**ABSTRACT**

LL-37 is the only antibacterial peptide of the cathelicidin family found in the human body. It has antimicrobial activity and is able to neutralize endotoxins, with no obvious toxic effects on probiotics. In the present study, we engineered *Bifidobacterium longum* (B. longum) as an experimentally constructed delivery system of LL-37 to treat bacterial diarrhea. The results showed that the engineered *B. longum* successfully secreted LL-37, at a level of 57 ± 6.8 μg/ml after fermentation for 42 h. The supernatant containing recombinant LL-37 significantly inhibited the growth of *Escherichia coli* (E. coli) and *Staphylococcus aureus* (S. aureus), and markedly decreased TNF-α levels in LPS-induced RAW264.7 cells. This modified *B. longum* was orally administered after bacterial diarrhea was induced with *E. coli*, and resulted in significant reduction of the proinflammatory cytokine TNF-α. The anti-inflammatory cytokine IL-10 and mucosal repair factor TGF-β were remarkably increased, and the intestinal bacteria *Lactobacillus* and *Bifidobacterium*, as well as *E. coli*, were well-regulated. Histological observations showed colonic protection with dose-modified LL-37. The results demonstrated effective resistance to bacterial diarrhea with LL-37–secreting *B. longum*.

**KEYWORDS** Bifidobacterium longum, LL-37, bacterial diarrhea, endotoxin, antibacterial activity, oral administration

**INTRODUCTION**

The only protein encoded by the human cathelicidin gene is hCAP-18, and LL-37 is a 37-amino-acid fragment of the hCAP-18 C-terminal. It is a 4.5-kDa, cationic, amphipathic R-helical antimicrobial peptide with the sequence LLGDFFRKKEIKGKEKRIVQRKDFLRLNLVPRTES1,2,3. In humans, LL-37 is extensively distributed throughout the skin, mouth, tongue, gums, salivary glands, sweat glands, spleen, liver, digestive tract, respiratory tract, urinary tract, reproductive tract, and in immune cells such as lymphocytes and monocytes3. LL-37 exerts a broad spectrum of positive effects against both Gram-negative and Gram-positive bacteria, various viruses, and fungi4-10. However, it has no obvious antibacterial effects on probiotics, and there is even evidence that probiotics can increase the release of LL-37 in the intestinal lumen11. Studies have also shown that LL-37 can inactivate lipopolysaccharide (LPS) and stimulate wound-healing, angiogenesis, cytokine release, and anti-tumor activity12,13.

*Bifidobacterium* are anaerobic microorganisms of the gastrointestinal tract that have many beneficial effects on human health, including balancing the intestinal microflora, fighting diarrhea and microbial infections, and enhancing immunity14,15. *Bifidobacterium* is generally regarded as beneficial and safe, and is widely used in the food industry. Recently, genetically engineered *Bifidobacteria* have been used as exogenous gene-delivery carriers for bowel disease, myocarditis, and cancer therapies, with studies suggesting that it has advantages as a vehicle for delivering LL-37 to the mucosa16-19.

Acute infectious diarrhea is a major cause of morbidity and mortality worldwide. Bacterial diarrhea is a common acute intestinal infectious disease, with mainly gastrointestinal clinical symptoms, including fever, vomiting, abdominal pain, nausea, and anorexia20. The most common bacterial diarrhea pathogens are *Escherichia coli*, *Salmonella*, *Shigella*, and *Staphylococcus aureus*21,22. *S. aureus* induces diarrhea mostly due to bacterial imbalances after long-term use of a large number of antibiotics23. Some *E. coli* strains cause infections of the gastrointestinal (GI) system while other pathotypes cause non-GI infections such as bacteremia, nosocomial pneumonia, and neonatal
meningitis. Prolonged diarrhea can cause severe dehydration and even death, and antibiotic therapy is the most effective method for the treatment of bacterial types. However, Gram-negative bacterial infections still have high incidence and mortality rates both because antibiotics cannot effectively neutralize endotoxins and because of growing numbers of drug-resistant pathogens. Pharmaceutical treatments with good antibacterial activity, excellent endotoxin neutralization, and to which pathogenic bacteria are not resistant, would be valuable in treating bacterial infectious diseases.

In this study, we constructed an expression system for B. longum to secrete the LL-37 protein. We used B. longum supernatant to assess its antibacterial activity and endotoxin-neutralizing ability in LPS-induced RAW264.7 cells. We also evaluated the antibacterial and endotoxin-neutralizing properties of B. longum-LL-37 in vivo using a murine model of E. coli-induced diarrhea. Our results demonstrated the feasibility of treating bacterial diarrhea with LL-37 secreting B. longum.

METHODS

Construction of B. longum-LL-37

E. coli ATCC11105 was purchased from American Type Culture Collection. B. longum HB25 strain was preserved in our laboratory. E. coli was grown in Luria-Bertani (LB) medium and B. longum HB25 was grown in de Man, Rogosa and Sharpe (MRS) medium supplemented with 0.05% (w/v) L-cysteine. All of the samples were anaerobically grown at 37°C. The hup promoter and amyB secretion signal peptide fragments were PCR-amplified from B. longum genomic DNA. The primers were as follows (underlined segments represent restriction sites):

- hup promoter F: 5’-CGGATCCCGTGCTTATTTT CATACGCCCTT-3’ (HindIII)
- hup promoter R: 5’-GGACTAGTACCAAGGCATCT CTTCTGGG-3’ (BamHI)
- hup terminator F: 5’-GGCCGGTGGGCCCCCTTC TGCTGTAGCGC (NotI)
- hup terminator R: 5’-GGAGCTCCGCGCTGAA CTAATCCGG-3’ (SacI)
- amyB signal peptide F: 5’-GGACTATGTTACATGG AACATCGGAAA CCGCACA-3’ (SpeI)
- amyB signal peptide R: 5’-GCTGACTAAATTG GGCCGTGCTGCGTGCGTCG-3’ (XbaI)

The LL-37 gene was synthesized by using codon optimization for Bifidobacterium, with the oligonucleotide as follows: GCAGATCT(XbaI)CTGCTGGCCCGGATTTCCTC AAAACGAAAGAAAAATCGG CAAAGAATCTAAGCAGCAGTG CAGCCGATCATGAAAGATTCCTGGCGCAGCAGG CACCCGAAGTGATTTGGCGCGGCGCGGCGGCGGCGGCGGCGG (NotI)TTT. The pMB1 fragment harboring a replicon of B. longum was cut from pDG7. This fragment was inserted into pBluescript II SK(−) in wells to yield the E. coli–B. longum shuttle expression plasmid vector pBs-LL-37. The general processes of digestion, ligation, and transformation of E. coli were performed as described, and the pBs-LL-37 plasmid was transformed into B. longum by electroporation as previously described. The transformants were screened by an agarized culture medium containing ampicillin with 0.5M sucrose and 20 g/ml penicillin (100 mg/L final concentration).

Expression and identification of LL-37

Transformed B. longum was added to Dulbecco’s Modified Eagle Medium (DMEM) when the optical density (OD) at 600 nm for the bacterial culture reached 0.6. Culture supernatants were collected at 0, 6, 12, 18, 24, 30, 36, and 42 h, and centrifuged at 3,000 rpm for 10 min. Secretion of LL-37 in B. longum cell supernatants was detected by Tricine SDS PAGE, while the comparison was a synthesized one. LL-37 ELISA kits were used to determine the LL-37 concentration, according to the manufacturer’s instructions (IBL-America, Minneapolis, MN, USA).

Determination of antibacterial and anti-endotoxin activity

Antibacterial activity was determined by the agar diffusion method. Briefly, 10 ml of agar gel was fully melted, poured into a sterile Petri dish, then cooled to solidification. The E. coli and S. aureus culture was poured into 12 ml of agar gel (final concentration: 4 × 10^8 CFU/ml) and mixed, then poured to cover the bottom gel. Next, 3-mm holes were punched in the upper agar, and the supernatants of B. longum-LL-37 and B. longum-pBs and the synthesized LL-37 (8 µl of 0.5 mg/ml) were placed dropwise into the holes. The sizes of the antibacterial circles were measured after an 18-h culture at 37°C.

RAW264.7 cells were obtained from the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM supplemented with 10% heat-inactivated FBS and 100 IU/ml of penicillin G under 5% CO₂ at 37°C, then stored until the experiments were performed. RAW264.7 cells were plated at a density of 5 × 10⁴ cells/ml. The cells were first incubated with the Bifidobacterium supernatants containing 8 µg/L of LL-37 for 1 h, then exposed to 30 ng/ml of LPS for another 24 h, after which the supernatants from the cells were collected. The TNF-α concentration in the cultured supernatants was analyzed by ELISA kits according to the manufacturer’s protocol (GenStar, China).

ANIMAL ASSAYS

Induction of bacterial diarrhea and oral administration of B. longum

The entire experiment was performed in the Animal Care Center of Jinan University. Sprague-Dawley (SPF) mice weighing 230–250 g were obtained from Sunshine University (Approval number: 44005800004319).
and housed in comfortable cages. They were allowed to acclimatize to standard lighting and temperature conditions and were given adequate food and water. The mice were randomly divided into four groups: control group, diarrhea group, pBs (dose-empty vector B. longum) group, and pBs-LL-37 (dose B. longum-LL-37) group. To induce diarrhea, the mice were injected intraperitoneally with 0.3 ml of E. coli daily for 3 days. After that, the pBs and pBs-LL-37 groups were orally administered B. longum-pBs and B. longum-pBs-LL-37 (1 × 10^9 CFU), respectively. The other groups were orally administered phosphate-buffered saline (PBS) daily. All doses were given once every 12 h.

**Detection of intestinal flora**

Six mice in each group were inoculated with 1 g of mixed and diluted intestinal contents. Intestinal flora were detected with the viable plate-count method. The *Bifidobacterium* and *Lactobacillus* culture medium was placed in an anaerobic tank in an anaerobic agent and cultured for 48–72 h at 37°C. The *E. coli* was cultured for 24–48 h at 37°C. The number of colonies on each plate was counted and recorded, and those that averaged 30–300 colonies were multiplied by the dilution factor.

**Measurement of TNF-α, IL-10, TGF-α, and LL-37 in the colon**

The mice were euthanized and colon tissues were collected for cytokine measurements. Colon samples were weighed and homogenized in PBS at pH 7.2 and centrifuged at 12,000 rpm for 10 min at 4°C. The cultured supernatants were harvested and the cytokines TNF-α, IL-10, and TGF-α were measured with ELISA kits. The levels of LL-37 in the colonic mucus were measured with LL-37 ELISA kits.

**Histological evaluations**

Each mouse colon was cut open and fixed in its entirety by immersion in 10% buffered formalin, then embedded in paraffin. These tissues were sectioned in 3-μm slices and stained with hematoxylin and eosin (H&E). Morphological changes in the colonic membranes were observed via microscopy.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism 5.0. All data in the figures and text were presented as arithmetic mean ± standard deviation (SD). Data were representative of three or more independent experiments. The significance of differences among groups was calculated with one-way ANOVA. Levels of LL-37 in colon mucus were analyzed using chi-square. In all cases, P < 0.05 was considered statistically significant.

**RESULTS**

**Construction and determination of Bifidobacterium secreting LL-37**

Although pDG7 is an *E. coli–B. longum* shuttle vector, it has few restriction sites and only contains a replicon fragment of *B. longum* pMB1; it was therefore not suitable for an expression system. Therefore, in order to construct a functionality vector, the pMB1 fragment was inserted into pBluescript II SK(-). The element hup promoter, *amyB* signal peptide, and a synthesized LL-37 oligonucleotide, as well as the hup terminator, were then introduced into pBluescript II SK(-) to achieve a high-expression plasmid pBs-LL-37 (Fig. 1).

Determination of the recombinant LL-37 in the modified *B. longum* was detected with ELISA and Tricine-SDS-PAGE. The determination of LL-37 is shown in Fig. 2.
A bright band of approximately 4.5 kDa was seen in the B. longum-LL-37, in perfect accordance with the synthetic LL-37, while the pBs B. longum group had no corresponding position. This indicated that LL-37 could secrete efficiently in B. longum-LL-37. We then shifted our attention to the expression of LL-37 in the supernatants of B. longum-LL-37. During the vigorous growth period, the levels of LL-37 were markedly increased, with an increased tendency to be relatively stable in the plateau stage. The concentration of LL-37 reached 48 ± 2.5 μg/ml in 24 h (Fig. 3).

**Antibacterial and endotoxin-neutralizing effects of B. longum-LL-37**

The agar diffusion method showed that different groups displayed different antibacterial activities against S. aureus and E. coli (Table 1). It has been previously proven that *Bifidobacteria* have antibacterial activity\(^{33}\); our data also showed that B. longum HB25 culture supernatants exerted visible antibacterial activity. The antibacterial activity increased significantly (\(P < 0.001\)) after B. longum expressed the LL-37 protein, but no significant variation (\(P > 0.05\)) was found compared to the synthesized group, indicating that recombinant LL-37 is better able to inhibit S. aureus and E. coli.

We also examined the effects of recombinant LL-37 on endotoxin neutralization using the LPS-induced murine macrophage cell line RAW264.7. The data showed that recombinant and synthetic LL-37 both were able to significantly inhibit TNF-α release (\(P < 0.01\)), but there was no significant difference between them (\(P > 0.05\)) (Fig. 4). These results indicated that recombinant LL-37 performed well at neutralizing endotoxins.

**Effects of B. longum-LL-37 against bacterial diarrhea**

Our research proved that recombinant LL-37 had good antibacterial and endotoxin-neutralizing activity *in vivo*, so we evaluated its effectiveness against bacterial diarrhea. We constructed a murine bacterial diarrhea model by intraperitoneally injecting E. coli; after 3 days, the mice had watery and shapeless feces, suggesting the successful construction of the model. The mice were given daily oral doses of B. longum, B. longum-LL-37, or saline for 7 days after bacterial diarrhea induction. The secretion of LL-37 in colonic mucus is shown in Fig. 5. In the pBs-LL-37 group, the LL-37 level increased more rapidly after 2 days; for the next 4 days it increased relatively slowly, and did not rise after the 7th day (3.2 ± 0.8 μg/ml). The pBs increased slowly for 7 days, the concentration was 1.6 ± 0.5 μg/ml after 7th day. LL-37 levels were higher in the pBs-LL-37 group than in the pBs group (\(P < 0.01\)).

Bacterial diarrhea is caused by damage to the intestinal mucosa, and changes in cytokines during the injury are crucial to this process. To evaluate their therapeutic values, the proinflammatory cytokine TNF-α, the anti-inflammatory cytokine IL-10, and the mucosal repair factor TGF-α were analyzed on colonic-segment supernatants. As shown in Fig. 6, on day 7 after oral administration, phlogosis driven by TNF-α was significantly attenuated (\(P < 0.01\)) in the B. longum-LL-37 group (117 ± 13.8 pg/ml) compared to the bacterial diarrhea group (398 ± 14.2 pg/ml). IL-10 was markedly increased.

![Fig. 3](image) Growth and LL-37 secretion of B. longum-LL-37 at indicated time-points (0, 6, 12, 18, 24, 30, 36, and 42 h).

![Fig. 4](image) Endotoxin-neutralizing effects of B. longum-LL-37 in LPS-induced RAW264.7 cells. *\(P < 0.05\), **\(P < 0.01\).

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>pBs</th>
<th>pBS-LL-37</th>
<th>Sy-LL-37+pBs</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>0.30 ± 0.23</td>
<td>6 ± 3.42</td>
<td>18 ± 2.14*</td>
<td>22 ± 1.49*</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>0.5 ± 0.17</td>
<td>3 ± 1.81</td>
<td>12 ± 3.62*</td>
<td>13 ± 2.94*</td>
</tr>
</tbody>
</table>

\(^*P < 0.001\) vs control group and pBs group.
(P < 0.01) in the B. longum-LL-37 group (710 ± 49.5 pg/ml) compared to the bacterial diarrhea group (213 ± 16.8 pg/ml). Significantly increased (P < 0.01) levels of TGF-β were observed in the B. longum-LL-37 group (94 ± 4.4 pg/ml) compared to the bacterial diarrhea group (21 ± 1.2 pg/ml). These results suggested that B. longum-LL-37 can improve bacterial diarrhea induced by E. coli in mice.

On day 7 after oral administration of B. longum-LL-37, we measured levels of Lactobacillus, Bifidobacterium, and E. coli. The results showed a 25.2% increase in the number of Lactobacilli and a 31.6% decrease in the number of pathogenic E. coli organisms in the B. longum-LL-37 group compared to the bacterial diarrhea group (Table 2). The amount of Bifidobacterium in the B. longum-LL-37 group was higher than in the pBs group. These results demonstrated that B. longum-LL-37 can relieve diarrhea by regulating intestinal microorganisms.

**Improvement of histological features**

To further evaluate the effects of B. longum-LL-37 on bacterial diarrhea, we observed the degree of histological damage to the colon. In the control group, the profile of the intestinal glands was clear and the colonic mucosa was intact. In contrast, mucosal erosion, serious

![Fig. 5](image_url)  
**Fig. 5** Level of LL-37 in colon mucus 7 days after oral administration of B. longum-LL-37. *P* < 0.01.

![Fig. 6](image_url)  
**Fig. 6** Effect of oral administration of B. longum-LL-37 on secretion of TNF-α, IL-10, and TGF-β in colon tissue on day 7. *P* < 0.05, **P** < 0.01.

![Fig. 7](image_url)  
**Fig. 7** Histological analysis of colonic samples (×200). (A) Control group: normal colonic histology. (B) Diarrhea group: devastated colonic mucosa. (C) pBs group: fractional destruction of epithelial mucosa. (D) pBs-LL-37 group: relatively normal colonic histology.
intestinal gland deformation, and inflammatory exudate on the colonic surfaces were observed in the E. coli diarrhea group. In the pBs-LL-37 group, intestinal glands were relatively clearly observed, but in a disordered arrangement, colon injury was remarkably reduced and most of the inflammatory exudate on the colonic surface villi disappeared compared with the pBs and control groups. These results showed that B. longum-LL-37 could alleviate colonic injury caused by bacterial diarrhea.

**DISCUSSION**

Bacterial infectious diseases continue to cause seriousness morbidity and mortality worldwide despite a variety of antimicrobial agents. Such agents are able to kill pathogenic microorganisms but cannot destroy the remaining endotoxins, which can lead to the host’s death. In addition, the overuse of antibiotics has led to the development of drug-resistant pathogenic organisms. Another factor is that while antimicrobial agents kill pathogenic microorganisms, they also kill beneficial microbes such as Lactobacillus and *Bifidobacterium*, which is a serious health threat. Therefore, the development of new pharmaceutical compounds is necessary to overcome these limitations.

LL-37 is a strong functional endogenous peptide with numerous effects. It exhibits broad antibacterial, antifungal, and antiviral properties. It modulates the immune response, influences cytokine release, promotes wound-healing, and is involved in tumor-cell apoptosis and proliferation. Among these unique functions, its antibacterial properties are quite distinctive and well-recognized. After dying, pathogenic microbes release the fatal endotoxin LPS. It has been reported that LL-37 can inactivate LPS by binding with it. For certain endotoxemias, disabling LPS is critical for eliminating deleterious effects. Additionally, some pathogenic bacteria form substantial biofilms to protect themselves from antibiotics and innate immunity, and to block wound-healing. However, LL-37 possesses antibiofilm-formation properties. For these reasons, LL-37 seems to be more powerful compared to common antibiotics.

Due to its distinct antibacterial properties, LL-37 may provide new possibilities for the development of new antimicrobials to treat infectious diseases. The ability to obtain a large quantity of LL-37 would be a useful foundation for its development as a medicine. In recent decades, many researchers have used engineered yeast to manufacture active LL-37. However, there are still many challenges with these systems. LL-37 is a robust antibacterial peptide that can kill yeast and *E. coli*, so the expressed LL-37 peptides must be inactive or in the form of a fusion protein. There, the activation processes have huge energy costs and require complex technological support. With respect to yeast and *E. coli*, the *Bifidobacterium* production system has a great advantage for practical applications. Studies have shown that antimicrobial peptides such as LL-37 have no significant toxic effects on intestinal probiotics, and there is even evidence that intestinal probiotics increase the release of LL-37 in the intestinal lumen. Moreover, since *Bifidobacterium* is a food-grade microorganism that grows in the colon, we anticipated that *B. longum*-LL-37 could be orally administered for convenient drug-delivery. Considering all of these factors, the *Bifidobacterium* expression system is superior for manufacturing LL-37.

In this study, we developed an oral delivery system using genetically engineered *Bifidobacterium* as a carrier of LL-37. The results showed that *B. longum*-LL-37 notably inhibited the growth of *E. coli* and *S. aureus*, and markedly decreased TNF-α release in LPS-induced RAW264.7 cells. Tests on *E. coli*-induced bacterial diarrhea in a mouse model showed that *B. longum*-LL-37 effectively alleviated bacterial diarrhea, based on significantly improved cytokine levels, well-regulated intestinal flora, and significantly attenuated tissue damage. Our findings suggest that LL-37 secreting *B. longum* is a workable pharmaceutical for fighting bacterial diarrhea.

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**REFERENCES**


