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ARTICLE INFORMATION	Fill in information in each box below
Article Title (within 20 words without abbreviations)	Effects of different inorganic:organic zinc ratios or combination of low crude protein diet and feed additives in weaned piglet diets
Running Title	Toward replacing high dose of ZnO in piglet diet
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ABSTRACT

Thirty-six weaned piglets with an initial body weight of 8.43±0.40kg (28 days of age, LYD) were randomly assigned to 6 treatments for a 2-week feeding trial to determine the effects of different inorganic (IZ), organic zinc (OZ) or combination of low crude protein diet (LP) and feed additives (MFA) on diarrhea score, nutrient digestibility, zinc utilization, blood profiles, organ weight, and fecal microflora in weaned piglet diet. The pigs were individually placed in 45×55×45 cm stainless steel metabolism cages in an environmentally controlled room (30±1℃). The dietary treatments included a negative control (NC), positive control (PC; Zinc Oxide, 1,000 mg/kg), T1 (IZ:OZ, 850:150), T2 (IZ:OZ 700:300), T3 (IZ:OZ, 500:500), and T4 (LP + MFA [0.1% Essential oils + 0.08% protease + 0.02% Xylanase]). The daily feed allowance was adjusted to 2.7 times the maintenance requirement for digestible energy (2.7×110 kcal of DE / kg $BW^{0.75}$). This allowance was divided into two equal parts, and the piglets were fed at 08:30 and 17:30 each day. Water was provided *ad libitum* through a drinking nipple. The diarrhea score was significantly decreased ($p < 0.05$) in NC treatment compared with other treatments. The apparent total tract digestibility (ATTD) of dry matter (DM), nitrogen (N), and gross energy (GE) was significantly increased ($p < 0.05$) in the T2 treatment compared with the PC and NC treatments at one week. At two weeks, the ATTD of DM, N, and GE was significantly decreased ($p < 0.05$) in the NC treatment compared with other treatments. The T3 treatment had significantly higher ($p < 0.05$) ATTD and apparent ileal digestibility of zinc than the PC and T1 treatments. The *E.coli* concentration in feces was significantly decreased in the T4 treatment compared with the NC and T2 treatments. The *Lactobacillus* concentration in feces was significantly increased in the T4 and T1 treatment compared with the T2 and T3 treatments. In conclusion, IZ:OZ 500:500 levels could improve nutrient digestibility and zinc utilization in weaned piglets, Moreover, MFA in LP diets could be used as a zinc alternative.

Key words: Zinc oxide, alternatives, diarrhea score, zinc excretion, nutrient digestibility

INTRODUCTION

Piglets frequently experience diarrhea due to various factors such as isolation from sows, dietary changes, the mixing of pigs from different pens, adaptation to a new environment, or intestinal morphologic changes after weaning [1]. Due to these stress factors, the proliferation of *Escherichia coli* in the intestine of weaned pigs is promoted through undigested proteins. The intestinal barrier is damaged by toxins from enterotoxigenic *E.coli*, causing post-weaning diarrhea (PWD) [2]. The pharmacological supplementation of weaned diet with high-dose zinc oxide (ZnO) can prevent PWD and promotes growth performance in the weaning period [1, 2]. However, it has recently been restricted in many countries including the European Union (EU) due to soil heavy metalization, accumulation in livestock products, and increased antimicrobial resistance [3]. The EU limits ZnO in weaned piglet diets to 150 mg/kg, and China limits it to 1,600 mg/kg [4]. In South Korea, the Zn content in compost is limited to 1,200 mg/kg, and a penalty is imposed on swine farms if this limit is exceeded. The pharmacological level of ZnO has been allowed to be added to piglet diets for two weeks after weaning in many countries to control PWD at this time [3, 4]. For this reason, studies on Zn dose control or elimination of dietary ZnO are being conducted to replace high-dose ZnO in the diet of weaned piglets during 2 weeks of post-weaning.

Studies on the effects of ZnO supplementation at doses lower than 2,500 mg/kg are limited and have shown distinctly different results [5, 6]. Piglet nutrition, breeding, management, and genetics have seen tremendous growth over the period since studies suggested that 2,500 mg/kg of dietary ZnO could control PWD and improve growth performance during 2 weeks of post-weaning [3, 7]. However, as described above, it is essential to investigate the effect of low-dose of dietary Zn than pharmacological dose of Zn on incidence of diarrhea and Zn excretion in weaned piglet diets. In our previous study, we conducted to evaluate the alternative forms of Zn, such as nano-particle size and Zn chelated with glycine, with lower level to replace high dose of inorganic Zn (IZ) in weaned piglet diets [8]. In our experiment, organic Zn (OZ) showed higher utilization than other forms of Zn such as inorganic Zn (IZ) and nanoparticle-sized Zn [8]. Many researchers reported that chelating Zn with an amino acid prevented precipitation and had high bioavailability through peptide or amino acid transport systems in the small intestine [9, 10]. OZ has greater stability than $ZnSO_4$ or ZnO [11], so has been suggested as an alternative to IZ.

In the search for replacing the pharmacological supplementation of ZnO, low-protein diets, essential oils, and enzymes are currently in the spotlight. The National Research Council (NRC) recommends 20 – 23% crude protein (CP) levels in weaned diets [12]. However, 3 – 4 week-old pigs cannot produce enough endogenous enzymes to digest that amount of protein, so some of the undigested protein reaches the large intestine, which can lead to PWD by proteolytic bacteria [13]. Furthermore, many researchers have reported that reducing CP levels in the weaned diet reduced the incidence of diarrhea [14, 15]. Essential oils (EOs) have powerful antimicrobial and immune-enhancing effects, improving growth performance and nutrient digestibility, intestinal morphology, and reducing PWD in weaned piglets [16]. Exogenous protease increased nutrient digestibility, particularly protein and amino acids, and increased digestive enzyme activity and growth performance in weaned pigs [17, 18]. Enzymes including protease and xylanase have various properties such as improving intestinal health and the immune system and growth performance [19]. In particular, it was reported to show benefits in improving gut health by inhibiting the growth of pathogenic microorganisms in the intestine [19].

Therefore, we hypothesized that different ratios of IZ and OZ at 1000 mg/kg or a low-protein diet with commercial feed additives containing either essential oils, protease and xylanase (MFA) could replace high-dose ZnO by preventing diarrhea and improving nutrient digestibility and gut health. Thus, we conducted this study to evaluate (1) the effects of different inorganic:organic Zn (IZ:OZ) ratios on diarrhea scores, nutrient digestibility, Zn utilization, blood profiles, organ weight, and fecal microflora toward replacing high-dose Zn oxide in weaned piglet diets and (2) whether 10% reduced protein diet with essential oils, protease, and xylanase could replace high-dose Zn oxide by showing similar effects.

Materials and methods

The experimental protocol for this study was reviewed and approved by the Institutional Animal Care and Use Committee of Chungbuk National University, Cheongju, Korea (approval #CBNUA-1530-21-01).

The organic Zn was chelated with glycine (containing 27 % of Zn) from Dr.Eckel Animal Nutrition GmbH & Co. KG (Anta®min; Niederzissen, Germany). The essential oils

(Avi®power, containing thymol 1.4 % and carvacrol 1.4 %; VetAgro SpA, Reggio Emilia. Italy), xylanase (Signis®, AB Vista, Marlborough, United Kingdom), and protease (PT125TM, an alkaline serine endopeptidase produced by *Streptomyces* spp.; Eugene-Bio, Suwon, Korea) were mixed feed additives supported by a Eugene-Bio.

Animals, Facilities and Dietary treatments

A total of 36 weaned piglets (Duroc × Landrace × Yorkshire; 28 day of old) were allotted to a completely randomized block design. The pigs (average initial body weight of 8.43 ± 0.40 kg) were individually placed in 45 cm × 55 cm × 45 cm stainless steel metabolism cages in an environmentally controlled room (30 ± 1 °C). There were one pig treatment in a cage and six replicate cage per treatments. The dietary treatments consisted of NC (negative control; no additional added ZnO in diet), PC (positive control; NC + 1,000 mg/kg ZnO), T1 (NC + IZ:OZ 850:150 mg/kg), and T2 (NC + IZ:OZ 700:300 mg/kg), T3 (IZ:OZ 500:500 mg/kg), and T4 (10% reduced protein diet [LP] + mixed additives [0.1% essential oil + 0.08% protease + 0.02% xylanase, MFA]). All diets were formulated to meet or exceed the NRC (Table 1). The daily feed allowance was adjusted to 2.7 times the maintenance requirement for digestible energy (DE; 2.7 × 110 kcal of DE/kg $BW^{0.75}$). This allowance was divided into two equal parts, and the piglets were fed at 08:30 h and 17:30 h each day. The diets were mixed with water in a 1:1 ratio (Wt/Wt) before feeding. Water was provided *ad libitum* through a drinking nipple. We individually weighed the pigs at the beginning of each period and recorded the amount of feed supplied and any residual feed quantity for each period. The subjective diarrhea scores were individually recorded at 09:00 h and 18:00 h from the same pigs on days 0 to 14 post weaning. The diarrhea score was assigned as follows: 0, diarrhea; 1, sloppy feces; 2 normal feces; and 3, well-formed feces. Scores were calculated as the average diarrhea score for each period (0 to 7 days; 7 to 14 days; overall period, 0 to 14 days) per group by summing the average daily diarrhea scores of each pig. The first experimental period consisted of a 4-day adaptation period, followed by a 3-day collection period to collect feces. The feed was the same during the second experimental period as that in the first experimental period. We set a 4-day feces collection period and alternated the feeding time between the day of slaughter and the previous 2 days so that pigs could be slaughtered within the designated time. The entire liver and spleen were weighed. The fecal collected by total collection method. The intestinal tract was incised along the abdominal gland to remove 20 cm from the end of the ileum. Then the contents were frozen in a plastic bag. The ileal digesta was freeze dried. Samples were finely crushed and stored at -20° C to measure Zn content. Feces were immediately collected as they appeared in the metabolism cages. They were stored in a freezer at -20° C until analyzed. Fecal samples were dried at 70° C for 72 hours in a forced-air oven and ground through a 1-mm screen. They were thoroughly mixed before a subsample was collected for chemical analysis.

Chemical analysis for diet and feces

Diets and feces were analyzed for dry matter (DM), nitrogen (N), and gross energy (GE) using AOAC methods (2007). For N of the diets and feces, we added 10 % concentrated sulfuric acid for nitrogen fixation. We analyzed the GE of the diets and feces using an adiabatic oxygen bomb calorimeter (Parr Instruments, Moline, IL, USA). Diets, feces, and ileal digesta samples were wet digested using nitric-perchloric acid and then diluted with deionized distilled water for mineral analysis. The concentration of Zn was analyzed using UV absorption spectrophotometry (UV-1201; Shimadzu, Tokyo, Japan). We calculated the apparent total tract digestibility (ATTD) of DM, N, GE, and Zn, as well as the average daily mineral intake, using the following equations: ATTD lrb%) $= ([DI \times NID - OF \times NIF]/[DI \times NID]) \times 100$; Average daily mineral intake $= ADFI \times MD$; DI is the DM intake (g), NID is the nutrient content (DM, N, GE, and Zn) of diet on a DM basis; OF is the output of feces (g); and NIF is the nutrient content of the feces on a DM basis. MD is the Zn content in the diet.

For the blood profiles, all pigs were sampled via an anterior vena cava puncture before the slaughter. Blood samples were collected into both nonheparinized tubes and vacuum tubes containing K_3EDTA (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) to obtain serum and whole blood. After collection, serum samples were centrifuged (3,000 g) for 20 min at 4° C. The red blood cells (RBC), white blood cells (WBC), lymphocyte, monocyte, eosinophil, basophil, glucose, cholesterol and blood urea nitrogen (BUN) levels in the whole blood were determined by using an automatic blood analyzer (ADVIA 120, Bayer, Tarrytown, NY, USA). The immunoglobulin G (IgG) and immunoglobulin M (IgM) were determined by using commercial enzyme-linked immunosorbent assay (ELISA, Bethyl Laboratories, Montgomery, TX, USA) kits. The Zn concentration of blood was determined according to the method described by Hill *et al*. [6]. The blood samples were diluted 1:7 with deionized water, and Zn concentration were determined by flame absorption spectrophotometry (Smith-Hieftje 4000, Thermo Jarrell Ash Corp., Franklin, MA)

Procedures of microbial shedding

Fecal samples were collected directly via massaging the rectum of all pigs in each treatment. They were then pooled and placed on ice for transportation to the lab. One gram of the composite fecal sample from each treatment was diluted in 9 mL of 1% peptone broth (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) and then homogenized. Viable bacteria in the fecal samples were then counted by placing serial 10-fold dilutions (in 1% peptone solution) onto MacConkey agar plates (Difco Laboratories, Detroit, MI, USA) and *lactobacilli* medium III agar plates (Medium 638, DSMZ, Braunschweig, Germany) to isolate the *Escherichia coli* and *Lactobacillus*. The *lactobacilli* medium III agar plates were then incubated for 48 hours at 39° C under anaerobic conditions. The MacConkey agar plates were incubated for 24 hours at 37° C. The *E. coli* and *Lactobacillus* colonies were counted immediately after removal from the incubator.

Statistical analysis

Data of growth performance, nutrient digestibility, Zn excretion, ATTD of Zn, AID of Zn, blood profiles, and organ weight were statistically analysed as a randomized complete block design using general linear models procedure of SAS (Statistical Analysis System 9.1, SAS Institute, Cary, NC, USA). The diarrhea score and fecal microflora were compared with a chi-squared test, using the FREQ procedure of SAS. The individual pig was used as the experimental unit. Orthogonal contrasts were used to compare the possible relationship about the effect of treatments: NC vs. other treatments; PC vs. T1, T2, T3; T4 vs. T1, T2, T3. Variability in the data was expressed as the pooled standard error, and $p < 0.05$ was considered statistically significant.

RESULTS

Diarrhea score

At 8 to 14 days, pigs fed the NC diet had higher ($p < 0.05$; contrast $p < 0.01$) diarrhea score than pigs fed the T1 and T3 diets (Table 2).

Nutrient digestibility and zinc utilization

The ATTD of DM, N, and GE were significantly ($p < 0.001$; contrast $p < 0.05$) decreased in the NC treatment compared with other treatments in weeks 1 and 2 (Table 3). In week 1, pigs fed the T2 diet had higher ($p < 0.05$) the ATTD of DM, N, and GE than the pigs fed the PC diet. The N intake and excretion were significantly decreased ($p < 0.001$; contrast $p < 0.05$) in the T4 treatment compared with other treatments in weeks 1 and 2. Pigs fed with the PC and T1 diets had significantly higher ($p < 0.05$) Zn intake than did pigs fed with the T2 and T3 diets in weeks 1 and 2. Pig fed with the T1, T2 and T3 diets had significantly lower ($p < 0.05$; contrast $p < 0.05$) Zn excretion in feces and higher the ATTD of Zn than pigs fed the PC treatment in week 1. Pigs fed with the T2 and T3 diets had significantly lower ($p < 0.05$; contrast $p < 0.05$) Zn excretion in feces compared with pigs fed with the PC and T1 diets in week 2. The ATTD of Zn was significantly increased ($p < 0.05$; contrast $p < 0.05$) in the T3 treatment compared with the PC treatment in the same period. The AID of Zn was significantly decreased ($p < 0.05$; contrast $p < 0.05$) in the PC treatment compared with the T1, T2 and T3 treatments, moreover, pigs fed with the T3 diet had significantly higher ($p < 0.05$; contrast $p < 0.05$) AID of Zn than pigs fed with the T1 diet.

Blood profiles

There was a high tendency for the blood concentration of lymphocyte in the T4 treatment compared with the NC, PC, T1 and T2 treatments (Table 4). The blood concentration of BUN was significantly decreased ($p < 0.05$; contrast $p < 0.05$) in the T4 treatment compared with the NC, PC, and T1 treatments (Table 5).

Organ weight

No significant differences were observed in the liver and spleen weight (Table 5).

Fecal microflora

The *E.coli* concentration in feces was significantly decreased ($p < 0.05$; contrast $p < 0.05$) in the T4 treatment compared with the NC and T2 treatments (Table 6). The *Lactobacillus* concentration in feces was significantly increased ($p < 0.05$; contrast $p < 0.05$) in the T4 and T1 treatments compared with the T2 and T3 treatments.

DISCUSSION

Zn is an essential mineral that has various enzymatic and co-enzymatic roles. It improves immunity and the composition of the body structure. It helps in developing the gastrointestinal tract, preventing diarrhea, and affecting the growth of pigs [10]. The Zn content in feedstuff is insufficient for pigs, and Zn is mainly added in an inorganic form, such as ZnO or $ZnSO_4$ [20]. The ZnO form has low reactivity and bioavailability, and the $ZnSO_4$ form is hygroscopic and reacts with rapid ions to form free radicals to accelerate the breakdown of fatty acids, vitamins, and other nutrients in the feed [21]. In addition, to prevent diarrhea in the weaning phase, Zn that cannot be absorbed by adding 2,000 to 3,000 mg/kg of ZnO to the weaning diets, is discharged in the feces, which is a major environmental problem [22]. The hypothesis of the present experiment was that there would be an additive effect of replacing inorganic Zn with organic Zn and LP diet with MFA, leading to reduced diarrhea, and improved nutrient digestibility, Zn utilization, and blood profiles. This would result in positive effects similar to pharmacologic levels of ZnO.

In the present study, IZ:OZ at ratios of 850:150 mg/kg and 500:500 mg/kg (T1, T3) decreased diarrhea scores, which means it reduced diarrhea compared to non- Zn diets (NC) but had no significant difference compared to 1,000 mg/kg ZnO (PC) at 8 to 14 days. Also, the diarrhea scores of pigs in the low-protein diet with MFA were similar to those of pigs treated with Zn. The supplementation of ZnO has usually led to better fecal scores and lower incidence of PWD and mortality [23]. Effective Zn sources can also be organic Zn forms [24]. Different Zn forms like Zn-methionine or Zn-lysine can increase Zn concentrations in the plasma more than ZnO or other inorganic Zn forms [25]. Reductions in diarrhea with increasing organic Zn levels can be explained by the increased bioavailability of organic Zn compared to inorganic Zn in the intestine. EOs have gained attention as ZnO alternatives for reducing PWD in animal diets [26]. They demonstrated many properties such as strong antimicrobial, antioxidant, and anti-inflammatory activity [16]. *E. coli*, known as the main etiological agent of PWD, proved to be susceptible to several EOs, including cinnamon, clove, and thyme oils, thereby leading to reduced fecal scores and incidence of diarrhea [27]. Also, supplementation with dietary enzymes including protease and xylanase could reduce diarrhea in pigs. These beneficial effects were attributed to the development of the digestive tract, an increase in enzymatic activity in the digestive system, and improvement in nutrient digestibility derived from the enzymes [28, 29]. The decrease in diarrhea from the addition of enzymes and EOs can be explained by the abovementioned mechanism.

In nutrient digestibility, pigs fed diets with different IZ:OZ ratios (PC, T1~T3) or LP diet with MFA had a higher ATTD of DM, N, and GE compared to the one and two-week NC treatments. These results were consistent with the results of Lei and Kim [30] who reported that the addition of Zn to the diet increased DM and N digestibility. Hu et al. [31] reported that dietary supplementation with ZnO could improve the activation of the digestive enzymes in the small intestine and pancreatic tissue, thereby improving the digestibility of nutrients. Other studies have reported that small intestine morphology was improved from pharmacological supplementation with ZnO [32]. Schlegel et al. [33] reported that the bioavailability of organic and inorganic Zn forms ranged from 85 to 117%. Unlike our hypothesis that improvements would be seen from increasing organic Zn ratios, the replacement of inorganic Zn with organic Zn did not make a dramatic difference among the treatments except for NC, but the IZ:OZ ratios of 500:500 mg/kg showed high digestibility. However, there was no significant difference in nutrient digestibility among the different dietary Zn levels [34]. This may have been due to the dosage or type of Zn. Additionally, environmental conditions, dietary ingredients, phosphorous levels, and nutritional composition may have caused these results. The LP diet with enzymes and EOs contributed to improving nutrient digestibility similar to the Zn treatments. At weaning, the high buffering capacity of hard diets and the low HCl production in the piglet stomach cause LP digestion [35]. The use of LP in this study led to improved digestibility due to the abovementioned mechanisms. Additionally, these improvements were attributed to feed additives like enzymes and EOs. Previously published studies reported the improved digestibility of energy and nutrients by supplementation with EOs [36, 37, 38]. Although studies on how EOs affect digestibility are handicapped by the complexity of EOs, we confirmed the results of the studies by Platel and Srinivasan [39], Zhai et al. [40]. They reported that these improvements could be explained by the enhanced secretion of bile and enzymes and altered gut peristalsis. The use of xylanase and protease in the swine diet improved nutrient digestibility [17].

Many researchers reported that the bioavailability of Zn was increased by organic Zn compared to the inorganic form of Zn sulfate owing to the amino acid or the peptide transport systems [41, 42]. These results were also observed in our study. We found that the ATTD and AID of Zn gradually increased as the ratio of organic Zn in the diets increased. The reasons for the improvements in Zn digestibility were considered to result from reduced fecal excretion and improved efficiency of the organic form. It was possible to confirm the effect of reducing diarrhea incidience, improving nutrient digestibility and Zn utilization when feeding OZ in a certain ratio rather than adding inorganic Zn alone. Also, piglets fed LP diet with MFA had higher ATTD and AID of Zn than piglets fed the NC diet. Diet acidification with formic, benzoic butyric, lactic, fumaric, and citric acids increased the ATTD of minerals with Ca and P in pigs [43]. Interestingly, dietary citric acid improved P utilization in growing pigs [22], and 1.5% citric acid improved the availability of other minerals in young pigs [44]. Sauer et al. [45] reported that the digestibility of minerals increased as benzoic acid levels in the diet increased. According to a recent study, the actions and mechanisms of EOs overlapped with those of benzoic acid, and some benzoic acid could be spared by the addition of EOs. Additionally, EOs increased the utilization rate of Zn and reduced the discharge of Zn [46].

Zn plays a critical role in the immune system of the host, and it affects various immune responses in different parts of the body, from innate immune functions to the skin barrier [47, 48]. Sun et al. [49] reported that when 400 – 600 mg/kg of nano-ZnO was supplied, IgM and IgG levels were increased. However, Ma et al. [50] showed that dietary supplementation with $ZnSO_4$, chitosan+$ZnSO_4$, and Zn chitosan chelate did not affect serum IgG levels in weaned piglets. Also, IgG levels remained unaffected by Zn-ASP supplementation to growing pigs [51]. Previous studies indicated that plasma Zn concentrations increased linearly with supplemental Zn [52]. Our results showed that there was no significant difference in blood profiles except for lymphocytes and BUN among the treatments. This discrepancy may have been due to the dosage or type of Zn, nutritional composition, or experimental period. BUN can be used to determine protein digestibility and be a parameter of protein utilization [53]. In the current study, BUN was decreased in the non- Zn pigs receiving a low CP diet with MFA, consistent with previous studies reporting that decreased protein levels resulted in lower BUN levels [14, 54, 55]. The lower BUN levels indicated improved protein utilization. The organ weight of pigs is used as an indicator to determine good health, disease-free status, and a resting state [56]. In the present study, there was no significant difference between the treatments. These results may have been because the dosage of Zn and the multiple feed additives was a safe dose for organ development.

Many researchers have shown that the addition of dietary ZnO improved the microbial composition in the intestine, thereby reducing pathogenic microorganisms and increasing beneficial bacteria [57, 58] However, similar results were not seen in our study. In the present study, the effect of Zn treatment was not different compared to the NC treatment. These results are consistent with the results of Li et al. [59] who reported that ZnO did not affect the *Enterobacteriaceae*, *Lactobacilli*, and *Clostridia* counts in the ileal digesta and feces in piglets. Additionally, supplementation with Zn, regardless of the form, had no effect on coliform bacteria and lactic acid bacteria counts in the small intestine or cecum [30]. The inconsistent intestinal microflora results may have been due to several reasons. First of all, the doses, forms, and duration of ZnO supplementation may have caused these differences. Also, different sampling areas in the intestine or feces and different analysis methods could have led to the differences and changes in the microbial communities [60, 61]. Interestingly, the LP diet with MFA resulted in increases in *Lactobacillus* and decreases in *E.coli* counts in the feces compared to the NC and Zn treatments. These improvements in intestinal bacterial composition could have been caused by several factors. The high-protein diets caused a higher acid-binding environment and increased the pH of the gastrointestinal tract to nearly neutral conditions, which provided a favorable environment for the proliferation of pathogenic bacteria, whereas the LP diet alleviated the negative effects of high protein and significantly lowered the number of *E.coli* in the ileum and colon [62]. EOs have strong antimicrobial action against pathogenic bacteria while not harming beneficial bacteria such as *bifidobacteria* and *lactobacilli*. Moreover, the increased number of *lactobacilli* and reductions in *E. coli* in the intestinal microbiota resulted in a decreased incidence of diarrhea in piglets [37]. In the present study, lower diarrhea in the T4 group was caused by the abovementioned mechanism. The *E. coli* and total anaerobe counts in the rectum were significantly reduced ($p < 0.05$) in pigs fed EOs, whereas the number of lactobacilli was slightly increased in the colon and rectum of pigs fed EOs. The effect of enzymes on the intestinal microbiota is related to changes in the physicochemical properties of the substrate in the intestine and the release of prebiotics and bioactive compounds [63]. Commercial xylanase may also contain feruloyl esterase produced by the microorganisms producing xylanase [64] that release phenolic compounds cross-linked to xylan [65, 66]. Studies have shown that phenolic compounds could modulate the intestinal microbiota by reducing ETEC K88 and F18+ growth in porcine feces [67]. Kim et al. [68] reported that the addition of multiple enzymes including xylanase, amylase, β-mannanase, protease, and phytase increased the *Lactobacillus* spp. count and decreased *E. coli* and *Clostridium* spp. counts in the digesta of the ileum and cecum.

CONCLUSION

Pigs in the LP+MFA group showed similar post-weaning diarrhea, ATTD of nutrients, and fecal microbiota as organic and inorganic Zn-supplemented treatments. During 1 week of post-weaning, 700:300 mg/kg of inorganic:organic Zn ratio could improve nutrient digestibility, and zinc utilization compared with 1,000 mg/kg ZnO. Likewise, in overall periods, a 500:500 mg/kg inorganic:organic Zn ratio showed improvements in ATTD/AID of Zn, and reductions in Zn excretion compared to 1,000 mg/kg ZnO. By partially replacing inorganic Zn with organic Zn, it showed the possibility of being presented as an alternative to high dose of ZnO in weaned piglet diets. In conclusion, reducing protein with essential oils, protease, and xylanase and a 700:300 or 500:500 mg/kg inorganic and organic Zn ratio were reduce Zn excretion and effective alternatives of high-dose of ZnO in weaned piglet diets.

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Table 1. Compositions of the weaning diets (as-fed basis)

Items	Basal diet	10% reduced CP diet
Ingredient, %		
Corn	34.43	38.34
Extruded corn	15.00	15.00
Lactose	10.00	10.00
Dehulled soybean meal, 51% CP[1)]	13.50	10.00
Soy protein concentrate, 65% CP[1)]	10.00	10.00
Plasma powder	6.00	4.50
Whey	5.00	6.00
Soy oil	2.20	2.20
Monocalcium phosphate	1.26	1.26
Limestone	1.40	1.40
L-Lysine-HCl, 78%	0.06	0.12
DL-Methionine, 50%	0.15	0.18
Choline chloride, 25%	0.10	0.10
Vitamin premix[2)]	0.25	0.25
Trace mineral premix[3)]	0.25	0.25
Salt	0.40	0.40
Total	100.00	100.00
Calculated value		
ME, kcal/kg	3508	3503
CP, %	20.78	18.70
Lysine, %	1.35	1.34
Metionine, %	0.39	0.40
Ca, %	0.82	0.82
P, %	0.65	0.65
Zn, %	0.01	0.01

[1)] CP, crude protein.

[2)] Provided per kg of complete diet: vitamin A, 11,025 IU; vitamin D_3, 1,103 IU; vitamin E, 44 IU; vitamin K, 4.4 mg; riboflavin, 8.3 mg; niacin, 50 mg; thiamine, 4 mg; d-pantothenic, 29 mg; choline, 166 mg; and vitamin B_{12}, 33 μg.

[3)] Provided per kg of complete diet without Zinc: Cu (as $CuSO_4{\bullet}5H_2O$), 12 mg; Mn (as MnO_2), 8 mg; I (as KI), 0.28 mg; and Se (as $Na_2SeO_3{\bullet}5H_2O$), 0.15 mg.

[4)] Values were calculated using National Swine Nutrition Guide(NSNG; V 2.0).

Table 2. Effects of different inorganic:organic zinc ratios or combination of low crude protein diet and feed additives on diarrhea scores in weaned piglet diets.

Treatment	NC	PC	T1	T2	T3	T4	SE	P-value
Inorganic:Organic zinc	0	1000:0	850:150	700:300	500:500	LP+MFA		
Diarrhea score[1]								
0 to 7 days	1.929	1.701	1.781	1.622	1.741	1.595	0.252	0.947
8 to 14 days [x]	1.164^{a}	0.778^{ab}	0.440^{b}	0.692^{ab}	0.464^{b}	0.833^{ab}	0.165	0.045
Overall period (0 to 14 days)	1.278	0.982	0.919	0.997	0.908	1.086	0.166	0.635

$^{a–b}$Means in the same row with different superscripts differ ($p < 0.05$).

NC, no additional added zinc oxide in diet (negative control); PC, NC+1000 mg/kg zinc oxide (positive control); T1, NC + inorganic:organic zinc 850:150 mg/kg ; T2, NC + inorganic:organic zinc 700:300 mg/kg; T3, NC + inorganic:organic zinc 500:500 mg/kg; T4, 10% reduced CP diet + 0.1% essential oil + 0.08% protease + 0.02% xylanase; LP + MFA, low protein diet + mixed feed additives; SE, standard error.

1)Diarrhea score was determined as follow: 0, well-formed feces; 1, normal feces; 2, sloppy feces; and 3, diarrhea

[x] contrast: NC vs other treatments ($p < 0.05$)

Table 3. Effects of different inorganic:organic zinc ratios or combination of low crude protein diet and feed additives on nutrient digestibility and zinc utilization in weaned piglets.

Treatment	NC	PC	T1	T2	T3	T4	SE	P-value
Inorganic:Organic zinc	0	1000:0	850:150	700:300	500:500	LP+MFA		
Nutrient digestibility								
One week ATTD, %								
Dry matter $^{x, y}$	86.7^{c}	88.2^{b}	88.9^{ab}	89.8^{a}	89.1^{ab}	89.2^{ab}	0.5	0.001
Nitrogen ^{x}	77.1^{c}	81.8^{b}	81.8^{b}	84.4^{a}	83.0^{ab}	81.6^{b}	0.8	0.001
Gross energy $^{x, y}$	82.8^{c}	85.1^{b}	86.7^{ab}	87.4^{a}	85.5^{b}	86.3^{ab}	0.6	0.001
Two week ATTD, %								
Dry matter ^{x}	87.6^{b}	89.8^{a}	89.7^{a}	89.9^{a}	90.5^{a}	90.3^{a}	0.4	0.001
Nitrogen ^{x}	77.8^{b}	81.0^{a}	80.4^{a}	81.3^{a}	82.2^{a}	81.7^{a}	0.8	0.017
Gross energy ^{x}	83.3^{b}	86.4^{a}	86.4^{a}	86.9^{a}	87.7^{a}	87.3^{a}	0.6	0.001
Zinc utilization								
One week								
Feed intake, g	340.0	340.0	340.0	340.0	340.0	340.0	0.0	1.000
Zinc intake, mg $^{x, y, z}$	34.0^{c}	382.5^{a}	374.0^{a}	340.0^{b}	340.0^{b}	34.0^{c}	3.1	0.001
Zinc excretion, mg $^{x, y, z}$	32.3^{d}	344.8^{a}	299.2^{b}	264.5^{c}	253.7^{c}	29.2^{d}	7.5	0.001
ATTD of Zinc $^{x, y, z}$	5.1^{d}	9.6^{c}	19.9^{ab}	22.3^{ab}	25.3^{a}	14.2^{bc}	2.7	0.001
Two week								
Feed intake, g $^{x, y, z}$	350.0^{c}	380.0^{a}	380.0^{a}	370.0^{b}	380.0^{a}	350.0^{c}	0.0	0.001
Zinc intake, g $^{x, y, z}$	35.0^{c}	427.5^{a}	418.0^{a}	370.0^{b}	380.0^{b}	35.0^{c}	2.4	0.001
Zinc excretion, mg $^{x, y, z}$	31.4^{c}	381.1^{a}	349.3^{a}	298.6^{b}	291.8^{b}	29.5^{c}	10.6	0.001
ATTD of Zinc $^{x, y}$	10.4^{b}	10.9^{b}	16.4^{ab}	19.3^{ab}	23.2^{a}	15.8^{ab}	3.2	0.045
AID of zinc, % $^{x, y}$	8.9^{c}	9.3^{c}	14.1^{b}	18.1^{ab}	21.1^{a}	14.1^{b}	1.5	0.001

$^{a–d}$Means in the same row with different superscripts differ ($p < 0.05$).

NC, no additional added zinc oxide in diet (negative control); PC, NC+1000 mg/kg zinc oxide (positive control); T1, NC + inorganic:organic zinc 850:150 mg/kg ; T2, NC + inorganic:organic zinc 700:300 mg/kg; T3, NC + inorganic:organic zinc 500:500 mg/kg; T4, 10% reduced crude protein diet + 0.1% essential oil + 0.08% protease + 0.02% xylanase; LP + MFA, low protein diet + mixed feed additives; SE, standard error; ATTD, apparent total tract digestibility; AID, apparent ileal digestibility.

^{x} contrast: NC vs other treatments ($p < 0.05$)

^{y} contrast: PC vs T1, T2, and T3 ($p < 0.05$)

^{z} contrast: T4 vs T1, T2, and T3 ($p < 0.05$)

Table 4. Effects of different inorganic:organic zinc ratios or combination of low crude protein diet and feed additives on blood profiles in weaned piglets.

Treatment	NC	PC	T1	T2	T3	T4	SE	P-value
Inorganic:Organic zinc	0	1000:0	850:150	700:300	500:500	LP+MFA		
Red blood cell, $10^6/\mu L$	7.32	7.37	7.14	7.52	7.64	7.59	0.40	0.949
White blood cell, $10^3/\mu L$	17.43	17.72	17.85	19.68	18.11	17.76	2.79	0.994
Lymphocyte, % ^{z}	49.88	49.35	49.18	50.48	57.68	66.58	4.58	0.065
Monocyte, %	3.48	4.63	2.47	4.33	3.88	4.77	0.73	0.252
Eosinophil, %	0.41	0.55	0.43	0.42	0.40	0.52	0.14	0.958
Basophil, %	0.40	0.35	0.33	0.40	0.55	0.43	0.09	0.548
Immunoglobulin G, mg/dL	174.0	160.3	185.2	148.5	185.0	162.3	23.2	0.816
Immunoglobulin M, mg/dL	47.3	43	47.7	50.7	48.0	44.0	3.7	0.774
Cholesterol, mg/dL	66.2	71.2	76.5	72.7	82.5	68.0	5.4	0.313
Glucose, mg/dL	109.7	107.3	108.2	111.2	106.0	109.7	9.6	0.999
Blood urea nitrogen, mg/dL ^{z}	6.83^a	6.83^a	6.83^a	6.33^{ab}	5.50^{ab}	5.00^b	0.48	0.049
Zinc, ug/dL	94.9	102.4	104.0	107.3	99.4	97.2	5.0	0.654

$^{a–b}$Means in the same row with different superscripts differ ($p < 0.05$).

NC, no additional added zinc oxide in diet (negative control); PC, NC+1000 mg/kg zinc oxide (positive control); T1, NC + inorganic:organic zinc 850:150 mg/kg ; T2, NC + inorganic:organic zinc 700:300 mg/kg; T3, NC + inorganic:organic zinc 500:500 mg/kg; T4, 10% reduced crude protein diet + 0.1% essential oil + 0.08% protease + 0.02% xylanase; LP + MFA, low protein diet + mixed feed additives; SE, standard error.

^{z} contrast: T4 vs T1, T2, and T3 ($p < 0.05$)

Table 5. Effects of different inorganic:organic zinc ratios or combination of low crude protein diet and feed additives on organ weight in weaned piglets.

Treatment	NC	PC	T1	T2	T3	T4	SE	P-value
Inorganic:Organic zinc	0	1000:0	850:150	700:300	500:500	LP+MFA		
Body weight, kg	10.0	10.1	10.3	10.6	10.7	10.0	0.2	0.399
Relative organ weight, %								
Liver	3.046	2.922	2.844	2.709	2.837	2.760	0.211	0.892
Spleen	0.205	0.282	0.231	0.208	0.268	0.214	0.026	0.257

NC, no additional added zinc oxide in diet (negative control); PC, NC+1000 mg/kg zinc oxide (positive control); T1, NC + inorganic:organic zinc 850:150 mg/kg ; T2, NC + inorganic:organic zinc 700:300 mg/kg; T3, NC + inorganic:organic zinc 500:500 mg/kg; T4, 10% reduced crude protein diet + 0.1% essential oil + 0.08% protease + 0.02% xylanase; LP + MFA, low protein diet + mixed feed additives; SE, standard error.

Table 6. Effects of different inorganic:organic zinc ratios or combination of low crude protein diet and feed additives on fecal microflora in weaned piglets.

Treatment	NC	PC	T1	T2	T3	T4	SE	P-value
Inorganic:Organic zinc	0	1000:0	850:150	700:300	500:500	LP+MFA		
E.coli, $\log_{10}$cfug$^{-1\ z}$	5.241^{a}	4.986^{ab}	4.897^{ab}	5.263^{a}	5.110^{ab}	4.742^{b}	0.139	0.087
Lactobacillus, $\log_{10}$cfug$^{-1\ z}$	6.969^{ab}	6.804^{b}	7.254^{a}	6.814^{b}	6.701^{b}	7.256^{a}	0.132	0.017

$^{a\text{–}b}$Means in the same row with different superscripts differ ($p < 0.05$).

NC, no additional added zinc oxide in diet (negative control); PC, NC+1000 mg/kg zinc oxide (positive control); T1, NC + inorganic:organic zinc 850:150 mg/kg ; T2, NC + inorganic:organic zinc 700:300 mg/kg; T3, NC + inorganic:organic zinc 500:500 mg/kg; T4, 10% reduced crude protein diet + 0.1% essential oil + 0.08% protease + 0.02% xylanase; LP + MFA, low protein diet + mixed feed additives; SE, standard error.

^{z} contrast: T4 vs T1, T2, and T3 ($p < 0.05$)