

Fructooligosaccharides improves growth performance and intestinal epithelium function in weaned pigs exposure to Enterotoxigenic *Escherichia coli*

Lei Liu

Institute of Animal Nutrition, Sichuan Agricultural University

daiwen Chen

Institute of Animal Nutrition, Sichuan Agricultural University

Bing Yu

Institute of Animal Nutrition, Sichuan Agricultural University

Yin Heng

Dalian Institute of Chemical Physics

Zhiqing Huang

Institute of Animal Nutrition, Sichuan Agricultural University

Yuheng Luo

Institute of Animal Nutrition, Sichuan Agricultural University

Ping Zheng

Institute of Animal Nutrition, Sichuan Agricultural University

Xiangbing Mao

Institute of Animal Nutrition, Sichuan Agricultural University

Jie Yu

Institute of Animal Nutrition, Sichuan Agricultural University

Junqiu Luo

Institute of Animal Nutrition, Sichuan Agricultural University

Hui Yan

Institute of Animal Nutrition, Sichuan Agricultural University

Jun He (✉ hejun8067@163.com)

Institute of Animal Nutrition, Sichuan Agricultural University <https://orcid.org/0000-0002-1963-9766>

Research

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Abstract

Background

This study was conducted to explore the protective potential of Fructooligosaccharides (FOS) against Enterotoxigenic *Escherichia coli* (ETEC)-induced inflammation and intestinal injury in weaned pigs.

Methods

Twenty-four weaned pigs were randomly assigned into three groups: (1) non-challenge (CON), (2) ETEC-challenge (ECON), and (3) ETEC challenge + 250 mg/kg FOS (EFOS). On day 19, non-challenged pigs were orally infused with sterilized culture while pigs in other groups were orally infused with ETEC (2.5×10^{10} colony-forming units). After 3 days, pigs were slaughtered for sample collection.

Results

We show that ETEC-challenged significantly reduced average daily gain (ADG); however, FOS improved the ADG ($P < 0.05$) and apparent digestibility of crude protein (CP), gross energy (GE), and ash in the ETEC-challenged pigs ($P < 0.05$). FOS reduced plasma concentrations of IL-1 β and TNF- α , and elevated the concentrations of immunoglobulin A (IgA) and immunoglobulin M (IgM) ($P < 0.05$). Interestingly, FOS elevated villus height in duodenum, and elevated the ratio of villus height to crypt depth in the duodenum and ileum in the ETEC-challenged pigs ($P < 0.05$). Moreover, FOS increased lactase activity in the duodenum and ileum ($P < 0.05$). The activities of sucrase and alkaline phosphatase (AKP) were higher in EFOS group than in the ECON group ($P < 0.05$). Importantly, FOS up-regulated the expressions of critical genes in intestinal epithelium function such as zonula occludens-1 (ZO-1), L-type amino acid transporter-1 (LAT1), and cationic amino acid transporter-1 (CAT1) in the duodenum and the expressions of ZO-1 and glucose transporter-2 (GLUT2) in the jejunum ($P < 0.05$). FOS also up-regulated the expressions of occludin, fatty acid transporter-4 (FATP4), sodium glucose transport protein 1 (SGLT1), and GLUT2 in the ileum ($P < 0.05$). FOS significantly increased the concentrations of acetic acid, propionic acid and butyric acid in the cecal digesta. Additionally, FOS reduced the populations of *Escherichia coli*, but elevated the populations of *Bacillus* and *Bifidobacterium* in the caecal digesta ($P < 0.05$).

Conclusions

These results suggested that FOS can improve the growth performance and intestinal health in weaned pigs upon ETEC challenge, which was associated with suppressed inflammatory responses and improved intestinal epithelium functions and microbiota.

Background

Small intestine is the main site of nutrient digestion and absorption, and can serve as an important defense barrier against exogenous and endogenous harmful substances or pathogens [1]. Previous studies indicated that a wide variety of enteric pathogens such as pathogenic bacteria and viruses can induce acute diarrhea in mammalian animals, especially at their weaning period, resulting in mortality, dehydration, weight loss, and growth retardation [2–4]. In the last decades, antibiotics have been widely used for the preventing of diarrhea; however, long-term or overdose utilization of antibiotics increased the risk of developing drug resistance [5, 6]. Therefore, alternatives for conventionally used antibiotic has attracted considerable research interest worldwide. The most widely studied alternatives include probiotics, prebiotics, enzymes, plant extracts, and nutraceuticals such as copper and zinc [7].

Oligosaccharides are carbohydrates of low degree of polymerization (DP) and low molecular weight composed of monosaccharides [8]. Oligosaccharides usually resist enzymatic hydrolysis and absorption in upper gastrointestinal tract, but can be fermented in large bowel by a number of bacteria [9]. Interestingly, non-digestible oligosaccharides have prebiotic activity, as they can promote body health by increasing populations of beneficial microbes and/or their metabolic activity [10]. FOS is composed of fructose and glucose units, which refers specifically to short chains (3–6 units) of fructose units bound by β -(2–1) linkages that are attached to a terminal glucose unit [11]. Oligosaccharides including the FOS have been previously reported to improve growth performance and gut health in a variety of animal species [12]. For instance, FOS not only promoted the growth of weaning pigs, but also effectively reduced the colonization of pathogenic bacteria in the intestine [10].

EPEC is a major cause of diarrhea in neonatal and weaning pigs [13]. EPEC colonized in the intestine can release enterotoxins, which stimulates secretion of fluid from the epithelial cells into the lumen, resulting in acute diarrhea [14]. On the other hand, weaning deprives neonatal pigs from passive immune protection of mother's milk, which increases their susceptibility to enterotoxigenic *E. coli* infection [15, 16]. Although, numerous studies indicated that FOS has prebiotic effects that can inhibit the growth of pathogenic bacteria [17], only few reports indicated the influences of FOS on the growth and intestinal health in weaned pigs exposure to EPEC challenge. In this study, we explored the effect of dietary FOS supplementation on growth performance, inflammatory response, intestinal epithelium function, and selected bacterial populations in weaned pigs upon EPEC challenge. This study will also provide convincing evidence on novel prebiotic effect of FOS and offer key insights into its potential mechanisms of action.

Methods

Animals, feeding and experimental design

All the procedures used in the animal experiment were approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University (no. 20181105). Twenty-four weaned pigs with an average initial body weight (6.30 ± 0.30 kg) pigs were randomly allotted into three treatment groups that included (1) non-challenged control (CON; pigs fed a basal diet and infused with sterilized Luria–Bertani culture);

(2) ETEC-challenged control (ECON; pigs fed the same basal diet and infused with ETEC); and (3) ETEC challenge + FOS treatment (EFOS, ETEC + FOS; pigs fed a basal diet supplemented with 250 mg kg⁻¹ FOS and infused with ETEC). FOS, purchased from Shanghai Lanpu Biotechnology CO., LTD., (FOS ≥ 20%). The basal diet (Table 1) was formulated to meet the nutrient requirements recommended by the National Research Council 2012 [18]. Pigs were individually housed in metabolism cages (0.7 m × 1.5 m) with room temperature maintained at 25–28 °C and relative humidity controlled at 55–65%. All pigs were given an ad libitum access to fresh water and feeding. The trial lasted for 21 d. On day 19, pigs in the challenged groups were orally administered 150 mL of Luria–Bertani culture containing approximately 2.5×10^{10} colony-forming units of ETEC (serotype O149:K91:K88ac; China Institute of Veterinary Drugs Control, Beijing, China) [19], while pigs in the non-challenged control group were administered an equal volume of sterilized Luria–Bertani culture. All pig's feeding consumption was measured daily and BW was measured on day 19 and 22 after 12-h fasting at the morning 8:00. The average daily body weight gain (ADG), average daily feed intake (ADFI) and gain-to-feed ratio (G : F) were calculated.

Table 1
Composition and nutrient level of experimental diet

Ingredients	%	nutrient level	contents
Corn	28.31	Digestible energy (calculated, MJ/kg)	14.78
Extruded corn	24.87	Crude Protein (%)	19.68
Soybean meal	8.50	Calcium (%)	0.81
Extruded full-fat soybean	10.30	Available phosphorus (%)	0.55
Fish meal	4.20	Lysine	1.35
Whey powder	7.00	Methionine	0.42
Soybean protein concentrate	8.00	Methionine + cysteine	0.60
Soybean oil	2.00	Threonine	0.79
Sucrose	4.00	Tryptophan	0.22
Limestone	0.90		
Dicalcium phosphate	0.50		
NaCl	0.30		
L -LysineHCl (78%)	0.47		
DL -Methionine	0.15		
L -Threonine (98.5%)	0.13		
Tryptophan (98%)	0.03		
Chloride choline	0.10		
Vitamin premix ¹	0.04		
Mineral premix ²	0.20		
Total	100		

¹ The vitamin premix provided the following per kg of diet: 9000 IU of VA, 3000 IU of VD 3, 20 IU of VE, 3 mg of VK 3, 1.5 mg of VB1, 4 mg of VB 2, 3 mg of VB6, 0.02 mg of VB12, 30 mg of niacin, 15 mg of pantothenic acid, 0.75 mg of folic acid, and 0.1 mg of biotin. ² 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 I, 0.3 mg Se.

Sample collection and preparation

At the beginning of the trial, representative feed samples of each group were sampled and stored at - 20 °C for chemical analysis. From day 12 to day 15 of the experiment, fresh fecal samples were collected immediately after excretion from eight randomly selected pigs in each group. After collection, the daily

excreta of each pig was weighted, and 10 mL of a 10% H₂SO₄ solution was added to each 100 g of wet fecal sample, and subsequently stored in a sealed plastic bag at - 20 °C. At the end of the 4-d period, all fecal samples of each pig were thawed at room temperature and mixed thoroughly, and then dried at 65 °C for 48 h, after which they were ground to pass through a 1-mm screen and stored at - 20 °C for chemical analyses including dry matter (DM), crude protein (CP), ether extract (EE), ash, and gross energy. Blood samples were collected by venepuncture at 8:00 after 12 h of fasting. Then the samples were centrifuged at 3500 × g at 4 °C for 10 min [20]. After centrifugation, the plasma samples were collected and frozen at - 20 °C until analysis. After blood collection, pigs were euthanized with an intravenous injection of sodium pentobarbital and then slaughtered by exsanguination protocols, the mid points of the duodenum, jejunum and ileum of each pig were harvested and fixed in 4% paraformaldehyde solution for morphological analyses. Besides, 2 g digesta from the cecum was immediately snap-frozen in liquid nitrogen, and stored at -80 °C for analysis of microbial DNA and Short-chain fatty acid. At the same time, mucosa samples were scraped with a scalpel blade from duodenum, jejunum and ileum segments and quickly-frozen using liquid N₂, followed by the preservation at - 80 °C until further analysis for related enzyme activity and gene expression.

Measurement of growth performance

Piglets were weighed before the morning feed on day 1, 19, and 22 after 12-h fasting at the morning 8:00. Feeding intake was recorded, orts were collected and weighed daily. All feed was mash. Average daily feed intake (ADFI), average daily gain (ADG), and the ratio of gain to feed intake (G:F) were calculated.

Determination of the apparent total tract digestibility

The apparent total tract digestibility (ATTD) was measured using acid-insoluble ash (AIA) as indicator [21]. The AIA in diets and faeces samples was determined by a method described by Chinese National Standard (GB/T23742). All samples were analyzed for DM, CP, EE, ash. The gross energy content of diets and fecal samples was determined using an adiabatic bomb calorimeter. Gross energy was determined using an automatic adiabatic bomb calorimeter (LECO, St. Joseph, Michigan, USA). The ATTD was calculated as $(100 - A1F2/A2F1 \times 100)$, where A1 represents the AIA content of the diet; A2 represents the AIA content of faeces; F1 represents the nutrient content of the diet; F2 represents the nutrient content of faeces.

Plasma biochemical analysis

Plasma concentrations of TNF- α , IL-1 β , IL-6, Immunoglobulin (Ig), including IgA, IgG and IgM were determined following the procedures outlined by the corresponding kit manufacturer using commercially available swine Enzyme-Linked Immunosorbent Assay (ELISA) kits (Jiangsu Jingmei Biotechnology Co., Ltd., Yancheng, China).

Intestinal morphology analysis

About 1-cm segment of the small intestine (duodenum, jejunum and ileum) were mixed in 10% neutral buffered formaldehyde. The mixed tissue samples were dehydrated with normal saline and then

embedded in paraffin. Cross sections of each sample were prepared, stained with haematoxylin and eosin (H&E) and then sealed by a neutral resin size. Ultrathin sections of the duodenal, jejunal and ileal samples were examined for the villus height and crypt depth with image processing and analysis system (Media Cybernetics, Bethesda, MD, USA). Villus height was calculated from the tip of the villi to the villus-crypt junction. Crypt depth was expressed as the invaginated depth between adjacent villi. A total of 10 intact, well-oriented crypt-villus units were analyzed in triplicate per segment. The ratio of villus height to crypt depth (V/C) was calculated from the values described above.

Determination of intestinal enzyme activities

After thawing, about 1 g of intestinal mucosa (including the duodenum, jejunum and ileum) was homogenized in ice-cold physiological saline at 1:9 and then centrifuged at $3000 \times g$, 4°C for 15 min. The supernatant was assayed for protein content using the Bradford method, followed by measurement of the sucrase, lactase maltase and alkaline phosphatase (AKP) activities, in accordance with the instructions accompanying the respective kit (Nanjing Jiancheng Bioengineering Institute). The results were normalised to protein concentration and expressed as U/mg protein.

Intestinal microbial populations measurement

Intestinal microbial populations measurement Microbial DNA was extracted from the caecal digesta using the EZNA® Stool DNA kit (Omega Bio-Tek, Doraville, CA, USA), according to the manufacturer's instructions. Based on the 16S rRNA sequences of maximum species of each genus encountered in the pig intestinal tract downloaded from the National Center for Biotechnology Information (GenBank), European Molecular Biology Laboratory and DNA Data Bank of Japan, The fluorescent quantitative specific primers and probe for total *E. coli*, *Lactobacillus*, *Bifidobacterium*, and *Bacillus* (Table S1) were obtained from the Fierer et. al [22]. and Qi et. al [23] and were commercially synthesised by Invitrogen (Shanghai, China). To quantify *Bacillus*, *Bifidobacterium*, *E. coli* and *Lactobacillus*, qPCR was carried out on a CFX96 Real-Time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA) using the RealMasterMix (Probe) kit (Tiangen Biotech Co., Ltd., Beijing, China). A volume of 25 μL , containing 12.5 μL of SuperReal PeMix (2.0 \times), 1 μL each of the forward and reverse primers, 1 μL of Fluorescent probe (20 \times), 1 μL of ROX Reference*3 Dye (50 \times), 2 μL of DNA and 6.5 μL of RNase-Free ddH₂O were used in each reaction. The reactions were subjected to 1 cycle at 95°C for 15 min, followed by 39 cycles at 95°C for 3 s, 58°C for 25 s and 72°C for 60 s. The cycle threshold (Ct) 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).

Volatile fatty acid (VFA) analysis

Samples of digesta from individual pigs were taken from the caecum to measure the VFA concentration. The VFA concentrations in the digesta were determined using gas liquid chromatography according to the method described by Pierce et al [24]. Approximately 1.0 g of thawed cecal digesta was suspended in 1.5 mL of sterile milli-Q water in a centrifuge tube for 30 min. The entire sample was centrifuged at

12,000 × g for 10 min. 1 mL supernatant was transferred to a sterile tube, and then mixed with 0.2 mL 25% metaphosphoric acid and 23.3 μL 210 mmol/L crotonic acid simultaneously, the sterile tubes were centrifuged again for 10 min after placed the sterile tubes in ice-bath for 30 min. The mixture of 500 μL supernatant and 500 μL methanol was homogenized for 10 min in another sterile tube. After that, the mixture was centrifuged at 12,000 × g for 10 min at 4°C. The supernatant was injected into a gas chromatographic system (VARIAN CP-3800, America) to separate and quantify the VFA.

RNA isolation, reverse transcription, and real-time quantitative PCR

Total RNA was extracted from duodenum, jejunum and ileum mucosa using the Trizol Reagent (TaKaRa, Dalian, China). Meanwhile, the concentration and purity of total RNA were assayed by spectrophotometer (Nan Drop, Gene Company Limited, Guangzhou, China) at 260 and 280 nm following manufacturer's guidelines. The ratio of OD 260/280 should vary between 1.8 and 2.0. Reverse transcription using the Prime Script RT reagent kit (TaKaRa Biotechnology, Dalian, China) was exploited following the manufacturer's instructions. The primers were synthesized commercially by Life Technologies Limited and were exhibited in (Table S1).

Quantitative real-time polymerase chain reaction (PCR) was conducted to analyse the mRNA expression abundance of ZO-1, Occludin, FATP1, FATP4, CAT1, LAT1, SGLT1 and GLUT2 in the small intestinal mucosa using the CFX-96 real-time PCR detection system (Bio-Rad) and SYBR Premix Ex Taq II (Tli RNaseH Plus) reagents (TaKaRa, Dalian, China). The PCR reaction was run in a 10 μL reaction volume, which contained 5 μL of SYBR Premix Ex Taq II (Tli RNaseH Plus), 0.5 μL of each primer, 1 μL of the cDNA sample, and 3 μL of nuclease-free water. The PCR cycling parameters were as follows: initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 57.5 °C for 30 s, and 72 °C for 5 min. A melting curve analysis was performed following each real-time quantitative PCR assay to confirm the Gene-specific amplification products had been generated. The housekeeping gene β-actin was used as an internal control for normalization. The target gene mRNA expression level was calculated using the $2^{-\Delta\Delta Ct}$ method [25]. Each sample was simultaneously performed on the same PCR plate and three replicates were set up.

Statistical analysis

All data were subjected to one-way analysis of variance for a completely randomised design using the general linear model procedure of SPSS 24.0 (SPSS, Inc.), with each pig representing one experimental unit. Statistical differences among the treatments were separated by Duncan's multiple-range test. The results are expressed as with their standard errors. Statistical significance was set at $P < 0.05$ and $0.05 < P < 0.10$ indicating a trend.

Results

Effect of FOS supplementation on growth performance and nutrient digestibility

As shown in Table 2, there were no differences ($P > 0.05$) in ADG, average daily feed intake (ADFI), and feed efficiency (G:F) among the three treatments during days 1–18 (pre-challenge). ETEC challenge (during days 19–21) significantly reduced the ADG ($P < 0.05$) in the weaned pigs; however, the ADG was significant higher in the EFOS group than in the ECON group ($P < 0.05$). As compared to the CON and ECON group, dietary FOS supplementation significantly elevated the apparent digestibility of CP, GE, and Ash ($P < 0.05$). FOS supplementation did not significant influence on the digestibility of DM (Table 3).

Table 2

Effect of FOS on growth performance in weaned pigs challenged with enterotoxigenic *Escherichia coli*

Items	Treatments			SEM	P-Value
	CON	ECON	EFOS		
pre-challenged (1–19 days)					
ADFI(g/day)	454.50	403.49	417.71	18.83	0.68
ADG (g/day)	296.56	270.67	285.00	11.23	0.56
F:G (g/g)	1.53	1.49	1.47	0.03	0.81
post-challenged (19–21 days)					
ADFI (g/day)	487.00	467.20	484.67	4.33	0.37
ADG (g/day)	396.00 ^a	330.00 ^b	381.80 ^a	19.33	0.12
¹ Values are means \pm SEM, (n = 6), non-challenged pigs (CON, fed with basal diet), ETEC-challenged pigs (ECON, fed with basal diet), and FOS and ETEC-treated pigs (EFOS, fed with basal diet containing 250 mg/kg FOS challenged by ETEC). ² ^{a, b, c} mean values within a row with unlike superscript letters were significantly different ($P < 0.05$). ³ ADFI = Average daily feed intake; ADG = Average daily gain; G/F = the ratio of gain to feed intake					

Table 3
Effect of FOS on ATTD of nutrients in weaned pigs

Item, %	Treatments				
	CON	ECON	EFOS	SEM	<i>P</i> -Value
DM	81.58	83.65	85.36	1.12	0.21
CP	83.38 ^b	85.33 ^b	90.95 ^a	1.20	0.01
GE	87.98 ^b	86.91 ^b	93.04 ^a	1.05	0.03
Crude fat	79.62 ^{ab}	78.38 ^b	88.60 ^a	2.03	0.07
Ash	74.19 ^b	68.81 ^b	79.54 ^a	1.26	0.00

Values are means \pm SEM, (n = 6), non-challenged pigs (CON, fed with basal diet), ETEC-challenged pigs (ECON, fed with basal diet), and FOS and ETEC-treated pigs (EFOS, fed with basal diet containing 250 mg/kg FOS challenged by ETEC). ² a, b, c mean values within a row with unlike superscript letters were significantly different ($P < 0.05$). ³ DM, dry matter; CP, crude protein; GE, gross energy.

Effect of FOS supplementation on concentrations of plasma cytokines and immunoglobulins in weaned pigs upon ETEC challenge

As shown in Table 4, ETEC challenge significantly increased the concentrations of TNF- α in the plasma ($P < 0.05$). However, dietary FOS supplementation significantly decreased the concentrations of plasma IL-1 β and TNF- α in the pigs upon ETEC challenge ($P < 0.05$). Moreover, the concentrations of plasma IgA and IgG were lower in the ECON group than in the CON group ($P < 0.05$). However, the concentrations of plasma IgA and IgM were significantly higher in the EFOS group than in the ECON group ($P < 0.05$).

Table 4

Effect of FOS on plasma Immunoglobulin and Cytokine Concentrations in weaned pigs challenged with enterotoxigenic *Escherichia coli*

Items	Treatments				
	CON	ECON	EFOS	SEM	P-Value
IL-1 β (ng/L)	456.20 ^a	508.93 ^a	392.40 ^b	16.56	0.01
IL-6 (ng/L)	1.34	1.44	1.35	0.07	0.82
TNF- α (pg/mL)	487.60 ^b	589.52 ^a	454.61 ^b	22.91	0.03
Ig A (ng/L)	432.48 ^a	357.52 ^b	481.92 ^a	17.66	0.00
Ig G (ng/L)	515.70 ^a	447.79 ^b	508.71 ^{ab}	13.82	0.08
Ig M (ng/L)	464.96 ^{ab}	393.21 ^b	518.15 ^a	19.67	0.02

¹ Values are means \pm SEM, (n = 6), non-challenged pigs (CON, fed with basal diet), ETEC-challenged pigs (ECON, fed with basal diet), FOS and ETEC-treated pigs (EFOS, fed with basal diet containing 250 mg/kg FOS challenged by ETEC). ² ^{a, b, c} mean values within a row with unlike superscript letters were significantly different ($P < 0.05$). ³ IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; IL-1 β , interleukin-1 β ; IL-6, interleukin-6 and TNF- α , tumor necrosis factor- α .

Effect of FOS supplementation on intestinal epithelium morphology in weaned pigs upon ETEC challenge

ETEC challenge significantly decreased the villus height, but increased the crypt depth in the duodenum (Table 5, Figure. 1). As compared to the villus height was significant higher in the EFOS group than in the ECON group ($P < 0.05$). Interestingly, ETEC significantly decreased the ratio of villus height to crypt depth (V/C) in the duodenum and ileum ($P < 0.05$). As compared to the ECON group, FOS supplementation significantly increase the V/C ratio in the duodenum and ileum ($P < 0.05$).

Table 5

Effect of FOS on intestinal morphology in weaned pigs challenged with enterotoxigenic *Escherichia coli*

Items	Treatments			SEM	P-Value
	CON	ECON	EFOS		
Duodenum					
Villus height (mm)	606.68 ^a	471.49 ^b	597.51 ^a	26.61	0.05
Crypt depth (mm)	273.66 ^a	356.23 ^b	287.65 ^a	13.46	0.01
V/C	2.22 ^a	1.32 ^b	2.08 ^a	0.16	0.05
Jejunum					
Villus height (mm)	421.21	398.47	422.01	9.79	0.57
Crypt depth (mm)	173.29	195.75	187.66	6.29	0.34
V/C	2.43	2.04	2.25	0.10	0.38
Ileum					
Villus height (mm)	371.82	330.30	359.15	12.70	0.11
Crypt depth (mm)	195.25	205.74	171.09	8.76	0.24
V/C	1.90 ^a	1.64 ^b	2.28 ^a	0.11	0.04
¹ Values are means ± SEM, (n = 6), non-challenged pigs (CON, fed with basal diet), ETEC-challenged pigs (ECON, fed with basal diet), and FOS and ETEC-treated pigs (EFOS, fed with basal diet containing 250 mg/kg FOS challenged by ETEC). ² ^{a, b, c} mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).					

Effect of FOS on enzyme activity of intestinal mucosa in weaned pigs upon ETEC challenge

As show in Table 6, ETEC challenge significantly decreased the mucosal activities of sucrase, lactase, and AKP in the duodenum ($P < 0.05$). The mucosal activity of lactase in the ileum was also lower in the ECON group than in the CON group ($P < 0.05$). However, FOS supplementation not only elevated duodenal the activities of sucrase, lactase, and AKP in the duodenum, but also elevated the ileal activity of lactase in the ETEC challenged pigs ($P < 0.05$).

Table 6

Effect of FOS on enzyme activity of small intestine in weaned pigs challenged with enterotoxigenic *Escherichia coli*

Items	Treatments				
	CON	ECON	EFOS	SEM	P-Value
Duodenum					
Sucrase, U/mg protein	155.07 ^a	83.3 ^b	156.70 ^a	14.08	0.04
Lactase, U/ mg protein	12.07 ^a	6.21 ^b	13.51 ^a	1.97	0.28
Maltase, U/mg protein	21.11	13.17	23.71	2.44	0.17
AKP, U/mg protein	1.44 ^a	0.65 ^b	1.51 ^a	0.16	0.04
Jejunum					
Sucrase, U/mg protein	536.76	475.36	526.50	40.71	0.83
Lactase, U/ mg protein	94.21	57.84	96.34	12.33	0.39
Maltase, U/mg protein	576.75	423.32	544.70	61.15	0.62
AKP, U/mg protein	1.21	1.15	1.22	0.05	0.83
Ileum					
Sucrase, U/mg protein	210.25	153.42	208.23	16.90	0.32
Lactase, U/ mg protein	35.19 ^a	15.37 ^b	32.38 ^a	3.66	0.04
Maltase, U/mg protein	131.49	110.36	135.41	10.59	0.62
AKP, U/mg protein	2.34	2.06	2.19	0.10	0.57
Values are means \pm SEM, (n = 6), non-challenged pigs (CON, fed with basal diet), ETEC-challenged pigs (ECON, fed with basal diet), and FOS and ETEC-treated pigs (EFOS, fed with basal diet containing 250 mg/kg FOS challenged by ETEC). ² ^{a, b, c} mean values within a row with unlike superscript letters were significantly different ($P < 0.05$). ³ AKP = alkaline phosphatase					

Effect of FOS supplementation on microbial metabolites and intestinal populations in weaned pigs upon ETEC challenge

As shown in Figure.2, ETEC challenge decreased the concentrations of acetic acid and propionic acid in cecal digesta ($P < 0.05$). However, FOS supplementation significantly elevated their concentrations in the ETEC challenged pigs ($P < 0.05$). The concentration of butyric acid was also higher in the EFOS group than in the ECON group ($P < 0.05$). ETEC challenge increased the abundance of *E. coli* in the cecal digesta; however, FOS supplementation significantly decreased the abundance of *E. coli* in the cecal digesta

(Table 7). Moreover, FOS supplementation significantly elevated the abundance of beneficial microflora such as the *Bifidobacterium* and *Bacillus* in the cecal digesta of ETEC-challenged pigs ($P < 0.05$).

Table 7

Effect of FOS on intestinal bacteria in the cecal digesta of weaned pigs challenged with enterotoxigenic *Escherichia coli*

Items	Treatments				P-Value
	CON	ECON	EFOS	SEM	
Total bacteria, 1 g (copies/g)	10.43	10.21	10.14	0.08	0.37
<i>Escherichia coli</i> 1 g (copies/g)	8.62 ^b	10.17 ^a	8.68 ^b	0.26	0.01
<i>Bifidobacterium</i> 1 g (copies/g)	6.86 ^a	5.24 ^b	6.54 ^a	0.30	0.05
<i>Bacillus</i> 1 g (copies/g)	8.57 ^a	8.03 ^b	8.59 ^a	0.10	0.01
<i>Lactobacillus</i> 1 g (copies/g)	5.23	4.57	5.03	0.15	0.18

¹ Values are means \pm SEM, (n = 6), non-challenged pigs (CON, fed with basal diet), ETEC-challenged pigs (ECON, fed with basal diet), FOS and ETEC-treated pigs (EFOS, fed with basal diet containing 250 mg/kg FOS challenged by ETEC). ² ^{a, b, c} mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

Effect of FOS supplementation on expressions of critical genes related to intestinal epithelium functions

As shown in Figure.3, ETEC challenge decreased the expression levels of tight-junction protein ZO-1 in the duodenum. However, FOS supplementation not only elevated the expression levels of ZO-1 in the duodenum and jejunum, but also elevated the expression levels of occludin and FATP4 in the ileum ($P < 0.05$). Moreover, FOS supplementation significantly elevated the expression levels of LAT1 and CAT1 in the duodenum and ileum of the ETEC-challenged pigs ($P < 0.05$). The expression level of SGLT1 in the ileum, and the expression levels of GLUT2 in the jejunum and ileum were both higher in the EFOS group than in the ECON group ($P < 0.05$).

Discussion

Antibiotics are widely used for preventing post-weaning diarrhea in pig production industry [7]. However, long-term or overdose utilization of antibiotics may lead to developing of bacterial resistance and drug

residues in the product [5, 6]. Fructooligosaccharides are highly interesting prebiotic fibres, resulting from the transfructosylating action of specific microbial enzymes on sucrose, composed of one molecule of glucose linked to 2 to 4 fructose units at position β -1,2 that has been shown to improve weaned pigs' intestinal morphology and growth performance [10, 12]. In the present study, we investigated the protective potential of FOS against ETEC-induced inflammation and intestinal injury in weaned pigs. We show that dietary FOS supplementation significantly improved the growth performance and apparent nutrient digestibility in pigs exposure to ETEC challenge. The result are consistent with previous studies on rats and pigs [26, 27].

Infection with ETEC is a major cause of diarrheal disease in livestock production, especially in nursery pig cultivation [28, 29]. TNF- α and IL-1 β are two major pro-inflammatory cytokines involved in the inflammatory responses [30]. In the present study, ETEC challenge elevated the plasma concentration of TNF- α ; however, FOS supplementation significantly decreased the concentrations of plasma IL-1 β and TNF- α in the ETEC-challenged pigs, which suggested an anti-inflammatory role of FOS in regulating the ETEC-induced inflammation. The result is also consistent with previous study that mannan oligosaccharides added in the sow diet enhance their offspring intestinal immunity by decreasing inflammation [31]. Moreover, the plasma concentrations of IgA and IgG were elevated by FOS in the ETEC-challenged pigs. Both the IgA and IgG are crucial index of body immune capacity and contribute to dealing with various pathogens. IgA is the main element of the humoral immune response that has been selected through evolution, together with innate mucosal defenses, to protest against microbial antigens at mucosal surfaces [32]. In general, IgG enhances mucosal homeostasis in addition to controlling non-invasive and invasive mucosal bacteria, and does so by reaching the lumen of mucosal organs upon binding to the epithelial transporter FcRn [33].

ETEC challenge significantly decreased the duodenal villus height, and reduced the ratio of V/C in the duodenum and ileum, indicating injury of the intestinal epithelium. The result is consistent with previous study that ETEC strains function through producing enterotoxins that act on the small intestines and lead to the secretion of fluids and electrolytes, causing diarrhea and intestinal injury [34]. However, dietary FOS supplementation attenuated the intestinal injury by increasing the villus height and the ratio of V/C. The result is probably due to the prebiotic effects of oligosaccharides. For instance, the mannan-oligosaccharides (MOS) were found to prevent the adhesion of enteric pathogens to intestinal epithelium, which significantly reduced their colonization and intestinal inflammation and injury [35]. Moreover, FOS was found to improve the intestinal morphology and significantly reduced the colonization of pathogenic bacteria in the intestine [36]. The beneficial effects of FOS supplementation on intestinal epithelium function has also been indicated by the mucosal enzyme activities, as FOS not only elevated duodenal the activities of sucrase, lactase, and AKP in the duodenum, but also elevated the ileal activity of lactase in the ETEC-challenged pigs. Sucrase and lactase are two important digestive enzymes involved in carbohydrate digestion, play crucial roles in the process of digestion in animals [37]. While, the AKP is an is another crucial endogenous enzyme expressed in the brush border, and is considered as an excellent marker enzyme involved in the primary digestive and absorptive processes of the small intestine [38].

In the present study, we also investigated the concentration of microbial metabolites and abundance of major bacterial populations in the cecum. As expected, FOS supplementation significantly elevated the concentrations of short chain fatty acids (SCFAs, e.g. acetic acid, propionic acid, and butyric acid) in the cecal digesta of ETEC-challenged pigs. The elevated SCFAs concentration is probably due to the fact that oligosaccharides cannot be digested in the upper digestive tract, but can be fermented by intestinal microorganisms in the lower digestive tract [39]. Both the acetic and propionic acids can be used as energy substrates for peripheral tissues. While the butyrate not only act as a critical energy source for intestinal epithelial cells, but can also promote cell proliferation and differentiation [40]. Importantly, FOS decreased the abundance of *E. coli*, but significantly increased the abundance of *Bifidobacterium* and *Bacillus* in the cecum. Previous study has already indicated that oligosaccharides can not be utilized by pathogenic bacteria, but can be efficiently utilized by beneficial bacteria such as the *Bifidobacterium* and *Bacillus* [41]. The result is also consistent with previous studies on pigs and other animal species [42].

To gain insights into the mechanisms underlying the FOS-regulated intestinal health, we further investigated the expression levels of several critical genes involved in the intestinal epithelium functions. Tight junctions play a critical role in maintaining the intestinal barrier integrity, which can prevent the paracellular diffusion of intestinal bacteria and other antigens across the epithelium [43]. Tight-junction proteins such as the ZO-1 and occludin are critical components for structural and functional organization of the tight junctions. In this study, FOS supplementation not only elevated the expression levels of ZO-1 in the duodenum and jejunum, but also elevated the expression levels of occludin and FATP4 in the ileum. FATP4 is a small molecule protein that is responsible for transportation of fatty acids across the cell membranes [44]. Moreover, FOS supplementation significantly elevated the expression levels of nutrient transporters (LAT1, CAT1, SGLT1, and GLUT2) in the ETEC-challenged pigs. The LAT1 and CAT1 are transporters that is responsible for transporting of the L-type and cationic amino acids, respectively [45]. While the SGLT1 and GLUT2 are closely associated with glucose absorption [46]. The elevated expressions of these nutrient transporters indicated an improved intestinal integrity and epithelium functions in pigs exposure to ETEC challenge.

Conclusions

The present study suggested that dietary FOS supplementation can attenuate the growth retardation in the weaned pigs exposure to ETEC challenge. Moreover, FOS can alleviate the ETEC-induced intestinal injury in the weaned pigs, which was associated with suppressing of the inflammatory responses and improving of the intestinal epithelium functions and intestinal microbiota. The beneficial effects of FOS on the growth performance and intestinal health should make it an attractive prebiotic that can be tentatively used for the substitution of antibiotics.

Abbreviations

FOS, Fructooligosaccharides; ETEC, Enterotoxigenic *Escherichia coli*; DP, degree of polymerization; ADG, average daily gain; ADFI, average daily feed intake; G/F, the ratio of gain to feed intake; AIA, acid-insoluble

ash; DM, dry matter; EE, ether extract; CP, crude protein; GE, gross energy; AKP, alkaline phosphatase; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; IL-1 β , interleukin-1 β ; IL-6, interleukin-6; TNF- α , tumor necrosis factor- α ; H&E, haematoxylin and eosin; VFA, Volatile fatty acid; rPCR, real-time polymerase chain reaction; ZO-1, zonula occludens-1; FATP1, Fatty acid transport protein-1; FATP4, Fatty acid transport protein-4; LAT1, L-type amino acid transporter-1; CAT1, cationic amino acid transporter-1; SGLT1, sodium glucose transport protein-1; GLUT2, glucose transporter-2.

Declarations

Data Availability and material

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

Conflict of Interest

The authors declare that there are no conflicts of interest.

Funding Statement

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Authors' contributions

LL, JH and HY conceived the study, performed the experiment, performed data analysis, and contributed to drafting the manuscript. LL carried out the animal experiment. DWC, BY, ZQH, YHL, PZ, XBM, JY, JQL, HY conceived the experiment and proofread the manuscript. All authors read and approved the final manuscript.

Ethics approval

All experimental procedures and animal care were carried out in compliance with the regulations of the Animal Care Committee of Sichuan Agricultural University (NO. 20181105)

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Figures

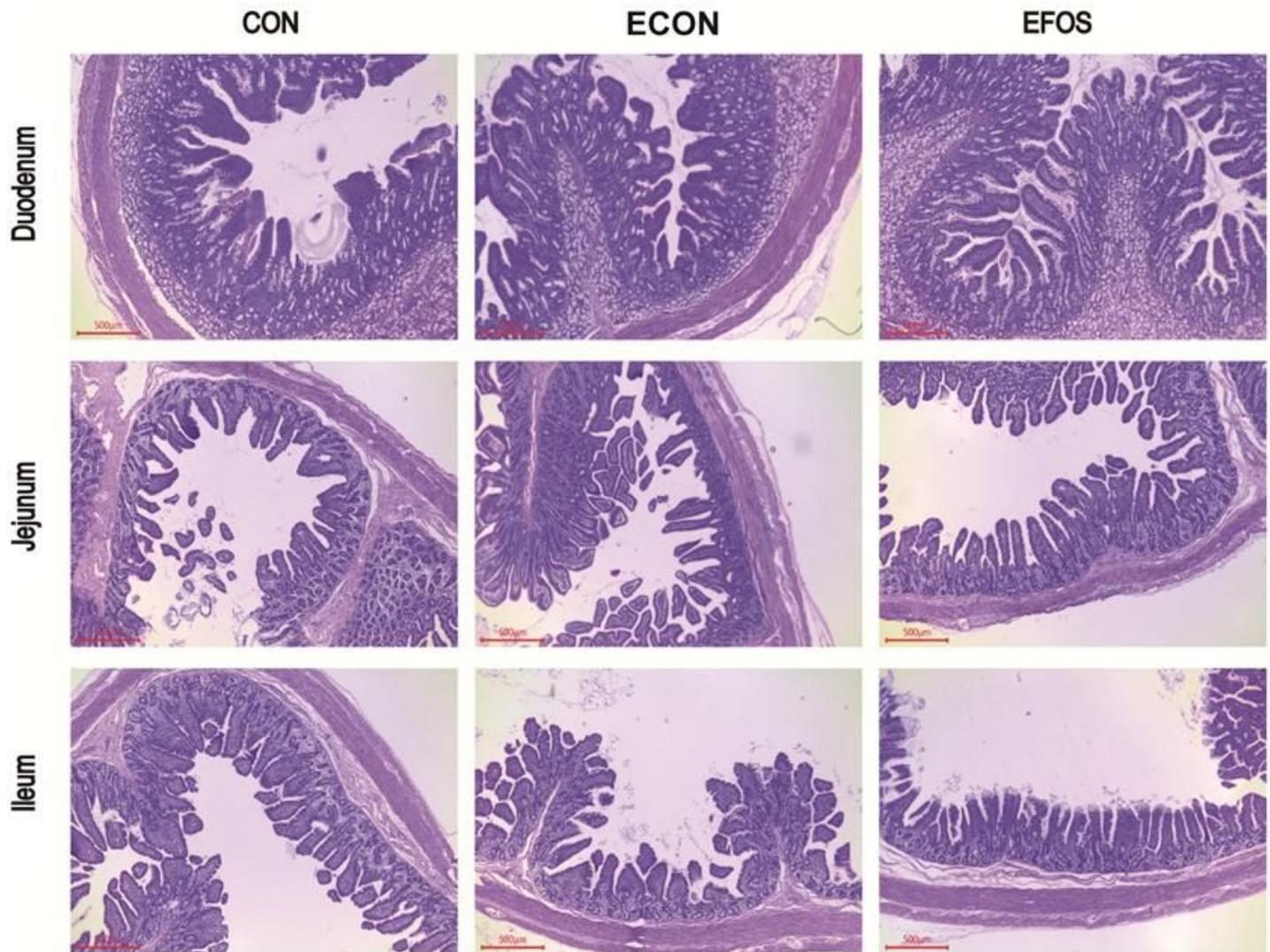


Figure 1

Effect of FOS on small intestinal morphology in weaned pigs. (H&E; $\times 40$). Non-challenged pigs (CON, fed with basal diet), ETEC-challenged pigs (ECON, fed with basal diet), FOS and ETEC-treated pigs (EFOS, fed with basal diet containing 250 mg/kg FOS challenged by ETEC).

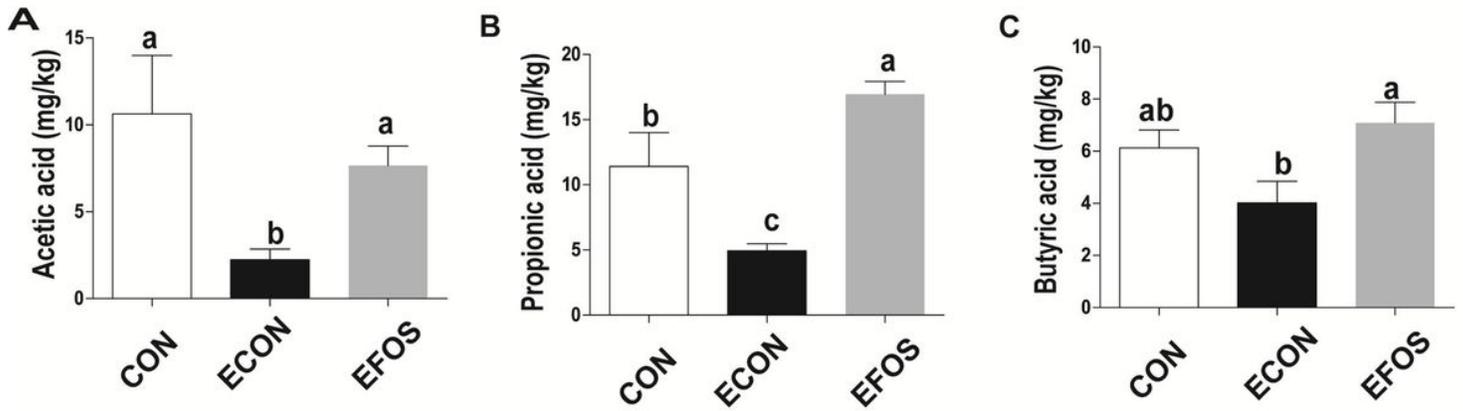


Figure 2

Effect of FOS on Concentration of Intestinal VFAs. Acetic acid concentration (A); Propionic acid concentration (B); Butyric acid (C); a, b, c Mean values with different letters on vertical bars indicate significant differences ($P < 0.05$). Non-challenged pigs (CON, fed with basal diet), ETEC-challenged pigs (ECON, fed with basal diet), FOS and ETEC-treated pigs (EFOS, fed with basal diet containing 250 mg/kg FOS challenged by ETEC).

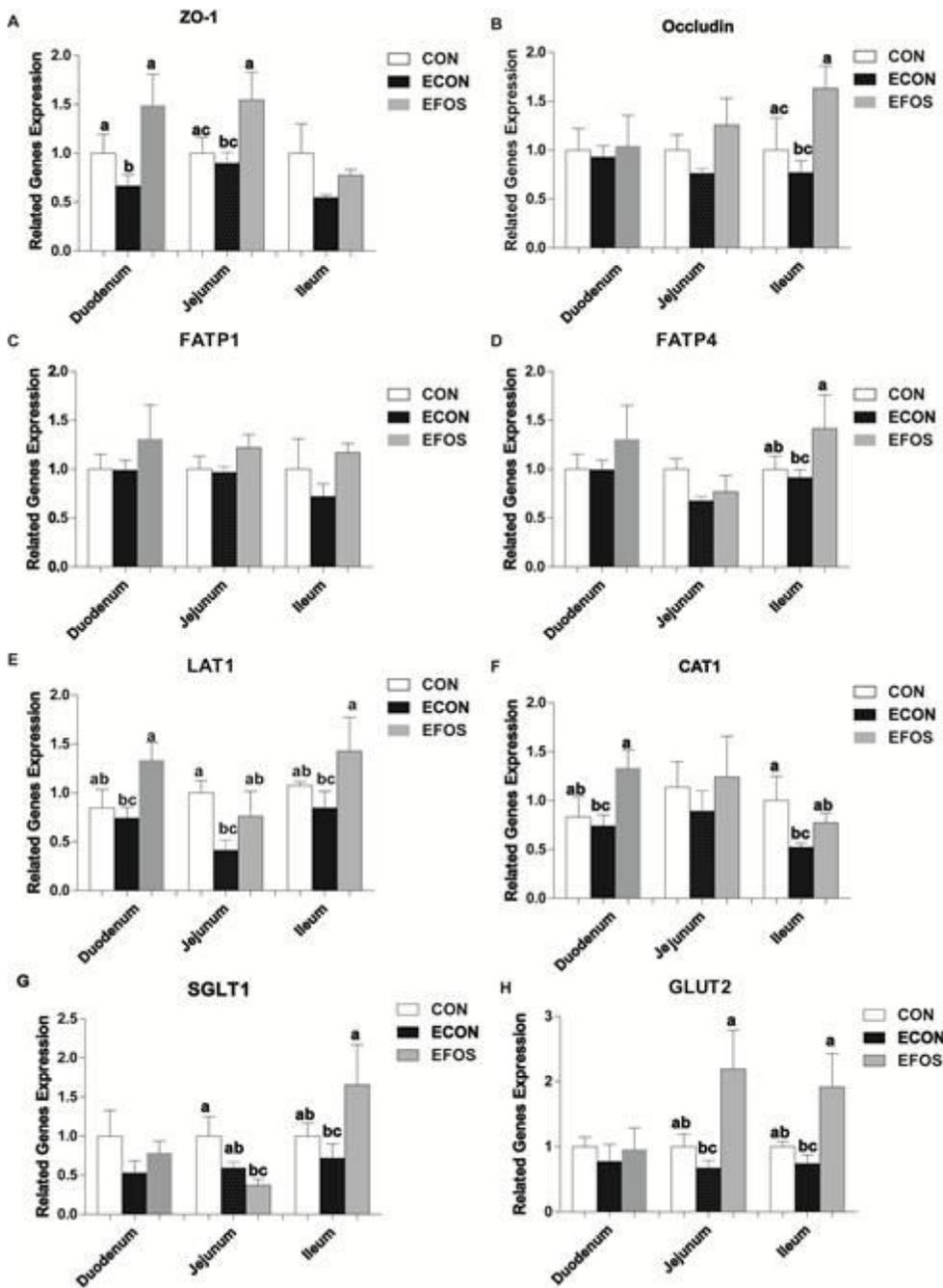


Figure 3

Relative expression levels of critical genes involved in the intestinal barrier functions. ZO-1, zonula occludens-1; FATP1, Fatty acid transport protein-1; FATP4, Fatty acid transportprotein-4; LAT1, L-type amino acid transporter-1; CAT1, cationic amino acid transporter-1; SGLT1, sodium glucose transport protein-1; GLUT2, glucose transporter-2; a,b,c Mean values with different letters on vertical bars indicate significant differences ($P < 0.05$). Non-challenged pigs (CON, fed with basal diet), ETEC-challenged pigs (ECON, fed with basal diet), FOS and ETEC-treated pigs (EFOS, fed with basal diet containing 250 mg/kg FOS challenged by ETEC).