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Dietary high protein-induced diarrhea and intestinal inflammation by activation of NF-κB signaling in piglets

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ABSTRACT

The present study aimed to investigate whether inflammation-associated responses in piglets are induced by high protein (HP) through activating nuclear factor kappa B (NF-κB) signaling. Sixteen piglets (35 d of age, Duroc × [Landrace × Yorkshire], weaned at d 21, initial BW = 9.70 ± 0.11 kg) were allocated to 18% and 26% CP (HP group) at random, comprising 8 replicate pens per treatment. The piglets were slaughtered to collect intestinal tissues when apparent, persistent, and stable diarrhea syndromes happened (on d 12). No significant differences were observed in their growth performance ($P > 0.05$), but reduction by 19.11%, 25.31%, 23.64% of ADFI, ADG, and G:F, respectively was detected in the HP group. The HP group had greater ($P = 0.002$) diarrhea rates. Furthermore, dietary HP had lower ileal villus height (VH; $P = 0.048$), ratio of villus height to crypt depth (VH/CD ratio; $P = 0.016$), and colonic CD ($P = 0.034$), as well as had the trend ($P = 0.075$) to reduce the ileal villus absorptive area. Moreover, HP diets significantly elevated the goblet cell numbers in the ileal villi ($P = 0.016$) and colonic crypts ($P < 0.001$) and up-regulated ($P = 0.012$) the mRNA expression of mucin2 (*Muc2*) in the ileum. In addition, HP diets increased the myeloperoxidase concentration in the ileum ($P = 0.002$) and colon ($P = 0.007$) of piglets. Dietary HP significantly down-regulated the mRNA expression of tumor necrosis factor-α (*TNF-α*; $P < 0.001$) in the ileum, induced nitric oxide synthase (*iNOS*; $P = 0.040$) and interleukin-22 (*IL-22*; $P = 0.008$) in the colon, and inclined to down-regulate interleukin-1β (*IL-1β*; $P = 0.076$) expression in the colon. The relative protein abundance of Galectin-3 ($P = 0.046$) in the colon and the ratio of phosphorylation NF-κB to NF-κB (p−NF−κB/NF-κB ratio) in the ileum of HP piglets were also greater ($P = 0.038$). These results suggest that dietary HP may cause diarrhea in piglets by activating NF-κB signaling induced intestinal inflammation.

1. Introduction

Piglets are faced with great growth challenges after weaning owing to nutritional, environmental, and physiological changes, and are susceptible to post-weaning diarrhea (Pluske et al., 1997). Among them, nutritional factors (protein, fiber, starch, and electrolyte levels) play a major role in piglets' post-weaning health (Gao et al., 2019). Protein is an indispensable nutrient for piglets. The National Research Council (NRC, 1998) recommended that the

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requirement of CP was 20% for weaned piglets, while according to the latest edition of NRC (1998), it is 2% to 4% lower than that stated in the former one. Previous studies suggested that dietary CP between 19% and 23% could satisfy the growth demands of weaned piglets (Htoo et al., 2007; Opapeju et al., 2009). As reported by Wu et al. (2015), pigs fed high protein (HP) diets showed better growth performance. However, other groups demonstrated that a high level of dietary protein may yield potentially toxic metabolites in the gut, which is closely associated with post-weaning nutrition diarrhea (PWND) (Pieper et al., 2014; Richter et al., 2014; Bikker et al., 2006; Kluess et al., 2010).

Lan et al. (2015) indicated that HP diets led to lower indices relevant to mucosal immune responses, predominantly in the ileum and colon of rats. Maladjustment of gut immunity may be involved in the pathogenic mechanism of inflammatory bowel disease (IBD) (Nenci et al., 2007). Wu et al. (2015) found that intestinal inflammatory responses contributed to diarrhea in weaned piglets. Yi et al. (2015) found that cathelicidin-BF could suppress intestinal inflammation by down-regulating the nuclear factor kappa B (NF-κB) signaling pathway to reduce diarrhea in weanling piglets. NF-κB is crucial for most immune and inflammatory responses. Yet, NF-κB hyperactivation contributes to inflammatory diseases, which is activated by IκB kinase (IKK) complex and IKK-mediated IκBα phosphorylation, thus resulting in IκBα degradation and translocation of NF-κB dimer to the nucleus (Sun, 2017). NF-κB is a key regulator of the pro-inflammatory process as well as sustaining homeostasis in intestinal epithelial cells and a decisive factor in preventing intestinal inflammation (Li and Verma, 2002; Karin and Greten, 2005).

Conventionally, piglets are given antibiotics to decrease PWND. However, the misuse of antibiotics leads to the development of antibiotic-resistant bacterial strains (Xiao et al., 2015). Accordingly, measures taken to control PWND are based on the etiology of diarrhea other than dietary antibiotics. Although substantial studies have been released on the influences of various protein sources and related contents on piglets, the etiology of diarrhea associated with higher protein in piglets is not fully understood. Hence, we hypothesized that HP induced diarrhea and inflammation-associated responses, which may be via activating NF-κB signaling in piglets. To this aim, effects of HP on morphological changes to gut architecture and intestinal inflammation-associated responses were dissected to provide a theoretical basis and scientific evidence to decrease PWND and regulate intestinal health.

2. Materials and methods

2.1. Animal ethics

All experiments in the present study were conducted according to the guidelines of animal welfare in China, and the experimental design and procedures were reviewed and approved (Approval number 2016-093) by the Animal Care and Use Committee of Hunan Normal University, Changsha, Hunan province, China (Yin et al., 2020).

2.2. Animals and diet

Sixteen crossbred piglets, 35 d of age (Duroc × [Landrace × Yorkshire], weaned at d 21), were grouped by initial BW of 9.70 ± 0.11 kg, assigned to 18% and 26% CP (HP) at random, and each group had 8 piglets. They were provided corn and soybean diets containing similar nutritional levels except for CP contents. Diet formulations (Table 1) satisfied piglets' nutrient demands (NRC, 2012), and lack of antibiotics or growth promoters. All pigs were housed individually, and had free access to clean water and assigned diets, and were fed four times per day at 08:00, 12:00, 16:00, and 20:00. The room temperature was maintained at around 28 °C by lamps.

Table 1
The composition and nutritional component of basal diet (%, as-fed basis).

Items	Dietary crude protein, %	
	18	26
Ingredient		
Corn, (8.5% CP)	69.07	54.29
Soybean meal, (46% CP)	13.00	15.00
Concentrated soy protein	0.00	9.00
Corn gluten meal, (50% CP)	6.00	8.00
Fish meal, (68% CP)	3.00	6.73
Whey powder	4.96	4.00
Lys, (98% CP)	0.72	0.00
DL–Met	0.09	0.00
L-Thr	0.17	0.00
L-Trp	0.07	0.00
Soybean oil	0.00	0.58
Limestone	0.70	0.50
Dicalcium phosphate	1.10	0.80
Choline chloride, 50%	0.10	0.10
NaCl	0.32	0.30
Vitamin and Mineral Premix[1]	0.70	0.70
Total	100	100
Calculated composition		
Crude protein (analyzed)	17.91	25.98
NE, MJ/kg	10.17	10.17
Ca	0.62	0.65
AP	0.37	0.38
Lys	1.34	1.34
Met	0.41	0.49
Met + Cys	0.69	0.88
Thr	0.79	0.98
Trp	0.22	0.26

[1] Vitamin and Mineral Premix supplied per kilogram of feed: 2,200 IU of Vitamin A, 220 IU of Vitamin D_3, 16 IU of Vitamin E, 0.5 mg of Vitamin K_3, 0.0175 mg of Vitamin B 12, 3.5 mg of riboflavin, 30 mg of niacin, 10 mg of D-pantothenic acid, 0.05 mg of biotin, 0.3 mg of folic acid, 1.0 mg of thiamine, 7 mg pyridoxine, and 4.0 mg ethoxyquin; 150 mg of Fe ($FeSO_4$), 100 mg of Zn ($ZnSO_4$), 30 mg of Mn ($MnSO_4$), 25 mg of Cu ($CuSO_4$), 0.5 mg of I (KIO_3), 0.3 mg of Co ($CoSO_4$), 0.3 mg of Se ($Na2SeO_3$), and 4.0 mg of ethoxyquin.

2.3. Sample collection and measurements

2.3.1. Sample collection

Piglets were slaughtered with 4% sodium pentobarbital solution (40 mg/kg BW) when obvious, persistent, and steady diarrhea symptoms occurred on d 12 (Ren et al., 2014). Briefly, the entire intestine was removed and divided into 2 segments: about a 10 cm section proximal to the ileocecal junction was regarded as the ileum; at 5 cm from the caecum, an approximately 10 cm segment was taken to represent the proximal colon. The middle portions of the ileum and colon (about 4 cm) segments were rinsed with 0.9% saline (pH = 7.0), and then an approximately 2 cm intestinal segment was fixed in 4% neutral-buffered formalin and stored at room temperature before morphology measurements, and the other intestinal tissues were quickly frozen in liquid nitrogen and transferred to -80 °C until mRNA expression and protein abundance analysis (He et al., 2013; Yan et al., 2018).

2.3.2. Growth performance and diarrhea rate

Piglets were weighed at the start (d 0) and end of the experiment (d 12), and the number of pigs who had diarrhea was monitored each day. Average daily gain (ADG), ADFI, and G:F were measured according to Yang et al. (2013). The incidence of diarrhea

in piglets was daily recorded thrice according to the method of Liu et al. (2008).

2.3.3. Intestinal morphology

Paraffin sections were prepared according to Wang et al. (2020b). Measurements were blindly executed for villus height (VH), crypt depth (CD), the ratio of VH to CD (VH/CD ratio), and villus width (VW) (Li et al., 2019). The VH/CD ratio and villus surface area were calculated (De Vos et al., 2014; Zong et al., 2019). Each value is the average of at least 30 well-oriented, complete villus-crypt structures.

2.3.4. Alcian blue-periodic acid-Schiff (AB-PAS) staining

Goblet cells were visualized by staining with AB-PAS (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the protocol, using the methods described by Deng et al. (2020a). The number of goblet cells were counted in at least 30 villi and crypts.

2.3.5. Enzyme-linked immuno-sorbent assay

Myeloperoxidase (MPO) concentrations in intestinal samples were determined using the enzyme-linked immuno-sorbent assay (ELISA) kit (Sangon Biotech Co., Ltd., China) according to the manufacturer's protocol, having a detection range of 5 to 180 ng/L. Intra and inter-assay precision coefficient of variation (CV) was about 9% and 15%. The ELISA kit employs a quantitative sandwich enzyme immunoassay technique.

2.3.6. RNA extraction and real-time quantitative PCR

Ileal and colonic tissues were ground into a fine powder over liquid nitrogen (Zhou et al., 2012). Total RNA extraction, cDNA synthesis and real-time quantitative PCR (RT-qPCR) were conducted according to Wang et al. (2020a). Primers were designed for the selected genes (Table 2) using Primer 5.0 (Premier Biosoft International, Palo Alto, CA, USA). The housekeeping gene (*β-actin*) was used to normalize the expression of the target gene, and calculated using the $2^{-\triangle\triangle CT}$ method (Xiong et al., 2015). All samples were in triplicate on each 384-well plate, and the mean value of each duplicate was calculated for subsequent statistical analyses.

Table 2
The primer sequences of intestinal inflammatory genes for PCR.

Genes[1]	Primers	Sequences (5′–3′)	Products length, bp
IL-1β	Forward	CCTGGACCTTGGTTCTCT	123
	Reverse	GGATTCTTCATCGGCTTCT	
IL-6	Forward	GGCAAAAGGGAAAGAATCCAG	87
	Reverse	CGTTCTGTGACTGCAGCTTATCC	
IL-10	Forward	GGGCTATTTGTCCTGACTGC	105
	Reverse	GGGCTCCCTAGTTTCTCTTCC	
COX-2	Forward	CCAGGTTTAAGATCTGATGTGGGGA	165
	Reverse	TGCCCTTCCATCATTACGAATCCTT	
IL-22	Forward	AGCAAGCGTGAAGGTGCGGTT	169
	Reverse	GCGGACATCTGGGAGCCCTTT	
TNF-α	Forward	ACAGGCCAGCTCCCTCTTAT	102
	Reverse	CCTCGCCCTCCTGAATAAAT	
IFN-γ	Forward	CCATTCAAAGGAGCATGGAT	146
	Reverse	GAGTTCACTGATGGCTTTGC	
TGF-β	Forward	CGAGCCCTGGATACCAACTA	164
	Reverse	AGGCTCCAGATGTAGGGACA	
iNOS	Forward	ACGAGCTTCTACCTCAAGCTATTGA	116
	Reverse	TGTTTCTATCTCCTTTGTTACCGCT	
Muc2	Forward	AGACGGGCGGAGACTTTGAATC	102
	Reverse	CTTGGATGGGAACGCTGGGATA	
β-actin	Forward	AGTTGAAGGTGGTCTCGTGG	216
	Reverse	TGCGGGACATCAAGGAGAAG	

[1] *IL* = interleukin; *COX* = cyclo-oxygenase; *TNF* = tumor necrosis factor; *TGF-β* = transforming growth factor-β; *iNOS* = inducible nitric oxide synthase; *Muc2* = mucin 2.

2.3.7. Western blotting analysis

Western blotting was performed according to Deng et al. (2020b). Primary antibodies used in this study were as follows: β-actin (Santa Cruz Biotechnology Inc., CA, USA; SC-47778) at 1:3,000 dilution; galectin-3 (LGALS3; Sangon Biotech Co., Ltd., Shanghai, China; D220661) at 1:500 dilution; phosphorylation NF-κB (p–NF–κB; Cambridge, MA, USA; ab86299) at 1:1,000 dilution; and NF-κB (Cambridge, MA, USA; ab16502) at 1:1,000 dilution. The secondary antibody (Santa Cruz Biotechnology Inc., CA, USA) was diluted at 1:3,000. Protein abundance was measured using the Image-Pro Plus version 6.0 software (Media Cybernetics, San Diego, CA, USA). The housekeeping protein, β-actin, served to normalize the expression of the target protein.

2.4. Statistical analysis

Data were analyzed using SPSS statistics 20 (SPSS Inc., Chicago, IL, USA). Any value that deviated more than 3 standard deviations from the standardized mean was eliminated. Non-parametric testing was performed if the data were not normally distributed (such as diarrhea rates). Values were expressed as mean ± SEM. Differences were assessed by independent-samples *T*-test, considered significant when $P < 0.05$ and tendencies when $P < 0.10$. All figures in this study were drawn using Graphpad Prism 6.0 (GraphPad Inc., San Diego, CA, USA).

3. Results

3.1. Growth performance, diarrhea rates

It was shown that there was no significant difference in growth performance between the 2 groups, but reduction by 19.11%, 25.31%, 23.64% of ADFI, ADG, G:F, respectively was observed in HP group (Table 3). Dietary HP increased diarrhea rates significantly ($P = 0.002$; Fig. 1).

3.2. Intestinal morphology, goblet cells, and MPO concentration

As shown in Fig. 2, ileal VH ($P = 0.048$), VH/CD ratio ($P = 0.016$) and colonic CD ($P = 0.034$) were significantly lower in the HP group. Furthermore, dietary HP moderately decreased ($P = 0.075$) the surface area of the ileal villus. Besides, the MPO concentrations were significantly increased both in the ileum ($P = 0.002$) and colon ($P = 0.007$; Fig. 3) of piglets fed with HP. Moreover, piglets fed with 26% CP had more goblet cells both in the villus of the ileum

Table 3
Effects of dietary crude protein on growth performance and diarrhea rate in piglets[1].

Item[2]	Dietary crude protein, %		*P*-value
	18	26	
IBW, kg	9.73 ± 0.16	9.69 ± 0.16	0.872
FBW, kg	13.05 ± 0.52	11.63 ± 0.88	0.184
ADFI, g/d	501.36 ± 48.72	405.56 ± 58.51	0.229
ADG, g/d	276.56 ± 44.68	206.55 ± 61.76	0.367
G:F, g/g	0.55 ± 0.07	0.42 ± 0.10	0.327

[1] Values are represented as means ± SEM of 8 piglets per treatment. The data were analyzed by independent-samples T-test. SEM: standard error of the mean.
[2] IBW = Initial body weight; FBW = Final body weight; ADFI = Average daily feed intake; ADG = Average daily gain; G:F = gain-to-feed ratio.

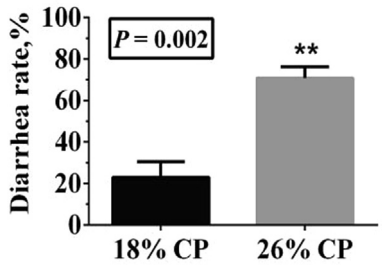

Fig. 1. Effects of dietary high protein on diarrhea rates of piglets. Diarrhea rate (%) = total number of pigs with diarrhea/(total number of pigs × experimental days) × 100%. The values in the figure are expressed as mean and SEM ($n = 8$); the differences were assessed by the non-parametric testing and denoted as follows: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

($P = 0.016$) and in the crypt of the colon ($P < 0.001$), and higher mRNA expression of ileal *Muc2* ($P = 0.012$; Fig. 4).

3.3. The mRNA expression of inflammatory genes in the intestine

Dietary HP significantly down-regulated the mRNA expression of ileal *TNF-α* ($P < 0.001$; Table 4), colonic *iNOS* ($P = 0.040$) and *IL-22* ($P = 0.008$; Table 5). It had the tendency to decrease the expression of *IL-1β* ($P = 0.076$; Table 5) in the colon.

3.4. Relative protein abundance of LGALS3 and NF-κB

As captured in Fig. 3, the relative protein abundance of LGALS3 was significantly elevated in the colon of piglets fed with HP ($P = 0.046$). For NF-κB signaling, its activation was assessed by the ratio of phosphorylated NF-κB to NF-κB (p–NF–κB/NF-κB ratio). It was found that the HP diet significantly enhanced ($P = 0.038$; Fig. 5) p–NF–κB/NF-κB ratio in the ileum.

4. Discussion

Dietary protein levels and sources may be associated with diarrhea in weaned piglets (Pluske et al., 2002). Many studies have investigated the impacts of dietary protein contents on the diarrhea index, growth performance, and morphological structure of weaned piglets (Bikker et al., 2006; Heo et al., 2015; Wu et al., 2015). We found that 26% CP could stably and significantly cause diarrhea, therefore, the present study selected this protein content as a high protein level compared with the actual production. The HP

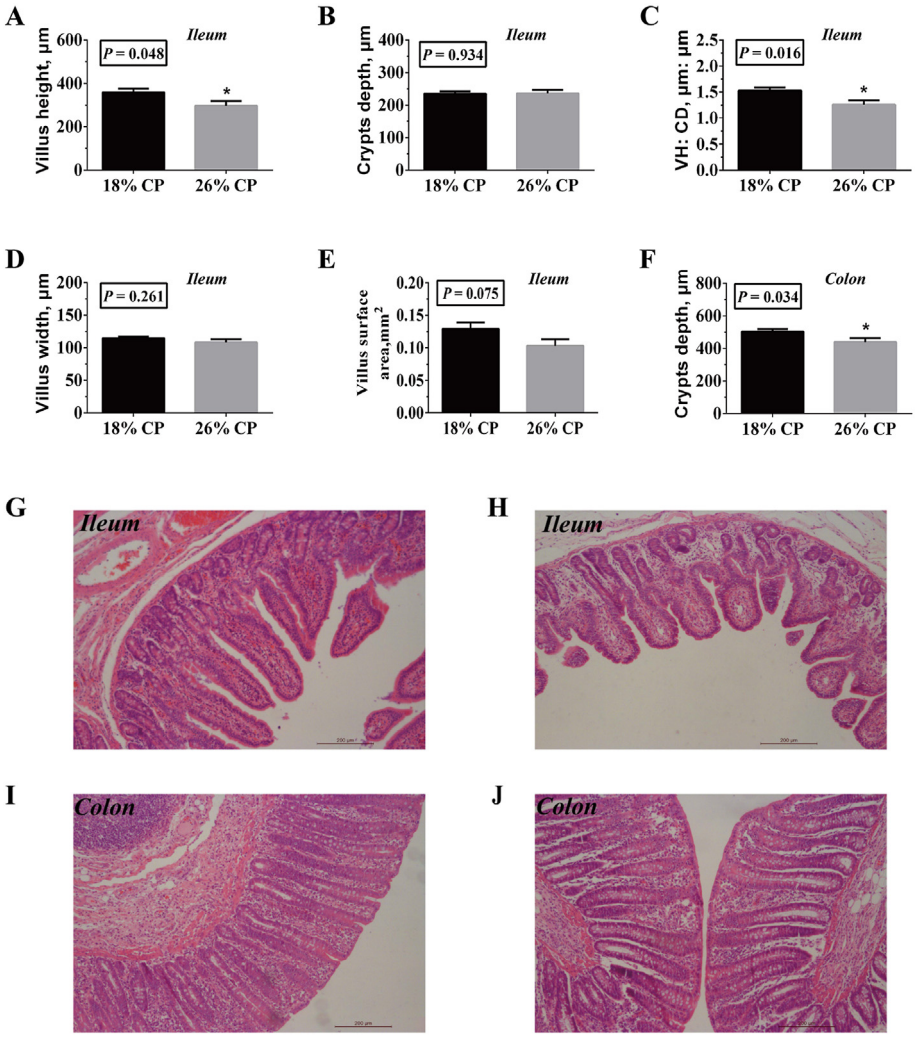

Fig. 2. Effects of high protein on intestinal morphology in piglets (A) Villus height (B) crypt depth (C) villus height to crypts depth ratio (D) villus width (E) villus surface area (F) colonic crypts depth. Representative images of (G and H) ileal and (I and J) colonic histomorphology in piglets. Images were taken at 10× magnification. Scale bars at 200 μm. The values in the figure are expressed as mean and SEM ($n = 8$); the differences were assessed by the independent-samples *T*-test and denoted as follows: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

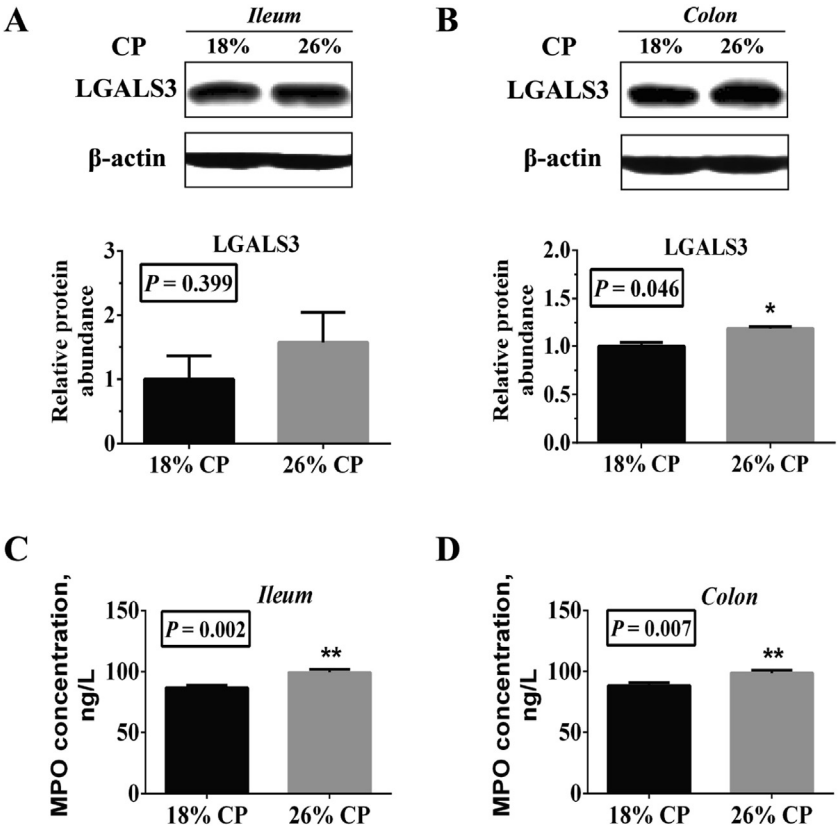

Fig. 3. Effects of dietary high protein on the protein abundance of (A and B) LGALS3 and (C and D) MPO concentration in ileum and colon. The protein abundance of LGALS3 was determined by western blotting and normalized using β-actin as an internal control. The values in the figure are expressed as mean and SEM ($n = 3$); the differences were assessed by the independent-samples *T*-test and denoted as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. LGALS3 = galectin-3; MPO = myeloperoxidase.

diets have been demonstrated to be associated with an increase of potentially toxic metabolites, thus increasing the incidence of post-weaning diarrhea (Pieper et al., 2012, 2016). In parallel, the present study found that dietary HP significantly increased diarrhea rates. Although no significant difference was observed in growth performance between 18% and 26% CP, HP diets reduced ADFI, ADG, G:F by 19.11%, 25.31%, 23.64%, respectively. These results indicated that the worse growth performance observed in the HP group might be because excessive protein was indigestible for piglets.

Morphometric indices, including VH and CD and their ratio, have commonly been used as indicators of piglets' intestinal health (Han et al., 2013; Heinritz et al., 2016). A previous study showed duodenal and jejunal VH and VH:CD ratio was reduced by higher protein levels (Wu et al., 2015). Decreased VH:CD ratio is considered to impair the digestive and absorptive capacity of the small intestine (Montagne et al., 2003). Our study showed that VH, VW, VH:CD ratio, and the villous surface area of the ileum narrowed under treatment with HP, which is in accordance with piglets' intestinal mucosa being more easily damaged when fed a high CP (van Beers-Schreurs et al., 1998; Wu et al., 2017), thus resulting in poor growth performance. A previous study demonstrated that CD was increased when pigs were fed with higher protein levels (Gao et al., 2020), conflicting with our finding that dietary HP decreased colonic CD, which could be because that research focused more on the ileum, while the change appeared in the colon in our study. Crypt depth may be dependent on the proliferation and shedding rate of epithelial cells (Hampson, 1986; Williams et al., 2013); however, more experiments are needed to further elucidate this notion.

Intestinal inflammation reflects the status of intestine health and is related to diarrhea, which includes the following criteria: epithelial hyperplasia and goblet cell depletion; leukocyte infiltration; and indicators of severe inflammation such as crypt edema, submucosal inflammation, and ulceration (Izcue et al., 2008). To better mirror intestinal inflammation, the present study measured the number of goblet cells, inflammatory cell infiltration, mRNA expression of inflammatory cytokine, and relative protein abundance of p–NF–κB/NF-κB ratio. Neutrophils secreted enzymes, MPO, whose concentration was often analyzed, which could be considered as the marker of neutrophil infiltration (Lan et al., 2015). LGALS3, also known as Mac2, is a marker molecule on the surface of macrophages; the expression level of intestinal mucosa directly reflects the number of macrophages. In our study, the MPO concentration in the ileum and colon, and protein abundance of LGALS3 in the colon were significantly elevated, suggesting that intestinal inflammation was induced, as also proved by previous studies (Lan et al., 2015; Yi et al., 2016). Moreover, mucus secreted by goblet cells is an essential element of the mucosal barrier, and mucus 2 (Muc2) is the most abundant intestinal mucus (Garrett et al., 2010). Our findings showed that goblet cells in the ileal villus and colonic crypts were significantly enhanced in the HP diet, as well as the ileal mRNA expression of *Muc2*, which may protect the intestine from inflammation.

Cytokines are vital for modulating the intestinal inflammatory response (Al-Sadi et al., 2009). Previous studies have shown that dietary HP significantly increased the jejunal expression of *IL-1β* and *IFN-γ* (Wu et al., 2015). Mu et al. (2016) showed that an up-regulation of pro-inflammatory genes was observed in rats fed

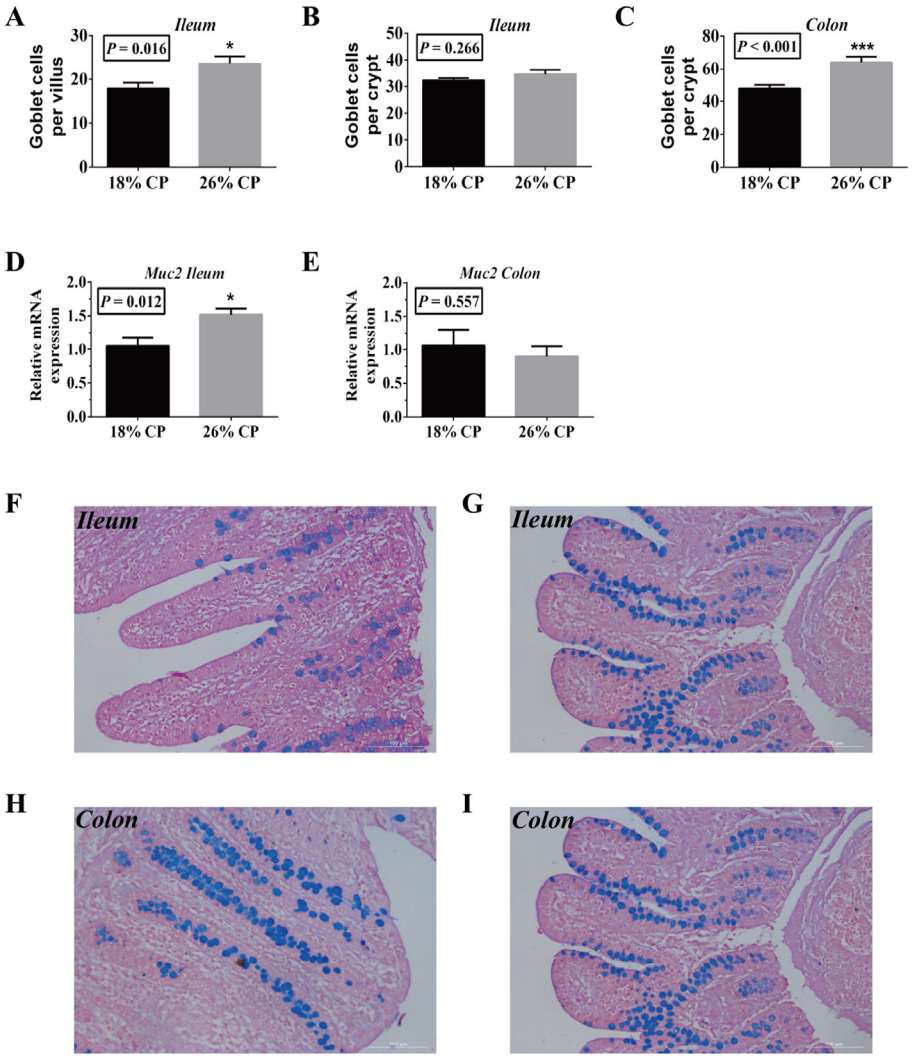

Fig. 4. Effects of dietary high protein on the number of (A, B and C) goblet cells and the mRNA expression of (D and E) *Muc2* of *ileum* and *colon* in piglets. Representative images of alcian blue-periodic acid-Schiff staining of piglets' (F and G) *ileum* and (H and I) *colon*. Goblet cells are blue. Images were taken at 20× magnification. Scale bars at 100 μm. The values in the figure are expressed as mean and SEM ($n = 8$); the differences were assessed by the independent-samples *T*-test and denoted as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. *Muc2* = mucin 2.

Table 4
Effects of dietary crude protein on expression of ileum inflammatory genes in piglets[1].

Item[2]	Dietary crude protein, %		*P*-value
	18	26	
IL-6	1.03 ± 0.08	0.90 ± 0.13	0.298
INOS	1.06 ± 0.12	0.69 ± 0.18	0.113
TNF-α	1.01 ± 0.05[a]	0.46 ± 0.06[b]	<0.001
IL-1β	1.06 ± 0.13	1.08 ± 0.34	0.952
IL-10	1.02 ± 0.07	0.84 ± 0.09	0.125
IL-22	1.14 ± 0.22	1.04 ± 0.29	0.806
TGF-β	1.03 ± 0.09	0.96 ± 0.07	0.595
IFN-γ	0.94 ± 0.13	0.77 ± 0.10	0.337
COX-2	0.96 ± 0.07	1.03 ± 0.08	0.486

[a, b] Within a variable, values with different superscripts differ significantly at $P < 0.05$.
[1] Values are expressed as mean ± SEM of 8 piglets per treatment. The data were analyzed by independent-samples *T*-test.
[2] *IL* = interleukin; *iNOS* = inducible nitric oxide synthase; *TNF-α* = tumor necrosis factor-α; *TGF-β* = transforming growth factor-β; *IFN-γ* = interferon-γ; *COX-2* = cyclo-oxygenase-2.

Table 5
Effects of dietary crude protein on expression of colon inflammatory genes in piglets[1].

Item[2]	Dietary crude protein, %		*P*-value
	18	26	
IL-6	0.95 ± 0.14	0.67 ± 0.11	0.144
INOS	1.16 ± 0.21[a]	0.55 ± 0.14[b]	0.040
TNF-α	0.94 ± 0.07	0.78 ± 0.10	0.226
IL-1β	1.16 ± 0.22	0.59 ± 0.20	0.076
IL-10	1.03 ± 0.09	0.94 ± 0.12	0.541
IL-22	1.07 ± 0.13[a]	0.49 ± 0.13[b]	0.008
TGF-β	0.96 ± 0.07	0.88 ± 0.08	0.510
IFN-γ	1.04 ± 0.10	0.84 ± 0.12	0.204
COX-2	1.54 ± 0.14	1.23 ± 0.13	0.124

[a,b] Within a variable, values with different superscripts differ significantly at $P < 0.05$ or show a tendency toward differing at $P < 0.10$.
[1] Values are expressed as mean ± SEM of 8 piglets per treatment. The data were analyzed by independent-samples T-test. SEM: standard error of the mean.
[2] *IL* = interleukin; *iNOS* = inducible nitric oxide synthase; *TNF-α* = tumor necrosis factor-α; *TGF-β* = transforming growth factor-β; *IFN-γ* = interferon-γ; *COX-2* = cyclo-oxygenase-2.

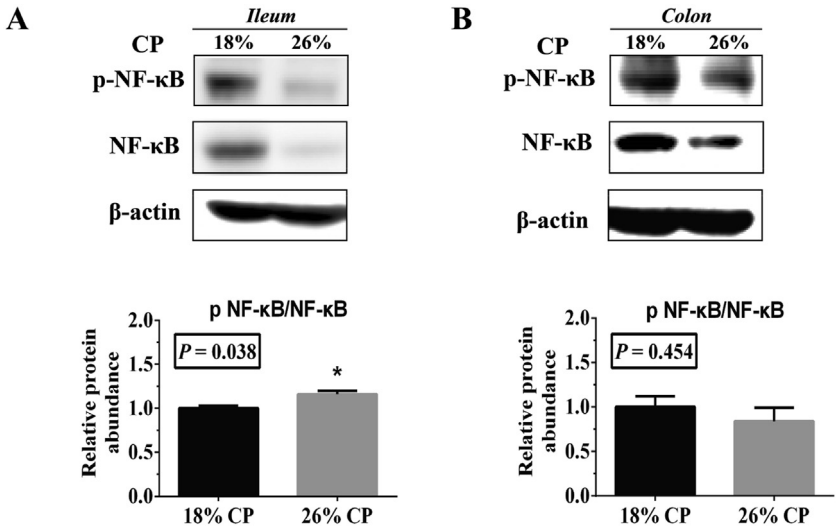

Fig. 5. Effects of dietary high protein on the activation of NF-κB signaling of the (A) ileum and (B) colon in piglets. Protein abundances of NF-κB and p−NF−κB were determined by western blotting. The values in the figure are expressed as mean and SEM ($n = 3$); the differences were assessed by the independent-samples *T*-test and denoted as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

with the HP diets. These results are inconsistent with our results that HP diets down-regulated the mRNA of expression of ileal *TNF-α*, and colonic *iNOS*, *IL-1β*, and *IL-22*. The current study suggested that HP diets affected both ileal and colonic goblet cells, increased the secretion of mucus, and lessened parameters relevant to basal intestinal inflammatory status. Increasing the mucus content may protect the intestinal immune cells from luminal antigenic stimulation, which decreases the expression of intestinal cytokines (Lan et al., 2015). Besides, Muc2 expression might be directly responsible for down-regulation of the pro-inflammatory cytokines, as it has been recently shown that this mucin attenuates pro-inflammatory cytokines produced by dendritic cells (Shan et al., 2013). In the colon, the present results showed that a HP diet slightly reduced the pro-inflammatory *IL-1β*, *IL-22*, and *iNOS* gene expression, which may be linked with an increased amount of short-chain fatty acids (SCFA) in the luminal colonic content (Lan et al., 2015); however, further studies are needed to confirm this. Notably, NF-κB signaling is crucial for maintaining epithelial integrity and intestinal homeostasis and has significant implications for comprehending the underlying mechanisms of pathogenesis in human IBD (Nenci et al., 2007). The present study revealed that HP diets may activate NF-κB signaling and aggravate intestinal inflammation. Further studies are required to explore the mechanism of HP regulating inflammation.

5. Conclusion

This study suggests that dietary supplementation with HP caused piglets' diarrhea through impaired intestinal morphology, lessened parameters related to basal gut inflammatory status and may activate NF-κB signaling to induce intestinal inflammation.

Author contributions

Lanmei Yin: Investigation, Data curation, Writing-original draft preparation. **Jun Li**: Visualization, Investigation. **Meiwei Wang**: Investigation. **Qiye Wang**: Conceptualization, Methodology. **Jianzhong Li**: Project administration. **Nengshui Ding**: Resources. **Huansheng Yang**: Conceptualization, Methodology, Software, Writing - review and editing. **Yulong Yin**: Supervision, Funding acquisition, Writing - review and editing.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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References

Al-Sadi R, Boivin M, Ma T. Mechanism of cytokine modulation of epithelial tight junction barrier. Front Biosci 2009;14:2765–78.
Bikker P, Dirkzwager A, Fledderus J, Trevisi P, le Huërou-Luron I, Lallès JP, et al. The effect of dietary protein and fermentable carbohydrates levels on growth performance and intestinal characteristics in newly weaned piglets. J Anim Sci 2006;84(12):3337–45.
De Vos M, Huygelen V, Willemen S, Fransen E, Casteleyn C, Van Cruchten S, et al. Artificial rearing of piglets: effects on small intestinal morphology and digestion capacity. Livest Sci 2014;159(1):165–73.
Deng Q, Shao Y, Wang Q, Li J, Li Y, Ding X, et al. Effects and interaction of dietary electrolyte balance and citric acid on the intestinal function of weaned piglets. J Anim Sci 2020a;98(5). skaa106.
Deng Q, Tan X, Wang H, Wang Q, Huang P, Li Y, et al. Changes in cecal morphology, cell proliferation, antioxidant enzyme, volatile fatty acids, lipopolysaccharide, and cytokines in piglets during the postweaning period. J Anim Sci 2020b;98(3). skaa046.
Gao J, Yin J, Xu K, Han H, Liu Z, Wang C, et al. Protein level and infantile diarrhea in a postweaning piglet model. Mediat Inflamm 2020;2020:1–15.
Gao J, Yin J, Xu K, Li T, Yin Y. What is the impact of diet on nutritional diarrhea associated with gut microbiota in weaning piglets: a system review. BioMed Res Int 2019;2019(2):1–14.
Garrett WS, Gordon JI, Glimcher LH. Homeostasis and inflammation in the intestine. Cell 2010;140(6):859–70.
Hampson DJ. Alterations in piglet small intestinal structure at weaning. Res Vet Sci 1986;40(1):32–40.
Han F, Hu L, Xuan Y, Ding X, Luo Y, Bai S, et al. Effects of high nutrient intake on the growth performance, intestinal morphology and immune function of neonatal intra-uterine growth-retarded pigs. Br J Nutr 2013;110(10):1819–27.

He L, Yang H, Hou Y, Li T, Fang J, Zhou X, et al. Effects of dietary L-lysine intake on the intestinal mucosa and expression of CAT genes in weaned piglets. Amino Acids 2013;45(2):383–91.
Heinritz SN, Weiss E, Eklund M, Aumiller T, Louis S, Rings A, et al. Intestinal microbiota and microbial metabolites are changed in a pig model fed a high-fat/low-fiber or a low-fat/high-fiber diet. PLoS One 2016;11(4):e0154329.
Heo JM, Kim JC, Yoo J, Pluske JR. A between-experiment analysis of relationships linking dietary protein intake and post-weaning diarrhea in weanling pigs under conditions of experimental infection with an enterotoxigenic strain of Escherichia coli. Anim Sci J 2015;86(3):286–93.
Htoo JK, Araiza BA, Sauer WC, Rademacher M, Zhang Y, Cervantes M, et al. Effect of dietary protein content on ileal amino acid digestibility, growth performance, and formation of microbial metabolites in ileal and cecal digesta of early-weaned pigs. J Anim Sci 2007;85(12):3303–12.
Izcue A, Hue S, Buonocore S, Arancibia-Cárcamo CV, Ahern PP, Iwakura Y, et al. Interleukin-23 restrains regulatory T cell activity to drive T cell-dependent colitis. Immunity 2008;28(4):559–70.
Karin M, Greten FR. NF-κB: linking inflammation and immunity to cancer development and progression. Nat Rev Immunol 2005;5(10):749–59.
Kluess J, Schoenhusen U, Souffrant WB, Jones PH, Miller BG. Impact of diet composition on ileal digestibility and small intestinal morphology in early-weaned pigs fitted with a T-cannula. Animal 2010;4(4):586–94.
Lan A, Andriamihaja M, Blouin JM, Liu X, Descatoire V, Desclée de Maredsous C, et al. High-protein diet differently modifies intestinal goblet cell characteristics and mucosal cytokine expression in ileum and colon. J Nutr Biochem 2015;26(1):91–8.
Li J, Yin L, Wang L, Li J, Huang P, Yang H, et al. Effects of vitamin B6 on growth, diarrhea rate, intestinal morphology, function, and inflammatory factors expression in a high-protein diet fed to weaned piglets1. J Anim Sci 2019;97(12):4865–74.
Li Q, Verma IM. NF-κB regulation in the immune system. Nat Rev Immunol 2002;2(12):725–34.
Liu P, Piao XS, Kim SW, Wang L, Shen YB, Lee HS, et al. Effects of chito-oligosaccharide supplementation on the growth performance, nutrient digestibility, intestinal morphology, and fecal shedding of Escherichia coli and Lactobacillus in weaning pigs1. J Anim Sci 2008;86(10):2609–18.
Montagne L, Pluske JR, Hampson DJ. A review of interactions between dietary fibre and the intestinal mucosa, and their consequences on digestive health in young non-ruminant animals. Anim Feed Sci Technol 2003;108(1–4):95–117.
Mu C, Yang Y, Luo Z, Guan L, Zhu W. The colonic microbiome and epithelial transcriptome are altered in rats fed a high-protein diet compared with a normal-protein diet. J Nutr 2016;146(3):474–83.
National Research Council. Nutrient requirements of swine. 10th revised edition. Washington, DC: National Academy Press; 1998.
National Research Council. Nutrient requirements of swine. 11th revised edition. Washington, DC: National Academy Press; 2012.
Nenci A, Becker C, Wullaert A, Gareus R, van Loo G, Danese S, et al. Epithelial NEMO links innate immunity to chronic intestinal inflammation. Nature 2007;446(7135):557–61.
Opapeju FO, Krause DO, Payne RL, Rademacher M, Nyachoti CM. Effect of dietary protein level on growth performance, indicators of enteric health, and gastrointestinal microbial ecology of weaned pigs induced with postweaning colibacillosis. J Anim Sci 2009;87(8):2635–43.
Pieper R, Boudry C, Bindelle J, Vahjen W, Zentek J. Interaction between dietary protein content and the source of carbohydrates along the gastrointestinal tract of weaned piglets. Arch Anim Nutr 2014;68(4):263–80.
Pieper R, Kröger S, Richter JF, Wang J, Martin L, Bindelle J, et al. Fermentable fiber ameliorates fermentable protein-induced changes in microbial ecology, but not the mucosal response, in the colon of piglets. J Nutr 2012;142(4):661–7.
Pieper R, Villodre Tudela C, Taciak M, Bindelle J, Pérez JF, Zentek J. Health relevance of intestinal protein fermentation in young pigs. Anim Health Res Rev 2016;17(2):137–47.
Pluske JR, Hampson DJ, Williams IH. Factors influencing the structure and function of the small intestine in the weaned pig: a review. Livest Prod Sci 1997;51(1–3):215–36.
Pluske JR, Pethick DW, Hopwood DE, Hampson DJ. Nutritional influences on some major enteric bacterial diseases of pig. Nutr Res Rev 2002;15(2):333–71.
Ren W, Yin J, Wu M, Liu G, Yang G, Xion Y, et al. Serum amino acids profile and the beneficial effects of L-arginine or L-glutamine supplementation in dextran sulfate sodium colitis. PLoS One 2014;9(2):e88335.
Richter JF, Pieper R, Zakrzewski SS, Günzel D, Schulzke JD, Van Kessel AG. Diets high in fermentable protein and fibre alter tight junction protein composition with minor effects on barrier function in piglet colon. Br J Nutr 2014;111(6):1040–9.
Shan M, Gentile M, Yeiser JR, Walland AC, Bornstein VU, Chen K, et al. Mucus enhances gut homeostasis and oral tolerance by delivering immunoregulatory signals. Science 2013;342(6157):447–53.
Sun SC. The non-canonical NF-κB pathway in immunity and inflammation. Nat Rev Immunol 2017;17(9):545–58.
van Beers-Schreurs HM, Nabuurs MJ, Vellenga L, Kalsbeek-van der Valk HJ, Wensing T, Breukink HJ. Weaning and the weanling diet influence the villous height and crypt depth in the small intestine of pigs and alter the concentrations of short-chain fatty acids in the large intestine and blood. J Nutr 1998;128(6):947–53.
Wang LX, Zhu F, Li JZ, Li YL, Ding XQ, Yin J, et al. Epidermal growth factor promotes intestinal secretory cell differentiation in weaning piglets via Wnt/β-catenin signalling. Animal 2020a;14(4):790–8.
Wang Z, Li J, Wang Y, Wang L, Yin Y, Yin L, et al. Dietary vitamin A affects growth performance, intestinal development, and functions in weaned piglets by affecting intestinal stem cells. J Anim Sci 2020b;98(2):skaa020.
Williams JM, Duckworth CA, Watson AJ, Frey MR, Miguel JC, Burkitt MD, et al. A mouse model of pathological small intestinal epithelial cell apoptosis and shedding induced by systemic administration of lipopolysaccharide. Dis Model Mech 2013;6(6):1388–99.
Wu F, Xiong X, Yang H, Yao K, Duan Y, Wang X, et al. Expression of proteins in intestinal middle villus epithelial cells of weanling piglets. Front Biosci 2017;22:539–57.
Wu Y, Jiang Z, Zheng C, Wang L, Zhu C, Yang X, et al. Effects of protein sources and levels in antibiotic-free diets on diarrhea, intestinal morphology, and expression of tight junctions in weaned piglets. Anim Nutr 2015;1(3):170–6.
Xiao H, Shao F, Wu M, Ren W, Xiong X, Tan B, et al. The application of antimicrobial peptides as growth and health promoters for swine. J Anim Sci Biotechnol 2015;6(1):19.
Xiong X, Yang HS, Wang XC, Hu Q, Liu CX, Wu X, et al. Effect of low dosage of chito-oligosaccharide supplementation on intestinal morphology, immune response, antioxidant capacity, and barrier function in weaned piglets. J Anim Sci 2015;93(3):1089–97.
Yan S, Long L, Zong E, Huang P, Li J, Li Y, et al. Dietary sulfur amino acids affect jejunal cell proliferation and functions by affecting antioxidant capacity, Wnt/β-catenin, and the mechanistic target of rapamycin signaling pathways in weaning piglets. J Anim Sci 2018;96(12):5124–33.
Yang HS, Fu DZ, Kong XF, Wang WC, Nyachoti CM, Yin YL. Dietary supplementation with N-carbamylglutamate increases the expression of intestinal amino acid transporters in weaned Huanjiang mini-pig piglets. J Anim Sci 2013;91(6):2740–8.
Yi H, Jiang D, Zhang L, Xiong H, Han F, Wang Y. Developmental expression of STATs, nuclear factor-κB and inflammatory genes in the jejunum of piglets during weaning. Int Immunopharm 2016;36:199–204.
Yi H, Yu C, Zhang H, Song D, Jiang D, Du H, et al. Cathelicidin-BF suppresses intestinal inflammation by inhibiting the nuclear factor-κB signaling pathway and enhancing the phagocytosis of immune cells via STAT-1 in weanling piglets. Int Immunopharm 2015;28(1):61–9.
Yin L, Li J, Wang H, Yi Z, Wang L, Zhang S, et al. Effects of vitamin B6 on the growth performance, intestinal morphology, and gene expression in weaned piglets that are fed a low-protein diet1. J Anim Sci 2020;98(2). skaa022.
Zhou X, Wu X, Yin Y, Zhang C, He L. Preventive oral supplementation with glutamine and arginine has beneficial effects on the intestinal mucosa and inflammatory cytokines in endotoxemic rats. Amino Acids 2012;43:813–21. https://doi.org/10.1007/s00726-011-1137-2.
Zong E, Yan S, Wang M, Yin L, Wang Q, Yin J, et al. The effects of dietary supplementation with hyodeoxycholic acid on the differentiation and function of enteroendocrine cells and the serum biochemical indices in weaned piglets. J Anim Sci 2019;97(4):1796–805.