Research paper

Effects of zinc-exchanged montmorillonite with different zinc loading capacities on growth performance, intestinal microbiota, morphology and permeability in weaned piglets

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ABSTRACT

Three zinc-exchanged montmorillonites (Zn-Mt1, Zn-Mt2, and Zn-Mt3, the amount of Zn adsorbed on Mt was 6.3×10^4, 5.1×10^4 and 4.2×10^4 mg/kg, respectively) were used to study the effects on growth performance, diarrhea, intestinal microbiota and barrier function in weaned piglets. A total of 144 piglets (Duroc × Landrace × Yorkshire), weaned at 21 ± 1 d with average initial body weight of 6.5 kg were allotted to four treatments groups for two weeks. Four treatments were: (1) control: basal diet; (2) ZM1: basal diet + 150 mg/kg Zn as Zn-Mt1; (3) ZM2: basal diet + 150 mg/kg Zn as Zn-Mt2; and (4) ZM3: basal

The aim of the present study is to investigate the effects of Zn-Mt with different Zn loading capacities on growth performance, intestinal microbiota, morphology and permeability in weaned piglets.

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2.1. Materials

Mt was obtained from the Inner Mongolia Autonomous Region, China. The content of the purified Mt was 99.0%. Zn-Mt was prepared by Zn^{2+}-exchanged reaction. Ten grams of the Mt was mixed with 0.1 L of 0.2 mol/L NaCl solution. The dispersion was agitated for 5 h on a magnetic stirrer (700 rpm) at 25 °C. The Na-Mt was then separated by centrifugation at a speed of 8000 *g* for about 15 min and washed with deionized water for three times. The washed Na-Mt was then added to $ZnSO_4$ solution at 0.20 mol/L, 0.15 mol/L and 0.10 mol/L, respectively. The dispersion was agitated at 60 °C for 6 h on a magnetic stirrer (700 rpm). After centrifugation at 8000 *g* for 5 min, the sediment was washed with deionized water for three times, dried at 80 °C for 24 h, then ground to a size less than 50 μm pore diameter. Zn concentration in the Zn-Mt1, Zn-Mt2 and Zn-Mt3, as measured by atomic absorption spectroscopy (ICE 3300, Thermo Fisher Scientific, Waltham, USA), was 6.3×10^4, 5.1×10^4 and 4.2×10^4 mg/kg, respectively.

2.2. Experimental design and sample collection

All procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang University. A total of 144 piglets (Duroc × Landrace × Yorkshire), weaned at 21 ± 1 d with average initial body weight of 6.5 kg were allotted to four treatments. The four treatments were: (1) control: basal diet; (2) ZM1: basal diet + 150 mg/kg Zn as Zn-Mt1; (3) ZM2: basal diet + 150 mg/kg Zn as Zn-Mt2; and (4) ZM3: basal diet + 150 mg/kg Zn as Zn-Mt3. Each treatment had six pens of six piglets. Basal diets were formulated according to the National Research Council (1998) (Table 1). All pigs were given ad libitum access to mash feed and water for two weeks. Average daily gain (ADG), average daily feed intake (ADFI), and feed/gain were measured. Post-weaning scour score was monitored for each pig according to Hu et al. (2012).

Tab e 1
Ingredient and composition of basal diets on an as-fed basis.

Ingredients, g/kg	
Maize	572.5
Soybean meal, crude protein 450 g/kg	257
Fish meal, crude protein 600 g/kg	50
Spray-dried plasma protein, crude protein 750 g/kg	25
Dried whey, crude protein 125 g/kg	45
Soybean oil	20
Dicalcium phosphate	11
Limestone	5
Sodium chloride	3

At 14 d after weaning, six piglets from each treatment were killed based on average diarrhea score. The gastrointestinal tract was quickly removed. Segments (1 cm) of the proximal jejunum were fixed in 10% formalin for morphology measurements. Adjacent jejunum and proximal colon were prepared for Ussing chamber studies. The intestinal contents from jejunum and proximal colon were collected, rapidly frozen in liquid nitrogen and stored at −80 °C for microbiota analysis.

2.3. Sample analysis

16S ribosomal RNA-based methods were used for the abundances of *Streptococcus suis* and *Escherichia coli* as described by Su et al. (2008). Briefly, total DNA was extracted from jejunal and colonic contents using a TIANamp Stool DNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. The amount and quality of DNA were measured at 260 and 280 nm using ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The primers of *S. suis* and *E. coli* were shown in Table 2. Real-time PCR was performed on 7500 real time PCR systems (Applied Biosystems, Foster City, USA) using Fast SYBR® Green Master Mix (Applied Biosystems, Foster City, USA). Each standard dilution and sample were assayed in triplicate in a 20 μL reaction mixture containing 10 μL of Fast SYBR Green Master Mix, 1 μL 25 pmol/μL of each primer and 7 μL Nuclease-free water and 1 μL 50 ng/μL DNA template. PCR amplification was performed with an initial denaturation step of 95 °C for 10 min, 40 cycles of 95 °C for 3 s, 60 °C for 1 min. For SYBR-Green® amplifications, a melting step was added to improve amplification specificity. Standard curves were generated as described by Li et al. (2009). The concentration of 16SrRNA gene abundance was plotted against the Cycle threshold value (CT value).

Three cross-sections for each jejunal sample were stained with hematoxylin and eosin using standard paraffin embedding procedures. Crypt depth and villus height were measured in at least ten well-oriented crypt–villus units using image analysis (Leica Imaging Systems Limited) and averaged for each sample.

Proximal jejunum and colon were stripped from the seromuscular layer in oxygenated Ringer's solution. Tissues were mounted in EasyMount Ussing chamber system (model VCC MC6, Physiologic Instruments, San Diego, CA, USA) as described previously (Hu et al., 2013a). Briefly, the clamps were connected to Acquire and Analyse software (Physiologic Instruments, San Diego, CA) for automatic data collection. After a 15-min equilibration period on Ussing chambers, transepithelial electrical resistance (TER) was recorded at 15-min intervals over a 1-h period. The epithelial barrier function was measured by the fluxes of fluorescein isothiocyanate dextran 4 kDa (FD4). The probe FD4 (Sigma-Aldrich, St. Louis, MO) was added to the mucosal side at the final concentration of 0.4 mg/mL. The samples were taken from the serosal side of tissues. The concentration of FD4 was measured by a fluorescence microplate reader (FLx800, Bio-Tek Instruments, Inc.).

2.4. Statistical analysis

One-way analysis of variance (ANOVA) was conducted using SPSS 9.0 statistical package (SPSS Inc., Chicago, IL). Differences among means were tested using Duncan's multiple range tests. Effects were considered significant at $P < 0.05$.

Tab e 3
Effects of zinc-exchanged montmorillonite on growth performance and diarrhea index of weaned piglets.

Items	Control	ZM1*	ZM2*	ZM3*	SEM[1]	P
Initial weight, kg	6.52	6.61	6.57	6.48	1.03	0.924
Final weight, kg	10.46	10.74	10.93	10.96	1.25	0.867
ADG[2], g/d	282[b]	295[ab]	312[a]	315[a]	9.06	0.035
ADFI[3], g/d	362 [b]	379 [ab]	386 [a]	390 [a]	8.30	0.002
Feed/gain ratio	1.28	1.28	1.25	1.24	0.02	0.822
Fecal scores	3.17[a]	2.50[b]	1.83[c]	1.50[c]	0.20	0.012

[a–b]Means within a row with different letters differ significantly ($P < 0.05$).
* Zinc-exchanged montmorillonite (Zn-Mt), Zn-Mt1, Zn-Mt2, and Zn-Mt3, the amount of Zn adsorbed on Mt was 6.3×10^4, 5.1×10^4 and 4.2×10^4 mg/kg, respectively.
[1] Standard error of means, $n = 6$.
[2] ADG = Average daily gain.
[3] ADFI = Average daily feed intake.

3. Re t

3.1. Growth performance and postweaning scour scores

Table 3 showed the growth performance and diarrhea index of weaned piglets. As compared with the control, supplementation with Zn-Mt2 or Zn-Mt3 increased ($P < 0.05$) ADG and ADFI, and decreased ($P < 0.05$) fecal scores. No significant difference ($P > 0.05$) was observed in the growth performance and scour scores between the Zn-Mt2 group and Zn-Mt3 group. The growth performance and scour scores of weaned pigs fed with Zn-Mt1 did not differ from those fed with the control diet. The feed/gain was not affected by dietary treatments ($P > 0.05$).

3.2. Intestinal microbiota

Supplementation with Zn-Mt2 or Zn-Mt3 reduced ($P < 0.05$) the number of *S. suis* and *E. coli* in the jejunum and proximal colon of weaned piglets as compared with the control (Table 4). No significant difference ($P > 0.05$) was observed between the Zn-Mt2 group and Zn-Mt3 group. The intestinal microbiota of weaned pigs fed with Zn-Mt1 did not differ from those fed with the control diet.

3.3. Intestinal morphology

Table 5 showed the jejunal morphology of weaned piglets. As compared with the control, supplementation with Zn-Mt2 or Zn-Mt3 increased villus height and the ratio of villus height and crypt depth ($P < 0.05$). No significant difference ($P > 0.05$) was observed between the Zn-Mt2 group and Zn-Mt3 group. The jejunal morphology of pigs fed Zn-Mt1 did not differ from those fed the control diet ($P > 0.05$).

3.4. Intestinal barrier function

Intestinal barrier function of weaned pigs, as reflected by the TER and paracellular flux of FD4, was presented in Table 6. As compared with the control, pigs fed Zn-Mt2 or Zn-Mt3 had a higher TER value ($P < 0.05$) and lower FD4 permeability in jejunum and colon ($P < 0.05$). No significant difference ($P > 0.05$) was observed between the Zn-Mt2 group and Zn-Mt3 group. The intestinal barrier function of weaned pigs fed with Zn-Mt1 did not differ from those fed the control diet ($P > 0.05$).

Tab e 4
Effect of zinc-exchanged montmorillonite on intestinal microbiota of weaned piglets[1].

Items	Control	ZM1*	ZM2*	ZM3*	SEM[2]	P
Jejunum						
E. coli	7.04[a]	6.67[ab]	6.45[b]	6.38[b]	0.15	0.025
Streptococcus suis	6.14[a]	5.78[ab]	5.49[b]	5.30[b]	0.20	0.037
Colon						
E. coli	8.32[a]	7.94[ab]	7.53[b]	7.39[b]	0.22	0.034
Streptococcus suis	7.47[a]	7.23[ab]	6.71[b]	6.56[b]	0.22	0.029

[a–b]Means within a row with different letters differ significantly ($P < 0.05$).
* Zinc-exchanged montmorillonite (Zn-Mt), Zn-Mt1, Zn-Mt2, and Zn-Mt3, the amount of Zn adsorbed on Mt was 6.3×10^4, 5.1×10^4 and 4.2×10^4 mg/kg, respectively.
[1] Bacterial numbers are expressed as $\log_{10}$ (16S rRNA gene copies g^{-1} wet weight).
[2] Standard error of the mean, $n = 6$.

Tab e 5
Effects of zinc-exchanged montmorillonite on jejunal morphology of weaned piglets.

Items	Control	ZM1*	ZM2*	ZM3*	SEM[1]	P
Villus height, μm	729[b]	754[ab]	779[a]	820[a]	19.6	0.022
Crypt depth, μm	375	361	350	347	12.1	0.377
Villus height:crypt depth	1.95[b]	2.09[b]	2.23[a]	2.36[a]	0.048	<0.001

[a–b]Means within a row with different letters differ significantly ($P < 0.05$).
* Zinc-exchanged montmorillonite (Zn-Mt), Zn-Mt1, Zn-Mt2, and Zn-Mt3, the amount of Zn adsorbed on Mt was 6.3×10^4, 5.1×10^4 and 4.2×10^4 mg/kg, respectively.
[1] Standard error of the mean, $n = 6$.

4. D c

In the present study, we found that providing 150 mg/kg Zn, supplementation of Zn-Mt2 or Zn-Mt3 alleviated postweaning diarrhea and improved growth performance in the weaned pigs. Additionally, feeding three Zn-Mts in diets gave variable results on the growth performance of weaned piglets, which indicated that the in vivo efficiency of the Zn-Mt with different Zn loading capacities was different. Preliminary experiments showed that weaned pigs fed Mt (the amount is equivalent to the Mt in the Zn-Mt1, Zn-Mt2, Zn-Mt3 treatment, respectively) had no significant effect on growth performance (data not shown). Our previous study had reported that supplementation with 500 mg/kg Zn from montmorillonite–ZnO hybrid or 600 mg/kg Zn from zeolite–ZnO hybrid could enhance growth performance and alleviate diarrhea of weaned pigs. The amount of Zn as ZnO adsorbed on Mt and zeolite is 25% and 28%, respectively (Hu et al., 2012, 2013b). It was suggested that Mt with lower Zn loading capacity may perform better in modifying the rate, time, or site of Zn release within the gastrointestinal tract and increased access of Zn to the intestinal tract, which would improve its biological roles in the hindgut and therefore benefit overall gut health and growth of animals.

Numerous investigations have found the imbalance of the intestinal microbiota in weaning piglets, which results in overgrowing pathogenic bacteria (*E. coli*, *S. suis)* and causes intestinal disorders (Castillo et al., 2007; Su et al., 2008; Xu et al., 2014). Previous researches have reported that Mt could be used as a carrier for inorganic antibacterial agents, such as Ag^+, Cu^{2+} and Zn^{2+}, which are loaded into Mt by ion exchange (Magana et al., 2008). The antibacterial properties of metallic ion-exchanged Mt on *E. coli* have been proved in vitro (Hu and Xia, 2006; Malachováa et al., 2011). The current study demonstrated for the first time that dietary Zn-Mt2 or Zn-Mt3 supplementation decreased the population of *E. coli* and *S. suis* in intestinal contents of weaned pigs. Two possible mechanisms for the antibacterial activity were proposed. One model involved the adsorption of the bacteria and immobilization

Tab e 6
Effect of zinc-exchanged montmorillonite on intestinal barrier integrity of weaned pigs.

Items	Control	ZM1*	ZM2*	ZM3*	SEM[3]	P
Jejunum						
TER[1], Ω cm^2	50.17[b]	53.17[a,b]	58.67[a]	59.83[a]	2.40	0.030
FD4[2] flux, μg cm^{-2} h^{-1}	2.58[a]	2.17[a,b]	1.68[b]	1.65[b]	0.25	0.046
Colon						
TER[1], Ω · cm^2	65.00[b]	69.17[a,b]	73.17[a]	75.33[a]	2.49	0.040
FD4[2] flux, μg cm^{-2} h^{-1}	2.18[a]	1.74[a, b]	1.39[b]	1.35[b]	0.22	0.047

[a–b]Means within a row with different letters differ significantly ($P < 0.05$).
* Zinc-exchanged montmorillonite (Zn-Mt), Zn-Mt1, Zn-Mt2, and Zn-Mt3, the amount of Zn adsorbed on Mt was 6.3×10^4, 5.1×10^4 and 4.2×10^4 mg/kg, respectively.
[1] TER = transepithelial electrical resistance.
[2] FD4 = fluorescein isothiocyanate dextran 4 kDa.
[3] Standard error of means, $n = 6$.

on the surface of the Zn-Mt. Alternatively, Zn was released from the Mt structure and directly exerted its antimicrobial effect on the bacteria. Therefore, the antibacterial effect of Zn-Mt may be explained by interactions of Mt (Magana et al., 2008).

There is evidence to suggest that a weaning transition of piglets is commonly associated with quick changes in the intestinal mucosa (Ke et al., 2014). A shortening of the villus decreases the surface area for nutrient absorption. The crypt is the area where stem cells divide to permit the renewal of the villus, and a large crypt indicates fast tissue turnover and a high demand for new tissue (Hu et al., 2013a). Changes in intestinal morphology, such as shorter villi and deeper crypts, can lead to poor nutrient absorption, increased secretion in the gut, diarrhea, reduced disease resistance, and impaired overall performance (Jiao et al., 2014). In the present study, increases in villus height and villus height:crypt depth ratio were observed in the small intestinal mucosa of weaned pigs fed the diet supplemented with Zn-Mt2 or Zn-Mt3. Similarly, Tang et al. (2014) reported that zinc-bearing clinoptilolite modulated intestinal morphology in broiler chickens. In our present study, Zn-Mt2 or Zn-Mt3 may partially improve the intestinal morphology via lowering the number of *E. coli* and *S. suis*.

An intact intestinal barrier plays a central role in preventing the translocation of intestinal bacteria, and the entering of toxic or allergenic substances from the gut into the body (Wijtten et al., 2011). Intestinal barrier function can be commonly assessed by many indices such as TER and the paracellular permeability of FD_4 (Jiao et al., 2014). It was reported that small-intestinal barrier function of pigs deteriorated after weaning (Smith et al., 2010; Wijtten et al., 2011). In the present study, consistent with improved intestinal morphology, increased TER and decreased FD4 flux were observed in the small intestinal mucosa of weaned pigs fed the diet supplemented with Zn-Mt2 or Zn-Mt3. Zn is known to play an important role in maintaining barrier function of the gastrointestinal tract (Hu et al, 2014). Mt has high surface area and standout adhesive ability. These inherent advantages make Mt effectively act by attaching Zn^{2+} to the mucus to reinforce the intestinal mucosal barrier and help in the regeneration of the epithelium (Hu et al., 2012).

5. C c

Providing 150 mg/kg Zn, the in vivo efficacy of Zn-Mt1, Zn-Mt2, and Zn-Mt3 was different in weaned piglets. Supplementation of Zn-Mt2 with 5.1×10^4 mg/kg Zn loading capacity or Zn-Mt3 with 4.2×10^4 mg/kg Zn loading capacity increased growth performance, alleviates postweaning diarrhea, and improved intestinal microflora and barrier function of weanling pigs. These positive effects of Zn-Mt may be attributed to Zn and montmorillonite interactions.

Ac ed e

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