Protective effects of selenium nanoparticle-enriched *Lactococcus lactis* NZ9000 against enterotoxigenic *Escherichia coli* K88-induced intestinal barrier damage in mice

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Abstract

Composite microecological agents have received widespread attention due to their advantageous properties, including safety, multi-effects, and low cost. This study was conducted to evaluate the protective effects of selenium (Se) nanoparticle-enriched *Lactococcus lactis* NZ9000 (*L. lactis* NZ9000-SeNPs) against enterotoxigenic *Escherichia coli* K88 (ETEC K88)-induced intestinal barrier damage in C57BL/6 mice. Oral administration of *L. lactis* NZ9000-SeNPs significantly increased the villi height and the number of goblet cells in the ileum, and reduced the levels of serum and ileal interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ), and increased the activities of thioredoxin reductase (TrxR) and glutathione peroxidase (GSH-Px) compared with the ETEC K88-infected group not treated with *L. lactis* NZ9000-SeNPs. In addition, *L. lactis* NZ9000-SeNPs significantly attenuated the reduction of the expression levels of occludin and claudin-1, dysbiosis of the gut microbiome, and the activation of toll-like receptor (TLR)/nuclear factor-kappa (NF-κB)-mediated signaling pathway induced by ETEC K88. These findings suggested that *L. lactis* NZ9000-SeNPs may be a promising and safe Se supplement for food or feed additives.

Importance

The beneficial effects of microecological agents have been widely proven. Se, which is nutritionally essential trace element for human and animals, is incorporated into selenoproteins that have a wide range of pleiotropic effects, ranging from antioxidant and anti-inflammatory effects. However, the sodium selenite, a common addition
form of Se in feed and food, has disadvantages such as strong toxicity and low bioavailability. We investigated the protective effects of \textit{L. lactis} NZ9000-SeNPs against ETEC K88-induced intestinal barrier injury in C57BL/6 mice. Our results show that \textit{L. lactis} NZ9000-SeNPs effectively alleviate ETEC-K88-induced intestinal barrier dysfunction. This study highlights the importance of developing a promising and safe Se supplement for the substitution of sodium selenite applied in food, feed and biomedicine.

**Keywords:** Probiotic; \textit{Lactococcus lactis} NZ9000; Intestinal barrier; Selenium nanoparticles.

1. \textbf{Introduction}

The intestine, as the largest immune and digestive organ, plays a crucial role in maintaining human and animal health (1). The intestinal barrier is the functional interface separating the luminal contents of the intestinal cavity and the intestinal mucosa harboring the intestine-associated immune system, which allows absorption of nutrients and fluids but concurrently prevents harmful substances like toxins and bacteria from invading the organism, and maintains intestinal homeostasis (2). Each part of the intestinal mucosal barrier has a corresponding structural basis (3). The integrity of the intestinal mucosal barrier is maintained by tight junction (TJ) proteins (4). TJ proteins are multi-protein complexes located around the top of the outer membrane of intestinal epithelial cells, mainly composed of occludin and claudin-1 (4, 5). Intestinal barrier dysfunction will result in the production of diarrhea in weaned piglets, necrotizing enteritis in poultry, and cause huge economic losses to the...
livestock and poultry industry. Therefore, it is urgently necessary to develop safe and efficient microecological agents to protect the integrity of the intestinal barrier.

Selenium (Se) is an essential trace element that is closely associated with multiple physiological functions, such as antioxidant activity, immunity enhancement, and promoting body metabolism (6). Se deficiency has become a problem in animal husbandry worldwide, which not only causes white muscle disease, but also abortion in ewes, and a decline in fertility and pregnancy rates (7). The chemical species of Se mainly includes inorganic Se (selenite, selenate, elemental Se, nano-selenium), and organic selenium (selenocysteine (Sec), selenomethionine (SeMet), selenoprotein, etc.)

To date, studies have confirmed that the order of toxicity of Se species is as follows: selenite > selenate > SeMet > selenium nanoparticles (SeNPs) (8). Se is involved in the regulation of immune responses (9). Crude Ulva lactuca polysaccharide (ULP)-modified SeNPs inhibited macrophage activation by inhibiting nuclear translocation of nuclear factor-kappa (NF-κB), thereby alleviating inflammation in a mouse model of colitis (10). In addition, it has also been shown that the regulatory effect of Se on Staphylococcus aureus induced-mastitis is mediated by inhibiting the NF-κB signaling pathway (11). Our previous studies showed that Lactococcus lactis (L. lactis) NZ9000 has a high capacity to convert toxic sodium selenite into SeNPs with red color when co-cultured with 0.6 mM Na₂SeO₃ for 48 h at 30 °C (12). Additionally, L. lactis can induce mucosal immunity and has adjuvant properties (13). Oral administration of L. lactis NZ9000 effectively improved the intestinal barrier function
in rats (14). However, the protective effects of SeNPs-enriched microorganisms on the function of the intestinal barrier remain to be elucidated.

In this study, we hypothesized that SeNPs-enriched *L. lactis* NZ9000 could play a dual role of probiotics and nano-Se, and produce a synergistic effect. This study aimed to evaluate the protective effects of SeNPs-enriched *L. lactis* NZ9000 against intestinal barrier injury and its association with the TLRs/NF-κB-mediated signaling pathway through the establishment of an enterotoxigenic *Escherichia coli* K88 (ETEC K88)-induced intestinal barrier dysfunction model in C57BL/6 mice.

2. Materials and Methods

2.1 Bacterial Strains and Experimental Animals

The *L. lactis* NZ9000 strain was purchased from MoBiTec Company (Goettingen, Germany). The ETEC K88 strain was kept in our laboratory. The forty healthy 4-week-old male C57BL/6 mice with weight of 20 ± 2 g used in this study were purchased from Keao Biotechnology Co., Ltd. (Xi’an, China).

2.2 Reagents

M17 broth, Luria-Bertani (LB) medium were purchased from Oxoid Ltd (Basingstoke, UK). Enzyme-linked immunosorbent assay (ELISA) kits for mouse tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin-1β (IL-1β) were purchased from Jianglaibio Co., Ltd. (Shanghai, China). The radioimmunoprecipitation assay (RIPA) lysis buffer and bicinchoninic acid (BCA) protein assay kit were purchased from Solarbio Life Sciences Co., Ltd. (Beijing, China). Thioredoxin Reductase (TrxR) and glutathione peroxidase (GSH-Px) assay kits were purchased from Nanjing...
Institute of Bioengineering (Jiangsu, China). Primary antibodies against occludin, claudin-1, β-actin, nuclear factor kappa-B (NF-κB)-p65, IκBα, p-IκBα and horseradish peroxidase (HRP)-labelled secondary antibody were purchased from Abclonal Biotechnology (Wuhan, China). Primary antibodies against toll-like receptor 2 (TLR2) and toll-like receptor 4 (TLR4) were purchased from Boster Biological Technology (Wuhan, China).

2.3 Bacteria Culture Conditions

*L. lactis* NZ9000 was incubated in M17 broth at 30°C for 24 h without shaking. The synthesis process of SeNPs-enriched *L. lactis* NZ9000 mainly included the following steps. First, monoclonal *L. lactis* NZ9000 was inoculated into M17 broth and cultured until an OD_{600 nm} = 0.5 was reached, after which the bacteria were inoculated into a new flask with fresh M17 broth and cultured at 30 °C for 24 h. Subsequently, a solution of sodium selenite (0.6 mM) was added into the *L. lactis* NZ9000 culture broth to stimulate the biosynthesis of the SeNPs. Then, the SeNPs-enriched *L. lactis* NZ9000 bacteria were obtained after co-culturing for 24 h at 30 °C. According to previous methods used in our laboratory, the ETEC K88 was cultured in LB medium with shaking at a speed of 120 rpm at 37 °C overnight. Bacteria were collected by centrifugation at 5,000 × g at 4 °C for 10 min, and then the pellets were washed with phosphate buffer saline (PBS, pH 7.4) (15). The obtained bacteria were suspended in the M17 broth medium. The concentration of the bacterial resuspension solutions of *L. lactis* NZ9000, *L. lactis* NZ9000-SeNPs, and ETEC K88 was adjusted to 1.0×10^9 CFU/mL, 1.0×10^9 CFU/mL, and 1.0×10^8 CFU/mL, respectively.
2.4 Protective effects of SeNPs-enriched L. lactis NZ9000 against ETEC K88-induced intestinal barrier dysfunction in C57BL/6 mice

The animal experimental protocol used in this study was approved by the Laboratory Animal Welfare and Ethics Committee of Northwestern Polytechnical University and the study was conducted strictly in accordance with the International Laboratory Animal Assessment and Accreditation Committee guidelines for the care and use of laboratory animals. During the entire experimental period, the forty healthy male C57BL/6 mice were maintained at the Experimental Animal Center of Northwestern Polytechnical University. The living conditions were as follows: ambient temperature of 25 ℃, relative humidity of 50 %, and under a 12 h light/dark cycle. The experimental scheme is depicted in Fig. 1A. After an adaptive period of 7 days, the mice were randomly assigned to four groups, with 10 mice per group, as follows: normal control group (M17 broth and LB medium), ETEC K88-infected model group (ETEC K88 resuspension solution), L. lactis NZ9000 treatment group (resuspension solution of L. lactis NZ9000 and ETEC K88), and SeNPs-enriched L. lactis NZ9000 treatment group (resuspension solution of L. lactis NZ9000-SeNPs and ETEC K88).

The experimental duration lasts for 14 days. The mice in the L. lactis NZ9000 treatment group were administered 100 μL of $1.0 \times 10^9$ CFU/mL of L. lactis NZ9000 resuspension solution per day by gavage. The mice in the L. lactis NZ9000-SeNPs treatment group were orally administered 100 μL of $1.0 \times 10^9$ CFU/mL of L. lactis NZ9000-SeNPs resuspension solution containing 0.5 mg/kg SeNPs per day. The mice in the other groups were given the same volume of M17 broth per day by gavage.
Mice in the ETEC K88-infected groups were orally administered 100 μL of 1.0×10^8 CFU/mL of ETEC K88 resuspension solution on days 8, 10, 12, and the other groups were orally administered with the same volume of LB broth. The body weight, diarrhea, behavior and mood were monitored and recorded daily. After the above treatments, all experimental mice were anesthetized with ether, and aliquots of their blood were rapidly drawn from their eyes for the isolation of the serum. The mice were then sacrificed, and organs in their abdominal cavities were dissected. Portions of the ileum (0.5 cm sections) were immediately excised and stored in 4% paraformaldehyde for the subsequent histological analysis. The appropriate amount of cecal content samples were aseptically collected into a 1.5 mL sterile centrifuge tube, and quickly frozen in liquid nitrogen. Finally, the remaining ileum tissue samples, cecum contents, and serum samples were stored at –80 °C for subsequent analysis.

2.5 Organ selenium content detection

The selenium content in the organs was measured by detected by inductively coupled plasma-mass spectrometry (ICP-MS). With surgical scissors clip about 200mg liver, kidney, and ileum, and then dissolved into nitric acid solution was colorless and transparent with 5 mL. The solution is then heated in boiling water (100 °C) for 2 hours to ensure that the acid is completely volatilized. Then, transfer the sample solution to a 10 ml volumetric flask to make the volume up to 10 ml. Before the test, prepare standard solutions with Se concentrations of 0 μg/mL, 5 μg/mL, 10 μg/mL, 20 μg/mL, 50 μg/mL, 100 μg/mL, 200 μg/mL, 500 μg/mL. Draw a standard curve based
on the content of selenium in the standard solution. Finally, determine the selenium content in the liver, kidney and ileum.

2.6 Evaluation of selenoenzyme activities

GSH-Px and TrxR are important antioxidant enzymes containing Se. The activity of GSH-Px and TrxR in serum was assayed by the corresponding kits.

2.7 Evaluation of intestinal morphology and goblet cells number in ileum tissue

The proximal ileum tissues samples fixed in 4% paraformaldehyde were dehydrated, paraffin-embedded, and cut into slices. Then, the tissue sections were stained with hematoxylin-eosin (H&E) and examined under a phase-contrast microscope for histological and morphological characteristics of the ileum. The villi height and crypt depth in the ileum were determined using the Image J analyzer software (National Institutes of Health (NIH), Bethesda, MD, USA). The number of goblet cells in the proximal ileum tissue was determined using Alcian Blue staining, and counting with a Nikon 80i fluorescence microscope.

2.8 Evaluation of the expression levels of TJ proteins by Western blot analysis.

About 50 mg of ileum tissue was suspended in 500 μL of RIPA lysis buffer solution containing 1% phenylmethylsulfonyl fluoride (PMSF), and homogenized on ice. Then, the supernatants were collected after centrifugation at 13,000 × g for 15 min at 4 °C. The total protein concentration of the supernatant was determined using a BCA kit, and the protein concentration of each group was adjusted to the same level with PBS. The expression levels of TJ proteins were evaluated by Western blot analysis as described in our previous study (16). Briefly, protein samples were first denatured at
95°C for 10 min using 5× loading buffer, and 30 μg of the denatured protein solution was loaded onto a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. Afterwards, the protein solution was transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with a 5% skim milk blocking buffer for 2 h, the membrane was washed three times with tris-buffered saline containing Tween 20 (TBST) for 5 min each time, and then incubated with primary antibodies against occludin, claudin-1, and β-actin overnight at 4 °C. Subsequently, the PVDF membrane was washed three times with TBST, followed by incubation with HRP-labelled secondary antibody at room temperature for 2 h. After washing three times with TBST, the expression levels of the proteins of interest were measured using the Clarity Western ECL substrate kit (BioRad Laboratories, Hercules, CA, USA), and the images of the blots were acquired using the Odyssey imaging system (LI-COR Biosciences, Lincoln, NE, USA). Densitometry of the images of the blots was performed using the Image J system (NIH) by normalizing to β-actin.

2.9 Analysis of mucin 2 (MUC2) and regenerating family member 3 gamma (Reg3γ) mRNA expression levels in ileum

The ileum segments were isolated and immediately stored in liquid nitrogen. Total RNA was extracted from ileum using TRIzol reagent (Invitrogen, USA). The quality and concentration of purified total RNA were detected with a nanophotometer (Implen, Germany). cDNA was synthesized using a PrimeScript™ II 1st Strand cDNA synthesis kit. Then, real-time PCR was performed on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA) according to the ChamQ™ SYBR® qPCR
Master Mix Kit. Primers used in the study are listed in Table 1. β-actin was chosen as an internal reference. Data analysis was performed using the $2^{-\Delta\Delta Ct}$ method.

2.10 Analysis of cytokines levels by ELISA

The levels of serum IL-1β, IFN-γ, and TNF-α, and ileal IL-1β were determined using the ELISA kits according to the manufacturer’s instructions.

2.11 Detection of TLRs/NF-κB signaling pathway-related proteins by Western blot analysis

The expression levels of NF-κB, p65, IκBα, p-IκBα, TLR2, TLR4 in the ileum tissue samples were determined by Western blot analysis described in section 2.8 above.

2.12 Analysis of bacterial diversity and community composition in the cecum

The bacterial diversity and community composition in cecal contents were analyzed by the 16S rRNA gene amplicon pyrosequencing technique using the Illumina MiSeq platform for sequencing by Shanghai Majorbio Co., Ltd. (Shanghai, China). Specific steps are as follows: The microbial community DNA was extracted using MagPure Stool DNA KF kit B (Magen, China) following the manufacturer's instructions. Variable regions V3–V4 of bacterial 16S rRNA gene was amplified with degenerate PCR primers, 341F (5’-ACTCCTACGGGAGGCAGCAG-3’) and 806R (5’-GGACTACHVGGGTWTCTAAT-3’). Libraries were qualified by the Agilent 2100 bioanalyzer (Agilent, USA). The validated libraries were used for sequencing on Illumina MiSeq platform following the standard pipelines of Illumina, and generating 2 × 300 bp paired-end reads.

2.13 Statistical Analysis

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All data were expressed as the mean ± standard error of the mean (S.E.M.). Statistical analysis was performed using GraphPad Prism 8 (Graph-Pad Software Inc., San Diego, CA, USA). Comparison of the mean values was performed using one-way analysis of variance (ANOVA). *P* < 0.05 was considered to be statistically significant.

### 3. Results

#### 3.1 Effects of *L. lactis* NZ9000 and *L. lactis* NZ9000-SeNPs on the body weight of mice

According to the overall trend observed in Fig. 1B, except for the normal control group, the body weight of mice in the other treatment groups decreased gradually at first, then increased slowly and finally didn’t change significantly. The average body weight of ETEC K88-infected mice was slightly lower than that of mice in the other treatment groups. Furthermore, the fecal material of mice from the ETEC K88-infected group was thin, soft, or irregularly shaped, and showing yellow-green color, which indicated symptoms of diarrhea compared with the normal control group. However, oral administration of *L. lactis* NZ9000 or *L. lactis* NZ9000-SeNPs effectively prevented the occurrence of diarrhea.

#### 3.2 Effects of *L. lactis* NZ9000 and *L. lactis* NZ9000-SeNPs on Se content in organs of mice

Compared with other groups, oral administration of *L. lactis* NZ9000-SeNPs significantly increased the Se levels in the liver, kidney and ileum of mice (Fig. 2A, B and C). SeNPs may be absorbed by the small intestine tissue and participate in the metabolic network. Se is the active site of many selenoenzymes such as GSH-Px and...
TrxR. Therefore, we detected the activity of GSH-Px and TrxR in serum of mice. As shown in Fig. 2D and E, L. lactis NZ9000-SeNPs supplementation significantly increased GSH-Px and TrxR activities in serum when compared with ETEC K88-infected group.

3.3 Effects of L. lactis NZ9000 and L. lactis NZ9000-SeNPs on the intestinal histology of mice

Infection with ETEC K88 caused an increase of the crypt depth (CD), and a decrease of the villus height (VH) in the ileum, as well as the decrease of the number of goblet cells in the ileum tissue when compared with the normal control group. However, orally administered L. lactis NZ9000 or L. lactis NZ9000-SeNPs significantly alleviated the ETEC K88-induced increase of the VH and CD, and the intestinal villi were more regularly distributed (Fig.3A and B). Moreover, oral administration of L. lactis NZ9000 or L. lactis NZ9000-SeNPs significantly inhibited ETEC K88 infection-induced decrease in the number of goblet cells in the ileum (Fig. 3C and D).

3.4 Effects of L. lactis NZ9000 and L. lactis NZ9000-SeNPs on the expression levels of TJ proteins in ileum

Occludin and claudin-1 are two key proteins that are involved in the maintenance of the intestinal barrier function. As shown in Fig. 4, ETEC K88 infection resulted in a significant decrease in the expression levels of occludin and claudin-1 in the ileum compared with the normal control group. Administration of L. lactis NZ9000 and L. lactis NZ9000-SeNPs significantly improved the expression levels of occludin and claudin-1 compared with the ETEC K88-infected group. Moreover, the up-regulatory
Effect of *L. lactis* NZ9000-SeNPs on the expression levels of TJ proteins was more pronounced than that of *L. lactis* NZ9000. Furthermore, the improvement effect of *L. lactis* NZ9000-SeNPs is significantly better than that of *L. lactis* NZ9000.

### 3.5 Effects of *L. lactis* NZ9000 and *L. lactis* NZ9000-SeNPs on the mRNA level of **MUC2** and **Reg3g**

The mRNA expression levels of **MUC2** and **Reg3g** in ileum were detected by Real-Time qPCR in ileum. As shown in Fig. 5A and B, compared with the normal control group, ETEC K88-infected can significantly reduce the expression of **MUC2** and **Reg3g**. However, compared with the group treated with ETEC K88 alone, pretreatment with *L. lactis* NZ9000 and *L. lactis* NZ9000-SeNPs significantly up-regulated the mRNA levels of **MUC2** and **Reg3g** in ileum, respectively.

### 3.6 Effects of *L. lactis* NZ9000 and *L. lactis* NZ9000-SeNPs on the levels of serum and ileum cytokines

As shown in Fig. 6A-D, compared with normal control group, infection with ETEC K88 caused the increase of the serum levels of IFN-γ, TNF-α, and IL-1β, and the increase ileal level of IL-1β. However, oral administration of *L. lactis* NZ9000 or *L. lactis* NZ9000-SeNPs significantly inhibited the increase of IFN-γ, TNF-α, and IL-1β levels in serum and ileum compared with that of ETEC K88-infected group.

### 3.7 Effects of *L. lactis* NZ9000 and *L. lactis* NZ9000-SeNPs on the TLRs/NF-κB signaling pathway
As shown in Fig. 7, compared with the normal control group, ETEC K88 infection resulted in the decrease of the expression levels of TLR2, TLR4, and NF-κB P65, and the increase of the expression levels of IκB and p-IκB in ileum of mice. Moreover, compared with the ETEC K88-infected group, administration of *L. lactis* NZ9000 or *L. lactis* NZ9000-SeNPs by gavage significantly improved the expression levels of TLR2 and TLR4, and decreased the expression levels of IκB and p-IκB. Furthermore, the improvement effect of *L. lactis* NZ9000-SeNPs is significantly better than that of *L. lactis* NZ9000.

### 3.8 Effects of *L. lactis* NZ9000 and *L. lactis* NZ9000-SeNPs on the cecum microbiome

Compared with the normal control group, orally administered ETEC K88 had no effect on α-diversity (Fig. 8A). Also, as shown in the community composition analysis-heatmap diagram (Fig. 8B), there were significant differences in intestinal flora structure among the experimental groups. A total of 531 operational taxonomic unit (OTU) were shared by each experimental group (Fig. 8C). The abundance analysis of the community composition at the levels of phylum revealed that compared with normal control group, ETEC K88 infection led to a marked decrease in the abundance of *Epsilonbacteraeota* in the ileum contents, while the *L. lactis* NZ9000 and *L. lactis* NZ9000-SeNPs could reverse this trend. Meanwhile *L. lactis* NZ9000 and *L. lactis* NZ9000-SeNPs could alleviate the increase in the abundance of *Actinobacteria* caused by ETEC K88 (Fig. 8D). The abundance analysis of the community composition at the levels of genus revealed that compared with the normal
control group, treatment with ETEC K88 significantly increased the abundance of
*g_norank_f_Muribaculaceae* and *Firmicutes_bacterium_M10-2*. However, compared
with the ETEC K88 group, supplementation of *L. lactis* NZ9000 or *L. lactis*
NZ9000-SeNPs reduced the abundance of these two bacteria (Fig. 8E).

### 4. Discussion

The intestinal barrier is a complex multi-layered structure that provides a physical and
functional barrier to the transport of luminal contents to the systemic circulation (17).
Therefore, maintaining the integrity of the intestinal barrier function is critical for
maintaining human and animal health. Alterations of the mucosal layer, as well as
goblet cell pathology, have been associated with inflammatory bowel disease (IBD)
(13). Numerous microbiotas inhabit in the intestine or gastrointestinal tract, which are
an indispensable component of the intestinal barrier. Under normal physiological
conditions, the relationship between microorganism and host is mutualistic and
symbiotic. Gut microbiota can harvest energy, protect against pathogens, and regulate
host immunity (14, 18-20). Since antibiotics are prohibited in animal feed, it is urgent
to develop safe and efficient nutrition regulation strategies. Under pathological
conditions, supplementation of exogenous probiotics is beneficial to regulate
intestinal microecological dysbiosis. Se, as an essential micronutrient is needed for
the biosynthesis of selenoproteins, which contribute to antioxidant defense and
immune function (21). The regulatory effects of dietary Se supplementation on
intestinal barrier function and immune responses are associated with its regulation of
gut microbiota (22). Many studies have shown that patients with IBD have a lower Se
level compared to healthy subjects (23, 24). An increase of dietary Se and Vitamin E was shown to relieve the impact of heat stress on the intestinal barrier integrity in pigs (25). The \textit{MUC2} secreted by goblet cells can form a mucous layer on the surface of the intestinal epithelium, which is one of the main intestinal mucosal mechanical barriers (16). In addition, \textit{Reg}^{3}g is closely related to the intestinal barrier function. Lack of \textit{Reg}^{3}g will increase the number of mucosa-associated bacteria, leading to intestinal barrier dysfunction (26). Oral administration of \textit{L. lactis} NZ9000-SeNPs or \textit{L. lactis} NZ9000 was found to increase the expression levels of TJ proteins and the mRNA levels of \textit{MUC2} and \textit{Reg}^{3}g in mice challenged by ETEC K88. The current results demonstrated that supplementation with exogenous \textit{L. lactis} NZ9000-SeNPs or \textit{L. lactis} NZ9000 both effectively alleviate ETEC K88-induced intestinal barrier dysfunction.

Dietary supplementation with Se is essential for maintaining the normal immune and antioxidant defense functions. Selenoproteins are involved in many physiological processes such as immune regulation and antioxidant defense. Se deficiency is strongly associated with an increased incidence of placental retention, white muscle disease (WMD), lower fertility rate, and increased susceptibility to infections. The prevention of these disorders can be achieved by adequate Se supplementation of the diet with Se (27). In this study, we found that oral administration of SeNPs-enriched \textit{L. lactis} NZ9000 attenuated the inflammatory response and enhanced the antioxidant capacity of C57BL/6 mice. Se plays a critical role in regulating the immune function, especially non-specific immune responses. Low level of Se was found to be closely
associated with the weakening of the immune system (28-30). Decreased Se concentration and impaired selenoproteins biosynthesis were observed in inflammatory diseases (31). The supplementation with Se alleviated the inflammatory response (30). Se deficiency was found to lead to the decrease of GSH-Px activity (32). To date, many genes which encode proteins associated with the antioxidant properties of Se have been identified (33). Also, Se supplementation was recently found to protect against oxidative stress in patients with Crohn disease (34).

Traditional Se supplements such as sodium selenite and selenomethionine, generally have low bioavailability and high toxicity compared to the other Se species, such as organic Se and nano-Se (8). Therefore, it is urgent and significant to develop an innovative supplement of Se with high bioavailability and low toxicity. The biological activities and toxic effects of Se are strongly associated with its dosage and its chemical form (35). Recent research indicates that SeNPs have numerous advantages as a food or feed additive (36). For instance, SeNPs can be synthesized using a chemical, physical, or biological approach (the so-called green synthesis) (37-40). Our previous studies demonstrated that Lactobacillus casei ATCC 393 and L. lactis NZ9000 effectively convert sodium selenite into SeNPs (12, 41). Biogenic SeNPs effectively relieve the intestinal barrier damage caused by oxidative stress and pathogenic bacteria infection. Moreover, SeNPs exhibit low toxicity compared with sodium selenite and selenomethine (41, 42). Also, SeNPs can theoretically pass through the intestinal epithelium in paracellular and transcellular ways (43). Our previous study indicated that SeNPs synthesized by Lactobacillus casei ATCC 393
could enter HepG2 cells by endocytosis (44).

SeNPs have higher antioxidant activity and lower toxicity than selenomethionine (SeMet) (45). Zhang et al. found that the activity of GSH-Px was higher in the liver of weanling pigs that were fed a diet with 0.5 or 1.0 mg Se/Kg in the form of SeNPs than that in the form of Na$_2$SeO$_3$ (46). In this study, we found that oral administration of *L. lactis* NZ9000-SeNPs increased the activities of GSH-Px and TrxR in serum of ETEC K88-infected C57BL/6 mice. The biological activity of Se is exerted through the functions of selenoproteins, into which it is cotranslationally incorporated, as the amino acid residue Sec, during their synthesis. Se deficiency induces intestinal mucosal injury by affecting mucosal morphology, S-IgA secretion, and GSH-Px activity (47). SeNPs synthesized by *Enterobacter cloacae* Z0206 attenuated the oxidative stress-induced intestinal barrier damage by activating the Nrf2 antioxidant signaling pathway (48). Se plays a crucial role in maintaining the epithelial barrier integrity, protecting against inflammation, and even against enteric infection (49). Supplementation with Se was found to restore the antioxidant capacity of the lungs, and further moderate the inflammatory response in respiratory distress syndrome patients (50).

In this study, we found that *L. lactis* NZ9000-SeNPs effectively relieve the increase of the serum levels of inflammatory cytokines in ETEC K88-infected mice. Moreover, administration of *L. lactis* NZ9000-SeNPs attenuates both ETEC K88-induced intestinal microbiota dysbiosis and activation of the TLRs/NF-κB signaling pathway. Specific bacteria of the *g_norank_f_Muribaculaceae* and
Firmicutes_bacterium_M10-2 family are correlated with intestinal barrier function and cytokine levels (51). The increase of these two bacteria will improve the intestinal inflammatory cytokine levels and damage the intestinal mucus layer barrier function. Similar results were observed in our study in which Muribaculaceae and Firmicutes were more prevalent in mice treated with ETEC K88. Although the relationship between Se supplementation and increased immunity against pathogens has not been fully elucidated, it is established that Se deficiency leads to immune suppression, and further increases the susceptibility to infections (52). Changed in the Se levels in the diet led to the alteration the composition of the gut microbiota in mice (53). Sufficient or supranutritional Se supplementation can optimize the gut microbiota for protection against intestinal dysfunctions (22). Se-induced downregulation of the NF-κB pathways was associated with the eicosanoid class-switching phenomenon to differentially regulate pathways of inflammation and resolution in immune cells (54). The biological activity of Se, exerted through various selenoproteins, effectively resolves inflammation by driving the production of prostaglandin D2 (PGD2) and its cyclopentenone prostaglandin (CyPG) metabolites that potentially modulate the NF-κB and peroxisome proliferator-activated receptor (PPAR)-dependent pathways (55). The protective effect of Se supplementation may be mediated through microbial metabolites that may not only impact the Se species selection but also assist in attenuating inflammation or enhancing resolution through the regulation of the host immune response.

At present, selenium-enriched probiotics not just have a wide range of applications
in the food industry, but also have great potential in the biomedical field in the future (56). Simona et al. found that *L. casei* strain is a potential SeNPs enriched probiotic, *L. casei* strain and *L. casei* strain SeNPs have the ability of annihilate the toxic effect of cadmium on the kidneys. *L. casei* strain SeNPs at 0.4 mg/kg significantly decreased gene expression of kidneys inflammatory markers (TNF-α, IL-6, NF-κB), compared with *L. casei* strain (57, 58). Yi et al, found that *Bifidobacterium longum* (BL) and Selenium-BL have protective effect on alcohol plus high fat diet (HFD) induced hepatic injury in mice. Selenium can play a synergistic effect with BL, and the effect is better than the BL group (59). In this study, we found that *L. lactis* NZ9000-SeNPs effectively relieve the increase of the serum levels of inflammatory cytokines in ETEC K88-infected mice. Moreover, administration of *L. lactis* NZ9000-SeNPs better attenuates both ETEC K88-induced intestinal microbiota dysbiosis and activation of the TLRs/NF-κB signaling pathway, and the effect has a significant advantage compared to *L. lactis* NZ9000.

5. Conclusion

Oral administration of SeNPs-enriched *L. lactis* NZ9000 and *L. lactis* NZ9000 were found to effectively alleviate ETEC-K88-induced intestinal barrier dysfunction in C57BL/6 mice. Additionally, it was also found that the protective mechanism may be related to their antioxidant activities and their downregulatory effects on the TLRs/NF-κB signaling pathway. Moreover, *L. lactis* NZ9000-SeNPs is more effective than *L. lactis* NZ9000 on regulating the expression of TJ proteins and the TLRs/NF-κB signaling pathway. These findings suggested that SeNPs-enriched
microorganisms may be a promising and safe Se supplement for use as a food or feed additive. However, it is still necessary to conduct further study on the effects of the application of SeNPs-enriched L. lactis NZ9000.

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Figure legends

Fig. 1. Experimental scheme and change of body weight of mice during the entire experimental period. (A) Experimental scheme. (B) Change of body weight of mice.
All data are presented as the mean ± S.E.M, n=10. ETEC K88 group means the mice were orally administered with ETEC K88 only. L. lactis NZ9000 + ETEC K88 group means the mice were pretreated with L. lactis NZ9000 before administration of ETEC K88. L. lactis NZ9000-SeNPs + ETEC K88 group means the mice were pretreated with L. lactis NZ9000-SeNPs before administration of ETEC K88.

Fig. 2. Effects of L. lactis NZ9000 and L. lactis NZ9000-SeNPs supplementation on the Se content. (A) Se content in liver. (B) Se content in kidney. (C) Se content in ileum. (D) The activity of TrxR in serum. (E) The activity of GSH-Px in serum. All data are presented as the mean ± S.E.M. n=6, *P < 0.05, **P < 0.01, ***P < 0.001.

Fig. 3. Effect of L. lactis NZ9000 and L. lactis NZ9000-SeNPs on the intestinal morphology in mice challenged with ETEC K88. (A) The histomorphology of the proximal ileum was observed by H&E staining. (B) Quantitative analysis of villus height and crypt depth. (C) Image of goblet cells in ileum using Alcian Blue staining. (D) Quantitative analysis of the number of goblet cells in the ileum of each experimental group. All data are presented as the mean ± S.E.M. n=6, **P < 0.01, ***P < 0.001.

Fig. 4. Effect of L. lactis NZ9000 and L. lactis NZ9000-SeNPs on the expression of tight junction (TJ) proteins in the ileum of mice challenged with ETEC K88. (A) The expression levels of TJ proteins were determined by Western blot analysis. (B) Statistical results of gray value quantification. All data are presented as the mean ± S.E.M. n=3, **P<0.01, ***P<0.001.
Fig. 5. Effect of *L. lactis* NZ9000 and *L. lactis* NZ9000-SeNPs on the mRNA level of *MUC2* and *Reg3g*. (A) mRNA levels of *MUC2* in ileum analyzed by real-time qPCR. (B) mRNA levels of *Reg3g* in ileum analyzed by real-time qPCR. All data are presented as the mean ± S.E.M. n=6, *P*<0.05, **P*<0.01.

Fig. 6. Effect of *L. lactis* NZ9000 and *L. lactis* NZ9000-SeNPs on serum and ileum cytokines levels in mice challenged by ETEC K88. (A) Serum level of IFN-γ. (B) Serum level of TNF-α. (C) Serum level of IL-1β. (D) Ileum level of IL-1β. All data are presented as the mean ± S.E.M. n=6, *P*<0.05, **P*<0.01.

Fig. 7. Effect of *L. lactis* NZ9000 and *L. lactis* NZ9000-SeNPs on the TLRs/NF-κB signaling pathway. (A) The expression levels of TLRs/NF-κB–related proteins were determined by Western Blot analysis. (B) Statistical results of gray value quantification. All data are presented as the mean ± S.E.M. n=3, *P*<0.05, **P*<0.01, ***P*<0.001.

Fig. 8. Effects of *L. lactis* NZ9000 and *L. lactis* NZ9000-SeNPs on the microbial community in cecum of mice challenged with ETEC K88. (A) α-diversity analysis. (B) Heatmap image at the phylum level in cecum. (C) Shared operational taxonomic unit (OTU) analysis of the different libraries in cecum. Venn diagram showing the unique and shared OTU (3% distance level) in the different libraries. (D) The relative abundance of intestinal microbiota at the phylum level and the genus level in cecum. (E) Selected genera (*g_norank_f_Muribaculaceae* and *Firmicutes_bacterium_M10-2*) whose relative abundance values were significantly altered by *L. lactis*.
NZ9000-SeNPs treatments. All data are presented as the mean ± S.E.M. n=6., *P<0.05, **P<0.01, ***P<0.001.

Table 1 The primers and sequences of qPCR
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<th>Genes</th>
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| β-actin | F: TCACCCACACTGTGCCCATCTACGA  
          | R: GGATGCCACAGGATTCCATACCCA |
| MUC2    | F: CTGACCAAGAGCGAACACAA  
          | R: CATGACTGGAAGCAACTIONGA   |
| Reg3g   | F: TCAGGACATCTTGTGTCTGTGCTC  
          | R: CATCCACCTCTGTTGGTTCA     |

**Abbreviations:** MUC2, mucin 2; Reg3g, regenerating family member 3 gamma.
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
A

Ocludin

Claudin 1

β-actin

ETEC K88  

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L. lactis NZ9000  

|       | - | - | + | - |

L. lactis NZ9000-SeNPs  

|       | - | - | - | + |

B

Relative Protein Expression Level

ETEC K88  

|       | - | + | + | + |

L. lactis NZ9000  

|       | - | - | + | - |

L. lactis NZ9000-SeNPs  

|       | - | - | - | + |