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Manno-oligosaccharide attenuates inflammation and intestinal epithelium injury in weaned pigs upon enterotoxigenic *Escherichia coli* K88 challenge

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Abstract

To explore the effect of manno-oligosaccharide (MOS) on intestinal health in weaned pigs upon enterotoxigenic *Escherichia coli* K88 (ETEC) challenge, thirty-two male weaned pigs were randomly assigned into four groups. Pigs fed with a basal diet or basal diet containing MOS (0·6 g/kg) were orally infused with ETEC or culture medium. Results showed that MOS significantly elevated the digestibility of crude protein and gross energy in both ETEC-challenged and non-challenged pigs ($P < 0·05$). MOS also elevated serum concentrations of IgA and IgM ($P < 0·05$), but decreased serum concentrations of TNF-α, IL-1β and IL-6 ($P < 0·05$) in ETEC-challenged pigs. Interestingly, MOS increased villus height and the ratio of villus height:crypt depth in duodenum and ileum ($P < 0·05$). MOS also increased duodenal sucrase and ileal lactase activity in ETEC-challenged pigs ($P < 0·05$). MOS decreased the abundance of *E. coli*, but increased the abundance of *Lactobacillus*, *Bifidobacterium* and *Bacillus* in caecum ($P < 0·05$). Importantly, MOS not only elevated the expression levels of zonula occludens-1 (ZO-1), claudin-1 and GLUT-2 in duodenum ($P < 0·05$) but also elevated the expression levels of ZO-1, GLUT-2 and L-type amino acid transporter-1 in ileum ($P < 0·05$) upon ETEC challenge. These results suggested that MOS can alleviate inflammation and intestinal injury in weaned pigs upon ETEC challenge, which was associated with suppressed secretion of inflammatory cytokines and elevated serum Ig, as well as improved intestinal epithelium functions and microbiota.

Key words: **Manno-oligosaccharide: Inflammation: Weaned pigs: Intestinal epithelium: Immunity**

Enterotoxigenic *Escherichia coli* (ETEC) is one of the major bacterial causes leading to post-weaning diarrhoea in pigs[1,2]. Currently, the pathogenesis of diarrhoea induced by ETEC has been studied in detail. For instance, several ETEC strains were found to function through producing enterotoxins that act on the small intestines and result in the secretion of fluids and electrolytes, causing diarrhoea and intestinal injury[3,4]. In last decades, antibiotics have been widely used to prevent various ETEC-induced diarrhoeas in pig production. Moreover, antibiotics also showed a significant growth-promoting effect on pigs and other domestic animals[5]. However, long-term utilisation or over-dose utilisation of antibiotics may result in residues in animal products and contribute to increased abundance of antibiotic resistant bacteria[5,6]. Therefore, developing of substitutes for conventionally used antibiotics has attracted considerable research interest worldwide.

Prebiotics are one of the favourable alternatives, as they can promote competitive exclusion of pathogenic microbes and selective colonisation by beneficial microbes[7]. Oligosaccharides were believed to be the main form of prebiotics, which is a type of carbohydrate of low degree of polymerisation and low molecular weight composed of monosaccharides[8,9]. Manno-oligosaccharides (MOS) are non-digestible oligosaccharides produced by enzymatic degradation of mannan polymers. Currently, most of the MOS products are derived from sugar polymers present in the cell wall of yeast[4]. The MOS were previously found to improve human health by promoting the growth of intestinal beneficial microflora, decreasing enteric pathogenic bacteria and

Abbreviations: CMOS, basal diet containing 3 g/kg manno-oligosaccharide product; CON, basal diet; ECON, basal diet and challenge by enterotoxigenic *Escherichia coli*; EMOS, basal diet containing 3 g/kg manno-oligosaccharide product and challenge by enterotoxigenic *Escherichia coli*; ETEC, enterotoxigenic *Escherichia coli*; LAT-1, L-type amino acid transporter-1; LB, Luria-Bertani; MOS, manno-oligosaccharide; V:C, villus height:crypt depth; ZO-1, zonula occludens-1.

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reducing fat absorption[4,10–12]. MOS has also been widely used in animal nutrition and feed industry. For instance, dietary supplementation of MOS was found to improve the growth performance and intestinal development in pigs and poultry[13–15].

Although numerous studies suggested a prebiotic effect of MOS on pigs and other animal species[14,16–18], few of them explored the effect of MOS on intestinal health in weaned pigs exposure to ETEC challenge. Moreover, most studies on pigs utilised the *Salmonella* as pathogenic bacteria, and the ETEC was only utilised *in vitro*[19,20]. As compared with the *Salmonella*, ETEC can specifically induce intestinal injury by adhesion to intestinal epithelial cells[3,4]. Therefore, the ETEC-challenged model was utilised in this study. We hypothesis that MOS may attenuate bacteria-induced inflammation, as the prebiotics are believed to beneficially manipulate the gut microflora and decrease enteric pathogenic bacteria[10–12]. In this study, we explored the effect of dietary MOS supplementation on nutrient digestibility, inflammatory response, intestinal epithelium function and selected bacterial populations in weaned pigs upon ETEC challenge. This study will also provide convincing evidence on the novel prebiotic effect of MOS and offer key insights into its potential mechanisms of action.

Materials and methods

All experimental protocols used in the animal experiment were approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University (no. 20181105). MOS (≥ 20 %) was purchased from the Shanghai Lanpu Bio. Co. Ltd. The product is derived from the degradation of konjac glucomannan by glucomannan hydrolase. It is a series of glucomannan oligosaccharides with degree of polymerisation 2–9, which is composed of glucose and mannose units through β-1,4 glycosidic bonds in a ratio of approximately 1:1·5.

Bacterial strains and culture

ETEC (serotype O149:K91: K88ac) was obtained from the China Institute of Veterinary Drugs Control (Beijing, China). Luria-Bertani (LB) and LB agar broth were prepared (yeast extract, 0·5 g; NaCl, 1 g; peptone, 1 g; double-distilled water, 50 ml; agar powder, 2 % for LB agar broth) and sterilised under 121°C, 0·11 MPa for 20 min (pH = 6). The bacteria were resuscitated in 3 ml of LB broth at 37°C for 24 h with shaking and plated onto LB agar. A single colony was inoculated into 50 ml of LB broth, cultured overnight at 37°C and 250 rpm and then subcultured and serially diluted on LB agar for bacterial enumeration[21].

Animal diets and experimental design

A total of thirty-two male Duroc × Landrace × Yorkshire pigs weaned at 21 d (with an average body weight of 6·48 (SE 0·14) kg) were randomly allotted into a 2 (MOS) × 2 (ETEC) factorial experiment of four treatments composed of CON (pigs were fed with a basal diet), CMOS (pigs were fed with basal diet containing 3 g/kg MOS product), ECON (pigs were fed with a basal diet and challenged by ETEC) and EMOS (pigs were fed with basal diet containing 3 g/kg MOS product and challenged by ETEC). As the purity of the MOS product is 20 %, the final concentration of the pure MOS in the diet is 0·6 g/kg. The number of pigs used in each group (n 8) meets this minimal requirement of statistic for digestible trials or mechanism studies (e.g. six replicates are acceptable)[21,22]. Pigs received the same parental nutrition and management (e.g. sows were fed with the same diet, synchronisation estrous). The trial lasted for 21 d. On 19th day, the challenge groups were orally treated with 100 ml of LB culture containing 1×10^{10} colony-forming units/ml of ETEC by using an orogastric tube lasted for 3 d, whereas the non-challenge groups were orally treated with equivalent amount of culture medium[23]. The basal diet (Table 1) was formulated to meet the swine nutrient requirements recommended by the National Research Council[24]. Pigs were individually housed in 1·5 × 0·7 m^2 metabolism cage and were given *ad libitum* access to feed and fresh water with the room temperature controlled between 25 and 28°C, relative humidity 65 % (SE 5) %.

Table 1. Experiment basal diet composition and nutrient level

Ingredients	%	Nutrient level	Contents
Maize	28·31	Digestible energy (calculated, MJ/kg)	14·78
Extruded maize	24·87	Crude protein (%)	19·68
Soyabean meal	8·50	Ca (%)	0·81
Extruded full-fat soyabean	10·30	Available P (%)	0·55
Fishmeal	4·20	Lysine	1·35
Whey powder	7·00	Methionine	0·42
Soyabean protein concentrate	8·00	Methionine + cysteine	0·60
Soyabean oil	2·00	Threonine	0·79
Sucrose	4·00	Tryptophan	0·22
Limestone	0·90		
Dicalcium phosphate	0·50		
NaCl	0·30		
L-Lysine HCl (78 %)	0·47		
DL-Methionine	0·15		
L-Threonine (98·5 %)	0·13		
Tryptophan (98 %)	0·03		
Chloride choline	0·10		
Vitamin premix*	0·04		
Mineral premix†	0·20		
Total	100		

* The vitamin premix provided the following per kg of diet: 2·7 mg of vitamin A, 75 μg of vitamin D_3, 20 mg of vitamin E, 3 mg of vitamin K_3, 1·5 mg of vitamin B_1, 4 mg of vitamin B_2, 3 mg of vitamin B_6, 0·02 mg of vitamin B_{12}, 30 mg of niacin, 15 mg of pantothenic acid, 0·75 mg of folic acid and 0·1 mg of biotin.

† The mineral premix provided the following per kg of diet: 100 mg Fe, 6 mg Cu, 100 mg Zn, 4 mg Mn, 0·30 mg iodine, 0·3 mg Se. The diet was formulated based on the recommendation of the National Research Council[24].

Sample collection

Feed samples were collected at the beginning of the experiment and stored at −20°C for nutrient analysis, during days 14–17 of the experiment; fresh faecal samples were collected immediately after excretion from pigs in each cage, weighed and added 10 ml 10 % H_2SO_4 solution to per 100 g of fresh faecal. The feed and faecal samples were dried at 65°C for 2 d, ground to pass through a 1-mesh screen and then stored at −20°C until measurement for nutrients digestibility. Blood samples were obtained on day 22 by jugular vein puncture and placed in two 10-ml vacuum tubes.

One tube for blood parameters was analysed, and the serum was collected after centrifugation of another tube at 3500 ***g*** and stored at −20°C until the serum indexes analysis. At the end of the trial, pigs were anaesthetised by intravenous injection with sodium pentobarbital (200 mg/kg body weight) and the tissues of the duodenum, jejunum and ileum were immediately isolated. At the same time, the duodenal, jejunal and ileal middle segments (4 cm obtained from the middle of each intestine) were gently flushed with ice-cold PBS, followed by fixation in 4 % paraformaldehyde solution for morphological analyses. Besides, digesta from caecum was collected, and the intestinal mucosa was obtained from the residual intestinal segments with a scalpel blade and placed in frozen tube and then frozen by immersion in liquid N_2 and stored at −80°C until analysis.

Growth performance evaluation

All pigs were weighed before feeding on days 1, 19 and 22. Feed intake and waste feed were recorded every day throughout the experiment which could be provided to calculate the average daily feed intake, average daily gain and the feed:gain ratio (the type of scale used in this study is electronic scale; 0–1, 0–20 kg, respectively).

Apparent total tract nutrient digestibility analysis

The frozen dried and milled feed and faecal samples were used for nutrient digestibility analysis, which uses acid insoluble ash as endogenous indicators, a method described by the Chinese National Standard (GB/T23742-2009). The DM, crude protein, ether extract and ash contents were determined according to AOAC[25], whereas the gross energy content was measured by an adiabatic bomb calorimeter (LECO) to calculate the apparent total tract digestibility. All contents were calculated by the following formula: $(100 - A1F2/A2F1 \times 100)$[26]. A1: digesta nutrient; A2: digesta acid insoluble ash; F1: diet acid insoluble ash; F2: digesta acid insoluble ash.

Serum pro-inflammatory cytokines and Ig detection

The concentration of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6)and Ig (IgG, IgM and IgA) in serum was determined by following the instructions of a commercially available porcine ELISA kits (Jiangsu Jingmei Biotechnology Co. Ltd). All procedures were guided by manuals of the kits. For quantification, the standards provided in the kits were used to generate standard curves.

Histomorphology analysis of each intestinal segment

The fixed intestinal segments were dehydrated through a graded series of ethanol and then embedded in paraffin[27]. Cross sections of each sample were prepared, stained with haematoxylin–eosin (H&E) and then sealed with neutral resin. Finally, ultrathin sections of the duodenal, jejunal and ileal samples were examined for crypt depth and villus height with image processing and analysis system (Image-Pro Plus 6.0). The method of measurement of crypt depth and villus height was followed by King's and Wan's. A total of ten intact, well-oriented, crypt-villus units were analysed in triplicate per intestinal segment[27,28]. The ratio of villus height:crypt depth (V:C) was calculated from the data described above.

Enzyme activity

The frozen duodenal, jejunal and ileal mucosa were homogenated in chilled saline at a ratio of 1:9 (w/v) for 15 min. The homogenate was centrifuged at 3500 ***g*** for 10 min at 4°C, and the supernatant was used in spectrophotometric assays for protein content, which was used to calculate the alkaline phosphatase and digestive enzymes (lactase, sucrase and maltase) activity following the instructions described by commercial kits (Jiancheng Bioengineering Ltd). Enzyme activity was defined as hydrolysis of 1 mol of the substrate per mg of protein tissue per minute under the condition of 37°C, pH = 6·0.

Caecal microbiological analysis

Approximately 200 mg caecal digesta was weighed and treated using the Stool DNA Kits (Omega Bio-Tek) following the manufacturer's instruction to extract total DNA for quantification real-time PCR, which was performed by conventional PCR on the Opticon DNA Engine (Bio-Rad). Total bacteria were detected by the reaction which runs in a volume of 25 μl with 1 μl of forward and 1 μl of reverse primers (100 nM), 12·5 μl SYBR Premix Ex Taq (2 × concentrated), 2 μl template DNA, 1 μl 50 × ROX Reference Dye*3 and 7·5 μl of RNase-Free ddH_2O. The SuperReal PreMix (Probe) kit (Tiangen Biotech Co. Ltd) was used for *Lactobacillus*, *E. coli*, *Bacillus* and *Bifidobacterium* detection, and primers and fluorescent oligonucleotide probes are presented in online Supplementary Table S1. Each reaction was run in a volume of 25 μl with 12·5 μl 2 × Super Real PreMix (Probe), 1 μl of forward and 1 μl of reverse primers (100 nM), 1 μl 50 × ROX Reference Dye*3, 1 μl probe (100 nM), 2 μl DNA and 6·5 μl of RNase-Free ddH_2O. All reaction protocols were composed of one cycle of pre-denaturation at 95°C for 15 min; forty cycles of denaturation at 95°C for 3 s; annealing and extension at 60°C for 30 s. The cycle threshold values and baseline settings were determined by automatic analysis settings, and the copy numbers of the target group for each reaction were calculated from the standard curves, which were generated by constructing standard plasmids by a 10-fold serial dilution of plasmid DNA (1×10^1 to 1×10^9 copies/μl).

Isolation and reverse transcription of RNA from intestinal mucosa and quantitative PCR

The frozen duodenal, jejunal and ileal mucosa samples (about 0·1 g) were ground in liquid N_2 and homogenised in 1 ml of RNAiso Plus (Takara Biotechnology Co. Ltd) to extract total RNA followed the manufacturer's instructions, and the purity and concentration of total RNA were detected by using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Inc.); samples which OD260:OD280 ratio ranged from 1·8 to 2·0 were deemed appropriate. Subsequently, a volume equivalent to 1 μg total RNA from each duodenal, jejunal and ileal sample was used for reverse transcription into cDNA, which was based on the

protocol of PrimeScript™ RT reagent kit with gDNA Eraser (Takara Biotechnology Co. Ltd). This process consists of two steps: I: 37°C for 15 min, II: 85°C for 5 s.

The expression level of the target gene in intestinal mucosa was quantified using quantitative PCR, the oligonucleotide primer sequences used in quantitative PCR are presented in online Supplementary Table S1 and quantitative PCR was performed with the SYBR® Green PCR I PCR reagents (Takara Bio Inc.) using a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories). All cDNA samples were detected in triplicate. The reaction mixture (10 μl) contained 5 μl SYBR Premix Ex Taq II (Tli RNaseH Plus), 0·5 μl forward primer, 0·5 μl reverse primer, 1 μl cDNA and 3 μl RNase-Free water. The protocol used in quantitative PCR was as follows: 95°C for 30 s, followed by 40 cycles: at 95°C for 5 s and 60°C for 34 s. The generated gene-specific amplification products were confirmed by melting curve analysis after each real-time quantitative PCR assay. The housekeeping gene *β*-actin was used to standardise the mRNA expression level of target genes, which was calculated based on the $2^{-\Delta\Delta Ct}$ method[29].

Sample size calculation and statistical analysis

The minimal sample size was calculated based on the experimental design (repeated-measures, between-factors ANOVA) determining the MOS × ETEC effect to the intestinal health as the primary outcome measure. We used G*Power software (version 3.1.9.2) for the power analysis with following variables; the power = 0·8, significant level = 0·05 and effect size = 0·35 in the experiment. The effect size was estimated based on the results from a preliminary study. Hence, the required minimal sample size was five pigs per group.

The data collected before the ETEC challenge were analysed by one-way ANOVA. After the challenge, the data were analysed by two-way ANOVA with the General Linear Model procedure of SPSS as a 2 (MOS) × 2 (ETEC) factorial design. *P* value < 0·05 was deemed to be significant and the *P* value between 0·05 and 0·1 to show a significant trend. Duncan's multiple range test was used based on the ANOVA, which showed a significant difference. All data were analysed by SPSS 24.0 (SPSS, Inc.) and expressed as means with their standard errors.

Results

Effect of manno-oligosaccharides on growth performance and nutrients digestibility in weaned pigs upon enterotoxigenic Escherichia coli *challenge*

As shown in Table 2, the average daily feed intake was significantly lower in the ECON group than in the CMOS group ($P < 0·05$). MOS supplementation did not affect the apparent digestibility of DM, ether extract and CA (Table 3). The apparent digestibility of crude protein and gross energy was higher in the CMOS group than in the CON group ($P < 0·05$). Interestingly, MOS supplementation also increased the apparent digestibility of crude protein and gross energy in the ETEC-challenged pigs ($P < 0·05$).

Effect of manno-oligosaccharides on serum Ig and inflammatory cytokines in weaned pigs upon enterotoxigenic Escherichia coli *challenge*

As shown in Fig. 1, there were no significant differences of serum Ig and inflammatory cytokines between the CON and EMOS groups ($P > 0·05$). ETEC challenge led to a significant reduction of serum concentrations of IgA, IgG and IgM in the weaned pigs (Fig. 1). However, the serum concentrations of IgA and IgG were significantly higher in the EMOS group than in the ECON group ($P < 0·05$). Additionally, the ETEC challenge significantly elevated the serum concentrations of inflammatory cytokines such as the TNF-*α*, IL-1*β* and IL-6 ($P < 0·05$). However, MOS supplementation significantly reduced their concentrations in the ETEC-challenged pigs (Fig. 1).

Effect of manno-oligosaccharides supplementation on intestinal morphology and mucosal enzyme activity in weaned pigs upon enterotoxigenic Escherichia coli *challenge*

There were no significant differences of villus height and the ratio of V:C between the CON and CMOS groups ($P > 0·05$). ETEC challenge decreased the villus height and the ratio of V:C in the duodenum and ileum (Table 4 and Fig. 2). However, MOS supplementation significantly elevated the villus height and the ratio of V:C in the ETEC-challenged pigs ($P < 0·05$). The crypt depth was also lower in the EMOS group than in the ECON group of ileum ($P < 0·05$). As shown in Table 5, the ETEC challenge not only decreased the activity of sucrase in the duodenal mucosa ($P < 0·05$) but also decreased the activities

Table 2. Effect of manno-oligosaccharide (MOS) supplementation on average daily feed intake (ADFI) and average daily gain (ADG) in weaned pigs upon enterotoxigenic *Escherichia coli* (ETEC) challenge
(Mean values with their standard errors; *n* 8)

	Treatments					*P*		
	CON	CMOS	ECON	EMOS	SEM	MOS	ETEC	Interaction
1–19 d								
ADFI (g/d)	430·72	440·41	426·02	434·39	18·96			
ADG (g/d)	292·22	305·89	289·00	301·44	10·63			
F:G	1·47	1·44	1·50	1·43	0·04			
19–21 d								
ADFI (g)	501·80[a]	505·87[a]	463·73[b]	488·60[b,c]	6·94	0·27	0·04	0·42
ADG (g)	396·00	412·67	364·00	393·33	12·94	0·40	0·35	0·82

F:G, feed:gain ratio; CON, basal diet; CMOS, MOS-containing diet (0·6 g/kg); ECON, basal diet and challenge by ETEC; EMOS, MOS-containing diet and challenge by ETEC.
[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P < 0·05$).

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Table 3. Effect of manno-oligosaccharide (MOS) supplementation on nutrient digestibility in weaned pigs
(Mean values with their standard errors; *n* 8)

	Treatments					
	CON	CMOS	ECON	EMOS	SEM	*P*
DM (%)	89·91	90·32	89·55	89·24	0·50	0·89
CP (%)	85·69^{b}	88·14^{a}	85·33^{b}	89·31^{a}	0·57	0·03
EE (%)	79·63	79·74	78·38	79·59	1·44	0·99
Ash (%)	70·06	73·50	68·41	73·73	1·18	0·35
GE (%)	86·96^{b}	90·96^{a}	86·91^{b}	91·03^{a}	0·75	0·04

CP, crude protein; EE, ether extract; CON, basal diet; CMOS, MOS-containing diet (0·6 g/kg); ECON, basal diet and challenge by ETEC; EMOS, MOS-containing diet and challenge by ETEC; ETEC, enterotoxigenic *Escherichia coli*; GE, gross energy.
^{a,b} Mean values within a row with unlike superscript letters were significantly different ($P < 0·05$).

of lactase and maltase in the ileal mucosa ($P < 0·05$). The duodenal sucrase activity and ileal lactase activity were both elevated by MOS in the ETEC-challenged pigs ($P < 0·05$).

Effect of manno-oligosaccharides supplementation on intestinal microbial populations in weaned pigs upon enterotoxigenic Escherichia coli *challenge*

As shown in Table 6, the ETEC challenge increased the abundance of *E. coli* in the caecum. However, MOS supplementation significantly decreased the abundance of *E. coli* in the caecum of ETEC-challenged pigs ($P < 0·05$). Interestingly, the abundance of beneficial bacteria such as the *Lactobacillus*, *Bifidobacterium* and *Bacillus* was higher in the EMOS group than in the ECON group ($P < 0·05$).

Effect of manno-oligosaccharides supplementation on expressions of critical genes involved in intestinal epithelium functions

As shown in Fig. 3, the ETEC challenge not only decreased the expression levels of tight-junction protein zonula occludens-1 (ZO-1) in the duodenum and ileum ($P < 0·05$) but also decreased the expression levels of claudin-1 in the duodenum and jejunum ($P < 0·05$). However, MOS significantly elevated the expression levels of ZO-1 and claudin-1 in the duodenum of ETEC-challenged pigs ($P < 0·05$). The expression levels of GLUT-2 and CAT-1 in the duodenum were significantly lower in the ECON group than in the CON group ($P < 0·05$). Moreover, the expression level of L-type amino acid transporter-1 was also lower in the ECON group than in the CON group in the ileum ($P < 0·05$). However, MOS supplementation not only elevated the expression levels of GLUT2 in the duodenum and ileum ($P < 0·05$) but also elevated the expression level of L-type amino acid transporter-1 in the ileum in the ETEC-challenged pigs ($P < 0·05$).

Discussion

Intestinal tract is the main site of nutrient digestion and absorption and can serve as the first line of defences against various harmful substances or pathogens[30]. For mammalian animals including the pigs, weaning is a critical developmental window which has been indicated by numerous morphological, enzymatic and inflammatory changes in the intestinal tract[31]. At this stage, pigs are susceptible to various bacterial pathogens, such as the ETEC, which may cause injury of the intestinal structure and integrity, consequently, decrease the rate of nutrient

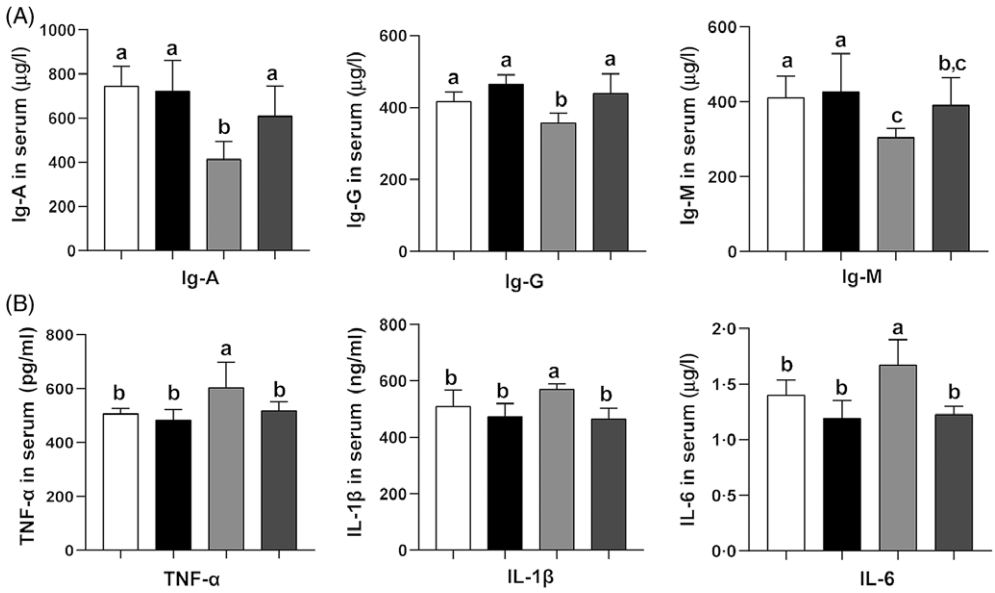

Fig. 1. Effect of manno-oligosaccharide (MOS) supplementation on serum concentrations of inflammatory cytokines and Ig in weaned pigs. (A) IgA, IgG and IgM; (B) TNF-α, IL-1β and IL-6. ^{a,b,c} Mean values within a row with unlike letters were significantly different ($P < 0·05$). CON, basal diet; CMOS, MOS-containing diet (0·6 g/kg); ECON, basal diet and challenge by enterotoxigenic *Escherichia coli* (ETEC); EMOS, MOS-containing diet and challenge by ETEC. (A: IgA) □, CON; ■, CMOS; ▒, ECON; ▓, EMOS; (A: IgG) □, CON; ■, CMOS; ▒, ECON; ▓, EMOS; (A: IgM) □, CON; ■, MOS; ▒, ECON; ▓, EMOS. (B: IgA) □, CON; ■, CMOS; ▒, ECON; ▓, EMOS; (B: IgG) □, CON; ■, CMOS; ▒, ECON; ▓, EMOS; (B: IgM) □, CON; ■, MOS; ▒, ECON; ▓, EMOS.

Table 4. Effect of manno-oligosaccharide (MOS) supplementation on intestinal morphology in weaned pigs upon enterotoxigenic *Escherichia coli* (ETEC) challenge
(Mean values with their standard errors; *n* 8)

	Treatments					*P*		
	CON	CMOS	ECON	EMOS	SEM	MOS	ETEC	Interaction
Duodenum								
Villus height (μm)	471·77[a]	495·92[a]	380·47[b]	468·72[a]	15·00	0·04	0·03	0·22
Crypt depth (μm)	288·52	278·19	343·24	286·98	11·73	0·16	0·18	0·32
V:C	1·64[a]	1·78[a]	1·10[b]	1·63[a]	0·10	0·03	0·02	0·34
Jejunum								
Villus height (μm)	493·19	496·27	450·72	489·06	14·11	0·49	0·41	0·56
Crypt depth (μm)	176·01	175·74	184·17	180·94	5·99	0·89	0·61	0·91
V:C	2·80	2·82	2·36	2·70	0·08	0·30	0·18	0·33
Ileum								
Villus height (μm)	415·06[a]	436·62[a]	317·75[b]	403·84[a]	17·32	0·09	0·04	0·29
Crypt depth (μm)	171·62[b,c]	156·45[b]	203·08[a]	165·91[b]	6·08	0·02	0·05	0·26
V:C	2·42[a]	2·79[a]	1·56[b]	2·43[a]	0·14	0·01	0·01	0·12

V:C, villus height:crypt depth; CON, basal diet; CMOS, MOS-containing diet (0·6 g/kg); ECON, basal diet and challenge by ETEC; EMOS, MOS-containing diet and challenge by ETEC.
[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P < 0·05$).

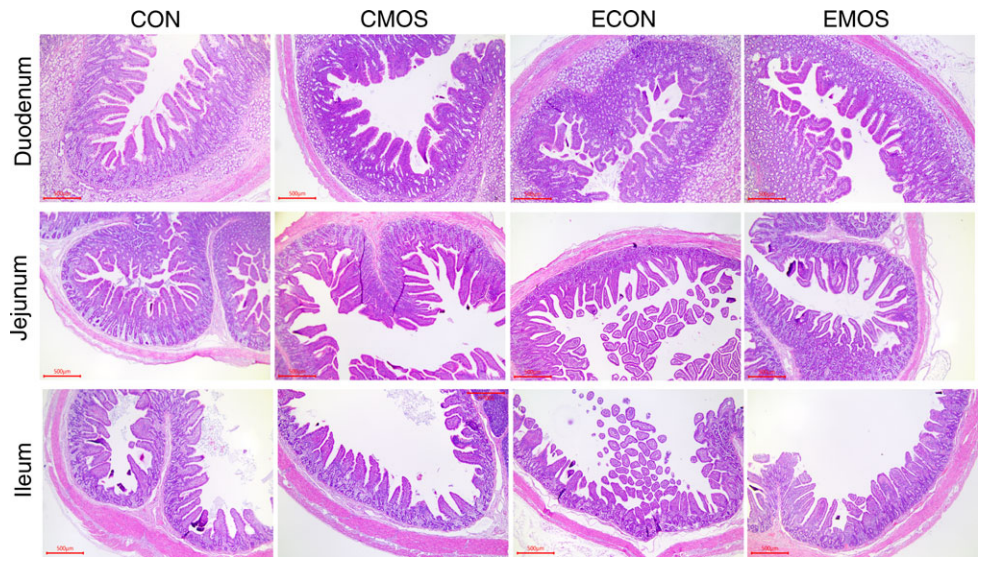

Fig. 2. Effect of manno-oligosaccharide (MOS) supplementation on intestinal morphology in weaned pigs upon enterotoxigenic *Escherichia coli* (ETEC) challenge (haematoxylin–eosin; 40×). CON, basal diet; CMOS, MOS-containing diet (0·6 g/kg); ECON, basal diet and challenge by ETEC; EMOS, MOS-containing diet and challenge by ETEC.

digestion and absorption[21,31,32]. In the present study, we explored the effect of dietary MOS supplementation on nutrient digestibility, inflammatory response and intestinal health in weaned pigs in response to ETEC challenge. We showed that MOS supplementation elevated the digestibility of crude protein and gross energy both in the non-challenged and ETEC-challenged pigs. The results are consistent with previous studies on pigs, broilers and laying hens. Both results indicated that MOS supplementation could improve the digestibility of protein and amino acids[14,26,33].

Pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, have been shown to mediate the host inflammatory process and initiate an effective inflammatory response to pathogen infection[34,35]. In the present study, the ETEC challenge elevated the serum concentrations of TNF-α, IL-1β and IL-6, which is consistent with previous reports that ETEC challenge induced abrupt inflammatory response in pigs[21]. However, MOS supplementation significantly attenuated the inflammatory response, as indicated by decreased serum concentrations of these pro-inflammatory cytokines. Previous studies indicated that dietary MOS supplementation has a beneficial effect on host immunity in weaned pigs and sows[36,37]. In the present study, we found that MOS significantly elevated the serum concentrations of IgA and IgM in the ETEC-challenged pigs. The result is also consistent with previous studies on pigs and turkeys that were challenged by porcine reproductive and respiratory syndrome virus and ETEC K88, respectively[38,39].

Table 5. Effect of manno-oligosaccharide (MOS) supplementation on mucosal enzyme activity in weaned pigs upon enterotoxigenic *Escherichia coli* (ETEC) challenge
(Mean values with their standard errors; *n* 8)

	Treatments					*P*		
	CON	CMOS	ECON	EMOS	SEM	MOS	ETEC	Interaction
Duodenum								
AKP (U/g protein)	1·19[a]	1·20[a]	0·62[a,b]	1·18[a]	0·12	0·44	0·09	0·47
Lactase (U/mg protein)	11·80	12·33	4·43	11·20	1·43	0·19	0·13	0·26
Sucrase (U/mg protein)	103·99[a]	123·45[a]	56·26[b]	102·06[a]	8·73	0·04	0·03	0·38
Maltase (U/mg protein)	16·22	16·53	12·00	13·34	1·03	0·49	0·25	0·83
Jejunum								
AKP (U/g protein)	1·21	1·21	1·15	1·20	0·05	0·81	0·76	0·83
Lactase (U/mg protein)	94·56	98·75	69·66	92·50	9·16	0·50	0·43	0·64
Sucrase (U/mg protein)	536·73	547·85	475·36	526·36	47·70	0·77	0·69	0·85
Maltase (U/mg protein)	517·62	523·32	423·32	519·45	34·18	0·49	0·50	0·54
Ileum								
AKP (U/g protein)	2·37	2·41	2·06	2·16	0·11	0·74	0·22	0·90
Lactase (U/mg protein)	30·91[a]	34·46[a]	15·27[b]	28·21[a]	2·34	0·03	0·006	0·19
Sucrase (U/mg protein)	205·17	210·78	149·09	202·38	19·44	0·48	0·44	0·56
Maltase (U/mg protein)	162·05[a]	191·26[a]	98·31[c]	152·96[b,c]	11·20	0·04	0·01	0·49

APK, alkaline phosphatase; CON, basal diet; CMOS, MOS-containing diet (0·6 g/kg); ECON, basal diet and challenge by ETEC; EMOS, MOS-containing diet and challenge by ETEC.
[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P < 0·05$).

Table 6. Effect of manno-oligosaccharide (MOS) supplementation on caecal microbial populations in weaned pigs upon enterotoxigenic *Escherichia coli* (ETEC) challenge
(Mean values with their standard errors; *n* 8)

	Treatments					*P*		
	CON	CMOS	ECON	EMOS	SEM	MOS	ETEC	Interaction
Caecum (lg(copies/g))								
Total bacteria	10·43[a,b]	10·49[a,b]	10·35[b]	10·77[a]	0·07	0·06	0·40	0·16
Lactobacillus	5·62[b,c]	5·99[a]	4·57[c]	5·74[a]	0·20	0·04	0·08	0·26
Escherichia coli	8·62[b]	8·42[b]	10·17[a]	9·06[b]	0·20	0·04	0·001	0·13
Bifidobacterium	6·68[a]	7·01[a]	5·49[b]	6·97[a]	0·22	0·04	0·07	0·09
Bacillus	8·57[b,c]	8·94[a]	8·13[c]	8·85[a]	0·10	0·002	0·09	0·27

CON, basal diet; CMOS, MOS-containing diet (0·6 g/kg); ECON, basal diet and challenge by ETEC; EMOS, MOS-containing diet and challenge by ETEC.
[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P < 0·05$).

The integrity of intestinal villus-crypt structure is crucial for nutrient digestion and absorption[28]. Disruption of the villus-crypt integrity (e.g. villous shedding, villus atrophy and crypt hyperplasia) may lead to invasion of pathogenic bacteria and growth retardation[32,40]. In the present study, the ETEC challenge significantly decreased the villus height and the ratio of V:C in the duodenum and ileum, indicating injury of the intestinal mucosa. The result is also consistent with previous studies on pigs challenged by ETEC[21,41]. Interestingly, MOS supplementation attenuated the mucosa injury by increasing the villus height and the ratio of V:C. This is probably due to decreased secretion of inflammatory cytokines in the ETEC-challenged pigs by MOS supplementation, as the inflammatory cytokines (e.g. TNF-α and IL-6) were found to be capable of inducing intestinal epithelial cell apoptosis[42]. The beneficial effect of MOS on intestinal morphology was also previously observed on pigs and broilers[13,43]. Moreover, the beneficial effects of MOS supplementation on intestinal heath were indicated by the mucosal enzyme activities, as MOS not only elevated duodenal sucrase activity but also elevated the ileal lactase and maltase activities in the ETEC-challenged pigs.

Oligosaccharides cannot be digested in the upper digestive tract, but can be utilised by beneficial micro-organisms in the lower digestive tract[9,14]. In this study, we also investigated major bacterial populations in the caecum and found that MOS elevated the abundance of beneficial bacteria such as the *Lactobacillus* and *Bacillus* in the non-challenged pigs. Importantly, MOS not only decreased the abundance of *E. coli* but also significantly increased the abundance of *Lactobacillus*, *Bifidobacterium* and *Bacillus* in the ETEC-challenged pigs. Previous studies indicated that oligosaccharides such as the MOS and fructo-oligosaccharides (FOS) not only reduced adhesion of ETEC to the intestinal epithelium[44,45] but also elevated the abundance of beneficial bacteria such as the *Lactobacillus* and *Bifidobacterium* in porcine intestine[46,47]. Moreover, the beneficial effect of MOS on the intestinal microbiota was also observed on other animal species[14,48].

To gain insights into the mechanisms underlying the MOS-regulated intestinal health, we further investigated the expression levels of several critical genes involved in the intestinal epithelium functions. Tight junctions, consisting of cytoplasmic

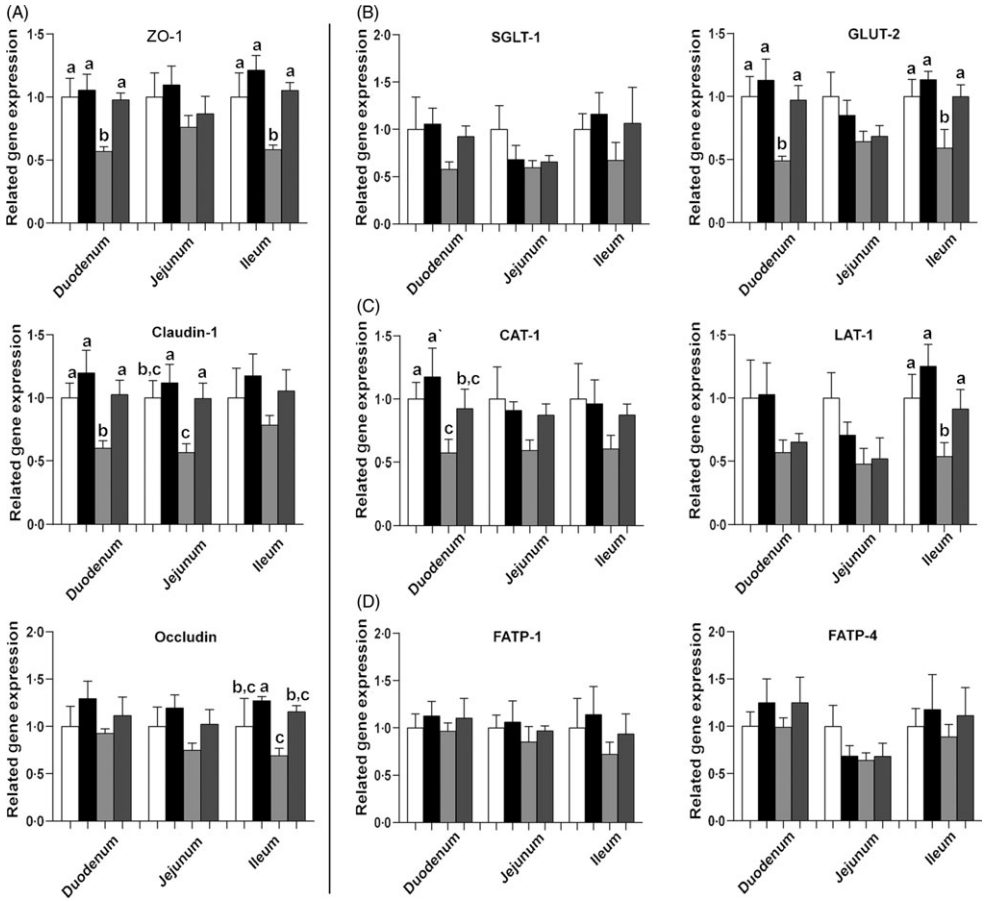

Fig. 3. Effect of manno-oligosaccharide (MOS) supplementation on mucosal gene expressions in weaned pigs upon enterotoxigenic *Escherichia coli* (ETEC) challenge. SGLT-1, sodium/glucose cotransporter-1; CAT-1, cationic amino acid transporter-1; LAT-1, L-amino acid transporter-1; FATP, fatty acid transport proteins; ZO-1, zonula occludens-1. [a,b,c] Mean values with unlike letters were significantly different ($P < 0{\cdot}05$). CON, basal diet; CMOS, MOS-containing diet (0·6 g/kg); ECON, basal diet and challenge by ETEC; EMOS, MOS-containing diet and challenge by ETEC. (A–D) ▭, CON; ■, MOS; ▭, ECON; ■, EMOS.

scaffold proteins such as ZO-1, claudins and occludin, play a critical role in maintaining the intestinal barrier integrity and permeability[49,50]. In this study, ETEC challenge not only decreased the expression levels of ZO-1 in the duodenum and ileum but also decreased the expression levels of claudin-1 in the duodenum and jejunum, indicating disruption of the intestinal tight junctions. The result is consistent with previous studies which indicated that ETEC infection negatively regulated the expression of tight junction proteins[21]. Interestingly, MOS supplementation not only elevated the expression levels of ZO-1 and claudin-1 but also elevated the expression levels of GLUT-2 and L-type amino acid transporter-1 in the ETEC-challenged pigs. GLUT-2 is one of the major transporters for glucose absorption[51]. While the L-type amino acid transporter-1 is responsible for the transportation of L-type amino acids[52]. The elevated expressions of these nutrient transporters indicated an improved intestinal integrity and epithelium functions in pigs' exposure to the ETEC challenge.

Conclusions

Dietary MOS supplementation alleviates ETEC-induced intestinal injury in the weaned pigs, which was associated with suppressing the inflammatory responses and attenuating ETEC-induced changes in microbial populations, gut morphology and gut functions. The beneficial effects of MOS on the intestinal integrity and epithelium functions could make it an attractive prebiotic in the use of animal nutrition and feed industry.

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J. H. conceived and designed the experiments. E. Y. performed the animal trial and wrote the manuscript. D. C., B. Y., X. M., P. Z., J. Y., H. Y. and Z. H. performed biochemical analysis. J. L., Y. L. and H. Y. gave constructive comments for the results and discussion of the manuscript. All authors read and approved the final manuscript.

The authors declare that there are no conflicts of interest.

Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

Supplementary material

For supplementary material referred to in this article, please visit https://doi.org/10.1017/S0007114520004948

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