



Protective Immunity Against Enterotoxigenic *Escherichia coli* by Oral Vaccination of Engineered *Lactococcus lactis*

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Abstract

Enterotoxigenic *Escherichia coli* (ETEC) is one of the leading causes of diarrhea in children globally, and thus suitable vaccines are desired. Antigen display on lactic acid bacteria is a reliable approach for efficient oral vaccination and preventing bowel diseases. To develop an oral vaccine against ETEC, the gene of the binding domain from heat-labile toxin (LTB), a key ETEC virulence factor, was codon-optimized and cloned into a construct containing a signal peptide and an anchor for display on *L. lactis*. Bioinformatics analysis showed a codon adaptation index of 0.95 for the codon-optimized gene. Cell surface expression of LTB was confirmed by transmission electron microscopy and blotting. White New Zealand rabbits were immunized per os (PO) with the recombinant *L. lactis*, and the antibody titers were assayed with ELISA. In vitro neutralization assay was performed using mouse adrenal tumor cells and rabbit ileal loop test was performed as the in vivo assay. ELISA results indicated that oral administration of the engineered *L. lactis* elicited a significant production of IgA in the intestine. In vitro neutralization assay showed that the effect of the toxin could be neutralized with 500 µg/ml of IgG isolated from the oral vaccine group. Furthermore, the dose of ETEC causing fluid accumulation in the ileal loop test showed a tenfold increase in rabbits immunized with either recombinant *L. lactis* or LTB protein compared to other groups. Our results imply that recombinant *L. lactis* could potentially be an effective live oral vaccine against ETEC toxicity.

Introduction

Pathogenic bacteria such as *Vibrio cholerae*, Enterotoxigenic *Escherichia coli* (ETEC), Shiga toxin-producing *E. coli* (STEC), enterohemorrhagic *E. coli* (EHEC), *Campylobacter* spp., *Shigella* spp., and *Salmonella* spp. are known to cause diarrhea in humans [1]. Among these bacteria, ETEC is the most common pathogen causing diarrhea in children. Unfortunately, ETEC-related diarrhea not only directly increases the risk of mortality in children but also causes growth disorders with additional complications in the community [2]. Moreover, high-level antibiotic resistance is being reported for these diarrheagenic strains [3].

Therefore, vaccine development is critical as an effective strategy to control enteric infections and to avoid their lasting consequences [1].

Two virulence factors are recognized in ETEC strains and are directly associated with diarrheal symptoms. These are heat-labile toxin (LT) and heat-stable type Ib toxin (STa) [4]. LT is a hexameric protein (A1B5) and includes an active domain (A) and five binding domains (B) organized to bind to galactose-containing receptors and gangliosides at the surface of eukaryotic cells [5]. Being an ADP-ribosylating toxin, LT irreversibly activates adenylate cyclase resulting in dysregulation of cAMP-sensitive ion transporter and water efflux in the intestinal lumen [6]. LTB is also known to have adjuvanticity and immunomodulatory properties and can impact the maturation of the IL-10 pathway in dendritic and Treg cells [7].

Development of ST-based vaccines is faced with certain limitations such as poor immunogenicity, potent toxicity, and cross-reactivity with human gastrointestinal peptides [8, 9]. Moreover, during the infection with ETEC majority of the humoral response is toward LT and colonization factors rather than the ST toxin [10]. Therefore, producing

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an immune response against LT for vaccine development against ETEC is a preferred approach.

Although there are several parenteral vaccines against acute enteric diseases such as typhoid and polio, mucosal immunization is still the most preferred and effective method for producing an effective immune response against non-inflammatory and non-invasive pathogens like ETEC and *V. cholerae* [11]. Considering the epidemiological panorama and the feasibility of incorporation into pediatric vaccination programs, oral vaccines are more advantageous compared to injectable vaccines. Mucosal vaccines are also cost-effective for use in lower-income countries as they do not require the injection process or costly manufacturing process [12]. Producing an effective immune response in oral vaccination demands prolonged access of the gut mucosal tissue to the antigens [13] and fast degradation of unprotected proteins leads to low immunogenicity. An ideal mucosal vaccine such as live vaccines could accomplish the tasks of antigen delivery and elicitation of secretory IgA and cellular immunity at the mucosal defense barrier [14], while soluble protein causes systemic tolerance in oral administration. Granulated antigens or microbes can stimulate an adequate response in gut-associated lymphoid tissue (GALT) after ingestion [15]. Among the limited options available for live mucosal adjuvants and antigen delivery mechanism, *L. lactis* is known for facilitating the interaction of the antigen and M cells in the epithelial intestine [13]. The lack of colonization ability of *L. lactis* circumvents the development of tolerance and avoids the production of low avidity antibodies caused by a short period of antigen presentation [16].

Several honored aspects of using *Lactococcus*-based vaccine are being regarded as GRAS (generally regarded as safe) organism and surviving under low pH of stomach and bile compounds [17]. Besides, inadequate colonization and temporary presence in the human intestinal tract, make *Lactococcus* favorable for consecutive administration as a vaccine vehicle. Furthermore, because of their low risk of spreading in the environment as a genetically modified organism (GMO) they are deemed as environmentally friendly [17, 18]. They could decrease large-scale production concerns via innate adjuvant-like characteristics [19], ease of storage without cold chain conditions, and missing the endotoxin LPS [20]. The effectiveness of *L. lactis* oral vaccines was showed in a study by Pasetti et al. that used these bacteria for expressing two proteins from *Shigella* virulence factors. This oral vaccine could induce antigen-specific IgG and IgA and about 90% protection against *S. flexneri* in a mice challenge experiment [21]. *Bifidobacterium infantis* was used to develop a live oral vaccine against ETEC. This live vaccine harbors an expression vector containing CFaB or LTb. The gene-specific antibody titers in serum and fecal

samples of the mixed vaccination group were significantly greater than either two groups with a single antigen-presenting bacteria [22].

There are several choices for antigen presentation in live vaccine design. Then, developing a successful approach for local expression of the antigen requires several considerations. Proteolytic degradation of the antigen in the environment's disruptive conditions and the low local concentration of antigen are the most common reasons for ineffective live oral vaccine [23–25]. Thus, anchoring systems are preferable due to their higher protection and increasing on-site concentration of antigens and are gaining more attention in recent years [26].

The surface localization of proteins highly depends on the original sequence and their motifs. The signal sequence at the N-terminal directs the protein to the secretory pathway. The signal sequence contains hydrophobic and negatively charged amino acids that facilitate interaction with the cell membranes and its final cleavage from the protein. The anchoring mechanism is located at the C-terminal of protein and includes about 30 amino acids that can adhere to the cell wall. Lactic acid bacteria (LAB), like other gram-positive bacteria, have a protein secretion and display system [27], which could be engaged to express heterologous protein on the cell surface [28]. Despite considerable efforts and achievements in understanding the protein structure and action mechanism of this system, there are still many unknown details regarding interactions between heterologous targets and autotransporters. These unknown aspects of protein secretion and display system make precise prediction of successful heterologous surface expression extremely difficult [29]. Considering the need for an oral vaccine against ETEC and the advantages of using *L. lactis* as an antigen presentation vehicle, our study is designed to assess the surface expression of LTb antigen and evaluate the efficacy of recombinant *L. lactis* as an oral vaccine for stimulation of mucosal immunity against ETEC.

Materials and Methods

Bacterial Strains, Plasmids, and Cell Line

An expression system from MoBiTec Company (Göttingen, Germany) was used for the surface display of the synthetic anchoring construct. The expression system includes NZ3900 *Lactococcus* strain which is deficient in lactose-metabolizing pathway and pNZ8149 as a food-grade vector with the lac F gene marker. All synthetic DNA fragments were synthesized by Biomatik Co. Services (Ontario, Canada). The pathogenic strain of ETEC used in this research was characterized and confirmed for secretion of LT toxin in our previous study [30]. *Y-1* (ATCC® CCL-79™) a mouse

adrenal tumor cell line was purchased from the Pasteur Institute of Tehran, Iran. The *L. lactis* NZ3900 strain was routinely cultured at 30 °C with 2–3% CO₂ in M17 broth containing 0.5% (wt/vol) glucose or lactose (G/LM17).

Design and Construction of Displaying Cassette

The displaying cassette is composed of a signal peptide for secretion (A. acids: 202–285), interval rejoins (286–303), anchor sequence (619–1047), and LTB coding sequence (304–618). The strategy to design the cassette and information required for the selection of the best anchor and signal peptide is described by Michon et al. [26]. The interval rejoins consist of nine amino acid residues in which two of them have negative charges to facilitate cleavage and improving secretion efficiency [31]. To achieve the best expression, the sequence of the cassette was codon-optimized based on *L. lactis* codon frequency and preference. The resulting plasmid, here named *pNZ8149-eltB*, contained DNA fragments encoding the signal sequence of the *usp45* gene and the C-terminal fragment of the cell wall anchored region in *SP6* with the same transcriptional orientation. These two fragments are found in most commercial pNZ vectors developed by MoBiTec Company and are originally found in gram-positive bacteria. (The *pNZ8149-eltB* map is presented in the supplementary data/Fig.S1.)

The synthesized cassette was inserted into the *pNZ8149* vector and transferred into *L. lactis* via the electroporation system of Bio-Rad's Gene Pulser Xcell with an exponential protocol condition of 2000 V/25 µF/200 Ω using a 2 mm cuvette. Positive clones were screened by culturing the transformed bacteria on *Elliker* agar medium containing bromocresol purple indicator that turns to yellow in the presence of acid metabolites produced from lactose consumption. Positive clones were verified by polymerase chain reaction (PCR) and digestion of the extracted plasmid with *XbaI* and *NcoI* restriction enzymes.

PCR was performed with a Bio-Rad Thermal Cycler and Sinaclon® reagents. All DNA manipulations, including DNA digestion, ligation, and agarose gel electrophoresis, were carried out using standard procedures. The forward and reverse primers used in PCR reactions were 5'-ACGCGA GCATAATAACGGC-3' and 5'-TCAACTGCTGCTTTT TGGCT, respectively, and were designed by Primer Premier 5 Software and confirmed by Primer3plus online server.

Development of Engineered *L. lactis*

For inducing protein expression on the surface of engineered *L. lactis*, the nisin-A was diluted to 100 ng/ml which is tested to be a sub-inhibitory concentration. The optimum concentration of nisin-A was determined by culturing 3 ml L/M17 tubes with equal amounts of engineered *L. lactis* and

a range of nisin-A concentrations (1–1000 ng/ml). After 4 h, the bacterial growth was measured at OD₆₀₀. Consequently, for surface expression of the target antigen 100 ng/ml of nisin-A was added to the bacteria cultured in M17 medium at the OD₆₀₀ ~ 0.4 and incubated for 5 h. Protein production was confirmed by blotting and immune electron microscopy (IEM) using mouse polyclonal anti-LTB sera.

Recombinant LTB in *pET28a* vector was expressed in *E. coli* BL21 and purified with Ni-NTA column. 20 µg per dose of the purified recombinant LTB was intraperitoneally injected into the mouse at days 0, 14, and 28, and the serum was collected 15 days after the last injection. This mouse serum was used as the detection antibody in all molecular techniques hereafter.

Blotting

For Dot blotting, a piece of PVDF membrane was spotted with cell lysate of induced and un-induced engineered *L. lactis*. The membrane was blocked overnight using 5% skimmed milk solution, then washed with washing buffer, and incubated with diluted (1:100) anti-LTB mouse antiserum for 2 h at room temperature. Then the membrane was incubated with (HRP)-conjugated goat anti-mouse IgG for 1 h. Eventually, the target–Ab complex was revealed by the addition of 3,3'-diaminobenzidine (peroxidase substrate). The membrane was washed after each step by floating in PBST (PBS plus 0.05% tween-20) for 15 min. The same staining protocol was employed for Western blotting.

Immunolectron Microscopy

Based on the method recommended by Lee, Jin-Yong, et al. [32], engineered *L. lactis* strain was grown in M17 broth at 30 °C, and LTB expression was induced by nisin-A at a concentration of 100 ng/ml. Bacterial cell suspension (1 × 10⁹ cells/ml) was transferred to Formvar-coated grids and left to air-dry. Then, 20 µl of LTB-immunized mouse antiserum per grid (1:50 dilution in 1% BSA-PBS) was added to the cells and incubated at 37 °C for 1 h. After washing five times with PBS, the cells were incubated with a 1:20 dilution of goat anti-mouse IgG conjugated with 10 nm gold particles (Sigma) at 37 °C for 30 min. The cells were rinsed twice with PBS and negatively stained with 2% uranyl acetate for 1 min. The stained cells were examined under a Zeiss EM10C electron microscope at 100 kV.

Rabbit Immunization

Female white New Zealand rabbits weighing 1.5–1.8 kg were used for animal immunization studies. Animals were rested in the animal care center of Shahed University for 1 week before the start of the experiment. Rabbits were

divided into six groups as detailed in Table 1 for the immunization procedure. These groups were chosen to assess the immune response after oral and subcutaneous (S.C.) administration of the developed live vaccine candidate in comparison with purified recombinant LTB protein injection. Incomplete Ferund adjuvant (1:1) was used for S.C. injection of the recombinant LTB protein. Serums were collected 12 days after each injection, and antibody production was monitored for about 15 weeks, starting 28 days after the first dose of the vaccine. For S.C. injections, bacterial suspensions were prepared as described for *nisA* induction, then washed three times, and diluted with PBS. For oral administration, the prepared suspension was stored at 4 °C and instantly combined with glucose and sodium bicarbonate as excipients.

Anti-LTB IgG and IgA Titers

Blood and feces samples of rabbits immunized with the engineered *L. lactis* or the recombinant protein were analyzed for their humoral and mucosal antibody response through indirect ELISA. Jet-Biofilm plates were coated overnight with 5 µg/well recombinant protein in a coating buffer (carbonate buffer pH 9.6) at 4 °C. After blocking and incubation with antiserum or extracted IgA [33], the goat anti-rabbit IgG-HRP (Sigma) or goat anti-rabbit IgA-HRP (Abcam) was added. Optical density at 450 nm was detected on BMG labtech, spectrostar nanoplate reader. The absorbance of pre-immune serum was measured and considered as the baseline to monitor the immune response during the three-stage injections.

Quantitative Estimation of Displayed LTB

Competitive indirect ELISA was carried out to measure the expression level of LTB antigen at the cell surface of engineered *L. lactis*. In brief, 5 µg/well recombinant LTB protein were coated and blocked with 5% skimmed milk in PBS containing 0.05% tween-20. A serial dilution of LTB from 0 to 800 ng/ml was prepared and incubated along with 1:250,000 diluted serum for 90 min at RT to draw the standard curve. The mixture was added to the wells and shaken

gently for 60 min. Simultaneously 10^9 engineered *L. lactis* were mixed and incubated with the diluted serum and added to separate wells. Finally, diluted anti-rabbit IgG was used, the HRP substrate was applied, and the absorbance signal was measured. Then, the equivalent amount of LTB in the cell mixture was estimated in a trendline equation.

Toxin Neutralization in Y-1 Mouse Adrenal Cell Line Culture

Toxin Production

Pre-cultured ETEC was inoculated into CAYE broth medium (2% Casamino acids, 0.6% yeast extract, 43 mM NaCl, 38 mM K₂HPO₄, 0.1% trace salt solution consisting of 203 mM MgSO₄, 25 mM MnCl₂, 18 mM FeCl₃) and incubated overnight at 37 °C and 170 rpm. The bacterial cells were harvested by centrifuging at 3000×g for 15 min. After sonic disruption, the cell debris was removed by centrifugation at 5000×g for 15 min at 4 °C. Supernatants, corresponding to cell-associated LT, were assayed immediately or stored at –20°C for up to 7 days. To provide different concentrations of LT toxin, the toxin extracts were serially diluted in the tissue culture medium (1/10, 1/20, ..., 1/640).

Total IgG Purification

To avoid serum interference in cell culture-based assays, total IgG from the last blood samples was purified using the protein G column according to the protocol given by the manufacturer. The concentration of IgG was estimated using the Bradford protein assay.

Cell Seeding and Toxin Neutralization

Y-1 mouse adrenal cells were grown at 37 °C in DMEM medium with 10% heat-inactivated fetal calf serum, 50 IU/mL penicillin, and 50 µg/mL streptomycin. For cytotoxicity assays, 5×10^4 *Y-1* cells were seeded in 96-well polystyrene flat-bottom plates and incubated for 18–24 h [34]. The wells were washed twice with PBS, treated with different

Table 1 Rabbits group for immunization program

Groups	G1	G2	G3	G4	G5	G6
Dose	5×10^9	5×10^9	1×10^9	1×10^9	400µg	–
Route	Oral	Oral	S.C.	S.C.	S.C.	S.C.
Antigen	<i>L.l.eltb</i> ¹	<i>L.lactis</i> ²	<i>L.l.eltb</i>	<i>L.lactis</i>	Rec-LTB	IF adjuvant
Administration Days	0,1,2,14,15,16,28,29,30	0,1,2,14,15,16,28,29,30	0,14,28	0,14,28	0,14,28	0,14,28

¹*L.l.eltb*: *L. lactis* containing pNZ8149-LTB

²*L.lactis*: *L. lactis* containing empty pNZ8149

concentrations of the toxin, and incubated at 37 °C for 30 min. Then, 150 µL of medium containing 2% fetal bovine serum was added per well, and the cytotoxicity was assessed microscopically after 12 h. A dose of the toxin that could change 50% of Y1 cells shape to spherical was considered the optimal concentration. Different concentrations of IgG, 50 to 800 µg/ml were treated with the toxin and shaken on an orbital shaker for 1 h at 37 °C, and then added to coated cells along with 100 µg/ml gentamycin.

Protection Challenge

The protection studies were performed using the ileal loop technique [34]. Immunized rabbits were fasted for 36 h and fed 10% glucose solution. Animals were anesthetized with 5 mg/kg xylazine and 40 mg/kg ketamine by S.C. injection and prolonged with 13 mg/kg ketamine if necessary. Three to four centimeters long loops from the terminal ileum region were segregated with tied non-absorbable 36 mm silk suture. Live ETEC suspension was prepared from overnight-incubated bacteria in CAYE. Different doses of bacteria from 10^5 to 10^8 CFU were tested in each animal, and 0.3 ml PBS was used as the negative control. Animals were sacrificed in the CO₂ chamber after 18 h, and all loops were weighted to quantify their swelling [35].

Statistical Analysis

Independent samples *t* test was used to show differences between the two groups. To compare more than two groups, One-way analysis of variance (ANOVA) was used. If there were significant differences among the groups, the Tukey post hoc test was applied to determine which group significantly differed. Differences were considered significant at $P < 0.05$. All experiments were carried out in SPSS Version 17, and graphs were plotted in Excel software.

Results

Construct Design and Cloning of the *eltB* Gene

For appropriate heterologous expression of the protein, the sequence was codon-optimized. The native and optimized sequences were analyzed for the GC content and codon adaptation index. GC percentage decreased from 39.96% to 32.76% and codon adaptation index increased from 0.65 to 0.95 after optimization. Furthermore, the codon frequency distributions were raised from 57 to 64% (graphs are presented as supplementary data/Fig.S4).

To prepare the *pNZ8149-eltB* expression vector, PGH plasmid was digested with *NcoI* and *XbaI* restriction enzymes, and the 843 bp extracted DNA fragment was

ligated into *pNZ8149*. After electroporation, several yellow transformant colonies were isolated on *Elliker* medium, and positive clones were confirmed by PCR (the electrophoresis diagrams are presented as supplementary data/Fig.S1–S3) and restriction digestion.

Assessment of LTB Expression

The optimum amount of inducer in LTB expression is the sub-inhibitory concentration of nisin-A and defined by the maximum amount of nisin that does not affect *L. lactis* cell viability. As shown in Fig S5, 100 ng/ml of nisin-A is the appropriate concentration for protein expression.

The metabolic burden of heterologous protein expression decreases bacterial proliferation, and thus the growth curve of engineered *L. lactis* containing *pNZ8149-eltB* and empty vector was compared (For detailed information, see the supplementary data/Fig.S6). The results indicate that a difference between growth rates and engineered *L. lactis* containing *pNZ8149-eltB* showed a slightly lower growth rate.

As described above, mouse anti-LTB serum was used for immuno-detection techniques. Dot blotting and Western blotting confirmed the successful expression of LTB in *L. lactis* (For detailed information, see the supplementary data/Fig.S7, S8).

Surface Expression Measurement

The competitive-indirect ELISA was optimized to measure the concentration of primary and secondary conjugated antibodies. Polyclonal serum antibody raised against recombinant LTB was applied as the primary antibody, and the standard curve was drawn for the optical density. Based on the optical density of the sample in competitive-indirect ELISA, the following logarithmic equation was driven which could approximately estimate LTB concentration in the samples:

$$Y = -0.039 \ln(X) + 0.9338$$

where Y is LTB concentration and X is the optical density of the sample. According to the equation, 2×10^{-5} pg LTB protein is displayed on each cell of *L. lactis* under the defined condition of induction

Mucosal and Serum Immunity Responses

The efficiency of the two vaccine formats was evaluated in rabbits model receiving S.C. injection or oral administration. The engineered live *L. lactis* expressing LTB on the surface and the purified LTB proteins are compared for immunostimulating effect of antigen and protectively of vaccines. Serum samples of rabbits were collected two weeks

after the third vaccination and were assayed for anti-LTB IgG. Fecal samples were also collected to determine anti-LTB IgA titers. The raise of antibody titers was observed in G1, G4, and G5 groups compared to their control group. A significant difference was detected between IgG titers after oral administration and S.C. injection ($P < 0.001$). The highest titer of IgG in serum was observed in G5, G1, and G4 groups, respectively. The analysis of IgG or IgA titers of rabbit groups showed the highest titer of IgA and IgG in G1 and G5 groups, respectively (Figs. 1, 2).

Toxin Neutralization Assay

Based on the results obtained from the toxin neutralization assay, the minimum IgG concentration from each group that was sufficient to block toxin was detected as follows: G5, 150 $\mu\text{g/ml}$; G1, 500 $\mu\text{g/ml}$; G4, G2, and G3, $>800 \mu\text{g/ml}$. They mirror the ranking of anti-LTB-specific IgG in serum (For detailed information, see the supplementary data/ Fig. S9).

Ileal Loop Challenge

The in vivo challenge was performed using a live isolate of ETEC at 13–15 weeks after the last vaccination. To specifically study the efficacy of the vaccine against the LT toxin and prevent pathogenicity caused by other toxins, only the strain-producing LT (not ST) was used. The results of rabbit ileal loop tests were expressed as a weight-to-length ratio of intestinal loops as a function of diarrhea and inflammation response for each inoculated dose of the bacteria. Based on the results, the effective bacterial dose for G1 and G5 rabbit groups was approximately tenfold higher than G4 and control groups (Fig. 3).

Discussion

Previous studies have shown that the expression of antigens on the surface of *Lactococcus* species improves the immune response against infectious diseases in the mucosal tissue [36, 37]. Since mucosal immunity is more advantageous than a systemic response in children [11], the present study aimed to investigate the immunogenicity of the LTB monomer displayed on the surface of *L. lactis*, which is considered the main species utilized as a food-grade oral vaccine. The engineered *L. lactis* could be a relevant candidate for a mucosal vaccine against diarrhea caused by ETEC. To our knowledge, this is the first successful oral vaccine against LTB through a surface display system.

Cell viability, protein secretion, and surface immobilization are the desired characteristics of any successful display system. Overloading of expression and secretion

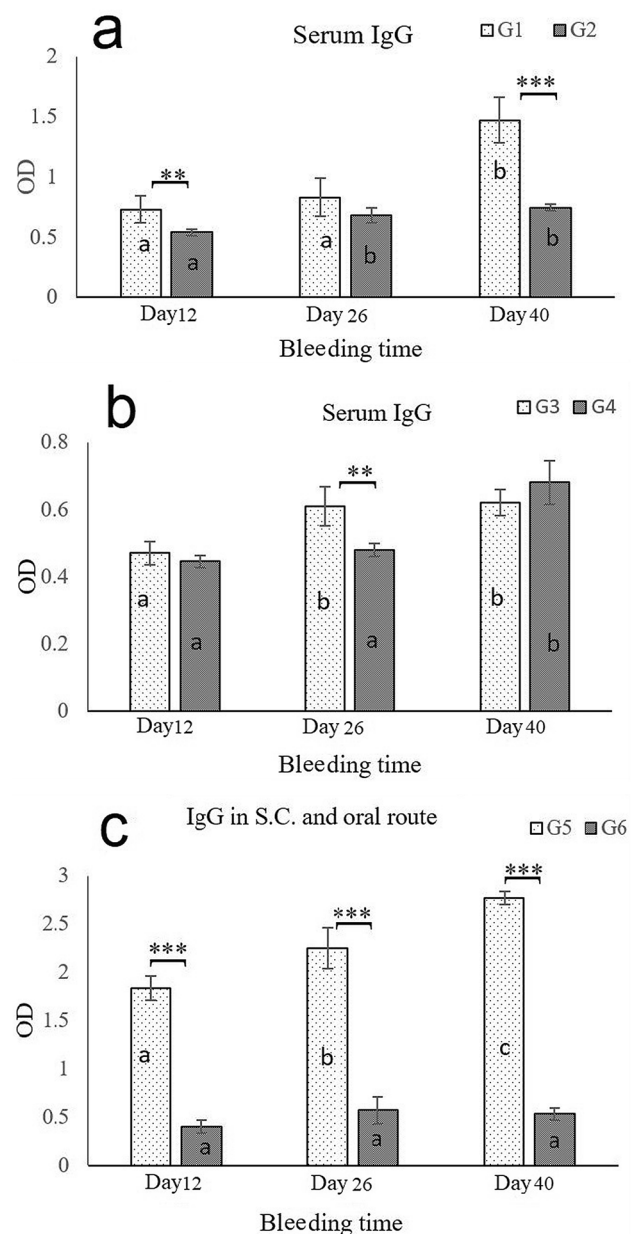


Fig. 1 Pairwise comparison of IgG levels in animal groups. The capital B represents bleeding time. **a** Oral administration of recombinant *L.lactis* and control strain. **b** S.C. administration of recombinant *L.lactis* and control strain. **c** S.C. administration of purified recombinant LTB protein and control group. Asterisks show a significant difference between the two columns. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Different characters (a, b, or c) show a significant difference between columns in the same group. The error bars on graph represent the mean \pm SD of data values

machinery may lead to induction of proteolytic pathways or reduce cell growth [38]. Generally, prediction of the efficiency of protein secretion for heterologous targets is difficult and could be affected by several factors like signal type, the combination of sequences, and cooperative rate of expression, folding, and secretion [39]. Thus, the

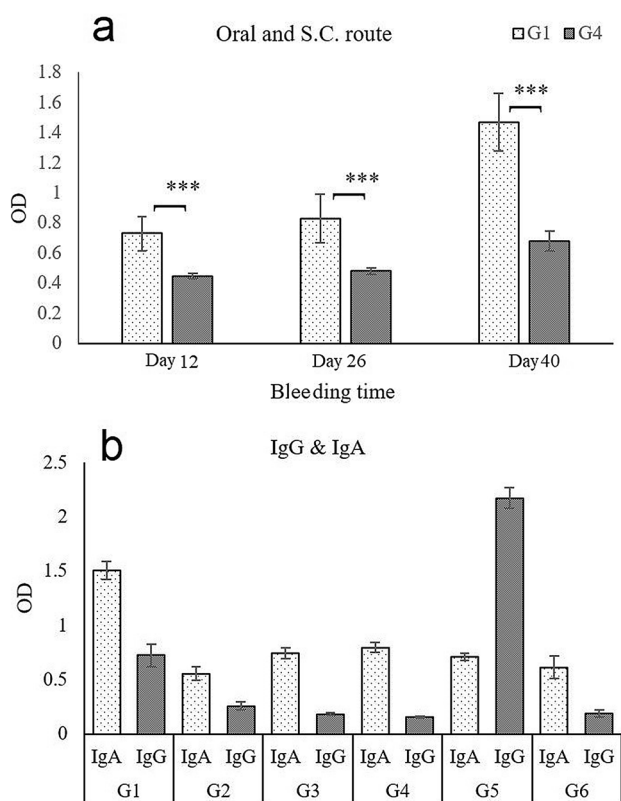


Fig. 2 A comparison of oral and S.C. administration. **a** IgG level from different immunization routes. S.C. and oral administration of recombinant *L. lactis* were compared in two groups. Asterisks show a significant difference between two routes of administration. Oral administration caused higher IgG levels (** $P < 0.001$). G1: oral, G4: S.C. **b** Specific serum IgG and fecal IgA in all immunized groups. The highest titer of IgA was observed in the G1 group ($P < 0.001$), whereas the G5 group showed the highest titer of IgG ($P < 0.001$). The error bars on graph represent the mean \pm SD of data values

selection of suitable signal peptide and anchoring protein greatly improves the success rate of surface display systems. M6 anchor protein from *Streptococcus pyogenes*, which was applied in LAB anchoring system, is known for preserving the structural stability of target protein [26]. For developing oral vaccine candidate against *Campylobacter*, M6 surface display system was used for expression of target antigen in *L. lactis* and surface-displayed cells showed superior results to cytoplasm-located target proteins [40].

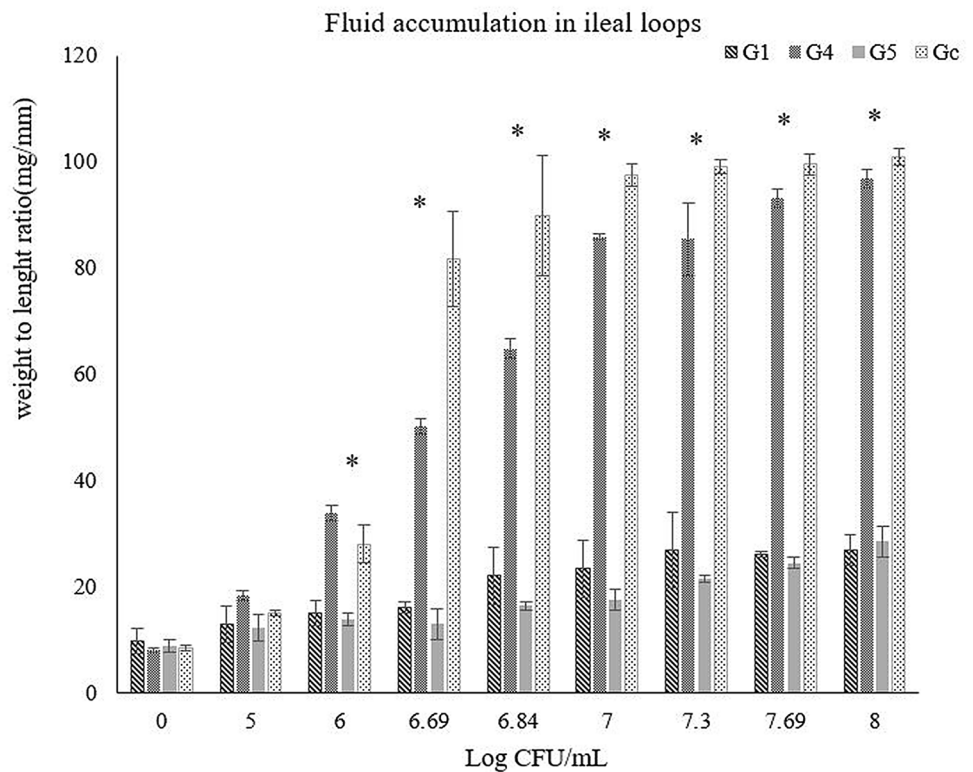
Despite all the successful reports, some researchers indicate that surface display systems might not pass all requirements of antigen presentation and vaccine delivery [41]. Our data confirmed that the use of *sp6* anchor in addition to *usp45* signal peptide and interval sequence can efficiently display LTB on the surface of *L. lactis*. The newly engineered construct presented here could be a useful tool to improve display systems in future studies.

Although *L. lactis* does not have the capacity of colonization in the host, the LTB-expressing bacteria induced local and systemic antibody responses after mucosal delivery [42]. Our results show that the secretory IgA (SIgA) produced by plasma cells in the lamina propria and transported into the lumen, could neutralize ETEC enterotoxin. The persistence of protective response induced after oral immunization is most likely directed by B memory cells located in Peyer's patches and lymphoid follicles of the intestine [11].

The animal challenge experiment showed a similar protective effect in engineered *L. lactis* (G1) and purified LTB (G5) vaccinated groups. High IgG titer in the G5 group may compensate for the low IgA titer in the mucosa. Previous studies showed that the IgG antibody-secreting cells scatter in the lamina propria (LP) and around intestinal glands [43]. The lumen immunoglobulins can be transmitted to the gut through the serum globulins catabolism route [44]. Despite all this, IgG has a limited role in mucosal protection due to its sensitivity to proteolysis and variable permeability of gut tissue. This may explain the result of low protection observed via S.C. injection of engineered *L. lactis* [11]. The LTB monomer expressed on the surface of *L. lactis* was highly immunogenic in rabbits via oral administration, inducing moderate serum IgG and high intestinal IgA titers. IgA titer was nearly 30-fold higher than those obtained from recombinant LTB protein injection despite having less than 1000-fold antigenic dose. The groups that received S.C. injection of engineered *L. lactis* did not show a significant increase in the serum IgG. Our finding is contrary to the result of Ricci et al. [45], who showed that the S.C. injection of *Streptococcus gordonii* expressing LTB on the surface induced a higher IgG response compared to the oral delivery approach in the mice model. In another research, LTB was expressed on *Bacillus subtilis* spore surface and administered intranasally to mice. However, this method failed to induce a significant humoral and mucosal response due to the low expression rate of antigen and elimination of spores by the mucosal immunity [42]. Zha et al. reported low levels of specific IgA and IgG with *L. lactis* NZ3900 as an oral vaccine [46]. They attributed the low antibody titer to the short exposure of gut tissue to live vaccine doses. We obtained high IgA levels with prolonged persistence, even 15 weeks after immunization (For detailed information, see the supplementary data/Fig.S10-S12).

Bignon et al. indicated that the LTB antigen promotes Treg cells and immature DCs after its presentation to mucosal surfaces which limits T-cell activities with consequent related autoimmune diseases. They suggested mucosal administration of LTB for the treatment of such mucosal inflammations [6]. We also suggest our engineered strain as a probiotic product for autoimmune disease treatment. Moreover, the malnutrition and limited food resources may change the gut microbiota of people in the endemic regions.

Fig. 3 Fluid accumulation in ileal loop test. All animal groups were challenged with ETEC strain for toxin neutralization test. The immunoglobulin secretion in the animal mucosa of G1 and G5 groups decreases fluid accumulation in segregated loops receiving 10^5 – 10^8 CFU bacteria per loop. G4 and control groups show detectable inflammation in loops receiving $10^6 \leq$ CFU bacterial doses. Asterisks show a significant difference between columns for each dose ($*P < 0.05$). The error bars on graph represent the mean \pm SD of data values



Thus, the application of probiotic strains for oral vaccine development could be a practical strategy to accomplish two goals at a time [13]. The combination of any target antigen with LT_B displayed on the *L. lactis* surface is a new strategy to improve the immunogenicity of antigens rather than other delivery vectors. LT_B can raise mucosal and systemic immunity and improve the delivery of target antigen to antigen-presenting cells, whereas it does not have the same potential in pulmonary vaccination [45]. Based on the induction of distinct cytokine profiles, the choice of specific LAB for oral administration seems crucial for the directed modulation of the systemic immune response [47].

We used rabbits which is an appropriated and highly recommended model to evaluate the vaccines against ETEC and LT toxicity but not ST [48]. Immunized rabbits with recombinant *L. lactis* are protected from fluid accumulation caused by LT-producing ETEC. The unknown interaction between LAB species, as well as lacking a suitable animal model, creates an obstacle in the way of testing new potential vaccine candidates. Therefore, food-grade vaccines seem more reliable for study in humans [1].

Due to low copy numbers of *pNZ8149*, the verification of expression level of the recombinant protein in the surface display system is not achieved through conventional methods used for cytoplasmic expression of heterologous proteins. For example, no specific protein bands could be observed in total protein extract on SDS-PAGE. However, the immunodetective methods like Western blotting and

immunoelectron microscopy are more appropriate methods for surface display evaluation [49]. We also realized that false-positive responses in the animal challenge could be avoided with adequate washing of the intestine and considering the location of rabbit ileal loops, which can critically change fluid accumulation since the IgG ASCs distribution along abdominal regions is different [43].

Conclusion

LT represents the most widely orally used mucosal adjuvant, which is delivered alone or in combination with other antigens. Due to its immunomodulatory capacity and protection of displayed antigens in the lumen environment, *L. lactis* is the most widely used LAB in the oral delivery of live vaccines. Considering the detection of high IgA titer in the gut mucosa, the engineered oral *eltB-L. lactis* could serve as an oral vaccine candidate against ETEC. This provides an opportunity to extend food-grade vaccines to fermented foods. Finally, based on its high immunoadjuvant activity and respect to GMO warnings, we hope that our findings can encourage researchers to use the developed *eltB-L. lactis* for the co-expression of new antigens.

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Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical Approval This research on animals was carried out due to animal welfare act in standard condition at the animal house of Shahed University and approved by ethics committee for research of Shahed University with letter No: HA939886002.

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