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

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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

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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 routesoffers 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′CCTCCGTCGACT**ATG**TCGTGGCAGACGT ACGTAGA3′) and the antisense primer K1 (5′ACTTCCCTGCAG**TTA**CATGCCCTGTTCG ACCAGGTAGT3′) (22, 24, 27). Deoxyribonucleic acid amplification of the ~399 base pair (bp) fragment was carried out in a 20 μL reaction mixture containing 2 μL of 10× PCR buffer, 1 μL deoxynucleoside triphosphate mixture (10 mM), 3 μL of MgSo4 (25 mM), 1 μL of each primer (10 pmol μL^{-1}), 2 μL of DNA, and 0.5 μL of *Pfu* polymerase (5 U μL^{-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 pnzr (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 OD_{600} = 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 (OD_{600}= 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 × 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 $\log_{10}$ 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 $CaCl_2$ and 1.2 g/l $NaHCO_3$ 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 - A1/A0) ×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% CO_2. 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% CO_2. 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		
L. lactis MG 1363	Subsp. cremoris, plasmid-free	MoBiTec GmbH, Germany (30)
L. lactis PNZche a 2	Subsp. cremoris, plasmid-free; carrying PNZche a 2	This study
E. coli BL21 (DE3)	DE3: carrying pET-32b(+)/Che a 2 expression vector	Novagen, NJ, USA
E.coli MC1061	araD139, Δ(ara, leu)7697, ΔlacX74, galU-, galK-, hsr-, hsm+, strA	MoBiTec GmbH, Germany
Plasmids		
pNZ3004	Cm^r Em^r; *E. coli-lactobacillus* shuttle vector; 4.9 kb	(31)
PNZche a 2	Cm^r Em^r; pBu003-containing *C. album 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).

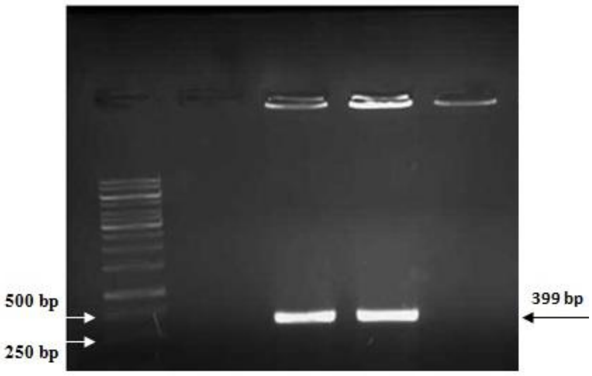

Fig. 1. Agarose gel electrophoresis of Che a 2 PCR products. Lane 1 is a 1 kbp Ladder, Lanes 2 and 3 are PCR products of DNase-treated RNA and synthesized cDNA as templates, respectively. Lane 4 is the product of direct-colony PCR of recombinant bacteria transformed with the plasmid alone as a negative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

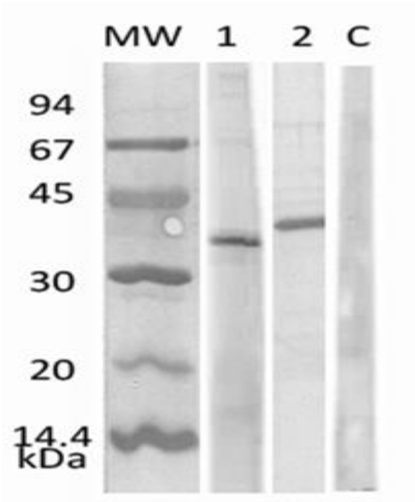

Fig. 2. Immunoblot analysis of Che a 2. Cell extracts from recombinant L. lactis (Lane 1), native L. lactis (Lane C) and Che a 2-expressing E. coli cells (Lane 2) were electrophoresed by SDS-PAGE and transferred to PVDF membranes. All lanes were incubated with pooled serum from Che a 2-allergic patients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

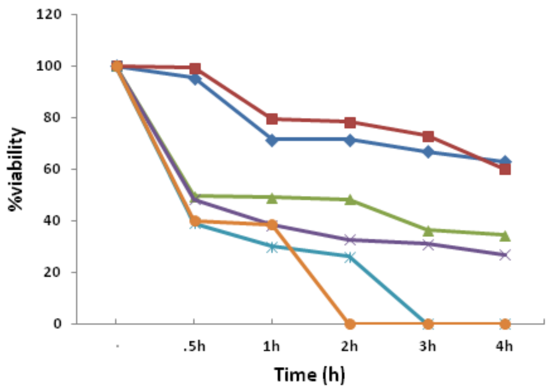

Fig. 3. Viability of recombinant and native L. lactis at various pH values after 0.5-4 hours of incubation. pH= 3: native (■), recombinant(♦); pH= 2.5: native (▲), recombinant (×); pH= 2.0: native (*), recombinant(●).

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).

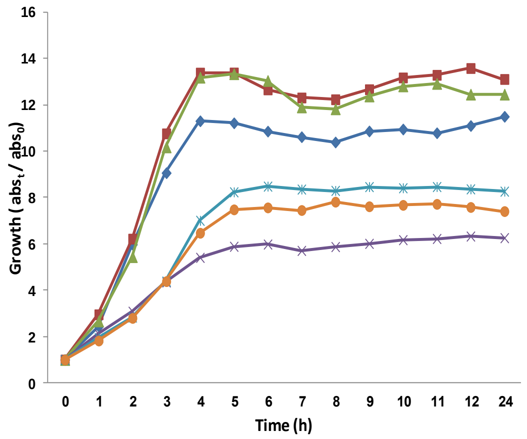

Fig. 4. Growth of native and recombinant L. lactis in 0, 0.3, and 2.0% ox bile extract. 0%: native (■), recombinant (▲); 0.3%: native (♦), recombinant (*); 2%: native (●), recombinant (×).

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).

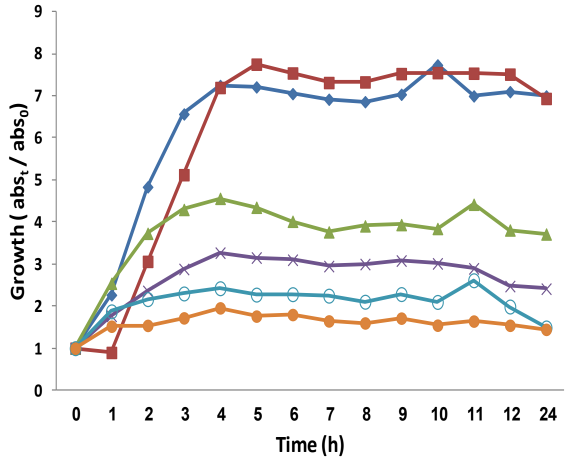

Fig. 5. Growth of native and recombinant L. lactis in taurocholate. 7 mmol l-1 native (■), recombinant (*), 14 mmol l-1 native (▲), recombinant (●), and 21 mmol l-1 native (♦), recombinant (×).

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 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	
L. lactis	Native	$1.49\pm0.2 \times 10^{13}$	$3.9\pm0.8 \times 10^{11}$	$1.4\pm0.2 \times 10^{6}$	$1\pm0.21 \times 10^{5}$	$5.9\pm0.1 \times 10^{3}$	11.79a
	Recombinant	$1.4\pm0.31 \times 10^{13}$	$8.7\pm0.5 \times 10^{10}$	$1.2\pm0.1 \times 10^{6}$	$8\pm0.13 \times 10^{2}$	$2\pm0.7 \times 10^{2}$	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	
L. lactis	Native	$1.49 \pm 0.4 \times 10^{13}$	$31.2 \pm 0.21 \times 10^{1}$	$0.63 \pm 0.6 \times 10^{1}$	$0.1 \pm 0.2 \times 10^{1}$	$0.1 \pm 0.7 \times 10^{1}$	5.2 [a]
	Recombinant	$1.4 \pm 0.31 \times 10^{13}$	$1 \pm 0.32 \times 10^{1}$	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
L. lactis (native)	2.11 ± 0.05 [b]	28.6 ±1.5 [b]
L. lactis (recombinant)	0.85 ± 0.11 [b]	23.7±0.55 [b]
L. rhamnosus 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.

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

		Bacteria	
		L. lactis	*L. lactis 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).

Discussion

Chenopodiacea pollens are significant allergens in the western USA and temperate areas of Europe (3). Among the *Chenopodiacea* 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;

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 by100%. 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.

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References

1. Kay AB. Allergy and allergic diseases. First of two parts. N Engl J Med. 2001 Jan;344(1):30-7.
2. de Weerd NA, Bhalla PL, Singh MB. Aeroallergens and pollinosis: Molecular and immunological characteristics of cloned pollen allergens. Aerobiologia (Bologna). 2002 Jun;18(2):87-106.
3. Barderas R, Villalba M, Lombardero M, Rodriguez R. Identification and characterization of Che a 1 allergen from *Chenopodium album* pollen. Int Arch Allergy Immunol. 2002 Jan;127(1):47-54.
4. Fereidouni M, Hossini RF, Azad FJ, Assarehzadegan MA, Varasteh A. Skin prick test reactivity to common aeroallergens among allergic rhinitis patients in Iran. Allergol Immunopathol (Madr). 2009 Mar-Apr;37(2):73-9.
5. Ezeamuzie CI, Thomson MS, Al-Ali S, Dowaisan A, Khan M, Hijazi Z. Asthma in the desert: spectrum of the sensitizing aeroallergens. Allergy. 2000 Feb;55(2):157-62.
6. Barderas R, Villalba M, Pascual CY, Batanero E, Rodriguez R. Profilin (Che a 2) and polcalcin (Che a 3) are relevant allergens of *Chenopodium album* pollen: isolation, amino acid sequences, and immunologic properties. J Allergy Clin Immunol. 2004 Jun;113(6):1192-8.
7. Amini A, sankian M, Assarehzadegan MA, Vahedi F, Varasteh A. *Chenopodium album* pollen profilin (Che a 2): homology modeling and evaluation of cross-reactivity with allergenic profilins based on predicted potential IgE epitopes and IgE reactivity analysis. Mol Biol Rep. 2011 Apr;38(4):2579-87.
8. Nouri HR, Sankian M, Vahedi F, Afsharzadeh D, Rouzbeh L, Moghadam M, et al. Diagnosis of *Chenopodium album* allergy

with a cocktail of recombinant allergens as a tool for component-resolved diagnosis. Mol Biol Rep. 2012 Mar;39(3):3169-78.
9. Canonica GW, Passalacqua G. Noninjection routes for immunotherapy. J Allergy Clin Immunol. 2003 Mar;111(3):437-48.
10. Levine MM, Dougan G. Optimism over vaccines administered via mucosal surfaces. Lancet. 1998 May;351(9113):1375-6.
11. Neutra MR, Kozlowski PA. Mucosal vaccines: the promise and the challenge. Nat Rev Immunol. 2006 Feb;6(2):148-58.
12. Bermudez-Humaran LG. Lactococcus lactis as a live vector for mucosal delivery of therapeutic proteins. Hum
Vaccin. 2009 Apr;5(4):264-7.
13. Wells JM, Mercenier A. Mucosal delivery of therapeutic and prophylactic molecules using lactic acid bacteria. Nat Rev Microbiol. 2008 May;6(5):349-62.
14. Mercenier A, Pavan S, Pot B. Probiotics as biotherapeutic agents: Present knowledge and future prospects. Curr Pharm Design. 2003 Jan;9(2):175-91.
15. Repa A, Grangette C, Daniel C, Hochreiter R, Hoffmann-Sommergruber K, Thalhamer J, et al. Mucosal co-application of lactic acid bacteria and allergen induces counter-regulatory immune responses in a murine model of birch pollen allergy. Vaccine. 2003 Dec;22(1):87-95.
16. Charng YC, Lin CC, Hsu CH. Inhibition of allergen-induced airway inflammation and hyperreactivity by recombinant lactic-acid bacteria. Vaccine. 2006 Aug;24(33-34):5931-6.
17. Schabussova I, Wiedermann U. Lactic acid bacteria as novel adjuvant systems for prevention and treatment of atopic diseases. Curr Opin Allergy Cl. 2008 Dec;8(6):557-64.
18. Bermudez-Humaran LG, Kharrat P, Chatel JM, Langella P. Lactococci and lactobacilli as mucosal delivery vectors for therapeutic proteins and DNA vaccines. Microb Cell Fact. 2011 Aug 30;10(Suppl 1):S4.
19. Braat H, Rottiers P, Hommes DW, Huyghebaert N, Remaut E, Remon JP, et al. A phase I trial with transgenic bacteria expressing interleukin-10 in Crohn's disease. Clin Gastroenterol Hepatol. 2006 Jun;4(6):754-9.
20. Sambrook J, Russell DW. Molecular Cloning: A laboratory manual. 2nd ed, New York: Cold Spring Harbor Laboratory Press; 1989 Dec.
21. Taheri HR, Moravej H, Tabandeh F, Zaghari M, Shivazad M. Screening of lactic acid bacteria toward their selection as a source of chicken probiotic. Poult Sci. 2009 Aug;88(8):1586-93.
22. Mason CK, Collins MA, Thompson K. Modified electroporation protocol for Lactobacilli isolated from the chicken crop facilitates transformation and the use of a genetic tool. J Microbiol Methods. 2005 Mar;60(3):353-63.
23. Bates EE, Gilbert HJ, Hazlewood GP, Huckle J, Laurie JI, Mann SP. Expression of a Clostridium thermocellum endoglucanase gene in Lactobacillus plantarum. Appl Environ Microbiol. 1989 Aug;55(8):2095-7.
24. Jarolim E, Tejkl M, Rohac M, Schlerka G, Scheiner O, Kraft D, et al. Monoclonal antibodies against birch pollen allergens: Characterization by immunoblotting and use for single-step affinity purification of the major allergen Bet v I. Int Arch Allergy Appl Immunol. 1989;90(1):54-60.
25. Ehrmann MA, Kurzak P, Bauer J, Vogel RF. Characterization of lactobacilli towards their use as probiotic adjuncts in poultry. J Appl Microbiol. 2002 May;92(5):966-75.26. Vizoso Pinto MG, Franz CM, Schillinger U, Holzapfel WH. Lactobacillus spp. with in vitro probiotic properties from human faeces and traditional fermented products. Int J Food Microbiol. 2006 Jun;109(3):205-14.
27. Coltart S. Survival of patients with cancer. BMJ. 1989 Dec;299(6712):1403.
28. Pelletier C, Bouley C, Cayuela C, Bouttier S, Bourlioux P, Bellon-Fontaine MN. Cell surface characteristics of Lactobacillus casei subsp. casei, Lactobacillus paracasei subsp. paracasei, and Lactobacillus rhamnosus strains. Appl Environ Microbiol. 1997 May;63(5):1725-31.
29. Schillinger U, Guigas C, Holzapfel WH. In vitro adherence and other properties of lactobacilli used in probiotic yoghurt-like products. Int Dairy J. 2005 Dec;15(12):1289-97.

30. Mierau I, Kleerebezem M. 10 years of the nisin-controlled gene expression system (NICE) in Lactococcus lactis. Appl Microbiol Biotechnol. 2005 Oct;68(6):705-17.
31. van Rooijen RJ, Gasson MJ, de Vos WM. Characterization of the Lactococcus lactis lactose operon promoter: contribution of flanking sequences and LacR repressor to promoter activity. J Bacteriol. 1992 Apr;174(7):2273-80.
32. Dunne C, O'Mahony L, Murphy L, Thornton G, Morrissey D, O'Halloran S, et al. In vitro selection criteria for probiotic bacteria of human origin: correlation with in vivo findings. Am J Clin Nutr. 2001 Feb;73(2):386s-92s.
33. Barderas R, Villalba M, Rodriguez R. Che a 1: Recombinant expression, purification and correspondence to the natural form. Int Arch Allergy Immunol. 2004 Dec;135(4):284-92.
34. Bjorksten B, Sepp E, Julge K, Voor T, Mikelsaar M. Allergy development and the intestinal microflora during the first year of life. J Allergy Clin Immunol. 2001 Oct;108(4):516-20.
35. Romagnani S. Immunologic influences on allergy and the TH1/TH2 balance. J Allergy Clin Immunol. 2004 Mar;113(3):395-400.
36. Daniel C, Repa A, Wild C, Pollak A, Pot B, Breiteneder H, et al. Modulation of allergic immune responses by mucosal application of recombinant lactic acid bacteria producing the major birch pollen allergen Bet v 1. Allergy. 2006 Jul;61(7):812-9.
37. Morello E, Bermudez-Humaran LG, Llull D, Sole V, Miraglio N, Langella P, et al. Lactococcus lactis, an efficient cell factory for recombinant protein production and secretion. J Mol Microbiol Biotechnol. 2008 Oct;14(1-3):48-58.
38. van Asseldonk M, Rutten G, Oteman M, Siezen RJ, de Vos WM, Simons G. Cloning of usp45, a gene encoding a secreted protein from Lactococcus lactis subsp. lactis MG1363. Gene. 1990 Oct;95(1):155-60.
39. van Asseldonk M, de Vos WM, Simons G. Functional analysis of the Lactococcus lactis usp45 secretion signal in the secretion of a homologous proteinase and a heterologous alpha-amylase. Mol Gen Genet. 1993 Sep;240(3):428-34.
40. Gasson MJ. Plasmid complements of Streptococcus lactis NCDO 712 and other lactic streptococci after protoplast-induced curing. J Bacteriol. 1983 Apr;154(1):1-9.
41. Kimoto H, Kurisaki J, Tsuji NM, Ohmomo S, Okamoto T. Lactococci as probiotic strains: adhesion to human enterocyte-like Caco-2 cells and tolerance to low pH and bile. Lett Appl Microbiol. 1999 Nov;29(5):313-6.
42. Klijn N, Weerkamp AH, de Vos WM. Genetic marking of Lactococcus lactis shows its survival in the human gastrointestinal tract. Appl Environ Microbiol. 1995 Jul;61(7):2771-4.
43. Norton PM, Brown HW, Le Page RW. The immune response to Lactococcus lactis: Implications for its use as a vaccine delivery vehicle. FEMS Microbiol Lett. 1994 Jul;120(3):249-56.
44. Todorov SD, Botes M, Danova ST, Dicks LM. Probiotic properties of Lactococcus lactis ssp. lactis HV219, isolated from human vaginal secretions. J Appl Microbiol. 2007 Sep;103(3):629-39.
45. Tuomola EM, Salminen SJ. Adhesion of some probiotic and dairy Lactobacillus strains to Caco-2 cell cultures. Int J Food Microbiol. 1998 May;41(1):45-51.
46. Gopal PK, Prasad J, Smart J, Gill HS. In vitro adherence properties of Lactobacillus rhamnosus DR20 and Bifidobacterium lactis DR10 strains and their antagonistic activity against an enterotoxigenic Escherichia coli. Int J Food Microbiol. 2001 Aug;67(3):207-16.47. Mäyrä-Mäkinen A, Manninen M, Gyllenberg H. The adherence of lactic acidbacteria to the columnar epithelial cells of pigs and calves. J Appl Bacteriol. 1983 Oct;55(2):241-5.48. Avall-Jaaskelainen S, Lindholm A, Palva A. Surface display of the receptor-binding region of the Lactobacillus brevis S-layer protein in Lactococcus lactis provides nonadhesive lactococci with the ability to adhere to intestinal epithelial cells. Appl Environ Microbiol. 2003 Apr;69(4):2230-6.
49. Vinderola CG, Medici M, Perdigon G. Relationship between interaction sites in the gut, hydrophobicity, mucosal immunomodulating capacities and cell wall protein profiles in indigenous and exogenous bacteria. J Appl Microbiol. 2004 Feb;96(2):230-43.