British Journal of Nutrition (2016), **115**, 1947–1957 doi:10.1017/S0007114516001100
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Fish oil enhances intestinal barrier function and inhibits corticotropin-releasing hormone/corticotropin-releasing hormone receptor 1 signalling pathway in weaned pigs after lipopolysaccharide challenge

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(Submitted 21 August 2015 – Final revision received 2 February 2016 – Accepted 18 February 2016 – First published online 15 April 2016)

Abstract

Stress induces injury in intestinal barrier function in piglets. Long-chain *n*-3 PUFA have been shown to exhibit potential immunomodulatory and barrier protective effects in animal models and clinical trials. In addition, corticotropin-releasing hormone (CRH)/CRH receptor (CRHR) signalling pathways play an important role in stress-induced alterations of intestinal barrier function. We hypothesised that fish oil could affect intestinal barrier function and CRH/CRHR signalling pathways. In total, thirty-two weaned pigs were allocated to one of four treatments. The experiment consisted of a 2 × 2 factorial design, and the main factors included immunological challenge (saline or lipopolysaccharide (LPS)) and diet (5 % maize oil or 5 % fish oil). On d 19 of the trial, piglets were treated with saline or LPS. At 4 h after injection, all pigs were killed, and the mesenteric lymph nodes (MLN), liver, spleen and intestinal samples were collected. Fish oil decreased bacterial translocation incidence and the number of translocated micro-organisms in the MLN. Fish oil increased intestinal claudin-1 protein relative concentration and villus height, as well as improved the intestinal morphology. In addition, fish oil supplementation increased intestinal intraepithelial lymphocyte number and prevented elevations in intestinal mast cell and neutrophil numbers induced by LPS challenge. Moreover, fish oil tended to decrease the mRNA expression of intestinal *CRHR1*, *CRH* and glucocorticoid receptors. These results suggest that fish oil supplementation improves intestinal barrier function and inhibits CRH/CRHR1 signalling pathway and mast cell tissue density.

Key words: **Lipopolysaccharides: Fish oil: Intestinal barrier function: Weaned pigs**

The small intestine plays an important role in the digestion and absorption of nutrients. At the same time, it constitutes a physical and immunological barrier against harmful materials including bacteria, viruses, parasites and allergenic macromolecules[1]. Intestinal barrier breakdown can increase intestinal permeability, which allows luminal antigenic agents to 'leak' across the mucosa, resulting in initiation or continuation of inflammatory processes and mucosal damage[2–4]. Several studies have shown that physical, psychological and chemical stresses cause injury to intestinal barrier function[5–7].

The mechanisms by which stress causes injury to intestinal barrier function have not been fully elucidated. However, several studies have demonstrated that stress-induced alterations in intestinal barrier function are mediated by the actions of corticotropin-releasing hormone (CRH) and subsequent activation of CRH receptors (CRHR) expressed locally in the gut[8–10]. One particular cell type that has been related to CRH/CRHR signalling pathways and stress-induced alterations in intestinal barrier function is the mast cell[11]. Mast cells are haematopoietic-derived immune cells that migrate to peripheral tissues to mature and regulate various effector functions. Mast cells are becoming well known as an important cell type mediating stress-induced intestinal disorders[12–14].

Some specific nutrients such as arginine, *n*-3 PUFA and glutamine have been shown to mitigate intestinal barrier dysfunction at weaning[15,16]. In recent years, *n*-3 PUFA have received considerable attention in both human and animal nutrition. *n*-3 PUFA such as EPA and DHA, which are rich in fish oil, exhibit potential immunomodulatory and barrier protective effects[17]. Moreover, *n*-3 PUFA have been shown to stimulate enterocyte differentiation and intestinal maturation, reduce transcellular permeability and stabilise the intestinal barrier function[18,19]. However, the exact molecular mechanisms by which *n*-3 PUFA exert this beneficial effect are poorly understood.

Lipopolysaccharide (LPS) is a membrane component of gram-negative bacteria. A large number of studies have shown

Abbreviations: CRH, corticotropin-releasing hormone; CRHR1, corticotropin-releasing hormone receptor 1; GR, glucocorticoid receptor; HPA, hypothalamo–pituitary–adrenall; IAP, intestinal alkaline phosphatase; IEL, intraepithelial lymphocytes; LPS, lipopolysaccharide; MLN, mesenteric lymph nodes.

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https://doi.org/10.1017/S0007114516001100 Published online by Cambridge University Press

https://doi.org/10.1017/S0007114516001100 Published online by Cambridge University Press

that LPS can cause damage to the intestinal barrier function[20–23]. LPS also is a potent activator of the hypothalamo–pituitary–adrenal (HPA) axis[24]. LPS exerts this effect principally by stimulating CRH and cortisol secretion[24,25]. Furthermore, a well-characterised response to stress is activation of the HPA axis resulting in adrenal cortisol release[26]. Therefore, LPS is a common tool used for inducing acute stress response and studying the effects of dietary regimens[24,27,28].

Accordingly, we hypothesised that *n*-3 PUFA would affect intestinal barrier function by regulating CRH/CRHR signalling pathways. In the current experiment, we made use of a well-established model to induce intestinal damage in weanling piglets by administering *Escherichia coli* LPS. Our objective was to determine whether dietary fish oil supplementation could alleviate the damage to intestinal barrier function caused by LPS through modulation of CRH/CRHR signalling pathways.

Methods

Animals care and experimental design

The experimental protocol used in this study was approved by the Animal Care and Use Committee of Hubei Province, People's Republic of China. In total, thirty-two pigs (Duroc × Large White × Landrace; barrows; 8·91 (SEM 0·74) kg initial body weight (BW), weaned at 28 (SEM 3) d of age) were randomly assigned to four treatment groups. Each treatment had eight replicate pens. The pigs were housed in 1·80 × 1·10-m stainless steel pens (one pig per pen). Pens contained a single-hole feeder and a nipple waterer to allow pigs *ad libitum* access to feed and water. The weaned pigs were fed a maize–soyabean basal diet with the addition of 5 % of fish oil (menhaden fish oil; Fujian Gaolong Company) or maize oil (Xiwang Food Company). Diets (Table 1) were formulated to meet or exceed NRC[29] requirements for all nutrients. The composition of fatty acids is presented in Table 2.

The experiment consisted of a 2 × 2 factorial arrangement of treatments with diet (5 % maize oil *v.* 5 % fish oil) and immunological challenge (saline *v.* LPS). On d 19 of the trial, half of the pigs (*n* 8) in each dietary treatment were injected intraperitoneally with *E. coli* LPS (*E. coli* serotype 055:B5; Sigma Chemical) at 100 µg/kg BW or the equivalent amount of 0·9 % NaCl solution. The dose of LPS was chosen in accordance with our previous experiment[25,30].

Collections of intestinal sample

Four hours after administration of LPS or sterile solution, pigs were killed with sodium pentobarbital (80 mg/kg BW). The abdominal cavity was opened and the mesenteric lymph nodes (MLN), liver and spleen were harvested to measure bacterial translocation. The jejunum and ileum were separated and flushed with 0·9 % NaCl solution. A 1–3-cm-long cross-section of intestinal tissue was obtained from the mid-jejunum and mid-ileum and fixed in 4 % paraformaldehyde in PBS for histological analysis. Mucosal samples were obtained by cutting segments lengthwise and scraping mucosa from the connective tissue, were immediately frozen in liquid N_2 and then stored at –80°C until measurement of mRNA abundance. Previous studies have shown that LPS caused acute intestinal morphological damage and a breakdown in intestinal barrier function in

Table 1. Ingredient composition of the diets (as-fed basis)

Items	Contents (g/kg)
Ingredients	
Maize	555
Soyabean meal (44 % crude protein)	220
Wheat bran	30
Fishmeal	55
Maize oil or fish oil	50
Soya protein concentrate	25
Milk-replacer powder	30
Limestone	7·0
Dicalcium phosphate	10
Salt	2·0
L-Lysine.HCl (78·8 % lysine)	2·7
Acidifier*	2·0
Butylated hydroquinone	0·5
Preservative†	0·5
Sweetener‡	0·3
Vitamin and mineral premix§	10
Nutrient composition	
Digestible energy‖ (MJ/kg)	14·0
Crude protein¶	203
Ca¶	8·9
Total P¶	6·9
Total lysine‖	13·6
Total methionine + cysteine‖	7·4

* A compound acidifier including lactic acid and phosphoric acid (Wuhan Fanhua Biotechnology Company).
† A compound mould inhibitor including calcium propionate, fumaric acid, fumaric acid monoethyl ester and sodium diacetate (Sichuan Minsheng Pharmaceutical Co. Ltd).
‡ A compound sweetener including saccharin sodium and disodium 5′-guanylate (Wuhan Fanhua Biotechnology Company).
§ The premix provided the following amounts per kilogram of complete diet: retinol acetate, 2700 µg; cholecalciferol, 62·5 µg; DL-α-tocopheryl acetate, 20 mg; menadione, 3 mg; vitamin B_{12}, 18 µg; riboflavin, 4 mg; niacin, 40 mg; pantothenic acid, 15 mg; choline chloride, 400 mg; folic acid, 700 µg; thiamin, 1·5 mg; pyridoxine, 3 mg; biotin, 100 µg; Zn, 80 mg ($ZnSO_4 \cdot 7H_2O$); Mn, 20 mg ($MnSO_4 \cdot 5H_2O$); Fe, 83 mg ($FeSO_4 \cdot H_2O$); Cu, 25 mg ($CuSO_4 \cdot 5H_2O$); I, 0·48 mg (KI); Se, 0·36 mg ($Na_2SeO_3 \cdot 5H_2O$).
‖ Calculated.
¶ Analysed.

Table 2. Fatty acid composition of fish or maize oil*

Fatty acids	Maize oil	Fish oil
% Total fatty acids		
4 : 0	2·27	2·73
14 : 0	0·04	8·89
16 : 0	12·81	21·06
16 : 1*n*-7	0·09	9·97
18 : 0	1·73	3·79
cis-18 : 1*n*-9	29·91	11·10
cis-18 : 2*n*-6	51·27	1·27
18 : 3*n*-3	0·75	0·75
20 : 4*n*-6	0·01	1·16
20 : 5*n*-3	ND	21·22
22 : 6*n*-3	ND	12·88
Total *n*-6 PUFA†	51·31	3·07
Total *n*-3 PUFA†	0·75	34·96
n-6/*n*-3	68·13	0·09

* The fatty acid profiles from 4 : 0 to 24 : 1*n*-9 were analysed in duplicate. Only the most abundant fatty acids are listed. The detection limit was 0·01 mg/g diet for each fatty acid.
† Total *n*-6 PUFA and total *n*-3 PUFA corresponded to the sum of all the *n*-6 or *n*-3 PUFA detected.

British Journal of Nutrition

https://doi.org/10.1017/S0007114516001100 Published online by Cambridge University Press

rats, mice and pigs within 3–6 h after injection[31–33]. Therefore, the time point of 4 h after LPS or saline injection was chosen for experimental measurements.

Bacterial translocation

Bacterial translocation analysis was based on the method of Yang *et al.*[34] and Chen *et al.*[35] with modifications. The collected MLN, spleen and liver were weighed and homogenised in ten volumes of ice-cold sterile saline. Aliquots of 50 µl of the homogenate from each tissue were plated onto blood and MacConkey's agar plates and examined after 24 h of aerobic incubation at 37°C. In fact, there are anaerobes and aerobes in the intestine, the former outnumbering the latter. However, most previous investigators have attributed the main role in bacterial translocation to aerobic and facultative gram-negative bacteria[36–38]. Therefore, we measured bacterial translocation in aerobic condition. The colonies were counted, and the results are expressed as colony-forming units (CFU) per gram of tissue. For each tissue, positive bacterial translocation was defined as at least four out of eight plates showing bacterial growth with CFU >50/g of tissue.

Histology

Fixed intestinal samples were dehydrated with graded ethanol solutions, cleared with xylene and embedded in paraffin. Histological slides were prepared from three cross-sections (4-µm thick) of each intestinal sample and stained with haematoxylin and eosin. The villus height and crypt depth were measured, and the villus:crypt ratio was calculated by dividing villus height by crypt depth. Villus area was quantitated from the perimeter and height of the villi[39]. The ten longest and straightest villi and their associated crypts from each segment were measured. The same villus and crypt were used to determine the number of intraepithelial lymphocytes (IEL) and goblet cells. These variables were expressed per 100 enterocytes.

The number of lamina propria cells, neutrophils and mast cells was counted in histological sections according to cellular and nuclear morphology and toludine blue staining (for mast cells). Cell counts were determined utilising image analysis programme and expressed as number of cells/mm^2. All cell counts and intestinal morphological measurements were performed by a histologist who was blinded to the treatments.

Western blot analysis

The method for protein immunoblot analysis in intestinal mucosa was the same as that described in the study by Hou *et al.*[40]. In brief, the intestinal samples (100–150 mg) (*n* 8) were homogenised and lysed in ice-cold lysis buffer. The homogenates were centrifuged at 12 000 ***g*** for 15 min at 4°C, and the supernatant was used for Western blot and protein assay. Protein concentration was determined using the bicinchoninic acid protein assay kit (Applygen Technologies Co. Ltd). Intestinal mucosal proteins were separated on a polyacrylamide gel and transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 3 % bovine serum albumin in Tris-buffered saline (TBS)-Tween-20 buffer at least for 60 min at room temperature (21–25°C). The membranes were incubated overnight (12–16 h) at 4°C with rabbit anti-claudin-1 (1:1000; no. 51-9000; Invitrogen Technology) or mouse anti-β-actin (1:10 000; no. A2228; Sigma Aldrich). The membranes were washed three times (for 5 min each) with TBS-T (1 × Tris-buffered saline including 0·1 % Tween-20) and incubated with goat anti-rabbit (no. ANT020) or mouse (no. ANT019) IgG horseradish peroxidase conjugated secondary antibody (1:5000; AntGene Biotech Co. Ltd) for 120 min at room temperature. Membranes were washed three times with TBS-T over 30 min. Blots were developed with enhanced Chemiluminescence Western blotting kit (Amersham Biosciences), visualised using a Gene Genome bioimaging system and analysed using GeneTools software (Syngene). The relative expression of claudin-1 protein was expressed relatively to β-actin protein.

Intestinal alkaline phosphatase activity analysis

Intestinal alkaline phosphatase (IAP) activity was determined by a microplate reader (SpectraMax M5; Molecular Devices) using a commercial kit (no. A059-2; Nanjing Jiancheng Bioengineering Institute) according to the instructions of the manufacturer. The protein concentrations of intestinal mucosa were determined using Coomassie Brilliant Blue G-250 reagent with bovine serum albumin as a standard. Specific activity of 1 King unit of IAP is defined as the amount of the enzyme that produces 1 mg *p*-nitrophenol/g of protein for 15 min at 37°C.

mRNA expression analysis by real-time PCR

Total RNA isolation, quantification, RT and real-time PCR were performed as previously described[30]. The primer pairs used are shown in online Supplementary Table S1. The expressions of the target genes relative to the housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase; *GAPDH*) were analysed by the $2^{-\Delta\Delta C_T}$ method of Livak & Schmittgen[41]. Our results demonstrated that *GAPDH* did not display any difference among treatments and tissues. Relative mRNA abundance of each target gene was normalised to the group receiving maize oil and treated with sterile 0·9 % NaCl solution.

Statistical analysis

The data were analysed by way of variance specific for repeated measurements using mixed procedure of SAS (SAS Institute Inc.). The model included the treatments, gut segment (jejunum and ileum) and their interactions. When a significant treatment × gut segment interaction occurred, comparisons among treatments in each segment (jejunum or ileum) were conducted. If the interaction was not significant, the arithmetic means of the gut segment (jejunum and ileum) were used to compare treatment effects. Effects of LPS and diet were analysed as a 2 × 2 factorial arrangement by ANOVA using the general linear model (GLM) procedures of SAS. The statistical model included the effects of immunological challenge (saline or LPS), diet (maize oil or fish oil) and their interactions. When significant

diet × LPS interaction or a trend for diet × LPS interaction occurred, *post hoc* testing was performed using Bonferroni's multiple comparison tests. When variances were heterogeneous, non-parametric ANOVA was conducted. Bacterial translocation (positive animals) data were analysed by one-way ANOVA and Fisher's exact test. Data are presented as mean values with pooled standard errors. Differences were considered to be significant if $P<0{\cdot}05$. Instances where $0{\cdot}05<P\leq0{\cdot}10$ were discussed as trends.

Results

Bacterial translocation

Pigs fed fish oil had lower bacteria translocation incidence ($P=0{\cdot}005$) in MLN compared with pigs fed maize oil among LPS-treated pigs, whereas there was no difference among saline-treated pigs (Table 3). Neither LPS nor diet affected bacterial translocation incidence in the spleen and liver. There was no interaction between LPS challenge and diet on bacterial translocation in the MLN, spleen and liver (Table 4). Pigs challenged with LPS had higher translocation micro-organisms in the liver ($P=0{\cdot}004$) and tended to have higher translocation micro-organisms in the MLN ($P=0{\cdot}092$) than those injected with saline. Moreover, pigs fed fish oil had lower translocation micro-organisms in the MLN ($P=0{\cdot}049$) than those fed maize oil. However, neither LPS nor diet affected translocation micro-organisms in the spleen.

Intestinal morphology

Pigs challenged with LPS displayed intestinal mucosal injury including lifting of epithelium at the tip of the villus and villous atrophy. Fish oil supplementation alleviated intestinal mucosal injury caused by LPS (Fig. 1). There was a treatment × segment interaction observed for villus height ($P=0{\cdot}05$). Pigs challenged with LPS had lower villus height in the jejunum ($P=0{\cdot}001$) than those injected with saline (Table 5). Pigs fed fish oil had higher villus height in the jejunum ($P=0{\cdot}044$) and lower villus height in the ileum ($P=0{\cdot}028$) than pigs fed maize oil.

No significant treatment × segment interaction was observed for crypt depth, villus height/crypt depth and villus areas. Overall, pigs challenged with LPS had lower villus area ($P=0{\cdot}001$) and crypt depth ($P=0{\cdot}015$) than those injected with saline. Moreover, pigs fed fish oil tended to have lower villus height/crypt depth ($P=0{\cdot}080$) and had higher crypt depth ($P=0{\cdot}006$). There was no LPS challenge × diet interaction observed for crypt depth, villus height/crypt depth and villus areas.

Intestinal tight junction protein claudin-1 expression

Claudin-1 tended to be higher in the ileum than in the jejunum ($P=0{\cdot}067$) (Table 6). A trend for treatment × segment interaction was observed for claudin-1 expression ($P=0{\cdot}078$). Pigs challenged with LPS had lower claudin-1 expression ($P=0{\cdot}042$) in the ileum than those injected with saline. Pigs fed fish oil had higher claudin-1 expression ($P=0{\cdot}034$) in the ileum and tended to have higher claudin-1 expression in the jejunum compared with those fed maize oil ($P=0{\cdot}053$). There was no LPS challenge × diet interaction observed for claudin-1 expression in the jejunum and ileum.

Intestinal alkaline phosphatase activity and mRNA expression

IAP activity was higher in the ileum than that in the jejunum ($P=0{\cdot}014$) (Table 6). No treatment × segment interaction was observed for IAP activity and mRNA expression. Overall, pigs challenged with LPS had lower IAP activity ($P=0{\cdot}012$) and mRNA expression ($P=0{\cdot}031$) than those injected with saline. There was no LPS challenge × diet interaction observed for IAP activity and mRNA expression.

Intestinal immune cells and lamina propria cells

The numbers of IEL ($P=0{\cdot}019$) and lamina propria cells ($P=0{\cdot}007$) were lower in the jejunum than those in the ileum (Table 7). There was an interaction ($P=0{\cdot}002$) between

Table 3. Effect of fish oil or maize oil supplementation on bacterial translocation incidences in weaned pigs after *Escherichia coli* lipopolysaccharide (LPS) challenge
(*n* 8 (1 pig/pen))

	Saline		LPS	
Items	Maize oil	Fish oil	Maize oil	Fish oil
MLN	2	2	8*	2
Spleen	2	2	4	3
Liver	2	2	4	3

MLN, mesenteric lymph node.
* $P<0{\cdot}05$, Maize oil + LPS *v.* fish oil + LPS (Fisher's exact test).

Table 4. Effect of fish oil or maize oil supplementation on translocation micro-organisms of weaned pigs after *Escherichia coli* lipopolysaccharide (LPS) challenge*
(Means values with their pooled standard errors; *n* 8 (1 pig/pen))

	Saline		LPS			*P*		
Items	Maize oil	Fish oil	Maize oil	Fish oil	SEM	Diet	LPS	Interaction
MLN	3·70	2·27	5·07	3·46	0·39	0·049	0·092	0·902
Spleen	3·97	3·49	4·63	4·13	0·29	0·434	0·305	0·990
Liver	2·63	2·33	4·79	4·51	0·38	0·665	0·004	0·996

MLN, mesenteric lymph node; CFU, colony-forming units.
* The values were expressed in $\log_{10}$ (CFU)/g of organ's weight.

https://doi.org/10.1017/S0007114516001100 Published online by Cambridge University Press

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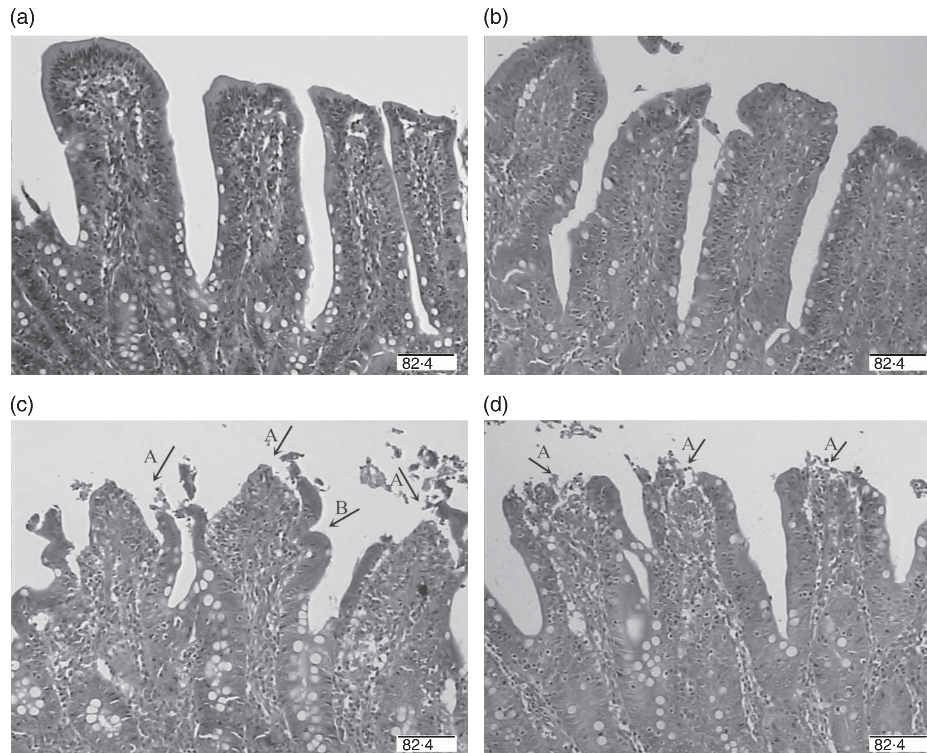

Fig. 1. Intestinal mucosal morphological characterisation of the jejunum (haematoxylin and eosin stained). (a) Pigs fed maize oil and injected with sterile saline. No obvious changes were found. (b) Pigs fed fish oil and injected with sterile saline. No obvious changes were found. (c) Pigs fed maize oil and challenged with lipopolysaccharide (LPS). Morphological changes associated with intestinal mucosal injury, such as lifting of epithelium at the tip of the villus (A) and villous atrophy (B). (d) Pigs fed fish oil and injected with LPS. Intestinal mucosal injury was significantly attenuated. Original magnifications 100×. Scale bars = 82·4 µm.

Table 5. Effect of fish oil or maize oil supplementation on the intestinal morphology of weaned pigs after *Escherichia coli* lipopolysaccharide (LPS) challenge
(Means values with their pooled standard errors; *n* 8 (1 pig/pen))

		T					*P**			*P*		
		Saline		LPS								
Items	S	Maize oil	Fish oil	Maize oil	Fish oil	SEM	T	S	T × S	Diet	LPS	Interaction
Villus height (μm)	Jejunum	265	288	219	246	7·45	0·003	0·613	0·050	0·044	0·001	0·832
	Ileum	269	251	266	211	8·49				0·028	0·186	0·255
Crypt depth (μm)	Jejunum	90	94	79	83	2·27	0·070	0·769	0·197	0·229	0·015	0·589
	Ileum	96	85	89	79	2·45						
Villus height/crypt depth	Jejunum	2·96	2·83	2·78	2·67	0·07	0·253	0·564	0·744	0·395	0·351	0·171
	Ileum	2·82	2·94	2·98	2·65	0·06						
Villus areas (×10^3 μm^2)	Jejunum	34	36	27	31	1·26	0·023	0·121	0·811	0·928	0·001	0·260
	Ileum	33	31	29	26	1·38						

T, treatment; S, segment.
* *P*-values obtained using treatment as the main effect and analysing data from the jejunum and ileum as repeated measurements.

treatment and segment on mast cells. Pigs challenged with LPS had higher mast cell number in the ileum ($P<0·001$) than those injected with saline (Fig. 2). A LPS × diet interaction ($P=0·002$) was observed for mast cell number in the jejunum, with pigs fed fish oil having lower mast cell number ($P<0·001$) compared with pigs fed maize oil among LPS-treated pigs, whereas there was no difference among saline-treated pigs.

No treatment × segment interaction was found for IEL, goblet cell, lamina propria cell and neutrophil numbers. Overall, pigs challenged with LPS had lower IEL ($P<0·001$) and lamina propria cell numbers ($P<0·001$) and higher neutrophil number ($P<0·001$) than those injected with saline. There was an interaction ($P=0·046$) between LPS challenge and diet on IEL number. Pigs fed fish oil had higher IEL number ($P=0·034$) compared with pigs fed maize oil among LPS-treated pigs, whereas there was no difference among saline-treated pigs. No LPS challenge × diet interaction was observed for goblet cells, lamina propria cells and neutrophils. Pigs fed fish oil had lower neutrophil number ($P=0·002$) (Fig. 3) compared with those fed maize oil. Neither LPS nor diet affected goblet cell number in the jejunum and the ileum.

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https://doi.org/10.1017/S0007114516001100 Published online by Cambridge University Press

Table 6. Effect of fish oil or maize oil supplementation on intestinal tight junction protein expressions, intestinal alkaline phosphatase (IAP) activity and gene expression in weaned pigs after *Escherichia coli* lipopolysaccharide (LPS) challenge
(Means values with their pooled standard errors; *n* 8 (1 pig/pen))

		T										
		Saline		LPS			*P**			*P*		
Items	S	Maize oil	Fish oil	Maize oil	Fish oil	SEM	T	S	T × S	Diet	LPS	Interaction
Claudin-1/β-actin	Jejunum	1·15	1·23	0·95	1·26	0·05	0·014	0·067	0·078	0·053	0·377	0·234
	Ileum	1·25	1·64	0·99	1·27	0·08				0·034	0·042	0·716
IAP activity (King unit/g protein)	Jejunum	132	151	63	71	8·57	0·02	0·014	0·541	0·501	0·012	0·907
	Ileum	154	138	142	108	7·97						
IAP gene expression	Jejunum	1·00	0·93	0·77	0·67	0·06	0·384	0·121	0·704	0·936	0·031	0·252
	Ileum	1·00	1·38	1·01	1·04	0·13						

T, treatment; S, segment.
* *P*-values obtained using treatment as the main effect and analysing data from the jejunum and ileum as repeated measurements.

Table 7. Effect of fish oil or maize oil supplementation on immune cells and lamina propria cells in the intestine of weaned pigs after *Escherichia coli* lipopolysaccharide (LPS) challenge
(Means values with their pooled standard errors; *n* 8 (1 pig/pen))

		T										
		Saline		LPS			*P**			*P*		
Items	S	Maize oil	Fish oil	Maize oil	Fish oil	SEM	T	S	T × S	Diet	LPS	Interaction
IEL (/100 enterocytes)	Jejunum	23·75	22·81	20·09	21·93	0·34	<0·001	0·019	0·885	0·689	<0·001	0·046
	Ileum	24·36	23·8	21·35	22·78	0·34						
Goblet cells (/100 enterocytes)	Jejunum	1·49	1·73	1·83	1·51	0·09	0·405	0·206	0·463	0·490	0·759	0·492
	Ileum	1·88	1·53	2·01	1·79	0·12						
Mast cells (mm^2)	Jejunum	52	49	60	45	1·34	<0·001	0·138	0·002	<0·001	0·281	0·002
	Ileum	45	41	58	53	1·69				0·058	<0·001	0·993
Lamina propria cells (×10^3 mm^2)	Jejunum	10·98	10·96	9·66	10·06	0·13	0·003	0·007	0·825	0·676	<0·001	0·934
	Ileum	11·49	11·69	10·62	10·47	0·23						
Neutrophils (mm^2)	Jejunum	4·06	3·24	5·85	4·73	0·23	<0·001	0·420	0·362	0·002	<0·001	0·721
	Ileum	3·83	3·26	4·98	4·64	0·20						

T, treatment; S, segment; IEL, intraepithelial lymphocyte.
* *P*-values obtained using treatment as the main effect and analysing data from jejunum and ileum as repeated measurements.

Corticotropin-releasing hormone receptor 1, corticotropin-releasing hormone, glucocorticoid receptor and tryptase mRNA expressions

The mRNA abundance of *CRHR1* in the jejunum was higher compared with the ileum ($P = 0·003$, Table 8). There was a treatment × segment interaction ($P = 0·03$) observed for the mRNA abundance of *CRHR1*. Pigs challenged with LPS had higher mRNA abundance of jejunal ($P = 0·002$) and ileal *CRHR1* ($P < 0·001$) than those injected with saline. Fish oil supplementation tended to decrease the mRNA abundance of *CRHR1* in the ileum ($P = 0·053$).

There was no treatment × segment interaction observed for *CRH*, glucocorticoid receptors (*GR*) and tryptase. Overall, pigs challenged with LPS had higher mRNA abundance of *CRH* ($P = 0·004$) and *GR* ($P = 0·022$) than those injected with saline. There was a trend for LPS challenge × diet interaction observed for *GR* ($P = 0·072$). Pigs fed fish oil had lower mRNA abundance of *GR* compared with pigs fed maize oil among LPS-treated pigs, whereas there was no difference among saline-treated pigs. There was no LPS challenge × diet interaction observed for *CRH* and tryptase. However, pigs fed fish oil tended to have lower mRNA abundance of *CRH* ($P = 0·065$) compared with those fed maize oil. Neither LPS nor diet affected tryptase mRNA abundance.

Discussion

Our previous studies have demonstrated that fish oil supplementation alleviated LPS-induced activation of the HPA axis and intestinal injury in a weaned piglet model[25,30]. In the current experiment, our aim was to investigate the effect of dietary fish oil supplementation on the intestinal barrier function in weaned pigs.

The intestinal mucosal barrier is the first line of defence against a hostile environment within the intestinal lumen[10]. The breakdown of this barrier may result in the crossing of viable bacteria and their products to MLN and more distant sites – a process known as bacterial translocation[42]. In the present study, we showed that bacteria translocation incidences and translocation micro-organisms in the MLN and the liver

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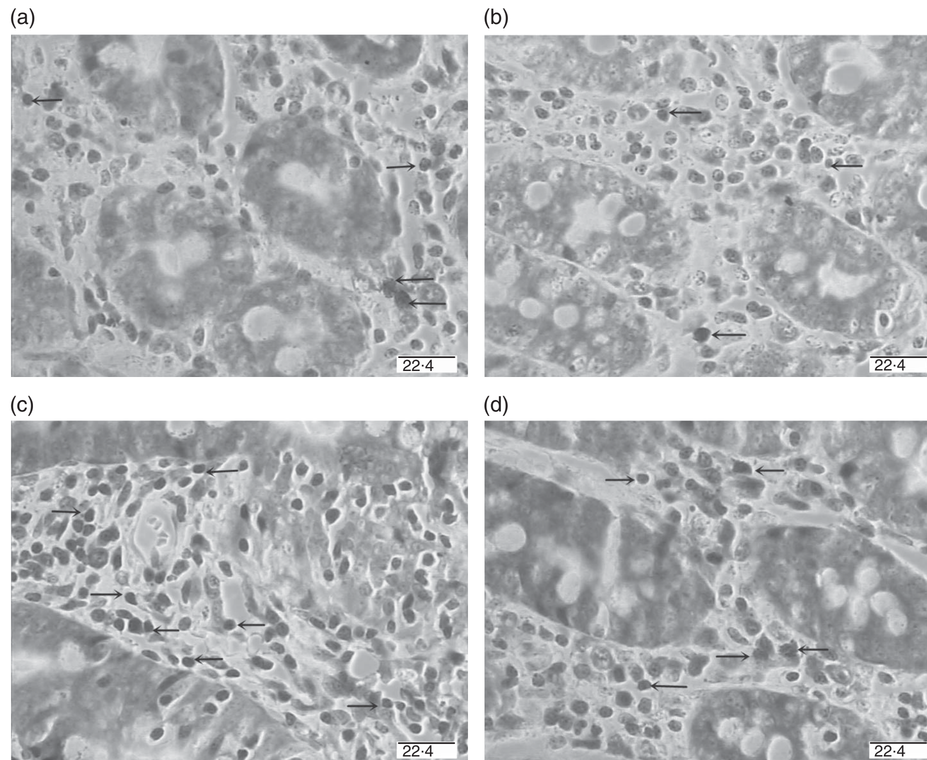

Fig. 2. Photomicrographs of pig jejunal mucosa showing mast cells stained with toluidine blue. (a) Pigs fed maize oil and injected with sterile saline. (b) Pigs fed fish oil and injected with sterile saline. (c) Pigs fed maize oil and challenged with lipopolysaccharide (LPS). (d) Pigs fed fish oil and injected with LPS. Arrows indicate toluidine blue-positive mast cells. Pigs fed fish oil had lower mast cell numbers ($P < 0·05$) compared with pigs fed maize oil among LPS-treated pigs, whereas there was no difference among saline-treated pigs. Original magnifications 400×. Scale bars = 22·4 μm.

increased or trended to increase in the pigs with LPS challenge, which indicates that intestinal obstruction really led to an increase in intestinal permeability. We also found that LPS caused the decrease of IAP activity and mRNA expression. In fact, IAP plays important roles in LPS dephosphorylation, reduction of LPS-induced intestinal inflammation and restriction of bacterial translocation[43,44]. Therefore, the decrease in IAP activity and mRNA expression reduced intestinal capacity to detoxify LPS, which in turn may lead to intestinal barrier dysfunction and inflammation. Fish oil reduced bacterial translocation incidences and translocation micro-organisms in the MLN. However, fish oil did not affect IAP activity and mRNA expression. In contrast with our findings, Nieto *et al.*[45] reported that 10 % fish oil supplementation decreased IAP activity in ulcerative rats compared with 10 % olive oil.

The intestinal barrier function is partly dependent on the mucosal structure of the intestine. Our histological study showed that LPS caused a significant morphological injury to the mucosa of the intestine, such as epithelium lifting and villous atrophy. Consistent with bacterial translocation, fish oil alleviated villous epithelium lifting caused by LPS. Similarly, Whiting *et al.*[46] reported that a diet enriched with *n*-3 PUFA (fish oil) enhanced epithelial barrier function and ameliorated several chronic inflammatory diseases.

The intestinal barrier function is maintained and regulated by the tight junctions between intact epithelial cells[47]. The formation of tight junctions requires the assembly of several proteins anchored directly or indirectly to the actin-based cytoskeleton. Tight junction proteins include occudin and members of a large class of proteins called claudins[34]. In the present study, fish oil increased claudin-1 expression in the ileum and tended to increase claudin-1 expression in the jejunum. In agreement with our finding, Li *et al.*[48] reported that *n*-3 PUFA improved tight junction formation and reduced transcelluar permeability. In the intestine, the dynamic renewal of the epithelium is characterised by cell production in the crypts followed by cell maturation and cell migration to the tip of the villi[49]. Many reports have shown that *n*-3 PUFA stimulate differentiation, support intestinal maturation and reduce transcellular permeability[18,19,50]. Our results indicate that fish oil protected the intestinal integrity and maintained barrier function partially by improving intestinal morphology and the expressions of tight junction proteins.

The main sites of the mucosal immune system in the intestine are gut-associated lymphoid tissue and immuno-associated cells such as IEL, mast cells and goblet cells[51]. IEL play an important role in maintaining the intestinal barrier function and as such form the first line of defence against infectious agents or allergens. It is well known that LPS causes gut barrier dysfunction and induces inflammatory response[52,53]. In the present study, LPS increased neutrophil infiltration and decreased IEL numbers in the intestine. However, fish oil increased IEL numbers and decreased neutrophil infiltration in the jejunum. Maeshima *et al.*[54] reported that adding fish oil to parenteral nutrition

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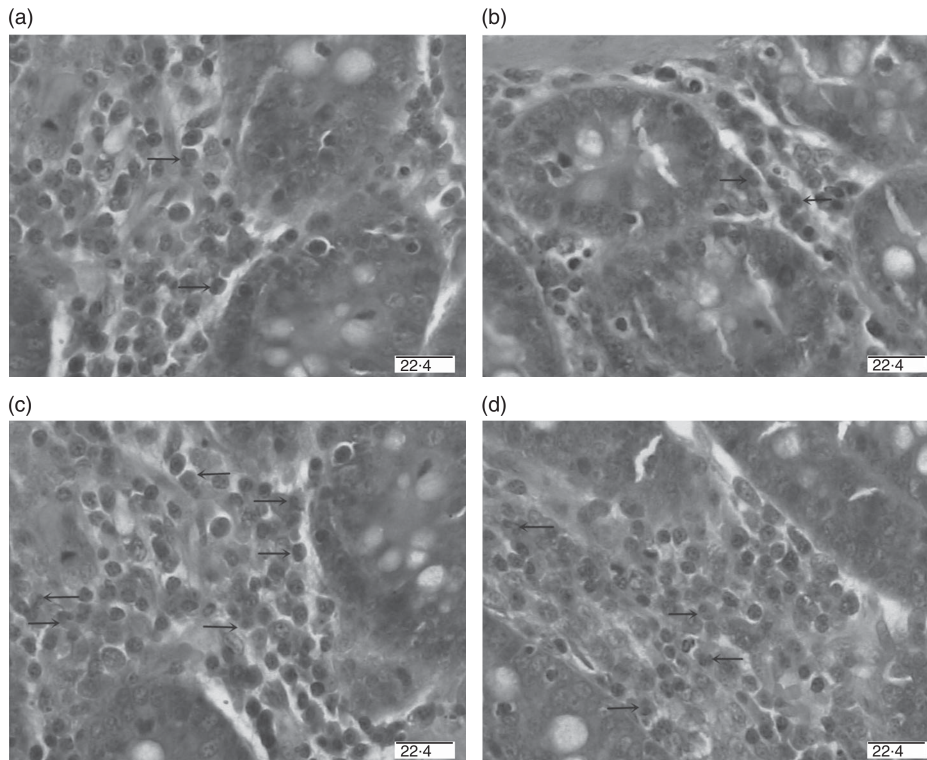

Fig. 3. Representative photomicrographs of pig jejunal mucosa showing neutrophils (haematoxylin and eosin stained). (a) Pigs fed maize oil and injected with sterile saline. (b) Pigs fed fish oil and injected with sterile saline. (c) Pigs fed maize oil and challenged with lipopolysaccharide (LPS). (d) Pigs fed fish oil and injected with LPS. Arrows indicate neutrophils. Pigs challenged with LPS had higher numbers of jejunal neutrophils ($P<0{\cdot}05$) than those injected with saline. Pigs fed fish oil had lower neutrophil numbers in the jejunum ($P<0{\cdot}05$) compared with those fed maize oil ($P=0{\cdot}058$). Original magnifications 400×. Scale bars = 22·4 µm.

Table 8. Effect of fish oil or maize oil supplementation on mRNA expressions of corticotropin-releasing hormone (*CRH*), glucocorticoid receptors (*GR*), tryptase and corticotropin-releasing hormone receptor 1 (*CRHR1*) in the intestine of weaned pigs after *Escherichia coli* lipopolysaccharide (LPS) challenge* (Means values with their pooled standard errors; *n* 8 (1 pig/pen))

		T										
		Saline		LPS			*P*†			*P*		
Items	S	Maize oil	Fish oil	Maize oil	Fish oil	SEM	T	S	T × S	Diet	LPS	Interaction
CRHR1	Jejunum	1·00	1·23	19·10	15·19	2·71	0·011	0·003	0·03	0·690	0·002	0·653
	Ileum	1·00	0·81	3·90	2·39	0·32				0·053	<0·001	0·125
CRH	Jejunum	1·00	0·868	2·15	1·198	0·16	0·01	0·910	0·460	0·065	0·004	0·115
	Ileum	1·00	1·09	1·87	1·40	0·13						
GR	Jejunum	1·00	0·98	1·67	1·02	0·08	<0·001	0·899	0·200	0·168	0·022	0·072
	Ileum	1·00	1·11	1·29	1·21	0·08						
Tryptase	Jejunum	1·00	0·94	0·98	0·99	0·08	0·94	0·639	0·949	0·769	0·610	0·851
	Ileum	1·00	0·91	1·12	1·08	0·09						

* All the data were acquired using real-time PCR. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was the housekeeping gene, and the pigs fed the maize oil diet and injected with saline comprised the calibrator samples.
† *P*-values obtained using treatment as the main effect and analysing data from the jejunum and ileum as repeated measurements.

partially reversed parenteral nutrition-induced IEL loss. In addition, dietary *n*-3 PUFA have been reported to protect intestinal epithelial cells from pro-inflammatory insults, alleviate the intestinal inflammatory response and accelerate recovery from inflammation[30,55]. Moreover, previous studies in mice have shown that the decrease in IEL number was associated with decreasing tight junction integrity in the intestine[56]. This finding supports the current finding that fish oil maintained epithelial barrier integrity by inducing the proliferation of IEL and alleviating the intestinal inflammatory response.

The mechanisms by which stress causes breakdown in intestinal barrier function are not been fully understood. Several studies have demonstrated that stress-induced alterations in intestinal barrier function are mediated by the release of central and peripheral stress mediators such as CRH and adrenal glucocorticoids[8–10]. Subsequent activation of CRHR and GR has been shown to trigger disturbances in the intestinal barrier[57,58]. Our previous studies also demonstrated that LPS-induced activation of the HPA axis, whereas fish oil attenuated the activation of the HPA axis[25]. In the present study, we observed that LPS increased

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mRNA abundance of *CRHR1*, *CRH* and *GR* in the intestine. Fish oil supplementation tended to alleviate the increase in mRNA abundance of *CRH* and *GR* caused by LPS. Moeser *et al.*[11] reported that CRH might be a more sensitive stress indicator of stress-induced intestinal dysfunction. Smith *et al.*[10] found that weaning-induced mucosal barrier dysfunction could be prevented by a CRH receptor antagonist, which suggests that CRH plays an important role in intestinal mucosal barrier integrity. CRH mediates its effects by binding to two CRH receptors (CRHR1 and CRHR2), which are expressed on multiple intestinal cell types, including enteric neurons, lamina propria immune cells and epithelial cells[59–61]. Moeser *et al.*[11] have shown that peripheral CRHR activation mediates intestinal mucosal disturbances induced by early weaning. On the basis of the CRHR antagonist experiment, Smith *et al.*[10] indicated that CRHR1 likely mediated impaired intestinal barrier dysfunction and hypersecretion in early-weaned pigs. Similarly, our study showed that LPS increased mRNA abundance of *CRHR1* in the intestine. Fish oil supplementation alleviated the increase of ileal mRNA abundance of *CRHR1* caused by LPS. However, fish oil did not affect jejunal mRNA abundance of *CRHR1*. The reason might be that the different segments of the intestine had different responses to fish oil supplementation. These results suggest that fish oil might improve intestinal barrier function and inhibit the CRH/CRHR1 signalling pathway.

In addition, mast cells are recognised as an important cell type mediating stress-related intestinal disorders[12,62,63]. Mast cells participate in the regulation of intestinal motility, gut barrier function and mucosal immune function[64]. Mast cells express CRHR. Mast cells can be activated either directly by CRH or indirectly by neuropeptides released from the CRH-stimulated neural process[65]. Our histological study showed that LPS increased mast cell number in intestinal mucosa. Mast cell number increased along with mast cell activation in the jejunum of diarrhoea-predominant irritable bowel syndrome patients[66]. Mast cell activation leads to degranulation and release of several mediators such as protease, histamine, eicosanoids, cytokines and chemokines, which results in injury to the intestinal structure and increase in intestinal permeability[67]. Our data showed that fish oil alleviated the increase in mast cell number induced by LPS. Similarly, Wang & Marianna[68] found that *n*-3 PUFA inhibited mast cell activation by disruption of FcεRI localisation and shuttling into lipid rafts. In present study, fish oil alleviated LPS-induced increase in mast cell number, which might decrease the release of protease, histamine, eicosanoids and cytokines from mast cells, thereby maintaining gut barrier function.

In conclusion, fish oil supplementation attenuates disruption of intestinal barrier function and inflammatory response induced by LPS. It suggests that fish oil supplementation improves intestinal barrier function and inhibits CRH/CRHR1 signalling pathways and mast cell tissue density.

Acknowledgements

This study was supported by National Science Foundation of China (31422053 and 31372318) and the project of the Hubei Province Department of Education (T201508).

The authors' contributions are as follows: Y. L., H. Z. and S. C. designed the study; H. Z., Y. L., S. C., X. W., D. P. and W. L. conducted the study; H. Z., Y. L., D. P. and W. L. analysed the data; H. Z., Y. L., D. P. and W. L. wrote the paper; Y. L. had primary responsibility for the final content. All the authors read and approved the final version of the manuscript.

The authors declare that there are no conflicts of interest.

Supplementary material

For supplementary material/s referred to in this article, please visit http://dx.doi.org/10.1017/S0007114516001100

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