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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-1 (TGF- 1) is an important component in the WPC, but whether TGF-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-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-, IL-6, IL-8 and IL-1 . Supplementation with WPC also increased ($P < 0·05$) TGF- 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- 1 canonical Smad and mitogen-activated protein kinase signalling pathways.

Whey protein concentrate: Intestinal integrity: Transforming growth factor- 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[1,2]. 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[2,3]. Recently, it has also been reported that WPC improves intestinal epithelial barrier function in vitro[4]. 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[5,6]. Among the most relevant substances in WPC are Ig, LF and growth factors (e.g. transforming growth factor (TGF-) 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[4].

Mammalian milk and WPC are rich in TGF- including TGF- 1[4,7]. TGF- 1 is also the most abundant isoform in the mucosa of the gut[8] and may play an important role in postnatal adaptation of the gastrointestinal tract in suckling animals[9].

TGF-β1 is of particular interest as it has known effects in remarkable number of biological processes, including epithelial cell growth and differentiation[10,11], restitution of intestinal epithelium after injury[9,12] and immune regulation[5]. Thus far, different signalling pathways have been reported to be involved in TGF-β action, including Smad-dependent and Smad-independent pathways[13]. The canonical TGF-β signalling pathway is mediated by Smad family proteins[13]. 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-β1 actions[14]. We hypothesised that TGF-β1 in WPC might be involved in those barrier-protection processes and would lead to changes of these TGF-β 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.

This experiment was approved by the Animal Care and Use Committee, Zhejiang University. A total of eighteen 35-d-old weaned barrows (Duroc × Landrace × 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 × 1·1 m^2 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[15]. After 19 d feeding with control or 5 % WPC diets, the

fe1g9 n an3(n)161(e)13.1(r)0(e.4(3(n)4.6(c)15.4(lu)t510.2(ts6.16r4)4.6(c)15.4(lu)barrie9o)14.6(n)]5.4(lu)j)1(d)-4(1(e)1r)-1rtedt1g9ed w-248.

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_2–5 % CO_2) 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 (Ω cm^2) 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 anal concentration of 0375 mg/ml. Mucosal-to-serosal ux of FD4 (μg/cm^2 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 uorescence microplate reader (FLx800; Bio-Tek Instruments Inc.). The ux over the 2-h period was calculated.

The TGF- 1 content in WPC was determined by ELISA according to the manufacturer's protocol (R&D Systems)[37]. TGF- 1 content was also assayed in the control group.

The Western blot analysis was performed according to the procedures outlined by Hu et al.[24]. In brief, after electrophoresis, the proteins were transferred to polyvinylidene diuoride 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-1, Smad2, phospho-Smad2, p38, phospho-p38, JNK, phospho-JNK (p-JNK), ERK, phospho-ERK 1/2 (p-ERK), -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. -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:-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.

The mRNA levels of TNF-, IL-1 , IL-6, IL-8 and TGF- receptors, as well as their downstream signal Smads (2,3,4,7), were determined by real-time PCR, as described by Liu et al.[17]. 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-specic ampli cation was determined by melting curve analysis and agarose gel electrophoresis. The $2^{-\Delta\Delta C_t}$ method

Table 2. [illegible]

[illegible]	[illegible]	[illegible]	( )
α	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
β	[illegible]	[illegible]	[illegible]
β I	[illegible]	[illegible]	[illegible]
β II	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]

β I [illegible] β [illegible] I β II [illegible] β [illegible] II *GAPDH* [illegible]

was used to analyse the relative changes in each target gene expression. The change (Δ) in C_t values in each group was compared with the C_t value of glyceraldehyde 3-phosphate dehydrogenase (GAPHH) (ΔC_t). Subsequently, $\Delta\Delta C_t$ was computed for each target gene from the treatment groups by subtracting the averaged ΔC_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.

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 $P < 0·05$.

WPC contains abundant TGF-β1 as much as 0·06 ng/mg. The content of TGF-β1 in the WPC diet was 3 μg/kg. We checked for the presence of TGF-β1 in the control diet but we obtained negative results. The reason may be that the TGF-β1 content in the control diet was too low to detect.

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.

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.

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. [illegible] (n [illegible])

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	P
V[illegible] (μm)	[illegible]	[illegible]	[illegible]	[illegible]	< [illegible]
[illegible] (μm)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
V[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible] (Ω [illegible])	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible] (μ[illegible])	[illegible]	[illegible]	[illegible]	[illegible]	< [illegible]

[illegible] ($P <$ [illegible]) [illegible] % [illegible] *Escherichia coli* [illegible] % [illegible] % [illegible]

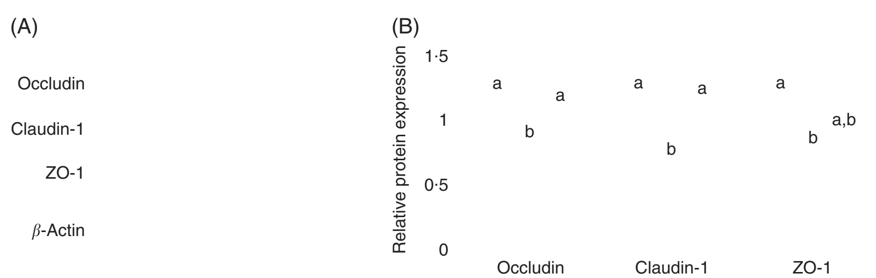

Fig. 1. [illegible] (A) [illegible] β [illegible] (B) [illegible] (n [illegible]) [illegible] ($P <$ [illegible]) [illegible] □ [illegible] ■ [illegible] ( ) ■ [illegible] % [illegible] *Escherichia coli* [illegible] % [illegible] % [illegible]

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.

Table 4 shows the pro-inammatory 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-, IL-1 , 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 and limited the LPS-induced increase in the mRNA expression of TNF- .

Fig. 2 and 3 present the protein expressions of TGF1- and Smad2 in the jejunal mucosa of piglets. Piglets challenged with LPS did not differ ($P > 0·05$) from the control group regarding TGF- 1 protein expression and Smad2 activation. Dietary supplementation with WPC increased ($P < 0·05$) the expression of TGF- 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 signicant increase in the mRNA expressions of TGF type I receptors (T RI), TGF type II receptors (T RII), Smad2, Smad3, Smad4 and Smad7. Supplementation with WPC signicantly improved ($P < 0·05$) the Smad4 and Smad7 mRNA expressions, whereas it did not change the mRNA expressions of RI, T RII, Smad2 and Smad3 in the jejunal mucosa of piglets when compared with the LPS group.

that have many beneficial effects on animal health[3,26,27]. 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[1,2]. 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[17,28]. In agreement with earlier reports[17,28], 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, Liu *et al.*[29] 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[7,30]. 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[17,19,21]. 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-, EGF, LF) that can counteract the effects of LPS, thus protecting the intestinal morphology and barrier integrity

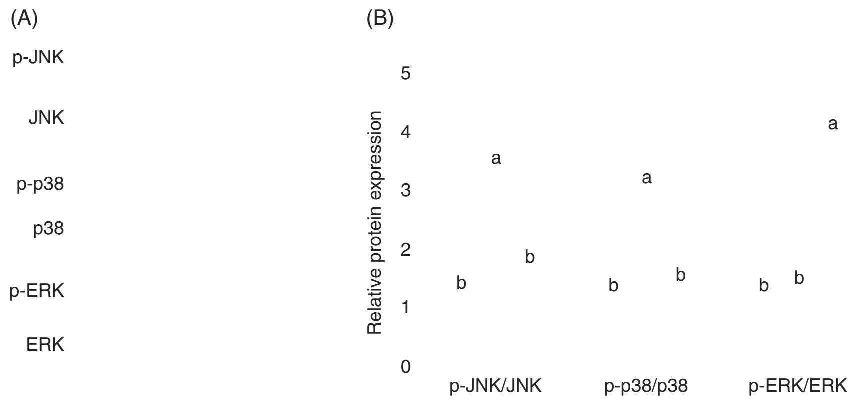

Fig. 4. ( ) ( ) ( ) ( ) ( ) (( )) (n) (P<). % ( ). *Escherichia coli* (%). %

Cytokines also participate in the regulation of the intestinal barrier integrity[38]. Over-production of pro-in ammatory cytokines has a negative in uence on gut integrity and epithelial function[39]. The TJ barrier disruptive actions of TNF- have been well established[40]. It has been reported that interferon-γ-induced reductions in epithelial barrier function are linked to decreases in the expressions of TJ proteins such as occludin and ZO-1[41]. In the present study, the expressions of pro-in ammatory cytokines such as TNF-, IL-1 , 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-, IL-1 , IL-6 and IL-8 gene expressions compared with the LPS-challenged group. In line with our ndings, Sprong et al.[1] reported that cheese whey protein protected rats against mild dextran sulphate Na-induced colitis by inhibiting the expressions of in ammatory cytokines. Whey product has demonstrated a number of anti-in ammatory effects including decreased cytokine release in rodent models after exposure to LPS[42]. It is possible that the protective effects of WPC supplementation on intestinal integrity were closely related to reducing the expressions of intestinal pro-in ammatory cytokines. According to the above results, we speculate that WPC may exert bene cial effects in intestinal damage by enhancing intestinal integrity and barrier function.

WPC contains abundant TGF- 1[5], but whether TGF- 1 plays a role in these protection processes is not clear. TGF- is of particular interest as it has known effects in a remarkable number of biological processes including epithelial cell growth and differentiation[11], restitution of intestinal epithelium after injury[12,43] and immune regulation[5]. TGF- has been reported to play an important role in the post-weaning adaptation process in the intestine of pigs[9,12]. In our present experiment, dietary supplementation with 5 % WPC increased the TGF- 1 protein expression in the jejunum mucosa of piglets compared with the LPS-challenged pigs. Similarly, Andújar et al.[44] found that TGF- 1 positively regulates gastrointestinal ulcer healing. TGF- was also reported to provide protection against dextran sodium sulphate-induced colitis and LPS-induced endotoxaemia/shock in rodents[45,46], and it is likely that the increased TGF- 1 concentration by supplementation with WPC is directly responsible for the protection. Therefore, our hypothesis is that the bene cial role exerted by WPC in intestinal barrier protection may be partially in uenced by TGF- 1 in the intestinal mucosa. We speculated that the increased expression of TGF- 1 may due to the TGF- 1 in WPC, as TGF- 1 in the diet can be absorbed by the intestine in vivo and TGF- retains biological activity when given as a supplement in infant formula[31,47,48]. It is possible that the higher presence of TGF- 1 is from dietary WPC by intestinal absorption.

To determine whether the TGF- 1 signalling pathway is involved in WPC exerting bene cial effects on LPS-induced intestinal injury, we next evaluated the canonical downstream substrate of TGF- signal. The canonical TGF- signalling pathway is mediated by Smad family proteins[13]. When TGF- ligands reach the membrane of target cells, they bind directly to T RII, which leads to the recruitment of T RI, and T RII then trans-phosphorylates T RI, enabling the T 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-, 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[13]. 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- 1 protects the immature gut from injury via Smad protein-dependent signalling pathways[49]. Ozawa et al.[31] have also found that TGF- in cows' milk provided protection against experimental

colitis and endotoxaemia by inducing Smad2 phosphorylation and the transcription of the TGF-/Smad target genes TGF- itself and Smad7 in vitro. It is also reported that TGF-1 enhancement of epithelial barrier function was mediated partly by Smad2/3 signalling pathways[50]. 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- 1 canonical Smad pathways directly or indirectly.

Apart from canonical Smad pathways, MAPK has been reported to be involved in TGF- actions[14]. 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[51]. 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[24]. Thus far, there are a few reports investigating the effects of WPC on MAPK signalling pathways[52]. 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 rst 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[53,54]. Cell apoptosis can disrupt intestinal mucosal integrity[55]. Activation of ERK pathways and inhibition of p38 and JNK pathways also improved intestinal barrier function in weaned pigs[56]. 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-1-induced modulation of TJ permeability and wound closure[56,57]. TGF- has been shown to attenuate IL-1-induced pro-in ammatory cytokine production in immature human intestinal epithelia cells by inhibiting ERK signalling[58]. 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-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[59]. Accumulating evidence has shown that EGF/EGF receptor signal improves healing of the gastrointestinal tract and enhances gut integrity and intestinal barrier function[32,60,61]. In addition, LF is a multifunctional glycoprotein present at high concentrations in milk that exerts antibacterial, immune-modulating and anti-in ammatory effects on intestinal health[33]. Studies have found that LF could directly induce enterocyte growth and proliferation and improve gut barrier function[34,62]. However, it is still unclear whether these constituents of WPC are the crucial biological factors that provide bene cial effects.

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

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