

Diosmectite–zinc oxide composite improves intestinal barrier function, modulates expression of pro-inflammatory cytokines and tight junction protein in early weaned pigs

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Abstract

The study evaluated whether feeding diosmectite–ZnO composite (DS–ZnO) at 500 mg Zn/kg to early weaned pigs would alleviate the weaning-related intestinal disorders as a substitute for high concentration of ZnO (2250 mg Zn/kg). The pigs weaned at an age of 21 ± 1 d were allotted to four treatment groups as follows: (1) control; (2) DS–ZnO, 500 mg Zn/kg diet; (3) ZnO, 2250 mg Zn/kg diet; and (4) mixture of 2.0 g DS/kg and 500 mg Zn/kg from ZnO (equal amount of DS and ZnO in the DS–ZnO treatment group). The results showed that, compared with the control on days 7 and 14 post-weaning, addition of DS–ZnO at 500 mg Zn/kg improved ($P < 0.05$) daily gain and feed intake, decreased ($P < 0.05$) post-weaning scour scores, increased ($P < 0.05$) jejunal villus height and the ratio of villus height and crypt depth, decreased ($P < 0.05$) jejunal paracellular permeability of fluorescein isothiocyanate dextran 4 kDa and up-regulated ($P < 0.05$) tight junction protein expression of occludin, claudin-1 and zonula occludens-1 in jejunal mucosa. The mRNA levels of *TNF- α* , *IL-6* and interferon- γ (*IFN- γ*) on day 7 post-weaning were also decreased ($P < 0.05$). The piglets fed DS–ZnO at 500 mg Zn/kg did not differ in the above parameters from those fed ZnO at 2250 mg Zn/kg, while they had better performance than those fed the mixture of DS and ZnO. Supplementation with DS–ZnO at 500 mg Zn/kg was effective in alleviating diarrhoea, barrier dysfunction and inflammatory cytokine expression and up-regulating tight junction protein expression.

Key words: Diosmectite–zinc oxide composite: Intestinal barrier: Pro-inflammatory cytokines: Tight junction protein: Weaned pigs

The early weaning process is commonly associated with intestinal barrier dysfunction and mucosal inflammation in weaned piglets, which are responsible for the stunted growth and diarrhoea observed in the first 2 weeks after weaning^(1–3). Zn is involved in anti-inflammation, anti-diarrhoea and restoration of mucosal barrier integrity^(4,5). Pharmacological addition of Zn (2000–4000 mg/kg of Zn as ZnO) in diets for weaned pigs is widely used in the pig industry worldwide due to its proven effects on alleviating post-weaning diarrhoea and improving performance^(3,6). However, high levels of ZnO in diets for weaner pigs has been criticised or banned because increased Zn excretion causes environmental pollution^(3,7). If the inclusion of ZnO is dramatically decreased and if the same effect as that of high levels of ZnO is achieved, then the Zn excretion will decrease and will be beneficial for sustaining swine production.

Diosmectite (DS) is an aluminosilicate clay mineral, which is composed of two outer tetrahedral silicon layers sandwiching an inner octahedral aluminum layer. Naturally occurring cations (i.e. Na^+) reside between the sheets to balance the overall negative surface charge of DS⁽⁸⁾. The ion exchange capacity of DS enables replacement of Na^+ with other organic and inorganic cations to add functionality, spurring research into the use of DS as a controlled-release carrier for various active ingredients in drug delivery systems^(8–10). DS intercalated by drug molecules delays and/or targets drug release, increases drug stability and modifies drug delivery patterns⁽¹¹⁾. On the other hand, DS can reinforce the intestinal mucosal barrier and epithelial regeneration⁽¹²⁾. It has anti-inflammatory and anti-diarrhoeal effects in rats, human subjects and pigs^(13–15).

ZnO acts largely within the gastrointestinal tract, as the majority of ingested Zn is excreted in faeces⁽¹⁶⁾. Controlling

Abbreviations: DS, diosmectite; DS–ZnO, diosmectite–ZnO composite; EGF, epidermal growth factor; FD4, fluorescein isothiocyanate dextran 4 kDa; IFN- γ , interferon- γ ; TER, transepithelial electrical resistance; ZO-1, zonula occludens-1; TJ, tight junction.

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the delivery and release of ZnO in the gastrointestinal tract may improve its effectiveness⁽³⁾. The ion exchange nature and biocompatibility of DS makes it a potential controlled-release carrier for ZnO delivery. DS–ZnO composite (DS–ZnO) has recently been synthesised by a sol–gel intercalation reaction^(17,18). Our previous dose–response test has shown that supplementation with DS–ZnO at 500 mg Zn/kg alleviated post-weaning diarrhoea and improved intestinal mucosal integrity⁽¹⁸⁾. In the present experiment, we evaluated the efficacy of DS–ZnO as a substitute for high concentration of ZnO (2250 mg Zn/kg). Additionally, a treatment of supplementing the mixture of 2.0 g DS/kg and 500 mg Zn/kg as ZnO (equal amount of DS and ZnO in the DS–ZnO treatment) was used to compare the DS–ZnO's effects. The growth performance, diarrhoea and intestinal morphology of weanling pigs were determined. Intestinal barrier function was assessed by transepithelial electrical resistance (TER) and paracellular flux of fluorescein isothiocyanate dextran 4 kDa (FD4) across the epithelium by the Ussing chamber technique. The expression of pro-inflammatory cytokines and tight junction (TJ) protein was also determined.

Materials and methods

Materials

DS was obtained from Chifeng WHTB Mining Company Limited. The DS content was 99.2%. The cation exchange capacity was 141 mmol/100 g DS. The DS–ZnO was synthesised using a sol–gel intercalation between the colloidal suspension of exfoliated DS nanosheets and the sol solution of ZnO reactants^(17–19). The aqueous solution of zinc chloride was mixed with NaOH solution at the molar ratio of $\text{Zn}^{2+}:\text{OH}^-$ of 1:15 and vigorously stirred at 70°C for 24 h. Then, the aqueous solutions of zinc chloride and NaOH were added into the colloidal suspension of exfoliated DS with continuous stirring at 70°C and reacted for 24 h. The DS–ZnO were separated by centrifugation at a speed of 10 000 g for about 15 min, washed three times with distilled water and dried at 150°C. The Zn content in DS–ZnO was 250 g/kg.

Experimental design and sample collection

All procedures were approved by the Zhejiang University Animal Care and Use Committee. A total of 144 early weaned piglets (Duroc × Landrace × Yorkshire), with an average weight of 5.9 (SEM 0.4) kg and weaned at an age of 21 ± 1 d, were allocated to four treatment groups. Each treatment group had six pens of six piglets. The dietary treatments were as follows: (1) control; (2) DS–ZnO, 500 mg Zn/kg diet; (3) ZnO (feed-grade sources of ZnO containing 78% Zn), 2250 mg Zn/kg diet; and (4) mixture of 2.0 g DS/kg diet and 500 mg Zn/kg diet from ZnO (equal amount of DS and ZnO in the DS–ZnO treatment). Diets were formulated to meet or exceed requirements suggested by the National Research Council⁽²⁰⁾ (Table 1). No antibiotic was included in the diets. The piglets were given *ad libitum* access to feed and water.

Table 1. Ingredients and chemical composition of the basal diet on an as-fed basis

Ingredients (g/kg)	
Maize	562.5
Soyabean meal	292
Fish meal	55
Dried whey	45
Soyabean oil	15
Limestone meal	5
Dicalcium phosphate	11
NaCl	3
L-Lys HCl	1
D,L-Met	0.5
Vitamin–mineral premix*	10
Analysed composition (g/kg)	
Digestible energy (MJ/kg)†	14.3
Moisture	81.2
Crude protein	228.7
Lys	14.1
Met	3.8
Ca	8.9
Total P	7.1
Zn (mg/kg)	125.4

* Provided per kg of diet: vitamin A, 5000 IU (1720 µg); vitamin E, 30 IU (30 mg); vitamin D₃, 400 IU (20 µg); vitamin K₃, 1.0 mg; biotin, 0.10 mg; riboflavin, 5.0 mg; thiamine, 2.0 mg; niacin, 30 mg; pantothenic acid, 20 mg; pyridoxine, 3.0 mg; folic acid, 0.6 mg; vitamin B₁₂, 0.03 mg; choline, 800 mg; Cu, 16 mg (CuSO₄·5H₂O); Fe, 125 mg (FeSO₄); Zn, 100 mg (ZnSO₄); Mn, 15 mg (MnSO₄·H₂O); Se, 0.3 mg (Na₂SeO₃); and I, 0.2 mg (KI).

† Digestible energy was calculated from data provided by Feed Database in China⁽³⁹⁾.

The feeding experiment lasted for 14 d. Average daily gain, average daily feed intake and gain:feed ratio were calculated from days 0 to 7 post-weaning and days 7 to 14 post-weaning.

Post-weaning scour score was monitored according to the previous faecal scoring system from 1 to 5⁽¹⁸⁾: 1 = hard faeces (rarely seen), 2 = normal consistency of faeces formed (no diarrhoea), 3 = soft, partially formed faeces (mild diarrhoea), 4 = loose, semi-liquid faeces (moderate diarrhoea) and 5 = watery faeces (severe diarrhoea). The faecal score of each piglet in a pen was recorded individually every day and the average scour scores per pen were calculated daily.

At 7 or 14 d after weaning, six piglets from each treatment (one pig from every pen) were killed based on average diarrhoea score. The gastrointestinal tract was quickly removed. Segments (20 cm) of the proximal jejunum were removed, immediately placed into bicarbonate Ringer's solution (145 mM-Na⁺, 6.3 mM-K⁺, 2 mM-Ca²⁺, 1 mM-Mg²⁺, 25 mM-HCO₃⁻, 128 mM-Cl⁻, 0.32 mM-PO₄³⁻ and 1 mM-SO₄²⁻; pH 7.4) and mounted in the Ussing chambers⁽²¹⁾. Adjacent specimens were fixed in 10% formalin for morphology measurements. Mucosal scrapings from the remaining jejunum were collected, rapidly frozen in liquid N₂ and stored at –80°C until expression analysis of pro-inflammatory cytokines and TJ protein.

Intestinal morphology

Three cross-sections for each jejunal sample were stained with haematoxylin 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.

Ex vivo Ussing chamber to measure intestinal barrier function

Ussing chamber procedures were performed as described by Hamard *et al.*⁽²¹⁾. Briefly, segments of jejunum were stripped from the seromuscular layer in oxygenated (95% O₂/5% CO₂) Ringer's solution and then mounted in the EasyMount Ussing chamber system with a multi-channel voltage–current clamp (model VCC MC6; Physiologic Instruments). They were bathed on each side with a bicarbonate Ringer's solution, with 10 mM-glucose and 10 mM-mannitol on the serosal and mucosal sides, respectively, and maintained at 37°C. The clamps were connected to Acquire and Analyse software (Physiologic Instruments) for automatic data collection. After a 30-min equilibration period on the Ussing chambers, TER ($\Omega\text{ cm}^2$) and short-circuit current ($\mu\text{A}/\text{cm}^2$) were recorded at 15-min intervals over a 2-h period and then averaged to derive the TER values for a given pig. FD4 (Sigma-Aldrich) was added on the mucosal side at a final concentration of 0.375 mg/ml. Mucosal-to-serosal flux of FD4 (ng/cm^2 per h) was monitored by sampling 100 μl of solution from the serosal side at 30-min intervals for 120 min. The concentrations of FD4 in the serosal side were measured by fluorescence microplate reader (FLx800; Bio-Tek Instruments, Inc.). The flux over the 2-h period was calculated.

Pro-inflammatory cytokine mRNA by real-time PCR

Relative mRNA abundance of *TNF- α* , *IL-6* and interferon- γ (*IFN- γ*) in jejunal mucosa was determined by real-time PCR, as described by Liu *et al.*⁽²²⁾. Briefly, total RNA was extracted using the TRIzol reagent (Invitrogen) and treated with DNase I (Invitrogen Life Technologies) following the manufacturer's guidelines. To amplify complementary DNA fragments, the following sequences of PCR primer were used: forward 5'-ATGGTGAAGGTCGGAGTGAAC-3', reverse 5'-CTCGCTCCTGGAAGATGGT-3' for glyceraldehyde-3-phosphate dehydrogenase (235 bp); forward 5'-CATCGCCGTCTCCTACCA-3', reverse 5'-CCCAGATTCAGCAAAGTCCA-3' for *TNF- α* (199 bp); forward 5'-GAGCCAAATTGTCTCCTTCTAC-3', reverse 5'-CGAAGTCATTGAGTTTCCCAG-3' for *IFN- γ* (140 bp); and forward 5'-CCTGTCCACTGGGCACATAAC-3', reverse 5'-CAAGAAACAACCTGGCTCTGAAAC-3' for *IL-6* (253 bp). Quantitative real-time RT-PCR was performed on a StepOne Plus real-time PCR system (Applied Biosystems) using a SYBR Green Master mix (Promega), according to the kit's instructions. Gene-specific amplification was determined by melting curve analysis and agarose gel electrophoresis. Each sample was run in triplicate. The average C_t was calculated for pro-inflammatory cytokine and GAPDH and the ΔC_t (C_t , cytokine- C_t , GAPDH) was determined, where C_t is the number of cycles required to reach an arbitrary threshold. The $2^{-\Delta\Delta C_t}$ method⁽²³⁾ was used to analyse the relative

expression (fold changes), calculated relative to the control group on day 7 post-weaning.

Tight junction protein immunoblot analysis

TJ protein expressions of zonula occludens-1 (ZO-1), occludin and claudin-1 were measured by Western blotting, as described by Ewaschuk *et al.*⁽²⁴⁾. Briefly, the total protein was extracted as directed by the instructions of a total protein extraction kit (Keygen Biotech). Equal amounts of protein from each treatment (20 μg) were separated by SDS-PAGE on 7.5% (ZO-1) and 10% (claudin-1 and occludin) polyacrylamide gels. Proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Millipore). The following primary antibodies were used: rabbit polyclonal anti-claudin-1 (1:300; Santa Cruz Biotechnology), rabbit polyclonal anti-occludin (1:450; Santa Cruz Biotechnology) and rabbit polyclonal anti-ZO-1 (1:400; Santa Cruz Biotechnology). Blots were also probed with anti-actin antibodies. The secondary antibody was horseradish peroxidase-conjugated anti-rabbit antibody (Santa Cruz Biotechnology). An enhanced chemiluminescence detection kit (Amersham) was used to detect the positive bands. Densitometric analysis for protein bands were carried out using ImageQuant software (Molecular Dynamics). The value of protein expression was the ratio of the densitometry units of TJ protein and β -actin. The control sample on day 7 post-weaning was used as the reference sample. The protein expression of all samples was expressed as fold changes, calculated relative to the control group on day 7 post-weaning.

Statistical analysis

Data were analysed using the SAS statistical package (version 8.1; SAS Institute)⁽²⁵⁾. A pen of pigs served as the experimental unit for all data. Differences among means were tested using Duncan's multiple range tests. Differences were considered significant at $P < 0.05$.

Results

Growth performance and post-weaning scour scores

Table 2 shows the growth performance and post-weaning scour scores of newly weaned pigs. As compared with the control, dietary addition of DS-ZnO at 500 mg Zn/kg or ZnO at 2250 mg Zn/kg improved ($P < 0.05$) average daily gain and average daily feed intake and decreased ($P < 0.05$) faecal scores on both days 0–7 post-weaning and days 7–14 post-weaning. The weaned piglets fed DS-ZnO at 500 mg Zn/kg did not differ in average daily gain, average daily feed intake and post-weaning scour scores from those fed ZnO at 2250 mg Zn/kg ($P > 0.05$), while they had better performance than those fed the mixture of equal amount of DS and ZnO. Supplementation with the mixture of DS and ZnO had no effect on post-weaning scour scores of piglets.

Table 2. Efficacy of diosmectite–zinc oxide composite (DS-ZnO) on growth performance and post-weaning scour scores of weanling pigs

(Mean values with their standard errors)

	Control	DS + ZnO*	500 mg Zn/kg as DS-ZnO†	2250 mg Zn/kg as ZnO	SEM
Days 0–7 post-weaning					
ADG (g)	155 ^b	163 ^b	182 ^a	178 ^a	4.8
ADFI (g)	186 ^b	195 ^b	216 ^a	214 ^a	6.0
Gain:feed (g/kg)	833	836	843	832	22.5
Faecal scores	3.97 ^a	3.75 ^a	1.81 ^b	1.61 ^b	0.12
Days 7–14 post-weaning					
ADG (g)	307 ^c	317 ^{b,c}	344 ^a	337 ^{a,b}	8.1
ADFI (g)	397 ^b	406 ^{a,b}	434 ^a	430 ^a	10.8
Gain:feed (g/kg)	773	781	793	784	18.1
Faecal scores	2.53 ^a	2.32 ^a	1.30 ^b	1.41 ^b	0.08

ADG, average daily gain; ADFI, average daily feed intake.

^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

* Supplemental mixture of 2.0 g DS/kg diet and 500 mg Zn/kg diet from ZnO (equal amount of DS and ZnO in the DS-ZnO group).

† DS-ZnO, DS–ZnO composite, containing 250 g Zn/kg DS-ZnO.

Jejunal morphology of weaned piglets

Table 3 shows jejunal morphology of piglets on days 7 and 14 post-weaning. Supplementation with DS-ZnO at 500 mg Zn/kg or ZnO at 2250 mg Zn/kg had both higher ($P < 0.05$) villus height and the ratio of villus height and crypt depth at the jejunal mucosa, as compared with the control. The weaned piglets fed DS-ZnO at 500 mg Zn/kg did not differ in jejunal morphology from those fed ZnO at 2250 mg Zn/kg ($P > 0.05$), while they had higher ($P < 0.05$) villus height and the ratio of villus height and crypt depth on day 7 post-weaning than those fed the mixture of DS and ZnO. Supplemental mixture of DS and ZnO had no ($P > 0.05$) effect on jejunal morphology.

Intestinal barrier function

Table 4 shows the intestinal barrier function of piglets on days 7 and 14 post-weaning, which was reflected by TER and paracellular flux of FD₄ measured in the Ussing chambers. As compared with the control, dietary addition of DS-ZnO at 500 mg Zn/kg or ZnO at 2250 mg Zn/kg decreased ($P < 0.05$) FD₄ flux on both days 7 and 14 post-weaning. The piglets fed DS-ZnO at 500 mg Zn/kg did not differ in FD₄ flux from those fed ZnO at 2250 mg Zn/kg ($P > 0.05$),

while they had lower ($P < 0.05$) FD₄ flux than those fed the mixture of DS and ZnO. Supplemental mixture of DS and ZnO had no ($P > 0.05$) effect on jejunal barrier function of weaned pigs. The TER and short-circuit current were not affected by dietary treatments ($P > 0.05$).

Pro-inflammatory cytokine mRNA

The mRNA levels of *TNF-α*, *IL-6* and *IFN-γ* in jejunal mucosa on days 7 and 14 post-weaning are presented in Table 5. As compared with control, supplementation with DS-ZnO at 500 mg Zn/kg or ZnO at 2250 mg Zn/kg both decreased ($P < 0.05$) mucosal mRNA of *TNF-α*, *IL-6* and *IFN-γ* on day 7 post-weaning. The piglets fed DS-ZnO at 500 mg Zn/kg did not differ in cytokine mRNA from those fed ZnO at 2250 mg Zn/kg ($P > 0.05$), while they had lower ($P < 0.05$) mRNA levels of *TNF-α* and *IL-6* on day 7 post-weaning compared with those fed the mixture of DS and ZnO. The mRNA levels of *TNF-α*, *IL-6* and *IFN-γ* on day 14 post-weaning were not affected by dietary treatments.

Tight junction protein expression

Fig. 1 shows the protein expression levels of occludin, claudin-1 and ZO-1 in jejunal mucosa on days 7 and 14

Table 3. Effects of diosmectite–zinc oxide (DS-ZnO) on jejunal morphology of weanling pigs

(Mean values with their standard errors)

	Control	DS + ZnO*	500 mg Zn/kg as DS-ZnO†	2250 mg Zn/kg as ZnO	SEM
On day 7 post-weaning					
Villus height (μm)	676 ^b	723 ^b	806 ^a	817 ^a	25.7
Crypt depth (μm)	382	368	351	347	12.5
Villus height: crypt depth	1.77 ^b	1.96 ^b	2.30 ^a	2.35 ^a	0.08
On day 14 post-weaning					
Villus height (μm)	761 ^b	785 ^{a,b}	845 ^a	830 ^{a,b}	26.9
Crypt depth (μm)	364	352	336	345	11.9
Villus height: crypt depth	2.09 ^c	2.23 ^{b,c}	2.51 ^a	2.41 ^{a,b}	0.09

^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

* Supplemental mixture of 2.0 g DS/kg diet and 500 mg Zn/kg diet from ZnO (equal amount of DS and ZnO in the DS-ZnO group).

† DS-ZnO, DS–ZnO composite, containing 250 g Zn/kg DS-ZnO.

Table 4. Effect of diosmectite–zinc oxide (DS-ZnO) on jejunal barrier function of weanling pigs (Mean values with their standard errors)

	Control	DS + ZnO*	500 mg Zn/kg as DS-ZnO†	2250 mg Zn/kg as ZnO	SEM
On day 7 post-weaning					
TER (Ω cm ²)	50.7	53.4	56.2	54.3	2.80
FD4 flux (μ g/cm ² per h)	2.46 ^a	2.18 ^a	1.61 ^b	1.45 ^b	0.13
Isc (μ A/cm ²)	13.0	13.2	12.7	12.5	0.34
On day 14 post-weaning					
TER (Ω cm ²)	52.5	54.5	58.7	59.2	3.02
FD4 flux (μ g/cm ² per h)	2.03 ^a	1.82 ^a	1.23 ^b	1.31 ^b	0.12
Isc (μ A/cm ²)	11.1	10.8	10.4	10.5	0.36

TER, transepithelial electrical resistance; FD4, fluorescein isothiocyanate dextran 4 kDa; Isc, short-circuit current.

^{a,b} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

* Supplemental mixture of 2.0 g DS/kg diet and 500 mg Zn/kg diet from ZnO (equal amount of DS and ZnO in the DS-ZnO group).

† DS-ZnO, DS–ZnO composite, containing 250 g Zn/kg DS-ZnO.

post-weaning. As compared with control, supplementation with DS-ZnO at 500 mg Zn/kg or ZnO at 2250 mg Zn/kg both increased ($P < 0.05$) protein expression levels of occludin, claudin-1 and ZO-1 on day 7 post-weaning, and protein expression of occludin and ZO-1 on day 14 post-weaning. The piglets fed DS-ZnO at 500 mg Zn/kg did not differ in TJ proteins expression from those fed ZnO at 2250 mg Zn/kg ($P > 0.05$), while they had higher ($P < 0.05$) protein expression levels of occluding and ZO-1 compared with those fed the mixture of DS and ZnO. Supplemental mixture of DS and ZnO had no ($P > 0.05$) effect on TJ proteins' expression.

Discussion

Early weaned piglets frequently encounter growth impairment and post-weaning diarrhoea, which induce major economic losses for pig producers^(2,3). The importance of Zn in the treatment of acute and persistent diarrhoea is well-known⁽⁵⁾. The addition of a high concentration of ZnO to the weaning diet (between 2000 and 4000 mg/kg of Zn as ZnO) alleviates post-weaning diarrhoea and increases growth, although the mechanisms underlying these effects have not been fully illuminated^(3,6,16). In the present study, supplementation with

ZnO at 2250 mg Zn/kg in weaned pigs improved feed intake and growth and decreased post-weaning diarrhoea, which is consistent with previous reports. The increased feed intake might have beneficial effects on the barrier function and inflammation, as weaning anorexia may contribute to local inflammation in the piglet small intestine⁽²⁶⁾.

In the present experiment, a supplemental mixture of 2.0 g DS/kg and 500 mg Zn/kg had no influence on post-weaning diarrhoea, inflammation and intestinal barrier function. In contrast to our findings, it was reported that DS was effective in anti-diarrhoea, anti-inflammation and intestinal mucosal restoration in human subjects and pigs^(13–15). DS may adhere to gastrointestinal mucous membranes and reinforce the physical mucous barrier⁽¹²⁾. Its anti-diarrhoeal mechanisms involve adsorption of toxins and bacteria, modifications of mucus rheological properties and modulatory action of cytokine production^(13,14). The possible reason for the ineffectiveness of DS in the present experiment may be that the inclusion of DS (2.0 g/kg) was too low. The dosage of DS in anti-diarrhoeal remedy was suggested to be 20–30 g/kg⁽¹⁵⁾. Gonzalez *et al.*⁽¹³⁾ found that the anti-inflammatory effect of DS was dose-dependent. The present result, that supplementation with ZnO at 500 mg Zn/kg had no influence on growth performance and post-weaning diarrhoeal of

Table 5. Relative mRNA levels of pro-inflammatory cytokines in jejunal mucosa of piglets* (Mean values with their standard errors)

	Control	DS + ZnO†	500 mg Zn/kg as DS-ZnO‡	2250 mg Zn/kg as ZnO	SEM
On day 7 post-weaning					
TNF- α	1.00 ^a	0.69 ^a	0.20 ^b	0.28 ^b	0.12
IL-6	1.00 ^a	0.81 ^a	0.34 ^b	0.30 ^b	0.15
Interferon- γ	1.00 ^a	0.77 ^{a,b}	0.41 ^b	0.32 ^b	0.16
On day 14 post-weaning					
TNF- α	0.36	0.25	0.26	0.20	0.07
IL-6	0.25	0.31	0.28	0.36	0.11
Interferon- γ	0.24	0.27	0.35	0.21	0.09

DS-ZnO, diosmectite–ZnO composite.

^{a,b} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

* The 2^{- $\Delta\Delta C_t$} method was used to analyse the relative expression (fold changes), calculated relative to the control group on day 7 post-weaning. Data are means of six pigs, each in triplicate.

† Supplemental mixture of 2.0 g DS/kg diet and 500 mg Zn/kg diet from ZnO (equal amount of DS and ZnO in the DS-ZnO group).

‡ DS-ZnO, DS–ZnO composite, containing 250 g Zn/kg DS-ZnO.

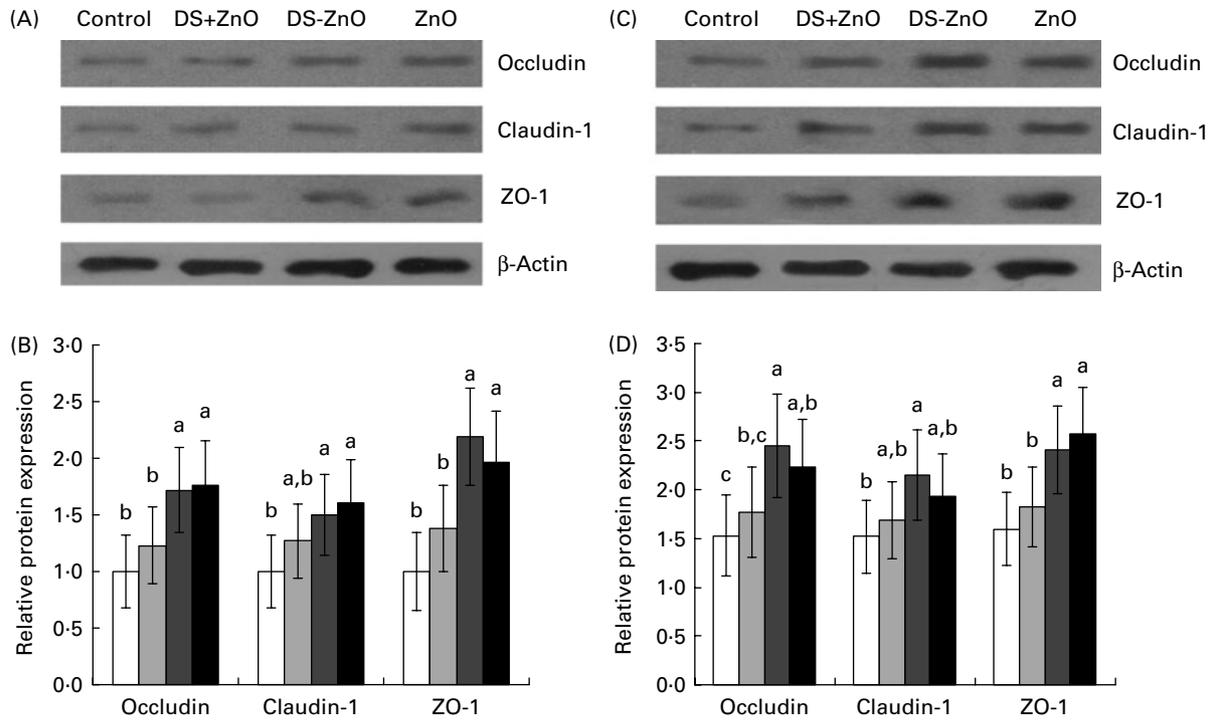


Fig. 1. Relative tight junction protein expressions in jejunal mucosa of piglets. (A) and (C) are representative blots of occludin, claudin, zonula occludens-1 (ZO-1) and β -actin on days 7 and 14 post-weaning, respectively. (B) and (D) show relative tight junction proteins expression on days 7 and 14 post-weaning, respectively. Values are means and standard deviations represented by vertical bars. ^{a,b,c} Mean values with unlike letters were significantly different ($P < 0.05$). DS-ZnO, diosmetite-zinc oxide composite, containing 250 g zinc/kg DS-ZnO. DS + ZnO, mixture of 2.0 g DS/kg diet and 500 mg zinc/kg diet from zinc oxide (equal amount of DS and zinc oxide in the DS-ZnO group). The value of protein expression was the ratio of the densitometry units of tight junction protein and β -actin. The control sample on day 7 post-weaning was used as the reference sample. The protein expression of all samples was expressed as fold changes, calculated relative to the control group on day 7 post-weaning. □, Control; ▤, DS+ZnO; ▥, DS-ZnO; ■, ZnO.

piglets, was consistent with previous research. Davis *et al.*⁽²⁷⁾ found that supplementing ZnO to provide 500 parts per million Zn in weaning pig diets did not result in any benefit. Hollis *et al.*⁽²⁸⁾ reported no growth improvement when supplementing ZnO at levels less than 1000 mg/kg in weaning pigs.

To decrease inclusion of ZnO and to achieve the same effect of high levels of ZnO, we used DS as a controlled-release carrier for ZnO delivery. DS has been researched as an effective drug delivery carrier for controlled-release of bioactive molecules, drugs and nutrients, such as epidermal growth factor (EGF)⁽⁸⁾, ibuprofen⁽⁹⁾ and vitamin B₆⁽¹⁰⁾. In the present study, we functionalised DS with ZnO via ion exchange reaction to create a composite (DS-ZnO). Khaorapong *et al.*⁽¹⁷⁾ found that ZnO was located between the inter-layer space and on the surface of DS. The present experiment demonstrated that the growth performance and post-weaning diarrhoea of weaned piglets fed DS-ZnO at 500 mg Zn/kg was comparable with those fed ZnO at 2250 mg Zn/kg, while significantly better than those fed the equivalent amount of the mixture of DS and ZnO. Zheng *et al.*⁽⁹⁾ intercalated ibuprofen into DS to synthesise an ibuprofen/DS composite as a drug release system. The *in vitro* release experiment showed that the release of ibuprofen from the ibuprofen/DS composite in simulated intestinal fluid was markedly higher than that in simulated gastric fluid. Vaiana *et al.*⁽⁸⁾ used DS as a delivery mechanism for EGF and found that the DS-EGF composite

enhanced both the haemostatic and the proliferative stages of epithelial wound healing. They observed a strong bond between EGF and DS after several days of incubation in complete media, suggesting that DS-EGF remains largely intact. Controlling the release of ZnO in the gastrointestinal tract may improve its effectiveness⁽³⁾. A lipid-coated ZnO has been claimed to prevent the absorption or chemical change of ZnO in the stomach, allowing it to enter the upper intestine where the lipid coating is broken down by lipase enzymes⁽³⁾. This releases the ZnO in the critical area of the gastrointestinal tract for maximum effectiveness⁽³⁾. DS served as a controlled-release carrier that might modify the rate and site of ZnO release. However, a better understanding of the binding and release properties of DS-ZnO is needed to fully characterise the mechanism of DS-ZnO.

Compromising alterations in villus-crypt structure are common in weaned pigs. Weaning is associated with villus atrophy and crypt hyperplasia^(3,29). The shorter villus and deeper crypt observed in the control treatment verified the deterioration of intestinal morphology induced by weaning. Dietary inclusion of DS-ZnO at 500 mg Zn/kg or ZnO at 2250 mg Zn/kg reduced the weaning-associated damage to small intestinal morphology. The small-intestinal epithelium serves as a barrier against noxious antigens and pathogens. Impaired intestinal barrier function may increase the translocation of intestinal bacteria and the entering of toxic

or allergenic substances from the gut into the body⁽²⁾. It has been reported that the early weaning process is associated with intestinal barrier dysfunction of piglets^(2,3,30). Zn supplementation improved mucosal repair in rats with experimental colitis⁽³¹⁾, and reduced intestinal permeability in children with acute diarrhoea and persistent diarrhoea syndromes⁽⁵⁾. However, little data are available regarding the addition of pharmacological doses of ZnO on intestinal permeability in weaned pigs. In the present experiment, the *ex vivo* Ussing chamber was used to monitor intestinal permeability. The flux of intact FD4 across the intestinal epithelium occurs mainly through paracellular pathways⁽²¹⁾. An increased flux of FD4 reflects an impaired intestinal barrier. Supplementation with DS-ZnO at 500 mg Zn/kg or ZnO at 2250 mg Zn/kg reduced the paracellular flux of FD4 across the epithelium, indicating that the intestinal barrier function of weaned pigs was improved. Our findings are similar to another study⁽³²⁾, which used urinary recovery of lactulose and mannitol to assess paracellular permeability and reported that addition of ZnO at 2000 mg Zn/kg decreased intestinal permeability of weaned piglets.

Weaning-associated intestinal inflammation and activation of the mucosal immune system have been described in the weanling rat⁽³³⁾. Pié *et al.*⁽¹⁾ also reported the up-regulated expression of pro-inflammatory cytokines in the intestine in newly weaned pigs. Over-production of pro-inflammatory cytokines has an adverse effect on intestinal mucosal integrity⁽²²⁾. Recent studies have indicated that most pro-inflammatory cytokines, such as TNF- α , IFN- γ , IL-1 β and IL-6, induce a pathologic opening of the intestinal TJ barrier and increase intestinal epithelial permeability⁽³⁴⁾. The cytokine TNF- α can act synergistically with INF- γ to induce structural changes in TJ⁽³⁵⁾. Controlling the release of intestinal pro-inflammatory cytokines may have potential benefits in alleviating gut disorders induced by weaning stress⁽²²⁾. *In vitro*, ZnO reduced inflammatory response of porcine epithelial IPEC J2 cells to enterotoxigenic *Escherichia coli* infection⁽⁴⁾, and counteracted the up-regulated expression of the inflammatory *IL-8*, *TNF- α* mRNA levels caused by *E. coli* K₈₈ using Caco-2 enterocytes⁽³⁵⁾. In the present study, dietary addition of DS-ZnO at 500 mg Zn/kg or ZnO at 2250 mg Zn/kg decreased mucosal mRNA of *TNF- α* , *IL-6* and *IFN- γ* on day 7 post-weaning, while it did not affect pro-inflammatory cytokine mRNA on day 14 post-weaning. It has been demonstrated that weaning is associated with a transient up-regulation of inflammatory cytokine mRNA content on days 3 to 4 post-weaning, and that the levels of most cytokines rapidly return to pre-weaning values after day 9 post-weaning⁽¹⁾. This might be why the mRNA levels of *TNF- α* , *IL-6* and *IFN- γ* on day 14 post-weaning were not affected by the dietary treatments in the present study. The down-regulation of pro-inflammatory cytokine on day 7 post-weaning in the presence of DS-ZnO indicated that weaning-induced inflammation was minimised. It has also been shown that activation of mast cells and release of mast cell proteases play a major role in the intestinal barrier dysfunction during the post-weaning period⁽³⁶⁾. Furthermore, Ou *et al.*⁽³⁷⁾ suggest a link between ZnO and mast cells in the porcine gut.

The intestinal barrier is mainly formed by a layer of epithelial cells joined together by TJ⁽³⁸⁾. TJ consist mainly of the transmembrane protein complexes (e.g. claudins and occludins) and the cytosolic proteins ZO (e.g. junctional adhesion molecule, ZO-1, ZO-2 and ZO-3)⁽³⁾. These proteins form a structure at the boundary of two adjacent cells, working as a barrier within the epithelial cell space. The TJ proteins are the rate-limiting step in the paracellular pathway and form a selectively permeable barrier⁽³⁸⁾. The early weaning-induced up-regulation of pro-inflammatory cytokine might elicit alterations of TJ protein expression, because pro-inflammatory cytokine have been shown to down-regulate TJ protein expression⁽³⁴⁾. The present study indicated that supplementation with DS-ZnO increased the protein expression of ZO-1, claudin-1 and occludin.

Overall, the results demonstrated that the weaned piglets fed DS-ZnO at 500 mg Zn/kg did not differ in growth performance, post-weaning diarrhoea and intestinal barrier function from those fed ZnO at 2250 mg Zn/kg, while they had better performance than those fed the equivalent amount of mixture of DS and ZnO. Dietary inclusion of DS-ZnO at 500 mg Zn/kg may alleviate diarrhoea and improve intestinal barrier function by down-regulation of pro-inflammatory cytokines and up-regulation of TJ proteins.

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