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# Subcutaneous Immunization with Recombinant *Lactococcus lactis* Expressing F1S1 Fusion Protein Induces Systemic and Mucosal Immune Responses in BALB/C Mice

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## Abstract

***Background:*** Lactic acid bacteria such as *Lactococcus (L.) lactis* are powerful tools that can function as live delivery vectors and heterologous protein expression hosts in development of novel vaccines. Pertussis toxin (PT) and filamentous hemagglutinin (FHA) are important virulence factors of *Bordetella* (*B.*) *pertussis* and constitute the major components of commercially available acellular pertussis (aP) vaccines. The purpose of the present study was to express F1S1 fusion protein, consisted of the N-terminal region of S1 subunit from PT and FHA type 1 immunodominant domain by *L. lactis* and to evaluate its immunogenicity.
***Methods:*** The fusion gene composed of sequences encoding the F1S1 and the signal peptide of usp45 fragments (SECF1S1) was codon optimized for protein production in *L. lactis* and was synthesized and inserted in-frame inside pNZ8149 plasmid. The resulting pNZ8149-SECF1S1 construct was introduced by electroporation into *L. lactis* cells (LL-F1S1). BALB/c mice were subcutaneously immunized with LL-F1S1 or commercial DTaP vaccine. The immune responses were investigated.
***Results:*** The LL-F1S1-immunized mice produced significant levels of specific IFN-g compared to their respective controls and DTaP-immunized mice. The F1S1- specific IgG antibody response was lower in LL-F1S1-immunized mice while the IgG2a/IgG1 ratio was higher in this group compared to the DTaP-immunized mice. Moreover, anti-F1S1 IgA antibodies were only detected in the lung homogenates of the LL-F1S1-immunized mice, suggesting the induction of a mucosal immune response.
***Conclusions:*** These results indicate the feasibility of expression of F1S1 fusion protein in *L. lactis.* This recombinant bacterium could induce mucosal and Th1-type systemic immune responses following subcutaneous administration.

**Keywords:** *Bordetella pertussis,* FHA, *Lactococcus lactis,* Pertussis toxin.

## Introduction

Studies on expression of heterologous proteins in nonpathogenic lactic acid bacteria (LAB) during the last two decades have shown their potency as interesting expression systems and delivery vehicles. LAB are used in the food industry for preparation of fermented products; hence, are classified as GRAS (generally regarded as safe) organisms (1). Lactococcus (L.) lactis is the best-known LAB and has been extensively manipulated for heterologous protein production (2-9). One of the most successful tools for regulated gene expression in L. lactis is Nisin-controlled gene expression system (NICE). Nisin which is a bacteriocin produced by L. lactis and a natural preservative in the food industry, is the inducer of protein expression in this system (10). These particular features of L. lactis make it an

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interesting host for the production of recombinant proteins for very different purposes. In addition, L. lactis exhibits immunomodulatory properties and has been used to inhibit the allergic responses in mice (3, 8) Moreover, the intrinsic Th1 adjuvant activity of L. lactis has been demonstrated in several studies (2, 3).

Pertussis (also known as whooping cough), is responsible for persistent respiratory infections in adults and remains one of the world's leading causes of vaccine-preventable deaths in susceptible infants. Despite the relative success of whole cell pertussis (wP) vaccines in mid-twentieth century, local and systemic reactogenicity cases incited a shift from wP to the more purified acellular (aP) versions, in high-income industrialized countries within recent decades (10). However, the high cost of purification of major antigens is a drawback, particularly for their widespread usage in the developing countries. Moreover, aP vaccines elicit Th2 type immune response which is not the optimum profile of the immune responses required for protection against the infection (11, 12). Among the several virulence factors of the bacterium, pertussis toxin and filamentous haemagglutinin (FHA) are considered to be very important and are components of almost all commercially available aP vaccines. PT is composed of five subunits, termed as S1 to S5; among them, S1 is the most immunogenic part (13, 14). This subunit and the 180 N-terminal amino acid residues of it have been reported to induce protective immune responses against pertussis (15, 16). FHA consists of two immunodominant domains, named type 1 and type 2, corresponding to the carboxyl and amino termini of the protein, respectively. The type 1 immunodominant domain is the most immunogenic portion and induces systemic and mucosal antibody responses in humans and mice, following the infection (17, 18).

We have previously used L. lactis for expression of a fusion protein, consisted of the N-terminal region of S1 subunit from PT and FHA type 1 immunodominant domain (LL. F1S1) and have evaluated its immunogenicity (19). In the present study, the systemic and mucosal immunogenicity of LL-S1F1 was assessed using BALB/c mice.

## Materials and methods

### *Constructions of the F1S1-expressing L. lactis*

The sequence encoding 456 amino acid residues of type I immunodominant domain of FHA (NCBI GenBank accession number X52156.1) was linked via a flexible linker (i.e. Pro-Gln-Asp-Pro-Pro) to the sequence encoding the N-terminal 180 amino acid residues of the mutant S1 (PT-9K/129G) subunit (NCBI GenBank accession number AJ920066.1), to construct F1S1 gene fragment. For the extracellular expression, the N-terminus of F1S1 was fused to SPUsp45 which is the signal peptide of Usp45, the major extracellular secretary protein in *L. lactis* subspecies cremoris MG1363 (NCBI GenBank accession number: EU382094.1)(20). The nucleotide sequence was optimized according to the codon bias of *L. lactis* using <http://www.jcat.de>/ website and was synthesized by GeneCust (Dudelange, Luxembourg).The synthesized fragment (SECF1S1) was inserted into pNZ8149 expression vector and the construct (i.e. to pNZ8149SEC-F1S1, Fig. 1) was transformed into *L. lactis* NZ3900 as previously described (19).

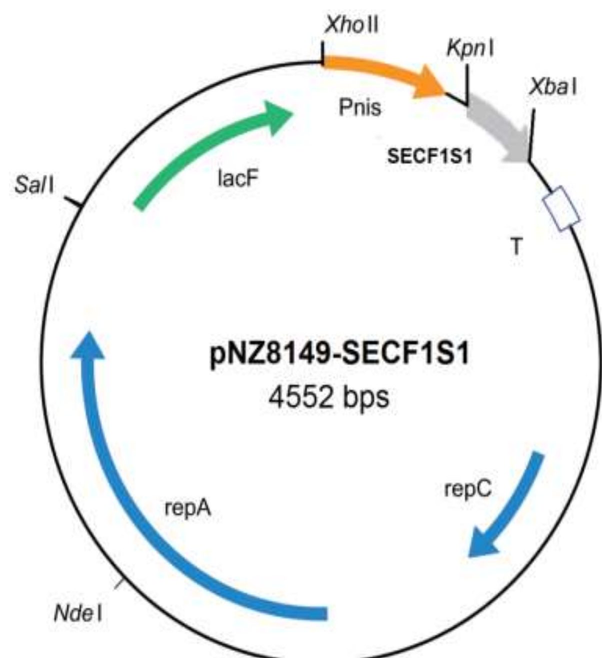



**Fig. 1** Schematic representation of *L. lactis* expression vector pNZ8149-SECF1S1. The pNZ8149-SECF1S1 vector carries the lacF: food-grade selection marker for growth on lactose; Pnis: nisin-inducible promoter; T: transcriptional terminator; repA and repC: replication elements; nisA.

### *Preparations of live bacteria inocula for immunization*

LL-F1S1, *as well as L. lactis* harboring a non-expressing pNZ8149 plasmid (LL; used as a negative control) were grown in LM17 broth medium (Merck, Germany), supplemented with 0.5% lactose (LM17) at 30°C without shaking. Cells were grown until optical density ($OD_{600}$) of 0.5 and then were inducted with 20 ng/mL of nisin (Sigma) for 4 h. The cellular pellets were harvested by centrifugation (5,000 x *g* at 4°C, 15 min) and were then washed twice with sterile phosphate-buffered saline (PBS). The pellets were adjusted to 2 x $10^{10}$ colony forming units (CFU)/ml in the same buffer and the inocula were immediately administered to the mice.

### *Animals and immunization procedures*

Female BALB/c mice (4–6-weeks-old) were purchased from animal facility of Production Complex of Pasteur Institute of Iran (Karaj, Iran). All animal experiments were performed in accordance with relevant institutional ethics guidelines, set by Ethical Committee of Pasteur Institute of Iran. Groups of 5 mice were subcutaneously immunized in the tail base with 2 x $10^9$ CFU of nisin-induced LL-F1S1 or LL for a total of 3 immunizations on days 0, 14 and 28. A third group of mice was immunized subcutaneously with a 1/4 of standard human dose of a commercial DTaP vaccine (containing 2 μg PT, 2 μg of FHA and 0.6 μg PRN; Boostrix, Belgium). Another group of mice were immunized with alum, using the same schedule.

### *Cytokine measurements*

Two weeks after the last immunization, the spleens were removed aseptically and the splenocytes from each individual mouse were collected and adjusted to a concentration of 2 x $10^6$/ml in RPMI 1640 medium (Sigma, Germany), supplemented with 10% fetal calf serum (FCS), 2 mM l-glutamine, 100 U/mL penicillin and 100 μg/ml streptomycin. The Cells were then restimulated with F1S1 (final concentration: 1 μg/ml) for 72 h at 37°C in a humidified 5% $CO_2$ incubator. Thereafter, the supernatants were collected and were assayed for the quantity of IFN-γ and IL-4 by commercially available ELISA kits (eBioscience, USA), according to the manufacturer's instructions.

### *F1S1-specific antibodies*

Two weeks after the last immunization, mice were bled for sera preparation. The lungs were excised from each mouse, washed with PBS and homogenized into 0.5 ml of cold PBS. The resulting suspensions were then centrifuged (5,000 x *g*, 4°C, 15 min) and the clarified supernatants were collected. The fresh fecal pellets (100 mg) from each mouse were suspended in 1 ml PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma, USA) and 1% BSA, and were incubated for 16 h at 4°C. The tubes were then centrifuged (10,000 x *g*, 4°C, 10 min) and the supernatants were used for detection of secretory IgA (sIgA). ELISA was used to determine the levels of anti-F1S1 specific IgG, IgG1 and IgG2a in serum of each individual mouse, using plates coated with purified F1S1. Similarly, the presence of IgA anti-F1S1 antibodies in the lung extracts of each individual mouse and in the fecal extracts of the different groups of mice were determined, using the methods described previously (19). Recombinant F1S1 had been produced using C-terminally histidine-tagged expression vector pET21a (Novagen, USA) in *E. coli* BL21 (DE3), as previously described (19).

### *Statistical analysis*

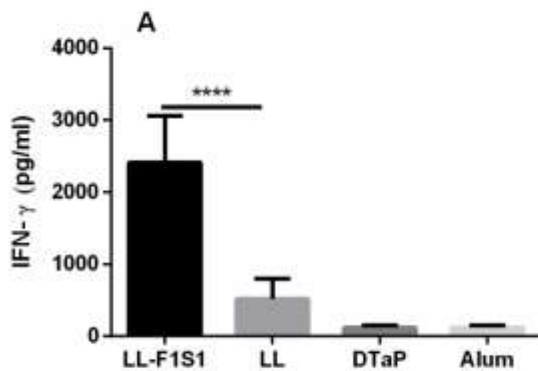
One-way analysis of variance (multiple comparisons Tukey's post-hoc test) was performed using GraphPad Prism 6.0 for Windows to detect statistical differences (GraphPad Software Inc, USA). *P*-values < 0.05 were considered to be statistically significant.

## Results

### *Cytokine responses*

Two weeks after the last immunization, mice were euthanized and the concentration of the main Th1- (IFN-γ) and Th2- (IL-4) type cytokines upon *in vitro* stimulation of the splenocytes with F1S1 protein were analyzed by ELISA. As shown in Fig. 2A, significant quantities of F1S1-specific IFN-γ were secreted by splenocytes, isolated from the mice that were immunized with LL-F1S1, compared to those of LL control (*P* < 0.0001). In response to stimulation with F1S1 protein, the splenocytes from DTaP group did not produce significant levels of IFN-γ. However; in this group,

significant amounts of IL-4 were detected, compared to the alum group (Fig. 2B; $P < 0.05$).



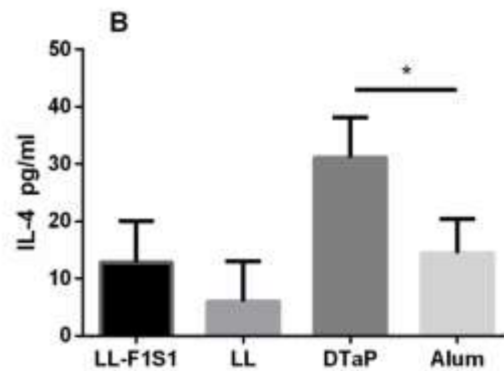




**Fig. 2.** Concentrations of IFN-γ (A) and IL-4 (B) in the supernatant of splenocytes of different groups stimulated with F1S1 measured by ELISA kits. mean+ SD (n = 5; *$P < 0.05$, **** $P < 0.0001$).

### *LL-S1F1 -specific antibody responses*

Two weeks after the last immunization, antigen-specific antibody responses against LL-F1S1 in the sera were analyzed and compared with those raised in the control groups. In LL-F1S1 group, very weak F1S1-specific IgG antibody response was induced; whereas significantly higher levels of specific IgG antibodies in DTaP- immunized mice was detected (Fig. 3A; $P < 0.0001$). Antigen-specific humoral responses were further analyzed for the levels of both IgG1 and IgG2a antibodies. As shown in Fig. 3B and 3C, significantly higher levels of IgG1 ($P < 0.0001$) and IgG2a ($P < 0.05$) were induced in DTaP-immunized mice, compared to the alum group. No significant levels of IgG1 and IgG2a were detected in LL-F1S1 group. The IgG2a/IgG1 ratio in LL-F1S1 and DTap groups were 1.36 and 0.69, respectively.

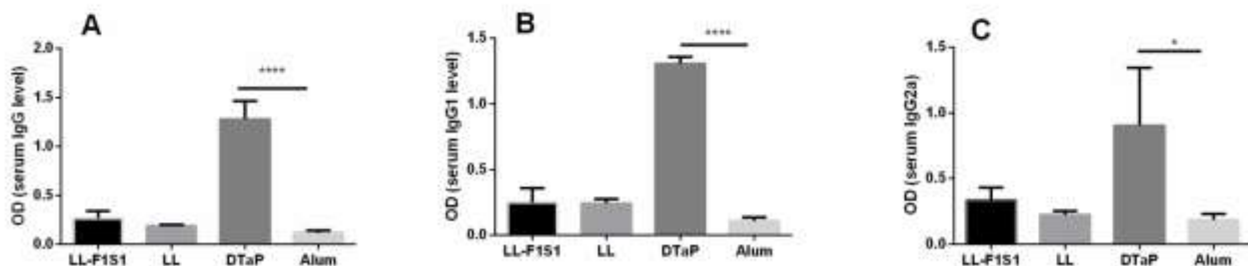



**Fig. 3.** Evaluation of anti-F1S1 IgG (A), IgG1 (B), and IgG2a (C) by ELISA. Results depict $A_{450}$ of sera diluted (1:100) and expressed as mean+ SD (n = 5; *$P < 0.05$, ****$P < 0.0001$).

We also evaluated whether immunization with LL-F1S1 was capable of inducing mucosal anti-F1S1 antibody responses. For this purpose, F1S1-specific IgA in the lung homogenates were determined 2 weeks after the last immunization. As shown in Fig. 4A, the levels of anti-F1S1 IgA were significantly higher in the lung homogenates of LL-F1S1 group, compared to LL group ($P < 0.001$). No significant levels of anti-F1S1 IgA were detected in the lung homogenates of DTaP group (Fig. 4A). In addition, the effects of the immunization in production of F1S1-specific fecal IgA were evaluated using fresh fecal pellets from the immunized mice. The mice immunized with LL-F1S1 showed significantly elevated levels of F1S1-specific fecal IgA response ($P < 0.05$), compared to the LL group. There was not any significant amount of IgA in fecal extracts of DTaP group (Fig. 4B).

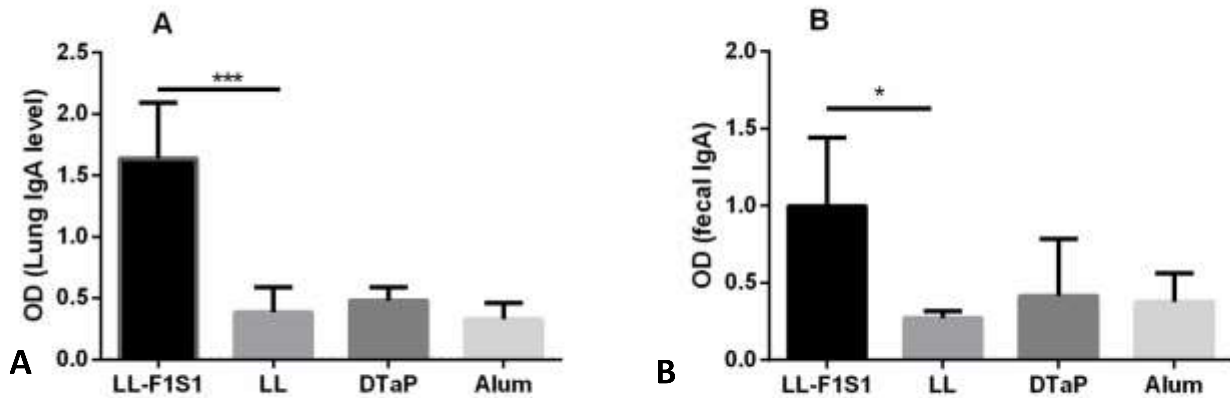



**Fig. 4.** Evaluation of anti-F1S1 IgA (A) in the lung homogenates and the feces (B) from different groups by ELISA. Results depict $A_{450}$ of the lung homogenate diluted 1:50 and the fecal extracts (100 mg/ml) as mean+ SD (n = 5; *$P < 0.05$, ***$P < 0.001$).

## Discussion

The reemergence of pertussis has motivated efforts to develop improved pertussis vaccines. Presently, the commercially wP vaccines suffer from undesirable local and systemic side-effects. Although aP vaccines are safer, their high costs of production and supply chains limit their worldwide application. Meanwhile, the replacement of natural proteins of *B. pertussis* with the pure recombinant ones in conventional prokaryotic expression systems will reduce the cost of production; however the pure proteins are less immunogenic and usually require powerful adjuvants to induce strong humoral and cellular immune responses (10). Furthermore, proteins which are produced in Gram-negative bacteria are contaminated with LPS which should be removed through a costly depyrogenation process before their parenteral administration (21).

Recent studies on expression of heterologous proteins in LAB such as *L. lactis* have shown their potency as versatile expression systems and delivery vehicles, capable of being engineered to express various proteins in a nonpathogenic and noninvasive manner (2-9). The food-grade selection marker lacF which confers *L. lactis* the ability to grow on media containing lactose as a carbon source (instead of the traditionally-used screening methods by antibiotic resistance genes) overcomes the potential risk of antibiotic-resistance in this system (22, 23). Another advantage of *L. lactis* is that the vector itself elicits weak immune responses; hence the major immune responses are directed against the expressed heterologous antigens (24).

In the present study, a previously-constructed recombinant *L. lactis* strain which could express FIS1 fusion protein, containing the most immunogenic parts of PT and FHA proteins from *B. pertussis* was used for subcutaneous immunization of BALB/c mice and the induced immune responses were evaluated and compared with the data from mice which were immunized with a commercial DTaP vaccine. Our results indicated that the administration of LL-F1S1 induced a Th1-type response. Similar to the natural pertussis infection, wP vaccine preferentially induces Th1 responses that favors cell-mediated immunity and is associated with the protection. In contrast, aP vaccine induces Th2 responses which do not appear to be as effective as the Th1 responses in clearance of *B. pertussis* from the respiratory tract (11, 12, 25). In comparison with DTaP-immunized mice, the highest levels of specific IFN-γ secretion were detected for the mice immunized with LL-F1S1 in our study. However, consistent with another study, no significant levels of F1S1-specific IFN-γ secretion were detected in mice that were subcutaneously immunized with a commercial DTaP vaccine (26).

*L. lactis* has generally been used for mucosal immunization; however, parenteral immunizations have also been practiced with different results. For instance, LACK and A2 antigens from *Leishmania,* C-repeat region (CRR) of M protein from *Streptococcus pyogenes,* fragment C of the tetanus

toxin and major birch pollen allergen (Bet v1) have been expressed in *L. lactis* and administered parentally (6-8, 27, 28). Subcutaneous immunization with recombinant *L. lactis* expressing LACK or A2 have been reported to induce high levels of antigen-specific serum antibodies (6, 7); however in contrast to our study, the responses in both studies were predominantly a Th2-type response, characterized by higher levels of antigen-specific IgG1 titers, compared to the levels of IgG2a. We observed that the F1S1-specific IgG antibody response in mice immunized with LL-F1S1 was lower than the response obtained by the DTaP-immunized mice; however, the ratio of IgG2a/IgG1 was higher in the former group which confirms the induction of a Th1-type immune response against F1S1 antigen, delivered by *L. lactis*. This was in agreement with another study in which *L. lactis* expressing Bet v1 induced significantly lower IgG1/IgG2a ratios, compared to purified Bet v1, in subcutaneous immunization (8). Moreover, the IL-5/IFN-γ ratio was lower in mice immunized with recombinant *L. lactis,* compared to the mice which had been immunized with the protein, indicating a shift toward Th1-like immune responses.

Considering that IgA is the key antibody of the mucosal immune response, and according to the important role of secretory IgA in controlling of *B. pertussis* infection (3, 29-31), we investigated the presence of IgA in lung extracts of the immunized mice. The results showed that in LL-F1S1-immunized mice, significant levels of IgA could be detected in the lung extracts while consistent with other studies (32), DTaP-immunized mice did not stimulate any mucosal immune responses (18). The induction of IgA response in the mucosa was in contrast to Mannam et al. (28) who did not find secretory IgA after subcutaneous immunization with recombinant *L. lactis* that expressed CRR. It appears that the difference in the immune responses induced by recombinant *L. lactis* expressing different proteins could be attributed to factors such as the route of administration, the nature of the proteins themselves and the differences in the proteins expression yields.

Considering the importance of the route of vaccination in determination of the immune responses, as well as the perceived practical advantages of mucosal delivery of vaccine antigens, full understanding of the potential benefits of *Lactococcus*-based vaccines such as LL-F1S1 merits further investigation. As an alternative to the commercially available whole cell- or toxin-based vaccines, LL-F1S1 which does not require antigen purification can serve as a basis for the development of a new generation of safe and inexpensive prophylactic measures against pertussis.

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