the higher relative abundance of *Limosilactobacilus* and *Agathobacter* may have contributed to the suppression of *Terrisporobacter* and stabilization of the intestinal environment, thereby enhancing the growth performance of pigs fed dietary coated $CuSO_4$ and ZnO than those fed CON and standard ZnO diets.

CONCLUSION

Our study demonstrated that dietary coated $CuSO_4$ and ZnO supplementation in a nursery diet improved growth performance and modulated the immune responses and gut microbiota in weaned pigs. These results indicate that improvement in growth performance and immune responses may be associated with changes in the fecal microbiota composition compared with CON group. In conclusion, dietary coated $CuSO_4$ and ZnO have positive effects in weaned pigs and represent potential alternatives to high levels of ZnO diets.

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