

Construction of a Recombinant Allergen-Producing Probiotic Bacterial Strain: Introduction of a New Line for a Live Oral Vaccine Against *Chenopodium album* Pollen Allergy

Leila Roozbeh Nasiraie¹, Farideh Tabatabaie¹, Mojtaba Sankian²,
Fakhri Shahidi¹, Abdolreza Varasteh*³

Abstract

Background: During the last two decades, significant advances have been made in the fields of lactococcal genetics and protein expression. *Lactococcus lactis* (*L. lactis*) is an effective vector for protein expression and can be used as an antigen delivery system. Hence, *L. lactis* is an ideal candidate for mucosal immunotherapy. Profilin (Che a 2), the major allergen in *Chenopodium album*, is one of the most important causes of allergic diseases in desert and semi-desert areas, especially in Iran, Saudi Arabia, and Kuwait that was cloned and expressed in *L. lactis* for the first time.

Methods: To construct *L. lactis* that expressed Che a 2, a DNA sequence was cloned and used to transform bacteria. Expression of Che a 2 was analyzed via monitoring of related RNA and protein. Hydrophobicity, adherence to HT-29 cells, antibiotic resistance, resistance to gastrointestinal contents, pH, and bile salt in recombinant and native *L. lactis* were evaluated.

Results: Immunoblot analyses demonstrated that recombinant Che a 2 is expressed as a 32 kDa dimeric protein immunological studies showed it can bind human IgE. Both native and recombinant bacteria were sensitive to low pH and simulated gastric conditions. Bacterial survival was reduced 80-100% after 2 h of exposure to pH 1.5-2. Both native and recombinant bacteria were able to grow in 0.3 and 2% bile salts. After incubation of recombinant *L. lactis* in simulated gastric and intestinal juices for one and two hours, respectively, cell survival was reduced by 100%. Adhesion capability in both strains was minimal and there were no significant differences in any of our tests between native and recombinant bacteria.

Conclusion: Successfully recombinant *L. lactis* with capability of expression Che a 2 was produced and revealed it is sensitive to gastrointestinal contents.

Keywords: *Chenopodium* pollen allergen, Oral vaccines, Probiotic bacteria, Recombinant *L. lactis*

Introduction

Type I allergy is a major health problem that affects more than 25% of the population in industrialized countries (1). Pollens from anemophilous plants are a

major problem in Type I allergy and the most predominant source of allergens in the outdoor environment (2). *Chenopodium album* (*C. album*, Lambs quarter) is a perennial plant that belongs to the Amaranthaceae/Chenopodiaceae family, and grows

1: Department of Food Science and Technology, Ferdowsi University of Mashhad, Mashhad, Iran

2: Immunobiochemistry Lab, Immunology Research Center, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

3: Immunobiochemistry Lab, Allergy Research Center, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

*Corresponding author: Abdolreza Varasteh; Tel: +98 511-7112611; Fax: + 98 511-7112596; E-mail: varastehA@mums.ac.ir

Received: May 22, 2013; Accepted: July 10, 2013

in all types of soils, even salty soil in temperate zones of southern Europe and the western United States (3). *C. album* pollen is one of the most important causes of allergic diseases in desert and semi-desert areas, with prevalences of 62.9%, 53%, and 70.7% in Iran, Saudi Arabia, and Kuwait, respectively (4, 5).

The major allergens in *C. album* are Che a 1, Che a 2, and Che a 3 (3, 6). Profilin (Che a 2), a 14.4 kDa protein, and polcalcin (Che a 3), a 9.5 kDa protein, have been cloned, purified, and characterized using immunochemical methods in our lab (7, 8). *C. album*-allergic patients showed 55 and 46% reactivities to Che a 2 and Che a 3, respectively (6).

In the past, allergen immunotherapy has been administered mainly by the parenteral injection of allergens, while more recently, mucosal delivery methods have been attempted (9). The administration of therapeutic molecules via mucosal routes offers several important advantages over systemic delivery such as reduction of secondary effects, easy administration, and the possibility to modulate both systemic and mucosal immune responses (10, 11). Moreover, it is important for molecules that exert their effects at mucosal surfaces to be delivered directly to the appropriate site. A major disadvantage of the mucosal administration route is that relatively large amounts of protein relative to other delivery systems must be administered due to the very small percentage of protein that survives degradation at mucosal surfaces such as the gastrointestinal tract. The use of live bacterial vectors to deliver antigens may allow the development of multivalent vaccines (12).

In this regard, Non-pathogenic lactic acid bacteria (LAB) such as certain species of lactococci and lactobacilli constitute attractive candidates for the development of live vectors for mucosal delivery of therapeutic proteins (13). Indeed, these bacteria have been used for centuries in the fermentation and preservation of food and are considered to be safe microorganisms with GRAS (Generally Recognized as Safe) status. Some strains have been reported to exert health benefits or probiotic effects (14). In addition, mucosal administration with genetically-engineered LAB has been shown to elicit both systemic and mucosal immunities (12, 13). Some studies that analyzed the immunological

effects of these recombinant LAB in animal models have shown that most of the tested antigens exposed to the surface of LAB yielded higher immune responses than non-surface-exposed antigens (13, 15-17).

In this way, *Lactococcus lactis* (*L. lactis*) is a homofermentative microorganism widely used in the dairy industry as a starter in milk fermentation, particularly in cheese-making, and is considered as the model LAB because many genetic tools have been developed for it and its complete genome has been sequenced (14). *L. lactis* can be genetically engineered to efficiently produce and secrete various proteins, a feature recently exploited by scientists to deliver therapeutic proteins to the mucosal tissues, specifically through the intranasal, oral, or genital mucosal surfaces. Presently, abundant data supports the use of recombinant LAB, in particular *L. lactis*, to deliver therapeutic proteins to mucosal tissues (12, 18). Moreover, a successful Phase I clinical trial with an *L. lactis* strain secreting interleukin-10 for Crohn's disease has opened new horizons for the use of genetically engineered LAB as delivery vehicles (19).

L. lactis is considered food-grade and endotoxin-free, and is able to secrete heterologous products together with native proteins. These characteristics make *L. lactis* a good candidate for mucosal immunotherapy. Chenopod pollen allergens play an important role in the sensitization of allergic patients. In this study, cloning and expression of profilin (Che a 2) of *C. album* pollen in *L. lactis* as a candidate for oral immunotherapy was described, and characteristics of natural and recombinant bacteria were compared.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *L. lactis* was grown at 30 °C in M17 medium (Difco) supplemented with 0.5% glucose. *Escherichia coli* (*E. coli*) MC1061 cells were cultured in Luria-Bertani (LB) broth at 37 °C. Solid media were produced by adding 1.5% agar to LB broth. To make selective media, the media were supplemented with 10 µg/ml and 400 µg/ml of erythromycin (Sigma-Aldrich, Missouri 63103 USA) for *L. lactis* and *E. coli*, respectively.

DNA and plasmid isolation

To access the Che a 2 coding sequence, *E. coli* BL21 CodonPlus (DE3) cells carrying the pET-32b(+)/Che a 2 expression vector were used, as described previously (7). Plasmid DNA was extracted from *E. coli* MC1061 cells by the alkaline lysis method (20). Restriction enzymes and T4 ligase were purchased from Fermentase Corporation (Fermentase GMBH, Germany).

Construction of expression plasmid and transformation

The Che a 2 sequence was amplified by RT-PCR with the sense primer E1 (5'CCTCCGTCGACTATGTCGTGGCAGACGTACGTAGA3') and the antisense primer K1 (5'ACTTCCCTGCAGTTACATGCCCTGTTCGACCAGGTAGT3') (22, 24, 27). Deoxyribonucleic acid amplification of the ~399 base pair (bp) fragment was carried out in a 20 μ L reaction mixture containing 2 μ L of 10 \times PCR buffer, 1 μ L deoxynucleoside triphosphate mixture (10 mM), 3 μ L of MgSo4 (25 mM), 1 μ L of each primer (10 pmol μ L⁻¹), 2 μ L of DNA, and 0.5 μ L of *Pfu* polymerase (5 U μ L⁻¹). The PCR started with heating at 95 °C for 3 min, followed by 35 cycles consisting of 95 sec at 95 °C, 1 min at 55 °C, and 90 sec at 72 °C, and a final segment at 72 °C for 3 min. The PCR products were digested with *Pst I* and *Sal I*. Subsequently, using T4 DNA ligase, the digested PCR products were ligated into to the LAB expression vector PNZ3004, and transferred into *E. Coli* MC1061 cells for amplification (7). Transformants were selected on LB agar plate containing erythromycin. The plasmid was extracted from transformed *E. coli* and sequenced using plasmid forward and reverse primers, pnzf (5'TAGGAGGTAGTCCAAATGGC3') and pnrz (5'TGATTTACTGTATTCAGGAGGAG3'), respectively. After a BLAST analysis and confirmation of the cloned fragment, the plasmid was electroporated into *L. lactis* as described previously (22). The electroporated *L. lactis* were cultured in M17 broth for 3 h, spread onto solid medium containing erythromycin, and incubated at ambient temperature until transformants appeared, generally about 24 to 48 h.

Reverse transcription PCR (RT-PCR) for detection of Che a 2 expression

Transformants were confirmed by direct colony PCR and RT-PCR with the E1/K1 primers. Recombinant bacteria were cultured in M17 media until OD₆₀₀ = 0.4, then induced with 2% lactose for 3 h before harvesting. Total RNA was extracted from Che a 2 transformants by the Pars Toos RNA extraction kit (Mashhad, Iran). cDNA was synthesized (RevertAid™ First Strand cDNA Synthesis Kit, Fermentas) with DNase-treated RNA and amplified by PCR with the E1/K1 primers. The PCR product was analyzed by agarose gel electrophoresis (Bioneer, Korea).

Determination of plasmid stability

The stability of the plasmid in recombinant cells was investigated by the Bates method (23). Briefly, transformed bacteria were grown in M17 broth without antibiotic and maintained in mid-log phase throughout 30 generations by refreshing bacteria. At the appropriate generation, bacteria were serially diluted and plated onto medium with or without erythromycin to determine the percentage of plasmid loss. Plasmid stability was confirmed by direct-colony PCR.

Immunological characterization of recombinant LAB in vitro

Che a 2, which was reported as a major allergen of *C. album* (6), was recognized by 81% (n = 26) of Iranian patient's sera as described previously (8). Sera from these patients was pooled for western blotting. Transformed bacteria were grown to the middle exponential phase (OD₆₀₀ = 0.4), induced with 2% lactose for 3 h, and harvested. Bacteria were washed with phosphate-buffered saline (PBS, 0.15 M, pH 7.4), resuspended in washing buffer, and disrupted by sonication for 10 min (3 sec pulse and 1 sec rest). All subsequent operations were performed at 4°C. Bacterial cell debris was removed by centrifugation, and cell extracts of recombinant and control strains were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer of proteins onto polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA) by electroblotting according to a standard protocol (24). In brief, after washing and blocking with 2% bovine serum albumin (BSA) for 16 h at

4°C, membranes were incubated with the pooled serum from the allergic patients or with control sera, diluted 1:5 in PBS for 3 h at room temperature (RT). Biotinylated anti-human IgE (KPL, Inc., MD, USA) (1:1000 v/v in 1% BSA) was added to the blotted membrane strips and incubated for 2 h at RT. Unbound antibodies were removed from the blots by washing with PBS, followed by incubation in 1:20000 v/v HRP-linked ExtrAvidin (SIGMA) in 1% BSA for 1 h at RT. The bound enzymatic activity of horseradish peroxidase was detected by enhanced chemiluminescence reagents (PIERCE, IL, USA) and documented with G-BOX Chemi-Doc (Syngene, Cambridge, UK).

Acidic pH tolerance test

Bacteria cultured anaerobically in M17 broth at 30 °C for 16 h and refreshed in 10 ml M17 broth for another 16 h. The bacteria were pelleted by centrifugation at $1700 \times g$ for 15 min at 4 °C and the pellets washed 2x with phosphate buffered saline (PBS). The washed samples were diluted 1/20 in PBS with pH values of, 2.0, 2.5 and 3.0. Incubation times were 0.5, 1, 2, 3, and 4 hours. Preparation and dilution of bacterial cells were performed according to Ehrmann *et al.* (2002) and the results were expressed as the mean of the log₁₀ of colony forming unit (CFU) (25).

Bile salt tolerance test

To determine bile salt tolerance, recombinant and native *L. lactis* were incubated in M17 broth containing 0.3 or 2% ox bile (Fluka, Sigma-Aldrich GmbH, Buchs; cat. 70168) or 0, 7, 14, or 21 mmol l⁻¹ sodium taurocholate (Fluka, Sigma-Aldrich GmbH, Buchs; cat. 86339). The optical densities (O.D.) were monitored over 12 h at 1 h intervals. Experiments were performed in four different series in 96 well plates.

Viability of recombinant and native *L. lactis* in simulated gastric juice

The method described M. G Vizoso Pinto *et al.* (2006) was used (26). The freshly harvested (1 g) of *L. lactis* cells were resuspended in 10 mL of sterile simulated gastric juice (6.2 g/l NaCl, 2.2 g/l KCl, 0.22 g/l CaCl₂ and 1.2 g/l NaHCO₃ pH 2.5) with 0.3% pepsin (Fluka, Germany) and incubated at 37 °C for 30, 60, 90, or 120 min. Surviving bacteria were

enumerated by pour plate counts in M17 agar after incubation at 30 °C. The counts were expressed as mean log cfu mL⁻¹.

Survival of bacteria cells in simulated intestinal juice after incubation in simulated gastric juice

The freshly harvested (1 g) *L. lactis* samples were placed in 10 mL of the described simulated gastric juice and incubated at 37 °C for 60 min (26, 27). After incubation, the samples were neutralized with NaOH (1%) solution. The bacterial cells were removed and placed in 9 mL of sterile simulated intestinal juice (1.28 g/l NaCl, 6.4 g/l NaHCO₃, 0.239 g/l KCl, pH 7.5) with 0.5% bile salt (Fluka, Sigma-Aldrich GmbH, Buchs; cat.70168) and 0.1% pancreatin (Fluka, Germany) . The tubes were then incubated at 37 °C for 30, 60, 90, or 120 min. After incubation, 1 ml of each sample was removed and enumerated in triplicate on M17 agar.

Hydrophobicity test

Microbial surface hydrophobicity was evaluated by adherence to non- polar solvents. N-hexadecane (Merck Schuchardt OHG, Hohenbrunn, Germany; cat. 8206330250) was used according to Pelletier *et al.* (28). Briefly, bacteria in stationary phase were pelleted by centrifugation as above, washed 2x with PBS, and their absorbance adjusted to 0.6 at 600 nm (A₀). One ml of N-hexadecane was added to 2 ml of adjusted cell suspension. After 10 min of incubation at RT, the suspension was stirred vigorously for 2 min. The phases were separated, the aqueous phase was collected and incubated for 30 min, and its absorbance measured at 600 nm (A₁). The percentage of hydrophobicity was calculated as $(1 - A_1/A_0) \times 100$. The probiotic strain *Lactobacillus rhamnosus GG* was used as a control.

Antibiotic resistance test

Bacterial antibiotic resistance was determined on solid M17 medium using vancomycin (30 µg), penicillin (10 µg), cefalexin (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), and streptomycin (10 µg) discs on each plate. The plates were incubated at 30 °C for 16 h in conditions suitable for the tested bacterial strains. Zones of inhibition were

measured in millimeters. Two strains with known antibiotic resistances (*Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212) were used as the controls.

Adherence to HT-29 cells

The adherence of *L. lactis* to HT-29 cells (National Cell Bank of Iran Code: C466; Pasteur Institute, Tehran, Iran) was examined essentially as described by Ulrich Schillinger et al. (29). Cells were grown in Roswell Park Memorial Institute medium (RPMI-1640, Gibco, Germany) supplemented with 2 g/L sodium bicarbonate, 10% heat-inactivated (30 min at 56 °C) fetal calf serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Sigma-Aldrich, St. Louis, MO, USA) at 37 °C in 5% CO₂. For the adherence assays, HT-29 cell monolayers were prepared in 24-well tissue culture plates (Gibco, Germany). Cells were inoculated at a concentration of 7×10^5 cells per well to obtain confluence and incubated for 21 days before the adhesion assay. Cell culture medium was changed on alternate days, and penicillin and streptomycin were omitted from the last two media changes.

Then overnight cultures of bacteria grown in RPMI-1640 supplemented with 2% (v/v) FCS were pelleted, washed, and resuspended in RPMI-1640. Viable counts were determined by plating on MRS agar. A 1 mL aliquot of the bacterial solution was added to each well of the tissue culture plate; the plates were pelleted by centrifugation at 2000 g for 2 min and incubated in 5% CO₂. After 1 h of incubation, viable counts of the supernatants were determined by plating serial dilutions on M17 agar. Cells were lysed by the addition of 0.05% Triton X-100 and the appropriate dilutions were again plated on M17 agar. Adhesion was calculated from the initial viable counts, those of the supernatants, and those of the cell lysates. Each determination was carried out in triplicate. The probiotic strain *Lactobacillus rhamnosus GG* was used as a control.

Statistical Analysis

Data analysis was performed using the ANOVA (SAS Institute and 2004). Treatment means were compared using Tukey's test, with significant level at P=0.05. In all growth studies, the mean of two to three repeated measurements yielded the value for each replicate.

Table 1. Bacterial strains and plasmids used in this study

Bacterium/Plasmid	Relevant feature (s)	Source and references
Bacteria		
<i>L. lactis</i> MG 1363	Subsp. cremoris, plasmid-free	MoBiTec GmbH, Germany (30)
<i>L. lactis</i> PNZche a 2	Subsp. cremoris, plasmid-free; carrying PNZche a 2	This study
<i>E. coli</i> BL21 (DE3)	DE3: carrying pET-32b(+)/Che a 2 expression vector	Novagen, NJ, USA
<i>E. coli</i> MC1061	araD139, Δ(ara, leu)7697, ΔlacX74, galU-, galK-, hsr-, hsm+, strA	MoBiTec GmbH, Germany
Plasmids		
pNZ3004	Cm ^r Em ^r ; <i>E. coli-lactobacillus</i> shuttle vector; 4.9 kb	(31)
PNZche a 2	Cm ^r Em ^r ; pBu003-containing <i>C. album</i> Che a 2 gene, 14.4 kb	This study

Results

Cloning and transformation of the expression plasmid into *L. lactis*

PNZche a 2 contained an open reading frame of 442 bp encoding a 147 amino acid polypeptide with a predicted molecular mass of 15.69 kDa and a calculated pI of 4.49. The expression plasmid

PNZche a 2 was electroporated into *L. lactis* with an efficiency of about 2×10^2 transformants per µg of DNA. The presence of Che a 2 gene in *L. lactis* was verified by direct-colony PCR. Plasmid primers (pnz set) amplified a 569 bp fragment containing the Che a 2 sequence (not shown). No band was visible on agarose gel electrophoresis following amplification of DNA from untransformed bacteria, and PCR of DNA from bacteria transformed with the plasmid alone amplified a product of 170 bps (not shown).

Detection of specific Che a 2 mRNA

The presence of Che a 2 mRNA was verified by the presence of a 399 bp band on agarose gel electrophoresis following RT-PCR (Fig. 1, Lane 3). A direct-colony PCR product was used as the positive control (Fig. 1, Lane 4). No bands were seen from negative controls (Fig. 1, lanes 2 and 5).

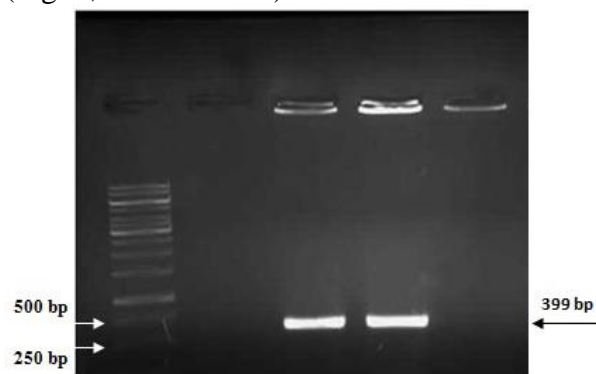


Fig. 1. Agarose gel electrophoresis of Che a 2 PCR products. Lane 1 is a 1 kbp Ladder, Lanes 2 and 3 are PCR products of DNase-treated RNA and synthesized cDNA as templates, respectively. Lane 4 is the product of direct-colony PCR of recombinant bacteria transformed with the plasmid alone as a negative control.

Plasmid stability

The stability of the recombinant plasmid in *L. lactis* was assessed in the absence of erythromycin. About 90% of *L. lactis* colonies that were grown in the absence of erythromycin remained resistant even after 30 generation. The stability of plasmid in *L. lactis* was confirmed by direct-colony PCR.

In vitro characterization of recombinant *L. lactis* producing Che a 2

Immunoblots with pooled sera from *C. album* pollen-allergic patients displayed similar binding of IgE to recombinant Che a 2 expressed in *E. coli* as a fusion 34 KDa-protein (Fig. 2, lane 2) and to Che a 2 expressed by the recombinant *L. lactis* as a dimer of 31.4 KDa (Fig. 2, lane 1). No IgE binding was detected using the control strain (Fig. 2, lane c). Sera of non-atopic or grass-pollen allergic control donors showed no IgE binding (data not shown).

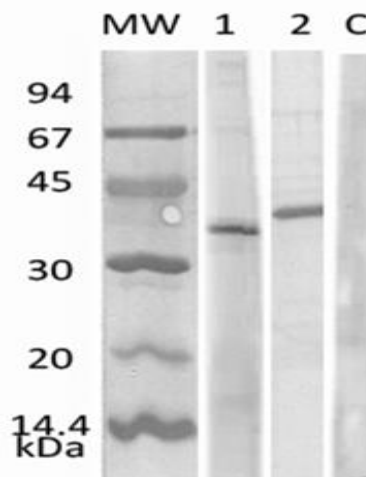


Fig. 2. Immunoblot analysis of Che a 2. Cell extracts from recombinant *L. lactis* (Lane 1), native *L. lactis* (Lane C) and Che a 2-expressing *E. coli* cells (Lane 2) were electrophoresed by SDS-PAGE and transferred to PVDF membranes. All lanes were incubated with pooled serum from Che a 2-allergic patients and then with an anti-human IgE secondary antibody.

pH tolerance test

Before reaching the intestinal tract, probiotic bacteria must first survive transit through the stomach where the pH can be as low as 2.0 (32). Fig. 3 shows the viability of different strains at various pH values. No significant difference was observed between native and recombinant *L. lactis* at the different pHs. The reduction in viability in both native and recombinant bacteria after 4 h of incubation at pH values of 3.0, 2.5 and 2.0 were 40, 70, and 100%, respectively.

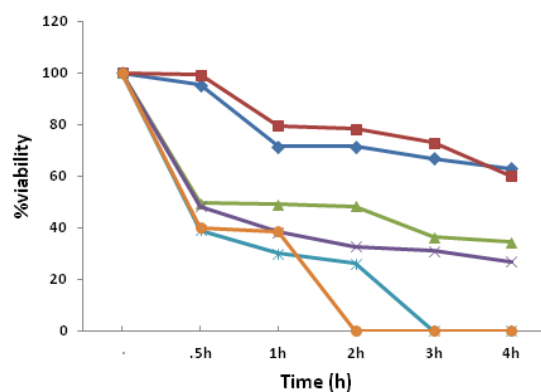


Fig. 3. Viability of recombinant and native *L. lactis* at various pH values after 0.5-4 hours of incubation. pH= 3: native (■), recombinant (◆); pH= 2.5: native (▲), recombinant (×); pH= 2.0: native (*), recombinant (●).

Growth in the presence of ox bile and taurocholate

Tolerance to bile salts is considered to be a prerequisite for the metabolic activity of bacteria in the host small intestine (27). Therefore, it is generally considered necessary to evaluate the ability of the bacteria to resist the effects of bile salts. In this study, growth of native and recombinant *L. lactis* strains was reduced in 0.3 and 2.0% ox bile (Fig. 4).

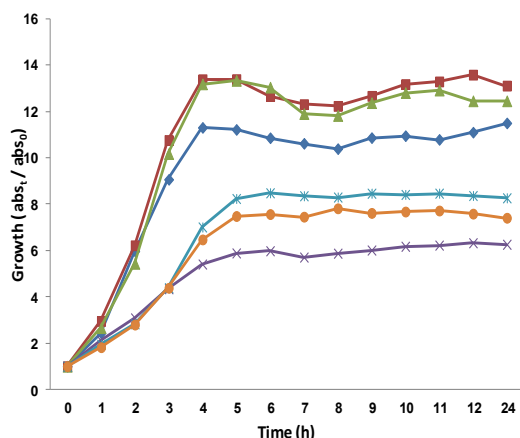


Fig. 4. Growth of native and recombinant *L. lactis* in 0, 0.3, and 2.0% ox bile extract. 0%: native (■), recombinant (▲); 0.3%: native (◆), recombinant (*); 2%: native (●), recombinant (×).

Survival of bacteria in simulated gastric juice

To determine whether production of recombinant protein affected cell survival in the digestive system, simulated acidic gastric juice was used. The viability of *L. lactis* was expressed as the destructive value (D-value), which is the time required to kill 90%, or one

The rate of reduction in 2% ox bile in the recombinants was significantly higher than in the native bacteria (P<0.05). Increasing concentrations of sodium taurocholate reduced growth of both native and recombinant bacteria; however, growth was significantly lower in recombinant than native *L. lactis* at all sodium taurocholate concentrations (Fig. 5).

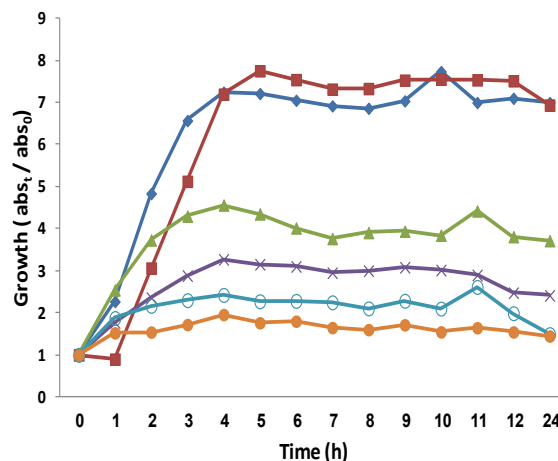


Fig. 5. Growth of native and recombinant *L. lactis* in taurocholate. 7 mmol l-1 native (■), recombinant (*), 14 mmol l-1 native (▲), recombinant (●), and 21 mmol l-1 native (◆), recombinant (×).

log cycle, of the organism (Table 2). No significant differences in D-values were observed between native and recombinant *L. lactis* in simulated gastric juice (Table 2). Viability of the native and recombinant cells decreased 71.39% and 82.5% after 2 h of exposure to the simulated gastric juice, respectively.

Table 2. Viable cells after exposure to simulated gastric juice at the times shown (cfu g⁻¹ bead)

		Time (min)					D-value (min)
		0	30	60	90	120	
<i>L. lactis</i>	Native	1.49±0.2 × 10 ¹³	3.9±0.8 × 10 ¹¹	1.4±0.2 × 10 ⁶	1±0.21 × 10 ⁵	5.9±0.1 × 10 ³	11.79a
	Recombinant	1.4±0.31 × 10 ¹³	8.7±0.5 × 10 ¹⁰	1.2±0.1 × 10 ⁶	8±0.13 × 10 ²	2±0.7 × 10 ²	10.09a

Values are mean ± standard error (n = 3). Values with the same letters are not significantly different.

Cell survival in simulated intestinal juice after incubation in simulated gastric juice

To determine the tolerance of the native and recombinant strains to the acidic pH of the stomach and simulated intestinal juice, an in vitro system was utilized. The results are shown in Table 3. The D-values of both native and recombinant *L. lactis*

incubated in simulated gastro-intestinal juice were similar, indicating that the transformation process has no effect on bacterial survival. In addition both native and recombinant *L. lactis* were highly sensitive to simulated intestinal juice and their viabilities were reduced nearly 100% within 30 min.

Table 3. Average number (mean) cfu g⁻¹ of survived cells and D-values of *L. lactis* cells after incubation at 37 °C for 60 min in simulated gastric juice and 37 °C for 2 h in simulated intestinal juice, pH 7.5 (n = 3).

		Time (min)					D-value (min)
		0	30	60	90	120	
<i>L. lactis</i>	Native	1.49±0.4 × 10 ¹³	31.2±0.21 × 10 ¹	0.63±0.6 × 10 ¹	0.1± 0.2 × 10 ¹	0.1±0.7 × 10 ¹	5.2 ^a
	Recombinant	1.4±0.31 × 10 ¹³	1±0.32 × 10 ¹	1±0.43	1 ± 0.5	1 ± 0.54	5.2 ^a

Values are mean ± standard error (n = 3). Values with the same letters are not significantly different.

Hydrophobicity and adherence to HT-29 cells

In this study the hydrophobicity of the bacterial outer membrane was evaluated photometrically in a hydrophilic environment. No hydrophobicity differences were observed between the native and

recombinant bacteria (P>0.05), while both were significantly different from the control strain (Table 4). The adherence to Caco-2 cells of both native and recombinant *L. lactis* was significantly lower than that of the control strain.

Table 4. Hydrophobicity and adhesion test.

Strain	%Adhesion	%Hydrophobicity
<i>L. lactis</i> (native)	2.11 ± 0.05 ^b	28.6 ± 1.5 ^b
<i>L. lactis</i> (recombinant)	0.85 ± 0.11 ^b	23.7 ± 0.55 ^b
<i>L. rhamnosus</i> GG	20.2 ± 0.6 ^a	58.6 ± 0.7 ^a

Values are mean ± standard error (n = 6). Values with the same letters are not significantly different (P<0.05).

Antibiotic resistant test

One important feature of probiotic strains of bacteria is their resistance to antibiotics, especially when they are used after antibiotic therapy. We observed differences between the experimental strains in their antibiotic resistance characteristics. The results are presented in Table 5. The native and recombinant *L. lactis* strains differed in their resistances to erythromycin and chloramphenicol, but not to cefalexin, vancomycin, streptomycin, or penicillin. The recombinant *L. lactis* carry plasmid PNZche a 2, which contains antibiotic resistance genes to erythromycin and chloramphenicol. Both the native and recombinant bacteria were least

sensitive to streptomycin and penicillin and more sensitive to the other antibiotics.

Discussion

Chenopodiaceae pollens are significant allergens in the western USA and temperate areas of Europe (3). Among the *Chenopodiaceae* family, *C. album* was selected for this study for two reasons; first, *C. album* pollen is one of the most important allergenic sources in desert and semi-desert areas especially in our country, Iran (4); second, Che a 2 is a member of the profilin family that has significant role in allergy (6, 33). Based on the assumption that lack of counter-regulatory immune responses may favor the development of type I allergies, the induction of allergen-specific Th1 responses has been proposed as a promising concept for treatment of Th2-biased hyper-responsiveness. According to recent studies adequate microbial intervention seems to constitute a promising approach to such treatment (34-36). Recently, LAB has increasingly been used to deliver bioactive compounds by various mucosal routes. *L. lactis* is the most widely used LAB in this regard (36). The major advantage of using *L. lactis* as a live vector for mucosal delivery of therapeutic proteins resides in its extraordinary safety profile;

Table 5. Susceptibility of native and recombinant *L. lactis* to antibiotics. Size of the growth inhibition areas are expressed in mm.

	Bacteria		
	<i>L. lactis</i>	<i>L. lactis</i> Recombinant	
Antibiotics	Cefalexin	7±0.6	7±0.2
	Vancomycin	8.25±0.42	9±0.31
	Streptomycin	0.5±0.2	0.5±0.23
	Chloramphenicol	13±0.12 ^{a**}	4.5±0.2 ^b
	Penicillin	1.45±0.14	1.25±0.1
	Erythromycin	15.25±0.2 ^{a*}	7±0.16 ^b

Values are mean ± standard error (n=3). Values with the same letters are not significantly different (P<0.05).

this bacterium is catalogued as non-invasive and non-pathogenic with GRAS status (37). Moreover, *L. lactis* is considered to be a good candidate for heterologous protein production because it secretes relatively few proteins and only one, Usp45, is sufficiently abundant to be detectable (38, 39). In this study, we used *L. lactis* strain MG1363. This bacterium is commonly used in laboratories, is plasmid-free, and produces no known extracellular proteases (40).

In this study we constructed recombinant strain of *L. lactis* MG 1363 to produce the airborne allergen Che a 2 with the aim of using this strain as live vector for specific prophylaxis of *Chenopodium* pollen allergy. The digested PCR product of a Che a 2 sequence was ligated into PNZ3004, the expression vector for LAB. The open reading frame of Che a 2 contains 442 bases encoding a putative 15.69 kDa protein.

The expression plasmid PNZche a 2 was electroporated into *L. lactis* for Che a 2 expression. Native and recombinant *L. lactis* was cultured in M17 media containing 2% lactose, total RNA was extracted, and cDNA was synthesized. The cDNA was used as a template to amplify a 399 bp product by PCR using the Che a 2 E1/K1 primers. This result confirmed Che a 2 mRNA expression by recombinant *L. lactis*. Pooled serum from allergic patients reacted with a 31.4 KDa band, likely representing a dimer.

Oral delivery of antigen via live bacteria is advantageous due to the nature of administration, side-effects reduction, specificity of target site, and persistence of the bacteria near the target site for a certain time period (19). It has been generally assumed that *Lactococcus* strains do not survive passage through the digestive system because of the low pH of the stomach and the presence of bile in the intestine (41). Consequently, in this study resistance of bacteria to low pH environments and response to different concentrations of bile salts and simulated gastrointestinal conditions was evaluated. Both native and recombinant *L. lactis* were sensitive to low pH (Fig. 3) and simulated gastric conditions (Table 2). Two h of exposure to pH 1.5-2 resulted in an 80-100% reduction in survival percentage. Both native and recombinant bacteria were able to grow in 0.3 and 2% bile salts (Fig. 4), while 60 min of incubation in the simulated gastric juice, followed by 2 h of

incubation in the simulated intestinal juice reduced cell survival by 100%. Klijn et al. studied the survival of *L. lactis* strain TC165.5 in the human gastrointestinal tract up to the feces and showed the cells recovered accounted for approximately only 1% of the cells ingested (42). Similarly, Norton et al. reported that orally-administered *L. lactis* MG 1363 did not survive passage through the gut in mice (43). Similar results were recorded for *L. lactis* HV219 that grew in MRS broth with initial pHs ranging from 6.0-11.0, but not at pHs of 3.0-5.0, and grew well in the presence of 0.3 and 0.6% ox bile, but not at higher concentrations (44).

In the present study, mucus-producing HT-29 cells were chosen to determine and compare the adhesion behavior of native and recombinant *L. lactis* and *L. rhamnosus* GG (with well-documented adhesion properties) as a control. No significant differences in adhesion were observed between the native and recombinant *L. lactis*, while the adhesion of *L. lactis* strains to Caco-2 cells were relatively low (0.8-2%) compared to that of the control strain (20%, Table 4). Tuomola and Salminen reported 3-14% binding of probiotic *Lactobacillus* strains, including *L. rhamnosus* GG, to Caco-2 cells (45); however, Gopal, Prasad, Smart, and Gill (2001) reported the adherence of two *L. acidophilus* and two *L. rhamnosus* strains to HT29-MTX cells to be two to three times higher than with HT29 and Caco-2 cells. These researchers suggested a higher affinity of the HT-29 MTX cell line to the lactobacilli than the others (46). Mayra-Makinen et al. (1983) observed that *L. lactis* isolated from plant materials, cultured milk, and cheese failed to adhere to cultured epithelial cells of pigs and calves (47). It has been shown that 3% of *L. lactis* HV219 cells adhered to Caco-2 cells in the first hour of incubation, but after 2 hours adherence increased to 7% (44). Moreover, another study showed that some *Lactococcus* strains, including strains of *L. lactis* subsp. *lactis* and subsp. *lactis* bv. *diacetylactis*, adhered to Caco-2 cells (41).

Several mechanisms are involved in the adhesion of microbial cells to intestinal and vaginal epithelial cells. Avall-Jaaskelainen et al. (2003) showed that the reduced adhesion of *L. lactis* vs. *Lactobacillus* spp. could be influenced by lack of an S-layer protein in these bacteria (48). Furthermore, high cell-surface hydrophobicity may favor the colonization of mucosal surfaces and play a role in the adhesion of

bacteria to epithelial cells and extracellular matrix (ECM) proteins (49). In this study a hydrophobicity analysis revealed considerable differences between the *L. lactis* and *L. rhamnosus* GG control strains, which agreed with the adhesion results (Table 4). According to some studies, strains with high cell-surface hydrophobicities generally adhere efficiently to mucosal cells. Hydrophobicity can contribute to adhesion, but is not a prerequisite. Cell surface hydrophobicity is one of the physico-chemical properties that facilitates the first contact between microorganisms and host cells. This non-specific initial interaction is weak and reversible and precedes the subsequent adhesion process mediated by more specific mechanisms involving cell-surface proteins and lipoteichoic acids. Therefore, the contribution of hydrophobicity to adhesion seems to be limited and may explain the lack of correlation between hydrophobicity and bacterial adhesion observed in several studies (48, 49).

No significant differences in antibiotic resistance were observed between native and recombinant *L. lactis* except that the recombinant was more resistant to erythromycin and chloramphenicol than the native strain due to the presence of plasmid PNZche a 2, which contains resistance genes to these antibiotics. Both native and recombinant bacteria were least

susceptible to streptomycin.

In this study we constructed a recombinant *L. lactis* that expresses *C. album* pollen profilin (Che a 2). The native and recombinant strains had similar properties. Viability of both strains was reduced in the simulated human gastrointestinal tract, which could be a problem inherent in the use of *L. lactis* as a delivery vehicle. Because resistance to low pH and elevated concentrations of bile salts are important for growth and survival of bacteria in the intestinal tract, we suggest microencapsulating the recombinant strain to investigate its immunotherapeutic properties. In addition, low adherence to epithelial cells will likely result in minimal colonization in the gastrointestinal tract, reducing the likelihood of anaphylactic shock during immunotherapy. We plan to study the effect of microencapsulation on viability of the recombinant strain during passage through the gastrointestinal tract, and treatment effects in vivo.

Acknowledgements

This work was supported by Ferdowsi University of Mashhad. The molecular experiments of this study were performed in the Immunobiochemistry lab in Bu-Ali research center of Mashhad University of Medical Sciences.

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