Dietary supplementation of fructooligosaccharides alleviates enterotoxigenic *E. coli*-induced disruption of intestinal epithelium in a weaned piglet model

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Abstract

Diarrhea caused by pathogens such as enterotoxigenic *E. coli* (ETEC) is a serious threat to the health of young animals and human infants. Here, we investigated the protective effect of fructooligosaccharides (FOS) on the intestinal epithelium with ETEC-challenge in a weaned piglet model. Twenty-four weaned piglets were randomly divided into three groups: (1) non-ETEC-challenged control (CON), (2) ETEC-challenged control (ECON), and (3) ETEC challenge + 2.5 g/kg FOS (EFOS). On day 19, the CON pigs were orally infused with sterile culture, while the ECON and EFOS pigs were orally infused with active ETEC (2.5×10^9 colony-forming units). On day 21, pigs were slaughtered to collect venous blood and small intestine. Result showed that the pre-treatment of FOS improved the antioxidant capacity and the integrity of intestinal barrier in the ETEC-challenged pigs without affecting their growth performance. Specifically, comparing with ECON pigs, the level of GSH-Px (glutathione peroxidase) and CAT (catalase) in the plasma and intestinal mucosa of EFOS pigs was increased (P<0.05), and the intestinal barrier marked by ZO-1 and plasmatic DAO was also improved in EFOS pigs. A lower level (P<0.05) of inflammatory cytokines in the intestinal mucosa of EFOS pigs might be involved in the inhibition of TLR4/MYD88/NF-κB pathway. The apoptosis of jejunal cells in EFOS pigs was also lower than that in ECON pigs (P<0.05). Our findings provide convincing evidence of possible prebiotic and protective effect of FOS on the maintenance of intestinal epithelial function under the attack of pathogens.

Keywords: ETEC, FOS, Intestinal epithelium, Weaned piglets

Introduction

Post-weaning diarrhea (PWD), associated with the proliferation of enterotoxigenic *E. coli* (ETEC) in the gut, is not only a severest threat to the viability of young pigs (1), but also a threat to the health of human infants. PWD always results in the increase of mortality, dehydration, weight loss, as well as growth retardation in young animals (2). Accumulating evidence indicates that these disorders can be triggered by weanling stress such as removal from the sow, abrupt changes in diet or adapting to a new environment. The histological changes in the small intestine, such as the height of villus or the depth of crypts with Paneth cells, caused by PWD may affect the immune responses of piglets and lead to an intestinal dysfunction (3-4). Although the utilization of antibiotics was once considered to be the most effective measure to control ETEC infection, a growing number of countries and regions have restricted the use of antibiotics in feed to minimize the spread of strains with antibiotic resistance (5). The development of alternatives of antibiotics thus becomes very urgent (6-7). At present, the supplementation of bioactive compounds, such as oligosaccharides, seem to be a feasible way to improve weaning related intestinal injury in post-weaned piglets (8-9).

Oligosaccharides are composed of monosaccharides with low degree (2-10 glycosidic bonds) of polymerization (DP) (10). Fructooligosaccharides (FOS) are composed of fructose and glucose units, specifically referring to a short chain of fructose units (3-6) connected by β-(2-1) bonds to the terminal glucose units (11). Such chemical structure of FOS makes it unable to be directly digested by animals but can be fermented by various microorganisms in the large intestine of pigs (12). The utilization of FOS by gut microbes may be beneficial to the resistance against pathogen infection, oxidative stress, mutagenicity and even the occurrence and development of colon cancer (11-13), which makes FOS become an attractive alternative of antibiotics in swine feed.

As the largest immune organ in animal body, gastrointestinal (GI) tract plays a vital role in activating the innate immunity and inducing the subsequent adaptive immune responses (14-15). ETEC is one of the main pathogens leading to the

symptomatic gastroenteritis in human infants and young animals (6). Once ETEC or its toxins enter into blood through the damaged intestinal epithelium, the general immune response is induced and the immune cells in tissues are activated by the recognition of bacterial ligands, resulting in a rapid burst of pro-inflammatory cytokines and the dysfunction of GI tract (16-17). Since FOS may be beneficial for improving the intestinal health in animals, several studies have been focused on its effect on the intestinal environment and immunological activity of weaned pigs (18)which are vulnerable to the early-life stress due to the underdeveloped GI tract and immune system, but the influence of FOS on the intestinal permeability, intestinal barrier, or even the apoptosis of intestinal epithelial cells still remains to be discussed. Therefore, a piglet model with ETEC infection was built in the current study to comprehensively investigate the effect of dietary FOS supplementation on the growth performance, inflammatory responses, function of intestinal epithelium, and anti-oxidative capacity in weaned piglets. Moreover, pigs share similar anatomic and physiological structures with humans (19), our results may also provide convincing evidence on the possible prebiotic effect of FOS and offer key insights into the underlying mechanisms.

Materials and Methods

Animal trial

All the procedures used in the animal experiment were approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University. The animal experimental design of the current study has been reported before (20). In brief, twenty-four healthy 21 day old weaned cross bred (Duroc × Landrace × Yorkshire) pigs with an average initial body weight (BW) of 6.49 kg were selected and randomly divided into three groups after a 3-day adaptation, including (1) non-ETEC-challenged control (CON; pigs fed a basal diet and infused with sterilized Luria-Bertani culture), (2) ETEC-challenged control (ECON; pigs fed a basal diet and infused with active ETEC), and (3) ETEC challenge + FOS treatment (EFOS; pigs fed a basal diet supplemented with 2.5 g/kg FOS and infused with active ETEC). The

FOS (≥ 20%) used in the current study, purchased from Shanghai Lanpu Biotechnology CO., LTD (Shanghai, China), is the fructosyltransferase hydrolysate of chicory. The FOS product is a series of oligosaccharides with 2-10 degree of polymerization, which is composed of glucose and fructose units bound together by β-1, 2 glycosidic bonds in a ratio of 1:2.5. The carrier of the FOS product is corn starch. The basal diet (Supplementary Table 1) was formulated to meet the nutrient requirements recommended by the National Research Council (21). Each group consisted of 8 pigs and each pig was individually housed in a metabolic cage (0.7 m× 1.5 m). Pigs were allowed access to food and water ad libitum with room temperature maintained at 25-28 ℃ and relatively controlled humidity (55-65%). The trial lasted for 21 d, and the FOS treatment was applied in the whole experimental period. On day 19, pigs in the ECON and EFOS groups were orally administered with 150 mL of Luria-Bertani culture containing active ETEC (2.5×10^9 CFU/mL, serotype O149: K91: K88ac; China Institute of Veterinary Drugs Control, Beijing, China), while pigs in the CON group were orally administered with an equal volume of sterilized Luria–Bertani culture (22). The feed intake of each pig was measured daily and the BW of each pig was measured on day 22 after 12-h fasting. The gain-to-feed ratio (G: F) of each pig was calculated according to the average daily gain (ADG) and average daily feed intake (ADFI).

Collection of blood and tissue samples

In the early morning of day 22, approximate 10 mL of jugular vein blood of each pig was collected into a centrifuge tube with heparin sodium anticoagulant by venepuncture after 12 h of fasting, and the blood sample was centrifuged at 3500 × g at 4 ℃ for 10 min to obtain plasma (23). All the prepared plasma samples were kept at -20 ℃ until analysis. After the collection of blood sample, each pig was euthanized with an intravenous injection of sodium pentobarbital at a dosage of 200 mg/kg BW and then slaughtered (24), and the abdomen was opened rapidly. Approximate 2 cm of each middle duodenum, jejunum and ileum was separated with sterile surgical scissors and fixed in 4% paraformaldehyde solution for the immunofluorescence

detection. Finally, the duodenal, jejunal and ileal mucosa of each pig was collected using a scalpel blade and stored at -80 °C for the analysis of antioxidant capacity and gene expression.

Detection of intestinal antioxidant capacity and cytokines

Plasma antioxidant enzymes such as the catalase (CAT) and glutathione (GSH), malondialdehyde (MDA), and the total antioxidant capacity (T-AOC) were measured by using commercial kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The activity of diamine oxidase (DAO) in the plasma was also measured by using commercial kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The concentrations of inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) in the intestinal mucosa were detected to by using an enzyme linked immunosorbent assay (ELISA) following the instructions of corresponding kits supplied by Jiangsu Jingmei Biotechnology Co., Ltd. (Yancheng, China). All the above kits are swine-specific. The minimum detectable concentration of DAO, TNF-α, IL-1β and IL-6 is 10 pg/m, 10pg/ml, ng/L and 50 ng/L, respectively. In addition, the minimum detectable concentration of CAT, GSH, MDA and T-AOC is 0.2 U/mL, 20 mg/mL, 0.5 nmol/mL, and 0.2 U/mL, respectively.

Immunofluorescence analysis and real-time PCR analysis

The localization of zonula occluden-1 (ZO-1) protein, one of the tight junction proteins, in the duodenum, jejunum and ileum of each pig was determined using immunofluorescence according to our described method (25). The fluorescence of each slide was investigated using a confocal scanning microscope (NIKON ECLIPSE TI-SR), and the images were analysed using the NIKON DS-U3 software. The total RNA of duodenal, jejunal and ileal mucosa from each pig was extracted using the Trizol Reagent (Ta KaRa, Dalian, China). The concentration and purity of the extracted RNA sample was assayed with a spectrophotometer (Nano Drop, Gene Company Limited, Guangzhou, China) at 260 and 280 nm. The ratio of OD (optical density) 260/280 should vary between 1.8 and 2.0. The reverse transcription of each

RNA sample was performed using the Prime Scripte RT reagent kit (TaKaRa Biotechnology, Dalian, China) following the manufacturer's instructions. The primers (Supplementary Table 2) for genes, Toll-like receptor 4 (TRL-4), nuclear factor kappa-B (NF-κB), myeloid differentiation factor 88 (MYD88), nuclear factor-erythroid 2 (Nrf2), heme oxygenase-1 (HO-1), Caspase-3, B-cell lymphoma-2 (BCL-2), BCL-2 associated x protein (Bax) and porcine β-defensin 1 (pBD129), were synthesized commercially by Takara Bio Inc. (Dalian, China). Real-time PCR was conducted by using the CFX-96 real-time PCR detection system (Bio-Rad). The reaction procedures and the preparation of reaction mixture system has been described before (25). The mRNA level of the target genes was calculated using the $2^{-\Delta\Delta Ct}$ method (26), and three replicates for each sample were simultaneously performed.

Flow cytometry assays

The percentage of cell apoptosis in the jejunum of each pig was determined by flow cytometry. In detail, and the intestinal sample of each pig was flushed gently with ice-cold PBS (for 1 litre: 8.00 g NaCl, 0.20 g KCl, 1.78 g $Na_2HPO_4 \cdot 2\ H_2O$, 0.27 g $KH2PO_4$, pH 7.4), and the washed intestinal serosa layer was spread on a sterile ice pack. The mucosal cells were then scraped with a glass slide. After adding moderate Roswell Park Memorial Institute (RPMI) 1640 medium (Hyclone, America), the cells were transferred to a new centrifuge tube and mixed using a vortex mixer. The mixed cells were then filtered into a flow tube with a 300-mesh filter cloth and centrifuged at 300 × g for 5 min. Then the supernatant was discarded and the sediment was washed again with PBS. The cells were resuspended with 200 μL of Binding Buffer (10 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) and the concentration of the cells was adjusted to 10^6 cells/ml with PBS at 4 °C. Finally, approximate 100 μL of the cell suspension was taken into a flow tube and then resuspend with 1 mL of Binding Buffer and centrifuged at 300 × g for 5 min to obtain sediment. To detect the apoptosis rate of the cells, 5 μL of Annexin V-FITC (fluorescein isothiocyanate, Invitrogen, Australia) was added into the tube for fluorescence staining (10 min), and then another 5 μL of PI (propidium

iodide) staining solution was applied into the tube for 5 min. After the incubation with 500 μL of Binding Buffer, the number of apoptosis cells was detected by CytoFLEX flow cytometry (Backman, America) and the data was analysed using the CytExpert software (Backman, America).

Statistical analysis

All data were subjected to one-way analysis of variance for a completely randomized design using the general linear model procedure of Statistical Product and Service Solutions (SPSS) 24.0 (SPSS, Inc.). Statistical differences among treatments were separated by Tukey's multiple-range test. The results were shown as means and standard error of means (SEM). The difference between groups was regarded as significant when $P < 0.05$, and a trend in difference was regarded when $0.05 < P < 0.10$.

Results

Effect of FOS supplementation on growth performance and antioxidant capacity in weaned piglets upon ETEC challenge

There were no differences ($P > 0.05$, Table 1) in the ADG, ADFI and F:G of pigs among the three groups throughout the experimental period. Comparing with CON pigs, ETEC challenge decreased the activity of GSH-Px and CAT, and increased the concentration of MDA in the plasma ($P<0.05$, Table 2). However, the activity of GSH-Px and CAT was increased and the concentration of MDA was decreased ($P<0.05$, Table 2) in the plasma of EFOS pigs when compared to ECON pigs, and the value of these parameters showed no differences between CON and EFOS pigs ($P>0.05$, Table 2).

Effect of FOS supplementation on intestinal permeability and the distribution of ZO-1

Comparing with the CON pigs, the concentration of plasma DAO was elevated in ECON pigs ($P<0.05$), while it was recovered in EFOS pigs ($P>0.05$, Table 2). Importantly, result of immunofluorescence analysis showed that the localization of

ZO-1 in the duodenum was not affected by dietary FOS. In the jejunum and ileum of ECON pigs (Figure 1A), the staining of ZO-1 was diffuse with little staining at the intercellular tight junction region, which was improved in EFOS pigs. Further analysis showed that the fluorescence intensity of ZO-1 in the duodenum and jejunum of ECON pigs was lower than that of ECON and EFOS pigs (P<0.05, Figure 1B).

Effect of FOS supplementation on cell apoptosis in intestinal mucosa

Result of flow cytometry showed an increase (P<0.05) of the early-stage, late-stage, and total apoptosis rate in the jejunum of ECON pigs (Figure 2B and E) comparing to CON pigs (Figure 2A and D), which was reduced (P<0.05) in EFOS pigs (Figure 2C and F) compared with ECON pigs.

Effect of FOS supplementation on intestinal mucosa immunity

The content of TNF-α in the small intestinal mucosa, IL-1β in the jejunal and ileal mucosa and IL-6 in the duodenal and ileal mucosa of ECON pigs showed elevated compared to CON pigs (P<0.05, Figure 3), while FOS supplementation decreased the contents of TNF-α and IL-1β in the intestinal mucosa of pigs challenged by ETEC when compared with ECON pigs (P<0.05).

Effect of FOS supplementation on the mucosal antioxidant capacity

Comparing with CON and EFOS pigs, the concentration of MDA in the duodenal mucosa of ECON pigs was higher (P<0.05, Table 3). Meanwhile, the activity of CAT, GSH-Px, and SOD in the jejunum mucosa, as well as the activity of GSH-Px and SOD in the ileum mucosa of ECON pigs was decreased compared to CON pigs (P<0.05), which was improved in EFOS pigs (P<0.05).

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

ETEC challenge upregulated the expressions of critical inflammation-related genes such as the TLR-4 and MYD88 in the jejunal mucosa (Figure 4). However, FOS supplementation decreased their expressions in the jejunual mucosa (P<0.05).

Moreover, FOS supplementation decreased the expressions of TLR-4 and NF-*k*B in the duodenal and ileal mucosa, respectively ($P<0.05$) but elevated the expression of pBD-129 in the duodenual and ileal mucosa ($P<0.05$). The expression of antioxidant genes, such as the Nrf-2 in the jejunum and HO-1 in the ileum, were higher in EFOS pigs than that in other pigs when compared with ECON pigs ($P<0.05$). FOS supplementation also elevated the expression of BCL-2 but decreased the expression of Caspase-3 in the jejunual mucosa ($P<0.05$).

Discussion

PWD caused by ETEC infection brings great economic loss to swine industry. In the present study, we proved that the short-term (21 d) supplement of FOS in the diet did not affect the growth performance of weaned pigs, and showed the protective effect of FOS against ETEC-induced intestinal injury in these animals, confirming the potential of FOS as a new healthy feed additive for post-weaning pigs.

Oxidative stress is a prerequisite condition of inflammatory responses. Excessive free radicals are harmful and scavenged by the antioxidant system including non-enzymatic components or a series of antioxidant enzymes (27-28). In the present study, dietary supplementation of FOS was found to maintain the activity of CAT and the concentration of GSH-Px in both plasma and intestinal mucosa of weaned piglets subject to the challenge of ETEC. Consistent results were also found on the mRNA level of Nrf2 and HO-1 genes in the intestinal mucosa of these animals. Nrf2 is a well-known critical transcription factor that can regulate the expression of genes involved in the production of a wide variety of antioxidant enzymes (i.e. glutathione peroxidase and catalase), as well as those genes related to detoxification or "stress-response" (29). HO-1 is one of the most important target genes of Nrf2, which can catalyze the rate limiting step in the degradation of heme and produce free iron, biliverdin, and carbon monoxide (30). Our findings suggest that the dietary supplementation of FOS may contribute to restore the reduced antioxidant capacity of piglets when encountering ETEC infection.

ETEC infection is usually characterized by extensive production of enterotoxins which is easy to penetrate the blood through the damaged intestinal epithelium (31-33). The intestinal barrier is composed of a layer of columnar epithelium and interepithelial tight junctions (TJs), a highly dynamic barrier structure that can selectively absorb water, ions and nutrients by adjusting the permeability among cells, playing a great role in the defense and immunity of the GI tract (34). In the current study, we found that the expression of ZO-1 protein, the most important TJ (35), was substantially decreased in the apical region of the epithelial cells in small intestine of the piglets by the ETEC challenge. However, the expression of ZO-1 in the small intestine of pigs fed FOS containing diet showed no difference compared to those healthy controls even if exposed to ETEC attack, indicating a possible protection of FOS on the intestinal barrier. As a highly active intracellular enzyme in the cytoplasm of intestinal mucosal upper villi, DAO is concentrated and has strong activity in the intestinal mucosal upper villi, but is less abundant and has very low activity in other tissues (36). When the intestinal mucosa is damaged, the intracellular DAO is released into the blood, leading to a sharp rise of DAO in the blood, which makes the activity of DAO a marker to evaluate the integrity and permeability of intestinal epithelium in the case of pathogenic infections (37). Here, we showed that ETEC challenge significantly elevated the concentrations of DAO in the plasma of weaned pigs, indicating a disruption of the intestinal epithelial barrier in these animals. Conversely, when FOS was supplemented into the diet, the concentration of DAO in the plasma of piglets was not affected even under the attack of ETEC, showing a noticeable protection of dietary FOS.

Intestinal epithelial cells (IECs) are in a state of continuous proliferation and renewal (38). During this homeostasis, apoptosis is beneficial to the regeneration and repair of IECs, and the interaction among the proliferation, renewal and apoptosis of IECs is responsible for the normal function of intestinal barrier (39-40). Yet, the excessive apoptosis of IECs leads to the abnormal increase of intestinal permeability and the dysfunction of intestinal barrier, as well as the subsequent diarrhea (41). ETEC infection has been proved to promote the apoptosis of IECs *in vitro* and *in vivo*

(42-43). In the current study, we confirmed that the challenge of ETEC increased the apoptosis rate of the jejunal epithelial cells of weaned pigs. Interestingly, the apoptosis rate of these cells in ETEC infected piglets was reduced to normal level by the pre-feeding of FOS containing diet. Theoretically, the apoptosis of IECs involves the activation of multiple proteins and genes. Of these proteins, Bcl-2 is found to specifically inhibit the expression of apoptotic proteins in the interstitial space and maintain the integrity of the mitochondrial membrane through binding to Bid, Bim or Bad and separating from Bax or Bak. It can also directly combine with the active factor of apoptotic protein-1 (Apaf-1) to form the Bcl-2/Apaf-1/Caspase-9 complex, blocking the initial activation of caspase, a family of common downstream effectors of multiple apoptosis pathways (44-45). The protease cascade reaction is the only way to directly induce the apoptosis of IECs in pigs (46-47). In the present study, we found that the ETEC challenge increased the mRNA level of the apoptosis-related marker gene, Caspase-3, in the small intestine of weaned piglets. But the pre-supplementation of FOS in the diet can prevent this increase of Caspase-3 and simultaneously enhance the expression of the two apoptosis repression-related marker genes, Bcl-2 and BAX, especially in the jejunum. These results provide a molecular basis for the interpretation of improved apoptosis of IECs in those pigs pre-fed with FOS containing diet.

During the development of intestinal inflammation caused by infectious pathogens, the activation of TLR4/NF-κB signaling pathway is regarded as a key incentive (48). As a typical pattern recognition receptor existing in intestinal epithelium, activated TLR4 can stimulate the activation of MyD88/NF-κB signaling pathway, leading to the release of various inflammatory cytokines, such as IL-6, TNF-α and IL-1. On the other hand, some self-produced molecules, such as β-defensins, can directly help IECs themselves fight against pathogens by killing microbes and activating inflammatory cells located in the infected site (49). We found that the pre-feeding of dietary FOS can reduce the concentration of TNF-α, IL-6 and IL-1β in the mucosa of small intestine of ETEC infected pigs to normal level, and such pre-feeding can even increase the expression of pBD129 gene in the duodenum and ileum of these pigs to a

level far higher than that of uninfected pigs. Similarly, when the maternal diet is supplemented with shor-chain FOS (scFOS) at 3.3g/kg during the last 4 weeks of gestation and 1.5g/kg during the 4 weeks of lactation, the TNF-α expression can be decrease in the visceral adipose tissue of their piglets (50). And the scFOS supplementation in the diet at 0.15% also reduces the concentration of TNF-α in the ileum of post-weaning pigs (51). The further real-time PCR analysis also indicate that the decreased expression of inflammatory cytokines in the small intestine of these ETEC infected pigs pre-fed may probably be achieved by the inhibition of TLR4/MyD88/NF-κB pathway. Previous studies also show similar mechanism of mannan-oligosaccharide and galacto-oligosaccharides inhibiting the inflammation in weaning pigs and human infants (52-53).

Conclusions

Our findings suggest that the dietary supplementation of FOS can attenuate the disruption of the intestinal mucosa caused by ETEC in weaned piglets, which was associated with the increase of anti-oxidative capacity and the improvement of intestinal barrier functions. These beneficial effects of FOS on the intestinal health make it an attractive prebiotic that can be tentatively used in the diet of young animals.

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Data Availability and materials

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

Authors' contributions

YL, LL and JH conceived the study, performed the experiment, performed data analysis, and contributed to drafting the manuscript. LL carried out the animal experiment. DC, BY, ZH, PZ, XM, JY, JL and HY conceived the experiment and proofread the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Ethics approval and consent to participate

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Sichuan Agricultural University and approved by the Animal Ethics Committee of Sichuan Agricultural University (Chengdu, China).

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Table 1. Growth performance of pigs from the three experimental groups

Items	Treatment			SEM	*P*-Value
	CON	ECON	EFOS		
Initial BW	6.49	6.5	6.48	0.12	1.00
ADFI (g/day)	459.14	412.59	427.28	16.08	0.51
ADG (g/day)	323.76	298.44	313.52	9.55	0.60
F:G	1.41	1.38	1.36	0.03	0.75

All the measured growth performance related parameters are for whole experimental period (1-21 days). Data are shown as means and SEM (standard error), n = 8. Non-challenged pigs (CON, fed with basal diet), ETEC-challenged pigs (ECON, fed with basal diet and challenged by ETEC), and FOS and ETEC-treated pigs (EFOS, fed with basal diet containing 2.5 g/kg FOS and challenged by ETEC). BW, body weight; ADFI, Average daily feed intake; ADG, Average daily gain; F:G, ADFI / ADG .

Table 2. Improvement of parameters related to antioxidant capacity in the plasma of ETEC challenged piglets by FOS supplement

Items	Treatment			SEM	*P*-Value
	CON	ECON	EFOS		
SOD (U/mL)	183.24	168.45	154.38	6.04	0.15
GSH-Px (mg/mL)	82.87[a]	67.75[b]	88.95[a]	2.87	< 0.01
T-AOC (U/mL)	0.55	0.43	0.52	0.06	0.78
MDA (nmol/mL)	1.95[b]	2.29[a]	2.04[b]	0.05	< 0.01
CAT (U/mL)	62.96[a]	36.64[b]	82.12[a]	6.89	0.01
DAO (U/L)	300.45[b]	420.55[a]	326.12[b]	20.39	0.03

Data are shown as means and SEM, n = 8. Non-challenged pigs (CON, fed with basal diet), ETEC-challenged pigs (ECON, fed with basal diet and challenged by ETEC), and FOS and ETEC-treated pigs (EFOS, fed with basal diet containing 2.5 g/kg FOS and challenged by ETEC). [a, b, c] Diverse superscript letters in the same row mean significantly difference ($P < 0.05$). GSH-Px, glutathione peroxidase; T-AOC, total antioxidant capacity, MDA, malondialdehyde; SOD, auperoxide dismutase; CAT, Catalase; DAO, diamine oxidase.

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Table 3. Improvement of parameters related to antioxidant capacity in small intestine of ETEC challenged piglets by FOS supplement

Items	Treatment			SEM	*P*-Value
	CON	ECON	EFOS		
Duodenum					
CAT (U/gprot)	32.94	27.41	40.07	4.54	0.56
MDA (nmol/mL)	0.83 [b]	1.48 [a]	0.72 [b]	0.15	0.08
GSH-Px (mg/gprot)	285.71	247.48	274.6	16.49	0.66
T-AOC (U/mgprot)	0.75	0.52	0.65	0.07	0.46
SOD (U/mgprot)	27.84	24.79	25.88	1.71	0.78
Jejunum					
CAT (U/gprot)	19.01 [ab]	15.73 [b]	32.46 [a]	3.09	0.04
MDA (nmol/mL)	1.08	1.26	0.93	0.14	0.68
GSH-Px (mg/gprot)	172.40 [a]	135.07 [b]	224.12 [a]	14.14	0.02
T-AOC (U/mgprot)	0.92	0.34	0.73	0.14	0.25
SOD (U/mgprot)	25.58 [a]	16.99 [b]	23.90 [a]	1.41	0.02
Ileum					
CAT (U/gprot)	45.45 [a]	20.46 [b]	48.33 [a]	4.00	< 0.01
MDA (nmol/mL)	2.ll [ab]	2.72 [a]	1.39 [b]	0.29	0.14
GSH-Px (mg/gprot)	240.86 [a]	207.86 [b]	273.53 [a]	11.75	0.06
T-AOC (U/mgprot)	0.50a	0.24	0.51	0.05	0.06
SOD (U/mgprot)	23.52	22.39	22.55	0.70	0.80

Data are shown as means and SEM, n = 8. Non-challenged pigs (CON, fed with basal diet), ETEC-challenged pigs (ECON, fed with basal diet and challenged by ETEC), and FOS and ETEC-treated pigs (EFOS, fed with basal diet containing 2.5 g/kg FOS and challenged by ETEC). [a, b, c] Diverse superscript letters in the same row mean significantly difference ($P < 0.05$). GSH-Px, Glutathione peroxidase; T-AOC, total antioxidant capacity, MDA, Malondialdehyde; SOD, Superoxide dismutase; CAT, Catalase.

Figure legends

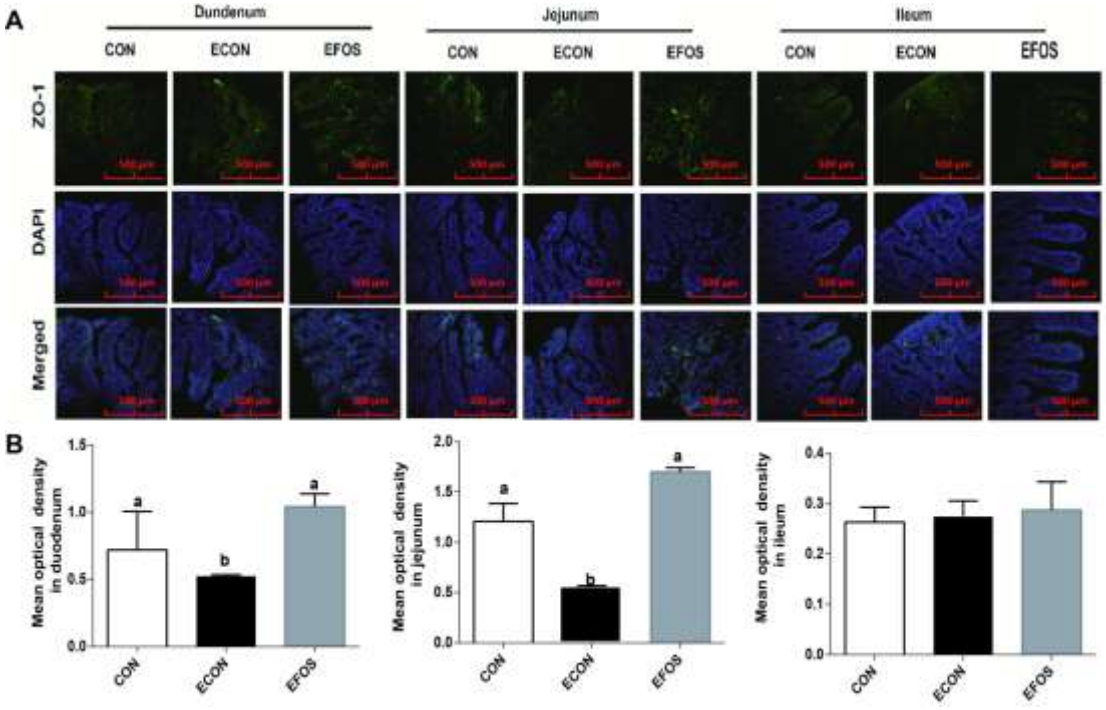

Figure 1. Effect of dietary FOS supplement on the distribution and localization of ZO-1 in the intestinal epithelium of pigs in different groups revealed by immunofluorescence. ZO-1 protein (Green), DAPI stain (blue) as well as merged ZO-1 protein and DAPI are presented. Non-challenged pigs (CON, fed with basal diet), ETEC-challenged pigs (ECON, fed with basal diet and challenged by ETEC), and FOS and ETEC-treated pigs (EFOS, fed with basal diet containing 2.5 g/kg FOS and challenged by ETEC). A, immunofluorescence image of duodenum, jejunum and ileum. B, Fluorescence intensity analysis corresponding to each intestinal segment.

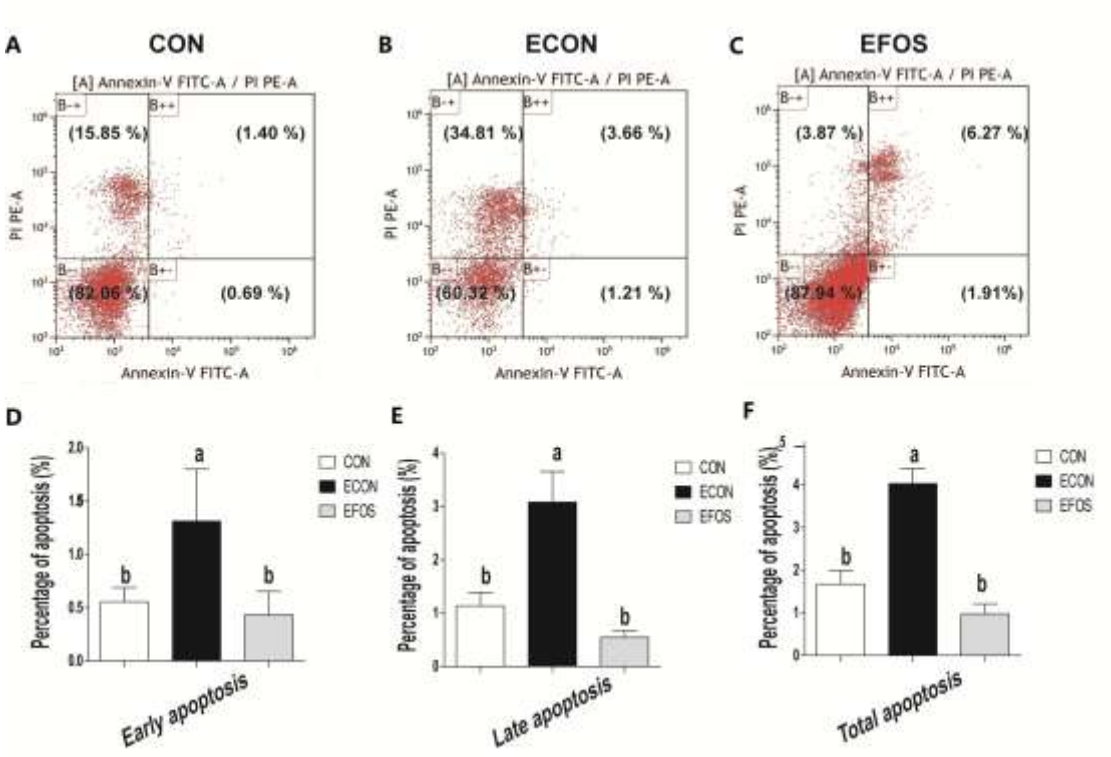

Figure 2. Effect of dietary FOS supplement on the apoptosis rate of IECs in the jejunum of pigs in different groups revealed by flow cytometry. A and D, percentage of apoptotic cells at early stage of apoptosis. B and E, percentage of apoptotic cells at late stage of apoptosis. D and F, the total apoptosis rate. In A, B and C, [A] means a total of 30000 cells were used in each acquisition reading. Frames were divided into 4 quadrants: B-+ represents necrotic cells, B++ represents late apoptotic and early necrotic cells, B-- represents early apoptotic cells, and B+- represents normal cells. Non-challenged pigs (CON, fed with basal diet), ETEC-challenged pigs (ECON, fed with basal diet and challenged by ETEC), and FOS and ETEC-treated pigs (EFOS, fed with basal diet containing 2.5 g/kg FOS and challenged by ETEC). ^{a,b,c} Diverse superscript letters on top of each bar mean significantly difference (P<0.05).

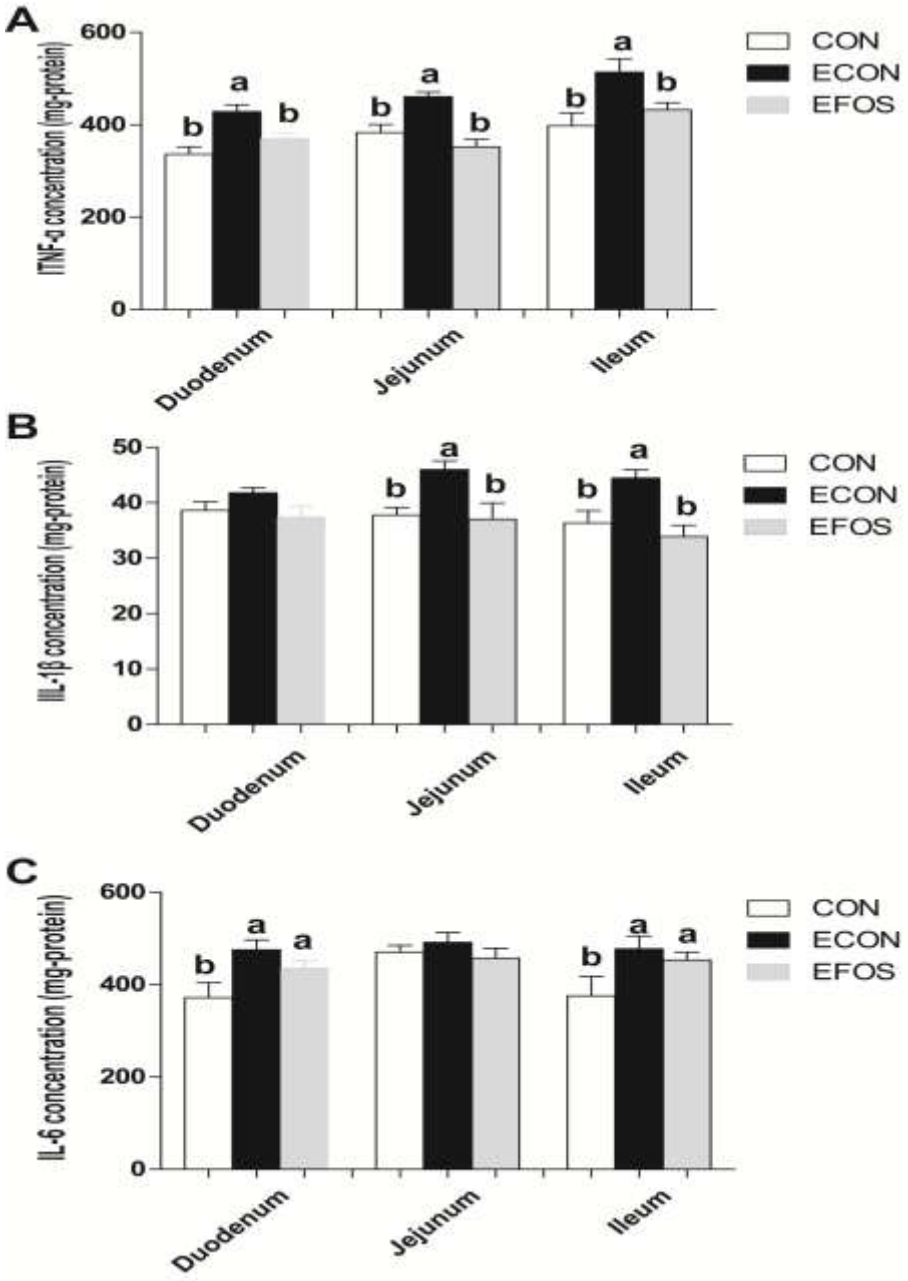

Figure 3. Effect of dietary FOS supplement on the concentration of cytokines in the mucosa of small intestine of pigs in different groups revealed by real-time PCR. TNF-α, tumor necrosis factor-α (A), IL-1β, interleukin -1β (B), IL-6, interleukin 6 (C), respectively; Non-challenged pigs (CON, fed with basal diet), ETEC-challenged pigs (ECON, fed with basal diet and challenged by ETEC), and FOS and ETEC-treated pigs (EFOS, fed with basal diet containing 2.5 g/kg FOS and challenged by ETEC); ^{a,b,c} Diverse superscript letters on top of each bar mean significantly difference (P<0.05).

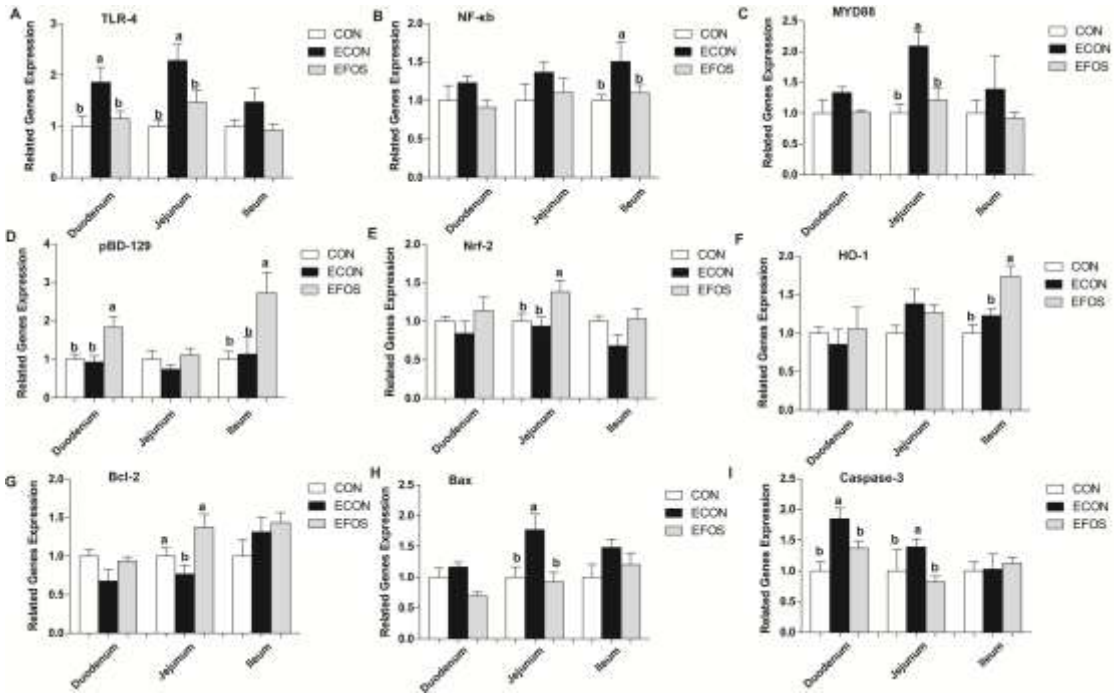

Figure 4. Effect of dietary FOS supplement on the relative expression of genes associated with inflammation, antioxidant capacity and apoptosis in the mucosa of small intestine of pigs in different groups. Toll-like receptor 4, TLR4; myeloid differentiation factor 88, MyD88; nuclear factor-κB, NF-κB; nuclear factor erythroid-derived 2-related factor 2, Nrf2; heme oxygenase-1, HO-1; B-cell lymphoma-2-associated X protein, BAX; B-cell lymphoma-2, BCL-2; cysteinyl aspartate-specific proteinase-3, caspase-3. Non-challenged pigs (CON, fed with basal diet), ETEC-challenged pigs (ECON, fed with basal diet and challenged by ETEC), and FOS and ETEC-treated pigs (EFOS, fed with basal diet containing 2.5 g/kg FOS and challenged by ETEC). ^{a,b,c} Diverse superscript letters on top of each bar mean significantly difference (P<0.05).