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Mucosal and systemic immune responses elicited by recombinant *Lactococcus lactis* expressing a fusion protein composed of pertussis toxin and filamentous hemagglutinin from *Bordetella pertussis*☆

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ABSTRACT

We constructed a food-grade expression system harboring a F1S1 fusion protein of *Bordetella pertussis* to be produced in *Lactococcus lactis* NZ3900 as a new oral vaccine model against whooping cough, caused by *B. pertussis*. F1S1 was composed of N-terminally truncated S1 subunit of pertussis toxin and type I immunodominant domain of filamentous hemagglutinin which are both known as protective immunogens against pertussis. The recombinant L. *lactis* was administered via oral or intranasal routes to BALB/c mice and the related specific systemic and mucosal immune responses were then evaluated. The results indicated significantly higher levels of specific IgA in the lung extracts and IgG in sera of mucosally-immunized mice, compared to their controls. It was revealed that higher levels of IgG2a, compared to IgG1, were produced in all mucosally-immunized mice. Moreover, immunized mice developed Th1 responses with high levels of IFN-γ production by the spleen cells. These findings provide evidence for *L. lactis* to be used as a suitable vehicle for expression and delivery of F1S1 fusion protein to mucosa and induction of appropriate systemic and mucosal immune responses against pertussis.

1. Introduction

Bordetella pertussis is the primary causative agent of pertussis or whooping cough, a severe disease in infants, young children and even in adolescents and adults [1]. The disease is initiated by adherence of the bacterium to ciliated respiratory epithelium of the upper respiratory tract. Several virulence factors including pertussis toxin (PT), adenylate cyclase toxin, dermonecrotic toxin, filamentous hemagglutinin (FHA) and fimbriae are involved in *B. pertussis* pathogenesis [2,3]. Whole cell pertussis (wP) vaccine which has been used for routine childhood vaccination confers protection against the disease and has significantly reduced the number of cases, as well as associated morbidity and mortality since its introduction in the 1950s. However, due to their high reactogenicity, wP vaccines were replaced in some countries by acellular pertussis (aP) vaccines which contain a few defined and purified antigens with reduced amounts of lipopolysaccharide [4]. The detoxified pertussis toxin (PT) and filamentous hemagglutinin (FHA) are the two main components of the commercially available aP vaccines. PT is one of the major virulence factors of *B. pertussis* and is composed of five subunits, termed as S1 to S5; among which S1 is the most immunogenic and protective part of the toxin [5]. The other major component of aP vaccines is FHA which mediates the bacterial attachment and colonization of the respiratory epithelia. This protein is a potent immunogen that induces systemic and mucosal antibody responses in humans and mice, following the infection [2,6]. The high cost of purification of these major antigens is a drawback, particularly for their widespread use in the developing countries. Moreover, aP vaccines elicit a Th2 type immune response which is not the most effective immune response required for protection against the infection [7,8].

B. pertussis enters and colonizes the body through the respiratory mucosa which is the first line of the host defense against this pathogenic organism. Several studies have suggested the importance of local secretory antibodies (IgA) and Th1-type immune responses during anti-*B. pertussis* immune responses [7–9]. Mucosally-administered vaccines can induce both the systemic and the mucosal immune responses while parenteral vaccines mainly activate the systemic immune response. Due to the fact that mucosally-administered soluble antigens are in general poorly immunogenic, several approaches, such as their encapsulation or expression in attenuated bacterial hosts, have been used to improve their immunogenicity. However, the attenuated bacterial hosts may still

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Table 1
Primers used for PCR amplification and cloning of constructs.

Primer	(5′–3′)	Restriction enzyme
esfs-F (forward)	GACT**GGTA**CCATGAAAAAAAAAATCATCTCAGCTATCCTTATG	*Kpn*I
esfs-R (reverse)	ACAG**TCTAGA**TTATGAAGTGTATGGGTTTGGGTTAGCAC	*Xba*I
f1s1-F (forward)	ACGT**GGATCC**TCACTTTACGCTGAACACGA	*BamH*I
f1s1-R (reverse)	ACGT**GAGCTC**AC<u>T</u>GAAGTGTATGGGTTTGG	*Sac*I

Restriction sites in each primer are in boldface. The underlined bases A and C in primer f1s1R were included to maintain an in-frame reading of F1S1 construct within the His-tagged expression vector.

be able to cause a limited infection in infants as well as the aged and immunocompromised people [10]. An attractive alternative to overcome this problem is the development of a new vaccine in association with lactic acid bacteria (LAB) which are safe mucosal delivery vehicles [11]. LAB are nonpathogenic and noninvasive Gram-positive organisms that produce lactic acid from carbohydrate fermentation. They have been widely consumed for centuries by humans in fermented foods; hence, are classified as GRAS (generally regarded as safe) organisms. *Lactococcus lactis* is the best-known LAB that has been extensively manipulated, and in particular has been used for heterologous protein expression and delivery vehicle via mucosal routes, reviewed in Refs. [12–14].

For development of a simplified, cost-effective and well-defined vaccine candidate against *B. pertussis*, we constructed a recombinant *L. lactis* to express and secrete a fusion protein composed of N-terminally truncated S1 subunit of pertussis toxin and type I immunodominant domain of filamentous hemagglutinin. The mentioned parts of S1 and FHA were selected because they are the most immunogenic and protective parts of the respective proteins [5,6]. The required changes to create a biologically-inactive mutant S1 (PT-9K/129G) were also implemented [15]. The recombinant L. *lactis* was used for immunization by oral and intranasal (i.n.) routes and the related immune responses were evaluated.

2. Materials and methods

2.1. Bacterial strains, plasmids, growth condition and animals

L. lactis NZ3900 (LL) and *L. lactis* food-grade expression vector pNZ8149 were from NIZO food research (Kernhemseweg, Netherlands). The lactobacilli were grown in M17 medium (Merck, Germany), supplemented with 0.5% glucose (GM17) or 0.5% lactose (LM17) at 30 °C without shaking. *E. coli* BL21 (DE3; Novagen, USA) was grown in Luria-Bertani (LB) medium at 37 °C with shaking at 220 rpm. Agar (15 g/l) was added to the media when agar plates were required. LB medium was supplemented with 100 μg/ml ampicillin as required. Female BALB/c mice (4–6 weeks old) were purchased from the animal facility of Production Complex of Pasteur Institute of Iran (Karaj, Iran). All animal experiments were performed according to protocols approved by Ethical Committee of Pasteur Institute of Iran.

2.2. Construction of transformant NZ3900/pNZ- SECF1S1 (LL-F1S1)

F1S1 gene fragment was composed of a sequence encoding 456 amino acid residues of type I immunodominant domain of FHA (residues 1656–2111) that was linked in-frame to a sequence encoding the N-terminal 180 amino acid residues of the mutant S1 (PT-9K/129G) subunit (NCBI GenBank accession numbers X52156.1 and AJ920066.1, respectively), via a flexible Pro-Gln-Asp-Pro-Pro linker. Extracellular expression of F1S1 was facilitated by its N-terminus fusion to the SPUsp45 which is the signal peptide of Usp45, the major extracellular secretary protein in *L. lactis* subsp. cremoris MG1363 (NCBI GenBank accession number: EU382094.1). Restriction enzyme sites for *Kpn*I and *Xba*I were added to the 5′ and 3′ ends of the designed fusion gene, accordingly. The nucleotide sequence was optimized according to the codon bias of *L. lactis* (http://www.jcat.de/) and was synthesized by a service provider (GeneCust, Dudelange, Luxembourg). The synthesized fragment (SECF1S1) was digested with *Kpn*I and *Xba*I restriction endonucleases (Thermo Fisher Scientific) and was inserted into the corresponding sites of the expression vector pNZ8149, giving rise to pNZ-SECF1S1. A mixture of 2 μl of pNZ- SECF1S1 and 40 μl of NZ3900 competent cells were mixed gently and kept on ice for 5 min. The electroporation was carried out using a Gene Pulsar II Electroporator (Bio-Rad, Richmond, CA, USA) at field strength of 2.00 kV/cm, capacitance of 25 μF and resistance of 200 Ω, using a 0.2 cm gap electroporation cuvette. Immediately after a single electric pulse, the cells were resuspended in GM17 broth, containing 20 mM $MgCl_2$ and 2 mM $CaCl_2$ for 5 min at 4 °C, then incubated for 2 h at 30 °C without any agitation to allow recovery. The cells were then plated onto Elliker agar plates (containing 20 g/l Tryptone, 5 g/l yeast extract, 4 g/l sodium chloride, 1.5 g/l sodium acetate, 0.5 g/l L(+) ascorbic acid, 15 g/l agar, 0.5% lactose and 0.004% Bromocresol purple) without any antibiotics, incubated for 1–2 days at 30 °C. The yellow colonies were selected for identification by PCR (primers sefs-F and sefs-R; Table 1), restriction enzyme analysis (*Kpn*I and *Xba*I) and DNA sequencing to confirm lack of mutations and correct ORF of the recombinant construct. The resulting LL-F1S1 cells were induced by 20 ng/ml of nisin for 4 h.

2.3. Expression evaluations by Western blot analysis

The expression and secretion of F1S1 by induced LL-F1S1 was investigated by Western blot analysis. To prepare extracts, cultures of nisin-induced LL-F1S1 were harvested by centrifugation at 5000 rpm for 15 min at 4 °C. The collected pellets and the supernatant fractions were treated separately as follows. The cell pellets were washed twice with sterile PBS and resuspended in SDS-PAGE sample buffer. The supernatant fractions were concentrated by cold trichloroacetic acid (TCA)/acetone precipitation method. In brief, the protein was precipitated by the addition of TCA (100% w/v) to a final concentration of 16%. The mixture was vortexed and placed for 15 min on ice, then centrifuged for 15 min at 10000 rpm at 4 °C. The pellet of protein was washed twice with cold acetone, dried and resuspended in SDS-PAGE sample buffer. The pellet and the supernatant were electrophoresed on 10% acrylamide gel and transferred onto PVDF membrane. The membrane was blotted with a polyclonal antiserum raised against DTaP vaccine (Boostrix, GSK, Belgium) in mouse. Horseradish peroxidase-labeled goat anti-Mouse IgG antibody (Sigma, Germany) was used as the secondary antibody.

2.4. Cloning, expression and purification of the recombinant F1S1

The F1S1 gene was amplified with f1s1-F and f1s1-R primers (Table 1). The resulting amplicon was double-digested with *BamH*I and *Sac*I (Thermo Fisher Scientific); and cloned in-frame into the *BamH*I and *Sac*I sites of a C-terminally histidine-tagged pET21a expression vector (Novagen, USA). The recombinant plasmid was transformed into *E. coli* BL21 (DE3) for protein expression by IPTG induction. The recombinant F1S1 protein was purified by Ni-NTA resin (Qiagen, Germany), according to the manufacturer's instruction.

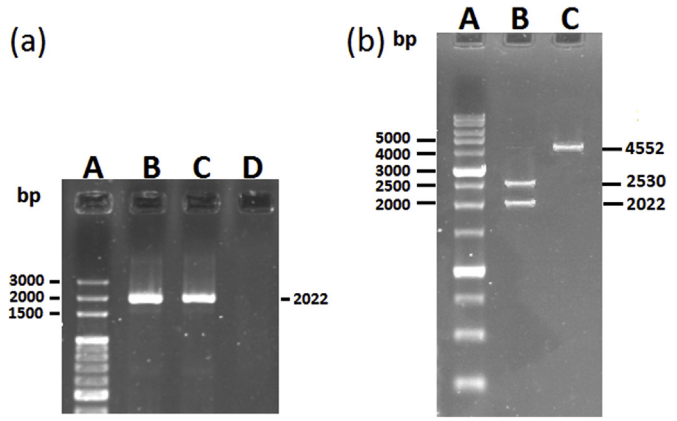

Fig. 1. Identification of the recombinant plasmid pNZ8149-SECF1S1 by PCR and restriction enzyme analysis (a) PCR amplification of F1S1gene. Lane A: DNA ladder; Lanes B and C: PCR products of the F1S1 gene amplification D: No template control. (b) Restriction analysis of the recombinant plasmid pNZ8149-SECF1S1. Lane A: DNA ladder; Lane B: pNZ8149-SECF1S1 double-digested with *Kpn*I and *Xba*I; C: pNZ8149-SECF1S1 digested with *Kpn*I.

2.5. Immunization protocol

Female BALB/c mice were used at 5–6 weeks of age. The approval for the experiments was confirmed by ethical committee of Pasteur Institute of Iran. All mice were fed a conventional balanced diet *ad libitum* during experiments. The bacteria were suspended in sterile PBS and were administered at a dose of 10^8 cells/day/mouse. For oral immunization, two groups of mice (6 mice in each group) were immunized by intra-gastric gavage of LL-F1S1 or LL using a standard gastric feeding tube on 3 consecutive days. This protocol was repeated twice in 2-week intervals.

For intranasal immunization 25 µl of inoculums were given by pipetting into each nostril on 3 consecutive days. This protocol was repeated twice in 2-week intervals.

2.6. Evaluation of antigen-specific antibody levels in serum and mucosal extracts

F1S1-specific serum IgG, IgG1, IgG2a, and lung extracts IgA antibodies were detected in each individual mouse by ELISA. For estimation of antigen-specific IgA antibodies in each group, freshly voided feces (2–4 pieces) from each mouse inside a group were collected and pooled on Days 15, 30, and 45. One hundred mg of pooled feces of each group were then added to 500 µl PBS containing complete protease inhibitor (Boehringer, Mannheim, Germany) and incubated for 16 h at 4 °C. The tubes were vortexed and centrifuged (5000 × rpm, 4 °C, 10 min). The supernatants were then collected and stored at −20 °C.

Serum samples and lung extracts of the mice were prepared two weeks after the last booster. The lungs were harvested and washed with PBS, grinded gently into 0.5 ml cold PBS individually and were then passed through steel mesh filters. The resulting suspension was clarified by centrifugation at 5000 × rpm, 4 °C for 15 min and the supernatants were stored at −20 °C until use.

In brief, ELISA plates (Greiner, Germany) were coated with 280 ng F1S1. Samples to be assayed including 100 µl of the sera (1:100) or the lung or the fecal extracts (1:50) were added to the wells. Goat anti-mouse IgG, rabbit anti-mouse IgG1, rabbit anti-mouse IgG2a, or goat anti-mouse IgA antibodies (Sigma, Germany), all conjugated to horseradish peroxidase, were used as secondary antibodies. The plates were developed with 3, 3′, 5, 5′-Tetramethylbenzidine (TMB; Sigma, Germany) and were read at 450 nm.

2.7. Cytokine measurements

Spleen cells were prepared by gentle flushing out the spleens with RPMI 1640 medium (Sigma, Germany). Erythrocytes were lysed for 5 min in ACK lysis buffer (0.15 M NH_4Cl 10 mM $KHCO_3$, and 0.1 mM EDTA) and the cells were washed two times in PBS. Single-cell suspensions from the splenocytes of individual experimental and control mice were prepared and adjusted to a concentration of 2×10^6/ml. The cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS; Gibco, Germany), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. The splenocytes were cultured at 37 °C in a humidified 5% CO_2 incubator in the presence or absence of purified recombinant F1S1 (final concentration: 1 µg/ml) and supernatants were collected after 72 h. The assessments of IFN-γ and IL-4 levels in cell culture supernatants were determined by quantitative ELISA using commercial kits from eBioscience, USA.

2.8. Statistical analysis

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

3. Results

3.1. Verification of LL-F1S1

The food-grade expression vector pNZ-SECF1S1 harboring the nisin-inducible promoter (PnisA) and Usp45 signal peptide was constructed. To verify successful cloning events, direct PCR amplification (Colony-touch PCR) of F1S1 gene (2022 bp) was conducted using the recombinant bacteria (yellow colonies) as templates. The amplicons were subjected to 1% agarose gel electrophoresis which revealed the presence of F1S1 fragment in the construct (Fig. 1a). The integrity of pNZ-SECF1S1 expression vector was further verified by restriction digestion analysis using *Kpn*I and *Xba*I restriction enzymes where expected fragments (corresponding to 2530 and 2022 bp) were obtained (Fig. 1b). The correct ORF insertion of the F1S1 fragment in the vector and the absence of alterations in the coding nucleotides were then verified by nucleotide sequencing (data not shown).

3.2. Expression and detection of F1S1 fusion protein

LL-F1S1 and its culture supernatant were used for detection of nisin-controlled expression and efficient secretion of F1S1 fusion protein, 4 h after the induction. Western blotting, using sera from mice immunized with DTaP vaccine revealed the presence of a F1S1-specific single band at ~73 kDa in the cell fraction of LL-F1S1 (Fig. 2, lanes B-D), corresponding to F1S1 precursor form (preF1S1); and another F1S1-specific band which was detected in the cell-free culture supernatant fraction of recombinant *L. lactis* (Fig. 2, lanes E and F), migrated at the expected size of mature F1S1 protein (~70 kDa). The larger Mw of the detected band in the cell fraction compared to the protein in the supernatant is due to presence of the signal peptide that is removed during the secretion.

3.3. Antigen-specific antibody levels in serum and mucosal extracts

Two weeks after the last booster, sera were tested for the induction of specific antibodies against F1S1 protein. As shown in Fig. 3, significantly higher amounts of specific IgG were produced by LL-F1S1-immunized mice via oral ($P < 0.0001$) and intranasal ($P < 0.001$) routes, compared to their respective control groups. The level of IgG was comparable between the two groups of mucosally-immunized mice with LL-F1S1. Investigation of antigen-specific antibody isotypes (IgG1

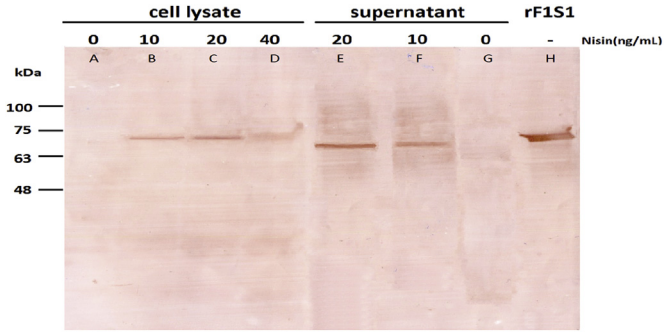

Fig. 2. Expression analysis of F1S1 by *LL*-F1S1.
Following induction with different concentrations of nisin (10, 20 and 40 ng/ml for 4 h), F1S1 protein production was confirmed by Western blot analysis using sera from mice immunized with anti-DTaP in the cell lysate (lanes A-D) and supernatant fractions (lanes E-G). Purified rF1S1 protein was used as a positive control (lane H). Cell extracts and supernatant fractions of non-induced cultures of recombinant *L. lactis* served as negative controls (lanes A and G, respectively).

and IgG2a) indicated that anti-F1S1 IgG1 in mice that were immunized intranasally with LL-F1S1 was not induced; however this group had significantly higher amounts of IgG2a, compared to the control mice which were immunized by i.n. route with LL ($P < 0.01$). In mice that were immunized orally with LL-F1S1, significantly higher amounts of IgG1 ($P < 0.01$) and IgG2a ($P < 0.0001$) were produced, compared to their counterpart controls. Moreover, mice that orally received LL-F1S1, produced significantly higher amounts of IgG1, compared to mice which received LL-F1S1, nasally ($P < 0.01$).

Two weeks after the last immunization, anti-F1S1 IgA levels were determined in the lung extracts from the different groups. Significant levels of anti-F1S1 IgA were detected in LL-F1S1-immunized groups, either by intranasal or oral ($P < 0.0001$) routes in comparison to their respective controls (Fig. 4a). No significant differences in anti-F1S1 IgA levels could be detected between the two groups of mucosally-immunized mice with LL-F1S1. Immunization via both routes induced significant levels of F1S1-specific IgA in feces, 15 days after the first immunization ($P < 0.0001$). Although, after the second booster (day 45), there were no significant changes in fecal IgA level in the i.n. group; there was a significant increase in fecal IgA the orally immunized mice between day 15 and day 45 (Fig. 4b; $P < 0.0001$).

3.4. Cytokine responses of splenocytes from the immunized mice

The concentration of secreted IL-4 and IFN-γ upon *in vitro* stimulation of the spleen cells with F1S1 protein were quantified by ELISA, two weeks after the last booster. As indicated in Fig. 5, mice immunized with LL-F1S1 by the oral route produced significant amounts of IFN-γ, compared to LL control group ($P < 0.01$). However, the highest levels of F1S1-specific IFN-γ secretion were detected for mice immunized intranasally, with LL-F1S1 ($P < 0.0001$). The difference between orally and intranasally immunized mice with LL-F1S1 was also significant ($P < 0.001$). No statistically-significant increase in IL-4 secretion was detected in none of the studied groups (data not shown).

4. Discussion

The mucosa represents the port of entry and the first line of the host defense against many pathogens including *B. pertussis* which colonizes the epithelium of the respiratory tract [16]. Stimulation of mucosal immunity is considered as an effective strategy against colonization of pathogens and prevention of their spreading into the systemic circulation. However, the parenteral route of vaccine administration confers a limited ability to induce mucosal immunity [17]. Hence, mucosal vaccination may be a more efficacious strategy for vaccine delivery against mucosal invaders. Low immunogenicity is one of the major disadvantages of protein antigens used in mucosal immunizations which has led to the development of vectors that deliver proteins to the target tissues [10]. During the last decade, several studies have confirmed *L. lactis* potential to express proteins and to induce mucosal immune responses [18]. In addition, the intrinsic Th1 adjuvant activity of *L. lactis* has been demonstrated in several studies [16,19]. All of these together make *L. lactis* a good mucosal delivery vehicle, capable of easily and directly expressing antigens in their native conformation for the low-cost oral or local administration.

In the present study, we successfully constructed a pNZ-SECF1S1 plasmid and transformed it into *L. lactis* to express and secrete F1S1 protein. It has been reported that fusion of a gene with the signal peptide of Usp45 in *L. lactis*, significantly increases the production yield of the secreted protein, probably due to recognition of the precursor by the secretion machinery, escaping from the intracellular degradation, or better translation efficiency [20]. Thus, we added the gene fragment encoding this signal peptide to the 5′ of the F1S1 gene. The fusion F1S1 protein expression with desirable immunogenicity was confirmed by Western blotting. The immunoreactivity of F1S1 protein with sera from mice which had been immunized by commercial DTaP vaccine containing native PT and FHA proteins indicated the maintenance of the immunodominant epitopes of these antigens in the recombinant fusion protein. Moreover, the immunogenicity of both parts of the fusion protein (F1 and S1) had been confirmed by immunoblotting using anti-F1S1 antiserum against *B. pertussis* strain Tohama I proteins (submitted).

Several studies suggest that both humoral and cell-mediated immunity are essential for protection against pertussis [21,22]. It has been shown that *B. pertussis* infection or immunization with wP vaccines cause primarily a Th1 response [7,8,23]. Th1 cytokines are strong activators of natural antimicrobial effector cells such as macrophages and neutrophils and are also inducers of IgG subclasses which promote opsonization, complement-fixation and bactericidal activities [22,24]. Moreover, secretory IgA has been suggested to play an important role in controlling *B. pertussis* infection [7,9,25]. On the other hand,

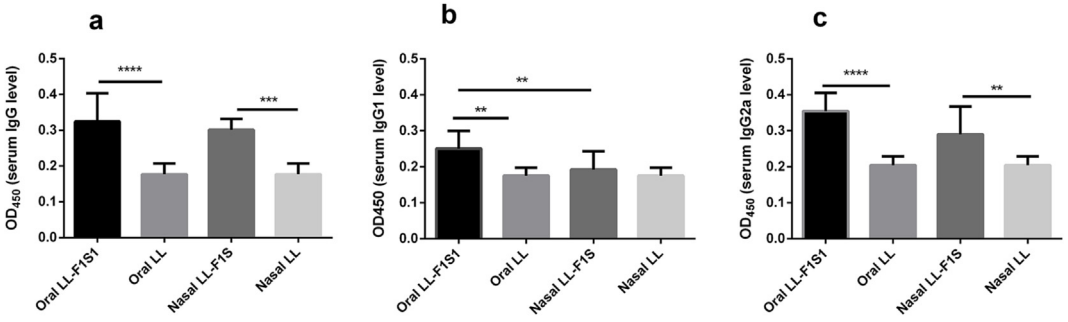

Fig. 3. Anti-F1S1 specific antibodies in sera, two weeks after the final immunization; anti- F1S1 IgG (a), IgG1 (b), IgG2a (c).Results were expressed as mean + SD (n = 5; $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$).

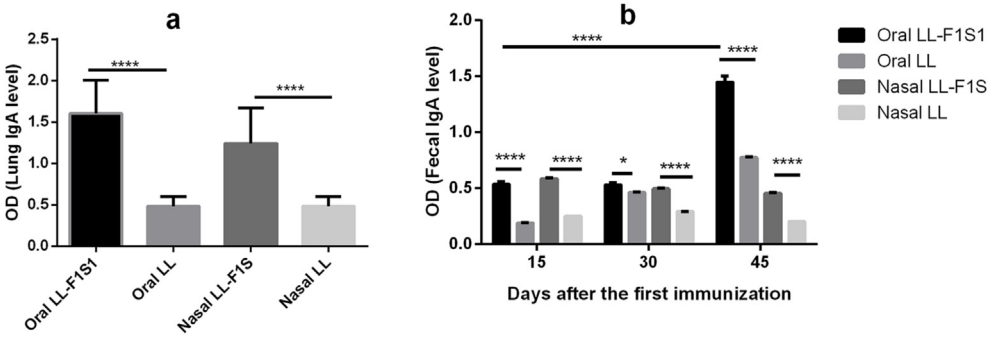

Fig. 4. Specific anti-F1S1 IgA levels. (a) Anti-F1S1 IgA levels were determined in the lung homogenates and the results were expressed as mean + SD (n = 5; ****P < 0.0001). (b) Anti-F1S1 IgA levels were determined in fecal extracts from pooled feces from 5 mice in each group and demonstrated as means + SD of ELISA duplicates (*P < 0.05, ****P < 0.0001).

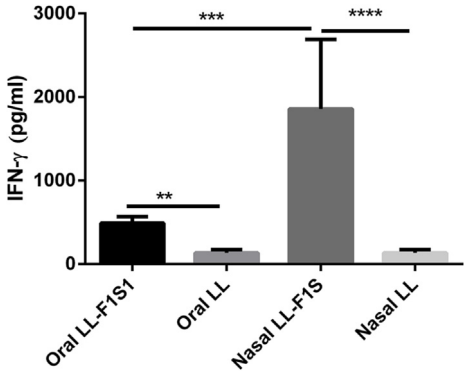

Fig. 5. Cytokine responses, two weeks after the final immunization. Mean + SD (pg/ml) of IFN-γ concentrations in the supernatants of splenocytes after stimulation with F1S1 are shown (n = 5; *P < 0.05,****P < 0.0001).

immunizations with aP vaccines which contain purified proteins of the bacterium and are mainly used in the developed countries promote a predominant Th2 response [8,26,27]. It seems that induction of strong Th1 responses and mucosal immunity should be the subject of future investigations. In an attempt to induce both mucosal and appropriate systemic immune responses, we used a live mucosal *L. lactis*-based vaccine which expressed and secreted F1S1 protein, composed of N-terminally truncated S1 subunit of PT and type I immunodominant domain of FHA from *B. pertussis*. These parts had the most potent ability to induce the immune responses. The results indicated that mucosal administration of LL-F1S1 to BALB/c mice fulfilled the proper immune responses, namely, induction of Th1 type response and IgA production at the mucosal surfaces. Two strategies for mucosal vaccination, oral and i.n., were investigated in this study. The administration of LL-F1S1 by both routes induced F1S1-specific mucosal and systemic immune responses in BALB/c mice. It was revealed that high levels of IgA in the lung extract and IgG in the sera, as well as higher level of IgG2a compared to IgG1 levels, were produced in all mucosally-immunized mice. Moreover, significant production of IFN-γ by splenocytes confirmed the induction of Th1 type response. These findings suggest that *L. lactis* is a suitable vehicle for the delivery of F1S1 fusion protein to mucosa and therefore this approach might provide a novel vaccine against pertussis.

The delivery route may influence the host immune responses. Both oral and i.n. routes have previously been used for mucosal immunization with different effectiveness. For instance, *L. lactis* transformant has been successfully used as a delivery vehicle to express tetanus toxin fragment for oral as well as i.n. inoculations against tetanus toxin challenge in a mouse model [28]. Pei et al. have reported high specific IgG induction in the sera of mice inoculated orally with *L. lactis* that expressed SARS-coronavirus nucleocapsid protein; however a very weak specific antibody could be detected after i.n. administration [29]. On the other hand, Daniel and colleagues have shown that i.n. administration of a recombinant *L. lactis* strain, secreting *Yersinia pseudotuberculosis* low-calcium response V antigen, elicited specific systemic and mucosal antibody and cellular immune responses while no immune response and no protection could be observed with oral administration [30]. They concluded that the antigen and the route of administration are important points that can influence antigen-specific immune responses, induced by *L. lactis*. The results of the present study indicated comparable levels of serum IgG and lung IgA between the orally and the intranasally immunized mice; while more IFN-γ and less IgG1 were induced in mice which received LL-F1S1 via i.n. route. Although the i.n. route seems to induce a stronger Th1 response, the priority of each route remains to be seen after challenging the immunized mice with *B. pertussis*.

Overall, we have demonstrated for the first time that *L. lactis* is a suitable vehicle for expression and delivery of F1S1 fusion protein via oral and i.n. routes. Nowadays, considerable efforts have been devoted to develop new mucosal vectors that could contribute to future vaccines development. In this regards, as an alternative to commercially available whole cell or acellular vaccines, the recombinant *L. lactis* secreting F1S1 fusion protein would be a promising host for the induction of an appropriate immune response against *B. pertussis*. In conclusion, this study, represent a step towards the further development of *L. lactis* as an environmentally safe vaccine against *B. pertussis* that can be delivered through mucosal administration.

Declarations of interest

None.

Conflicts of interest

Authors have no conflict of interest.

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