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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 $6.3 \times 10^4$, $5.1 \times 10^4$ and $4.2 \times 10^4$ 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 diet + 150 mg/kg Zn as Zn-Mt3. Results showed that, compared with the control, supplementation of Zn-Mt2 or Zn-Mt3 improved ($P < 0.05$) average daily gain (ADG) and average daily feed intake (ADFI), and lowered ($P < 0.05$) fecal scores. Supplemental Zn-Mt2 or Zn-Mt3 increased ($P < 0.05$) villus height and the ratio of villus height and crypt depth at the jejunal mucosa. Supplementation of Zn-Mt2 or Zn-Mt3 decreased ($P < 0.05$) the number of *Escherichia coli* and *Streptococcus suis*, the paracellular permeability of fluorescein isothiocyanate dextran 4 kDa (FD4), but increased transepithelial electrical resistance (TER) in jejunum and colon of weaned piglets. No difference was observed between the Zn-Mt1 group and the control group. The results indicated that the in vivo efficacy of three Zn-Mts was different. Supplementation of Zn-Mt2 was as effective as Zn-Mt3 in enhancing growth performance, alleviating diarrhea, as well as improving intestinal microbiota, barrier function of weaned pigs under our trial conditions.



## 1. Introduction

Weaning is the most significant event in the life of pigs and weaning process is commonly associated with microbial imbalance and intestinal barrier dysfunction, which are responsible for the stunted growth and diarrhea observed in the first 2 weeks after weaning (Moeser et al., 2007; Hu et al., 2013c; Xiao et al., 2014). Feeding pharmacological level of Zn (2000–4000 mg/kg) is used in the pig industry to alleviate post-weaning diarrhea and improve performance (Hu et al., 2014). However, the strategy has been criticized because high level of Zn results in large quantities of Zn excreted and poses an environmental problem (Carlson et al., 2004). Therefore, it is essential to find an alternative to reduce Zn supplementation for sustaining swine production.

Special attention has been paid to natural clay minerals, which are used as a potential carrier for release of active ingredients in controlled drug delivery systems (Rodrigues et al., 2013; Ke et al., 2014). A few researches have reported that zinc-bearing clinoptilolite may be used as an antibiotic growth promoter substitution for broiler chicken (Wang et al., 2012; Tang et al., 2014). Zinc-exchanged montmorillonite (Zn-Mt) is prepared by an ion-exchange reaction and studies are mainly focused on the physical and chemical properties in vitro (Malachováa et al., 2011). However, there are no data regarding the biological effects of Zn-Mt in weaned pigs so far. Additionally, it has been reported that Zn-Mts with different Zn loading capacities had different physical and chemical properties. Churakov and Dähn (2012) found that whether Zn was adsorbed on montmorillonite (Mt) external surface or within its interlayer depended on the amount of Zn loading. Dähn et al. (2011) found that Zn-Mt with low or medium Zn loading capacity had different structures. Shi et al. (2010) reported that the Zn content in the interlayer space of Mt had an effect on the antibacterial activity of Zn-Mt. So we hypothesize that the different Zn loading capacities will affect in vivo efficiency.

*Abbreviations:* ADFI, average daily feed intake; ADG, average daily gain; SEM, standard error of the mean; Zn-Mt, zinc-exchanged montmorillonite; FD4, fluorescein isothiocyanate dextran 4 kDa; TER, transepithelial electrical resistance.

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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.

## 2. Materials and methods

### 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 $6.3 \times 10^4$, $5.1 \times 10^4$ and $4.2 \times 10^4$ 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).

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.

**Table 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 |
| L-Lysine HCl, 780 g/kg | 1 |
| DL-Methionine, 990 g/kg | 0.5 |
| Vitamin–mineral premix[a] | 10 |
| Analyzed composition, g/kg | |
| Digestible energy[b], MJ/kg | 14.38 |
| Moisture | 85.7 |
| Crude protein | 224.1 |
| Lysine | 14.1 |
| Methionine | 3.8 |
| Calcium | 8.6 |
| Total phosphorus | 6.7 |
| Zn (mg/kg) | 127.3 |

[a] Supplied per kilogram of diet: vitamin A, 6000 IU; vitamin $D_3$, 600 IU; vitamin E, 50 IU; vitamin $K_3$, 1.5 mg; riboflavin, 8.0 mg; thiamine, 2.0 mg; niacin, 30 mg; pyridoxine, 3.0 mg; pantothenic acid, 20 mg; choline, 800 mg; folic acid, 0.6 mg; biotin, 0.10 mg; vitamin $B_{12}$, 0.04 mg; Zn ($ZnSO_4$), 100 mg; Cu ($CuSO_4 \cdot 5H_2O$), 16 mg; I (KI), 0.2 mg; Fe ($FeSO_4$), 125 mg; Mn ($MnSO_4 \cdot H_2O$), 15 mg; Se ($Na_2SeO_3$), 0.3 mg.
[b] Digestible energy was calculated from data provide by Feed Database in China (2012).

### 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 $P < 0.05$.

**Table 2**
PCR primers for the quantification of intestinal microbiota by real-time PCR.

| Target organism | Forward/reverse | Sequence 5′–3′ |
|---|---|---|
| *E. coli* | F | CATGCCGCGTGTATGAAGAA |
| | R | CGGGTAACGTCAATGAGCAAA |
| *Streptococcus suis* | F | CAGTATTTACCGCATGGTAGATAT |
| | R | GTAAGATACCGTCAAGTGAGAA |