

# Whey protein concentrate enhances intestinal integrity and influences transforming growth factor- $\beta$ 1 and mitogen-activated protein kinase signalling pathways in piglets after lipopolysaccharide challenge

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## Abstract

Whey protein concentrate (WPC) has been reported to have protective effects on the intestinal barrier. However, the molecular mechanisms involved are not fully elucidated. Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) is an important component in the WPC, but whether TGF- $\beta$ 1 plays a role in these processes is not clear. The aim of this study was to investigate the protective effects of WPC on the intestinal epithelial barrier as well as whether TGF- $\beta$ 1 is involved in these protection processes in a piglet model after lipopolysaccharide (LPS) challenge. In total, eighteen weanling pigs were randomly allocated to one of the following three treatment groups: (1) non-challenged control and control diet; (2) LPS-challenged control and control diet; (3) LPS + 5 %WPC diet. After 19 d of feeding with control or 5 %WPC diets, pigs were injected with LPS or saline. At 4 h after injection, pigs were killed to harvest jejunal samples. The results showed that WPC improved ( $P < 0.05$ ) intestinal morphology, as indicated by greater villus height and villus height: crypt depth ratio, and intestinal barrier function, which was reflected by increased transepithelial electrical resistance and decreased mucosal-to-serosal paracellular flux of dextran (4 kDa), compared with the LPS group. Moreover, WPC prevented the LPS-induced decrease ( $P < 0.05$ ) in claudin-1, occludin and zonula occludens-1 expressions in the jejunal mucosae. WPC also attenuated intestinal inflammation, indicated by decreased ( $P < 0.05$ ) mRNA expressions of TNF- $\alpha$ , IL-6, IL-8 and IL-1 $\beta$ . Supplementation with WPC also increased ( $P < 0.05$ ) TGF- $\beta$ 1 protein, phosphorylated-Smad2 expression and Smad4 and Smad7 mRNA expressions and decreased ( $P < 0.05$ ) the ratios of the phosphorylated to total c-jun N-terminal kinase (JNK) and p38 (phospho-JNK:JNK and p-p38:p38), whereas it increased ( $P < 0.05$ ) the ratio of extracellular signal-regulated kinase (ERK) (phospho-ERK:ERK). Collectively, these results suggest that dietary inclusion of WPC attenuates the LPS-induced intestinal injury by improving mucosal barrier function, alleviating intestinal inflammation and influencing TGF- $\beta$ 1 canonical Smad and mitogen-activated protein kinase signalling pathways.

**Key words:** Whey protein concentrate: Intestinal integrity: Transforming growth factor- $\beta$ 1: Mitogen-activated protein kinase: Piglets

Whey protein concentrate (WPC) is a protein-enriched powder made from whey during the process of cheese making. It is commonly used in the manufacturing of foods for infants and young children. Emerging evidence has demonstrated that WPC is useful for the treatment of a wide variety of gastrointestinal disorders such as inflammatory bowel disease and necrotising enterocolitis<sup>(1,2)</sup>. It has been found that the beneficial role of WPC in the intestine is closely related to its numerous bioactive compounds including functional amino acids, lactoferrin (LF) and growth factors, which is largely attributed to the stimulation of mucin synthesis and modification of immune response<sup>(2,3)</sup>. Recently, it has also been reported that WPC improves intestinal epithelial barrier function *in vitro*<sup>(4)</sup>. However, the molecular mechanisms underlying the protective effects are poorly understood.

WPC contains abundant bioactive compounds that are vital for immune and gut development early in life<sup>(5,6)</sup>. Among the most relevant substances in WPC are Ig, LF and growth factors (e.g. transforming growth factor  $\beta$  (TGF- $\beta$ ) and epidermal growth factor (EGF)). Nevertheless, investigations directly examining the role of WPC in affecting the barrier integrity *in vivo* have not been reported. It is also of great interest to investigate whether bioactive compounds in WPC can be partly involved in WPC-induced prevention of intestinal epithelial barrier disruption. Until now, there are little data about the role of WPC in restitution of intestinal epithelium after injury<sup>(4)</sup>.

Mammalian milk and WPC are rich in TGF- $\beta$  including TGF- $\beta$ 1<sup>(4,7)</sup>. TGF- $\beta$ 1 is also the most abundant isoform in the mucosa of the gut<sup>(8)</sup> and may play an important role in postnatal adaptation of the gastrointestinal tract in suckling animals<sup>(9)</sup>.

**Abbreviations:** EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FD4, fluorescein isothiocyanate dextran 4 kDa; JNK, c-jun N-terminal kinase; LF, lactoferrin; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; TER, transepithelial electrical resistance; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; WPC, whey protein concentrate; ZO-1, zonula occludens-1.

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TGF- $\beta$ 1 is of particular interest as it has known effects in remarkable number of biological processes, including epithelial cell growth and differentiation<sup>(10,11)</sup>, restitution of intestinal epithelium after injury<sup>(9,12)</sup> and immune regulation<sup>(5)</sup>. Thus far, different signalling pathways have been reported to be involved in TGF- $\beta$  action, including Smad-dependent and Smad-independent pathways<sup>(13)</sup>. The canonical TGF- $\beta$  signalling pathway is mediated by Smad family proteins<sup>(13)</sup>. Besides the canonical Smad pathways, there have been a number of non-Smad signalling pathways described, including mitogen-activated protein kinase (MAPK pathways) (extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK) and p38 MAPK pathways) in TGF- $\beta$ 1 actions<sup>(14)</sup>. We hypothesised that TGF- $\beta$ 1 in WPC might be involved in those barrier-protection processes and would lead to changes of these TGF- $\beta$  signalling pathways. In the present study, we used a piglet model challenged with lipopolysaccharide (LPS) to investigate the beneficial effects of WPC on intestinal epithelial barrier function. Moreover, intracellular signals through which WPC and its active components might exert beneficial barrier effects were studied.

## Methods

### Animal care and experimental design

This experiment was approved by the Animal Care and Use Committee, Zhejiang University. A total of eighteen 35-d-old weaned barrows (Duroc  $\times$  Landrace  $\times$  Yorkshire, weaned at 21 d of age), with an average weight of 9.5 kg, were allocated to three groups, each consisting of six animals. One group served as the control group, whereas the other two groups were subjected to intestinal injury by injecting LPS. Animals were fed diets according to their groups: (1) control group (piglets fed the control diet); (2) LPS group (piglets fed the control diet and LPS); (3) LPS+WPC group (piglets fed the diet inclusion of 5%WPC; WPC was provided by Open Country Dairy Ltd). Pigs were individually housed in pens with dimensions of 1.8  $\times$  1.1 m<sup>2</sup> in an environmentally controlled nursery barn. The room temperature was maintained at 25–27°C. Each pen contained a feeder and a nipple waterer to allow piglets *ad libitum* access to feed and water. There were six replicate pens for each treatment. Diets were formulated to meet or exceed requirements as suggested by the National Research Council (2012) (Table 1). The crude protein, Ca and total P contents in diet were analysed according to the method of Association of Official Analytical Chemists<sup>(15)</sup>. After 19 d feeding with control or 5%WPC diets, the two challenged groups (the LPS group and the LPS + WPC group) were intraperitoneally injected with *Escherichia coli* LPS (*E. coli* serotype 055: B5; Sigma Chemical) at 100  $\mu$ g/kg body weight (BW), and the unchallenged group was injected with the same amount of 0.9% NaCl solution. The dose of LPS was chosen to cause acute intestinal injury in weaned pigs according to Liu *et al.*<sup>(16–18)</sup>.

### Sample collection

Four hours following the injection of LPS or saline, piglets were killed under anaesthesia with an intravenous injection of

**Table 1.** Ingredients and chemical composition of the weaned diets on an as-fed basis

Items	Control diet	5%WPC
<b>Ingredients (g/kg)</b>		
Maize	633.5	611.9
Soyabean meal	265	236
Fishmeal	55	53.3
WPC (crude protein 350 g/kg)		50
Limestone meal	5	5
Dicalcium phosphate	11.5	12
Sodium chloride	3	3
L-Lysine HCl	1.5	1.3
DL-Methionine	0.5	0.5
Vitamin–mineral premix*	10	10
Soyabean oil	15	17
<b>Composition (g/kg)</b>		
Digestible energy† (calculated) (MJ/kg)	14.33	14.32
Crude protein (measured)	219.6	219.5
Lysine (measured)	13.7	13.9
Methionine (measured)	4.4	4.5
Ca (measured)	8.2	8.3
Total P (measured)	6.8	6.6
TGF- $\beta$ 1 (measured) ( $\mu$ g/kg)		3

WPC, whey protein concentrate; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1.

\* Provided per kilogram of diet: vitamin A, 3.3 mg; vitamin E, 33.4 mg; vitamin D<sub>3</sub>, 0.015 mg; vitamin K<sub>3</sub>, 1.5 mg; biotin, 0.10 mg; riboflavin, 8.0 mg; thiamine, 2.0 mg; niacin, 30 mg; pantothenic acid, 20 mg; pyridoxine, 3.0 mg; folic acid, 0.6 mg; vitamin B<sub>12</sub>, 0.04 mg; choline, 800 mg; Cu (CuSO<sub>4</sub>·5H<sub>2</sub>O), 16 mg; Fe (FeSO<sub>4</sub>), 125 mg; Zn (ZnSO<sub>4</sub>), 100 mg; Mn (MnSO<sub>4</sub>·H<sub>2</sub>O), 15 mg; Se (Na<sub>2</sub>SeO<sub>3</sub>), 0.3 mg; I (KI), 0.2 mg.

† Digestible energy was calculated from data provided by Feed Database in China (2011).

sodium pentobarbital (40 mg/kg BW). The reason for the choice of measurement at one point (4 h) after LPS injection was because previous studies have demonstrated that, within 3–6 h after injection, LPS caused acute intestinal morphological damage and a breakdown in intestinal barrier function in rats, mice and pigs<sup>(16–22)</sup>. Jejunum is one of the most susceptible intestinal segment to damage from endotoxins (LPS)<sup>(23)</sup>. Segments of the mid-jejunum were harvested immediately after the animals were killed and prepared for Ussing chamber studies. Adjacent specimens were fixed in buffered 10% formalin until morphological measurements. Mucosal scrapings from the remaining jejunum samples were collected, rapidly frozen in liquid N<sub>2</sub> and stored at –80°C.

### Intestinal morphology and barrier function

After fixation, the intestinal segments were dehydrated, embedded in paraffin, sectioned (5  $\mu$ m) and stained with haematoxylin and eosin<sup>(24)</sup>. Villus height and crypt depth were measured in three intestinal cross-sections per animal with at least ten well-oriented crypt–villus units for each cross-section using image analysis (Leica Imaging Systems Limited) and averaged for each sample. The transepithelial electrical resistance (TER) and mucosal-to-serosal permeability to 4-kDa FITC dextran (fluorescein isothiocyanate dextran 4 kDa (FD4); Sigma-Aldrich) were determined *in vitro* in a Ussing chamber system, according to the procedures outlined by Moeser *et al.*<sup>(25)</sup>. In brief, segments of the jejunum were stripped from the seromuscular layer in oxygenated (95% O<sub>2</sub>/5% CO<sub>2</sub>) Ringer's solution and then mounted in the Easy Mount Ussing

chamber system with a multi-channel voltage-current clamp (model VCC MC6; Physiologic Instruments). Tissues were bathed on the serosal and mucosal sides with 5 ml Ringer's solution. The serosal bathing solution contained 10 mM glucose, which was osmotically balanced on the mucosal side with 10 mM mannitol. Bathing solutions were oxygenated (95% O<sub>2</sub>–5% CO<sub>2</sub>) and circulated in water-jacketed reservoirs maintained at 37°C. The clamps were connected to Acquire and Analyze software (Physiologic Instruments) for automatic data collection. After a 30-min equilibration period on the Ussing chambers, TER ( $\Omega$  cm<sup>2</sup>) was recorded at 15-min intervals over a 2-h period and then averaged to derive the TER values for a given pig. FD4 was added on the mucosal side at a final concentration of 0.375 mg/ml. Mucosal-to-serosal flux of FD4 ( $\mu$ g/cm<sup>2</sup> per h) was monitored from the serosal side at 30-min intervals for 120 min. The concentrations of FD4 in the serosal side were measured using a fluorescence microplate reader (FLx800; Bio-Tek Instruments Inc.). The flux over the 2-h period was calculated.

#### Transforming growth factor- $\beta$ 1 content in whey protein concentrate by ELISA measurements

The TGF- $\beta$ 1 content in WPC was determined by ELISA according to the manufacturer's protocol (R&D Systems)<sup>(37)</sup>. TGF- $\beta$ 1 content was also assayed in the control group.

#### Protein expression analysis by Western blot

The Western blot analysis was performed according to the procedures outlined by Hu *et al.*<sup>(24)</sup>. In brief, after electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Millipore). The membranes were incubated overnight at 4°C with primary Antibody (Ab) and then with the secondary

Ab for 120 min at room temperature. The primary Ab (occludin, claudin-1, zonula occludens-1 (ZO-1), TGF- $\beta$ 1, Smad2, phospho-Smad2, p38, phospho-p38, JNK, phospho-JNK (p-JNK), ERK, phospho-ERK 1/2 (p-ERK),  $\beta$ -actin rabbit mAb) were purchased from Santa Cruz Technology Inc. The secondary Ab was HRP-conjugated anti-rabbit antibody (Cell Signaling Technology). Western blot was detected using an enhanced chemiluminescence detection kit (Amersham), photographed by a ChemiScope 3400 (Clinx Science Instruments) and analysed using Quantity One software.  $\beta$ -Actin was used as an internal control, which exhibited no difference among each group. The relative abundance of each target protein was expressed as target protein:  $\beta$ -actin protein ratio or ratio of phosphorylated protein:total protein. The protein expression of all samples was expressed as fold changes, calculated relative to the control group.

#### mRNA expression analysis by real-time PCR

The mRNA levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8 and TGF- $\beta$  receptors, as well as their downstream signal Smads (2,3,4,7), were determined by real-time PCR, as described by Liu *et al.*<sup>(17)</sup>. In brief, total RNA was extracted from jejunal mucosa using TRIzol reagent (Invitrogen) following the manufacturer's guidelines. The purity and concentration of all RNA samples were measured using a NanoDrop spectrophotometer (ND-2000; NanoDrop Technologies). Reverse transcription using the PrimeScript RT reagent kit (Takara Biotechnology) was carried out following the manufacturer's instructions. Quantitative analysis of PCR was carried out on a StepOne Plus real-time PCR system (Applied Biosystems) using SYBR Green Master mix (Promega) according to the manufacturer's instructions. The primers used are given in Table 2. Gene-specific amplification was determined by melting curve analysis and agarose gel electrophoresis. The 2<sup>– $\Delta\Delta C_t$</sup>  method

**Table 2.** GenBank accession numbers, sequences of forward and reverse primers and fragment sizes used for real-time PCR

Target	GenBank number	Primer sequence	Size (bp)
TNF- $\alpha$	NM_214022.1	F:5'CATCGCCGCTCTCTACCA3' R:5'CCCAGATTGAGCAAAAGTCCA3'	199
IL-6	NM_001252429.1	F:5'CCTGTCCACTGGGCACATAAC3' R:5'CAAGAAACACCTGGCTCTGAAAC3'	253
IL-8	M86923	F:5'TGGCAGTTTTCTGCTTTCT 3' R:5'CAGTGGGGTCCACTCTCAAT 3'	154
IL-1 $\beta$	NM_214055	F:5'CAAAGGCCGCCAAGATATAA 3' R:5'GAAATTCAGGCAGCAACAT 3'	147
T $\beta$ RI	AF461808	F:5'CTGTGTCTGTCCACCATTCATTG3' R:5'CAACTTTGCTATGTCTGTCTCCCC3'	496
T $\beta$ RII	X70142	F:5'CATCTCCTGCTAATGTTGTTGCC3' R:5'CGGTTCTAAATCCTGGGACACG3'	324
Smad2	BP437096	F:5'GAAGAGAAGTGGTGTGAGAAAGCAG3' R:5'AATACTGGAGGCCAAAACCTGGTGT3'	428
Smad3	AB052738	F:5'TGGAGGAGGTGGAGAAATCAGAAC3' R:5'CACACTCGCTTGCTCACTGTAATC3'	541
Smad4	NM_214072	F:5'CCTGAGTATTGGTGTTCATTGC3' R:5'TGATGCTCTGCCCTGGGTAATC3'	598
Smad7	AW359979	F:5'TACTGGGAGGAGAAGACGAGAGTG3' R:5'TGGCTGACTTGATGAAGATGGG3'	241
GAPDH	NM001206359	F:5'ATGGTGAAGGTCGGAGTGAAC3' R:5'CTCGCTCCTGGAAGATGGT3'	235

T $\beta$ RI, transforming growth factor- $\beta$  receptor I; T $\beta$ RII, transforming growth factor- $\beta$  receptor II; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

was used to analyse the relative changes in each target gene expression. The change ( $\Delta$ ) in  $C_t$  values in each group was compared with the  $C_t$  value of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) ( $\Delta C_t$ ). Subsequently,  $\Delta\Delta C_t$  was computed for each target gene from the treatment groups by subtracting the averaged  $\Delta C_t$  for the control group. The final fold differences were computed as  $2^{-\Delta\Delta C_t}$  for each target gene. The results showed that GAPDH exhibited no difference between the three groups.

### Statistical analysis

Data were analysed using the SAS statistical package (SAS Institute), with each animal considered an experimental unit. Results were statistically analysed by one-way ANOVA. Differences between the means were tested using Duncan's multiple range tests. Differences were considered significant at  $P < 0.05$ .

## Results

### Content of transforming growth factor- $\beta$ 1 in whey protein concentrate and whey protein concentrate diet and control diet

WPC contains abundant TGF- $\beta$ 1 as much as 0.06 ng/mg. The content of TGF- $\beta$ 1 in the WPC diet was 3  $\mu$ g/kg. We checked for the presence of TGF- $\beta$ 1 in the control diet but we obtained negative results. The reason may be that the TGF- $\beta$ 1 content in the control diet was too low to detect.

### Effects of dietary whey protein concentrate on growth performance

Throughout the 19-d trial (pre-challenge), there were no differences in initial (9.5 (SD 0.6) kg) and final BW (19.25 (SD 1.2) kg), daily gain (513 (SD 32) g) ( $P = 0.741$ ), daily feed intake (823 (SD 61) g) ( $P = 0.362$ ) and the gain:feed ratio (0.62 (SD 0.07)) ( $P = 0.641$ ) between the WPC and control groups.

### Effects of dietary whey protein concentrate on the intestinal mucosal morphology and barrier function of piglets after lipopolysaccharide challenge

Table 3 shows the jejunum morphology and barrier function of piglets. Compared with the control group, the pigs challenged with LPS had shorter ( $P < 0.05$ ) villus height and lower villus height:crypt depth ratio in the jejunum. LPS challenge also increased the FD4 flux and lowered ( $P < 0.05$ ) TER. However, dietary WPC significantly prevented the LPS-induced decrease ( $P < 0.05$ ) in villus height:crypt depth ratio and TER and limited the LPS-induced decrease in villous height and the LPS-induced increase in FD4 flux.

### Effects of dietary whey protein concentrate on the tight-junction proteins in the jejunal mucosa of piglets after lipopolysaccharide challenge

Fig. 1 shows the protein expressions of occludin, claudin-1 and ZO-1 in the jejunal mucosa of piglets. Compared with the control group, LPS challenge decreased ( $P < 0.05$ ) protein

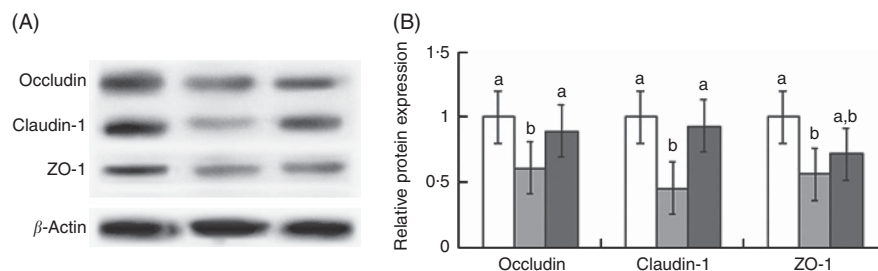
**Table 3.** Effects of dietary whey protein concentrate (WPC) on the jejunum morphology and barrier function of piglets\* (Mean values with their standard errors;  $n$  6 pigs)

Items	Control	LPS	LPS + WPC	SEM	$P$
Villus height ( $\mu$ m)	385 <sup>a</sup>	334.2 <sup>c</sup>	359.6 <sup>b</sup>	6.16	<0.001
Crypt depth ( $\mu$ m)	115.33	124.17	117.92	4.32	0.23
Villus height: crypt depth	3.36 <sup>a</sup>	2.71 <sup>b</sup>	3.06 <sup>a</sup>	0.1	0.001
TER ( $\Omega$ cm <sup>2</sup> )	67.83 <sup>a</sup>	49.25 <sup>b</sup>	59.08 <sup>a</sup>	3.04	0.002
FD4 flux ( $\mu$ g/cm <sup>2</sup> per h)	1.15 <sup>c</sup>	2.32 <sup>a</sup>	1.49 <sup>b</sup>	0.11	<0.001

LPS, lipopolysaccharide; FD4, mucosal:serosal flux of fluorescein isothiocyanate dextran (4 kDa); TER, transepithelial electrical resistance.

<sup>a,b,c</sup> Mean values within a row with superscript letters were significantly different ( $P < 0.05$ ).

\* Control (non-challenged control), piglets receiving a control diet and injected with 0.9% sterile saline; LPS (LPS-challenged control), piglets receiving the same control diet and injected with *Escherichia coli* LPS; LPS + WPC (LPS challenged + 5% WPC), piglets receiving a 5% WPC diet and injected with LPS.



**Fig. 1.** Effects of dietary whey protein concentrate (WPC) on protein expressions of occludin, claudin-1 and zonula occludens-1 (ZO-1) in jejunal mucosa of piglets. (A) Representative blots of occludin, claudin, ZO-1 and  $\beta$ -actin in the jejunal mucosa of piglets. (B) Relative tight-junction protein expressions in the jejunal mucosa of piglets. Values are means ( $n$  6), and standard deviations represented by vertical bars. <sup>a,b</sup>Mean values with unlike letters were significantly different ( $P < 0.05$ ). The control sample was used as the reference sample. The protein expression of all samples was expressed as fold changes, calculated relative to the control group. □, Control; ■, lipopolysaccharide (LPS); ■, LPS + WPC. Control (non-challenged control), piglets receiving a control diet and injected with 0.9% sterile saline; LPS (LPS-challenged control), piglets receiving the same control diet and injected with *Escherichia coli* LPS; LPS + WPC (LPS challenged + 5% WPC), piglets receiving a 5% WPC diet and injected with LPS.



expressions of occludin, claudin-1 and ZO-1. Dietary WPC prevented the LPS-induced decrease ( $P < 0.05$ ) in occludin, claudin-1 and ZO-1 protein expressions.

### Effects of dietary whey protein concentrate on pro-inflammatory cytokine mRNA expressions in the jejunal mucosa of piglets after lipopolysaccharide challenge

Table 4 shows the pro-inflammatory cytokine mRNA expressions in the jejunal mucosa of piglets. Compared with the control group, piglets challenged with LPS had higher ( $P < 0.05$ ) mRNA expressions of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8 levels in the jejunal mucosa. Dietary WPC prevented ( $P < 0.05$ ) the LPS-induced increase in mRNA expressions of IL-6, IL-8 and IL-1 $\beta$  and limited the LPS-induced increase in the mRNA expression of TNF- $\alpha$ .

### Effects of dietary whey protein concentrate on transforming growth factor- $\beta$ 1 expression and its canonical Smad signalling pathway in the jejunal mucosa of piglets after lipopolysaccharide challenge

Fig. 2 and 3 present the protein expressions of TGF- $\beta$ 1 and Smad2 in the jejunal mucosa of piglets. Piglets challenged with

**Table 4.** Effects of dietary whey protein concentrate (WPC) on cytokine mRNA levels on the jejunal mucosa of piglets\* (Mean values with their standard errors;  $n$  6 pigs)

Items	Control	LPS	LPS + WPC	SEM	$P$
TNF- $\alpha$	1.00 <sup>c</sup>	4.15 <sup>a</sup>	2.34 <sup>b</sup>	0.45	0.001
IL-6	1.00 <sup>b</sup>	3.26 <sup>a</sup>	1.59 <sup>b</sup>	0.36	0.001
IL-8	1.00 <sup>b</sup>	3.82 <sup>a</sup>	1.31 <sup>b</sup>	0.41	<0.001
IL-1 $\beta$	1.00 <sup>b</sup>	2.64 <sup>a</sup>	1.27 <sup>b</sup>	0.32	0.006

LPS, lipopolysaccharide.

a, b, c Mean values within a row with unlike superscript letters were significantly different ( $P < 0.05$ ).

\* Control (non-challenged control), piglets receiving a control diet and injected with 0.9 % sterile saline; LPS (LPS-challenged control), piglets receiving the same control diet and injected with *Escherichia coli* LPS; LPS + WPC (LPS challenged + 5 %WPC), piglets receiving a 5 %WPC diet and injected with LPS. The  $2^{-\Delta\Delta C_T}$  method was used to analyse the relative expression (fold changes), calculated relative to the values in samples from the control pigs.

LPS did not differ ( $P > 0.05$ ) from the control group regarding TGF- $\beta$ 1 protein expression and Smad2 activation. Dietary supplementation with WPC increased ( $P < 0.05$ ) the expression of TGF- $\beta$ 1 and phosphorylated-Smad2 compared with the LPS group.

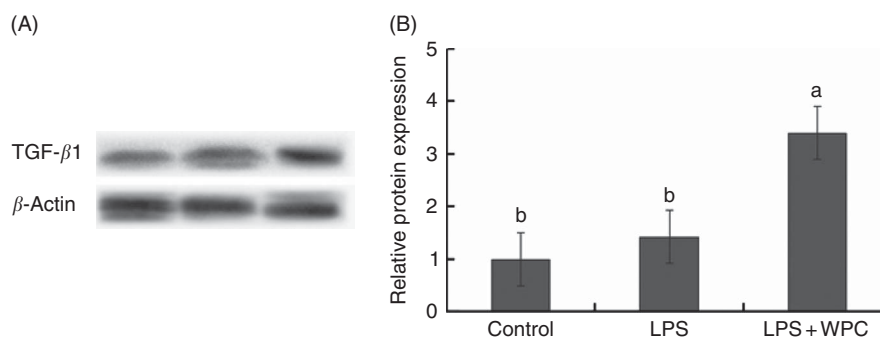
Table 5 shows the mRNA expressions of canonical Smad signals in the jejunal mucosa of piglets. Compared with the control group, LPS challenge did not ( $P > 0.05$ ) activated Smad signals as indicated by no significant increase in the mRNA expressions of TGF $\beta$  type I receptors (T $\beta$ RI), TGF $\beta$  type II receptors (T $\beta$ RII), Smad2, Smad3, Smad4 and Smad7. Supplementation with WPC significantly improved ( $P < 0.05$ ) the Smad4 and Smad7 mRNA expressions, whereas it did not change the mRNA expressions of T $\beta$ RI, T $\beta$ RII, Smad2 and Smad3 in the jejunal mucosa of piglets when compared with the LPS group.

### Effects of dietary whey protein concentrate on mitogen-activated protein kinase signalling pathways of piglets in the jejunal mucosa after lipopolysaccharide challenge

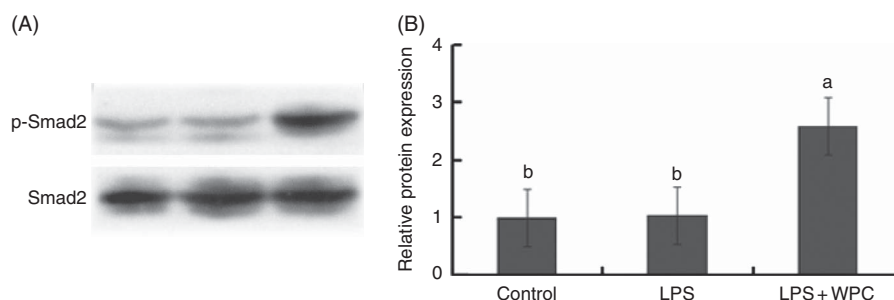
There was no difference in the total protein levels of the MAPK between groups (data not shown). Fig. 4 shows the activation of the MAPK signalling pathways in jejunal mucosa of piglets. Compared with the control group, LPS challenge significantly increased ( $P < 0.05$ ) the ratios of the phosphorylated forms to the total levels of JNK and p38 (p-JNK:JNK and p-p38:p38). Supplementation with WPC significantly decreased ( $P < 0.05$ ) the ratios of the phosphorylated forms to the total levels of JNK and p38 (p-JNK:JNK and p-p38:p38), whereas WPC significantly increased ( $P < 0.05$ ) the ratio of the phosphorylated forms to the total levels of ERK (p-ERK:ERK) compared with the LPS group.

## Discussion

WPC is a protein-enriched powder derived from whey of cow's milk, which is widely used in the food industry as a functional and nutritional ingredient. It contains not only essential nutrients but also a number of natural bioactive substances such as  $\alpha$ -lactalbumin, LF, lactoperoxidase, lysozyme, EGF and TGF



**Fig. 2.** Effects of dietary whey protein concentrate (WPC) on protein expression of transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) in the jejunal mucosa of piglets. (A) Representative blots of TGF- $\beta$ 1 expression and  $\beta$ -actin. (B) Relative TGF- $\beta$ 1 protein expression in the jejunal mucosa of piglets. a, b Mean values with unlike letters were significantly different ( $P < 0.05$ ). Values are means ( $n$  6), and standard deviations represented by vertical bars. The control sample was used as the reference sample. The protein expression of all samples was expressed as fold changes, calculated relative to the control group. Control (non-challenged control), piglets receiving a control diet and injected with 0.9 % sterile saline; lipopolysaccharide (LPS) (LPS-challenged control), piglets receiving the same control diet and injected with *Escherichia coli* LPS; LPS + WPC (LPS challenged + 5 %WPC), piglets receiving a 5 %WPC diet and injected with LPS.



**Fig. 3.** Effects of dietary whey protein concentrate (WPC) on Smad2 activation in the jejunal mucosa of piglets. (A) Representative blots from one of the six pigs. (B) Values calculated as the ratio of the phosphorylation level (p-Smad2):total level of Smad2. The control sample was used as the reference sample. The protein expression of all samples was expressed as fold changes, calculated relative to the control group. Values are means ( $n$  6), with standard deviations represented by vertical bars. <sup>a,b</sup>Mean values with unlike letters were significantly different ( $P < 0.05$ ). Control (non-challenged control), piglets receiving a control diet and injected with 0.9% sterile saline; LPS (lipopolysaccharide-challenged control), piglets receiving the same control diet and injected with *Escherichia coli* LPS; LPS + WPC (LPS challenged + 5% WPC), piglets receiving a 5% WPC diet and injected with LPS.

**Table 5.** Effects of dietary whey protein concentrate (WPC) on mRNA expressions of smad signals in the jejunal mucosa of piglets\* (Mean values with their standard errors;  $n$  6 pigs)

Items	Control	LPS	LPS + WPC	SEM	<i>P</i>
TβRI	1.00	1.12	1.25	0.17	0.573
TβRII	1.00	1.24	1.31	0.20	0.651
Smad2	1.00	1.24	1.33	0.21	0.573
Smad3	1.00	1.32	1.27	0.15	0.302
Smad4	1.00 <sup>b</sup>	1.41 <sup>b</sup>	2.54 <sup>a</sup>	0.39	0.033
Smad7	1.00 <sup>b</sup>	1.24 <sup>b</sup>	3.43 <sup>a</sup>	0.53	0.009

LPS, lipopolysaccharide; TβRI, transforming growth factor-β receptor I; TβRII, transforming growth factor-β receptor II.

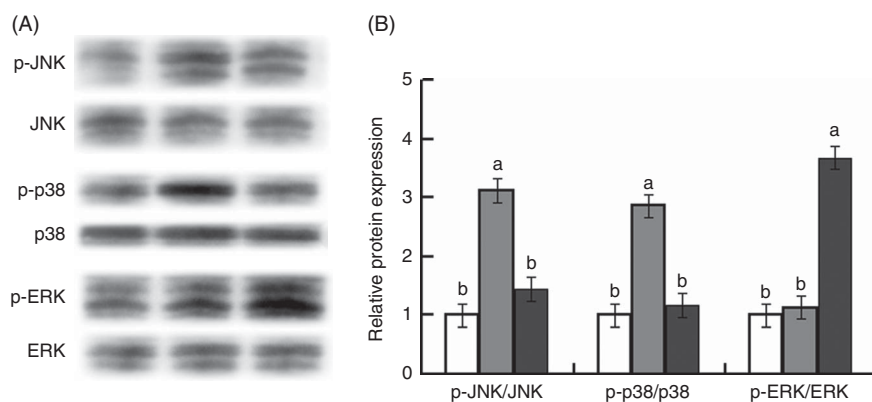
<sup>a,b</sup> Mean values within a row with unlike superscript letters were significantly different ( $P < 0.05$ ).

\* Control (non-challenged control), piglets receiving a control diet and injected with 0.9% sterile saline; lipopolysaccharide (LPS) (LPS-challenged control), piglets receiving the same control diet and injected with *Escherichia coli* LPS; LPS + WPC (LPS challenged + 5% WPC), piglets receiving a 5% WPC diet and injected with LPS. The  $2^{-\Delta\Delta C_t}$  method was used to analyse the relative expression (fold changes), calculated relative to the values in samples from the control pigs.

that have many beneficial effects on animal health<sup>(3,26,27)</sup>. Substantial evidence has shown that WPC exerts beneficial effects on a wide variety of gastrointestinal disorders such as inflammatory bowel disease and necrotising enterocolitis in animal models and clinical trials<sup>(1,2)</sup>. On the basis of this, we investigated the protective effect of supplementation with 5% WPC on intestinal morphology and barrier function after a 4-h *E. coli* LPS challenge using a piglet model. LPS-induced intestinal injury in piglets is one of the well-established animal models for studying infant nutrition and gastrointestinal physiology<sup>(17,28)</sup>. In agreement with earlier reports<sup>(17,28)</sup>, the present study showed that LPS challenge decreased jejunal villus height and villus height:crypt depth ratio at 4 h after LPS challenge, which suggests that LPS caused acute intestinal mucosal damage. However, supplementation with 5% WPC ameliorated LPS-induced intestinal injury by increased jejunal villus height and villus height:crypt depth ratio, which indicated that WPC improved intestinal morphology after damage. Similarly, Li *et al.*<sup>(29)</sup> found that feeding WPC pigs had greater villus heights for preterm pigs. Enteral supplementation with colostrum has also been reported to increase villus growth in newborn pigs<sup>(7,30)</sup>. The gut is a target during endotoxin challenge. LPS could excessively activate

inflammatory response and induce nitric oxide synthase production and lipid peroxidation, which finally led to local intestinal damage such as morphological damage and a breakdown in intestinal barrier function<sup>(17,19,21)</sup>. We conjectured that endotoxin-induced intestinal epithelial injury may be a result of oxygen-related free radical damage and inflammatory response. When exposed to LPS, the villi were frequently noted to have epithelial vacuolisation, lifting, sloughing and focal necrosis, which would lead to villi shortening as a result. Our study also demonstrated that LPS can damage the intestinal morphology and barrier integrity. WPC contains abundant bioactive substances such as anti-inflammatory cytokines and antioxidative factors (e.g. TGF-β, EGF, LF) that can counteract the effects of LPS, thus protecting the intestinal morphology and barrier integrity<sup>(4,31–35)</sup>. In the present experiment, consistent with improved intestinal morphology, WPC improved intestinal barrier function, indicated by increased TER and decreased FD4 flux in the jejunum of piglets compared with the LPS group. Our data were supported by the results of Hering *et al.*<sup>(4)</sup>, who reported that WPC protects against interferon-γ-induced barrier impairment by increased TER and decreased fluorescein permeability in HT-29/B6 cells. Moreover, WPC also reduced intestinal permeability in preterm pigs<sup>(29)</sup>. To our knowledge, the present study is the first to determine the effects of dietary supplementation with WPC on the attenuation of LPS-induced intestinal injury in weaned piglets.

The tight-junction (TJ) is a major cellular component for maintenance of tissue integrity and barrier function. It has a complex molecular composition that forms the continuous intercellular barrier against external agents in the intestine. The integral membrane components of TJ include claudins, occludins and ZO<sup>(24,36)</sup>. In our present study, consistent with the improved intestinal morphology and barrier function, WPC prevented the LPS-induced decrease in claudin-1, occludin and ZO-1 expressions. Our observations were supported by Hering *et al.*<sup>(4)</sup> and Visser *et al.*<sup>(37)</sup>, who reported that milk protein components improved intestinal barrier function, in part, by altering the expressions and functions of claudins. In our present study, WPC may have partially improved the intestinal barrier function via improving the expressions of intestinal TJ proteins.



**Fig. 4.** Effects of whey protein concentrate (WPC) on mitogen-activated protein kinases (MAPK) signal pathways in the jejunal mucosa of piglets. The three MAPK are the c-Jun NH2-terminal kinase (JNK), p38 MAPK and extracellular regulated kinases (ERK 1/2). (A) Representative blots from one of the six pigs. (B) Values calculated as the ratios of their phosphorylation levels (phospho-JNK:phospho-p38:phospho-ERK (p-JNK:p-p38:p-ERK)) to the total levels of MAPK. The values in samples from the control were used as the reference sample. The protein expression of all samples was expressed as fold changes, calculated relative to the values from the control group. Values are means ( $n$  6), with standard deviations represented by vertical bars. <sup>a,b</sup>Mean values with unlike letters were significantly different ( $P < 0.05$ ). □, Control; ■, lipopolysaccharide (LPS); ■, LPS + WPC. Control (non-challenged control), piglets receiving a control diet and injected with 0.9% sterile saline; LPS (LPS-challenged control), piglets receiving the same control diet and injected with *Escherichia coli* LPS; LPS + WPC (LPS challenged + 5 % WPC), piglets receiving a 5 % WPC diet and injected with LPS. JNK, c-jun N-terminal kinase; p-JNK, phospho-JNK; ERK, extracellular regulated kinases; p-ERK, phospho-ERK.

Cytokines also participate in the regulation of the intestinal barrier integrity<sup>(38)</sup>. Over-production of pro-inflammatory cytokines has a negative influence on gut integrity and epithelial function<sup>(39)</sup>. The TJ barrier disruptive actions of TNF- $\alpha$  have been well established<sup>(40)</sup>. It has been reported that interferon- $\gamma$ -induced reductions in epithelial barrier function are linked to decreases in the expressions of TJ proteins such as occludin and ZO-1<sup>(41)</sup>. In the present study, the expressions of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8 were elevated in the jejunal mucosa of piglets subjected to LPS challenge. Consistent with the improved intestinal integrity by WPC supplementation, WPC decreased the TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8 gene expressions compared with the LPS-challenged group. In line with our findings, Sprong *et al.*<sup>(1)</sup> reported that cheese whey protein protected rats against mild dextran sulphate Na-induced colitis by inhibiting the expressions of inflammatory cytokines. Whey product has demonstrated a number of anti-inflammatory effects including decreased cytokine release in rodent models after exposure to LPS<sup>(42)</sup>. It is possible that the protective effects of WPC supplementation on intestinal integrity were closely related to reducing the expressions of intestinal pro-inflammatory cytokines. According to the above results, we speculate that WPC may exert beneficial effects in intestinal damage by enhancing intestinal integrity and barrier function.

WPC contains abundant TGF- $\beta$ 1<sup>(5)</sup>, but whether TGF- $\beta$ 1 plays a role in these protection processes is not clear. TGF- $\beta$ 1 is of particular interest as it has known effects in a remarkable number of biological processes including epithelial cell growth and differentiation<sup>(11)</sup>, restitution of intestinal epithelium after injury<sup>(12,43)</sup> and immune regulation<sup>(5)</sup>. TGF- $\beta$  has been reported to play an important role in the post-weaning adaptation process in the intestine of pigs<sup>(9,12)</sup>. In our present experiment, dietary supplementation with 5 %WPC increased the TGF- $\beta$ 1 protein expression in the jejunum mucosa of piglets compared with the LPS-challenged pigs. Similarly, Andújar *et al.*<sup>(44)</sup> found that TGF- $\beta$ 1 positively regulates gastrointestinal ulcer healing.

TGF- $\beta$  was also reported to provide protection against dextran sodium sulphate-induced colitis and LPS-induced endotoxaemia/shock in rodents<sup>(45,46)</sup>, and it is likely that the increased TGF- $\beta$ 1 concentration by supplementation with WPC is directly responsible for the protection. Therefore, our hypothesis is that the beneficial role exerted by WPC in intestinal barrier protection may be partially influenced by TGF- $\beta$ 1 in the intestinal mucosa. We speculated that the increased expression of TGF- $\beta$ 1 may due to the TGF- $\beta$ 1 in WPC, as TGF- $\beta$ 1 in the diet can be absorbed by the intestine *in vivo* and TGF- $\beta$  retains biological activity when given as a supplement in infant formula<sup>(31,47,48)</sup>. It is possible that the higher presence of TGF- $\beta$ 1 is from dietary WPC by intestinal absorption.

To determine whether the TGF- $\beta$ 1 signalling pathway is involved in WPC exerting beneficial effects on LPS-induced intestinal injury, we next evaluated the canonical downstream substrate of TGF- $\beta$  signal. The canonical TGF- $\beta$  signalling pathway is mediated by Smad family proteins<sup>(13)</sup>. When TGF- $\beta$  ligands reach the membrane of target cells, they bind directly to T $\beta$ RII, which leads to the recruitment of T $\beta$ RI, and T $\beta$ RII then trans-phosphorylates T $\beta$ RI, enabling the T $\beta$ RI kinase domain to act on cytoplasmic Smad proteins, and thereby propel downstream signalling actions. Smad 2 and 3 are receptor-regulated Smad. Following stimulation by TGF- $\beta$ , Smad2 and Smad3 become phosphorylated. Phosphorylated Smad2 and Smad3 can complex with Smad4 (the common mediator Smad) and then translocate to the nucleus and regulate gene expression<sup>(13)</sup>. In the present study, we observed that supplementation with WPC promoted phosphorylated-Smad2 expression and mRNA expressions of Smad4 and Smad7 in the jejunal mucosa compared with the LPS-challenged pigs fed the control diet, which indicates that canonical Smad signalling pathways were activated. In line with our study, a recent study has revealed that oral administration of TGF- $\beta$ 1 protects the immature gut from injury via Smad protein-dependent signalling pathways<sup>(49)</sup>. Ozawa *et al.*<sup>(31)</sup> have also found that TGF- $\beta$  in cows' milk provided protection against experimental

colitis and endotoxaemia by inducing Smad2 phosphorylation and the transcription of the TGF- $\beta$ /Smad target genes TGF- $\beta$  itself and Smad7 *in vitro*. It is also reported that TGF- $\beta$ 1 enhancement of epithelial barrier function was mediated partly by Smad2/3 signalling pathways<sup>(50)</sup>. Consequently, in the present study, we speculated that the WPC may have protected the intestinal epithelial barrier from LPS-induced intestinal injury partially through TGF- $\beta$ 1 canonical Smad pathways directly or indirectly.

Apart from canonical Smad pathways, MAPK has been reported to be involved in TGF- $\beta$  actions<sup>(14)</sup>. The three primary MAPK signalling pathways are the ERK 1/2, p38 and JNK. It has been demonstrated that MAPK become activated when stimulated by LPS<sup>(51)</sup>. Moreover, a recent study showed that weaning stress activates p38, JNK and ERK 1/2 signalling pathways in the intestine, which may be an important mechanism of weaning-associated enteric disorders of piglets<sup>(24)</sup>. Thus far, there are a few reports investigating the effects of WPC on MAPK signalling pathways<sup>(52)</sup>. In the present study, we observed an increase in the phospho-p38 and p-JNK in the jejunum of LPS-challenged pigs and we demonstrated, for the first time, that dietary WPC decreased the relative protein levels of phosphorylated p38 and JNK, while increasing p-ERK 1/2 protein levels, indicating that WPC inhibited the JNK and p38 signalling pathways while activating ERK 1/2 signalling in LPS-challenged pigs. In general, ERK delivers a survival signal, whereas JNK and p38 are associated with the induction of cell apoptosis under stressful conditions<sup>(53,54)</sup>. Cell apoptosis can disrupt intestinal mucosal integrity<sup>(55)</sup>. Activation of ERK pathways and inhibition of p38 and JNK pathways also improved intestinal barrier function in weaned pigs<sup>(56)</sup>. The ERK 1/2 cascade can be activated by growth factors and preferentially regulates cell growth and differentiation. There is evidence that activation of the ERK 1/2 signalling is linked to the TGF- $\beta$ 1-induced modulation of TJ permeability and wound closure<sup>(56,57)</sup>. TGF- $\beta$  has been shown to attenuate IL-1 $\beta$ -induced pro-inflammatory cytokine production in immature human intestinal epithelia cells by inhibiting ERK signalling<sup>(58)</sup>. In our study, it is possible that the protective effects of WPC on intestinal integrity after exposure to LPS are also related to activation of ERK 1/2 and inhibition of JNK and p38 MAPK signalling pathways.

Other bioactive substances than TGF- $\beta$ 1 in WPC may be implied in the effects of WPC. EGF is another important growth factor in WPC that has protective barrier effects on intestinal epithelia and has often been related to effects on cell proliferation and/or epithelial restitution, which could indirectly or secondarily affect the TJ<sup>(59)</sup>. Accumulating evidence has shown that EGF/EGF receptor signal improves healing of the gastrointestinal tract and enhances gut integrity and intestinal barrier function<sup>(32,60,61)</sup>. In addition, LF is a multifunctional glycoprotein present at high concentrations in milk that exerts antibacterial, immune-modulating and anti-inflammatory effects on intestinal health<sup>(33)</sup>. Studies have found that LF could directly induce enterocyte growth and proliferation and improve gut barrier function<sup>(34,62)</sup>. However, it is still unclear whether these constituents of WPC are the crucial biological factors that provide beneficial effects.

In summary, the present study demonstrated that dietary supplementation with WPC attenuates LPS-induced intestinal

injury via improving mucosal barrier function, alleviating intestinal inflammation and influencing TGF- $\beta$ 1, Smad and MAPK signalling pathways.

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The authors declare that they have no conflicts of interest.

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