Effects of dietary supplementation with the antimicrobial peptide WK3 on growth performance and intestinal health in diarrheic weanling piglets

Chunyu Cao*, Jianan Li*, Qiuyuan Ma, Licong Zhang and Anshan Shan

Institute of Animal Nutrition, Northeast Agricultural University, Harbin, People’s Republic of China

ABSTRACT
This study aimed to determine the effects of the antimicrobial peptide WK3, as an alternative to antibiotics, on diarrheic piglets. Before treatment, all pigs were orally challenged with 10⁸ CFU/ml enterotoxigenic Escherichia coli (ETEC) K88 for 3 days. Piglets were randomly divided into 3 treatments of eight piglets each, namely, the control treatment, antibiotic treatment and antimicrobial peptide treatment. The experimental results show that the addition of the antibacterial peptide WK3 to the diet can significantly reduce the chance of diarrhea. Compared with the CON group, average daily gain (ADG) and average daily feed intake (ADFI) of the WK3 group (P < 0.05) increased. Compared with that with the control treatment and antibiotic treatment, the level of GSH-Px in the jejunum significantly increased (P < 0.05) with WK3 supplementation. The numbers of bacteria, Lactobacillus spp. (P < 0.01) and Bifidobacteria spp. (P < 0.01), were higher in the WK3 group, but Enterobacterium spp. in digesta of the cecum were depleted (P < 0.01). The WK3 group decreased the expression levels of the inflammatory factors IL-1α (P < 0.05) and TLR-4 (P < 0.01) in the jejunal mucosa contrast to the control treatment.

Introduction
Piglets face various types of stressors that decrease their resistance to pathogens, making them susceptible to various diseases after weaning. Postweaning diarrhea is a difficult problem for piglet breeding that is caused by Escherichia coli and results in a decrease in growth performance (Rhouma et al. 2016). Therefore, it is necessary to search for possible methods to control the negative effects of diarrhea in piglets. To decrease the susceptibility of piglets to diarrhea, antibiotics are widely used (Sen et al. 2012). Nevertheless, indiscriminate use of antibiotics has resulted in the emergence of bacterial resistance and drug residues in animal-derived products, thereby endangering human health (Yoon et al. 2012). In response to this situation, various antibiotic substitutes have been developed.

Antimicrobial peptides (AMPs), also referred to as host defense peptides, are small peptides with broad-spectrum antimicrobial activity against bacteria, viruses, fungi, cancer cells, and parasites and are important components of biological innate immunity (Lai and Gallo 2009; Thennarasu et al. 2005). The antimicrobial activity of AMPs is particularly related to their amino acid composition and physical and chemical properties, such as positive net charge, flexibility, size, amphipathicity, and hydrophobicity (da Cunha et al. 2017). The mechanism of action of AMPs against pathogens involves mainly membrane permeabilization, which completely differs from the mechanism of antibiotics (Sierra et al. 2017); thus, bacterial resistance is highly unlikely to develop. It has been reported that antimicrobial peptides play an active role in growth performance, the intestinal flora, immune function, nutrient digestibility and intestinal morphology (Pié et al. 2004; Tossou et al. 2016; Wu et al. 2012; Yi et al. 2016., Yu et al. 2017). A battery of linear trpzip-like β-hairpin antimicrobial peptides was prepared by positioning paired Trp residues at non-hydrogen-bonded sites and Lys residues at hydrogen-bonded sites on the basis of the sequence template (WK)nDPG(KW)n-NH2 (n = 1, 2, 3, 4 or 5). Antimicrobial peptides for which n = 3 have the best killing effect against both gram-negative bacteria and gram-positive bacteria. WK3 is composed of 14 amino acids and has good antibacterial activity and protease stability, and its hemolytic activity and cytotoxicity are low (Xu et al. 2015).

E. coli is a predominant species of facultative anaerobe in the gut of pigs that can cause diarrhea (Li et al. 2016). Enterotoxigenic E. coli (ETEC) K88 is one of the major pathogens causing diarrhea in piglets, colonizes the small intestine, and releases enterotoxins to induce liquid secretion into the lumen of the gut through stimulating epithelial cells, thereby causing diarrhea (Wu et al. 2012). Piglets can no longer obtain passive immunity from sows after weaning, which increases their susceptibility to enterotoxigenic E. coli infections. In this research, ETEC K88 was used to create a pig model of inflammation to investigate whether the
antimicrobial peptide WK3 had antibacterial activity in vivo and affected both intestinal health and growth performance, and it provided a theoretical basis for antimicrobial peptides to replace antibiotics.

**Material and methods**

All animal experiments were performed in accordance with the guidelines for the care and use of laboratory animals approved by the Institutional Animal Care and the institutional Committee (2011–19).

Random allocation was performed to divide 24 pigs into three groups. The treatments included a corn-soybean meal-based basal diet that did not contain any antibiotics or medicine (CON), and similar diets were supplemented with antibiotics (CC, basal diet + 500 mg/kg chlorotetracycline + 200 mg/kg colistin sulfate) or an antimicrobial peptide (WK3, basal diet + 50 mg/kg WK3). Twenty-four Duroc × Landrace × Yorkshire barrows (6 ± 1 kg) were from a local commercial swine farm (Longitude 125° East and Latitude 44° North) (Harbin, China). The piglets were weaned at 21 d of age. Before the experiment, the houses and cages were thoroughly cleaned and sterilized. Individual metabolic cages were used for housing all piglets in a temperature-controlled nursery room (23–25°C). After an acclimation period of 4 d, all piglets were infected orally with 100 mL of 10^8 CFU/mL ETEC K88 for 3 d before the formal test. Piglet diarrhea was observed and recorded. The model was established successfully since all the piglets became diarrheic.

Based on previous experimental data, the level of WK3 supplementation was chosen, and analysis of its progression point showed that supplementation with WK3 at a dose of 50 mg/kg may increase body weight (unpublished data). The treatment lasted for 6 d. Diets were formulated according to the National Research Council 2012 requirements for various nutrients (Table 1). Feed and water were allowed on an ad libitum basis. Growth performance results, such as ADG, ADFI, and F:G, were determined for each pen.

The antimicrobial peptide WK3 was synthesized by solid-phase chemical synthesis by Shanghai (China). Reverse antibacterial peptide synthesis was performed after reversed-phase high-performance liquid chromatography (RP-HPLC) and electrospray mass ionization spectrometry (ESI-MS) for the purification and identification of peptide molecules. The peptides were then stored at -20°C.

ETEC K88 was obtained from Beijing (China). The bacteria were resuscitated with shaking in 3 mL of Luria-Bertani (LB) medium at 37°C for 24 h and then plated onto LB agar. A single colony was inoculated in 50 mL of Luria Bertani (LB) medium. Incubation at 37°C and 250 RPM overnight was applied to cultivate dilutions on LB agar in order to determine bacterial counts. A final concentration of 10^8 CFU/mL was used in this experiment.

All piglets were weighed at the beginning of the experiment, and the same was done at the end of the experiment. Feed intake was recorded daily during the whole experimental period. Piglets were anesthetized by intravenous injection of pentobarbital sodium (50 mg/kg BW), and then bloodletting was performed. Approximately 20 cm of the mid-jejunum was longitudinally cut open, and precooled saline was flushed over the tissue. Slides were used to scrape down quickly over the intestinal mucous membrane, frozen in liquid nitrogen, and then transferred to a -80°C freezer. The remaining jejunum tissue samples were stored in a freezer at -20°C for determination of the antioxidant index. Approximately 2 g of the cecum contents were packed into a 1.5 ml EP tube, rapidly frozen with liquid nitrogen, and then transferred to a freezer at -80°C for storage. Intestinal microorganisms were tested. The contents of approximately 2-cm duodenal, jejunal and ileum segments were removed and fixed in 10% formaldehyde fixative solution, which was used to make intestinal tissue slices.

The diarrheal index was scored according to a fecal consistency scoring system (0, normal; 1, soft feces; 2, mild diarrhea; and 3, severe diarrhea). A fecal score of 2 or 3 was considered clinical diarrhea (Bhandari et al. 2008). The diarrhea index = the sum of diarrhea scores for each group of piglets during the trial period/(the number of days tested × the number of piglets per group); the diarrhea incidence (%) = the number of piglets with diarrhea per treatment during the trial period/ (the number of days tested × the number of piglets per group) × 100.

The duodenum, jejunum and ileum were fixed with 10% formaldehyde fixative solution, and after washing, transparency induction, waxing, paraffin embedding, trimming, sectioning and dewaxing, hematoxylin eosin staining, dehydration and sectioning were carried out. Observation was carried out under an optical microscope (EVOS, USA). A typical visual field (i.e., 5 visual fields with complete villi and a straight morphology) was selected from each intestinal tissue section to take photos. Image software, Micron (EVOS, v2.0), was used to measure and analyze villous height and crypt depth, and the ratio of villous height to crypt depth was calculated. Villous height is the midpoint distance where the fluffy and

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Table 1. Composition and nutrient levels of basal diet (as-fed basic).

| Item                  | Ingredient, % | Nutritional content, % | DE (Kcal/kg) |
|-----------------------|---------------|------------------------|--------------|
| Corn                  | 69.56         |                       |              |
| Soybean meal          | 17.65         |                       |              |
| Fish meal             | 3.0           |                       |              |
| Soybean oil           | 1.5           |                       |              |
| Wheat bran            | 5.0           |                       |              |
| Dicalcium phosphate   | 0.8           |                       |              |
| Limestone             | 0.78          |                       |              |
| Salt                  | 0.35          |                       |              |
| L-Lysine-HCl, 98%     | 0.26          |                       |              |
| Vitamin and mineral premix¹ | 1.0 |                       |              |
| Choline chloride      | 0.1           |                       |              |
| Nutritional content, % |              |                       |              |
| DE (Kcal/kg)          | 3330          |                       |              |
| ME (Kcal/kg)          | 3080          |                       |              |
| Crude protein (CFP)   | 16.65         |                       |              |
| Calcium               | 0.65          |                       |              |
| Total phosphorus      | 0.56          |                       |              |
| Lysine                | 1.06          |                       |              |
| Methionine            | 0.28          |                       |              |
| Lysine + Methionine   | 0.55          |                       |              |

¹Provided the following per kilogram of diet: vitamin A, 11,000 IU; vitamin D3, 1100 IU; vitamin E, 16IU; vitamin K, 1 mg; vitamin B1, 0.6 mg; vitamin B2, 0.03 mg; pantothenic acid, 6 mg; nicotinic acid, 10 mg; Zn (ZnSO4), 95 mg; Fe (FeSO4), 100 mg; Cu (CuSO4), 16.5 mg; Mn (MnSO4), 3.7 mg; KI (Kl), 140 μg; Se (Na2SeO3), 286 μg.

Nutrient levels were calculated values.
Table 2. Primers used for quantitative real-time PCR to detect bacterial numbers.

| Targeted bacterial group (amplicon size) | Primer sequence (5'-3') | Annealing temperature (°C) |
|-----------------------------------------|--------------------------|---------------------------|
| Total eubacteria (200 bp)               | F:CCGGC(T/T)CCAGACTCTCTCCGGG  | 58                        |
| Lactobacillus spp. (341 bp)             | R:CTCTACGAGACTCAAGCTTGCGG  | 62                        |
| Enterobacteriaceae spp. (195 bp)        | R:CCACATCAGG(A/G)ATCCAC   | 60                        |
| Bifidobacteria spp. (243 bp)            | F:CTGCGTCGTCGTGAAGAG      | 58                        |
| Enterococcus spp. (144 bp)              | F:CCCATATTTGTCGTGCACTATT  | 60                        |

F: Forward primer, R: Reverse primer.

Table 3. Sequences of the primers used for quantitative real-time PCR.

| Genes1 | GenBank number | Primer sequence (5'-3') | Product length (bp) |
|--------|----------------|--------------------------|---------------------|
| IL-1α  | NM_214029      | F:CTGGAAGAAGACGGGTAGGAGG | 162                 |
| IL-1β  | NM_214055      | R:GGAGTCTGGGTAGTGAG     | 70                  |
| IL-8   | NM_213867      | R:GTCCATCTCATCATTGCCCTTC | 163                 |
| β-actin| AY550069       | R:ATGCTCTAGGGGGACTGCT    | 211                 |
| TLR-4  | NM_0012933161  | R:CCATCACGACCTGTTGCATT   | 113                 |
| Occludin| NM_001163647.2| R:GAAGTACTTGCGCTGAG      | 173                 |
| Claudin-4| NM_001161637.1| R:ACACAGCTGAGGCTGAGTCT   | 109                 |
|ZO-1    | XM_005659811.1 | R:GCTCTTGGCGGACTGTTGAG   | 217                 |

1: IL-1α, interleukin-1α; IL-1β, interleukin-1β; IL-8, interleukin-8; TLR-4, Toll-like receptors 4; ZO-1, zonula occludens-1.
2: F: Forward primer, R: Reverse primer.

fossae villi join on both ends (crypt - villous junctions), and crypt depth is the midpoint of where crypt villi join on both ends and the distance from mucosa to the basal level.

Analysis of the jejunal antioxidant index proceeded as follows. Approximately 1 g of jejunum tissue was placed in 10 mL EP tubes, 9 mL of physiological saline homogenate was added, and a 10% tissue homogenate was made. The solution was centrifuged at 3500 rpm for 10 min, the supernatant was removed, and the concentrations of malondialdehyde (MDA), the activity of total superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) and total antioxidant capacity (T-AOC) were measured. All antioxidant parameters were measured by assay kits supplied by Nanjing Jiancheng Bioengineering Institute, following the manufacturer’s instructions.

Piglet cecum content DNA was extracted with an E.Z.N.A. stool DNA kit from OMEGA Inc. (USA). A NanoPhotometer-P330 spectrophotometer (Implen GmbH, Munich, Germany) was used to assess the quantity and quality of DNA. According to the microbial 16S RNA operon gene sequence published in GenBank, primers were designed and synthesized by Sangon Bioengineering (Shanghai) Co., Ltd. (Table 2). The obtained PCR product was purified, and an E.Z.N.A. cycle-pure kit was purchased from OMEGA (USA). The test procedures were conducted in strict accordance with the kit instructions. The concentration of the purified product was determined, the product was diluted by a gradient, and then PCR was performed. A standard curve was made according to the PCR amplification results of a standard substance with a known gradient, and the starting copy number of the sample was calculated according to the Ct value of the sample.

Jejunum mucosa samples were taken from the freezer at -80°C and placed in liquid nitrogen. Approximately 0.1 g was quickly ground in a mortar and placed in a 1.5 mL EP tube for RNA extraction. An E.Z.N.A. cetyl-pure Kit was purchased from OMEGA (USA). RNA was extracted and then reverse transcribed. A kit was purchased from Bao Biological Reagent Company (Bao Biological Reagent Co., Ltd., China) to produce cDNA, and the cDNA was used for RT-PCR. A SYBR Green I RT-PCR Kit (TaKaRa® Bio) was used to measure the mRNA expression of cytokines (IL-1α, IL-1β, IL-8, TLR-4, Occludin, Claudin-4, and ZO-1) relative to the expression of the β-actin endogenous control. Specific primers were designed by using Primer Express® software (Applied Biosystems) and were synthesized by Shanghai Sangon Biological Engineering Co., Ltd. (Table 3). Relative gene expression levels were determined by the 2^-ΔΔCt method.

For data (n = 8), individually slaughtered piglets were considered the experimental unit for statistical analysis. The test data were first collated and calculated by Microsoft Excel 2003, then one-way ANOVA was performed by SPSS 19.0 statistical software (Chicago, USA), and the Duncan method was used for multiple comparisons. Tukey’s test was used to determine differences between means. The results were expressed as the mean ± SEM, with P < 0.05 as the criterion for judging
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Villus height to crypt depth

Crypt depth (Villus height (Duodenum

Table 4. Effects of WK3 on growth performance and diarrhea incidence of piglets.

| Item | CON | CC | WK3 | SEM | P-value |
|------|-----|----|-----|-----|---------|
| ADG (kg) | 0.23<sup>a</sup> | 0.43<sup>b</sup> | 0.35<sup>b</sup> | 0.289 | 0.006 |
| ADFI (kg) | 0.85<sup>a</sup> | 1.05<sup>b</sup> | 1.13<sup>b</sup> | 0.045 | 0.019 |
| F/G | 4.09<sup>b</sup> | 2.50<sup>b</sup> | 3.42<sup>ab</sup> | 0.244 | 0.016 |
| Diarrhea incidence (%) | 83.33 | 33.33 | 50 |

<sup>a,b</sup> means within a row with no common superscripts differ significantly (p < 0.05).

All the values are expressed as the means ± SEM.

1ADG, average daily weight gain; ADFI, average daily feed intake; F/G, feed conversion ratio.

Results

We obtained the effects of the antimicrobial peptide WK3 added to the diet on the villus height and crypt depth of piglets (Table 5). As seen from the table, compared with the control condition, dietary supplementation with WK3 significantly increased the height of ileum villi (P < 0.05) and the villus height-to-crypt depth ratio in the ileum (P < 0.05), significantly reduced the depth of ileum crypts (P < 0.05), and had no significant effect on the height of duodenum and jejunal villi, crypt depth or the ratio of the two (P > 0.05). Dietary supplementation with antibiotics significantly increased the height of the jejunal villi and villus height-to-crypt depth ratio in the ileum (P < 0.05). Compared with that in the antibiotic group, the villus height-to-crypt depth ratio in the ileum in the antimicrobial peptide group was significantly higher (P < 0.05), but there was no significant difference in ileal villus height or crypt depth (P > 0.05).

It is shown that dietary supplementation with the antimicrobial peptide WK3 in piglets increased GSH-Px activity in the jejunal mucosa of piglets (Figure 1). The ADG (P < 0.05) and ADFI (P > 0.01) or F/G (P > 0.05). Administration of WK3 effectively alleviated the incidence of diarrhea in ETEC K88-challenged piglets.

![Figure 1. Effects of WK3 on the mRNA expression of cytokines in the jejunal mucosa of piglets.](Image 319x83 to 553x213)

Figure 1. Effects of WK3 on the mRNA expression of cytokines in the jejunal mucosa of piglets. a, b means within a row with no common superscripts differ significantly (p < 0.05). IL-1α, interleukin-1α; IL-1β, interleukin-1β; IL-8, interleukin-8; TLR4, Toll-like receptors 4.

Table 5. Effects of WK3 on villous height and crypt depth in small intestine of piglets.

| Items | CON | CC | WK3 | SEM | P-value |
|------|-----|----|-----|-----|---------|
| Duodenum | | | | | |
| Villus height (µm) | 156.83 | 187.32 | 153.81 | 7.17 | 0.102 |
| Crypt depth (µm) | 108.27 | 111.67 | 91.40 | 5.96 | 0.351 |
| Villus height to crypt depth ratio | 1.46 | 1.83 | 1.78 | 0.14 | 0.529 |
| Jejunum | | | | | |
| Villus height (µm) | 174.39 | 203.44 | 189.21 | 8.80 | 0.429 |
| Crypt depth (µm) | 115.84 | 92.83 | 110.32 | 5.43 | 0.203 |
| Villus height to crypt depth ratio | 1.54<sup>a</sup> | 2.37<sup>b</sup> | 1.75<sup>ab</sup> | 0.16 | 0.094 |
| Ileum | | | | | |
| Villus height (µm) | 164.12<sup>ab</sup> | 189.78<sup>b</sup> | 194.68<sup>b</sup> | 5.25 | 0.027 |
| Crypt depth (µm) | 103.82<sup>a</sup> | 93.10<sup>ab</sup> | 77.43<sup>a</sup> | 3.91 | 0.011 |
| Villus height to crypt depth ratio | 1.59<sup>a</sup> | 2.07<sup>b</sup> | 2.59<sup>c</sup> | 0.13 | 0.001 |

<sup>a,b</sup> means within a row with no common superscripts differ significantly (p < 0.05).

All the values are expressed as the means ± SEM.

Results

We obtained the effects of WK3 on growth performance and diarrhea rate (Table 4). The ADG (P < 0.05) and ADFI (P < 0.01) of piglets in the WK3 group were higher than those of piglets in the control group. Dietary supplementation with WK3 did not significantly affect the feed/gain ratio (F/G) compared with that of the control group (P > 0.05). There was no significant difference between the WK3 and CC groups for ADG (P > 0.05), ADFI (P > 0.01) or F/G (P > 0.05). Administration of WK3 effectively alleviated the incidence of diarrhea in ETEC K88-challenged piglets.

The effects of WK3 on antioxidant capacity in the jejunal mucosa of piglets (Table 6). Dietary supplementation with antibiotics significantly lowered the concentration of MDA (P < 0.05) and increased the SOD activity (P < 0.05), the GSH-Px content (P < 0.01) and T-AOC level (P < 0.05). We obtained the cecal microbial populations of total eubacteria, Lactobacillus spp., Bifidobacteria spp., Enterobacterium spp. and Enterococcus spp. (Table 7). Dietary supplementation with the antimicrobial peptide WK3 in piglets increased the numbers of bacteria, Lactobacillus spp. and Bifidobacteria spp.
(P < 0.01) and decreased the abundance of Enterobacterium spp. in the digesta of the cecum (P < 0.01) compared with those in the control and CC groups. There was no significant effect on total eubacteria and Enterococcus spp. in the digesta of the cecum (P > 0.05).

The mRNA concentrations of IL-1α (P < 0.05) and TLR-4 (P < 0.01) in the jejunum mucosa from the WK3 group were lower than those in the control group (Figure 1). There were no significant differences in the expression of IL-1β and IL-8 in the jejunum mucosa between the group treated with WK3 and the control group (P > 0.05). Treatment with antibiotics also significantly decreased the mRNA expression of TLR-4 (P < 0.01) in the jejunum mucosa compared to that in the control group.

We obtained the results of analysis of the expression of tight junction proteins (Figure 2). The results illustrated that dietary supplementation with the antimicrobial peptide WK3 in piglets did not significantly affect the mRNA expression of claudin-4, occludin and zonula occludens-1 (ZO-1) in the intestinal mucosa (P > 0.05). However, dietary supplementation with antibiotics significantly increased the expression of occludin mRNA in the intestinal mucosa but did not significantly affect the mRNA expression of ZO-1 or claudin-4.

Discussion

In this study, compared with those in other groups, piglets treated with *E. coli K88* in the control group had lower stool viscosity and a higher incidence of diarrhea. Piglets fed diets that included antibiotics or WK3 both showed better fecal viscosity and a lower incidence of diarrhea than piglets fed a control diet. This finding may be related to the bacteriostatic activity of the antibacterial peptide WK3 on ETEC. However, the treatment effect of the antibiotic is better than that of WK3, which may be caused by the enzymolysis of the antimicrobial peptide WK3 in the intestine and stomach, which has been previously reported (Han and Thacker 2012; Stewart et al. 2010).

Many previous studies have also shown that dietary addition of antimicrobial peptides can improve the growth performance in weaned piglets, and our findings were similar. For example, Wang (Wang et al. 2011) found that supplementation of piglet diets with antimicrobial peptides could improve ADG and F/G, and in Jin’s study, the growth performance of piglets increased linearly with the addition of antimicrobial peptides (Jin et al. 2008). In the current experiments, we evaluated the effect of antibiotic and antimicrobial peptide dietary therapeutics on the performance of weaned piglets after challenge with *E. coli K88*. In addition, in this study, we found that the WK3 and antibiotic treatments both relieved the condition of piglets after *E. coli K88* challenge.

Intestinal morphology plays a vital role in nutrient absorption. Weaning stress can cause changes in intestinal morphology in mammals, including villus atrophy, crypt hyperplasia, and decreased digestion and absorption abilities (Qin et al. 2019). The ratio of villus height to crypt depth is a comprehensive manifestation of intestinal development and functional status. An increase in the ratio indicates that the intestinal mucosal function is improved, digestion and absorption capacity is enhanced, animal growth and development is accelerated, intestinal flora is stable, and animal diarrhea rate is reduced. When the ratio decreases, the results are the opposite (Caspary 1992). In this study, it was found that the antimicrobial peptide WK3 can significantly increase the height of ileac villi, reduce the depth of crypts, and increase the ratio of the two. If the ratio decreases, the result reverses. Humphrey et al. (Humphrey et al. 2002) showed that adding antimicrobial peptides to a broiler chicken diet can improve the height of villi in the duodenum. The intestinal morphological structure can reflect the integrity of the mechanical structure of the intestinal barrier and ensure the function of the intestinal barrier, which is closely related to the presence of enterotoxins in the digestive tract (Gislason et al. 1993). Therefore, the improvement of villus height/crypt depth by the antimicrobial peptide WK3 may be related to the reduction in the number of pathogenic bacteria.

Oxidative stress and inflammation are highly correlated. Furthermore, disorder of the total number of bacteria in the intestine can lead to oxidative stress and cause inflammation. The body itself has an antioxidant system; antioxidant enzymes are the first layer of defense in the body’s antioxidant system and play a primary role by removing reactive oxygen species (ROS) or preventing the formation of ROS. The main antioxidant enzymes in the body are SOD and GSH-Px. However, oxidative stress leads to the production of MDA. A previous study has clearly demonstrated that antibacterial peptides can increase the GSH-Px content, SOD activity and total antioxidant capacity in the serum of piglets (Tang et al. 2009). However, the results of our study showed only that dietary supplementation with WK3 significantly increased the content of GSH-Px in the jejunum, and the effect on the MDA content, SOD activity and T-AOC level was not significant. The results showed that the antimicrobial peptide WK3 had an influence on some antioxidant indexes. The effect of WK3 on the antioxidant capacity of piglets needs to be studied further.

A balanced intestinal flora is one of the most important factors for the healthy growth of piglets (Mathew et al. 1998). An important factor in preventing exogenous pathogens from colonizing the stomach and intestines is a barrier established by the native microbial community. This phenomenon is referred to as colonization resistance (Brassart and Schiffirn 1997). The intestinal microbiota also plays a vital role in directing the development of the immune system and host metabolism (Brown et al. 2012). In our study, dietary supplementation with WK3 significantly increased populations of...
Lactobacillus spp. and Bifidobacteria spp. in the piglet cecum. This result is consistent with previous studies in which antimicrobial peptides could increase the number of beneficial bacteria (Chen et al. 2009). Meanwhile, WK3 reduced the populations of Enterobacteriaceae spp. compared with those in both the control and CC groups, indicating that the antimicrobial peptide WK3 can kill intestinal pathogens, thereby reducing the diarrhea rate of piglets, as the piglet diarrhea rate may be related to the number of bacteria in the intestinal flora (Callesen et al. 2007). Both Lactobacillus and Bifidobacteria are known to have the potential to block pathogen colonization by competing for nutrient and epithelial binding sites. They can also block pathogen invasion by producing antibacterial factors such as lactic acid and bacteriocins. This finding is in conjunction with previous studies on the number of beneficial bacteria (Blake et al. 2003; Chen et al. 2009).

There is no denying that the function of the intestinal epithelium is a physical barrier to intestinal material. The intestinal tract can regulate the high concentration of resident microorganisms through different systems to protect the mucosal surface from pathogens, so the intestinal tract is also a kind of immune tissue (Abraham and Medzhitov 2011). Improper immunization protection responses can lead to serious immunopathogenesis; however, a healthy body can prevent this outcome (Worbs et al. 2006). In healthy situations, T cells seem to be activators but not stimulants. They are ready to be stimulated and secrete various cytokines. Once invaded, as a part of the nonspecific immune system, the epithelium can act through expressing TLRs and secreting cytokines (Cario et al. 2002; Maaser and Kagnoff 2002). Hence, although various factors may cause diarrhea, intestinal inflammation remains the common symptom of this disorder. Levels of proinflammatory cytokines such as IL-6 and TNF-α are usually elevated in the small intestine of weaned pigs (Pié et al. 2004). Previous reports have suggested that reduced expression of inflammatory cytokine genes may lead to a more balanced ecosystem, thereby preventing the proliferation of specific bacterial populations (Walsh et al. 2013). Recent studies have shown that most proinflammatory cytokines, such as IL-1α, IL-1β, and IL-8, induce a purposeful disease-induced increase in intestinal epithelial permeability (Al-Sadi et al. 2009). In this study, we demonstrated that WK3 inhibited intestinal inflammation by downregulating the mRNA expression of IL-1α and TLR-4 in the jejunal mucosa. The first function of TLR4 is to recognize exogenous molecules from pathogens; in addition, TLR4 also involves the recognition of endogenous molecules released by damaged tissues and necrotic cells. Similar to our results, previous studies have shown that cathelicidin-BF significantly decreases the expression of IL-6, IL-8 and IL-22 (Yi et al. 2015). In addition, Song et al. reported that cathelicidin-BF pretreatment significantly reduces TNF-α mRNA levels compared to those in LPS-treated mice (Song et al. 2015). Collectively, our results illustrate that the mechanisms by which WK3 inhibits inflammation could be regulated by the downregulation of the expression of inflammatory factors. This effect may explain why WK3 and antibiotics have similar inhibitory effects on intestinal inflammation in diarrheal piglets.

Intestinal mucosal barrier function is closely related to the tight junction between small intestinal epithelial cells. An increase in proinflammatory cytokine secretion is often accompanied by damage to intestinal tight junctions, resulting in a change in the structure of tight junctions. Previously, studies have shown that antimicrobial peptides can increase the expression of tight junction protein (ZO-1, occludin and claudin-1) genes (Han et al. 2015). However, the expression of claudin-4, occludin and ZO-1 mRNA in the jejunal mucosa of piglets was not significantly affected by the addition of the antimicrobial peptide WK3 in our study. This finding is inconsistent with previous results. However, dietary supplementation with antibiotics significantly increased the expression of occludin mRNA, indicating that the bacterial peptide WK3 does not achieve the same effect as antibiotics in intestinal immunity in piglets.

Conclusions

The results of the present study show that the AMP WK3 has the ability to improve growth performance and reduce the incidence of diarrhea in piglets challenged with ETEC K88. These findings may be due to the antibacterial activity of WK3 in vivo, reducing the immune response by regulating the secretion and expression of cytokines. Moreover, WK3 has a positive effect on the intestinal microflora, intestinal morphology and oxidative damage in piglets. Therefore, it appears that the AMP WK3 has the potential to be utilized as an alternative to antibiotics for dietary supplementation of weaned piglets.

Disclosure statement

The authors declare no conflict of interest.

Funding

This project was supported by the Natural Science Foundation of China (32030101, 31872368 and 31472104), the Natural Science Foundation of Heilongjiang Province (TD2019C001) and the China Agriculture Research System (CARS-35).

ORCID

Anshan Shan http://orcid.org/0000-0003-2830-7509

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