1 **Oral immunization of mice against *Clostridium perfringens* epsilon toxin with a**
2 ***Lactobacillus casei* vector vaccine expressing epsilon toxoid**

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ABSTRACT

Clostridium perfringens type D infects ruminants and causes the enterotoxemia disease by ϵ -toxin. A mutated ϵ -toxin gene lacking toxicity was designed, synthesized, and cloned into the pT1NX vector and electroporated into *Lactobacillus casei* competent cells to yield LC-pT1NX- ϵ recombinant strain. BALB/c mice, immunized orally with this strain, highly induced mucosal, humoral, and cell-mediated immune responses and developed a protection against 200 MLD/ml of the activated ϵ -toxin. This study showed that the LC-pT1NX- ϵ could be a promising vaccine candidate against the enterotoxemia disease.

Keywords: *Clostridium perfringens*, *Lactobacillus casei*, Vaccine, Toxoid, Enterotoxemia

1 **Introduction**

2 *Clostridium perfringens* (*C. perfringens*) causes numerous gastrointestinal infections in most
3 animals. Enterotoxemia caused by *C. perfringens* type D (overeating or pulpy kidney disease)
4 has great economic and sanitary importance for farming small ruminants worldwide.
5 Enterotoxaemia is a condition caused by the absorption of large amounts of toxins from the
6 intestines into the general circulation [1]. The most virulent toxin produced by *C. perfringens*
7 type D is epsilon toxin [2].

8 The best effective method to control enterotoxaemia is vaccination [3]. Development and
9 production of current conventional clostridial vaccines have some problems such as time
10 consuming production processes, detoxification, purification, antigen concentration, quality
11 control steps, etc. [4, 5]. Thus, the production of these vaccines, despite their efficacy, has
12 some limitations.

13 A genetically engineered live vaccine (GELV) system is an effective method for vaccine
14 delivery [6]. Recombinant toxoid vaccines, as GELV, are considered alternative candidates
15 for the conventional clostridial vaccines. Non-toxicity, more stability, and high-yielding
16 process with superior biosafety characteristics are among the advantages of these vaccines
17 [5]. For the GELV production, non-pathogenic bacteria like probiotics have been
18 recommended to be used as a vaccine carrier, since they enhance the safety of vaccines [6].

19 Among probiotics, lactic acid bacteria (LAB) have been generally recognized safe with
20 intrinsic immunogenicity and offer several advantages over current systemic vaccinations.
21 They have a significant potential for oral and mucosal immunity. Expression of bioactive
22 compounds on the cell surface of LAB can stimulate appropriate immune responses [7-11].
23 Stimulation of the immune system by *Lactobacillus casei* (*L. casei*) has been extensively
24 studied [12-16]. This bacterium has been previously used for gene delivery and expression of

protective antigens against different microorganism pathogens [17-19] such as clostridial antigens, fragment C of the tetanus toxin [20], and alpha toxin of *C. perfringens* [21].

The aim of the present study was, first, to make a safe and non-toxic enterotoxemia vector vaccine for expressing ϵ -toxoid on the cell surface of *L. casei* and, second, to evaluate its protective capacity in a mouse model.

Materials and Methods

Bacterial strains and growth conditions

L. casei ATCC 393 (purchased from Pasteur Institute of Iran) and recombinant *L. casei* expressing ϵ -toxoid (LC-pT1NX- ϵ) were anaerobically grown in deMan, Rogosa, and Sharpe (MRS) medium (Himedia, India) at 37°C without shaking. *Lactococcus lactis* subsp. *cremoris* MG1363 was grown in M₁₇ medium (Micromaster Laboratories, India) containing 0.5% glucose (GM₁₇) at 30°C without shaking. Plating of bacteria were performed on the respective media with 1.5% agar. MRS and GM₁₇ media were supplemented with 5 µg/ml erythromycin (Ery) when necessary.

Plasmids and gene synthesis

The pT1NX vector was obtained from BCCM/LMBP plasmid collection of University of Ghent, Belgium (<http://bccm.belspo.be/about/lmbp.php>). This expression vector was designed for the expression of cloned genes in Gram-positives, particularly LAB species, under the control of the lactococcal P1 constitutive promoter [22]. The pT1NX vector carries the lactococcal usp45 secretion signal sequence and the sequence encoding the cell wall anchor of *Staphylococcus aureus* protein A (spaX). The encoded protein can be anchored to

the cell wall and displayed at the cell surface by the insertion of the coding region between the unique *NaeI* and *BamHI* sites.

The gene encoding epsilon toxoid of *C. perfringens* (based on gene bank: AY858558.1) was modified to eliminate the toxicity by the substitution of proline₁₀₆ for histidine₁₀₆ (H₁₀₆ → P₁₀₆) using site-directed mutagenesis [23]. Moreover, *NaeI* and *BamHI* restriction sites and a dendritic cell-targeting peptide sequence were added to this modified gene [24]. This gene was synthesized and cloned in pGH plasmid by Generay Biotechnology Company (Shanghai, China).

Cloning and recombinant procedures

Nucleic acid manipulation and general cloning procedures were performed according to the laboratory manuals [25]. Briefly, the pGH-epsilon and pT1NX vectors were digested with *BamHI* and *NaeI* (Jena Bioscience, Germany) and, then, a gel-purified epsilon toxoid gene was ligated into pT1NX and electroporated into *L.casei* ATCC 393.

For the preparation of competent *L.casei*, an overnight culture was inoculated 1:50 in MRS broth containing 1% glycine and 0.5 M sucrose and incubated at 37°C until the OD₆₀₀ of 0.3 was obtained. The culture was maintained on the ice for 15 min and the bacteria were collected by centrifugation at 4000 g for 10 min at 4°C. The pellet was washed three times in a washing solution (10 mM MgCl₂). The bacteria were resuspended 1:100 in 10% glycerol and the volume of 100 µL was used for electroporation.

Electroporation was carried out as previously described [26]. Ten microliters of ligation mixture or empty pT1NX vector (approximately, 150 ng), as the control, was added to the competent cells. The competent bacteria were electroporated at 1.75 kV, 200 Ω, 25 µF in 0.2 cm cuvettes using a Gene PulserTM apparatus (Bio-Rad Laboratories, Richmond, CA). Then, the electroporated bacteria were resuspended in MRS broth containing 0.3 M sucrose, 20 mM

MgCl₂ and 2 mM CaCl₂ and incubated anaerobically at 37 °C for 2.5 h. Afterwards, the bacteria were plated in MRS containing 0.3 M sucrose supplemented with Ery and incubated at 37 °C for 3 days under anaerobic conditions.

Recombinant *L. casei* strains were obtained by screening the Ery-resistant clones. Plasmid extraction was performed same as Klaenhammer protocol [27]. These clones were investigated for the presence of the insert of interest by PCR and sequencing, using specific primers: Forward 5' TGGGAACTTCGATACAAGCA 3' and Reverse 5' CCACTTACTTGTCTACTAAC 3'. PCR reaction was performed on the extracted plasmid as previously described [28]. The expected size of the amplified fragment corresponded to 238 bp. Positive clones were frozen and stored in MRS broth containing glycerol (final concentration 25%) at -80°C.

Protein expression

For the expression analysis of the modified ε-toxoid, the recombinant LC-pT1NX-ε and *L. casei* as the negative control were grown in MRS medium at 37°C until OD₆₀₀ = 1.5 was achieved. Bacterial cells were collected by centrifugation at 4000 g for 10 min at 4°C. The pellets were suspended in a lysis buffer (50 mM Glucose, 25 mM Tris-HCl, 10 mM EDTA, 20 mg/ml Lysozyme, pH 8.0) and exposed to three consecutive freeze-thaw cycles. The protein fractions were processed as previously described [29, 30].

The cell protein extracts of LC-pT1NX-ε and *L. casei* were fractionated by a 12% SDS-PAGE gel. After the electrophoresis, the proteins were transferred to a nitrocellulose membrane and probed with the polyclonal *C. perfringens* epsilon antitoxin (NIBSC, UK) and horseradish peroxidase (HRP)-conjugated goat anti-Horse IgG antibody (KPL, USA). Colorimetric detection was performed using the 4-chloro-1-naphthol substrate. The

exposition of ϵ -toxoid on LC-pT1NX- ϵ surface was confirmed with FITC- *C. perfringens* epsilon antitoxin using immunofluorescence.

Immunization

Female BALB/c mice, 6-8 weeks old, and 20-25 g in weight (10 mice per group), were used according to the local guidelines for the animal care. The study was approved by Animal Experimentation Ethics Committee of Kerman University of Medical Sciences. The mice in the vaccinated group orally received the recombinant LC-pT1NX- ϵ and the mice from two control groups received *L. casei* carrying the empty vector (LC-pT1NX) or PBS. The bacteria grown overnight ($OD_{600} \geq 2$) were collected by centrifugation (2500 g, 10 min at room temperature), washed twice with PBS and resuspended in PBS until 1×10^9 CFU/ml. The amount of 200 μ l of the cell suspensions (suspension in PBS alone) was inoculated at three doses on three consecutive days via gastric gavage with each cycle being started on days 0, 16, and 37.

Cytokine profile

Interleukin-2 (IL-2), IL-4, IL-10, and interferon- γ (IFN γ) concentrations were measured in sera from the vaccinated mice with LC-pT1NX- ϵ and controls (LC-pT1NX and PBS groups) using a mouse Th1/Th2 ELISA Ready-SET-Go Kit (eBioscience, San Diego, CA) according to the manufacturer's instructions.

Challenge

The immunized mice were challenged subcutaneously by the injection of activated ϵ -toxin (with trypsin) from the overnight culture of *C. perfringens* type D reference strain (CN409, RVSRI, Islamic Republic of Iran) after 17 days of the last booster. Minimum lethal dose

(MLD) was calculated according to the standard operating procedure (ANB.0024.SOP, Razi Vaccine and Serum Research Institute, Iran). The challenge was performed with 1 MLD, 2 MLD and 10 MLD of the activated toxin.

Detection of anti-specific ϵ -toxin antibody

The mice were bled on days -1, 14, 30, 51, and 63 through tail vein. Intestinal washes were collected from sacrificed mice seven days post-challenge. Anti-epsilon IgG, IgA, and sIgA (secretory IgA) antibodies were detected from sera and intestinal washes by indirect ELISA using the international reference preparation of *C. perfringens* epsilon toxoid (NIBSC, UK) as the coating antigen (100 μ g/ml) and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:1000) (Serotec, UK) or anti-mouse IgA (1:2500) (KPL, USA). The final absorbance was read at 450 nm.

Statistical analysis

The statistical significance was calculated using Pearson's Chi-square test ($P \leq 0.05$ was considered the significance level) in SPSS 21.0 software (SPSS, Inc., Chicago, IL).

Results

Construction of the pT1NX- ϵ plasmid

The modified ϵ -toxoid gene was synthesized and cloned in the pT1NX vector after digesting with appropriate restriction endonucleases (*Bam*HI and *Nae*I) and the pT1NX- ϵ plasmid was constructed (Fig. 1).

Transformants were selected by screening as described above and confirmed by the PCR reaction [28]. This PCR product (a 238 bp amplicon) was purified and sequenced for

confidence by Macrogen (Korea). The sequence was precisely the same as the designed sequence.

Expression of the modified ϵ -toxoid on *L. casei*

The fractionated protein extracts from LC-pT1NX- ϵ and its control (*L. casei*) were analyzed by western blotting. The results indicated that the modified ϵ -toxoid was successfully expressed. Fusion of the designed ϵ -toxoid sequence (~ 31 kDa) with the USP45 (~ 2 kDa) and anchoring peptide sequences (~ 20 kDa) produced a band of 53 kDa, which could be observed in the cytoplasmic extracts of LC-pT1NX- ϵ (Fig. 2).

Surface expression of the modified ϵ -toxoid

The expression of cell wall anchored toxoid was confirmed by immunofluorescence. LC-pT1NX- ϵ was treated with FITC- *C. perfringens* epsilon antitoxin and the fusion ϵ -toxoid protein was shown on the surface of *L. casei* cells, as demonstrated in Fig. 3. These results indicated that *L. casei* carrying pT1NX- ϵ produced the ϵ -toxoid and displayed it on the cell surface.

Induction of anti- ϵ antibodies by oral immunization

The yields of LC-pT1NX- ϵ and control bacteria after the overnight culture were about 2×10^9 CFU/ml. LC-pT1NX- ϵ was inoculated at three doses of 1×10^9 CFU/ml on three consecutive days via gastric gavage. The control groups received either LC-pT1NX or PBS. Analysis of anti- ϵ -toxin IgG and IgA in sera or sIgA in intestinal washes revealed that LC-pT1NX- ϵ was able to induce high levels of IgG and IgA antibodies by gastric gavage (Figs. 4 and 5). As can be observed in Fig. 5, gastric inoculation with LC-pT1NX- ϵ induced specific anti- ϵ sIgA in

pooled intestinal washes. No differences between the levels of anti- ϵ sIgA antibodies were observed in the intestinal washes collected from the animals receiving LC-pT1NX or PBS.

Cytokine profile

Finally, the effects of LC-pT1NX- ϵ on the stimulation of immune responses were analyzed by measuring serum concentrations of interleukin-2 (IL-2), interferon- γ (IFN γ), IL-4, and IL-10 in the vaccinated mice at the end of the immunization. It was found that the vaccinated mice with LC-pT1NX- ϵ had higher IFN γ serum levels (1750 Pg/100 μ l) compared to the vaccinated mice with LC-pT1NX (630.0 Pg/100 μ l), and PBS group (187.5 Pg/100 μ l), higher IL-4 levels (62.5 Pg/100 μ l) than the vaccinated mice with LC-pT1NX (56.5 Pg/100 μ l), and PBS group (43.0 Pg/100 μ l), higher IL-10 levels (312.5 Pg/100 μ l) than the vaccinated mice with LC-pT1NX (250.0 Pg/100 μ l), and the PBS group (218.8 Pg/100 μ l). In contrast, the mice vaccinated with LC-pT1NX- ϵ and LC-pT1NX had the same IL-2 serum levels (10.7 Pg/100 μ l) than the PBS group (6.6 Pg/100 μ l). With respect to the controls, the results identified that the vaccinated mice with LC-pT1NX- ϵ had significantly higher IFN γ concentrations ($P < 0.05$). This indicated that LC-pT1NX- ϵ construct induced higher levels of Th1-promoting cytokines than Th2-promoting cytokines.

Challenge

In the current study, the enterotoxemia candidate vaccine expressing the modified ϵ -toxoid was protective against the lethal challenge with 2 MLD (200 MLD/ml) of the activated ϵ -toxin. The calculated MLD for ϵ -toxin of *C. perfringens* type D reference strain was 100 MLD/ml. Complete protection was achieved when 1 MLD and 2 MLD of ϵ -toxin were used, whereas the mice challenged with 10 MLD of ϵ -toxin were not completely protected ($P < 0.05$). The percentages of total survived mice were 100, 100, and 50 for the immunized LC-

pT1NX-ε groups challenged with 1, 2, or 10 MLD of the activated ε-toxin, respectively, and 33.3 and 0.0 for the LC-pT1NX and the control groups challenged with 1 MLD of the activated ε-toxin, respectively ($P < 0.05$) (Fig. 6).

Discussion

Oral immunization against gastrointestinal infections is a desirable method to induce both local and systemic immune responses. The present study was undertaken to develop an effective oral enterotoxemia vaccine using a safe live vector vaccine. LC-pT1NX-ε was therefore tested here as a vaccine in a mouse model against the challenge with ε-toxin. We showed that the immune responses were elicited by this oral vaccination.

To date, there are some limited studies on oral immunization against *C. perfringens* antigens by live bacterial vectors. Different bacteria have been used such as *L. casei* [10, 21], *Salmonella enterica* [31-33], and *Bacillus subtilis* [34] as the antigen delivery systems. *L. casei* has the potential immune-modulatory properties and is widely used for expressing several heterologous antigens as vaccines in animal models with promising results [19, 20, 35-41]. To our best knowledge, the current study is the first description for showing an effective immunity response for ε-toxoid of *C. perfringens* following oral administrative of this *L. casei*-based vaccine.

It has been previously shown that the mutation of histidine residue 106 to a proline results in a protein that is non-toxic in mice. This mutated ε-toxin is stable and has protective epitopes [23]. Due to the strong toxicity of the activated ε-toxin (LD_{50} of 110 ng/kg) [42] and the absence of a detoxification step in our oral immunization schedule, we evaluated this modified ε-toxoid as a potential antigen to develop a vaccine against *C. perfringens* type D. In our study, no toxicity was observed for this modified antigen at the administrated dose for the immunization of mice.

1 In the present work, we used a recombinant *L. casei* strain that expressed the modified ϵ -
2 toxoid anchored to cell wall by the pT1NX expression vector. This vector contained a spaX
3 gene product as an anchor to express antigens on the cell wall. The spaX is considered a good
4 choice for surface expressing in *L. casei* [19, 29]. Meanwhile, the expression of susceptible
5 antigens for mucosal immunization with live vectors can elicit a better immune response
6 when the expressed proteins anchor to a cell wall [10, 32, 38]. As shown in Figure 3, the
7 modified ϵ -toxoid was successfully expressed on the cell wall of *L. casei*.

8 The oral immunization of the mice with LC-pT1NX- ϵ induced a higher level of sera IgG and
9 IgA antibodies, specifically IgA, in the vaccinated group than in the control groups,
10 indicating that the immune system was properly stimulated by the modified ϵ -toxoid.
11 Moreover, the constitutive expression of this toxoid on the cell-wall might contribute to the
12 high antibody titers obtained in this study. Better immunity responses to expressed antigens
13 depend on the appropriate presentation of proteins and their capturing by antigen-presenting
14 cells such as dendritic cells (DCs) [32]. Mohamadzadeh et al. reported the DC-targeting
15 strategy to induce higher immunity via *Lactobacillus* vector vaccines [24, 43]. Here, a DC-
16 targeting peptide sequence (FYPSYHSTPQRP) was added to the modified ϵ -toxoid for better
17 immune responses. Moreover, *L. casei* has an ability to induce elevated levels of sIgA
18 responses in feces and small intestine [9]. Our results showed that the vaccinated mice
19 displayed higher concentrations of anti- ϵ IgA in the intestinal washes.

20 The balance or ratio between Th1 and Th2 cells is important for a healthy immune response
21 [44]. We examined the Th1-type (IL2 and IFN- γ) and Th2-type (IL-4 and IL10) cytokine
22 responses by the ELISA assay. In the current study, we showed a relative Th1/Th2 balance in
23 the cytokine production. The cytokine profiling results demonstrated the importance of the
24 cellular immune responses. Higher levels of IFN- γ were detected in response to the
25 stimulation with modified ϵ -toxoid in the mice immunized with LC-pT1NX- ϵ as compared to

the levels in the control groups. In adaptive immunity responses, IFN- γ is produced by the CD4⁺ T helper 1 (Th1) subset, which promotes inflammatory responses, clearance of intracellular pathogens, and class-switching of IgG [45].

In summary, our findings demonstrated that the BALB/c mice orally vaccinated with the recombinant LC-pT1NX- ϵ were able to induce more potent mucosal, humoral, and cell-mediated immune responses and protect mice against the challenge with the activated ϵ -toxin. This issue showed a correlation between antibody production and death inhibition at the challenge [39] and suggested that the recombinant LC-pT1NX- ϵ strain is a potential candidate against the enterotoxemia disease. Furthermore, this surface display system could be used to design vaccines against other toxins and potential pathogens.

Acknowledgements

This work was supported by a grant from Iran National Science Foundation (INSF) under grant number 920238680. We are grateful to Dr. Jalil Abshenas for his experimental support. We also thank Dr. Mehrdad Shamsaddini Bafti for his helpful advice. The authors declare no conflict of interest.

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Figure Legends:

Figure 1: Schematic presentation of the constructed pT1NX- ϵ plasmid. The epsilon toxoid gene (green), USP45 signaling, and cell wall anchor protein (SpaX) sequences were shown.

Figure 2: Detection of ϵ -toxoid on *L. casei* by western blotting. Fractionated extracts from 10^9 cells of the recombinant LC-pT1NX- ϵ strain that expressed the 53 kDa ϵ -toxoid probed with a polyclonal antibody as the primary antibody and a horseradish peroxidase-conjugated goat anti-horse IgG antibody as the secondary antibody. Lane M; Prestained protein ladder (PR901641, Sinaclon, Iran), lane 1; Cytoplasmic protein of *L. casei*, lane 2; Cytoplasmic protein of LC-pT1NX- ϵ

Figure 3: Detection of cell wall anchored ϵ -toxoid in LC-pT1NX- ϵ cells. All the cells were probed with FITC-conjugated polyclonal *C. perfringens* epsilon antitoxin. Wild type *L. casei* was used as a negative control.

Figure 4: Induction of IgG and IgA by gastric immunization of mice with LC-pT1NX- ϵ . Sera from the test and control groups were analyzed by indirect ELISA for anti- ϵ antibodies using a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:1000) or anti-mouse IgA (1:2500). Animals receiving the *L. casei* carrying the pT1NX empty vector (LC-pT1NX) or PBS were used as controls.

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Figure 5: Analysis of the induction of sIgA in the intestinal washes at the end of immunization. Samples from the test (LC-pT1NX-ε) and control (LC-pT1NX and PBS) groups were analyzed by ELISA for anti-ε antibodies.

Figure 6: The percentages of the total survived mice were subcutaneously challenged with 1, 2, or 10 MLD of the activated ε-toxin of *C. perfringens* type D reference strain orally immunized with LC-pT1NX-ε. Mice orally given LC-pT1NX or PBS were used as controls.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6