Animal Nutrition 7 (2021) 770–778

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

Original Research Article

Low crude protein diets supplemented with casein hydrolysate enhance the intestinal barrier function and decrease the pro-inflammatory cytokine expression in the small intestine of pigs

Huisong Wang [a, b], Junhua Shen [a, b], Chunlong Mu [a, b], Kan Gao [a, b], Yu Pi [a, b], Weiyun Zhu [a, b, *]

[a] Laboratory of Gastrointestinal Microbiology, Jiangsu Key Laboratory of Gastrointestinal Nutrition and Animal Health, College of Animal Science and Technology, Nanjing Agricultural University, Nanjing, 210095, China
[b] National Center for International Research on Animal Gut Nutrition, Nanjing Agricultural University, Nanjing, 210095, China

ARTICLE INFO

Article history:
Received 21 August 2020
Received in revised form
29 December 2020
Accepted 8 March 2021
Available online 26 May 2021

Keywords:
Casein hydrolysate
Immunity
Intestinal barrier function
Low crude protein diet
Pig

ABSTRACT

To reduce nitrogen excretion and lower feeding costs, low crude protein (CP) diets are sometimes proposed, however, a great reduction of dietary CP concentration (>4% reduction vs. recommended concentration), even supplemented with essential and nonessential amino acids (AA) can detrimentally affect small intestinal barrier function and immunity, possibly due to the excessive lack of peptides. Here we hypothesize that with an extremely low CP concentration diet, protein-derived peptides, rather than AA supplementation, can improve intestinal barrier development and health. To test this hypothesis, 21 growing pigs (19.90 ± 1.00 kg body weight) were randomly assigned to 3 treatments with control diet (16% CP), or low CP diets (13% CP) supplemented with AA (LCPA) or casein hydrolysate (LCPC) for 28 days. In comparison with the control diet, the LCPA diet decreased the protein expression level of jejunal barrier factor zonula occludens-1 (ZO-1) and stem cell proliferation factor leucine-rich repeat-containing G-protein-coupled receptor-5, whereas the LCPC diet enhanced intestinal barrier function by increasing the protein expression level of jejunal occludin and ZO-1 and ileal mucin-2. The LCPA diet reduced *Lactobacillus* counts, whereas the LCPC diet increased *Lactobacillus* counts and reduced *Escherichia coli* counts in the ileum. The LCPA diet also increased protein expression levels of pro-inflammatory cytokine interleukin-6 (IL-6) and IL-22, whereas the LCPC diet decreased protein expression levels of pro-inflammatory IL-1β, IL-17A and tumor necrosis factor-α in the ileum. Collectively, the casein hydrolysate supplementation of low CP diets showed beneficial effects on the small intestinal barrier, bacterial community, and immunity in pigs, pointing to the important role of protein-derived peptides in small intestinal health in cases of low crude protein diets.

1. Introduction

In order to reduce nitrogen excretion and the feeding costs, low crude protein (LCP) diets are sometimes proposed, with the amino acid (AA) gap caused in the LCP diets completely filled to meet AA requirement. Previous studies have shown that moderately LCP diets (≤4% reduction of crude protein [CP] concentration vs. NRC (2012)) supplemented with essential AA considered to be an effective strategy to support intestinal health and protein utilization and to reduce nitrogen excretion without damaging the growth performance of pigs (Wu et al., 2015; Zhang et al., 2017; Kerr et al., 2006). In order to further reduce the feeding cost and

* Corresponding author.
E-mail address: zhuweiyun@njau.edu.cn (W. Zhu).
Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

https://doi.org/10.1016/j.aninu.2021.03.003

nitrogen excretion, a further reduction of CP with extremely LCP diets (>4% reduction of CP concentration vs. NRC (2012)) has been proposed. However, extremely LCP diets can lead to detrimental effects on intestinal barrier development, intestinal bacterial community, and mucosal immunity even when supplementing essential and nonessential AA (Chen et al., 2018; Peng et al., 2017; Fan et al., 2017; Che et al., 2017). In addition to AA, protein also releases peptides during digestion. Researchers have considered that the main reason for the detrimental effects of extremely LCP diets may be due to the excessive lack of protein-derived peptides in extremely LCP diets (Che et al., 2017; Wang et al., 2019; Deng et al., 2007).

Peptides are considered to be the preferred and efficient source of protein nutrition for animals compared with AA (Rérat et al., 1992; Monchi and Rérat, 1993; Silk et al., 1979). Moreover, peptides can also display a variety of bioactive functions, such as immunomodulation and promotion of intestinal development (Malinowski et al., 2014; Hou et al., 2017). Our previous studies have shown that the supplementation of casein hydrolysate as the peptides source in extremely LCP diets has increased the proportion of generally beneficial *Lactobacillus* and enhanced the mucosal defense capability by increasing the mucin-4 expression and activating the humoral immunity in the colon of pigs as compared with the supplementation of AA (Wang et al., 2019). The small intestine is exposed to orally feed ingredients (earlier than the colon) and the major site where AA and peptides are digested and absorbed. Dietary nitrogen nutrients are major fuels for small intestinal mucosa to maintain barrier functional integrity and to promote immune system maturation (Wu, 1998; Hughes, 1999). However, it remains unclear whether casein hydrolysate supplementation to extremely LCP diets shows more benefits on the small intestinal barrier and immunity in pigs as compared with AA supplementation.

In this study, therefore, we hypothesize that supplementation of protein-derived peptides, instead of solely supplementing with AA to fill the AA gap between the extremely LCP and AA requirement, can be used to improve the small intestinal health in pigs. To test this hypothesis, we evaluated the small intestinal barrier function, immunity and bacterial community in pigs fed extremely LCP diets, supplemented with AA or casein hydrolysate, aiming to investigate whether the supplementation of casein hydrolysate as the peptides source, compared with only AA supplementation, can improve the small intestinal health under an extremely LCP concentration.

2. Materials and methods

The experimental procedures were approved by the Animal Care and Use Committee of Nanjing Agricultural University (Nanjing, China).

2.1. Experimental design and sample collection

The present study was a part of series of studies designed to investigate whether supplementation of casein hydrolysate as a peptide source can improve the intestinal health under extremely LCP concentration in pigs, and the detailed experimental design has been described previously (Wang et al., 2019). Briefly, 21 crossbred (Duroc × Landrace × Yokshire, 63 ± 1 days of age, initial body weight 19.90 ± 1.00 kg) pigs (castrate males) were housed individually in metabolism cages and randomly assigned to 3 groups ($n = 7$), and fed with control diet (CP 16%), low CP supplemented AA diet (CP 13%, LCPA), and low CP supplemented casein hydrolysate diet (CP 13%, LCPC), respectively. The compositions and calculated nutrients of 3 diets are shown in Appendix Table 1, and have been reported previously (Wang et al., 2019). The casein hydrolysate added in the LCPC diet is an enzymatically hydrolyzed casein (CP 89%; Haibo Biotech Inc., Tsingtao, China), with 46% free AA and 54% peptides (as CP basis). The supplemental dosage of casein hydrolysate in the LCPC diet was 4.5%, based on a previous study (Che et al., 2017). Synthetic crystalline AA were added in the 3 diets to balance dietary essential and nonessential AA, ensuring that the 3 diets had similar standardized ileal digestible (SID) AA. Except for CP concentration, all the contents of the other nutrients were similar and met the nutrient requirements recommended by NRC (2012).

Pigs had free access to water via a low-pressure nipple drinker. Pigs were fed ad libitum with 2 meals daily, and feed refusals were recorded. The room temperature was maintained at 24 ± 2 °C. Pigs were weighed at the beginning and end of the experiment. The health condition of each animal was closely monitored.

The experiment lasted for 28 days. On day 29, all pigs were anesthetized by intravenous injection of sodium pentobarbital (50 mg/kg body weight) and sacrificed by exsanguination. Then the small intestine was removed and each segment (duodenum, jejunum and ileum) was identified, ligated and separated. The middle section, including the digesta and tissue, of each small intestinal segment was collected and used for subsequent sampling. The digesta from the jejunum and ileum were collected and stored at −80 °C for extraction of bacterial genomic DNA. The intestinal tissue was divided into 2 parts. The first part of intestinal tissue (rinsed in sterile PBS, pH = 7.4, 4 °C) was collected after mincing and stored at −80 °C for the isolation of RNA and protein. The second part (2 cm long) was fixed in 4% paraformaldehyde solution (Sigma, USA) for subsequent histological analysis.

2.2. Small intestinal gene expression

Frozen intestinal tissues (50 to 100 mg) were crushed under liquid nitrogen, and total RNA was extracted with TRIzol reagent (TaKaRa, Japan). RNA concentration was quantified by using a Nano-Drop 2000 spectrophotometer (Thermo, USA). RNA (1 μg) was then reverse-transcribed by using a PrimeScript RT Reagent Kit (TaKaRa, Japan) with guidance from the manufacturer's instructions.

Gene expression levels of intestinal barrier and immune factors were detected by real-time quantitative PCR (qPCR) with Sybr-Green reagents (TaKaRa), and fluorescence was detected by using ABI 7300 sequence detector (Applied Biosystems, USA). The specific primers are shown in Appendix Table 2. For detailed methods refer to our previous study (Zhang et al., 2017). Beta-actin was used as the reference gene, and the expression of target genes was calculated relative to the reference gene with $2^{-\Delta\Delta Ct}$ method.

2.3. Immunohistochemical and periodic acid-Schiff (PAS) staining

After an overnight fixation, the intestinal tissues were embedded in paraffin wax and 4-μm transversal sections of jejunal and ileal tissues were cut for PAS and immunohistochemical staining. For detailed methods of PAS stain (including the staining and the signal statistic) refer to the procedures of Lan et al. (2015). The quantification of PAS signal was the number of goblet cells per mm of intestinal crypt depth. The expressions of jejunal occludin and zonula occludens (ZO)-1 and ileal mucin-2 (MUC-2) were detected by using immunohistochemical staining with primary antibodies for occludin (1:400; 66378-1-ig, Proteintech, USA), ZO-1 (1:200; SC-33725, Santa Cruz, USA), MUC-2 (1:200; ab134119, Abcam, UK) and HRP-conjugated secondary antibody (Thermo Fisher Scientific, USA) according to a previous study (Pi et al., 2020). Image J software (NIH, USA; http://rsb.info.nih.gov/ij/) was used to analyze the mean optical density of each specimen.

2.4. Western blot analysis of intestinal stem cell (ISC) proliferation factor

Protein expression levels of ISC proliferation marker leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5) in the jejunum and ileum were detected by using Western blot analysis as described previously (Zhong et al., 2018). Total protein of intestinal tissues was extracted by Tissue Protein Extraction Reagent (Thermo Pierce, 78510), and the expressions of Lgr5 and β-actin proteins were detected using primary antibodies against Lgr5 (1:1500; PA523000, eBioscience, USA) and β-actin (1:1500; SC-47778, Santa Cruz, USA) and Goat anti-Rabbit IgG (H + L) secondary antibody (1:5000; 31210, Thermo Pierce, USA). Image J software (NIH, USA) was used to quantify the Western blot signal.

2.5. Intestinal bacterial community analysis

Total bacterial genomic DNA in the jejunal and ileal digesta was extracted using the DNA Stool Mini Kit (Qiagen, Germany). Total bacteria, Firmicutes, Bacteroidetes, *Clostridium* cluster IV/XIV groups, *Lactobacillus*, *Bifidobacterium* and *Escherichia coli* were quantified by using qPCR (ABI StepOne platform, Applied Biosystems, USA) with specific primers (Appendix Table 2) according to our previous method (Mu et al., 2017).

2.6. Intestinal cytokine analysis

The protein expression levels of ileal cytokines and immunoglobulin A were detected by enzyme-linked immunosorbent assay (ELISA), using commercially porcine specific high-sensitivity kits according to manufacturer's instructions. For pre-treatment of intestinal tissue, samples (50 mg) were homogenized in sterile PBS (450 μL; pH = 7.4, 4 °C) and centrifuged at 3,000 × *g* for 15 min at 4 °C, then supernatants were collected and sub-packaged. The protein concentration of supernatants was detected by Nano-Drop 2000 spectrophotometer (Thermo, USA). The detected cytokines included helper T cell 1 (Th1) type-cytokines, which were tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-1β and IL-12 p40 (ELISA Kit brand: R&D Systems, USA), Th2-cytokines, which were IL-4, IL-6, and IL-10 (R&D Systems), Th17-cytokines, which were IL-17A (eBioscience, USA) and IL-22 (BioAim, Canada), and transforming growth factor (TGF)-β (R&D Systems). Evaluating the variations of different Th-cytokines can help us to deeply understand the variation of immune response (Neurath et al., 2002).

2.7. Statistical analysis

A completely randomized experimental design was used in this study. Each individual pig was considered as an experimental unit. The dietary treatment was the fixed effect, and the pig was considered as a random effect. Data analysis was performed using SPSS 21.0 (Chicago, IL, USA), and subjected to one-way ANOVA with Tukey's honestly significant difference. Data were presented as group means ± SEM, differences were considered significant at $P < 0.05$. The correlation analyses were conducted by Pearson's correlation test using the GraphPad Prism version 7.00 (GraphPad Software, USA).

3. Results

All pigs showed no clinical signs of diarrhea or health impairment during the whole experimental period. LCPC diet increased the average daily gain of pigs as compared with the control diet and LCPA diet, and increased the average daily feed intake as compared with LCPA diet, but showed no effect on feed-to-gain ratio; the detailed results on growth performance have been reported previously (Wang et al., 2019; also, Appendix Fig. 1).

3.1. Small intestinal barrier function and intestinal stem cell proliferation

LCPA diet and LCPC diet had different effects on the gene expression of small intestinal barrier factors (Fig. 1). LCPA diet downregulated ($P < 0.05$) the gene expression levels of β-defensin-1, *ZO-1* and claudin-1 in jejunum as compared with the control and LCPC diets. LCPC diet upregulated ($P < 0.05$) gene expression levels of occludin and *ZO-1* in the jejunum and *MUC-2* and *MUC-4* in the ileum compared with the control and LCPA diets. Moreover, we also observed increased ($P < 0.05$) protein expression levels of occludin and ZO-1 in the jejunum and MUC-2 in the ileum in LCPC group compared with the control group and LCPA group (Fig. 2A). Based upon the increased expressions of mucins, we further measured the goblet cell numbers in the ileum. Compared with control diet and LCPA diet, LCPC diet increased ($P < 0.05$) the goblet cell numbers in the ileum (Fig. 2B).

Intestinal barrier development and goblet cell proliferation are driven by intestinal stem cell (ISC) proliferation. Lgr5 is a biomarker of intestinal stem cells. In the jejunum, compared with the control diet, LCPA diet decreased ($P < 0.05$) protein expression level of Lgr5, whereas LCPC diet increased ($P < 0.05$) protein expression level of Lgr5 (Fig. 3). In the ileum, LCPC diet increased ($P < 0.05$) protein expression level of Lgr5 as compared with LCPA diet (Fig. 3). Overall, these results indicate that LCPA diet down-regulated the expressions of intestinal barrier and ISC proliferation factors and LCPC diet showed opposite effects.

3.2. Small intestinal bacterial community

To understand whether casein hydrolysate supplementation affects the small intestinal microbes, qPCR was performed to quantify the major representative bacterial groups. Compared with the control diet, LCPA diet reduced ($P < 0.05$) the counts of *Lactobacillus*, and LCPC diet increased ($P < 0.05$) the counts of *Lactobacillus* and *Clostridium* cluster IV and reduced ($P < 0.05$) the counts of *E. coli* in the ileum (Fig. 4). Compared with LCPA diet, LCPC diet increased ($P < 0.05$) the counts of *Clostridium* cluster IV in the jejunum and Firmicutes, *Lactobacillus* and *Clostridium* cluster IV in the ileum, and reduced ($P < 0.05$) the counts of *E. coli* in the ileum (Fig. 4). These results indicated that the effects of LCPA and LCPC diets on the small intestinal bacterial community mainly occurred in the ileum; and LCPC diet increased the counts of bacteria generally regarded as beneficial (*Lactobacillus* and *Clostridium* cluster IV) and decreased the counts of *E. coli* compared with LCPA diet.

3.3. Intestinal immune response

The effects of LCPA diet and LCPC diet on intestinal Toll-like receptor (TLR) signaling pathway also mainly occurred in the ileum (Fig. 5). Compared with the control diet, LCPA diet upregulated ($P < 0.05$) gene expression level of *TLR2*, and LCPC diet downregulated ($P < 0.05$) gene expression levels of *TLR4* and nuclear factor kappa B (*NF-κB*). Compared with LCPA diet, LCPC diet downregulated ($P < 0.05$) gene expression levels of *TLR2*, *TLR4* and *NF-κB*. Based upon the changes in TLR signaling pathway, we further detected protein expression levels of cytokines in the ileum (Fig. 6). Compared with the control diet, LCPA diet increased ($P < 0.05$) protein expression levels of IL-6 and IL-22, LCPC diet

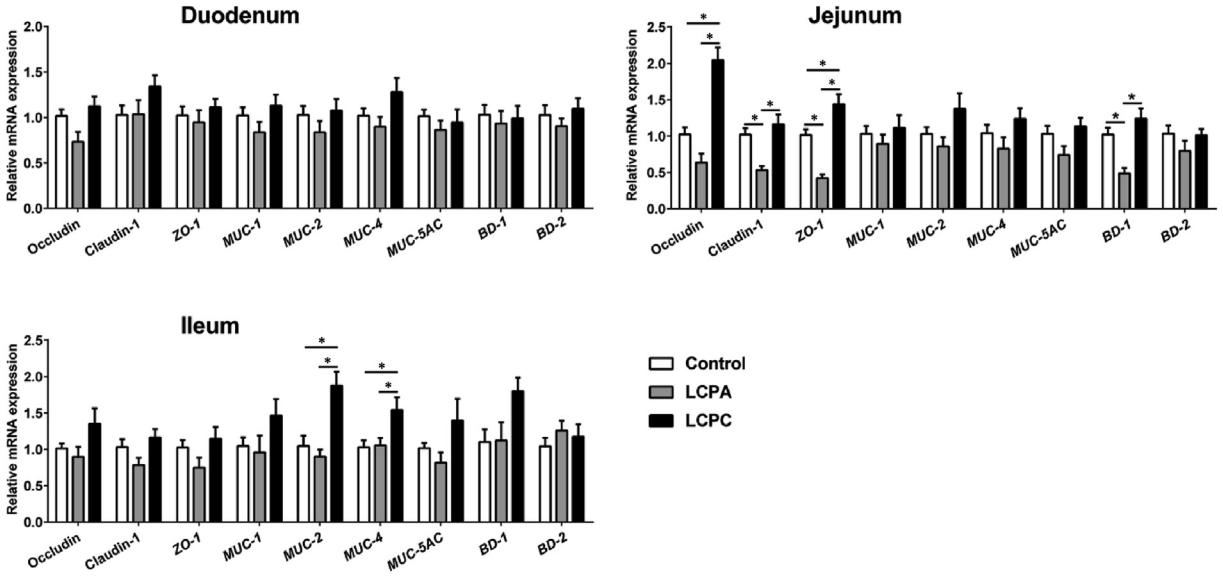

Fig. 1. Gene expression levels of small intestinal barrier factors in pigs. Values in the histogram are means ± SEM ($n = 7$), *$P < 0.05$. *MUC* = mucin; *ZO-1* = zonula occludens-1; *BD* = β-defensins; LCPA = low crude protein diets supplemented with amino acids; LCPC = low crude protein diets supplemented with casein hydrolysate.

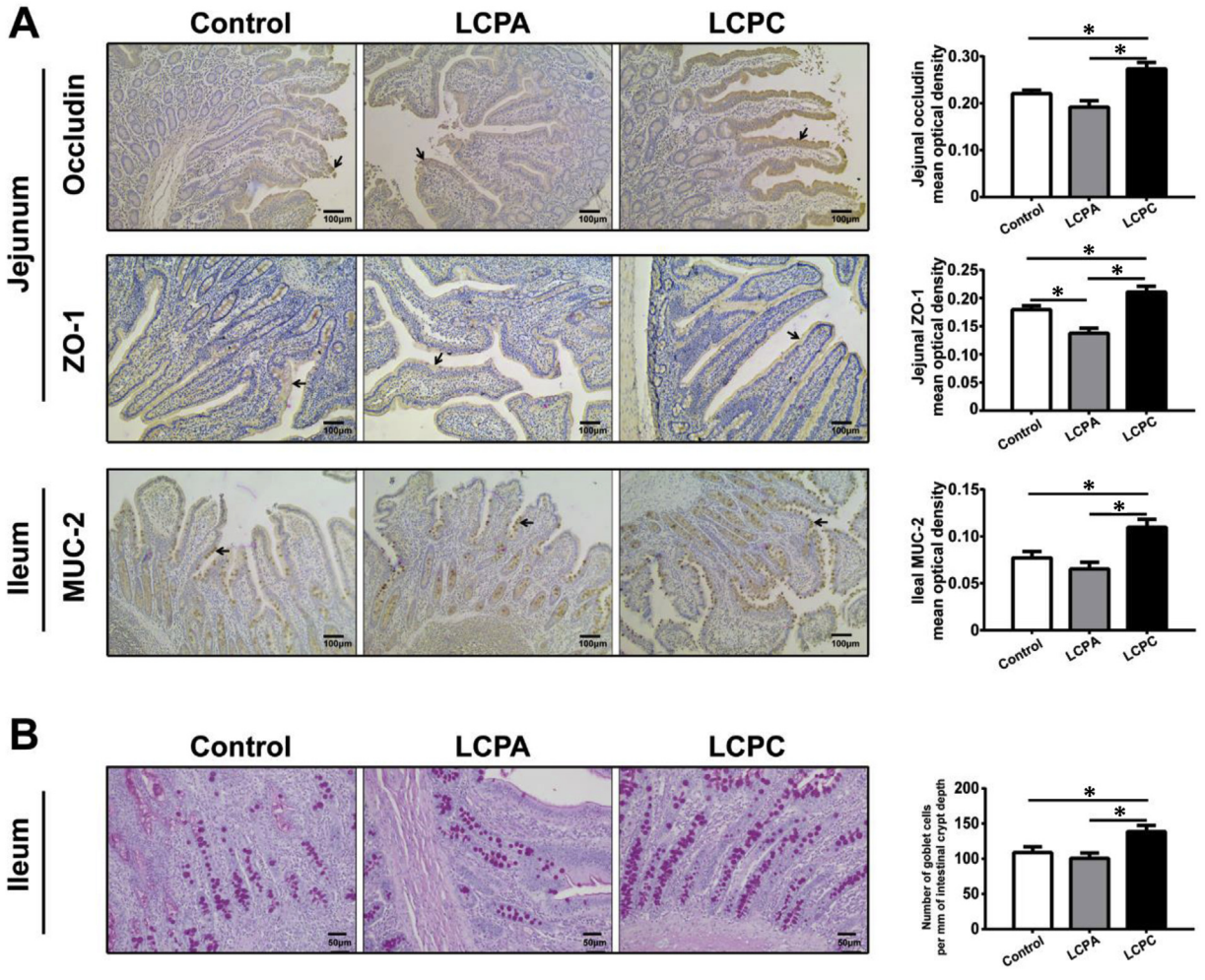

Fig. 2. Representative photos of immunohistochemical and periodic acid-Schiff (PAS) staining. (A) The immunohistochemical staining of zonula occludens-1 (ZO-1) and occludin in jejunum and mucin-2 (MUC-2) in ileum (magnification 100 ×). (B) The PAS staining of ileal tissues and the quantification of PAS signal. Values in histogram are means ± SEM ($n = 7$), *$P < 0.05$. LCPA = low crude protein diets supplemented with amino acids; LCPC = low crude protein diets supplemented with casein hydrolysate.

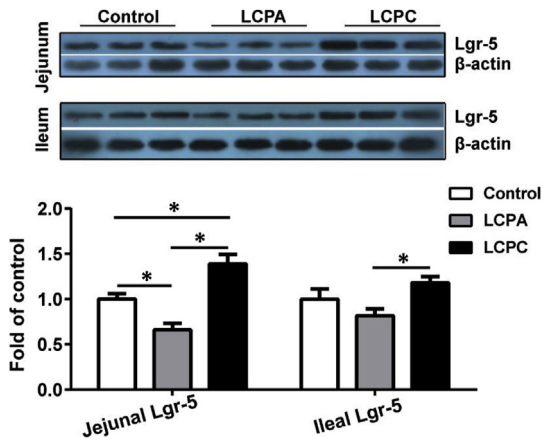

Fig. 3. Immunoblotting of leucine-rich repeat-containing G-protein-coupled receptor-5 (Lgr5) protein in jejunum and ileum of pigs. Values in histogram are means ± SEM ($n = 7$), *$P < 0.05$. LCPA = low crude protein diets supplemented with amino acids; LCPC = low crude protein diets supplemented with casein hydrolysate.

decreased ($P < 0.05$) protein expression levels of IL-1β, TNF-α and IL-17A. Compared with LCPA diet, LCPC diet decreased ($P < 0.05$) protein expression levels of IL-1β, TNF-α, IL-6, IL-17A and IL-22. These results indicate that LCPC diet decreased expressions of pro-inflammatory cytokines as compared with LCPA diet.

3.4. Correlation analysis of intestinal barrier, bacterial and immune parameters

The correlation analysis of intestinal barrier, bacterial and immune parameters is shown in Fig. 7. In LCPA group, gene and protein expression levels of ZO-1 were positively correlated ($P < 0.05$) with protein expression level of Lgr5 in the jejunum. Protein expression levels of Lgr5 and ZO-1 in the jejunum were positively correlated ($P < 0.05$) with ileal *Lactobacillus* counts. Gene expression level of *TLR2* was positively correlated ($P < 0.05$) with protein expression levels of IL-6 and IL-22 in the ileum.

In comparison with LCPA group, LCPC group showed more correlations between barrier function, microbes and immunity. Similar to in the LCPA group, in LCPC group, gene and protein expression levels of ZO-1 and occludin were positively correlated ($P < 0.05$) with protein expression level of Lgr5 in the jejunum. Protein expression levels of jejunal Lgr5, ZO-1 and occludin and ileal MUC-2 were positively correlated ($P < 0.05$) with ileal *Lactobacillus* counts. Furthermore, *Lactobacillus* and *Clostridium* cluster IV counts were negatively correlated ($P < 0.05$) with *E. coli* counts in the ileum. *E. coli* counts were positively correlated ($P < 0.05$) with gene expression levels of *NF-κB* and *TLR4* and protein expression levels of IL-1β and TNF-α in the ileum. The protein expression level of MUC-2 was negatively correlated ($P < 0.05$) with gene expression levels of *NF-κB* and *TLR4* in the ileum. Gene expression levels of *NF-κB* and *TLR4* were positively correlated ($P < 0.05$) with protein expression

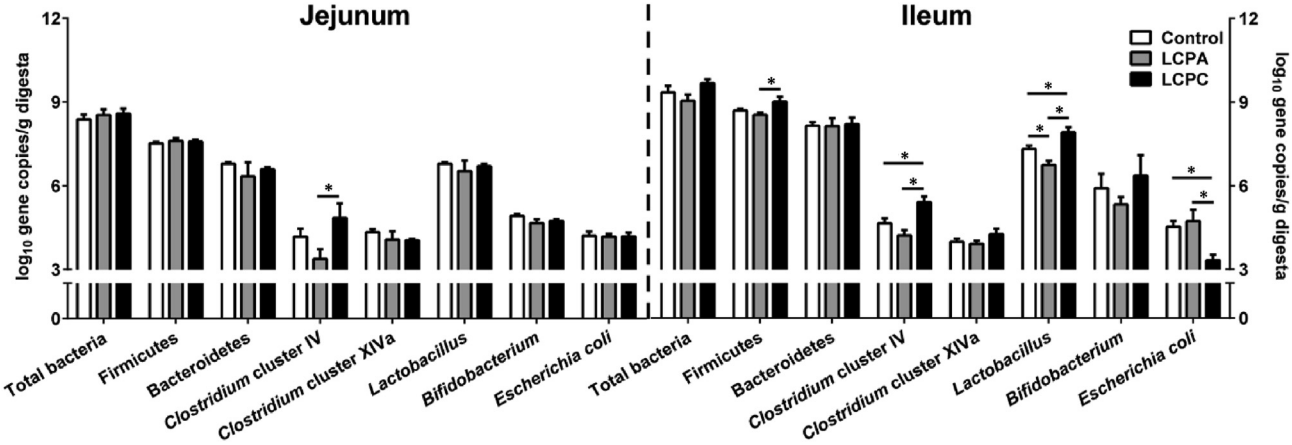

Fig. 4. The dominated bacterial taxonomic groups in jejunum and ileum of pigs. Values in the histogram are means ± SEM ($n = 7$), *$P < 0.05$. LCPA = low crude protein diets supplemented with amino acids; LCPC = low crude protein diets supplemented with casein hydrolysate.

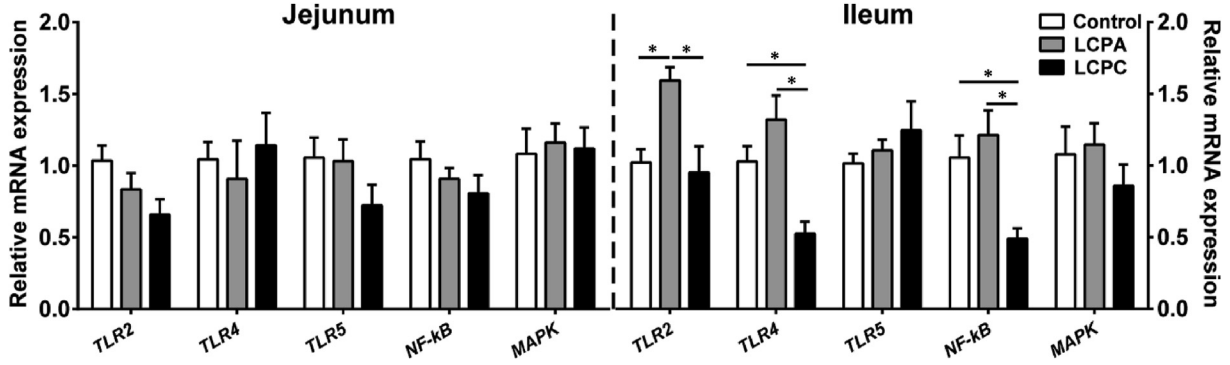

Fig. 5. Gene expression levels of toll-like receptors (*TLR*) and their downstream signaling pathways in jejunum and ileum of pigs. Values in the histogram are means ± SEM ($n = 7$), *$P < 0.05$. LCPA = low crude protein diets supplemented with amino acids; LCPC = low crude protein diets supplemented with casein hydrolysate; *NF-κB* = nuclear factor kappa B; *MAPK* = mitogen-activated protein kinase.

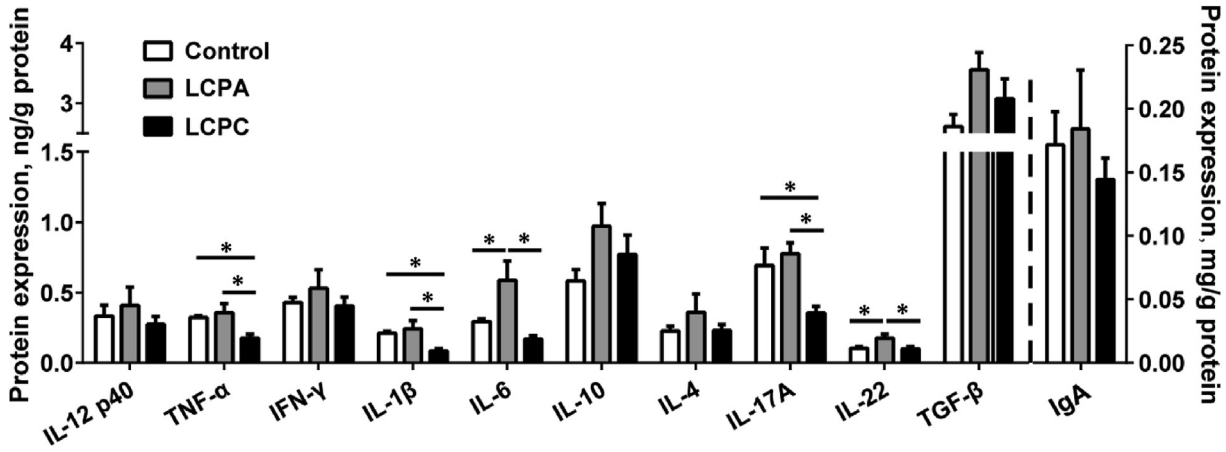

Fig. 6. Protein expression levels of ileal cytokines in pigs. Values in the histogram are means ± SEM ($n = 7$), *$P < 0.05$. LCPA = low crude protein diets supplemented with amino acids; LCPC = low crude protein diets supplemented with casein hydrolysate; IL = interleukin; TNF-α = tumor necrosis factor-α; IFN-γ = interferon-γ; TGF-β = transforming growth factor-β; IgA = immunoglobulin A.

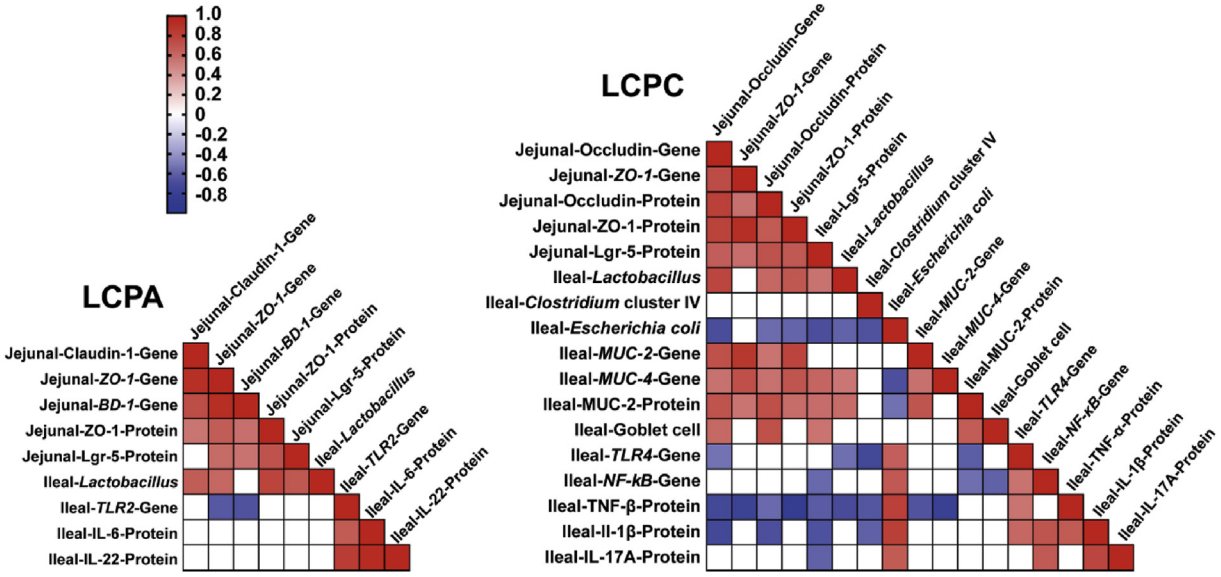

Fig. 7. Correlation analysis of intestinal barrier, stem cell proliferation and immune factors and dominated bacterial communities counts in jejunum and ileum. Only the significantly changed factors in LCPC and LCPA groups (vs. control) were presented. The blue represents the negative correlation ($P < 0.05$), the red represents the positive correlation ($P < 0.05$), and the white represents that the correlation was not significant ($P \geq 0.05$). LCPC = low crude protein diets supplemented with casein hydrolysate; LCPA = low crude protein diets supplemented with amino acids; ZO-1 = zonula occludens-1; *BD-1* = β-defensin-1; Lgr5 = leucine-rich repeat-containing G-protein-coupled receptor 5; IL = interleukin; *TLR* = toll-like receptor; MUC = mucin; TNF-α = tumor necrosis factor-α; NF-kB = nuclear factor kappa B.

level of IL-1β in the ileum. Altogether, these suggest that the changes in intestinal barrier factors were correlated with the alteration of the ISC proliferation factor. The alterations in ISC and intestinal barrier factors were correlated with the alterations of *Lactobacillus* counts. The decreased expression levels of pro-inflammatory factors were correlated with the reduced *E. coli* counts and the increased intestinal barrier factors expressions.

4. Discussion

Extremely LCP diets, even supplemented with essential and nonessential AA, can damage small intestinal development and health in pigs (Che et al., 2017; Deng et al., 2007; Peng et al., 2016). The present study showed that in comparison with AA supplementation, casein hydrolysate supplementation of extremely LCP diets to fill their AA gap with the AA requirement, favored the small intestinal barrier function, bacterial community, and immunity. Our findings highlight the essential importance of protein-derived peptides for small intestinal health in pigs fed extremely LCP diets.

In this study, 16% CP concentration in the control diet was a moderately low CP concentration for 20 to 50 kg pigs (the recommended CP concentration is 18% according to the NRC (2012)), which has been shown to reduce N excrement without damaging gut health and growth performance (Chen et al., 2018; Peng et al., 2017); 13% CP concentration in both LCPA and LCPC diets was extremely LCP concentration. The adverse changes in intestinal environment induced by LCPA diet were consistent with results of previous studies on extremely LCP diets (Peng et al., 2017; Fan et al., 2017; Che et al., 2017). On the contrary, the supplementation of casein hydrolysate in LCP diets showed beneficial effects on the intestinal health.

4.1. Casein hydrolysate supplementation enhanced the small intestinal barrier function in comparison with AA supplementation in low crude protein diets

Small intestinal barrier functions can be greatly affected by the dietary protein nutrition (Fan et al., 2017; Liu et al., 2017). Intestinal tight junctions, including occludin, claudin-1 and ZO-1, are responsible for maintaining the integrity of intestinal epithelium. In the present study, relative to the control group, the decreased expressions of ZO-1 and claudin-1 in LCPA group are likely due to the certainty that the dietary protein or peptide nutrition provided by LCPA diet is inadequate for maintaining the integrity of intestinal epithelium; casein hydrolysate supplementation may fill the gap of the nitrogen nutrition in LCP diets. An accumulation of data has indicated that casein-derived peptides have a potential promoting role in intestinal development and epidermal integrity by increasing the expressions of tight junction factors (Martínez-Augustin et al., 2014; Yasumatsu and Tanabe, 2010). Therefore, the adequate peptide nutrients in LCPC diet and the potential bioactive functions of casein-derived peptides may have contributed to the enhanced intestinal epidermal barrier, as reflected by increased expressions of occludin and ZO-1 in LCPC group compared with other 2 groups. In addition to the tight junctions, mucins are another important intestinal barrier factor. Mucins are secreted mainly by goblet cells distributed along the intestine and make up the skeleton of the intestinal mucus layer (Corfield, 2015). Indeed, in the present study, compared with LCPA group and control, LCPC diet showed greater numbers of goblet cells in the ileum, which may contribute to the increased expression of MUC-2 in the ileum.

The intestinal barrier function undergoes constitutive renewal, which can be driven by the proliferation of actively cycling Lgr5 ISC (daily intestinal maintenance) (Westphalen et al., 2014; Obata et al., 2018; Barker et al., 2007). Intestinal stem cell proliferation requires adequate dietary protein supply (Obata et al., 2018). In this study, relative to the control group, the decreased expression of jejunal Lgr5 in LCPA group suggests that the protein nutrient supply in LCPA diet may not be enough to meet the need of daily maintenance of ISC proliferation. Fan et al. (2017) reported that extremely LCP diets can decrease the protein expression levels of small intestinal stem cell proliferation factors in pigs. The decreased ISC proliferation may partly explain the decreased expressions of intestinal barrier factors in LCPA group. In contrast, the supplementation of casein hydrolysate in LCP diets may not only meet the requirement of protein or peptide nutrition for daily intestinal maintenance, but also exhibit a promoting effect on ISC proliferation, as reflected by the increased expression of jejunal Lgr5 in LCPC group compared with the other 2 groups. Further, the increased expression of Lgr5 may contribute to an increased expression of intestinal barrier factors in the LCPC group, and vice versa in the LCPA group; this was supported by correlation analysis that showed the positive correlations between Lgr5 and barrier function factors. However, whether the changed ISC proliferation played a decisive role in the changed barrier function remains to be further studied. Collectively, the supplementation of casein hydrolysate in LCP diets shows a beneficial effect on small intestinal barrier development, which may be associated with increased ISC proliferation.

4.2. Casein hydrolysate supplementation increased Lactobacillus counts and decreased E. coli counts in the small intestine in comparison with AA supplementation in low crude protein diets

Dietary protein composition can greatly influence the intestinal bacterial community. *Lactobacillus* are important members of commensal bacteria in the small intestine and are regarded as beneficial bacteria to maintain/promote the intestinal bacterial community homeostasis. LCPC diet may promote the bacterial community homeostasis, as reflected by the increase of *Lactobacillus* counts in LCPC group compared with the control group, while LCPA diet showed an opposite effect with a decrease of *Lactobacillus* counts. *Lactobacillus* can preferentially utilize exogenous peptides for protein synthesis, as they have a limited ability to hydrolyze intact protein (Pridmore et al., 2004; Zhang et al., 2011; Davila et al., 2013) and have the kinetic advantages of peptide-uptake systems (Davila et al., 2013). Therefore, relative to both the control and LCPA groups, the increased counts of *Lactobacillus* in LCPC group may be associated with increased available peptides. Moreover, given the preferential utilization of *Lactobacillus* to the peptide, we speculate that the increased *Lactobacillus* count could also happen in response to other hydrolyzed protein (peptides source); which, of course, needs to be studied further.

In addition to *Lactobacillus*, *Clostridium* are also important intestinal commensal bacteria in pigs. The mechanisms responsible for the increase of *Clostridium* cluster IV counts in LCPC group, relative to the control and LCPA groups, are unknown, but the increase of *Clostridium* cluster IV may also benefit the intestinal environment as this bacterial group has been regarded to be beneficial to gut health (Lopetuso et al., 2013; Atarashi et al., 2011). These commensal or generally beneficial bacteria can competitively colonize gut adhesion sites, utilize nutrients, and produce lactic acid; these effects can effectively restrict the growth of opportunistic pathogens. Consistently, in this study, *Lactobacillus* and *Clostridium* cluster IV counts were negatively correlated with *E. coli* counts as revealed by correlation analysis; therefore, the increase of commensal *Lactobacillus* and *Clostridium* cluster IV may be one of the contributing factors for the decrease of *E. coli* in LCPC group. Furthermore, *Lactobacillus* spp. can stimulate the proliferation of ISC via NADPH oxidase 1 mediated generation of reactive oxygen species in mice (Jones et al., 2014). *Lactobacillus* spp. have also been shown to increase mucin expression in vitro (pig intestinal cell line IPEC-J2) (Liu et al., 2010) and in pigs (Yi et al., 2018; Liu et al., 2013). In the present study, *Lactobacillus* were positively correlated with Lgr5 and MUC-2; therefore, the increased *Lactobacillus* counts in LCPC group may partly contribute to the enhanced intestinal barrier development. Collectively, the supplementation of casein hydrolysate in LCP diets shows beneficial effects on the small intestinal bacterial community, which could contribute to the small intestinal health.

4.3. Casein hydrolysate supplementation decreased the pro-inflammatory cytokines expressions in comparison with AA supplementation in low crude protein diets

Dietary protein composition can affect intestinal immunity. Cytokines are important signal molecules for regulating the immune cells, and their expression can represent the immune response status. TNF-α and IL-1β belong to the pro-inflammatory Th1-type cytokine and IL-17A and IL-22 are typical Th17 cytokines which are major contributors to intestinal mucosal inflammation (Bettelli et al., 2008). Although IL-6 is a Th2 cytokine, it plays an important role in promoting the Th17 immune response (Korn et al., 2008; Zhou et al., 2007). Relative to the control group, the increased expression of IL-6 and IL-22 in LCPA group suggests an increased risk of intestinal inflammation. But this potential risk was reduced with LCPC diet, as reflected by the decreased expression of IL-17A, TNF-α and IL-1β.

The decreased expressions of pro-inflammatory Th1- and Th17-cytokines in LCPC group may be associated with the inhibited TLR4-NF-κB signaling pathway; this was supported by correlation

analysis that showed the positive correlations between pro-inflammatory cytokines and TLR4-NF-κB. The pattern recognition receptor signal comes from its binding to the specific ligands. Lipopolysaccharide, the main cell wall constituent of *E. coli*, is a major ligand for TLR4. Moreover, the intestinal mucus layer can spatially segregate the luminal antigen ligands from the epithelial immune receptors to avoid unnecessary immune signaling. In this study, NF-κB and TLR4 were positively correlated with *E. coli* and negatively correlated with MUC-2 as revealed by correlation analysis. Therefore, the decreased expressions of TLR4-NF-κB in the LCPC group may also be contributed to the decreased *E. coli* counts and the increased MUC-2 expression. Collectively, the supplementation of casein hydrolysate in LCP diets shows benefits for the small intestinal immune response homeostasis, which may be associated with the enhanced intestinal barrier and the reduced *E. coli* counts.

In the present study, compared with AA supplementation, the supplementation of casein hydrolysate as a peptide source may have filled the gap of the protein or peptide nutrition in extremely LCP diets, which benefited the small intestinal barrier, bacterial community, and immunity. Moreover, the bioactive functions of many peptides may also have contributions to the intestinal health. Thus, we believe that the beneficial effects of LCPC diet, compared with LCPA diet, comes mainly from the supplementation of peptide nutrition under extremely LCP diets. Therefore, we speculate that with extremely LCP diets, other peptides sources (e.g., soy or whey protein hydrolysate, etc.) supplementation may also have a beneficial effect on intestinal health as compared with AA supplementation. Nevertheless, further research is needed to support this viewpoint.

5. Conclusions

In comparison with the 16% CP diet, the 13% CP diet supplemented with AA to fully fill the AA gap with the AA requirement, detrimentally affected the small intestinal barrier function and immunity, whereas the 13% CP diet supplemented with casein hydrolysate favored the small intestinal barrier function, bacterial community, and immunity in pigs. These observations provide novel insights into the role of peptides in the nutritional intervention in pigs, suggesting that a certain quantity of protein-derived peptides is critically necessary for maintaining or even favoring small intestinal health in pigs under extremely low crude protein diets.

Author contributions

Huisong Wang: conceptualization, methodology, investigation, formal analysis, writing - original draft, writing - review & editing; **Junhua Shen**: conceptualization, investigation, formal analysis, writing - review & editing; **Chunlong Mu**: writing - review & editing; **Kan Gao**: investigation; **Yu Pi**: investigation; **Weiyun Zhu**: conceptualization, methodology, funding acquisition, supervision, writing - review, editing and finalizing manuscript.

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.

Acknowledgments

This study was supported by Natural Science Foundation of China (31430082) and National Key Basic Research Program of China, 973 Program (2013CB127300).

Appendix

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2021.03.003.

References

Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, et al. Induction of colonic regulatory T cells by indigenous Clostridium species. Science 2011;331:337–41.
Barker N, Van Es J, Kuipers J, Kujala P, van den Born M, Cozijnsen M, et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 2007;449:1003–7.
Bettelli E, Korn T, Oukka M, Kuchroo V. Induction and effector functions of TH17 cells. Nature 2008;453:1051–7.
Che L, Peng X, Hu L, Wu C, Xu Q, Fang ZF, et al. The addition of protein-bound amino acids in low-protein diets improves the metabolic and immunological characteristics in fifteen- to thirty-five-kg pigs. J Anim Sci 2017;95:1277–87.
Chen X, Song P, Fan P, He T, Jacobs D, Levesque CL, et al. Moderate dietary protein restriction optimized gut microbiota and mucosal barrier in growing pig model. Front Cell Infect Microbiol 2018;8:246.
Corfield A. Mucins: a biologically relevant glycan barrier in mucosal protection. Biochim Biophys Acta 2015;1850:236–52.
Davila A, Blachier F, Gotteland M, Andriamihaja M, Benetti PH, Sanz Y, et al. Intestinal luminal nitrogen metabolism: role of the gut microbiota and consequences for the host. Pharmacol Res 2013;68:95–107.
Deng D, Huang R, Li T, Wu G, Xie M, Tang Z, et al. Nitrogen balance in barrows fed low-protein diets supplemented with essential amino acids. Livest Sci 2007;109:220–3.
Fan P, Liu P, Song P, Chen X, Ma X. Moderate dietary protein restriction alters the composition of gut microbiota and improves ileal barrier function in adult pig model. Sci Rep 2017;7:43412.
Hou Y, Wu Z, Dai Z, Wang G, Wu G. Protein hydrolysates in animal nutrition: industrial production, bioactive peptides, and functional significance. J Anim Sci Biotechnol 2017;8:24.
Hughes D. Diet and the maturation of the immune system. Food Agric Immunol 1999;11:279–85.
Jones RM, Luo L, Ardita CS, Richardson AN, Kwon YM, Mercante JW, et al. Symbiotic lactobacilli stimulate gut epithelial proliferation via Nox-mediated generation of reactive oxygen species. EMBO J 2014;32:3017–28.
Kerr BJ, Ziemer CJ, Trabue SL, Crouse JD, Parkin TB. Manure composition of swine as affected by dietary protein and cellulose concentrations. J Anim Sci 2006;84:1584–92.
Korn T, Mitsdoerffer M, Croxford A, Awasthi A, Dardalhon V, Galileos G, et al. IL-6 controls Th17 immunity in vivo by inhibiting the conversion of conventional T cells into Foxp3 regulatory T cells. Proc Natl Acad Sci U S A 2008;105:18460–5.
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;26:91–8.
Liu F, Li G, Wen K, Wu S, Zhang Y, Bui T, et al. *Lactobacillus rhamnosus* GG on rotavirus-induced injury of ileal epithelium in gnotobiotic pigs. J Pediatr Gastroenterol Nutr 2013;57:750–8.
Liu F, Li G, Wen K, Bui T, Cao D, Zhang Y, et al. Porcine small intestinal epithelial cell line (IPEC-J2) of rotavirus infection as a new model for the study of innate immune responses to rotaviruses and probiotics. Viral Immunol 2010;23:135–49.
Liu Y, Wang X, Hou Y, Yin Y, Qiu Y, Wu G, et al. Roles of amino acids in preventing and treating intestinal diseases: recent studies with pig models. Amino Acids 2017;49:1277–91.
Lopetuso L, Scaldaferri F, Petito V, Gasbarrini A. Commensal Clostridia: leading players in the maintenance of gut homeostasis. Gut Pathog 2013;5:23.
Malinowski J, Klempt M, Clawin-Rädecker I, Lorenzen PC, Meisel H. Identification of a NFκB inhibitory peptide from tryptic β-casein hydrolysate. Food Chem 2014;165:129–33.
Martínez-Augustin O, Rivero-Gutiérrez B, Mascaraque C, Sánchez de Medina F. Food derived bioactive peptides and intestinal barrier function. Int J Mol Sci 2014;15:22857–73.
Monchi M, Rérat AA. Comparison of net protein utilization of milk protein mild enzymatic hydrolysates and free amino acid mixtures with a close pattern in the rat. JPEN J Parenter Enteral Nutr 1993;17:355–63.
Mu C, Yang Y, Zhen L, Zhu W. Temporal microbiota changes of high-protein diet intake in a rat model. Anaerobe 2017;47:218–25.
Neurath MF, Finotto S, Glimcher LH. The role of Th1/Th2 polarization in mucosal immunity. Nat Med 2002;8:567–73.
NRC. Nutrient requirements of swine. 11th ed. Washington, DC: Natl Acad Press; 2012.

Obata F, Tsudasakurai K, Yamazaki T, Nishio R, Nishimura K, Kimura M, et al. Nutritional control of stem cell division through s-adenosylmethionine in drosophila intestine. Dev Cell 2018;44:741–51.
Peng X, Hu L, Liu Y, Yan C, Fang ZF, Lin Y, et al. Effects of low-protein diets supplemented with indispensable amino acids on growth performance, intestinal morphology and immunological parameters in 13 to 35 kg pigs. Animal 2016;10:1812–20.
Peng Y, Yu K, Mu C, Hang S, Che L, Zhu W. Progressive response of large intestinal bacterial community and fermentation to the stepwise decrease of dietary crude protein level in growing pigs. Appl Microbiol Biotechnol 2017;101:5415–26.
Pi Y, Mu C, Gao K, Liu Z, Peng Y, Zhu W. Increasing the hindgut carbohydrate/protein ratio by cecal infusion of corn starch or casein hydrolysate drives gut microbiota-related bile acid metabolism to stimulate colonic barrier function. mSystems 2020;5. e00176-20.
Pridmore RD, Berger B, Desiere F, Vilanova D, Barretto C, Pittet AC, et al. The genome sequence of the probiotic intestinal bacterium *Lactobacillus johnsonii* NCC 533. Proc Natl Acad Sci U S A 2004;101:2512–7.
Rérat A, Simoes-Nuñes C, Mendy F, Vaissade P, Vaugelade P. Splanchnic fluxes of amino acids after duodenal infusion of carbohydrate solutions containing free amino acids or oligopeptides in the non-anaesthetized pig. Br J Nutr 1992;68:111–38.
Silk DB, Chung YC, Berger KL, Conley K, Beigler M, Sleisenger MH, et al. Comparison of oral feeding of peptide and amino acid meals to normal human subjects. Gut 1979;20:291–9.
Wang H, Shen J, Pi Y, Gao K, Zhu W. Low-protein diets supplemented with casein hydrolysate favor the microbiota and enhance the mucosal humoral immunity in the colon of pigs. J Anim Sci Biotechnol 2019;10:79.
Westphalen C, Asfaha S, Hayakawa Y, Takemoto Y, Lukin DJ, Nuber AH, et al. Long-lived intestinal tuft cells serve as colon cancer-initiating cells. J Clin Invest 2014;124:1283–95.
Wu G. Intestinal mucosal amino acid catabolism. J Nutr 1998;128:1249–52.
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;1:170–6.
Yasumatsu H, Tanabe S. The casein peptide Asn-Pro-Trp-Asp-Gln enforces the intestinal tight junction partly by increasing occludin expression in Caco-2 cells. Br J Nutr 2010;104:951–6.
Yi H, Wang L, Xiong Y, Wen X, Wang Z, Yang X, et al. Effects of *Lactobacillus reuteri* LR1 on the growth performance, intestinal morphology, and intestinal barrier function in weaned pigs. J Anim Sci 2018;96:2342–51.
Zhang C, Yu M, Yang Y, Mu C, Su Y, Zhu W. Differential effect of early antibiotic intervention on bacterial fermentation patterns and mucosal gene expression in the colon of pigs under diets with different protein levels. Appl Microbiol Biotechnol 2017;101:2493–505.
Zhang Q, Ren J, Zhao H, Zhao M, Xu J, Zhao Q. Influence of casein hydrolysates on the growth and lactic acid production of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. Int J Food Sci Technol 2011;46:1014–20.
Zhou L, Ivanov I, Spolski R, Min R, Shenderov K, Egawa T, et al. IL-6 programs TH-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. Nat Immunol 2007;8:967–74.
Zhong X, Yu J, Frazier K, Weng X, Li Y, Cham C, et al. Circadian clock regulation of hepatic lipid metabolism by modulation of m6A mRNA methylation. Cell Rep 2018;25:1816–28.