British Journal of Nutrition (2022), 128, 1526–1534 doi:10.1017/S0007114521004451

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

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(Submitted 1 April 2021 – Final revision received 29 September 2021 – Accepted 9 November 2021 – First published online 12 November 2021)

Abstract

Diarrhoea 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 fructo-oligosaccharides (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 × 109 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, compared with ECON pigs, the level of GSH peroxidase and catalase in the plasma and intestinal mucosa of EFOS pigs was increased ($P < 0·05$), and the intestinal barrier marked by zonula occluden-1 and plasmatic diamine oxidase 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.

Key words: **ETEC: FOS: Intestinal epithelium: Weaned piglets**

Post-weaning diarrhoea, 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. Post-weaning diarrhoea always results in the increase of mortality, dehydration, weight loss, as well as growth retardation in young animals[2]. These disorders can be induced by various weaning stresses 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 post-weaning diarrhoea may affect the immune responses of piglets and lead to an intestinal dysfunction[3,4]. Although the utilisation 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 minimise 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 polymerisation[10]. Fructo-oligosaccharides (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 micro-organisms in the large intestine of pigs[12]. The utilisation 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],

Abbreviations: BAX, BCL-2-associated x protein; BCL-2, B-cell lymphoma-2; Caspase, cysteinyl aspartate-specific proteinase; CAT, catalase; CON, non-ETEC-challenged control; DAO, diamine oxidase; ECON, ETEC-challenged control; EFOS, ETEC challenge + FOS treatment; ETEC, enterotoxigenic E. coli; FOS, fructo-oligosaccharides; GSH-Px, GSH peroxidase; IEC, intestinal epithelial cells; ZO-1, zonula occluden-1.

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which makes FOS become an attractive alternative of antibiotics in swine feed.

As the largest immune organ in animal body, gastrointestinal 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 gastrointestinal 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 gastrointestinal 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 antioxidative capacity in weaned piglets. Moreover, pigs share similar anatomic and physiological structures with humans[19], and 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-d-old weaned crossbred (Duroc × Landrace × Yorkshire) pigs with an average initial body weight of 6·49 kg were selected and randomly divided into three groups after a 3-d adaptation, including: (1) non-ETEC-challenged control (CON; pigs fed a basal diet and infused with sterilised 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, is the fructosyltransferase hydrolysate of chicory. The FOS product is a series of oligosaccharides with 2–10 degree of polymerisation, 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 maize starch. The basal diet (online Supplementary Table 1) was formulated to meet the nutrient requirements recommended by the National Research Council[21]. Each group consisted of eight 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°C 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 ($2{\cdot}5 \times 10^9$ CFU/ml, serotype O149: K91: K88ac; China Institute of Veterinary Drugs Control), while pigs in the CON group were orally administered with an equal volume of sterilised Luria–Bertani culture[22]. The feed intake of each pig was measured daily, and the body weight of each pig was measured on day 22 after 12-h fasting. The gain-to-feed ratio of each pig was calculated according to the average daily gain and average daily feed intake.

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 Na anticoagulant by venepuncture after 12 h of fasting, and the blood sample was centrifuged at 3500 × g at 4°C for 10 min to obtain plasma[23]. All the prepared plasma samples were kept at −20°C until analysis. After the collection of blood sample, each pig was euthanised with an intravenous injection of Na pentobarbital at a dosage of 200 mg/kg body weight and then slaughtered[24], and the abdomen was opened rapidly. Approximately, 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 GSH, malondialdehyde (MDA), and the total antioxidant capacity (T-AOC) were measured by using commercial kits purchased from Nanjing Jiancheng Bioengineering Institute. The activity of diamine oxidase (DAO) in the plasma was also measured by using commercial kits purchased from Nanjing Jiancheng Bioengineering Institute. The concentrations of inflammatory cytokines such TNF-α, IL-1β and IL-6 in the intestinal mucosa were detected by using an ELISA following the instructions of corresponding kits supplied by Jiangsu Jingmei Biotechnology Co., Ltd. All the above kits are swine-specific. The minimum detectable concentrations of DAO, TNF-α, IL-1β and IL-6 are 10 pg/m, 10 pg/ml, ng/l and 50 ng/l, respectively. In addition, the minimum detectable concentrations of CAT, GSH, MDA and T-AOC are 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 localisation 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 (TaKaRa). The concentration and purity of the extracted RNA sample were assayed with a spectrophotometer (Nano Drop, Gene Company Limited) at 260 and 280 nm. The ratio of optical density (OD) 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) following the manufacturer's instructions. The primers (online Supplementary Table 2) for genes, toll-like receptor 4 (TRL-4), NF-κB, myeloid differentiation factor 88 (MYD88), nuclear factor-erythroid 2 (Nrf2), haem oxygenase-1 (HO-1), cysteinyl aspartate-specific proteinase-3 (Caspase-3), B-cell lymphoma-2 (BCL-2), BCL-2-associated x protein (Bax) and porcine β-defensin 1 (pBD129), were synthesised commercially by Takara Bio Inc.. Real-time PCR was conducted using the CFX-96 real-time PCR detection system (Bio-Rad). The reaction procedures and the preparation of reaction mixture system have 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, 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), 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, 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) was added into the tube for fluorescence staining (10 min), and then another 5 µl of 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) and the data were analysed using the CytExpert software (Backman).

Statistical analysis

All data were subjected to one-way ANOVA for a completely randomised 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. The difference between groups was regarded as significant when $P < 0{\cdot}05$, and a trend in difference was regarded when $0{\cdot}05 < P < 0{\cdot}10$.

Table 1. Growth performance of pigs from the three experimental groups (Mean values and their standard errors of the mean, *n* 8)

	Treatment				
Items	CON	ECON	EFOS	SEM	*P*
Initial BW	6·49	6·5	6·48	0·12	1·00
ADFI (g/d)	459·14	412·59	427·28	16·08	0·51
ADG (g/d)	323·76	298·44	313·52	9·55	0·60
F:G	1·41	1·38	1·36	0·03	0·75

CON, non-ETEC-challenged control; ECON, ETEC-challenged control; EFOPS, ETEC challenge + FOS treatment; BW, body weight; ADFI, average daily feed intake; ADG, average daily gain; F:G, ADFI/ADG.
All the measured growth performance-related parameters are for whole experimental period (1–21 d).
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).

Table 2. Improvement of parameters related to antioxidant capacity in the plasma of ETEC-challenged piglets by FOS supplement (Mean values and their standard errors of the mean, *n* 8)

	Treatment				
Items	CON	ECON	EFOS	SEM	*P*
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

ETEC, enterotoxigenic *E. coli*; FOS, FOS, fructo-oligosaccharides; CON, non-ETEC-challenged control; ECON, ETEC-challenged control; EFOPS, ETEC challenge + FOS treatment; SOD, superoxide dismutase; GSH-Px, GSH peroxidase; T-AOC, total antioxidant capacity, MDA, malondialdehyde; CAT, catalase; DAO, diamine oxidase.
^{a,b,c}Diverse superscript letters in the same row mean significant differences ($P < 0$ 05).
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).

Results

Effect of fructo-oligosaccharide supplementation on growth performance and antioxidant capacity in weaned piglets upon enterotoxigenic E. coli *challenge*

There were no differences ($P > 0{\cdot}05$, Table 1) in the average daily gain, average daily feed intake and gain-to-feed ratio of pigs among the three groups throughout the experimental period. Compared with CON pigs, ETEC challenge decreased the activity of GSH peroxidase (GSH-Px) and CAT and increased the concentration of MDA in the plasma ($P < 0{\cdot}05$, Table 2). However, the activity of GSH-Px and CAT was increased and the concentration of MDA was decreased ($P < 0{\cdot}05$, Table 2) in the plasma of EFOS pigs when compared with ECON pigs, and the value of these parameters

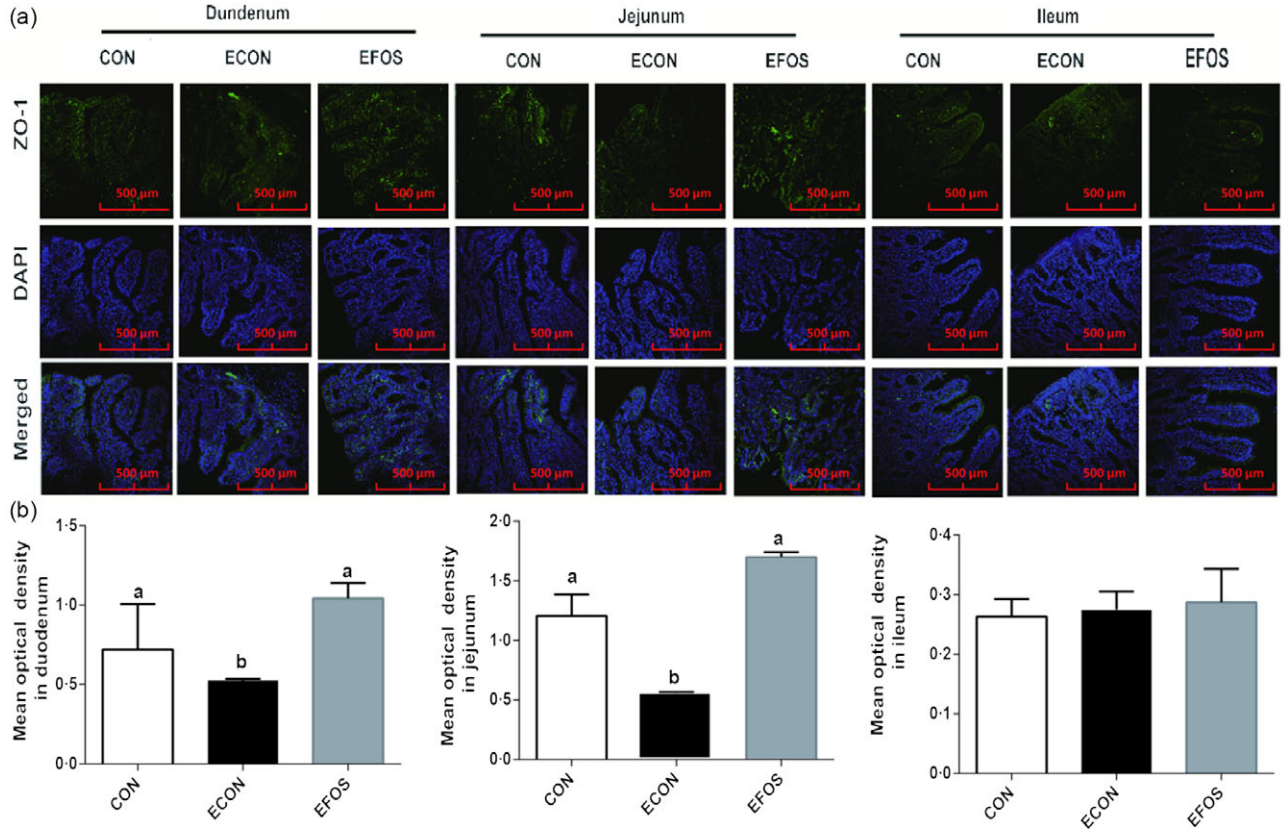

Fig. 1. Effect of dietary FOS supplement on the distribution and localisation of ZO-1 in the intestinal epithelium of pigs in different groups revealed by immunofluorescence. ZO-1 protein (green), DAPI stain (blue) as well as merged ZO-1 protein and DAPI are presented. Non-challenged pigs (CON, fed with 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. ETEC, enterotoxigenic *E. coli*; FOS, fructo-oligosaccharides; ZO-1, zonula occluden-1.

showed no differences between CON and EFOS pigs ($P > 0{\cdot}05$, Table 2).

Effect of fructo-oligosaccharide supplementation on intestinal permeability and the distribution of zonula occluden-1

Compared with the CON pigs, the concentration of plasma DAO was elevated in ECON pigs ($P < 0{\cdot}05$), while it was recovered in EFOS pigs ($P > 0{\cdot}05$, Table 2). Importantly, result of immunofluorescence analysis showed that the localisation of ZO-1 in the duodenum was not affected by dietary FOS. In the jejunum and ileum of ECON pigs, the staining of ZO-1 was diffuse with little staining at the intercellular tight junction region, which was improved in EFOS pigs (Fig. 1a). Further analysis showed that the fluorescence intensities of ZO-1 in the duodenum and jejunum of ECON pigs were lower than that of ECON and EFOS pigs (Fig. 1b).

Effect of fructo-oligosaccharide supplementation on cell apoptosis in intestinal mucosa

Result of flow cytometry showed an increase ($P < 0{\cdot}05$) of the early-stage, late-stage and total apoptosis rate in the jejunum of ECON pigs compared with CON pigs, which was reduced ($P < 0{\cdot}05$) in EFOS pigs compared with ECON pigs (Fig. 2).

Effect of fructo-oligosaccharide 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 with CON pigs ($P < 0{\cdot}05$, Fig. 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 ($P < 0{\cdot}05$).

Effect of fructo-oligosaccharide supplementation on the mucosal antioxidant capacity

Compared with CON and EFOS pigs, the concentration of MDA in the duodenal mucosa of ECON pigs was higher ($P < 0{\cdot}05$, Table 3). Meanwhile, the activities of CAT, GSH-Px and superoxide dismutase in the jejunum mucosa as well as the activities of GSH-Px and superoxide dismutase in the ileum mucosa of ECON pigs were decreased compared with CON pigs ($P < 0{\cdot}05$), which were improved in EFOS pigs ($P < 0{\cdot}05$).

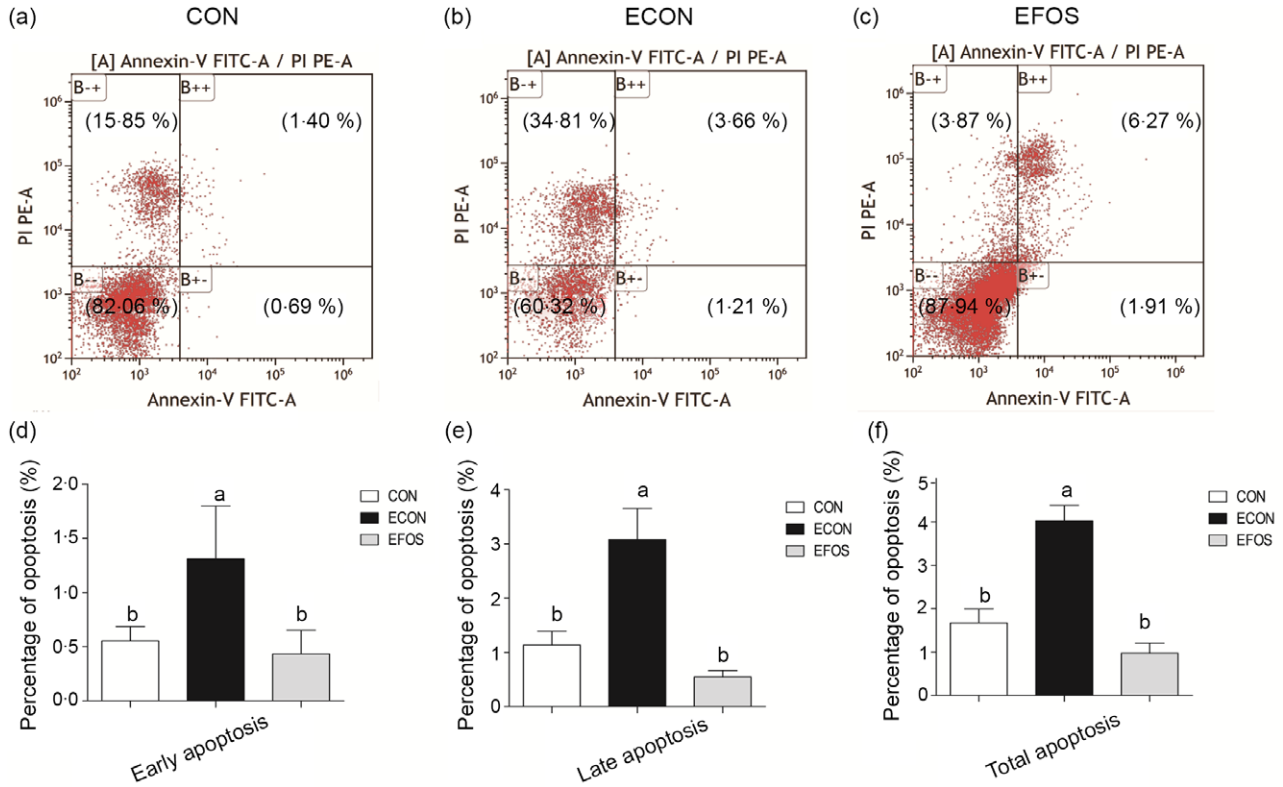

Fig. 2. Effect of dietary FOS supplement on the apoptosis rate of IEC in the jejunum of pigs in different groups revealed by flow cytometry. (a and d) Percentage of apoptotic cells at early stage of apoptosis. (b and e) Percentage of apoptotic cells at late stage of apoptosis. (d and f) The total apoptosis rate. (a, b and c) [A] means a total of 30 000 cells were used in each acquisition reading. Frames were divided into four 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 significant differences ($P < 0·05$). ETEC, enterotoxigenic *E. coli*; FOS, fructo-oligosaccharides; IEC, intestinal epithelial cells.

Effect of fructo-oligosaccharide 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 (Fig. 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

Post-weaning diarrhoea 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. GSH-Px and CAT), 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 catalyse the rate-limiting step in the degradation of haem and produce free Fe, 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 characterised 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

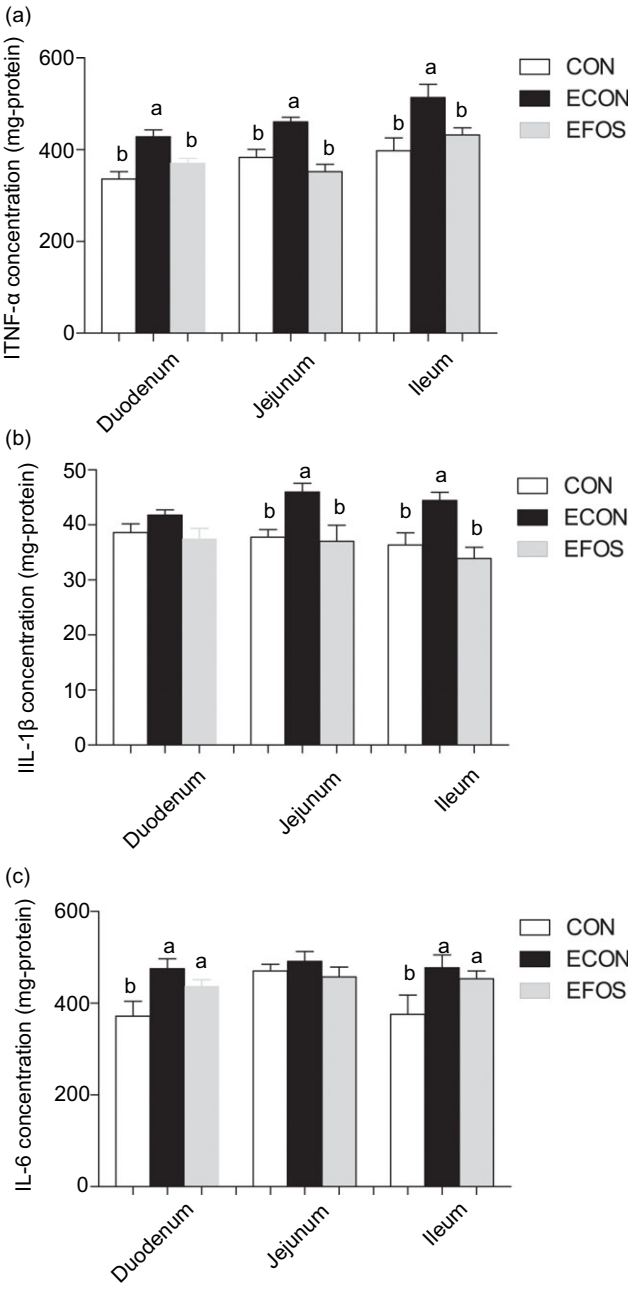

Fig. 3. Effect of dietary FOS supplement on the concentration of cytokines in the mucosa of small intestine of pigs in different groups revealed by real-time PCR. TNF-α (A), IL-1β (B), IL-6 (C). Non-challenged pigs (CON, fed with basal diet), ETEC-challenged pigs (ECON, fed with basal diet and challenged 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 significant differences ($P < 0·05$). ETEC, enterotoxigenic *E. coli*; FOS, fructo-oligosaccharides.

tight junctions, 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 defence and immunity of the gastrointestinal tract[34]. In the current study, we found that the expression of ZO-1 protein, the most important tight junction[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 with 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 (IEC) are in a state of continuous proliferation and renewal[38]. During this homoeostasis, apoptosis is beneficial to the regeneration and repair of IEC, and the interaction among the proliferation, renewal and apoptosis of IEC is responsible for the normal function of intestinal barrier[39,40]. Yet, the excessive apoptosis of IEC leads to the abnormal increase of intestinal permeability and the dysfunction of intestinal barrier, as well as the subsequent diarrhoea[41]. ETEC infection has been proved to promote the apoptosis of IEC *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 IEC 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 IEC 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 IEC 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 signalling 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 signalling pathway, leading to the release of various inflammatory cytokines, such as IL-6, TNF-α and IL-1. On the other hand, some

Table 3. Improvement of parameters related to antioxidant capacity in small intestine of ETEC-challenged piglets by FOS supplement (Mean values and their standard errors of the mean)

Items	Treatment			SEM	*P*
	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[a,b]	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.II[a,b]	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

ETEC, enterotoxigenic *E. coli*; FOS, FOS, fructo-oligosaccharides; CON, non-ETEC-challenged control; ECON, ETEC-challenged control; EFOPS, ETEC challenge + FOS treatment; CAT, catalase; MDA, malondialdehyde; GSH-Px, GSH peroxidase; T-AOC, total antioxidant capacity; SOD, superoxide dismutase.
[a,b,c]Diverse superscript letters in the same row mean significant differences ($P < 0.05$).
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).

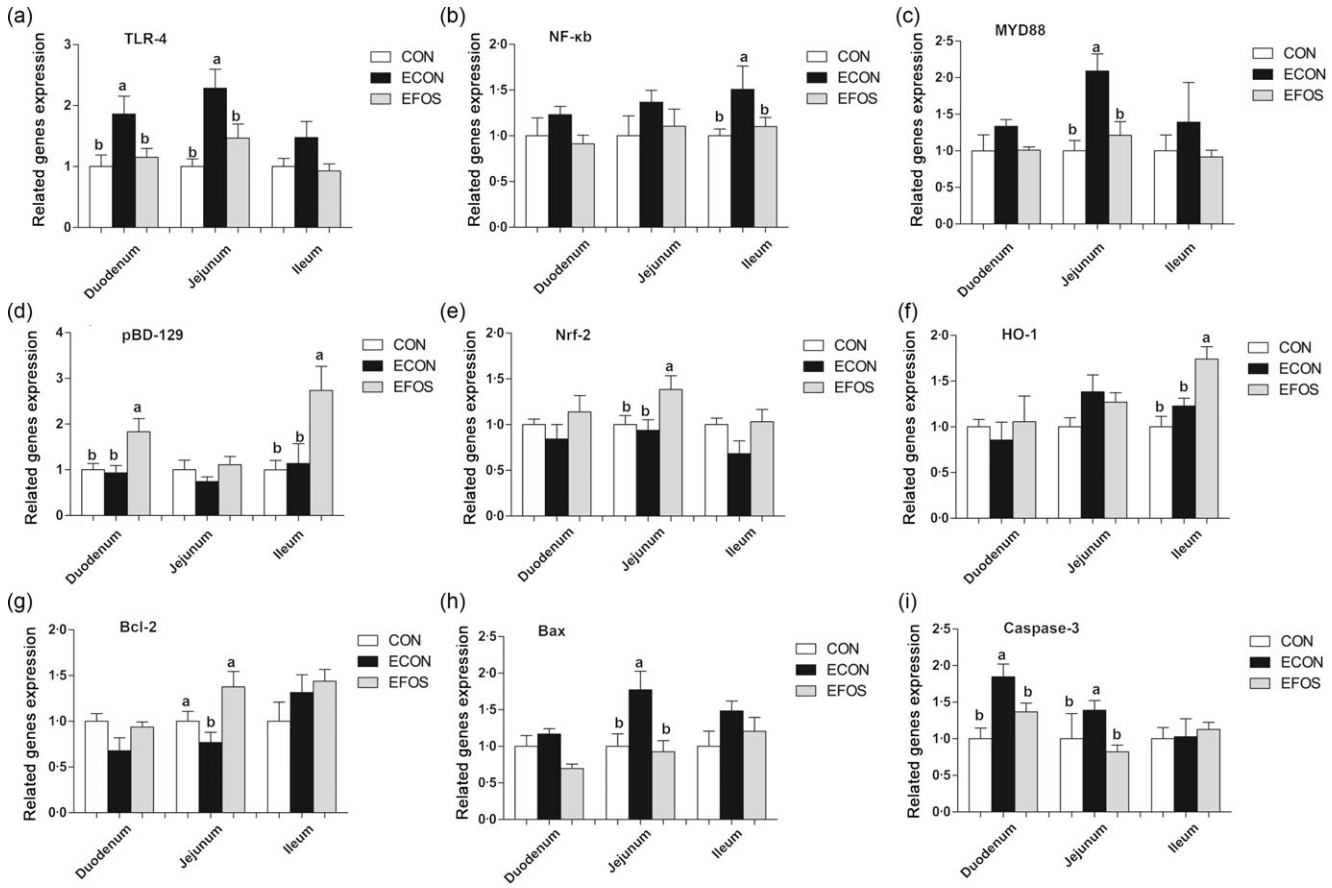

Fig. 4. Effect of dietary FOS supplement on the relative expression of genes associated with inflammation, antioxidant capacity and apoptosis in the mucosa of small intestine of pigs in different groups. Non-challenged pigs (CON, fed with basal diet), ETEC-challenged pigs (ECON, fed with basal diet and 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 significant differences ($P < 0·05$). ETEC, enterotoxigenic *E. coli*; FOS, fructo-oligosaccharides; TLR4, toll-like receptor 4; MyD88, myeloid differentiation factor 88; Nrf2, nuclear factor erythroid-derived 2-related factor 2; HO-1, haem oxygenase-1; BAX, B-cell lymphoma-2-associated X protein; BCL-2, B-cell lymphoma-2; Caspase-3, cysteinyl aspartate-specific proteinase-3.

self-produced molecules, such as β-defensins, can directly help IEC 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 short-chain FOS at 3·3 g/kg during the last 4 weeks of gestation and 1·5 g/kg during the lactation, the TNF-α expression decreases in the visceral adipose tissue of their piglets[50]. And the short-chain FOS 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 indicates 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 in antioxidative 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.

Acknowledgements

The authors thank Wang Huifen, Wu Fali and Yu En for their help during the animal trial and sample collections.

This work was supported by the Key Research and Development Programs of Sichuan Province (20ZDYF0003 and 2018NZDZX0005), and the National Natural Science Foundation of China (31972599).

Y. L., L. L. and J. H. conceived the study, performed the experiment, performed data analysis and contributed to drafting the manuscript. L. L. carried out the animal experiment. D. C., B. Y., Z. H., P. Z., X. M., J. Y., J. L. and H. Y. conceived the experiment and proofread the manuscript. All authors read and approved the final manuscript.

The authors declare that there are no conflicts of interest.

Supplementary material

For supplementary material/s referred to in this article, please visit https://doi.org/10.1017/S0007114521004451

References

1. Nagy B & Fekete PZ (2005) Enterotoxigenic *Escherichia coli* in veterinary medicine. *Int J Med Microbiol* **295**, 443–454.
2. Rhouma M, Fairbrother JM, Beaudry F, *et al.* (2017) Post weaning diarrhea in pigs: risk factors and non-colistin-based control strategies. *Acta Vet Scand* **59**, 31.
3. Bhandari SK, Xu B, Nyachoti CM, *et al.* (2008) Evaluation of alternatives to antibiotics using an *Escherichia coli* K88+ model of piglet diarrhea: effects on gut microbial ecology1. *J Anim Sci* **86**, 836–847.
4. Burkey TE, Skjolaas K & Minton J (2009) Board-invited review: porcine mucosal immunity of the gastrointestinal tract. *J Anim Sci* **87**, 1493–1501.
5. Owusu-Asiedu A, Nyachoti C & Marquardt R (2003) Response of early-weaned pigs to an enterotoxigenic *Escherichia coli* (K88) challenge when fed diets containing spray-dried porcine plasma or pea protein isolate plus egg yolk antibody, zinc oxide, fumaric acid, or antibiotic. *J Anim Sci* **81**, 1790–1798.
6. Kiarie E, Bhandari S, Scott M, *et al.* (2011) Growth performance and gastrointestinal microbial ecology responses of piglets receiving *Saccharomyces cerevisiae* fermentation products after an oral challenge with *Escherichia coli* (K88). *J Anim Sci* **89**, 1062–1078.
7. Thacker PA (2003) Alternatives to antibiotics as growth promoters for use in swine production: a review. *J Anim Sci Biotechnol* **4**, 35.
8. Bornet F, Brouns F, Tashiro Y, *et al.* (2002) Nutritional aspects of short-chain fructooligosaccharides: natural occurrence, chemistry, physiology and health implications. *Dig Liver Dis* **34**, S111–S120.
9. Nishizawa-Yokoi A, Yabuta Y & Shigeoka S (2008) The contribution of carbohydrates including raffinose family oligosaccharides and sugar alcohols to protection of plant cells from oxidative damage. *Plant Signaling Behav* **3**, 1016–1018.
10. Le Bourgot C, Ferret-Bernard S, Blat S, *et al.* (2016) Short-chain fructooligosaccharide supplementation during gestation and lactation or after weaning differentially impacts pig growth and IgA response to influenza vaccination. *J Funct Foods* **24**, 307–315.
11. Sabater-Molina M, Larqué E, Torrella F, *et al.* (2009) Dietary fructooligosaccharides and potential benefits on health. *J Physiol Biochem* **65**, 315–328.
12. Schokker D, Fledderus J, Jansen R, *et al.* (2018) Supplementation of fructooligosaccharides to suckling piglets affects intestinal microbiota colonization and immune development. *J Anim Sci* **96**, 2139–2153.
13. Gao F, Jiang Y, Zhou G, *et al.* (2001) Effect of fructooligosaccharides (FOS) on growth, metabolism and immune in weaning piglets. *China Anim Husb Vet Med* **33**, 8–9.
14. Oswald IP (2006) Role of intestinal epithelial cells in the innate immune defence of the pig intestine. *Vet Res* **37**, 359–368.
15. Pié S, Lallès JP, Blazy F, *et al.* (2004) Weaning is associated with an upregulation of expression of inflammatory cytokines in the intestine of piglets. *J Nutr* **134**, 641–647.
16. Nyachoti C, Kiarie E, Bhandari S, *et al.* (2012) Weaned pig responses to *Escherichia coli* K88 oral challenge when receiving a lysozyme supplement. *J Anim Sci* **90**, 252–260.
17. Bosi P, Casini L, Finamore A, *et al.* (2004) Spray- dried plasma improves growth performance and reduces inflammatory status of weaned pigs challenged with enterotoxigenic *Escherichia coli* K88. *J Anim Sci* **82**, 1764–1772.
18. Csernus B & Czeglédi L (2020) Physiological, antimicrobial, intestine morphological, and immunological effects of fructooligosaccharides in pigs. *Arch Anim Breed* **63**, 325–335.
19. Kraemer JG, Ramette A, Aebi S, *et al.* (2018) Influence of pig farming on the human nasal microbiota: key role of airborne microbial communities. *Appl Environ Microbiol* **84**, e02470–e02417.

20. Liu L, Chen D, Yu B, *et al.* (2020) Fructooligosaccharides improve growth performance and intestinal epithelium function in weaned pigs exposed to enterotoxigenic *Escherichia coli*. *Food Funct* **11**, 9599–9612.
21. National Research Council (2012) *Nutrient Requirements of Swine*, 11th ed. Washington, DC: National Academies Press.
22. Xiao D, Wang Y, Liu G, *et al.* (2014) Effects of chitosan on intestinal inflammation in weaned pigs challenged by enterotoxigenic *Escherichia coli*. *PLOS ONE* **9**, e104192.
23. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
24. Liu L, Wu C, Chen D, *et al.* (2020) Selenium-enriched yeast alleviates oxidative stress-induced intestinal mucosa disruption in weaned pigs. *Oxid Med Cell Longevity* **2020**, 1–11.
25. Wan J, Zhang J, Chen D, *et al.* (2018) Alginate oligosaccharide enhances intestinal integrity of weaned pigs through altering intestinal inflammatory responses and antioxidant status. *RSC Adv* **8**, 13482–13492.
26. Livak KJ & Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**, 402–408.
27. Bolzán AD, Bianchi MS & Bianchi NO (1997) Superoxide dismutase, catalase and glutathione peroxidase activities in human blood: influence of sex, age and cigarette smoking. *Clin Biochem* **30**, 449–454.
28. Lightfoot TJ, Skibola CF, Smith AG, *et al.* (2006) Polymorphisms in the oxidative stress genes, superoxide dismutase, glutathione peroxidase and catalase and risk of non-Hodgkin's lymphoma. *Haematologica* **91**, 1222–1227.
29. Yang C, Ferket P, Hong Q, *et al.* (2012) Effect of chito-oligosaccharide on growth performance, intestinal barrier function, intestinal morphology and cecal microflora in weaned pigs. *J Anim Sci* **90**, 2671–2676.
30. Kim Y-M, Pae H-O, Park JE, *et al.* (2011) Heme oxygenase in the regulation of vascular biology: from molecular mechanisms to therapeutic opportunities. *Antioxid Redox Signaling* **14**, 137–167.
31. Luo Q, Kumar P, Vickers TJ, *et al.* (2013) Enterotoxigenic *Escherichia coli* secretes a highly conserved mucin-degrading metalloprotease to effectively engage intestinal epithelial cells. *Infect Immun* **82**, 509–521.
32. Gao Y, Han F, Huang X, *et al.* (2013) Changes in gut microbial populations, intestinal morphology, expression of tight junction proteins, and cytokine production between two pig breeds after challenge with *Escherichia coli* K88: a comparative study. *J Anim Sci* **91**, 5614–5625.
33. Tang Y, Tan B, Xiong X, *et al.* (2015) Methionine deficiency reduces autophagy and accelerates death in intestinal epithelial cells infected with enterotoxigenic *Escherichia coli*. *Amino Acids* **47**, 2199–2204.
34. Dokladny K, Zuhl MN & Moseley PL (2016) Intestinal epithelial barrier function and tight junction proteins with heat and exercise. *J Appl Physiol* **120**, 692–701.
35. Fanning AS, Jameson BJ, Jesaitis LA, *et al.* (1998) The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. *J Biol Chem* **273**, 29745–29753.
36. Maria Paola A-C & Autuori F (2018) *Localization of Diamine Oxidase in Animal Tissues. Structure and Functions of Amine Oxidases*. Boca Raton, FL: CRC Press.
37. Thompson JS, Vaughan W, Forst C, *et al.* (1987) The effect of the route of nutrient delivery on gut structure and diamine oxidase levels. *J Parenter Enteral Nutr* **11**, 28–32.
38. Vereecke L, Beyaert R & van Loo G (2011) Enterocyte death and intestinal barrier maintenance in homeostasis and disease. *Trends Mol Med* **17**, 584–593.
39. Fujimoto K, Iwakiri R, Wu B, *et al.* (2002) Homeostasis in the small intestinal mucosa balanced between cell proliferation and apoptosis is regulated partly by the central nervous system. *J Gastroenterol* **37**, 139–144.
40. Bernstein H, Holubec H, Warneke JA, *et al.* (2002) Patchy field defects of apoptosis resistance and dedifferentiation in flat mucosa of colon resections from colon cancer patients. *Ann Surg Oncol* **9**, 505–517.
41. Souza HS, Tortori CJ, Castelo-Branco MT, *et al.* (2005) Apoptosis in the intestinal mucosa of patients with inflammatory bowel disease: evidence of altered expression of FasL and perforin cytotoxic pathways. *Int J Colorectal Dis* **20**, 277–286.
42. Ren W, Yin J, Chen S, *et al.* (2016) Proteome analysis for the global proteins in the jejunum tissues of enterotoxigenic *Escherichia coli*-infected piglets. *Sci Rep* **6**, 1–9.
43. Xia Y, Bin P, Liu S, *et al.* (2018) Enterotoxigenic *Escherichia coli* infection promotes apoptosis in piglets. *Microb Pathog* **125**, 290–294.
44. Neuzil J, Wang X-F, Dong L-F, *et al.* (2006) Molecular mechanism of 'mitocan'-induced apoptosis in cancer cells epitomizes the multiple roles of reactive oxygen species and Bcl-2 family proteins. *FEBS Lett* **580**, 5125–5129.
45. Yuan Z, Liu S, Yao J, *et al.* (2016) Expression of Bcl-2 genes in channel catfish after bacterial infection and hypoxia stress. *Dev Comp Immunol* **65**, 79–90.
46. Budihardjo I, Oliver H, Lutter M, *et al.* (1999) Biochemical pathways of caspase activation during apoptosis. *Annu Rev Cell Dev Biol* **15**, 269–290.
47. Riedl SJ & Shi Y (2004) Molecular mechanisms of caspase regulation during apoptosis. *Nat Rev Mol Cell Biol* **5**, 897–907.
48. Sabroe I, Parker LC, Dower SK, *et al.* (2008) The role of TLR activation in inflammation. *J Pathol* **214**, 126–135.
49. Pomorska-Mo M & Markowska-Daniel I (2011) Porcine cathelicidins and defensins. *Med Weter* **67**, 20–24.
50. Le Bourgot C, Ferret-Bernard S, Apper E, *et al.* (2019) Perinatal short-chain fructooligosaccharides program intestinal microbiota and improve enteroinsular axis function and inflammatory status in high-fat diet-fed adult pigs. *FASEB J* **33**, 301–313.
51. Le Bourgot C, Le Normand L, Formal M, *et al.* (2017) Maternal short-chain fructo-oligosaccharide supplementation increases intestinal cytokine secretion, goblet cell number, butyrate concentration and Lawsonia intracellularis humoral vaccine response in weaned pigs. *Br J Nutr* **117**, 83–92.
52. Scholtens PA, Alliet P, Raes M, *et al.* (2008) Fecal secretory immunoglobulin A is increased in healthy infants who receive a formula with short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides. *J Nutr* **138**, 1141–1147.
53. Che T, Song M, Liu Y, *et al.* (2012) Mannan oligosaccharide increases serum concentrations of antibodies and inflammatory mediators in weanling pigs experimentally infected with porcine reproductive and respiratory syndrome virus. *J Anim Sci* **90**, 2784–2793.

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