



Injury and mechanism of recombinant *E. coli* expressing STa on piglets colon

Yang LV¹⁾, Xueni LI¹⁾, Lin ZHANG²⁾, Yutao SHI¹⁾, Linxiao DU¹⁾, Binying DING¹⁾, Yongqing HOU¹⁾, Joshua GONG^{1,3)} and Tao WU^{1)*}

¹⁾Hubei Key Laboratory of Animal Nutrition and Feed Science, Wuhan Polytechnic University, Wuhan 430023, China

²⁾Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Wuhan, 430070, China

³⁾Guelph Research and Development Centre, Agriculture and Agri-Food Canada, Guelph, N1G 5C9, Canada

ABSTRACT. *Enterotoxigenic Escherichia coli* (ETEC) is primary pathogenic bacteria of piglet diarrhea, over two thirds of piglets diarrhea caused by ETEC are resulted from STa-producing ETEC strains. This experiment was conducted to construct the recombinant *E. coli* expressing STa and study the injury and mechanism of recombinant *E. coli* expressing STa on 7 days old piglets colon. Twenty-four 7 days old piglets were allotted to four treatments: control group, STa group (2×10^9 CFU *E. coli* LMG194-STa), LMG194 group (2×10^9 CFU *E. coli* LMG194) and K88 group (2×10^9 CFU *E. coli* K88). The result showed that *E. coli* infection significantly increased diarrhea rates; changed DAO activity in plasma and colon; damaged colonic mucosal morphology including crypt depth, number of goblet cells, density of lymphocytes and lamina propria cell density; substantially reduced antioxidant capacity by altering activities of GSH-Px, SOD, and TNOS and productions of MDA and H₂O₂; obviously decreased AQP3, AQP4 and KCNJ13 protein expression levels; substantially altered the gene expression levels of inflammatory cytokines. Conclusively, STa group had the biggest effect on these indices in four treatment groups. These results suggested that the recombinant strain expressed STa can induce piglets diarrhea and colonic morphological and functional damage by altering expression of proteins connect to transportation function and genes associated with intestinal injury and inflammatory cytokines.

KEY WORDS: colon, heat-stable enterotoxin A, piglet, recombinant *escherichia coli*

J. Vet. Med. Sci.

80(2): 205–212, 2018

doi: 10.1292/jvms.17-0528

Received: 1 October 2017

Accepted: 21 November 2017

Published online in J-STAGE:

29 November 2017

Piglet diarrhea is one of the most challenging problems what pig breeding industry has faced at home and abroad, which causes a huge economic loss annually. *Enterotoxigenic Escherichia coli* (ETEC) is the primary pathogenic bacteria of piglet diarrhea, whose pathogenicity depends on the co-effect of adhesion and enterotoxin [10]. Among the six recognized diarrheagenic classifications of *Escherichia coli* (*E. coli*), ETEC is the most ordinary etiologic agent [29].

ETEC isolates secrete two primary toxins (either one or both): the heat-stable (ST) and the heat-labile (LT) enterotoxin [23]. ST enterotoxin is a low-molecular-weight peptide and has no immunogenicity, while LT consists essentially of one A and five B subunits, which is a hexameric protein with strong immunogenicity [22]. There are two varieties of STs, which can be clearly characterised by structure and function: soluble in carbinol, resistant to proteolytic enzyme and binding of guanylyl cyclase-C-STa (also referred to as STI); insoluble in carbinol and sensitive to proteolytic enzyme-STb (STII) [19]. Over two thirds of piglets diarrhea caused by ETEC are resulted from STa-producing ETEC strains [25], so the most heated studies of ETEC are frequently focused on STa [31].

In general, ETEC induces piglets diarrhea and intestinal damage via simultaneous effects of several enterotoxins. Therefore, it is extremely difficult to research the effect of each enterotoxin respectively. In order to study the mechanism of STa and evaluate the effects of drugs or nutrients to the piglet diarrhea caused by STa, the host strain *E. coli* LMG194 and the plasmid-pBAD202/D-topo were utilized in this research to construct a recombinant *E. coli* strain named LMG194-STa, which expressed heat-stable enterotoxin A (STa). Furthermore, LMG194-STa recombinant strain was utilized to investigate its effects as well as mechanism on colonic injury, transport activity and antioxidant capacity of 7 days old piglets.

*Correspondence to: Wu, T.: wtao05@163.com

©2018 The Japanese Society of Veterinary Science



This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License. (CC-BY-NC-ND 4.0: <https://creativecommons.org/licenses/by-nc-nd/4.0/>)

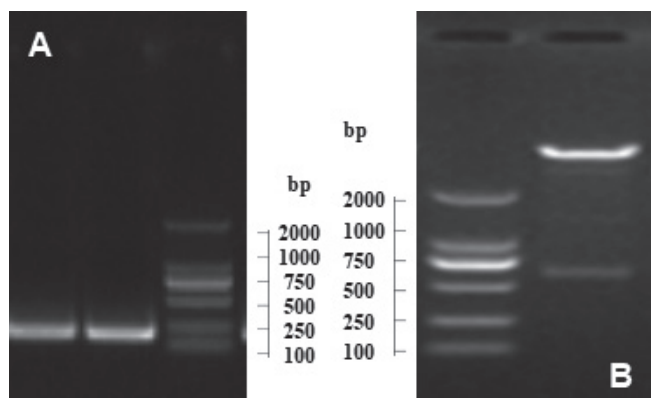


Fig 1. Construction of the recombinant *E. coli* LMG194-STa.
A) Amplification of STa by PCR (216 bp) M: DL2000 Marker.
B) Verification of pBAD-STa by enzyme digestion (393+216 bp)
M: DL2000 Marker.

Table 1. Nutrition-allocated proportion of milk-substitutes

Composition	Contents	Composition	Contents
Crude Protein /%	≥20.0	Crude Fiber /%	≤0.3
Ether Extract /%	≤10.0	Calcium /%	0.4–1.1
Crude Ash /%	≤9.0	Total Phosphorus /%	≥0.3
Water /%	≤10.0	Lysine /%	≥1.4

MATERIALS AND METHODS

Construction and verification of recombinant *E. coli* strain LMG194-STa

STa gene *estA* cloned and expressed in nonpathogenic *E. coli* strain LMG194. Enterotoxin clone DH5a-STa with *estA* gene of ETEC was presented by 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 supplier's instructions. The PCR product was examined by gel electrophoresis and sequence. The STa fragments were purified from agarose gels using PCR cleanup and gel extraction kit (Takara). The plasmid pBAD-STa was constructed according to the manual of the pBAD202 Directional TOPO® Expression Kit (Invitrogen, Carlsbad, CA, U.S.A.) and verified by enzyme digestion. The recombinant *E. coli* LMG194 which expression STa of ETEC was constructed by transferring the recombinant plasmid pBAD-STa into the *E. coli* LMG194 component cell and cultivated on LB agar with kanamycin (30 µg/ml) according 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 primer previously used.

The results showed that 219 bp fragments got when the *estA* gene was amplified by PCR with the DH5a-STa as template (Fig. 1A). Nucleic acid sequence analysis demonstrated that the fragment was 100% homologous to the *estA* gene of ETEC. The verification of the recombinant strain shown that the same size fragments got when the *estA* gene was amplified by PCR with the positive clones as template. The plasmid was extracted from recombinant strain LMG194-STa and identified by enzyme digestion, the result showed the correct size (393+216 bp) fragments got (Fig. 1B). These results means that the recombinant strain expressed STa constructed correctly, named LMG194-STa.

Animals experimental

Approval of animal use protocol for this experiment was authorized by the Animal Care and Use Committee of Hubei Province. Twenty-four healthy crossbred (duroc × landrace × yorkshire) 7-day-old piglets were assigned to 4 groups at random: (1) control group, 6 piglets were fed with commercially available milk-substitutes (artificial milk); (2) STa group, 6 piglets were fed with artificial milk and challenged with 2×10^9 CFU recombinant *E. coli* LMG194-STa; (3) LMG194 group, 6 piglets were fed with artificial milk and challenged with 2×10^9 CFU *E. coli* LMG194; and (4) K88 group, 6 piglets were fed with artificial milk and challenged with 2×10^9 CFU *E. coli* K88. The challenge doses and duration were determined by a preliminary experiment before. ETEC strains K88 was used as positive control (State Key Laboratory of Agricultural Microbiology, Wuhan, China) and LMG194 was used as negative control.

The basal diet in this experiment used commercially available artificial milk (Anyou Feed Technology Co., Wuhan, China), and the nutrition-allocated proportion of the milk-substitutes is presented in Table 1. Each piglet was individually housed in a 1.10×1.20 m² steel metabolic cage with six replicate cages in each group. The challenge of *E. coli* started on day 5, 1×10^9 CFU twice a day (in morning and evening). Piglets were killed by jugular puncture, and blood and colon samples were collected on day 7. In the whole process of the experiment the diarrhea was observed and diarrhea rate was calculated.

Blood and colon samples collection

Blood samples (anterior vena cava) were centrifuged at 3,000 rpm for 10 min at 4°C to acquire plasma, and that was stored at -80°C. After mesentery was separated, 5 to 10 cm colon were fixed in 10% formalin solution for assessments of colonic mucosal morphology, the colonic mucosa was rapidly collected, froze in liquid nitrogen and stored at -80°C until analyses.

Table 2. Primers for Real-Time PCR analysis

Gene	Primers	Gene code
RPL4	5'-GAGAAACCGTCGCCGAAT-3' 5'-GCCACCAGGAGCAAGTT-3'	XM_005659862
Villin	5'-TATTATTGGTGTTCGTGCTA-3' 5'-TCTGGAGGAATAGGATACTAA-3'	YMD14268
MMP3	5'-GATGTTGGTTACTTCAGCAC-3' 5'-ATCATTATGTCAGCCTCTCC-3'	YMD14303
I-FABP	5'-AGATAGACCGCAATGAGA-3' 5'-TCCTTCTGTGTAATTATCATCAGT-3'	YMD14286
IL-4	5'-AGGAGCCACACGTGCTTGA-3' 5'-TTGCCAAGCTGTTGAGATTCC-3'	M77120
IFN- γ	5'-TCTGGGAAACTGAATGACTTCG-3' 5'-GACTTCTCTCCGCTTTCTTAGGTT-3'	YMD14356
CCL2	5'-CATAAGCCACCTGGACAAGAAAA-3' 5'-GGGTATTAGGGCAAGTTAGAAGGA-3'	NM-214214
CXCL9	5'-CTTGCTTTTGGGTATCATCTTCCT-3' 5'-TCATCCTTTGGCTGGTGTG-3'	NM_001114289
HSPH-1	5'-ATGAGCACGGCTTCATTTC-3' 5'-GGGCTTTTCCGACTTTCCA-3'	NM_001007518
VNN1	5'-ATGGATGTGTCGCTGGTCTTT-3' 5'-CACAACTGCTTGCCTTACGAG-3'	NM_004666

Assessments of colonic mucosal morphology

Morphological analysis of colonic mucosa was performed on hematoxylin and eosin (H&E)-stained sections. Morphological examination was carried out using a light microscope (American Optical Co., New York, NY, U.S.A.). The crypt depth, number of goblet cells and lymphocytes, and density of lymphocytes and lamina propria cell were measured and calculated by Leica Application Suite image analysis software (Leica, Wetzlar, Germany).

Analysis of colonic barrier function and antioxidant capacity

Activity of diamine oxidase (DAO) in colon, which is a biomarker of intestinal injury, is used as an assessment of colonic barrier function. DAO activity in colonic mucosa and plasma was analyzed using commercial enzyme kits (Jiancheng Bioengineering Institute, Nanjing, China) by spectrophotometry, which was described by Hosoda *et al.* [14].

Activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and total nitric oxide synthase (TNOS), as well as productions of malondialdehyde (MDA) and hydrogen peroxide (H₂O₂) in colonic samples, which are used as an assessment of colonic antioxidant capacity, were analyzed by utilizing commercial enzyme kits (Jiancheng Bioengineering Institute, Nanjing, China). Each colon sample was carried out in triplicate.

Analysis of colonic transport function

The expression levels of three protein, aquaporin 3 (AQP3), aquaporin 4 (AQP4), and potassium inwardly-rectifying channel subfamily J member 13 (KCNJ13), which are used as assessment of colonic transport function, were performed by western blotting [32]. The primary antibodies: AQP3, AQP4 and KCNJ13 (rabbit monoclonal antibodies, 1:1,000 in dilution buffer; Cell Signaling Technology, Inc., Danvers, MA, U.S.A.), β -actin (mouse monoclonal antibody, 1:2,000 in dilution buffer; Sigma-Aldrich Inc., St. Louis, MO, U.S.A.). The secondary antibody: anti-rabbit (mouse monoclonal antibody, 1:5,000 in dilution buffer; Zhongshan Golden Bridge Biological Technology Co., Ltd., Beijing, China). Blots were carried out by utilizing a chemiluminescence kit (Amersham Biosciences, Uppsala, Sweden) and image forming system (Alpha Innotech, New York, NY, U.S.A.).

Analysis of gene expression levels

The gene expression levels in colonic mucosa samples: villin, I-FABP and MMP3 which are associated with intestinal injury, as well as IL-4, CCL-2, CXCL9, IFN- γ , HSPH1 and VNN1 which are associated with inflammatory cytokines of the intestine, were quantitated by the method of real-time PCR [15]. The real-time PCR was carried out with primers (Table 2) of these genes as well as reference gene ribosomal protein L 4 (RPL4) and the SYBR[®] Premix Ex TaqTM (Takara, Dalian, China) on 7,500 Fast Real-Time PCR System (Foster City, CA, U.S.A.). Data was analyzed by the 2^{- Δ Ct} method [30].

Statistical analysis

Data were analyzed using one-way analysis of variance to analysis, expressed as mean values \pm SEM. All experimental data was analysed using SPSS (Version 17.0). A *P*-value of <0.05 was considered statistically significant.

Table 3. The rate of piglet diarrhea

Items	Control group	STa group	LMG194 group	K88 group
Before challenge (%)	22.2	5.6	5.6	11.1
After challenge (%)	25.0 ^{a)}	91.7 ^{b)}	33.3 ^{a)}	91.7 ^{b)}

Data are mean ± SEM, n=6. a, b) Different letters differ significant ($P<0.05$).

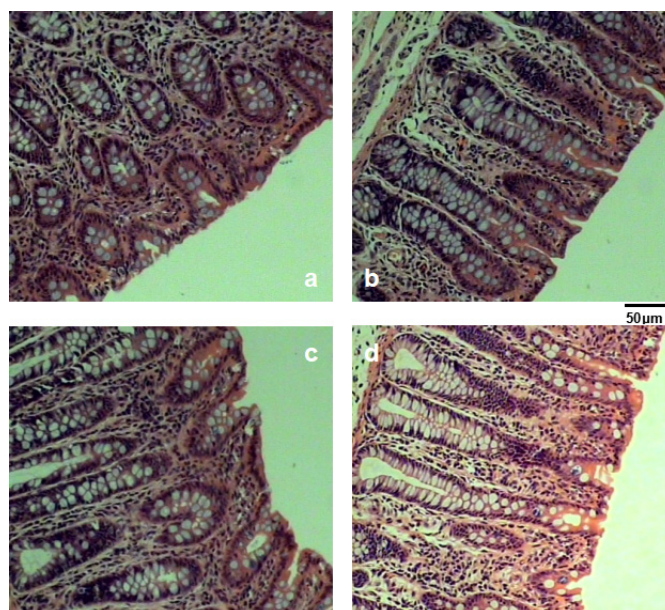


Fig 2. Colonic mucosal morphology. a) control group, b) STa group, c) LMG194 group and d) K88 group.

RESULTS

The rate of piglet diarrhea

The diarrhea rate was listed in Table 3. Compare to the diarrhea rate with different group, there is no significant difference before challenge ($P>0.05$), but the diarrhea rate of LMG194-STa group and K88 group increased sharply and was much higher than the LMG194 group and control group after challenge ($P<0.05$). However, there is no significant difference between the LMG194-STa group and K88 group ($P>0.05$).

Colonic mucosal morphology

Colonic mucosal morphology was showed in Fig. 2 and Table 4. Relative to the control group, both STa and K88 group significant increased the depth of crypt, the number of goblet cells and lamina propria cell density in colonic mucosa ($P<0.05$). Three treatments all reduced the density of lymphocyte ($P<0.05$), and at the same time, STa group had more serious impacts than LMG194 group particularly.

DAO level and gene expression associated with intestinal integrity

The result showed that, both STa group and LMG194 group had a higher DAO activity in plasma ($P<0.05$), and a lower activity of that in colonic mucosa ($P<0.05$) than the control group (Table 5). Nevertheless, STa group had a bigger impact than LMG194 group.

The mRNA levels of these three genes in STa group were lower ($P<0.05$) than that in control group. Similarly, villin and MMP3 in K88 group were lower ($P<0.05$) than in control group, but LMG194 group had a contrary effect on MMP3 and I-FABP (Fig. 4).

Transport function

Relative to the control group, STa group as well as LMG194 group significantly decreased AQP3, AQP4 and KCNJ13 expression ($P<0.05$) in colon (Fig. 3). K88 group also significantly reduced AQP3 expression ($P<0.05$) in colon.

Antioxidant capacity and inflammatory cytokines

Relative to the control group, STa group noticeably decreased the activities of SOD, GSH-Px, and TNOS ($P<0.05$), and raised the productions of MDA and H_2O_2 ($P<0.05$) in colon (Table 5). At the same time, LMG194 group as well as K88 group decreased the activities of GSH-Px, TNOS and iNOS ($P<0.05$).

Relative to the control group, LMG194-STa increased expression of CCL-2 and HSPH-1 gene and decreased expression of IL-4, IFN- γ , CXCL-9 and VNN1 gene (Fig. 5). Moreover, other two group except IL-4 in LMG194 and VNN1 in K88 had the same effect as STa group.

DISCUSSION

The large intestine (also called the colon) is very important segment in vertebrates, which is one portion of the eventual section of digestion, and also a large tunnel that conveys waste from the body. The function of colon mainly consist of absorption,

Table 4. Index of colonic mucosal morphology

Items	Control group	STa group	LMG194 group	K88 group
Depth of crypt (μm)	411.3 \pm 52.4 ^b	679.3 \pm 41.6 ^a	517.6 \pm 52.3 ^b	716.2 \pm 61.1 ^a
Number of goblet cells	7.0 \pm 1.0 ^b	17.0 \pm 1.7 ^a	9.5 \pm 1.5 ^b	16.5 \pm 2.2 ^a
Number of lymphocytes	59.5 \pm 5.8	58.8 \pm 6.2	57.3 \pm 7.7	58.3 \pm 6.7
Lamina propria cell density (/mm ²)	527.4 \pm 54.3 ^a	674.4 \pm 43.8 ^c	550.2 \pm 58.9 ^a	694.7 \pm 24.8 ^b
Density of lymphocyte (/mm ²)	711.9 \pm 61.7 ^b	546.7 \pm 55.9 ^a	613.5 \pm 48.1 ^a	595.5 \pm 56.7 ^a

Data are mean \pm SEM, n=6. a–c) Different letters differ significant ($P < 0.05$).

Table 5. DAO activities in plasma and colon and antioxidant capacity in colon

Items	Control group	STa group	LMG194 group	K88 group
DAO in plasma (U/ml)	5.26 \pm 1.05 ^a	16.42 \pm 1.49 ^d	10.48 \pm 2.99 ^b	13.48 \pm 1.60 ^c
DAO in colon (U/ml)	0.74 \pm 0.16 ^a	0.52 \pm 0.09 ^b	0.59 \pm 0.12 ^b	0.68 \pm 0.13 ^{ab}
SOD (U/mg)	36.52 \pm 4.41 ^{ab}	31.16 \pm 5.42 ^c	33.11 \pm 5.23 ^{bc}	38.32 \pm 4.61 ^a
GSH-Px (U/mg)	197.29 \pm 50.58 ^a	140.85 \pm 22.52 ^b	132.15 \pm 17.89 ^b	136.98 \pm 23.26 ^b
H ₂ O ₂ (nmol/mg)	9.34 \pm 2.64 ^b	12.93 \pm 2.51 ^a	10.57 \pm 2.19 ^{ab}	8.73 \pm 1.65 ^b
MDA (nmol/mg)	15.97 \pm 5.91 ^a	21.90 \pm 5.42 ^b	20.54 \pm 5.62 ^b	13.52 \pm 4.78 ^a
TNOS (U/mg)	1.59 \pm 0.36 ^a	0.91 \pm 0.28 ^b	0.81 \pm 0.15 ^b	0.87 \pm 0.27 ^b

a–d) Different letters differ significant ($P < 0.05$).

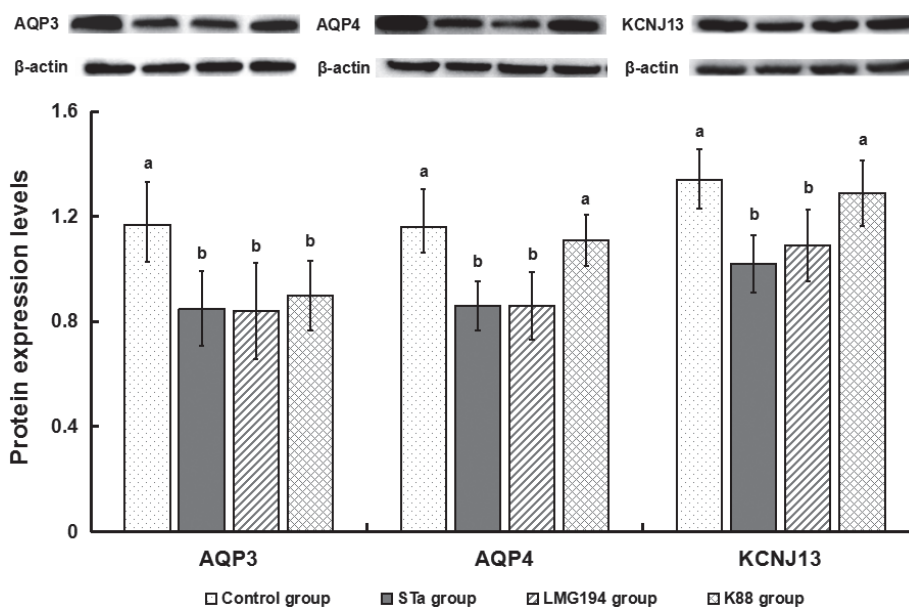


Fig. 3. Protein expression levels of AQP3, AQP4 and KCNJ13 in colonic mucosa. Data are mean \pm SEM, n=6. a–c) Different letters differ significant ($P < 0.05$), the same as below.

sercretion, digestion and excretion [11]. There are plenty of calciform cells in colon, which can serecte alkaline liquid to protect mucosa and help excretion. Colon has no digestion function in itself, but bacteria which live in colon have that. Moreover, by means of peristalsis, colon eliminates waste after nutrients are removed from it [26].

During the experiment, the diarrhea rate of LMG194-STa group and K88 group increased sharply and was much higher than the LMG194 group and control group, but there is no significant difference between the LMG194-STa group and K88 group, it indicated that *E. coli* LMG194-STa and K88 had similar effects on diarrhea which was greater than *E. coli* LMG194. Of note, the diarrheal rate is 22.2% in the control group before the inoculation, and the group inoculated with the host strain alone showed a significant increase of diarrhea rate, this condition might be mainly caused by weaning prematurely and stress reaction during inoculating.

The elongation of crypts caused by bacteria or viruses may have symbolized a irritation of the intestinal repairation process, which was obvious as a compensatory pathway for the wastage of crypts in the proximal mucosa concomitant injury to the gut [7]. The mucus layer covering the epithelia is secreted by the calciform cells, which facilitates the removal of alimentary canal contents. In addition, it affords the first defendand line against physical and chemical injury caused by intake food, bacteria and the

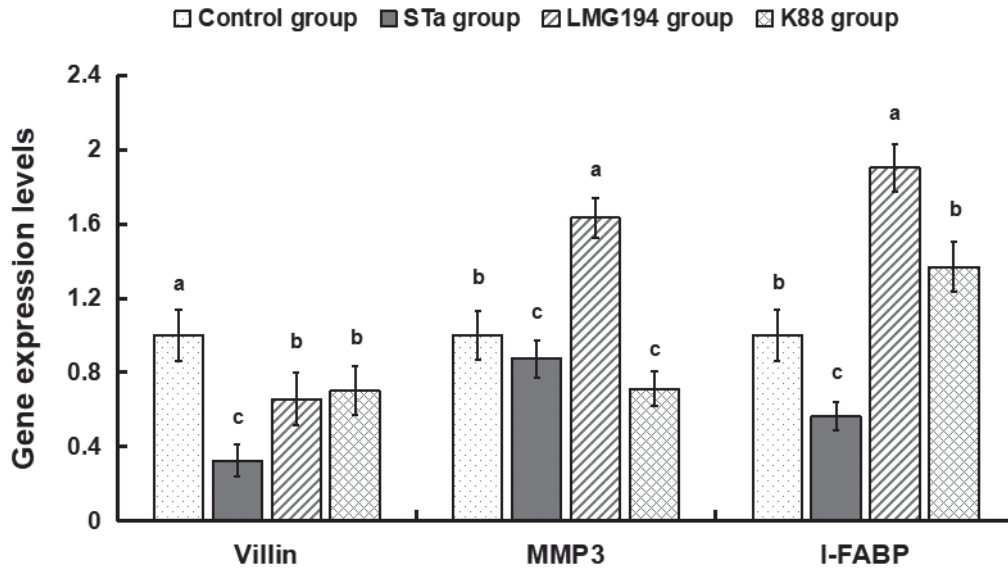


Fig 4. Gene expression levels of villin, I-FABP and MMP3 in colonic mucosa.

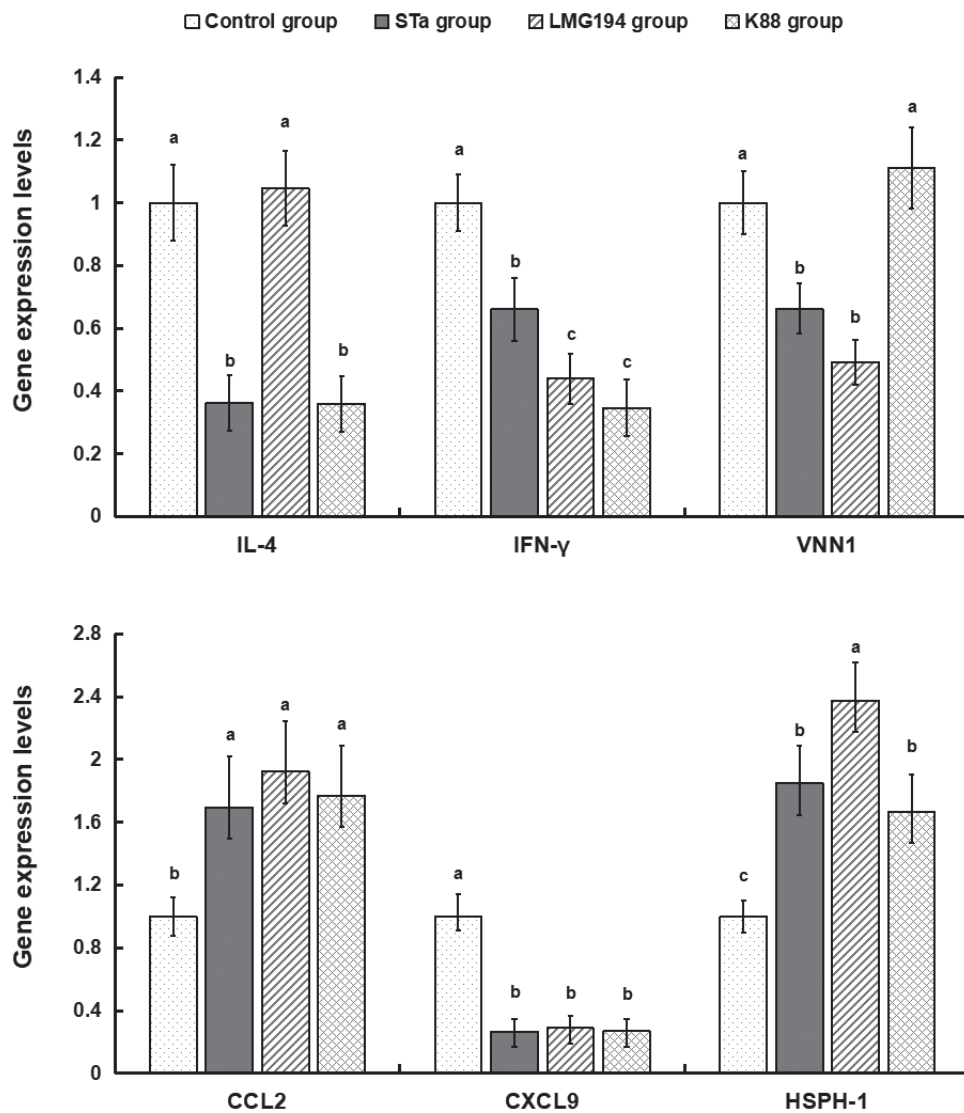


Fig. 5. Gene expression levels of inflammatory cytokines in colonic mucosa.

bacteria products [17]. In this study, both STa and K88 group significantly increased the depth of crypt, the number of goblet cells and lamina propria cell density in colonic mucosa ($P < 0.05$). Three treatments all reduced the density of lymphocyte, this result showed that these three kinds of *E. coli* could simultaneously induce colonic mucosal damage. Particularly, recombinant *E. coli* LMG194-STa and *E. coli* K88 had a bigger impact than *E. coli* LMG194.

DAO activity is frequently used as a noninvasive biomarker of alterations in the function and structure of intestinal mucosa [2, 16]. Under certain conditions, cells in intestinal mucosa experience necrosis, and slough off into the enteric entocoel, resulting in a decline of DAO levels in intestinal mucosa and an increase of DAO levels in circulation [18]. The data in this research, which STa group and LMG194 group relative to the control group had a higher activity of DAO in plasma and lower that in colonic mucosa, implied that both recombinant *E. coli* LMG194-STa and *E. coli* LMG194 could reduce the colonic barrier function. Moreover, recombinant *E. coli* LMG194-STa had a greater influence than *E. coli* LMG194.

The injury and slow growth as well as the barrier function reducing of colonic mucosa were probably associated with some genes such as villin, I-FABP and MMP3. Villin is one kind of actin binding protein and a marker of villus cell differentiation, which conduces to prop up the microfilaments of the microvilli of the mucosal villus [6]. 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 [4]. MMP3 as well as their inhibitors (TIMPs) play a crucial role in the repairment of extracorporeal matrix homeostasis, which is expressed at high levels in the intestine of clinical IBD and celiac diseases [5]. In this research, mRNA levels of these three genes in STa group were simultaneously less than that in control group, these data showed that LMG194-STa could obviously cause the damage of the colon.

AQP3 and AQP4 are two of the most important water channel proteins which regulate the water homeostasis in the central nervous system [1]. All functions of them are to afford fast water transport as well as support self-balanced within the CNS [20]. KCNJ13 is an ATP-dependent potassium channel that transports potassium out of cells, which plays a very considerable role in potassium homeostasis [12, 13]. In this study, STa group as well as LMG194 group remarkably decreased AQP3, AQP4 and KCNJ13 expression in colon, it revealed that recombinant *E. coli* LMG194-STa and *E. coli* LMG194 could substantially reduce colonic transport function.

Oxidative stress reflects the imbalance between the systematic phenomenon of reactive oxygen species and the capacity of the biosystem to readily detoxicate the reactive intermediaries or to renovate the resulting injury [8, 21]. Whereas, cells protect themselves from hydroxyl radicals and other oxygenants by antioxidant enzymes, including SOD, GSH-Px and CAT [27, 28]. MDA can induce noxious stress in cells and constitute homopolar protein adducts known as advanced lipoxidation end-products (ALEs), which is usually utilized as a marker to evaluate the oxidative stress levels in a biosome [9]. NOS catalyzes the production of NO, helps modulate insulin secretion, vascular and airway tone, and is actively involved in neural and angiogenesis development [15]. In this research, STa group significantly reduced the activities of SOD, GSH-Px and TNOS, and increased the productions of H_2O_2 and MDA in colon. This result indicated that recombinant *E. coli* LMG194-STa can induce oxidative stress, and reduce the colonic antioxidant capacity. Furthermore, LMG194 group as well as K88 group also decreased the activities of GSH-Px and TNOS, it showed that *E. coli* LMG194 and *E. coli* K88 could reduce the colonic antioxidant capacity as well.

Oxidative stress has been implicated in the development of many chronic inflammatory disorders, such as enteritis, myocarditis and thyroiditis [3]. Antioxidant defense systems may be impaired as a consequence of excessive oxidative stress, and inflammatory responses can be partially mediated by oxidative stress [24]. The genes associated with inflammatory cytokines of the intestine such as interleukin 4 (IL-4), chemokine ligand 2 (CCL-2), chemokine ligand 9 (CXCL9), Interferon gamma (IFN- γ), heat shock protein h 1 (HSPH1), vanin 1 (VNN1) were altered when the piglets challenged by *E. coli*. In present study we found that relative to the control group, LMG194-STa noticeably increased expression of CCL-2 and HSPH-1 gene and decreased expression of IL-4, IFN- γ , CXCL-9 and VNN1 gene. Moreover, LMG194 group as well as K88 group had similar effect on inflammatory cytokines. These results of these genes related to inflammatory cytokines declared that *E. coli* infection caused intestinal inflammatory reaction. Accordingly, these genes might be the potential inducement of oxidative stress.

In this research, ETEC strains K88 was used as positive control which was confirmed by PCR genotyping as genes expressing K88 fimbrial antigen. Results showed that *E. coli* K88 had analogous impact to LMG194-STa on piglet diarrhea, colonic injury and inflammatory reaction; but less influence on colonic antioxidant capacity than LMG194-STa. Therefore, the similarities and difference between *E. coli* LMG194-STa and K88 has become a new topic worthy of inquiry.

In conclusion, the recombinant *E. coli* expressing heat-stable enterotoxin A (LMG194-STa) constructed correctly, and it can induce colonic mucosal damage, decrease barrier, immune and transport function and antioxidant capacity of seven days old piglets. A further mechanistic study revealed that the injury, dysfunction and oxidative stress of colon were induced by altering expression of proteins connected with transportation function and genes associated with injury and inflammatory cytokines when piglets challenged by *E. coli*.

CONFLICT OF INTEREST. We declare that we have no conflict of interest.

ACKNOWLEDGMENTS. This work was supported by Hubei Provincial Technology and Innovation Program (2017AHB062), National Key Research and Development Program of China (2017YFD0500505), National Natural Science Foundation of China (Grant No. 31302089) and Agriculture and Agri-Food Canada A-base project (J-001391).

REFERENCES

1. Agúndez, J. A., Ayuso, P., Cornejo-García, J. A., Blanca, M., Torres, M. J., Doña, I., Salas, M., Blanca-López, N., Canto, G., Rondon, C., Campo, P., Laguna, J. J., Fernández, J., Martínez, C. and García-Martín, E. 2012. The diamine oxidase gene is associated with hypersensitivity response to non-steroidal anti-inflammatory drugs. *PLOS ONE* **7**: e47571. [Medline] [CrossRef]
2. Arnsperg, E. C., Sundbye, S., Nelson, W. J. and Nejsum, L. N. 2013. Aquaporin-3 and aquaporin-4 are sorted differently and separately in the trans-Golgi network. *PLOS ONE* **8**: e73977. [Medline] [CrossRef]
3. Besnard, P., Niot, I., Poirier, H., Clément, L. and Bernard, A. 2002. New insights into the fatty acid-binding protein (FABP) family in the small intestine. *Mol. Cell. Biochem.* **239**: 139–147. [Medline] [CrossRef]
4. Bhuvaramurthy, V., Kristiansen, G. O., Johannsen, M., Loening, S. A., Schnorr, D., Jung, K. and Staack, A. 2006. In situ gene expression and localization of metalloproteinases MMP1, MMP2, MMP3, MMP9, and their inhibitors TIMP1 and TIMP2 in human renal cell carcinoma. *Oncol. Rep.* **15**: 1379–1384. [Medline]
5. Boutten, A., Goven, D., Artaud-Macari, E. and Bonay, M. 2011. [Protective role of Nrf2 in the lungs against oxidative airway diseases]. *Med. Sci. (Paris)* **27**: 966–972. [Medline] [CrossRef]
6. Chantret, I., Barbat, A., Dussaulx, E., Brattain, M. G. and Zweibaum, A. 1988. Epithelial polarity, villin expression, and enterocytic differentiation of cultured human colon carcinoma cells: a survey of twenty cell lines. *Cancer Res.* **48**: 1936–1942. [Medline]
7. Del Rio, D., Stewart, A. J. and Pellegrini, N. 2005. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutr. Metab. Cardiovasc. Dis.* **15**: 316–328. [Medline] [CrossRef]
8. Doke, N. 1983. Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthora infestans*, and to the hyphal wall components. *Physiol. Plant Pathol.* **23**: 345–357. [CrossRef]
9. Drozdowski, L. and Thomson, A. B. 2006. Intestinal mucosal adaptation. *World J. Gastroenterol.* **12**: 4614–4627. [Medline] [CrossRef]
10. Fasina, Y. O., Moran, E. T., Ashwell, C. M. and Conner, D. E. 2007. Effect of dietary gelatin supplementation on the expression of selected enterocyte genes, intestinal development and early chick performance. *Int. J. Poult. Sci.* **6**: 944–951. [CrossRef]
11. Fu, W. X., Liu, Y., Lu, X., Niu, X. Y., Ding, X. D., Liu, J. F. and Zhang, Q. 2012. A genome-wide association study identifies two novel promising candidate genes affecting *Escherichia coli* F4ab/F4ac susceptibility in swine. *PLoS One* **7**: e32127. [Medline] [CrossRef]
12. Hames, C. A. C. and Hopkin, S. P. 2010. The structure and function of the digestive system of terrestrial isopods. *J. Zool. (Lond.)* **217**: 599–627. [CrossRef]
13. Hejtmancik, J. F., Jiao, X., Li, A., Sergeev, Y. V., Ding, X., Sharma, A. K., Chan, C. C., Medina, I. and Edwards, A. O. 2008. Mutations in KCNJ13 cause autosomal-dominant snowflake vitreoretinal degeneration. *Am. J. Hum. Genet.* **82**: 174–180. [Medline] [CrossRef]
14. Hosoda, N., Nishi, M., Nakagawa, M., Hiramatsu, Y., Hioki, K. and Yamamoto, M. 1989. Structural and functional alterations in the gut of parenterally or enterally fed rats. *J. Surg. Res.* **47**: 129–133. [Medline] [CrossRef]
15. Hou, Y., Wang, L., Ding, B., Liu, Y., Zhu, H., Liu, J., Li, Y., Wu, X., Yin, Y. and Wu, G. 2010. Dietary α -ketoglutarate supplementation ameliorates intestinal injury in lipopolysaccharide-challenged piglets. *Amino Acids* **39**: 555–564. [Medline] [CrossRef]
16. Hou, Y., Yao, K., Wang, L., Ding, B., Fu, D., Liu, Y., Zhu, H., Liu, J., Li, Y., Kang, P., Yin, Y. and Wu, G. 2011. Effects of α -ketoglutarate on energy status in the intestinal mucosa of weaned piglets chronically challenged with lipopolysaccharide. *Br. J. Nutr.* **106**: 357–363. [Medline] [CrossRef]
17. Jiang, H. B. and Ichikawa, Y. 1999. Neuronal nitric oxide synthase catalyzes the reduction of 7-ethoxyresorufin. *Life Sci.* **65**: 1257–1264. [Medline] [CrossRef]
18. Kim, Y. S. and Ho, S. B. 2010. Intestinal goblet cells and mucins in health and disease: recent insights and progress. *Curr. Gastroenterol. Rep.* **12**: 319–330. [Medline] [CrossRef]
19. Kitanaka, J., Kitanaka, N., Tsujimura, T., Terada, N. and Takemura, M. 2002. Expression of diamine oxidase (histaminase) in guinea-pig tissues. *Eur. J. Pharmacol.* **437**: 179–185. [Medline] [CrossRef]
20. Li, J. Y., Lu, Y., Hu, S., Sun, D. and Yao, Y. M. 2002. Preventive effect of glutamine on intestinal barrier dysfunction induced by severe trauma. *World J. Gastroenterol.* **8**: 168–171. [Medline] [CrossRef]
21. Liu, M., Ruan, X., Zhang, C., Lawson, S. R., Knudsen, D. E., Nataro, J. P., Robertson, D. C. and Zhang, W. 2011. Heat-labile- and heat-stable-toxoid fusions (LTR₁₉₂G-StaP₁₃F) of human enterotoxigenic *Escherichia coli* elicit neutralizing antitoxin antibodies. *Infect. Immun.* **79**: 4002–4009. [Medline] [CrossRef]
22. Oklinski, M. K., Skowronski, M. T., Skowronska, A., Rützler, M., Nørgaard, K., Nieland, J. D., Kwon, T. H. and Nielsen, S. 2016. Aquaporins in the Spinal Cord. *Int. J. Mol. Sci.* **17**: 2050. [Medline] [CrossRef]
23. Qadri, F., Svennerholm, A. M., Faruque, A. S. G. and Sack, R. B. 2005. Enterotoxigenic *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. *Clin. Microbiol. Rev.* **18**: 465–483. [Medline] [CrossRef]
24. Qi, H. and Liu, X. L. 2016. Research progress of expression systems of *Escherichia coli* and Yeast. *J. Anhui Agri. Sci.* **44**: 4–6.
25. Ringseis, R., Piwek, N. and Eder, K. 2007. Oxidized fat induces oxidative stress but has no effect on NF- κ B-mediated proinflammatory gene transcription in porcine intestinal epithelial cells. *Inflamm. Res.* **56**: 118–125. [Medline] [CrossRef]
26. Rocha, L. B., Ozaki, C. Y., Horton, D. S., Menezes, C. A., Silva, A., Fernandes, I., Magnoli, F. C., Vaz, T. M., Guth, B. E. and Piazza, R. M. 2013. Different assay conditions for detecting the production and release of heat-labile and heat-stable toxins in enterotoxigenic *Escherichia coli* isolates. *Toxins (Basel)* **5**: 2384–2402. [Medline] [CrossRef]
27. Su, P. and Gong, G. L. 2017. Research progress on optimizing the expression of exogenous proteins in *Escherichia coli*. *Biotechnol. Bull.* **33**: 16–23.
28. Svennerholm, A. M. and Lundgren, A. 2012. Recent progress toward an enterotoxigenic *Escherichia coli* vaccine. *Expert Rev. Vaccines* **11**: 495–507. [Medline] [CrossRef]
29. Uzar, E., Ozay, R., Evliyaoglu, O., Aktas, A., Ulkay, M. B., Uyar, M. E., Ersoy, A., Burakgazi, A. Z., Turkay, C. and Ilhan, A. 2012. Hydroxycycloquine-induced oxidative stress on sciatic nerve and muscle tissue of rats: a stereological and biochemical study. *Hum. Exp. Toxicol.* **31**: 1066–1073. [Medline] [CrossRef]
30. Wang, M. Z., Ding, L. Y., Wang, J. F., Yu, L. H. and Wang, H. R. 2012. Dietary effects of n-6:n-3 polyunsaturated fatty acid ratios on the antioxidant status of the liver in goslings. *J. Anim. Feed Sci.* **21**: 372–382. [CrossRef]
31. Wang, R. R. and Ma, Y. P. 2010. Progress in development of recombinant human Enterotoxigenic *Escherichia coli* vaccine. *Chin. J. Biol.* **23**: 544–548.
32. Yao, K., Yin, Y. L., Chu, W., Liu, Z., Deng, D., Li, T., Huang, R., Zhang, J., Tan, B., Wang, W. and Wu, G. 2008. Dietary arginine supplementation increases mTOR signaling activity in skeletal muscle of neonatal pigs. *J. Nutr.* **138**: 867–872. [Medline]