View Article Online
View Journal

Food & Function

Linking the chemistry and physics of food with health and nutrition

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: L. Liu, D. Chen, B. Yu, H. Yin, Z. Huang, Y. Luo, P. Zheng, X. Mao, J. Yu, J. Luo, H. Yan and J. He, *Food Funct.*, 2020, DOI: 10.1039/D0FO01998D.

This is an Accepted Manuscript, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this Accepted Manuscript with the edited and formatted Advance Article as soon as it is available.

You can find more information about Accepted Manuscripts in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this Accepted Manuscript or any consequences arising from the use of any information it contains.

View Article Online
DOI: 10.1039/D0FO01998D

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

Lei Liu[1,2], Daiwen Chen[1,2], Bing Yu[1,2], Heng Yin[3*], Zhiqing Huang[1,2], Yuheng Luo[1,2], Ping Zheng[1,2], Xiangbing Mao[1,2], Jie Yu[1,2], Junqiu Luo[1,2], HuiYan[1,2], Jun He[1,2*]

[1]Institute of Animal Nutrition, Sichuan Agricultural University, Sichuan Province, Chengdu, 611130, P. R. China

[2]Key Laboratory of Animal Disease-resistant Nutrition, Sichuan Province, Chengdu, 611130, P. R. China

[3]Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Liaoning Province, Dalian, 116023, P. R. China.

[*]Corresponding author: Institute of Animal Nutrition, Sichuan Agricultural University, Chengdu, Sichuan 611130, People's Republic of China; Tel: 86-28-86291781; Fax: 86-28-86290922; E-mail: yinheng@dicp.ac.cn and hejun8067@163.com.

View Article Online
DOI: 10.1039/D0FO01998D

Abstract

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. Twenty-four weaned pigs were randomly assigned into three groups: (1) non-challenge (CON, fed with basal diet), (2) ETEC-challenge (ECON, fed with basal diet), and (3) ETEC challenge + FOS treatment (EFOS, fed with basal diet plus 2.5 g/kg FOS). On day 19, CON group was orally infused with sterilized culture while pigs in ECON group and EFOS group were orally infused with ETEC (2.5×10^{11} colony-forming units). After 3 days, pigs were slaughtered for sample collection. We showed that ETEC-challenged significantly reduced average daily gain (ADG), however, FOS improved the ADG ($P<0.05$), apparent digestibility of crude protein (CP), gross energy (GE), ash and reduced the diarrhea incidence ($P<0.05$). FOS reduced plasma concentrations of IL-1β and TNF-α, down-regulated ($P < 0.05$) the mRNA expression of IL-6 and TNF-α in jejunum and ileum as well as IL-1β and TNF-α in duodenum. Elevated the concentrations of plasma immunoglobulin A (IgA), immunoglobulin M (IgM) and secreted IgA (SIgA) in the jejunum ($P<0.05$). Interestingly, FOS elevated villus height in duodenum, and elevated the ratio of villus height to crypt depth in the

View Article Online
DOI: 10.1039/D0FO01998D

duodenum and ileum in the EFOS group 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). These results suggested that FOS could 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.

Keywords: *Escherichia coli*, FOS; Intestine, Inflammation, Microbiota, Weaned pigs

View Article Online
DOI: 10.1039/D0FO01998D

Introduction

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 have 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]. Fructooligosaccharides 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

View Article Online
DOI: 10.1039/D0FO01998D

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].

ETEC is a major cause of diarrhea in neonatal and weaning pigs [13]. ETEC 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 have 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 ETEC 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 ETEC challenge. This study could 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

View Article Online
DOI: 10.1039/D0FO01998D

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 2.5 mg/kg 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 ECON and EFOS group were orally administered 150 mL of Luria–Bertani culture containing approximately 2.5×10^9 CFU/mL of ETEC (serotype O149:K91:K88ac; China Institute of Veterinary Drugs Control, Beijing, China) [19], while pigs in the CON 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.

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

View Article Online
DOI: 10.1039/D0FO01998D

selected pigs in each group. After collection, the daily excreta of each pig was weighted, and 10 mL of a 10% H_2SO_4 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 DM, CP, ether extract (EE), ash, and GE. 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 were prepared by using heparin sodium anticoagulant and frozen at −20 °C until analysis. After blood collection, pigs were euthanized with an intravenous injection of sodium pentobarbital at a dose of 200 mg/kg BW [21] 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 ℃ 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-freezed using liquid N_2, following by the preservation at −80°C until further analysis for related enzyme activity and gene expression.

Measurement of growth performance

View Article Online
DOI: 10.1039/D0FO01998D

Piglets were weighed before the morning feed on day 1, 19, and 22 after 12 h fasting at the morning 8:00. Feed intake was recorded, orts were collected and weighed daily. All feed was mash. ADFI, ADG, and 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 [22]. 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. GE 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/A2F1×100), 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 D-lactate, 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

View Article Online
DOI: 10.1039/D0FO01998D

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 SIgA-Positive Cells

Paraffin-embedded jejunal tissues were cut into 4 μm thin and deparaffinized with xylene and then dehydrated using a descending alcohol gradient. Sections were incubated with 3% hydrogen peroxide for 10 minutes at room temperature and then rinsed three times for 5 minutes with phosphate-buffered saline (PBS). After that, sections were heated up to a boil in 10 mM citrate buffer (pH 6.0), washed 2 times in PBS for 5 minutes, and incubated with 10% goat serum albumin for 20 min at room temperature. Subsequently, sections were incubated at 4℃ overnight with primary antibodies for anti-SIgA. PBS instead of primary antibody was employed as negative control. After being washed with PBS, the sections were incubated with biotinylated goat anti-rabbit secondary antibodies (Beijing ZhongShan Golded Bridge Biotechnology Co., Ltd., Beijing, China) for 30 min at 37 ℃. Sections were washed three times with PBS for 5 minutes and incubated with 3,3-diaminobenzidine (DAB)

View Article Online
DOI: 10.1039/D0FO01998D

to visualize immune complexes. The sections were then counterstained with hematoxylin and mounted with glycerol gelatin. Images were obtained using BA400 Digital microscope (Motic China Group Co., Ltd.). The integrated optical density was detected using Image-Pro Plus 6.0 image analysis system (Media Cybernetics, Bethesda, MD, USA), and the protein expression was reflected by the mean value of the integrated optical density.

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 × 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 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

View Article Online
DOI: 10.1039/D0FO01998D

fluorescent quantitative specific primers and probe for total *E. coli, Lactobacillus, Bifidobacterium,* and *Bacillus* (Table S1) were obtained from the Fierer et. al [23]. and Qi et . al [24] and 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×), 1 μL each of the forward and reverse primers, 1 μL of Fluorescent probe (20×), 1 μL of ROX Reference*3 Dye (50×), 2 μL of DNA and 6.5 μL of RNase-Free ddH_2O 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 [25]. 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

View Article Online
DOI: 10.1039/D0FO01998D

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℃. 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 (Nano 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 Scripte 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 TNF-α, IL-1β, IL-6, 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

View Article Online
DOI: 10.1039/D0FO01998D

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 [26]. 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 treatments were separated by Tukey'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, ADFI, and G:F among the three treatments during days 1–18 (pre-challenge). ETEC challenge

View Article Online
DOI: 10.1039/D0FO01998D

(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 ($P < 0.05$). Moreover, FOS supplementation significantly decreased the diarrhea incidence in the ETEC-challenge pigs (Table 2). 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).

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-1β and TNF-α in the pigs upon ECTC 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 ($P < 0.05$).

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

ETEC challenge significantly decreased the duodenal villus height, increased the duodenal crypt depth in the ECON group compared to the CON group (Table 5, Figure. 1). The duodenal villus height was significant higher in the EFOS group than

View Article Online
DOI: 10.1039/D0FO01998D

ECON ($P < 0.05$). Interestingly, ETEC significantly decreased the duodenal ratio of villus height to crypt depth (V/C) in the ECON group compared to the CON group ($P < 0.05$). As compared to the ECON group, FOS supplementation significantly increased the V/C ratio in the duodenum and ileum ($P < 0.05$).

Effect of FOS on intestinal permeability and the mucosal immunity

As shown in Figure. 2A, compared to ECON group, the D-lactate content in the plasma were significantly decreased ($P < 0.05$) in the EFOS group pigs. Moreover, we determined the jejunal SIgA secretion by the immunohistochemical analysis, indicating that FOS supplementation increased the counts of SIgA cell in the jejunum of pigs (Fig. 2B). Besides, we detected the mRNA expression levels of cytokines in the small intestine of pigs. As shown in Fig. 3, FOS supplementation down-regulated ($P < 0.05$) the mRNA expression of IL-6 and TNF-α in jejunum and ileum as well as IL-1β and TNF-α in duodenum.

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

As shown 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 ($P < 0.05$).

View Article Online
DOI: 10.1039/D0FO01998D

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

As shown in Figure.4, 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 ($P < 0.05$).

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

As shown in Figure.5, 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 elevated the expression levels of LAT1 in the duodenum and ileum ($P < 0.05$). The expression level of SGLT1 in the ileum, and the expression levels of GLUT2 in the jejunum and ileum were higher in the EFOS group than in the ECON group ($P < 0.05$).

View Article Online
DOI: 10.1039/D0FO01998D

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 have 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 showed that dietary FOS supplementation significantly improved the growth performance, apparent nutrient digestibility and reduced the diarrhea incidence in pigs exposure to ETEC challenge. The results are consistent with previous studies on pigs [27,28].

An integrated intestinal morphological structure is of critical importance for nutrient digestion and absorption. D-Lactic acid is a metabolite produced by fermentation of bacteria in the gastrointestinal tract that is rarely absorbed into the bloodstream under normal circumstances [29]. However, large amounts of D-lactic acid can be absorbed into the bloodstream through the damaged intestinal mucosa, so plasma D-lactic acid can serve as a marker of maturity, integrity, and functional status of intestinal epithelial cells [30,31]. In the present study, we found that FOS supplementation reduced the D-lactic acid content in the plasma, indicating that FOS can alleviate the detrimental effects of ETEC in pig's intestine which might be related

View Article Online
DOI: 10.1039/D0FO01998D

to the FOS enhanced the maturity, integrity, and functional status of intestinal. 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 [32]. 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 FOS. For instance, the FOS were found to prevent the adhesion of enteric pathogens to intestinal epithelium, which significantly reduced their colonization and intestinal inflammation and injury [33]. Moreover, FOS were found to improve the intestinal morphology and significantly reduced the colonization of pathogenic bacteria in the intestine [34]. The beneficial effects of FOS supplementation on intestinal epithelium function have also been indicated by the mucosal enzyme activities, as FOS not only elevated duodenal the activities of sucrase, lactase, and AKP, 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 [35]. While, the AKP is an another crucial endogenous enzyme expressed in the brush border, and considered as an excellent marker enzyme involved in the primary digestive and absorptive processes of the small intestine [36].

SIgA is a 400 kDa molecule composed of the secretory components, J-chain and dimeric IgA [37]. As a major component of the intestinal immune barrier, SIgA plays

View Article Online
DOI: 10.1039/D0FO01998D

an important role in maintaining intestinal health by clearing pathogenic microorganisms and interacting with intestinal commensal microorganisms [38,39]. Furthermore, the host may discriminate symbionts from pathogens by recognizing the coating of commensal bacteria by SIgA [40]. Thus, enough SIgA secretion in the gut is essential for the intestinal homeostasis. However, piglets, especially weaning piglets, are unable to get maternal immunoglobulins and usually cannot secret enough SIgA because of their underdeveloped intestinal immune system [41]. Interestingly, in the current study, results of the immunohistochemistry analyses confirmed that FOS supplementation increased the jejunal SIgA levels in EFOS group piglets, which could be associated with a key role of supplementation of FOS stimulating the development and maturation of the intestinal mucosal.

Cytokines act as crucial roles in the immune and inflammatory responses [42]. Over-production of TNF-α, IL-1β and IL-6 have shown a series of proinflammatory functions in inflammatory intestinal mucosa, further induces the dysfunction of the intestinal barrier, causing the death of intestinal cells [43,44]. In the present study, FOS supplementation down-regulated the mRNA expression of IL-6 and TNF-α in jejunum and ileum as well as IL-1β and TNF-α in duodenum, which suggested an ant-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 [45]. Both these results suggested a beneficial role of FOS in regulating the intestinal

View Article Online
DOI: 10.1039/D0FO01998D

immune function of pigs under ETEC injection. Our results are in line with the present study that FOS suggest a positive effect on mucosal immunity. [46].

Both the IgA and IgG are crucial index of body immune capacity and contribute to dealing with various pathogens. IgA is the most abundant immunoglobulin in the body and is of critical importance in body immunity, plays a critical role in maintaining intestinal mucosal immunity and preventing intestinal infection [47]. In general, IgG enhances mucosal homeostasis and controls non-invasive and invasive mucosal bacteria by reaching the lumen of mucosal organs upon binding to the epithelial transporter FcRn [48]. Moreover, the plasma concentrations of IgA and IgG were elevated by FOS in the ETEC-challenged pigs, which might be related to FOS stimulate the growth and development of immune organs, promote the differentiation of lymphocyte and enhance the cellular immunity and humoral immunity [49], our result was consistent with the present study that FOS supplementation elevate the IgA and IgG in the plasma [50].

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 diegstive tract [51]. Both the acetic and propionic acids can be used as energy substrates for peripheral tissues.

View Article Online
DOI: 10.1039/D0FO01998D

While the butyrate not only acts as a critical energy source for intestinal epithelial cells, but can also promote cell proliferation and differentiation [52]. In the present study, we found FOS decreased the abundance of *E. coli*, but significantly increased the abundance of *Bifidobacterium* and *Bacillus* in the cecum. This is probably due to the fact that pathogenic bacteria such as the *E. coli* can not utilize the prebiotics [53,54]. Moreover, the fermented products by beneficial bacteria provide an acidic environment that is important for inhibiting the growth of harmful bacteria [55]. The result is consistent with previous reports that FOS can regulate the balance of intestinal microbial flora [56]. Both these results suggested a beneficial role of prebiotics in regulating the intestinal microbial ecology and health.

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 [57]. 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 [58]. Moreover, FOS supplementation significantly elevated the expression levels of nutrient transporters (LAT1, CAT1, SGLT1, and GLUT2) in the EFOS group. The LAT1 and CAT1 are transporters that is responsible for transporting of the L-type and cationic amino acids, respectively [59]. While the

SGLT1 and GLUT2 are closely associated with glucose absorption [60]. 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.

Funding Statement

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

Data Availability and materials

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

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

View Article Online
DOI: 10.1039/D0FO01998D

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.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Consent for publication

Not applicable.

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 (No. 20181105).

Acknowledgments

We thank Wang Huifen, Wu Fali, and Yu En for their helps during the animal trial and sample collections.

View Article Online
DOI: 10.1039/D0FO01998D

References

[1] Turner, J.R., Intestinal mucosal barrier function in health and disease. Nature Reviews Immunology, 2009. 9(11): p. 799-809.

[2] Do T, Stephens C, Townsend K, et al. Rapid identification of virulence genes in enterotoxigenic Escherichia coli isolates associated with diarrhoea in Queensland piggeries[J]. 2010.

[3] S K, Bhandari, B, Xu, C M, Nyachoti, et al. Evaluation of alternatives to antibiotics using an Escherichia coli K88+ model of piglet diarrhea: effects on gut microbial ecology.[J]. Journal of Animal Science, 2008, 86(4):836-847.

[4] Knudsen K E B. Animal nutrition and metabolism group symposium on quality inputs for quality foods: Development of antibiotic resistance and options to replace antimicrobials in animal diets[J]. Proceedings of The Nutrition Society, 2001, 60(3):291-299

[5] Walsh, A.M, Sweeney, et al. Effect of dietary laminarin and fucoidan on selected microbiota, intestinal morphology and immune status of the newly weaned pig[J]. British Journal of Nutrition, 2013.

[6] Guan G , Ding S , Yin Y , et al. Macleaya cordata extract alleviated oxidative stress and altered innate immune response in mice challenged with enterotoxigenic Escherichia coli[J]. Science China Life Sciences, 2019.

[7] P. A. Thacker. Alternatives to antibiotics as growth promoters for use in swine production: a review, J. Anim. Sci. Biotechnol., 2013, 4, 35

[8] Ayako, Nishizawa-Yokoi, Yukinori, et al. The contribution of carbohydrates including raffinose family oligosaccharides and sugar alcohols to protection of plant cells from oxidative damage[J]. Plant Signaling & Behavior, 2014.

[9] Engfer M B, Stahl B, Finke B , et al. Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract[J]. American Journal of Clinical Nutrition, 2000,

[10] Le Bourgot C, Ferret-Bernard, Stéphanie, Blat S , et al. Short-chain fructooligosaccharide supplementation during gestation and lactation or after weaning differentially impacts pig growth and IgA response to influenza vaccination[J]. Journal of Functional Foods, 2016, 24:307-315.

[11] M, Sabater-Molina, E,et al. Dietary fructooligosaccharides and potential benefits on health[J]. Journal of Physiology & Biochemistry, 2009.

[12] Feng G , Yun J , Guang Z , et al. Effect of fructooligosaccharides (FOS) on growth,metabolism and immune in weaning piglets[J]. Animal Husbandry & Veterinary Medicine, 2001.

[13] Francis DH, Grange PA, Zeman DH, et al. Expression of mucin-type glycoprotein K88 receptors strongly correlates with piglet susceptibility to K88 (+) enterotoxigenic Escherichia coli, but adhesion of this bacterium to brush borders does not.[J]. Infection & Immunity, 1998, 66(9):4050.

[14] Gaastra, W., and F. Graaf. 1982. Host-specific fimbrial adhesions of noninvasiveenterotoxigenic Escherichia coli strains. Microbiol. Rev. 46:129–161.

[15] Yuan S L , Piao X S , Li D F , et al. Effects of dietary Astragalus polysaccharide on growth performance and immune function in weaned pigs[J]. Animal Science, 2006, 82(04):501---507.

[16] C, S, Pohl, et al. Early weaning stress induces chronic functional diarrhea, intestinal barrier defects, and increased mast cell activity in a porcine model of early life adversity[J]. Neurogastroenterology & Motility, 2017.

[17] Barrangou, Rodolphe, Altermann,et al. Functional and comparative genomic analyses of an operon involved in fructooligosaccharide utilization by Lactobacillus acidophilus.[J]. Proceedings of the National Academy of Sciences of the United States of America, 2003.

[18] USA, N.C., Nutrient requirements of swine. Nutrient Requirements of Swine, 2012. 44(3).

[19] Dingfu X , Yongfei W , Gang L , et al. Effects of Chitosan on Intestinal Inflammation in Weaned Pigs Challenged by Enterotoxigenic *Escherichia coli*[J]. PLoS ONE,9,8(2014-8-4), 2014, 9(8):e104192.

[20] M.M. Bradford. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1–2) (1976) 248–254

[21] Chen, H, Mao,et al. Dietary fibre affects intestinal mucosal barrier function and regulates intestinal bacteria in weaning piglets[J]. BRITISH JOURNAL OF NUTRITION, 2013.

[22] Chemists AOOA. Official Methods of Analysis. Official Methods of Analysis. 1950.

[23] Fierer N , Jackson J A , Vilgalys R , et al. Assessment of Soil Microbial Community Structure by Use of Taxon-Specific Quantitative PCR Assays[J]. Applied and Environmental Microbiology, 2005, 71(7):4117-4120.

[24] Qi H , Xiang Z , Han G , et al. Effects of different dietary protein sources on cecal microflora in rats[J]. 2013.

[25] Pierce J P , Stefanick M L , Flatt S W , et al. Greater Survival After Breast Cancer in Physically Active Women With High Vegetable-Fruit Intake Regardless of Obesity[J]. Journal of Clinical Oncology, 2007, 25(17):2345-2351.

[26] Analysis of relative gene expression data using real-time quantitative PCR and the 2(-DeltaDelta C(T)) Method. Methods 25(4):402-408.

[27] R. Nemcová, Bomba A , S. Gancaríková, et al. Effects of the Administration of Lactobacilli, Maltodextrins and Fructooligosaccharides upon the Adhesion of E. coli O8:K88 to the Intestinal Mucosa and Organic Acid Levels in the Gut Contents of Piglets[J]. Veterinary Research Communications, 2007, 31(7):791-800.

[28] Tsukahara T , Iwasaki Y , Nakayama K , et al. Stimulation of butyrate production in the large

intestine of weaning piglets by dietary fructooligosaccharides and its influence on the histological variables of the large intestinal mucosa.[J]. Journal of Nutritional ence & Vitaminology, 2003, 49(6):414-421.

[29] Nieto, N., Experimental Ulcerative Colitis Impairs Antioxidant Defense System in Rat Intestine. Digestive Diseases & Sciences, 2000. 45(9): p. 1820-1827.

[30] Smith, S.M., R.H. Eng, and F. Buccini, Use of D-lactic acid measurements in the diagnosis of bacterial infections. Journal of Infectious Diseases, 1986. 154(4): p. 658-664.

[31] Wertz, E, Berge, L L, Walker, P M, Early weaning and postweaning nutritional management marbling score to feed efficiency.[J]. Journal of Animal Science, 79(7).

[32] James W P T , Drasar B S , Miller C . Physiological mechanism and pathogenesis of weanling diarrhea[J]. American Journal of Clinical Nutrition(6):564-71.

[33] Pan X D , Chen F Q , Tian-Xing W U , et al. Prebiotic oligosaccharides change the concentrations of short-chain fatty acids and the microbial population of mouse bowel[J]. Journal of Zhejiang University ENCE B, 2009, 10(4):258-263.

[34] Donalson L M , Mcreynolds J L , Kim W K , et al. The influence of a fructooligosaccharide prebiotic combined with alfalfa molt diets on the gastrointestinal tract fermentation, Salmonella enteritidis infection, and intestinal shedding in laying hens.[J]. Poultry ence, 2008, 87(7):1253.

[35] Gang T , Linhui H , Xiaohua S , et al. Effects of Chitooligosaccharides on Growth Performance,Antioxidant Capacity and Nutrient Digestion and Transport Capacity of Jejunum of Oxidative Stress Piglets[J]. Chinese Journal of Animal Nutrition, 2018.

[36] TA S W H , B J H K , C C L , et al. Intestinal alkaline phosphatase ameliorates experimental colitis via toll-like receptor 4-dependent pathway[J]. European Journal of Pharmacology, 2018, 820:156-166.

[37] Woof J M , Russell M W . Structure and function relationships in IgA[J]. Mucosal Immunology, 2011, 4(6):590-597.

[38] Chairatana P , Nolan E M . Defensins, lectins, mucins, and secretory immunoglobulin A: microbe-binding biomolecules that contribute to mucosal immunity in the human gut[J]. Crc Critical Reviews in Biochemistry, 2017, 52(1):45-56.

[39] Lindner C , Wahl B , Fohse L , et al. Age, microbiota, and T cells shape diverse individual IgA repertoires in the intestine[J]. Journal of Experimental Medicine, 2012, 209(2):365-377.

[40] Sansonetti P J . To be or not to be a pathogen: that is the mucosally relevant question[J]. Mucosal Immunology, 2011, 4(1):8-14.

[41] Ushida K , Kameue C , Tsukahara T , et al. Decreasing Traits of Fecal Immunoglobulin A in Neonatal and Weaning Piglets[J]. Journal of Veterinary Medical ence, 2008, 70(8):849.

585 [42] Rana A S . Mechanism of cytokine modulation of epithelial tight junction barrier.[J]. Frontiers in
586 Bioence, 2009, 14(14):2765-2778.

587 [43] Pié S, Lallès JP, Blazy F , et al. Weaning is associated with an upregulation of expression of
588 inflammatory cytokines in the intestine of piglets.[J]. Journal of Nutrition, 2004, 134(3):641-7.

589 [44] Brabers N A C H , Nottet H S L M . Role of the pro-inflammatory cytokines TNF-α and IL-1β in
590 HIV-associated dementia[J]. European Journal of Clinical Investigation, 2010, 36(7):447-458.

591 [45] Che T M, Song M, Liu Y, et al. Mannan oligosaccharide increases serum concentrations of
592 antibodies and inflammatory mediators in weaning pigs experimentally infected with porcine
593 reproductive and respiratory syndrome virus[J]. Journal of Animal ence, 2012, 90(8):2784-2793.

594 [46] Scholtens P A M J , Alliet P , Raes M , et al. Fecal secretory immunoglobulin A is increased in
595 healthy infants who receive a formula with short-chain galacto-oligosaccharides and long-chain
596 fructo-oligosaccharides.[J]. Journal of Nutrition, 2008, 138(6):1141-1147.

597 [47] Annelot, Breedveld, Marjolein,et al. IgA and FcαRI: Pathological Roles and Therapeutic
598 Opportunities.[J]. Frontiers in Immunology, 2019.

599 [48] Rath T , Baker K , Pyzik M , et al. Regulation of Immune Responses by the Neonatal Fc Receptor
600 and Its Therapeutic Implications[J]. Frontiers in Immunology, 2015, 5(3):664

601 [49] Manhart N , Spittler A , Bergmeister H , et al. Influence of fructooligosaccharides on Peyer's patch
602 lymphocyte numbers in healthy and endotoxemic mice[J]. Nutrition, 2003, 19(7-8):657-660.

603 [50] Czech A , Grela E R , Mokrzycka A , et al. Efficacy of mannanoligosaccharides additive to sows
604 diets on colostrum, blood immunoglobulin content and production parameters of piglets.[J]. Polish
605 Journal of Veterinary ences, 2010, 13(3):525.

606 [51] M B, Engfer, Stahl, et al. Human milk oligosaccharides are resistant to enzymatic hydrolysis in
607 the upper gastrointestinal tract.[J]. The American journal of clinical nutrition, 2000

608 [52] Tremaroli V , Backhed F . Functional interactions between the gut microbiota and host
609 metabolism[J]. Nature, 2012, 489(7415):p.242-249.

610 [53] Dominguez L S , Rodrigues L G R , Lima N M , et al. An Overview of the Recent Developments
611 on Fructooligosaccharide Production and Applications[J]. Food and Bioprocess Technology,
612 2014, 7(2):324-337.

613 [54] Rastall R A , Gibson G R . Recent developments in prebiotics to selectively impact beneficial
614 microbes and promote intestinal health.[J]. Curr Opin Biotechnol, 2015, 32:42-46.

615 [55] Maria Elena Rodríguez-Cabezas, Desirée Camuesco, Belén Arribas, et al. The combination of
616 fructooligosaccharides and resistant starch shows prebiotic additive effects in rats[J]. Clinical
617 Nutrition, 2010, 29(6):832-839.

618 [56] Schokker D , Fledderus J , Jansen R , et al. Supplementation of fructooligosaccharides to suckling
619 piglets affects intestinal microbiota colonization and immune development1[J]. Journal of Animal
620 ence, 2018, 96 (Pt 24):2139-2153.

621 [57] Fanning, A. S., B. J. Jameson, L. A. Jesaitis, and J. M. Anderson. 1998. The tight junction protein
622 ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. J.
623 Biol. Chem. 273: 29745-29753.

624 [58] Jia Z , Moulson C L , Pei Z , et al. FATP4 is the principal very long-chain fatty acyl-CoA
625 synthetase in skin fibroblasts[J]. Journal of Biological Chemistry, 2007, 21(5):A607-A608.

626 [59] Rotoli B M , Closs E I , Barilli A , et al. Arginine transport in human erythroid cells:
627 discrimination of CAT1 and 4F2hc/y+LAT2 roles[J]. Pflügers Archiv European Journal of
628 Physiology, 2009, 458(6):1163.

629 [60] Pia V. Röder, Geillinger K E , Zietek T S , et al. The Role of SGLT1 and GLUT2 in Intestinal
630 Glucose Transport and Sensing[J]. Plos One, 2014, 9(2):e89977.

631

View Article Online
DOI: 10.1039/D0FO01998D

Figure legends

Fig. 1 Effect of FOS on small intestinal morphology in weaned pigs. (H&E; × 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 2.5 g/kg FOS challenged by ETEC).

Fig. 2 Effect of FOS on intestinal permeability and the mucosal immunity 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 2.5 g/kg FOS challenged by ETEC). [2 a, b, c] Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$)

Fig. 3 Effects of FOS on mRNA levels of intestinal inflammatory cytokines. Duodenum (A); Jejunum (B), and Ileum (C). 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 2.5 g/kg FOS challenged by ETEC). [2 a, b, c] Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$); IL-1β, interleukin-1β; IL-6, interleukin-6; TNF-α, tumour necrosis factor-α.

Fig. 4 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).

View Article Online
DOI: 10.1039/D0FO01998D

653 Non-challenged pigs (CON, fed with basal diet), ETEC-challenged pigs (ECON, fed
654 with basal diet), FOS and ETEC-treated pigs (EFOS, fed with basal diet containing
655 2.5 g/kg FOS challenged by ETEC).

656 **Fig. 5 Relative expression levels of critical genes involved in the intestinal barrier**
657 **functions.** ZO-1. zonula occludens-1; FATP1, Fatty acid transport protein-1; FATP4,
658 Fatty acid transportprotein-4; LAT1, L-type amino acid transporter-1; CAT1, cationic
659 amino acid transporter-1; SGLT1, sodium glucose transport protein-1; GLUT2,
660 glucose transporter-2; [a,b,c] Mean values with different letters on vertical bars indicate
661 significant differences (P<0·05). Non-challenged pigs (CON, fed with basal diet),
662 ETEC-challenged pigs (ECON, fed with basal diet), FOS and ETEC-treated pigs
663 (EFOS, fed with basal diet containing 2.5 g/kg FOS challenged by ETEC).

664

View Article Online
DOI: 10.1039/D0FO01998D

Tables

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.

672 **Table 2**. Effect of FOS on growth performance in weaned pigs challenged with
673 enterotoxigenic *Escherichia coli*

Items	Treatments			*P-Value*
	CON	ECON	EFOS	
pre-challenged (1–19 days)				
ADFI(g/day)	430.70 ± 30.80	403.49 ± 26.27	417.71 ± 38.79	0.96
ADG (g/day)	296.56 ± 14.63	270.67 ± 16.74	285.00 ± 27.32	0.68
F:G (g/g)	1.45 ± 0.05	1.60 ± 0.16	1.47 ± 0.04	0.76
post-challenged (19–21 days)				
ADFI (g/day)	501.40 ±13.40	463.73 ± 3.45	484.67 ± 8.10	0.05
ADG (g/day)	396.00 ± 23.98 [a]	314.67 ± 48.42 [b]	381.80 ± 22.14 [a]	0.04
Diarrhea incidence (%)	33.00 ± 2.15 [b]	61.07 ± 5.89 [a]	23.33 ± 4.76 [b]	< 0.01

674 [1] Values are means ± SEM, (n = 6), non-challenged pigs (CON, fed with basal diet), ETEC-challenged
675 pigs (ECON, fed with basal diet), and FOS and ETEC-treated pigs (EFOS, fed with basal diet
676 containing 2.5 g/kg FOS challenged by ETEC). [2 a, b, c] mean values within a row with unlike superscript
677 letters were significantly different ($P < 0.05$). [3] ADFI = Average daily feed intake; ADG = Average
678 daily gain; G/F =the ratio of gain to feed intake. [4] Incidence of diarrhea was calculated as follows:
679 diarrhea incidence (%)=(total number of pigs with diarrhea)/(number of pigs×3)×100%, where the
680 number of pigs with diarrhea was the summation of the number of pigs with diarrhea every day.

681 .

View Article Online
DOI: 10.1039/D0FO01998D

682 **Table 3.** Effect of FOS on ATTD of nutrients in weaned pigs

Item, %	Treatments			*P-Value*
	CON	ECON	EFOS	
DM	81.58 ± 1.41	83.65 ± 2.51	85.36 ± 0.61	0.21
CP	83.38 ± 2.40 [b]	85.33 ± 0.84 [b]	90.95 ± 0.86 [a]	0.01
GE	87.98 ± 2.31 [b]	86.91 ± 0.59 [b]	93.04 ± 0.58 [a]	0.03
Crude fat	79.62 ± 2.82	78.38 ± 5.06	88.60 ± 0.70	0.07
Ash	74.19 ± 0.66 [b]	68.81 ±1.16 [b]	79.54 ± 0.38 [a]	< 0.01

683 Values are means ± SEM, (n = 6), non-challenged pigs (CON, fed with basal diet), ETEC-challenged
684 pigs (ECON, fed with basal diet), and FOS and ETEC-treated pigs (EFOS, fed with basal diet
685 containing 2.5 g/kg FOS challenged by ETEC). [2] [a, b, c] mean values within a row with unlike superscript
686 letters were significantly different ($P < 0.05$). [3] DM, dry matter; CP, crude protein; GE, gross energy.

687

688 **Table 4.** Effect of FOS on plasma Immunoglobulin and Cytokine Concentrations in
689 weaned pigs challenged with enterotoxigenic *Escherichia coli*

Items	Treatments			*P-Value*
	CON	ECON	EFOS	
IL-1β (ng/L)	456.20 ± 8.28 [a]	508.93 ± 31.71 [a]	392.40 ± 10.19 [b]	< 0.01
IL-6 (ug/L)	1.34 ± 0.14	1.44 ± 0.13	1.35 ± 0.08	0.76
TNF-α (pg/mL)	487.60 ± 11.09 [b]	589.52 ± 52.31 [a]	454.61 ± 13.27 [b]	0.03
Ig A (ug/L)	432.48 ± 5.78 [a]	357.52 ± 15.60 [b]	481.92 ± 32.26 [a]	< 0.01
Ig G (ug/L)	515.70 ± 28.15	447.79 ± 17.40	508.71 ± 14.55	0.08
Ig M (ug/L)	464.96 ± 26.63 [ab]	393.21 ± 21.40 [b]	518.15 ± 30.48 [a]	0.02

690 [1] Values are means ± SEM, (n = 6), non-challenged pigs (CON, fed with basal diet), ETEC-challenged
691 pigs (ECON, fed with basal diet), FOS and ETEC-treated pigs (EFOS, fed with basal diet containing
692 2.5 g/kg FOS challenged by ETEC). [2] [a, b, c] mean values within a row with unlike superscript letters
693 were significantly different ($P < 0.05$). [3] IgA, immunoglobulin A; IgG, immunoglobulin G; IgM,
694 immunoglobulin M; IL-1β, interleukin-1β; IL-6, interleukin-6 and TNF-α, tumor necrosis factor-α.

695

Table 5. Effect of FOS on intestinal morphology in weaned pigs challenged with enterotoxigenic *Escherichia coli*

Items	Treatments			*P-Value*
	CON	ECON	EFOS	
Duodenum				
Villus height (μm)	486.81 ± 22.81 [a]	424.46 ± 62.95 [b]	524.71 ± 67.59 [a]	0.02
Crypt depth (μm)	345.09 ± 18.10 [b]	372.68 ± 17.11 [a]	301.78 ± 5.95 [b]	0.04
V/C	1.42 ± 0.07 [a]	1.12 ± 0.10 [b]	1.73 ± 0.11 [a]	< 0.01
Jejunum				
Villus height (μm)	430.81 ± 14.48	399.71 ± 12.26	438.98 ± 12.79	0.29
Crypt depth (μm)	173.29 ± 8.39	194.45 ± 11.36	194.97 ± 11.82	0.29
V/C	2.50 ± 0.11	2.28 ± 0.09	2.10 ± 0.18	0.13
Ileum				
Villus height (μm)	341.94 ± 26.81	357.14 ± 20.34	366.00 ± 7.03	0.69
Crypt depth (μm)	178.42 ± 6.23 [b]	203.97 ± 6.12 [a]	159.78 ± 9.65 [b]	< 0.01
V/C	1.92 ± 0.13 [ab]	1.84 ± 0.17 [b]	2.32 ± 0.11 [a]	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 2.5 g/kg FOS challenged by ETEC). [2 a, b, c] mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

703 **Table 6**. Effect of FOS on enzyme activity of small intestine in weaned pigs
704 challenged with enterotoxigenic *Escherichia coli*

Items	Treatments			*P*-Value
	CON	ECON	EFOS	
Duodenum				
Sucrase, U/mg protein	155.07 ± 20.71 [a]	83.30 ± 22.67 [b]	156.70 ± 16.11 [a]	0.04
Lactase, U/ mg protein	12.07 ± 3.59 [a]	6.21 ± 1.88 [b]	13.51 ± 4.16 [a]	0.28
Maltase, U/mg protein	21.11 ± 2.65	13.17 ± 2.23	23.71 ± 6.33	0.17
AKP, U/mg protein	1.44 ± 0.35 [a]	0.65 ± 0.08 [b]	1.50 ± 0.19 [a]	0.04
Jejunum				
Sucrase, U/mg protein	536.76 ± 43.76	475.36 ± 88.10	526.5 ± 84.74	0.83
Lactase, U/ mg protein	94.21 ± 26.96	57.84 ± 13.22	96.34 ± 21.48	0.39
Maltase, U/mg protein	576.75 ± 78.80	423.32 ± 113.97	544.7 ± 131.62	0.76
AKP, U/mg protein	1.21 ± 0.09	1.15 ± 0.08	1.22 ± 0.09	0.83
Ileum				
Sucrase, U/mg protein	210.25 ± 34.41	153.42 ± 31.10	208.23 ± 17.69	0.32
Lactase, U/ mg protein	35. 19 ± 6.69 [a]	15. 37 ± 3.10 [b]	32. 38 ± 5.39 [a]	0.04
Maltase, U/mg protein	131.49 ± 18.75	110.36 ± 25.63	135.41 ± 8.82	0.62
AKP, U/mg protein	2.34 ± 0.20	2.06 ± 0.21	2.19 ± 0.11	0.57

705 Values are means ± SEM, (n = 6), non-challenged pigs (CON, fed with basal diet), ETEC-challenged
706 pigs (ECON, fed with basal diet), and FOS and ETEC-treated pigs (EFOS, fed with basal diet
707 containing 2.5 g/kg FOS challenged by ETEC). [2 a, b, c] mean values within a row with unlike superscript
708 letters were significantly different ($P < 0.05$). [3] AKP = alkaline phosphatase

709

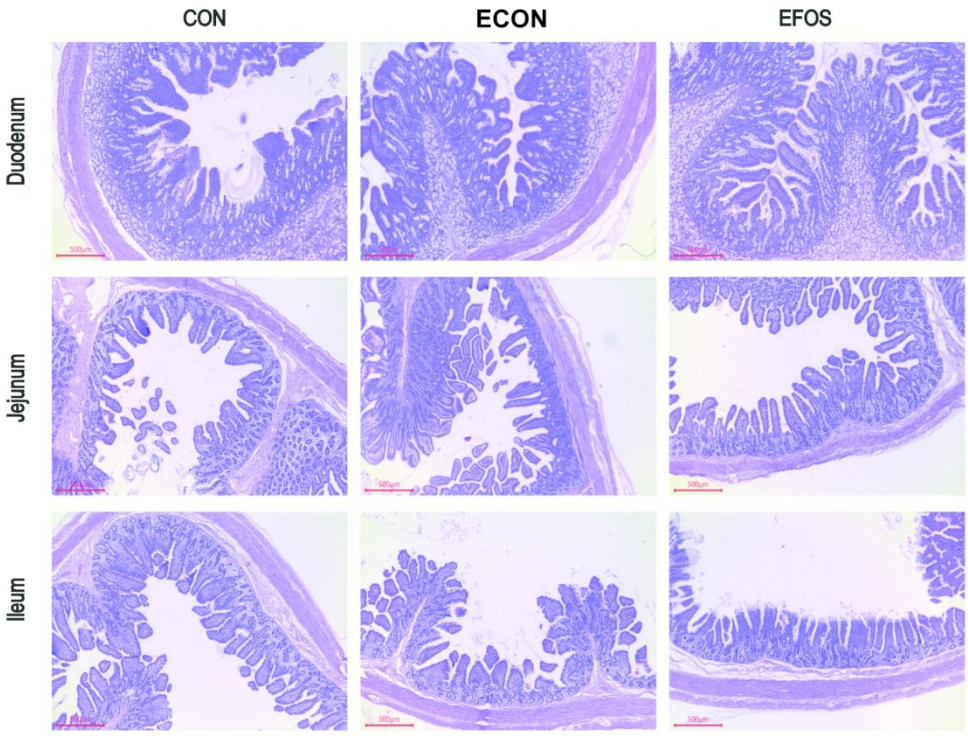
View Article Online
DOI: 10.1039/D0FO01998D

710 **Table 7**. Effect of FOS on intestinal bacteria in the cecal digesta of weaned pigs
711 challenged with enterotoxigenic *Escherichia coli*

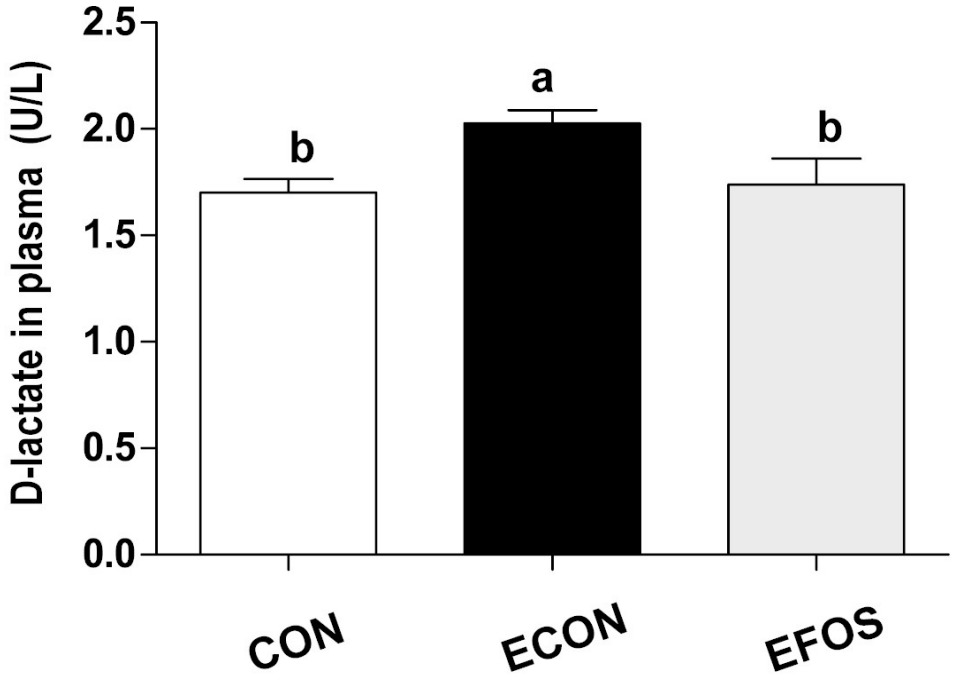
Items	Treatments			*P-Value*
	CON	ECON	EFOS	
Total bacteria 1g (copies/g)	10.43 ± 0.13	10.21 ± 0.08	10.14 ± 0.19	0.37
Escherichia coli 1g (copies/g)	8.62 ± 0.05 [b]	10.17 ± 0.23 [a]	8.68 ± 0.26 [b]	0.01
Bifidobacterium 1g (copies/g)	6.86 ± 0.47 [a]	5.24 ± 0.42 [b]	6.54 ± 0.39 [a]	0.046
Bacillus 1g (copies/g)	8.57 ± 0.17 [a]	8.03 ± 0.06 [b]	8.59 ± 0.10 [a]	0.01
Lactobacillus 1g (copies/g)	5.21 ± 0.37	4.57 ± 0.23	5.03 ± 0.12	0.17

712 [1] Values are means ± SEM, (n = 6), non-challenged pigs (CON, fed with basal diet), ETEC-challenged
713 pigs (ECON, fed with basal diet), FOS and ETEC-treated pigs (EFOS, fed with basal diet containing
714 2.5 g/kg FOS challenged by ETEC). [2 a, b, c] mean values within a row with unlike superscript letters
715 were significantly different ($P < 0.05$).

View Article Online
DOI: 10.1039/D0FO01998D

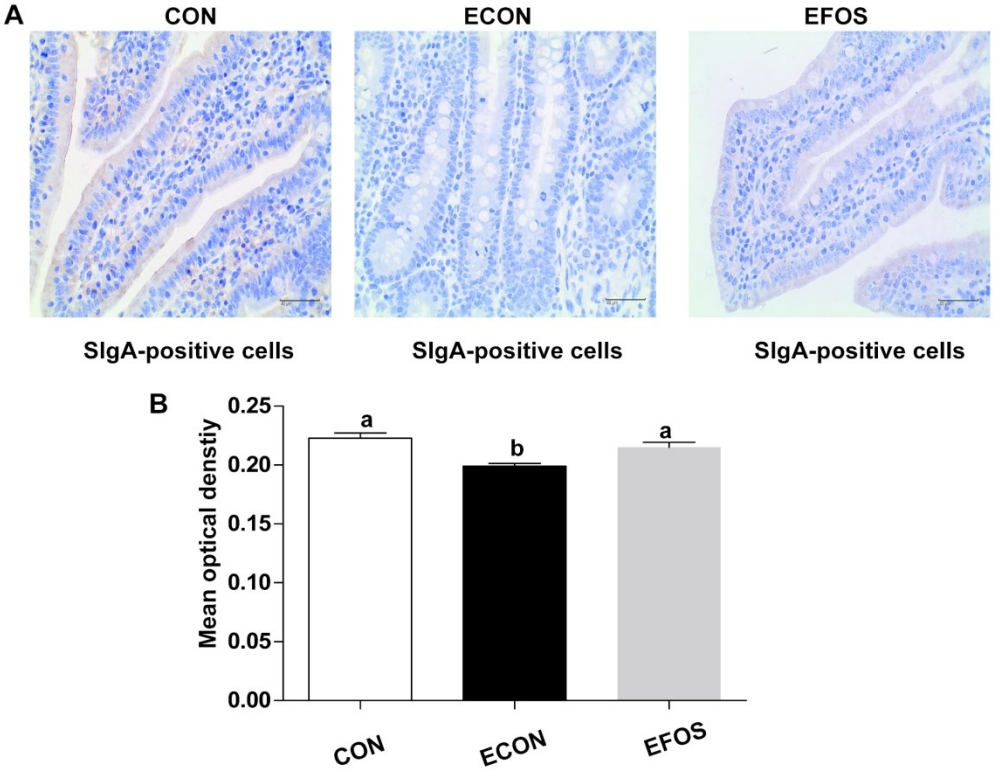


View Article Online
DOI: 10.1039/D0FO01998D



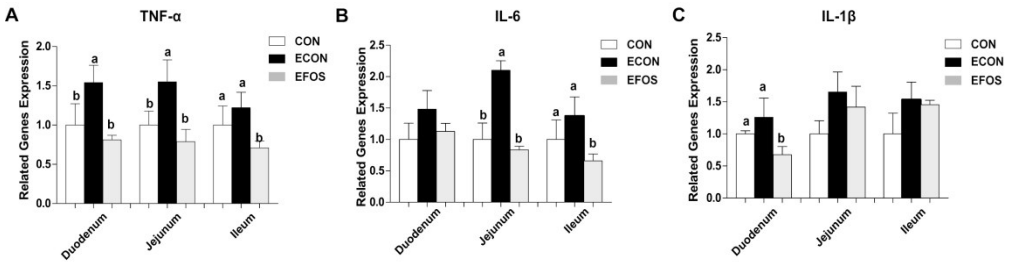
95x65mm (300 x 300 DPI)

View Article Online
DOI: 10.1039/D0FO01998D



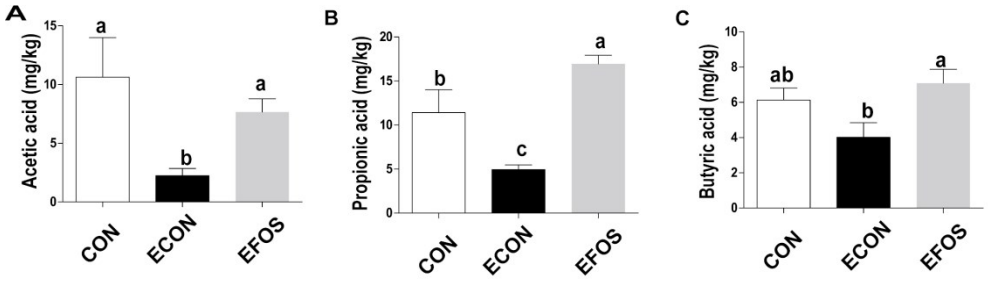
177x141mm (300 x 300 DPI)

View Article Online
DOI: 10.1039/D0FO01998D



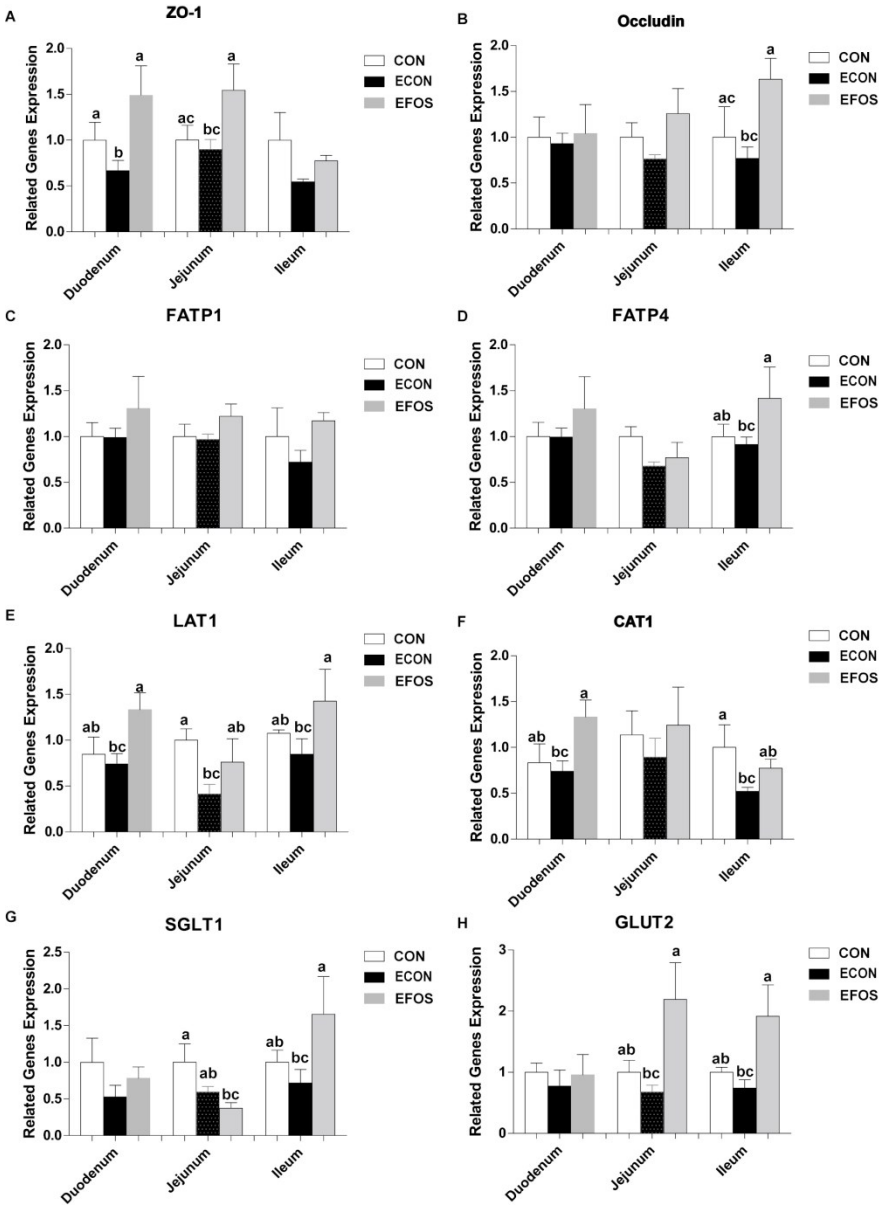
259x70mm (300 x 300 DPI)

View Article Online
DOI: 10.1039/D0FO01998D



204x60mm (300 x 300 DPI)

View Article Online
DOI: 10.1039/D0FO01998D



245x327mm (200 x 200 DPI)