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Establishment of a recombinant *Escherichia coli*-induced piglet diarrhea model

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1. ABSTRACT

Enterotoxigenic *Escherichia coli* strains induce human and animal intestinal dysfunction and injury, and cause diarrhea in weanling pigs. A recombinant *E.coli* strain LMG194-STa which expressed single toxin STa of ETEC was constructed by directly inserting the STa gene of ETEC into the expression vector pBAD202 and then transferring the recombinant plasmid pBAD-STa into the *E.coli* LMG19 host strain. Diarrhea and intestinal injury in piglets were induced by oral administration of recombinant strain LMG194-Sta. *In vitro*, the recombinant strain LMG194-Sta had the same toxicity to the IPEC cell as wild type strain K88, and higher toxicity than the host strain LMG194. *In vivo*, LMG194-STa caused severe diarrhea in piglets as K88, the diarrhea rate of LMG194-STa and K88 groups was higher than that in the LMG194 and control groups. Both K88 and LMG194-STa induced intestinal inflammation, injury, and atrophy, while adversely affecting the expression of genes for cytokines, transporters and ion channels, and nutrient metabolism. Thus, we established a porcine model of recombinant *E. coli* strain LMG194-STa-induced diarrhea for future nutritional and mechanistic studies of intestinal dysfunction.

2. INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) strains are the most common bacterial pathogens that cause diarrhea. Diarrhea is one of the most important diseases in young farm animals and also the second leading cause of death in children under 5 years old (1-3). The enterotoxigenic *Escherichia coli* (ETEC)-induced postweaning diarrhoea (PWD) of piglets mainly occurs within the first week after weaning, resulting in great economic losses to the

swine industry due to reduced growth performance as well as increased morbidity and mortality (4,5). The disease is largely caused by ETEC which colonize on the gut epithelium through adhesion to specific receptors on the brush border membrane. Following the colonization, the excretion of different enterotoxins induces intestinal inflammatory responses and causes diarrhea (4-6). The key virulence factors of ETEC in diarrhea are bacterial adhesins and enterotoxins (7). Enterotoxins produced by ETEC strains associated with diarrhea include heat-labile toxin (LT) and heat-stable toxins (ST), the latter includes type I (STa), type II (STb), and enteroaggregative heat-stable toxin 1 (EAST1) (7-9).

LT, STb, and STa have been identified as virulence determinants in diarrhea (7,10-13). The heat-stable toxin type I (STa) disrupts fluid homeostasis in host small intestinal epithelial cells to cause hyper-secretion of electrolyte-rich fluid through the activation of intracellular guanylate cyclase, leading to diarrhea (14).

Using molecular biology techniques, we cloned the Sta gene. It was expressed in *E. coli* to produce the heat-stable toxin type I protein (STa) . This work established a new model of piglet diarrhea caused by a single toxin of ETEC. The recombinant toxin may be used as a platform for drug and vaccine development to prevent and treat diarrhea in swine and other animals.

3. CONSTRUCTION OF RECOMBINANT E.COLI STRAIN LMG194-STa

3.1. General study protocol

To study the pathologic effects of enterotoxigenic *E.coli* (ETEC) and the mechanism of ETEC-induced piglet diarrhea, a heat-stable *Escherichia* coli enterotoxin gene *estA was* cloned and expressed in nonpathogenic *E.coli* LMG194. Enterotoxin clone DH5a-STa with *estA* gene of enterotoxigenic *E.coli* (ETEC), which was obtained from Agriculture and Agri-food Center of Canada (AAFC), was cultivated at 37°C in Luria-Bertani (LB) broth or agar. The *estA* gene was amplified from the DH5a-STa by PCR using the primers 5'- CACCATGAAAAAGCTAATGTT -3' and 5'- ATAACATCCAGCACAGGCA -3' and DNA polymerase (Takara, Dalian, China) according to the manufacturer's instructions. The PCR products was checked by gel electrophoresis and sequencing. The STa fragments were purified from agarose gels using PCR cleanup and gel extraction kit (Takara). The plasmid pBAD-STa was constructed according the manual of the pBAD202 Directional TOPO® Expression Kit (Invitrogen) and verified by enzyme digestion. The recombinant *E.coli* LMG194 which expressed STa of ETEC was constructed by transferring the recombinant plasmid pBAD-STa into the *E.coli* LMG194 component cell and then cultivating the recombinant *E.coli* on LB agar with kanamycin (30μg/mL) according to the manual of the pBAD202 Directional TOPO® Expression Kit (Invitrogen). The positive clones were cultivated in LB broth with kanamycin (30 μg/ml) and checked by PCR using the primers noted previously.

3.2. Verification of recombinant *E.coli* strain LMG194-STa

The results of our study showed that 219bp fragments were obtained when amplifing the *estA* gene by PCR using the DH5a-STa as template, Nucleic acid sequence analysis demonstrated that the fragment was 100% homologous to the *estA* gene of ETEC. The verification of the recombinant strain showed that the same size of fragments was gotten when amplifying the *estA* gene by PCR using the positive clones selected as template. The recombinant strain that expressed STa was constructed successfully. The correct recombinant strain was named LMG194-STa. In this study, pBAD202/D-TOPO® vector and the *E.coli* strain LMG194 were chosen to express the toxin gene. The pBAD Directional TOPO® Expression Kit utilizes a highly efficient, fast cloning strategy ("TOPO® Cloning") to directionally clone a blunt-end PCR product into a vector which can regulate gene expression and simplify the purification of soluble proteins in *E. coli*. Blunt-end PCR products were cloned directionally at greater than 90% efficiency without ligase, post-PCR procedures, or restriction enzymes. In addition, pBAD202/D-TOPO® vector contains the His-Patch (HP) thioredoxin leader for increasing translation efficiency and solubility of recombinant fusion proteins. Expression in *E.coli* is driven by the araBAD promoter (PBAD). The AraC gene product encoded in the pBAD202/D-TOPO® vector positively regulates this promoter. The *E.coli* strain LMG194 is included in the kit to allow for low basal levels of toxic genes. This strain can grow in minimal medium (RM medium), which allows repression of PBAD by glucose.

4. *IN VITRO* EVALUATION OF THE TOXICITY OF LMG194-Sta

4.1. General study protocol

In vitro toxicity of the recombinant strain was evaluated by co-culturing with IPEC-1 cells and then measuring lactate dehydrogenase activity in culture medium. The porcine neonatal jejunal epithelial cell line (IPEC-1) cells were grown in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (1:1) (Invitrogen) supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100 μg/ml and amphotericin B (0.2.5 μg/ml) and maintained in an atmosphere of 5% CO_2 at 37°C for cultures and assays. IPEC-1 cells were seeded in six-well plates at

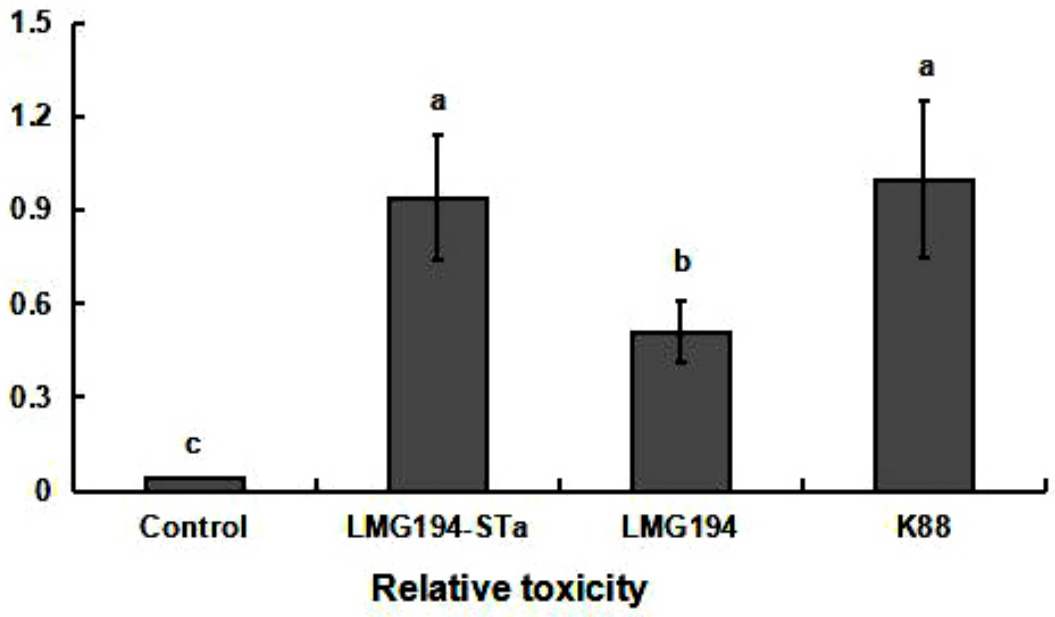

Figure 1. Relative toxicity of the LMG194-Sta. Values are means ± SD, n = 6. [a, b, c] Means with different letters differ ($P < 0.0.5$).

2 × 10^5 cells per well in a 1.0. ml volume of DMEM and grown overnight (12–16 h) to reach 50% confluence. *Escherichia coli* K88 was used as a positive control, LMG194 used as a negative control. LMG194/pBAD-STa was grown in a Luria–Bertani broth at 37°C for 12h, then washed and adjusted to the concentration of 10^8 CFU/ml with PBS buffer. After a rinse with pre-warmed DMEM (with 1% FBS) without antibiotics, IPEC-1 cells were treated with LMG194/pBAD-STa, LMG194 and K88 at 2 × 10^7 CFU/ml in 1 ml of the same medium and co-cultured for 2.5. h at 37°C. The cultures were then transferred to tubes and centrifuged at 300×g for 5 min, the cells were discarded and the supernatants were transferred to the new tubes and then centrifuged at 21000×*g* for 5 min, the bacteria was discarded and the supernatant fluid was transferred to the new tube. IPEC-1 cell death induced by *E.coli* was assessed by testing the activity of LDH of the supernatant using Lactate Dehydrogenase Activity Assay Kit (Nanjing Jiancheng Bioengineering Instigute). The toxicity of LMG194-STa strains relative to K88 positive control group was calculated.

4.2. LMG194-STa exhibited toxicity to IPEC-1

The toxicity of the recombinant strain to IPEC-1 was evaluated by measuring the supernatant lactate dehydrogenase activity of the medium post co-culturation of IPEC-1 and *E.coli*. The relative toxicity was listed in Figure 1. The results showed that the toxicity of the recombinant strain LMG194-STa was almost the same as the *E.coli* wide type strain K88, and higher than that of the host strain LMG194. The results demonstrated that the recombinant strain LMG194-STa had the same toxicity to IPEC-1 cells as K88.

5. *IN VIVO* EVALUATION OF THE TOXICITY OF LMG194-STA

5.1. General study protocol

Twenty four 7-day-old healthy crossbred piglets (Durc × Landrace × Yorkshire, no *E.coli* related diseases immunized) reared by sows were chosen and then fed with milk replacer. After 3-day adaptation, piglets were randomly divided into 4 groups (6 pigs/group): LMG194-STa group (piglets were challenged with *E.coli* LMG194-STa), LMG194 group (piglets were challenged with *E.coli* LMG194), K88 group (a positive group, piglets were challenged with *E.coli* K88), and control group (a negative control, piglets were treated with sterile saline). Each group had 6 pigs according to a single-factor experimental design.

At the 5th day of the experiment, the piglets of the LMG194-STa, LMG194 and K88 groups were orally inoculated with *E.coli* LMG194-STa, LMG194 and K88, respectively, at the dose of 2 × 10^9CFU/pig per day (i.e., administration in the morning and evening). Piglets of the control group were treated with sterile saline in the same way as for other groups. Piglet diarrhea was observed two days after challenge, and diarrhea rate was calculated. On the 7th day of the trial, 10% D-xylose (1 ml/kg BW) was orally administrated to all piglets, and the D-xylose absorptive test was performed to measure intestinal absorption

capacity and mucosal integrity (14). At 1 h after the administration of D-xylose, blood samples were collected from the anterior vena cava into heparinized vacuum tubes (Becton-Dickinson Vacutainer System, Franklin Lake, NJ, USA), as described by Kang *et al.* (15). Blood samples were centrifuged at 600 x *g* for 10 min at 4 °C to obtain plasma (15,16), which was stored at −80 °C until analysis. After blood sampling, all pigs were killed under sodium pentobarbital anesthesia (50 mg/kg BW, iv) to obtain the small intestine mucosal samples (17, 18).

All assays were performed using the previously published methods (19, 20). To determine intestinal morphology, three paraformaldehyde-fixed intestinal samples (duodenum, jejunum and ileum) were dehydrated and embedded in paraffin. Five-μm sections were cut and then stained with hematoxylin and eosin stain. Intestinal morphology was determined using a light microscope (Leica, Germany) with the Leica Application Suite image analysis software (Leica, Germany). Villus area was quantitated from the perimeter and height of the villi (21). The villus height: crypt depth ratio (VCR) was calculated. The 10 longest and straightest villi and their associated crypts from each segment were measured. The same villus and crypt were used to determine the number of intraepithelial lymphocytes (IELs) (21). The variables were expressed per 100 enterocytes. Goblet cells were counted in 10 villi and 10 crypts on two intestinal sections combination stained with the periodic acid Schiff (PAS)/alcian blue pH 2.5. procedure (22). The goblet cell numbers were determined per 100 enterocytes. The mean value of each sample was calculated.

Concentrations of biochemical parameters such as aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), creatine kinase (CK), blood urea nitrogen (BUN), total bilirubin (TBIL), cholesterol (CHOL) and creatinine in plasma were measured with corresponding kits using a Hitachi 7060 Automatic Biochemical Analyzer (Hitachi, Japan) (23). D-xylose in plasma was determined as described by Hou *et al.* (17). Briefly, 50 μL of the collected plasma was added to 5 mL of the phloroglucinol color reagent solution (Sigma Chemical Inc., St. Louis, MO, USA) and heated at 100 °C for 4 min. The samples were allowed to cool to room temperature in a water bath. A xylose standard solution was prepared by dissolving d-xylose in saturated benzoic acid (prepared in deionized water) to obtain 0, 0.7, 1.3, and 2.6mmol/L. A D-xylose standard solution or sample was added to the color reagent solution. Absorbance of the resultant mixture at 554 nm was measured using a spectrophotometer (Model 6100, Jenway LTD., Felsted, Dunmow, CM6 3LB, Essex, England, UK). The standard solution of 0 mmol/L d-xylose was considered as the blank.

Diamine oxidase (DAO) activities in plasma were determined using spectrophotometry as described by Hosoda *et al.* (24). The assay mixture (3.8 mL) contained 3 mL of phosphate buffer (0.2 M, pH 7.2), 0.1 mL (0.04%) of horseradish peroxidase solution (Sigma Chemicals), 0.1 mL of *o*-dianisidine-methanol solution (0.5% of *o*-dianisidine (Sigma Chemicals) in methanol), 0.5 mL of plasma, and 0.1 mL of substrate solution (0.1, 75% of cadaverine dihydrochloride, Sigma Chemicals). This mixture was incubated for 30 min at 37 °C, and absorbance at 436 nm was measured to indicate DAO activity (25). Protein was measured as described by Hou *et al.* (17). DAO was used as a marker of intestinal injury (26).

Plasma and intestinal mucosae were used for the analyses of anti-oxidative enzymes and related products. Catalase (CAT) activities, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) concentrations were determined as described by Hou *et al.* (20). Activities of CAT, as well as MDA and H_2O_2 were determined using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocols. Assays were performed in triplicate.

The transcriptional response of the mucosa of small intestines to the recombinant strain was quantified using real-time RT-PCR (27-29). Approximately 100 mg of each frozen mucosal sample was powdered and homogenized, and total RNA was isolated using the Trizol Reagent protocol (Invitrogen, Carlsbad, CA). Total RNA was quantified using the NanoDrop® ND-1000A UV-VIS spectrophotometer (Thermo Scientific, Wilmington, DE, USA) at 260 nm, and its purity was assessed by determining the OD260/OD280 ratio (1.8 to 2.1). Total RNA was reverse-transcribed using a PrimeScript® RT reagent kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer's instruction. cDNA was synthesized and stored at -20 until use.

To amplify cDNA fragments, the primers pairs (Table 1) were used for qPCR. To minimize amplification of potentially contaminating genomic DNA, the primers were designed to span introns and intron-exon boundaries. The qPCR was performed using the SYBR® Premix Ex Taq™ (Takara, Dalian, China) on an Applied Biosystems 7500 Fast Real-Time PCR System (Foster City, CA). The total volume of PCR reaction system was 50 μL. In brief, the reaction mixture contained 0.2 μM of each primer, 25 μL of SYBR® Premix Ex Taq™ (2 ×) and 4 μL of cDNA in a 50-μL reaction volume. All PCRs were performed in triplicate on a 96-well real-time PCR plate (Applied Biosystems) under the following conditions (two-step amplification): 95 for 30 sec, followed by 40 cycles of 95 for 5 sec and 60 for 30 sec. A subsequent melting curve (95 for 15 sec, 60 for 1 min and 95 for 15 sec) with continuous

Table 1. Strains, plasmids and primer used in the study

Strain, plasmid and primer	Relevant characteristics	Source of reference
E.coli		
E.coli Top10	Cloning vehicle	Takara (Dalian, China)
E.coli K88	K88 fimbrial antigen positive,STa^{+},STb^{+} LT^{+}	State key laboratory of agricultural microbiology of China
LMG194	Expression host	Invitrogen (USA)
Plasmid		
pCR®TOPO-STa	Clone plasmid containing STa gene of ETEC	Provided by AAFC (CAD)
pBAD202/D-TOPO Vector	Expression vector; kan ^{r}	Invitrogen (USA)
pBAD -STa	Expression plasmid containing STa gene of ETEC	This work
Primers (Synthesized in Sangon, Shanghai, China)		
P1	5'- CACCATGAAAAAGCTAATGTT -3' upstream primer with STa gene initiator codon of ETEC	This work
P2	5'- ATAACATCCAGCACAGGCA -3'downstream primer with STa gene termination codon of ETEC	This work

fluorescence measurement was constructed, followed by setting at 25. The specificity of the qPCR reactions was assessed by analyzing the melting curves of the products and size verification of the amplicons (30). To ensure the sensitivity and accuracy of the results obtained by qPCR, samples were normalized internally using simultaneously the average cycle threshold (Ct) of ribosomal protein L4 (RPL4) as the reference genes in each sample to avoid any artifact of variation in the target gene (29,31). Results were analyzed by the $2^{-\Delta Ct}$ method (28,29). Each biological sample was run in triplicate.

According to the $2^{-\Delta Ct}$ method, the mean value of gene expression in the control group was set to 1.0.0. A fold-change less than 1 ($P < 0.0.5$) means down-regulation. Conversely, a fold change higher than 1 ($P < 0.0.5$) means up-regulation (32). Additionally, the normality and constant variance for all data were tested by the Levene's test (33). Differences among treatment means were determined by the Duncan's multiple comparison test. All statistical analyses were performed by the SPSS 13.0. software (Chicago, IL, USA). Probability values of < 0.0.5 were taken to indicate statistical significance (34).

5.2. LMG194-STa caused severe diarrhea in piglets as enteropathogenic E. coli K88

During the experimental period, diarrhea was observed and the diarrhea rate was calculated (Figure 2). Comparing the diarrhea rates among different groups before and after the challenge, there is no significant difference in the diarrhea rate before challenge. However, after challenge, the diarrhea rates in LMG194-STa group and K88 group increased markedly and were much higher than those in the LMG194 group and control group. There is no significant difference between the LMG194-STa and K88 groups.

5.3. Alterations of intestinal morphology in piglets challenged with LMG194-STa

The morphological structure of the pig intestine is shown in Figure 3, and the intestine morphology indexes such as crypt depth, villus height, width, surface area and the ratio of villus height to crypt depth are summarized in Table 2. The duodenum, jejunum and ileum of piglets in the control group (Figure 3 a, e, and i) were normal in morphological structure. However, in the duodenum, jejunum and ileum of piglets challenged by LMG194-STa (Figure 3 b, f, and j), LMG194 (Figure 3 c, g, and k) and K88 (Figure 3 d, h, and l), the abnormal histomorphological changes that were associated with intestinal mucosal injury were observed, especially in the LMG194-STa and K88 groups.

The intestine morphology indexes of the duodenum showed that LMG194-STa challenge reduced villus height, crypt depth, villus surface area and the ratio of villus height to crypt depth, but had no effect on the villus width. K88 challenge had similar effects on these indexes, but the villus height was shorter and the villus surface area was smaller under the K88 challenge than under the LMG194-STa challenge. These results indicated that the injury to the duodenum caused by K88 may be more serious. LMG194 challenge decreased villus height, crypt depth and villus surface area, but the

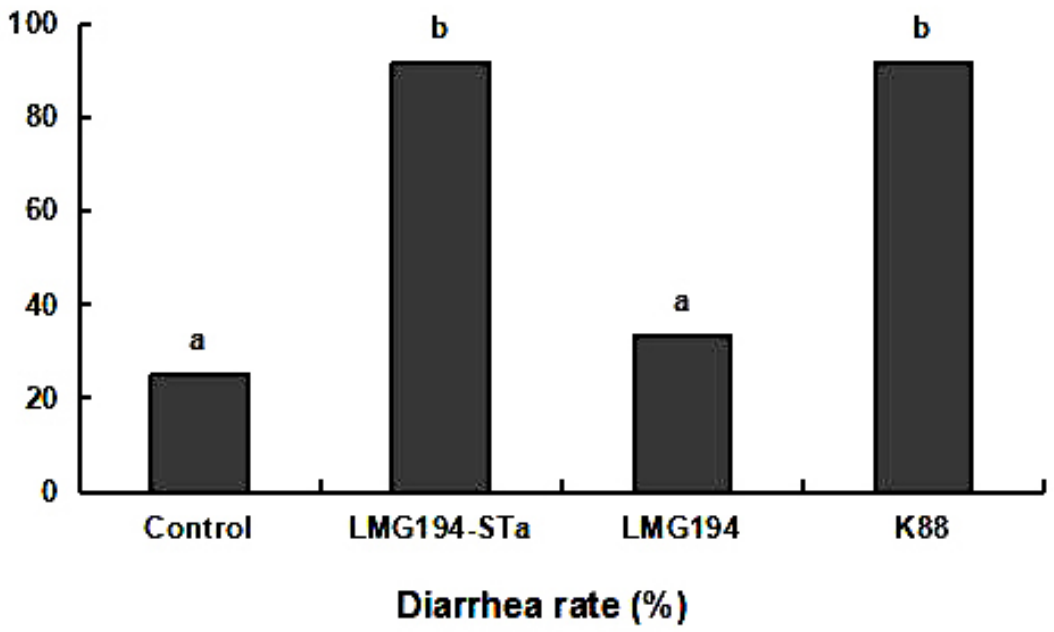

Figure 2. Diarrhea rate of piglets after LMG194-STa challenge. Values are means ± SD, n = 6. [a, b] Means with different letters differ ($P < 0.0.5$).

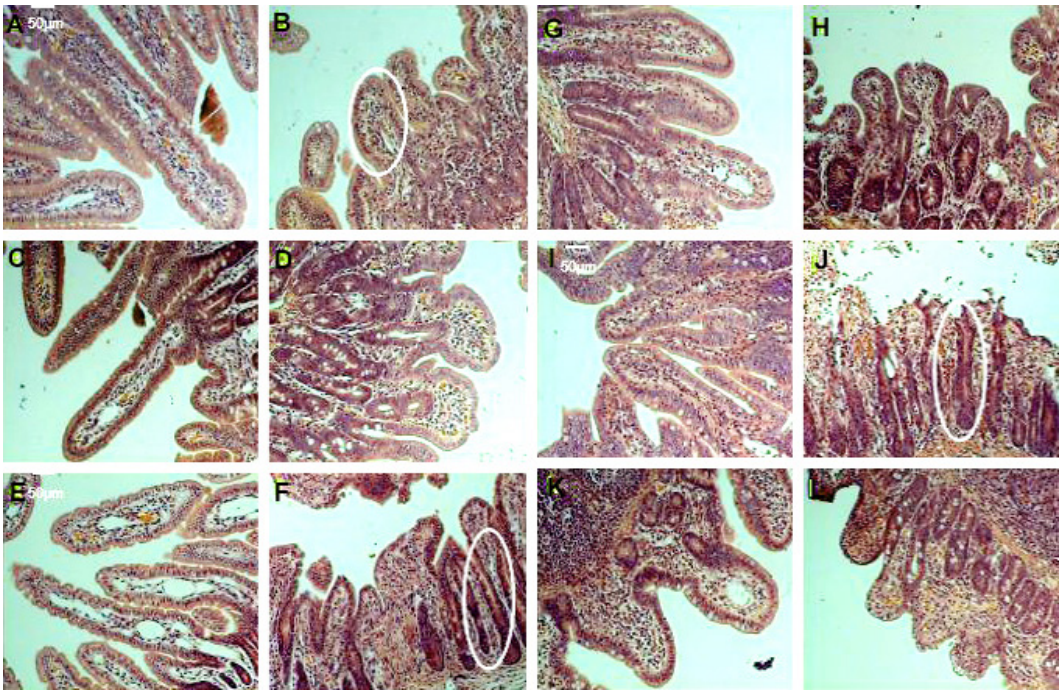

Figure 3. Intestinal morphological characterization in piglets. Duodenum (a), jejunum (e) and ileum (i) of piglets receiving oral administration of sterile saline: no obvious morphological changes were observed. Duodenum (b), jejunum (f) and ileum (j) of piglets challenged with *E.coli* LMG194-STa. Duodenum (c), jejunum (g) and ileum (k) of piglets challenged with *E.coli* LMG194. Duodenum (d), jejunum (h) and ileum (l) of piglets challenged with *E.coli* k88. Morphological changes of the LMG194-STa and K88 groups were associated with intestinal mucosal injury, such as lifting of the epithelium at the tip of the villus.

ratio of villus height to crypt depth and villus surface area was increased in comparison with the LMG194-STa challenge. The jejunum morphology indexes revealed that LMG194-STa and K88 challenge resulted in villus height shortening and villus surface area reduction, but had no significant effects on the other indexes. The villus surface area in K88 group was larger than that in the LMG194-STa group.

Table 2. Indexes of the intestinal morphology in piglets

Items	Control	LMG194-STa	LMG194	K88	*P* value
Morphological indexes of duodenum					
Villus height, μm	255±29^{c}	193±37^{a}	239±26^{a}	186±35^{a}	<0.0.34
crypt depth, μm	144±17^{b}	128±30^{a}	126±32^{a}	125±26^{a}	0.0.12
Ratio of villus height to crypt depth	1.8.1±0.2.5^{b}	1.6.4±0.3.3^{a}	1.7.9±0.3.4^{b}	1.5.3±0.2.8^{a}	<0.0.01
Villus width, μm	120±26	119±21	116±21	115±22	0.7.50
Villus surface area	41751±7814^{a}	30497±6940^{b}	31200±7399^{b}	23819±5767^{a}	<0.0.01
Morphological indexes of jejunum					
Villus height, μm	168±27^{b}	133±39^{a}	154±34^{a}	136±25^{b}	0.0.15
crypt depth, μm	88±15	82±14	83±15	84±16	0.2.60
Ratio of villus height and crypt depth	1.9.5±0.4.1^{b}	1.6.6±0.5.4^{a}	1.9.0±0.4.6^{b}	1.6.8±0.4.3^{a}	0.0.21
Villus width, μm	112±18	113±29	108±26	111±15	0.1.09
Villus surface area	27174±4892^{c}	19912±3300^{a}	26703±3561^{c}	23112±2706^{b}	<0.0.01
Morphological indexes of ileum					
Villus height, μm	232±32^{b}	182±32^{a}	224±26^{b}	173±33^{a}	0.0.17
crypt depth, μm	137±23^{d}	102±22^{b}	123±17^{c}	68±13^{a}	<0.0.01
Ratio of villus height and crypt depth	1.8.6±0.3.3^{b}	1.5.8±0.2.4^{a}	1.7.4±0.2.1^{b}	1.5.9±0.1.9^{a}	<0.0.01
Villus width, μm	135±22^{b}	127±20^{ab}	135±16^{b}	122±17^{a}	0.0.07
Villus surface area	43614±7178^{d}	31070±4442^{b}	34234±5008^{c}	18188±3104^{a}	<0.0.01

Values are means ± SD, n = 6.

LMG194 challenge had no influence on the jejunum morphology indexes. The ileum morphology indexes showed that LMG194-STa challenge caused villus height shortening, crypt shallowing, and villus surface area reduction. but had no effects on the ratio of villus height to crypt depth or villus width. K88 challenge resulted in similar changes in intestinal morphology to those in the LMG194-STa challenge, but the villus height was much shorter, the crypt depth was much lower and the villus surface area was much smaller than the LMG194-STa challenge. These results indicate that the injury to ileum caused by K88 may be more serious than that by LMG194-STa. LMG194 challenge decreased villus height, crypt depth and villus surface area, but the villus height, crypt depth and villus surface area were increased in comparison with the LMG194-STa challenge. Based on the results of small intestine morphology indexes, a conclusion can be drawn that LMG194-STa could cause small intestine injury to the same extent as that of K88, especially in the ileum. In the current study, we found that *E.coli* challenge caused epithelium lifting and villous atrophy. This is in accordance with most of the published reports regarding the effect of *E.coli* infection or other toxins of *E.coli*, such as LPS, on intestinal morphology (35, 36).

5.4. Variations of intestinal mucosal immunocyte of piglets challenged with LMG194-STa

The indexes of intestinal mucosal cells, such as goblet cells, leukomonocytes, cell density of lamina propria and density of lymphocytes are shown in Table 3. The number of goblet cells in the duodenum decreased sharply in piglets challenged with *E.coli,* especially the K88, but there was no significant difference between the LMG194 and LMG194-Sta. In the duodenum of piglets challenged with *E. coli*, the number of leukomonocyte, cell density of lamina propria, and density of lymphocytes were not affected, as compared with the control group. Jejunal goblet cell and lymphocyte numbers decreased when the piglets were challenged by *E. coli,* especially k88. Cell density of jejunum lamina propria decreased in the LMG194-Sta and K88 groups. The density of jejunum lymphocytes decreased when piglets were challenged with *E.coli*, and ileal goblet cell number and cell density of lamina propria decreased when the piglets were challenged with *E. coli*. Density of ileum lymphocyte decreased when piglets were challenged with K88 or LMG194-Sta. Increases in the number and density of the lymphocytes indicated inflammation, whereas decreases in the number of goblet cells and lamina propria cell density indicated intestinal injury. The changes in intestinal mucosal

Table 3. Number of intestinal mucosal cells in piglets

Items	Control	LMG194-STa	LMG194	K88	P value
Duodenum					
Number of goblet cells per mm^2	7.5.±1.5.^{a}	4.7.±1.2.^{b}	6.8.±1.3.^{a}	2.0.±1.0.^{c}	0.0.12
Number of lymphocytes per mm^2	21.2.±5.8.6	19.4.±4.8.	20.4.±5.8.	18.8.±5.1.	0.2.75
Lamina propria cell density permm^2	8190±709	7571±430	7968±712	7513±741	0.3.41
Density of lymphocytes per mm^2	985±115	1123±171	1069±290	1293±277	0.5.19
Jejunum					
Number of goblet cells per mm^2	7.8.±0.9.^{a}	6.8.±0.8.^{a}	7.0.±1.5.^{a}	3.3.±1.2.^{b}	<0.0.01
Number of lymphocytes per mm^2	21.2.±3.3.^{a}	21.4.±4.8.^{a}	20.8.±3.5.^{a}	17.0.6±3.0.^{b}	0.0.05
Lamina propria cell density per mm^2	10699±924^a	9061±491^b	10683±1234^a	8605±1310^b	0.0.09
Density of lymphocytes per mm^2	1012±280	847±179	979±154	876±25	0.1.62
Ileum					
Number of goblet cells per mm^2	10.4.±1.8.^{a}	7.3.±1.0.^{b}	9.6.±2.1.^{a}	6.2.±1.1.^{b}	0.0.16
Number of lymphocytes per mm^2	27.0.±3.3.	27.4.±5.9.	29.8.±4.8.	28.8.±4.7.	0.4.17
Lamina propria cell density per mm^2	12651±983^a	8942±953^{bc}	10188±930^b	7612±1104^c	<0.0.01
Density of lymphocytes per mm^2	1817±192^a	1375±294^b	1948±218^a	1419±314^b	0.0.01

Values are means ± SD, n = 6.

Table 4. Blood variables in piglets

Items	Control	LMG194-STa	LMG194	K88	*P* value
Blood cells indexes					
NEU (10^9/L)	3.5.9±1.1.2^b	1.9.9±0.4.2^a	2.7.3±0.5.2^{ab}	2.8.8±0.9.4^{ab}	0.0.23
NEUR (%)	43.2.±15.5.^{b}	27.5.±4.3.^{a}	28.3.±3.1.^{a}	28.7.±5.1.^{a}	0.0.13
LYMR (%)	48.8.±14.0.^{a}	65.2.±3.7.^{b}	61.2.±7.0.^{b}	62.8.±4.9.^{b}	0.0.13
MCHC (g/L)	307±9^a	307±0.7.^{a}	315±3^{ab}	323±12^b	0.0.04
RDW (%)	19.4.±0.7.^{b}	18.1.±2.4.^{ab}	16.3.±0.3.^{a}	16.8.±1.8.^{a}	0.0.11
Plasma biochemical indexes					
ALT (U/L)	44.4.±1.2.^{c}	40.5.±4.3.^{b}	37.5.±1.2.^{ab}	36.6.±1.8.^{a}	<0.0.01
AST (U/L)	38.3.±9.8.^{a}	46.3.±3.2.^{ab}	55.9.±17.0.^{b}	36.8.±8.2.^{a}	0.0.22
BUN (mmol/L)	1.3.1±0.1.0^a	1.3.1±0.1.1^a	3.8.1±2.2.8^b	3.1.6±2.1.0^{ab}	0.0.20
Concentration of D-xylose in the plasma					
D-xylose(mmoL/L)	0.4.3±0.1.2^b	0.3.0±0.0.5^a	0.4.1±0.0.5^b	0.2.8±0.0.6^a	<0.0.01
Concentration of diamine oxidase in the plasma					
DAO (U/mL)	5.2.6±1.0.5^a	16.4.2±1.4.9^d	10.4.8±2.9.9^b	13.4.8±1.6.0^c	<0.0.01

Values are means ± SD, n = 6.

immunocytes demonstrated that LMG194-STa and K88 challenge induced intestinal inflammatory responses and injury in piglets. It is well known that IELs and goblet cells are the major intestinal immunocompetent cells which are positioned strategically at the epithelial barrier, signifying their important roles in mucosal surveillance. Our histological study showed that *E.coli* infection decreased the number of goblet cells in the small intestine and lowered the density of lymphocytes, indicating increases in intestinal damage and inflammation. These results are in accordance with previous studies reporting that *E.coli* infection or other toxins of *E.coli,* such as LT, affected intestinal morphology (35, 36).

5.5. Changes of hematological indexes in piglets challenged with LMG194-STa

All measured haematological indexes in piglets are listed in Table 4. The results showed that

the number and the ratio of neutrophilic granulocytes in the blood of piglets challenged with *E. coli* decreased, especially with LMG-STa, whereas the lymphocyte ratio increased when piglets were challenged with *E. coli*. Mean corpuscular hemoglobin concentration (MCHC) was obviously higher in piglets challenged with *E.coli* k88 than in other three groups of pigs. Red cell distribution width (RDW) decreased when piglets were challenged with *E.coli*. Except for these indexes with differences, other indexes such as WBC, LYM, MONO, EOS, BASO, MONOR, EOSR, BASOR, RBC, HGB, HCT,MCV, MCH, PLT, MPV, PCT, PDW were not affected by *E. coli* treatment.

Plasma ALT and AST activities serve as sensitive indicators of damage in tissues, particularly the liver (37,38). Our results showed that plasma ALT in piglets challenged with *E.coli* decreased, but there was no significant difference between the LMG194-STa and K88 groups. Plasma AST activity in the piglets challenged with *E.coli* k88 did not differ from that in the control group. Plasma AST activity increased when piglets were challenged with *E.coli* LMG194 or LMG194-STa in comparison with the control group, but there was no significant difference between the *E.coli* LMG194 or LMG194-STa groups. BUN concentrations in piglets challenged with *E.coli* K88 or LMG194-STa were higher than those in the control and LMG194 groups. Except for the three indexes with difference (Table 3), the other plasma indexes, such as TBIL, TP, ALB, CHOL, TG, ALP, Crea, GLU, CK, GGT, did not differ among the four groups of pigs. The results of blood indexes reflected whole-body infection by *E.coli.* The increase in plasma ALT and AST activities indicated liver injury in pigs challenged with *E. coli* infection, which is in consistence with the previous reports on bacteremia (39).

E.coli challenge caused a decrease in plasma D-xylose and an increase in plasma DAO activity (Table 4). There were no significant differences in plasma D-xylose concentrations between piglets challenged with *E.coli* LMG194-STa and K88. The levels of these variables were elevated in the *E.coli* LMG194-STa and K88 groups than in the control and LMG194 groups. Plasma DAO activity in the piglets challenged with *E.coli* increased, especially in the LMG194-STa and K88 groups.

Plasma D-xylose concentration is a simple, specific, and sensitive indicator of intestinal absorption capacity. In healthy pigs, D-xylose is readily absorbed by the small intestine. However, under conditions of malabsorption, the entry of D-xylose from the intestinal lumen to the portal vein is impaired, thereby reducing D-xylose concentrations in both blood and urine (40, 41). Additionally, plasma DAO can serve as a monitor of the severity of mucosal injury (40, 42). In mammals, DAO is abundantly expressed in the upper part of the intestinal mucosa. Under certain circumstances, intestinal mucosal cells undergo necrosis and are sloughed off into the intestinal lumen, leading to an increase in circulating levels of DAO (43). According to the results of our present study, we concluded that LMG194-STa and K88 challenges could cause severe injury to the intestinal mucosal integrity, thereby increasing intestinal permeability in piglets.

5.6. Variations of antioxidant indexes in the plasma and intestinal mucosa of piglets challenged with LMG194-STa

Levels of anti-oxidative enzymes and related products in the plasma and intestinal mucosa of piglets are summarized in Table 5. LMG194-STa challenge caused increases in plasma MDA and decreases in TNOS relative to the control group. LMG194 challenge caused decreases in plasma TNOS relative to the control. K88 challenge caused decreases in plasma CAT and TNOS relative to the control group. Plasma TNOS in the LMG194-STa group increased in comparison to the LMG194 group. Plasma TNOS and CAT in the LMG194-STa group increased in comparison to the K88 group. There was no significant difference in the other index among the four groups.

In the duodenal mucosa, the concentration of MDA increased, but the concentrations of H_2O_2, GSH-PX and TNOS decreased relative to the control group when piglets were challenged with LMG194-STa. H_2O_2, GSH-PX and TNOS decreased relative to the control group when piglets were challenged with LMG194. SOD and CAT increased and H_2O_2, GSH-PX and TNOS decreased relative to the control group when piglets were challenged with K88. Mucosal MDA was higher in the LMG194-STa group compared to the LMG194 group. MDA, MPO and GSH-PX increased and SOD decreased in the LMG194-STa group relative to the K88 group. There was no difference in other duodenal indexes among four groups of pigs.

In the jejunal mucosa, the amounts of H_2O_2 and TNOS increased and GSH-PX decreased relative to the control group when piglets were challenged with LMG194-STa. SOD GSH-PX and TNOS increased relative to the control group when piglets were challenged with K88. SOD and TNOS increased relative to the control group when piglets were challenged with LMG194. GSH-PX activity decreased in the LMG194-STa group relative to the LMG194 group. SOD and GSH-PX decreased in the LMG194-STa group relative to the K88 group. There was no significant difference in other ileal antioxidant indexes among the four groups of pigs.

In the ileal mucosa, the amounts of H_2O_2 and SOD increased, but MPO, GSH-PX and TNOS decreased relative to the control group when piglets

Table 5. Anti-oxidative enzymes and related products in the plasma of piglets

Items	Control	LMG194-STa	LMG194	K88	*P* value
Anti-oxidative enzymes and oxidation-relevant products in plasma					
SOD (U/mL)	62.4.±5.2.	61.6.±6.5.	60.6.±7.0.	62.7.±6.4.	0.4.75
MDA (mmol/mL)	3.6.9±1.7.2^{a}	5.9.6±1.4.7^{b}	4.9.5±2.1.9^{ab}	4.9.7±2.0.9^{ab}	0.0.35
CAT (U/mL)	2.3.1±0.7.2^{a}	2.9.8±0.5.4^{a}	2.4.3±0.8.3^{a}	0.9.6±0.2.3^{b}	0.0.08
H_2O_2 (mmol/mL)	11.2.±2.2.1	9.0.9±2.5.4	8.4.7±1.0.9	10.6.±2.2.9	0.2.96
MPO (U/mL)	0.3.3±0.1.3	0.4.2±0.2.1	0.4.5±0.1.5	0.4.5±0.2.6	0.4.94
GSH-PX (U/mL)	0.3.5±0.1.1	0.4.8±0.0.6	0.3.8±0.0.7	0.4.1±0.0.7	0.1.91
TNOS (U/mL)	11.2.±0.8.2^{a}	9.3.4±1.0.2^{b}	6.3.1±1.1.3^{c}	5.8.9±1.2.^{c}	<0.0.01
iNOS (U/mL)	9.5.8±2.5.1	8.3.6±2.7.8	8.8.5±2.7.3	6.7.5±1.7.9	0.0.66
Anti-oxidative enzymes and oxidation-relevant products in duodenal mucosa					
SOD (U/mL)	26.5.±2.3.0^{a}	28.8.±5.5.6^{a}	28.1.±7.6.2^{a}	33.7.±5.7.6^{b}	0.0.23
MDA (mmol/mL)	20.4.±3.7.7^{a}	35.6.±7.1.9^{b}	18.2.±6.0.1^{a}	23.6.±5.8.9^{a}	0.0.32
CAT (U/mL)	11.4.±2.4.4^{a}	16.7.±3.8.7^{ab}	19.0.±5.1.0^{ab}	23.4.±5.0.6^{b}	<0.0.01
H_2O_2 (mmol/mL)	31.1.±4.0.4^{a}	21.9.±4.5.4^{b}	19.1.±3.1.6^{b}	19.2.±4.0.3^{b}	0.0.04
MPO (U/mL)	0.7.1±0.2.4^{ab}	0.8.7±0.2.7^{b}	0.6.4±0.2.8^{ab}	0.4.6±0.2.0^{a}	0.0.3
GSH-PX (U/mg)	306±48^{a}	206±40^{b}	192±5.6.^{bc}	145±5^{c}	0.0.01
TNOS (U/mL)	2.3.3±0.8.5^{a}	0.8.4±0.3.2^{b}	0.6.6±0.2.4^{b}	0.6.9±0.0.9^{b}	0.0.03
iNOS (U/mL)	0.3.1±0.1.7	0.3.0±0.1.8	0.2.5±0.1.2	0.2.1±0.1.4	0.3.72
Anti-oxidative enzymes and oxidation-relevant products in jejunal mucosa					
SOD (U/mL)	19.6.±2.9.2^{a}	21.0.±3.7.3^{ab}	23.4.±4.5.7^{b}	27.8.±4.5.7^{c}	0.0.01
MDA (mmol/mL)	10.2.±2.6.9	11.2.±2.7.0	10.1.±4.3.1	13.1.±3.5.0	0.4.85
CAT (U/mL)	17.2.±2.2.5	14.2.±1.7.1	16.5.±3.3.6	18.8.±4.3.2	0.3.24
H_2O_2 (mmol/mL)	13.2.±3.0.8^{a}	22.4.±5.5.^{b}	18.5.±4.0.5^{ab}	18.9.±4.1.5^{ab}	0.0.11
MPO (U/mL)	0.8.1±0.3.9	0.6.3±0.1.2	0.6.2±0.2.1	1.5.3±0.6.6	0.1.39
GSH-PX (U/mL)	120±25^{a}	66±14^{b}	117±25^{a}	144±28^{c}	0.0.01
TNOS (U/mL)	0.5.9±0.2.8^{a}	1.3.8±0.1.7^{b}	1.0.1±0.3.8^{b}	0.8.5±0.2.1^{b}	0.0.34
iNOS (U/mL)	0.8.0±0.1.9	0.7.4±0.2.6	0.7.5±0.2.7	0.5.7±0.1.5	0.1.84
Anti-oxidative enzymes and oxidation-relevant products in ileal mucosa					
SOD (U/mL)	28.9.±5.4.6^{ab}	32.6.±5.0.4^{bc}	34.4.±5.7.1^{c}	30.3.±3.0.7^{ab}	0.0.01
MDA (mmol/mL)	12.5.±4.7.5^{a}	12.7.±3.9.6^{a}	45.9.±7.3.1^{c}	28.6.±4.8.4^{b}	<0.0.01
CAT (U/mL)	25.5.±3.9.8^{b}	24.2.4±1.2.2^{ab}	13.4.4±3.0.9^{a}	16.5.1±2.8.6^{ab}	0.0.4
H_2O_2 (mmol/mL)	13.7.±2.9.4^{a}	18.4.±3.0.1^{b}	13.8.±3.2.7^{a}	13.9.±2.5.6^{a}	<0.0.01
MPO (U/mg)	1.0.1±0.4.2^{a}	0.5.1±0.1.6^{b}	1.0.1±0.1.9^{a}	0.5.6±0.1.4^{b}	0.0.14
GSH-PX (U/mL)	174±46^{a}	146±37^{b}	70±25^{c}	85±20^{c}	<0.0.01
TNOS (U/mL)	1.0.6±0.3.7^{a}	0.5.3±0.1.7^{b}	0.2.1±0.0.8^{b}	0.4.4±0.1.3^{b}	0.0.01
iNOS (U/mL)	0.5.6±0.1.4	0.3.3±0.1.4	0.4.0±0.1.3	0.6.2±0.2.4	0.1.35

Values are means ± SD, n = 6.

were challenged with LMG194-STa. MDA increased, but CAT, GSH-PX and TNOS decreased relative to the control group when piglets were challenged by K88. SOD and MDA increased, but CAT, GSH-PX and TNOS decreased relative to the control group when piglets were challenged with LMG194. H_2O_2 and GSH-PX increased, but MDA and MPO decreased in the LMG194-STa group relative to the LMG194 group. H_2O_2 and GSH-PX increased, but MDA decreased in the LMG194-STa group relative to the K88 group. There was no significant difference in other ileal antioxidant indexes among the four groups of pigs.

Oxidative stress is one of the major factors that impair the integrity of the gastrointestinal barrier and increase intestinal permeability (44). Antioxidant enzymes are an important part of the antioxidant system, and the antioxidant capacity of the body can be assessed by the determination of antioxidant enzyme activities (45). MDA is an end-product of free radicals-induced lipid peroxidation (46), an important indicator of oxidative damage (47, 48), and a useful biomarker of *in vivo* oxidative stress (49,50).

GSH-Px, SOD, and CAT are regarded as the first line of the anti-oxidant enzyme systems against reactive oxygen species (ROS) generated during metabolism and oxidative stress (51). These anti-oxidative enzymes can cooperatively convert ROS into water and O_2 (37, 52, 53). ROS, such as H_2O_2 are produced primarily by the mitochondria of cells during conversion of molecular oxygen to water (20, 48). However, cells possess scavenging mechanisms against ROS and other oxidants. H_2O_2 is degraded to water by anti-oxidative enzymes including CAT, which catalyzes the reduction of H_2O_2 to water (54). The results of the anti-oxidative enzymes and oxidation relevant products in the plasma and intestinal mucosa of piglets indicated that the *E. coli* challenge impaired the function of the antioxidant system.

5.7. Variations of gene expression profiles in the intestinal mucosa of piglets challenged with LMG194-STa

Gene expression profiles in the intestinal mucosa of piglets among different groups of pigs are summarized in Table 6. The results showed that the genes associated with intestinal injury such as villin, I-FABP and MMP3 were altered when piglets were challenged with *E. coli*. Villin is a marker of villus cell differentiation (54, 55). Thus, enhanced villin expression implies that more villus cells undergo differentiation (56, 57). Our results further demonstrated that the villin mRNA level in the LMG194-Sta group decreased in the jejunum and ileum, the villin mRNA level in the LMG194 group decreased in the jejunum, and the villin mRNA level in the K88 group decreased in the ileum. Intestinal fatty acid-binding protein (I-FABP) is also detectable in the blood during enterocyte death (58). I-FABP is located mainly in the enterocytes of the small intestine, and is released into the blood stream after intestinal ischemia and cell disruption (59,60). Therefore, I-FABP is considered as another marker of intestinal cell damage. In the present study, we found that the I-FABP mRNA level in the LMG194-Sta group decreased in the jejunum and ileum, the I-FABP mRNA level in the LMG194 group decreased in the jejunum and ileum, and the I-FABP mRNA level in the k88 group decreased in the jejunum and ileum. Matrix metalloproteinase-3 (MMP3) is considered as another marker of intestinal cell injury, and MMP3 is expressed at high levels in the intestine of clinical IBD and celiac diseases (61). Of interest, LPS challenge increased mRNA levels for MMP3 in piglets (62). In our study, we found that MMP3 mRNA levels in the jejunum of LMG194-treated pigs increased, while those in the LMG194-Sta and K88 groups decreased in comparison to the control group.

Furthermore, MMP3 mRNA levels in the ileum of the LMG194-Sta group decreased, while those in the LMG194 and K88 groups increased in comparison to the control group. These results demonstrated that LMG194-Sta challenge caused the intestinal injury in piglets.

The expression of genes associated with inflammatory cytokines in the intestine such as IL-1β, IL-4, CCL-2 (MCP-1), CXCL9 (MIG), IFN-γ, HSPH1, and VNN1, were altered when piglets were challenged with *E. coli*. In our study, we found that, relative to the control group, in the jejunum, the LMG194-Sta challenge increased the expression of IL-4, CCL-2, CXCL9 and IFN-γ but decreased the expression of VNN1; the LMG194 challenge increased the expression of IL-1β, CCL-2 and CXCL9 but decreased the expression of IFN-γ, HSPH1 and VNN1; and the K88 challenge increased the expression of VNN1 but decreased the expression of CXCL9, IFN-γ and HSPH1. In the ileum, the LMG194-Sta challenge increased the expression of VNN1 but decreased the expression of IL-4, IL-1β, CXCL9 and IFN-γ; the LMG194 challenge increased the expression of IL-1β and CCL-2 but decreased the expression of IL-4 and IFN-γ; and the K88 challenge increased the expression of IL-1β, IL-4, CCL-2, CXCL9, and IFN-γ but decreased the expression of VNN1. These results indicated that the LMG194-Sta challenge induced intestinal inflammatory responses in piglets.

The expression of genes associated with transporters and ion channels in the intestine, such as AQP8, AQP10, $b^{0,+}$AT, SGLT-1 and KCNJ13, were altered when piglets were challenged with *E. coli*. In our study, we found that, relative to the control group, in the jejunum, the LMG194-Sta challenge increased the expression of AQP10 but decreased the expression of AQP8, $b^{0,+}$AT, SGLT-1 and KCNJ13; the LMG194 challenge decreased the expression of AQP8, $b^{0,+}$AT, SGLT-1 and KCNJ13, whereas the K88 challenge increased the expression of APQ8 but decreased the expression of KCNJ13. In the ileum, the LMG194-Sta challenge increased the expression of AQP10 and SGLT-1 but decreased the expression of AQP8; the LMG194 challenge increased the expression of AQP8; and the K88 challenge increased the expression of APQ8 but decreased the expression of AQP10, $b^{0,+}$AT, SGLT-1 and KCNJ13. SGLT-1 is the major route for the transport of dietary glucose from the lumen into enterocytes (63), and Na^+/K^+-ATPase is responsible for transporting Na^+ into the intestinal cells in exchange for the efflux of K^+ (64). CFTR is located on the apical membrane of epithelial cells and is responsible for the

Table 6. Gene expression profiles in small intestines

Items	Control	LMG194-STa	LMG194	K88
Jejunum				
Villin	1.0.00±0.2.37^{b}	0.4.59±0.1.22^{c}	0.5.57±0.1.07^{c}	1.2.77±0.2.09^{a}
MMP3	1.0.00±0.1.93^{b}	0.8.74±0.2.08^{bc}	1.6.32±0.1.05^{a}	0.7.13±0.0.91^{c}
I-FABP	1.0.00±0.1.96^{a}	0.2.90±0.0.80^{c}	0.4.32±0.1.17^{c}	0.7.15±0.1.03^{b}
CCL2	1.0.00±0.1.87^{c}	1.5.18±0.2.75^{b}	2.5.85±0.5.40^{a}	0.8.89±0.2.21^{c}
IL-1β	1.0.00±0.1.79^{b}	0.7.51±0.1.94^{b}	3.1.18±0.7.88^{a}	0.7.62±0.0.62^{b}
IL-4	1.0.00±0.2.45^{b}	5.3.94±1.0.70^{a}	1.2.98±0.2.89^{b}	0.9.58±0.1.85^{b}
CXCL9	1.0.00±0.1.91^{b}	1.3.59±0.1.82^{a}	1.2.80±0.2.24^{a}	0.7.28±0.1.85^{c}
HSPH-1	1.0.00±0.1.27^{a}	0.9.81±0.2.38^{a}	0.6.99±0.0.90^{b}	0.6.42±0.1.71^{b}
IFN-γ	1.0.0±0.2.23^{b}	1.4.89±0.1.47^{a}	0.7.18±0.1.58^{c}	0.5.77±0.1.07^{c}
VNN1	1.0.0±0.2.58^{b}	0.5.82±0.0.44^{c}	0.3.70±0.0.45^{d}	1.4.97±0.2.11^{a}
AQP8	1.0.00±0.1.54^{b}	0.1.64±0.0.19^{c}	0.3.91±0.0.75^{c}	4.1.18±0.4.63^{a}
AQP10	1.0.00±0.2.51^{b}	0.6.09±0.1.39^{c}	0.4.88±0.0.94^{c}	1.3.17±0.2.91^{a}
$b^{0,+}$AT	1.0.00±0.2.43^{a}	0.7.50±0.0.57^{b}	0.5.79±0.0.72^{b}	1.2.08±0.3.07^{a}
SGLT-1	1.0.00±0.1.86^{ab}	0.5.03±0.0.58^{c}	0.8.36±0.1.27^{b}	1.0.73±0.1.63^{a}
KCNJ-13	1.0.00±0.1.93^{a}	0.1.40±0.0.21^{d}	0.4.21±0.1.11^{c}	0.6.26±0.1.04^{b}
INSR	1.0.00±0.0.81^{a}	0.6.39±0.1.02^{b}	0.7.26±0.0.92^{bc}	0.5.43±0.1.06^{c}
LPL	1.0.00±0.2.63^{bc}	1.2.36±0.1.00^{ab}	0.8.62±0.0.78^{c}	1.1.55±0.1.80^{a}
PCK1	1.0.00±0.2.39^{b}	0.3.00±0.0.68^{c}	0.3.53±0.0.78^{c}	1.8.03±0.3.44^{a}
HIF-1	1.0.00±0.2.00^{a}	0.8.37±0.1.12^{ab}	0.9.18±0.1.78^{ab}	0.7.76±0.1.22^{b}
TLR4	1.0.00±0.2.18^{b}	1.2.50±0.3.23^{ab}	1.3.24±0.1.58^{a}	1.0.56±0.1.76^{ab}
NF-κB	1.0.00±0.0.87	1.0.16±0.2.07	1.0.62±0.2.30	0.9.32±0.2.44
pBD-1	1.0.00±0.2.24^{b}	1.0.53±0.2.14^{b}	1.4.55±0.3.91^{a}	0.6.95±0.3.64^{b}
REG3G	1.0.00±0.1.50^{b}	0.1.79±0.0.42^{c}	0.3.97±0.0.62^{c}	4.3.24±0.4.99^{a}
NOX2	1.0.00±0.0.99^{a}	0.5.80±0.0.73^{c}	0.7.29±0.0.77^{b}	0.7.43±0.1.52^{b}
Nrf-2	1.0.00±0.2.13^{b}	0.7.57±0.1.20^{b}	1.7.73±0.3.90^{a}	0.9.46±0.1.68^{b}
GSTO2	1.0.00±0.2.35^{b}	1.0.73±0.2.73^{b}	1.4.19±0.2.90^{a}	1.1.39±0.2.68^{ab}
Ileum				
Villin	1.0.00±0.2.31^{a}	0.6.57±0.1.49^{b}	1.0.41±0.1.94^{a}	0.5.21±0.1.40^{b}
MMP3	1.0.00±0.2.28^{b}	0.5.69±0.1.20^{c}	1.7.73±0.4.02^{a}	1.9.54±0.2.99^{a}
I-FABP	1.0.00±0.1.44^{a}	0.8.67±0.1.51^{a}	0.8.90±0.1.92^{a}	0.2.95±0.0.56^{b}
CCL2	1.0.00±0.2.52^{c}	0.9.32± 0.0.70^{c}	3.7.39±0.3.69^{a}	1.4.27±0.3.32^{b}
IL-1β	1.0.00±0.1.74^{c}	0.5.06±0.1.09^{c}	2.8.48±0.7.02^{b}	3.9.95±1.0.13^{a}
IL-4	1.0.00±0.1.03^{b}	0.1.85±0.0.36^{d}	0.5.41±0.1.20^{c}	1.7.80±0.3.92^{a}
CXCL9	1.0.00±0.1.89^{b}	0.6.23±0.0.80^{c}	0.8.55±0.1.89^{bc}	3.1.35±0.3.65^{a}
HSPH-1	1.0.00±0.2.50	0.9.79±0.2.41	0.8.17±0.1.48	0.8.90±0.1.51
IFN-γ	1.0.00±0.1.14^{b}	0.1.56±0.0.17^{d}	0.4.91±0.1.12^{c}	1.7.75±0.4.31^{a}
VNN1	1.0.00±0.2.58^{b}	1.4.21±0.3.60^{a}	0.8.23±0.1.41^{b}	0.3.21±0.0.93^{c}
AQP8	1.0.00±0.1.57^{c}	0.3.90±0.0.66^{d}	2.0.18±0.4.72^{b}	4.8.65±0.4.67^{a}
AQP10	1.0.00±0.1.64^{b}	1.5.52±0.3.26^{a}	1.0.67±0.0.99^{b}	0.2.73±0.0.54^{c}

$b^{0,+}AT$	1.0.00±0.1.04[a]	0.9.19±0.1.04[a]	1.0.24±0.1.12[a]	0.2.72±0.0.44[b]
SGLT-1	1.0.00±0.1.70[b]	1.2.66±0.3.49[a]	1.1.37±0.1.48[ab]	0.2.06±0.0.46[c]
KCNJ-13	1.0.00±0.1.58[a]	1.1.35±0.1.99[a]	1.0.21±0.0.99[a]	0.2.80±0.0.64[b]
INSR	1.0.00±0.2.41[a]	1.2.57±0.2.92[a]	1.0.35±0.2.07[a]	0.5.40±0.0.81[b]
LPL	1.0.00±0.2.25[b]	0.5.47±0.0.47[c]	0.9.27±0.2.27[b]	1.4.72±0.3.52[a]
PCK1	1.0.00±0.2.45[b]	1.9.82±0.4.42[a]	1.2.85±0.2.13[b]	0.1.48±0.0.28[c]
HIF-1	1.0.00±0.0.72[a]	0.8.51±0.1.48[b]	0.8.33±0.1.22[b]	0.9.14±0.0.74[ab]
TLR4	1.0.00±0.2.37[c]	0.8.63±0.1.64[c]	1.3.04±0.1.37[b]	1.6.05±0.3.29[a]
NF-κB	1.0.00±0.1.31	1.0.89±0.1.05	1.0.75±0.1.69	1.0.61±0.1.32
pBD-1	1.0.00±0.1.93[b]	1.4.92±0.2.94[a]	1.2.61±0.3.42[ab]	0.9.92±0.2.32[b]
REG3G	1.0.00±0.1.60[c]	0.3.24±0.0.56[d]	1.8.89±0.4.47[b]	2.7.97±0.2.38[a]
NOX2	1.0.00±0.2.34[a]	0.5.71±0.1.33[bc]	0.5.96±0.1.10[b]	0.3.98±0.0.95[c]
Nrf-2	1.0.00±0.1.71[b]	0.8.28±0.1.89[b]	1.1.08±0.0.62[b]	1.8.44±0.4.25[a]
GSTO2	1.0.00±0.1.66[c]	1.1.69±0.1.90[bc]	1.3.84±0.1.17[a]	1.2.77±0.2.31[a]

Values are means ± SD, n = 6.

transport of chloride and bicarbonate ions into and out of the cell (65,66). AQP8 and AQP10 are the major water transporters in the gastrointestinal tract (67). Our results of the changes in the expression of genes for the transport of fluids and electrolytes, such as AQP8, AQP10, $b^{0,+}AT$, SGLT-1 and KCNJ13 in ETEC-infected intestinal segments, were consistent with the reports of previous studies on ETEC *E. coli* infection (68,69). These findings clearly demonstrated a role of STa toxin in the disturbance of water and electrolyte transport after ETEC infection.

The expression of genes associated with innate immunity, such as TLR4, NF-κB, pBD-1 and REG3G, were altered when piglets were challenged with *E. coli*. In our study, we found that, relative to the control group, in the jejunum, the LMG194-Sta challenge increased the expression of pBD1 but decreased the expression of REG3G; the LMG194 challenge decreased the expression of pBD1 but decreased the expression of REG3G; and the K88 challenge increased the expression of REG3G but decreased the expression of pBD1. In the ileum, the LMG194-Sta challenge increased the expression of pBD1 but decreased the expression of REG3G; the LMG194 challenge increased the expression of TRL4 and REG3G; and the K88 challenge increased the expression of TRL4 and REG3G (70). Regenerating islet-derived protein 3 gamma is a protein encoded by the REG3G gene in humans and is produced by intestinal paneth cells via stimulation of toll-like receptors (TLRs) by pathogen-associated molecular patterns (PAMPs). REG3 gamma specifically targets Gram-positive bacteria by binding to their surface peptidoglycan layer (71). In our study, we found that the expression levels of the genes associated with innate immunity were different among various intestinal segments in pigs. This indicated that the E.coli challenge affected intestinal immune function.

The expression of genes associated with oxidation, such as NOX2, Nrf-2, HIF-1 and GSTO2, was altered when piglets were challenged with *E. coli*. In our study, we found that, relative to the control group, in the jejunum, the LMG194-Sta challenge decreased the expression of NOX2; the LMG194 challenge increased the expression of Nrf-2 and GSTO2 but decreased the expression of NOX2; and the K88 challenge decreased the expression of HIF-1 and NOX2. In the ileum, the LMG194-Sta challenge decreased the expression of HIF-1 and NOX2; the LMG194 challenge increased the expression of GSTO2 but decreased the expression of HIF-1 and NOX2; and the K88 challenge increased the expression of GSTO2 and Nrf-2 but decreased the expression of NOX2. Hypoxia-inducible factor 1 (HIF-1) activates the transcription of genes encoding proteins that mediate adaptive responses to reduced oxygen availability. The HIF-1β subunit is constitutively expressed, whereas the HIF-1α subunit is subject to ubiquitination and proteasomal degradation, a process that is inhibited under hypoxic conditions (72). A counterpart of NF-κB, nuclear factor-erythroid 2-related factor-2 (Nrf2), which is a redox sensitive transcription factor, plays a protective role in inflammation and responds to pro-inflammatory stimuli and therefore rescues cells from inflammatory injuries. Nrf2 modulates cellular defense against oxidative and electrophilic insults by rapid induction of antioxidative and phase-II detoxifying enzymes and related stress-response proteins (73). NADPH oxidase (NOX) family members are important sources for ROS generation. In our study, we found that the *E. coli* challenge altered the expression of genes associated with oxidation in different segment of the intestinal tract.

The expression of genes associated with nutrient metabolism, such as PCK1, INSR and LPL, was altered when piglets were challenged with *E. coli*. In our study, we found that, relative to the control group, in the jejunum, the LMG194-Sta or LMG194 challenge decreased the expression of INSR and PCK1; the K88 challenge decreased the expression of INSR but increased the expression of LPL and PCK1. In the ileum, the LMG194-Sta challenge decreased the expression of LPL but increased the expression of PCK1, whereas the K88 challenge increased the expression of LPL but decreased the expression of INSR and PCK1. Based on these results, we conclude that the *E. coli* challenge can affect the intestinal metabolism of nutrients, including amino acids, glucose and lipids. Our findings have very important implications for intestinal nutrition and health, because these nutrients are essential for cell signaling, protein synthesis, and animal growth (74,75).

6. CONCLUSION AND PERSPECTIVE

A well-established model of porcine diarrhea and intestinal injury provides a very important approach to studying the mechanisms responsible for diarrhea and nutritional modulation of intestinal function. However, an animal model of diarrhea is generally difficult to establish because the pathogenesis of the disease is complex and pathogens (stimuli) may include multiple factors which can induce intestinal injury and dysfunction. A LPS-induced diarrhea model was well used to study the protective effects of different nutrients on intestinal injury (17, 18, 23, 29, 37, 40). In our research, we have established a useful model of piglet diarrhea and intestinal injury in response to oral administration of a recombinant *E.* coli strain LMG194-Sta which expressed a single toxin STa of ETEC. *In vitro* experiments indicated that the recombinant strain LMG194-Sta had the same toxicity to the IPEC cells as wide type Strain K88, and higher toxicity than that of the host strain LMG194. *In vivo* experiments showed that the LMG194-STa challenge could cause severe diarrhea in piglets as did K88 and that the diarrhea rates in the LMG194-STa and K88 groups were higher than those in the LMG194 and control groups. Further investigation of the mechanisms responsible for LMG194-STa-induced intestinal dysfunction identified that the recombinant strain LMG194-STa challenge adversely altered intestinal morphology, mucosal immunocyte numbers, plasma and mucosal antioxidant indexes, hematological indexes, and the expression profiles of mucosal genes for the transport of water, ions and nutrients (including amino acids). Collectively, these results demonstrated that a model of porcine diarrhea and intestinal injury was successfully established to study the protective effects of different nutritional supplements on alleviating toxin-induced intestinal injury.

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Abbreviations: ETEC: Enterotoxigenic Escherichia coli; STa: type I heat-stable toxins; LT: heat-labile toxin; ALB: albumin: ALP: alkaline phosphatase; ALT: alanine transaminase; AQP: aquaporin; AST: aspartate transaminase; $b^{0,+}$AT: $B^{0,+}$ neutral amino acid transporter; BASOR: basophilic granulocyte rate; BUN: blood urea nitrogen; CAT: catalase; CCL-2: C-C motif chemokine ligand 2; CHOL: cholesterol; CK: creatine kinase; Crea: creatinine; CXCL9: Chemokine (C-X-C motif) ligand 9; DAO: diamine oxidase; EOSR: eosnophils rate; GGT: gamma-glutamyl transpeptidase; GLU: glucose; GSH-PX: glutathione peroxidase; GSTO2: glutathione S-transferase omega 2; HCT: hematocrit value; HGB: hemoglobin; HIF-1: hypoxia-inducible factor 1; HSPH1: heat shock protein family H (Hsp110) member 1; I-FABP: intestinal fatty acid-binding protein; IFN-γ: interferon-γ; IL-4: interleukin-4; KCNJ13: potassium channel subfamily J, member 13; LYM: lymphocyte; MCH: mean corpuscular hemoglobin; MCV: mean corpuscular volume; MDA: malondialdehyde; MMP3: matrix metalloproteinase 3; MONOR: monocyte rate; MPO: myeloperoxidase; MPV: mean platelet volume; NOX: non- phagocytic cell oxidase; NF-κB: nuclear factor κB; Nrf-2: nuclear factor erythroid-2-related factor-2; pBD-1: porcine β-defensins 1; PCT: procalcitonin; PDW: platelet distribution width; PLT: blood platelet; RBC: red blood cell; REG3G: regenerating islet-derived protein 3 gamma; ROS: reactive oxygen species; RPL4: ribosomal protein L4; SGLT-1: sodium glucose co-transporters 1; SOD: superoxidde dismutase; TBIL: total bilirubin; TG: triglyceride; TLR4: toll-like receptor 4; TNOS: total nitric oxide synthase; TP: total protein; VNN1: vanin 1; WBC: white blood cells.

Key Words: Recombinant Escherichia coli, Piglet Diarrhea, Intestinal injury, Review

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