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ORIGINAL ARTICLE

Clinical and microbiological response of mice to intranasal inoculation with *Lactococcus lactis* expressing Group A *Streptococcus* antigens, to be used as an anti-streptococcal vaccine

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ABSTRACT

Protein subunit vaccines are often preferred because of their protective efficacy and safety. Lactic acid bacteria expressing heterologous antigens constitute a promising approach to vaccine development. However, their safety in terms of toxicity and bacterial clearance must be evaluated. Anti-*Streptococcus pyogenes* (*S. pyogenes*) vaccines face additional safety concerns because they may elicit autoimmune responses. The assessment of toxicity, clearance and autoimmunity of an anti-streptococcal vaccine based on *Lactococcus lactis* (*L. lactis*) expressing 10 different M protein fragments from *S. pyogenes* (*L. lactis*-Mx10) is here reported. Clearance of *L. lactis* from the oropharynges of immunocompetent mice and mice devoid of T/B lymphocytes mice was achieved without using antibiotics. The absence of autoimmune responses against human tissues was demonstrated with human brain, heart and kidney. Assessment of toxicity showed that leucocyte counts and selected serum biochemical factors were not affected in *L. lactis*-Mx10-immunized mice. In contrast, mice immunized with *L. lactis* wild type vector (*L. lactis*-WT) showed increased neutrophil and monocyte counts and altered histopathology of lymph nodes, lungs and nasal epithelium. Two days after immunization, *L. lactis*-Mx10-immunized and *L. lactis*-WT-immunized mice weighed significantly less than unimmunized mice. However, both groups of immunized mice recovered their body weights by Day 6. Our results demonstrate that *L. lactis*-WT, but not the vaccine *L. lactis*-Mx10, induces alterations in certain hematologic and histopathological variables. We consider these data a major contribution to data on *L. lactis* as a bacterial vector for vaccine delivery.

Key words anti-streptococcal vaccine, clinical response, *Lactococcus lactis*.

Despite ongoing efforts, no vaccine for preventing infections caused by *S. pyogenes* (Group A *Streptococcus)* and their complications is currently available. We are developing an intranasal bacterial-vectored vaccine based on 10 strains of *Lactococcus lactis*, each expressing a different M protein fragment from *S. pyogenes*. We have previously reported the immunogenicity and efficacy of an *L. lactis* strain expressing M9 antigen (1)

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List of Abbreviations: *L. lactis*-Mx10, *L. lactis* expressing 10 different M protein fragments from *S. pyogenes*; *L. lactis*-WT, *L. lactis* wild type vector; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; WT, wild type.

and, more recently, nine additional strains of *L. lactis* expressing nine different M protein fragments individually (M1, M2, M3, M4, M6, M12, M22, M28 and M77) and all of these fragments combined in a 10-valent vaccine, named *L. lactis*-Mx10 (2). This vaccine elicits serum and mucosal specific antibodies against most M types included in the vaccine and protects against lethal challenge with virulent *S. pyogenes* M28 in mice.

Protein subunit vaccines delivered parenterally are often preferred because of their protective efficacy and safety. Lactic acid bacteria expressing heterologous antigens constitute a promising approach to vaccine development (3–5). However, many safety concerns related to toxicity and bacterial clearance from the host have yet to be evaluated. Additionally, anti-*S. pyogenes* vaccines face additional safety concerns related to the possibility of them eliciting autoimmune responses. Regions of the M protein, other than the hypervariable one, mimic human epitopes and are responsible for autoimmune disorders (6). Identification of these regions has made it possible to design safe vaccines that exclude these harmful regions and are relatively unlikely to cross-react with human tissues (7). In spite of all these precautions, the absence of autoimmune antibodies must be demonstrated.

One of the most significant safety concerns associated with live-vectored vaccines is the fact that some commensal bacteria are able to persist in their hosts even in the presence of antibiotics (8, 9). Infections caused by *L. lactis* are classified as rare in humans (10). However, some reports have recently raised concerns about increasing consumption of organic unpasteurized dairy products (10). We aimed to demonstrate that the host can spontaneously eliminate our *L. lactis*-based vaccine.

Evaluation of toxicity is very complex and varies with the type of vaccines. In the case of our vaccine, several studies are required to investigate inflammatory reactions, effects on lymph nodes, histopathological changes in pivotal organs, and systemic toxicity on various hematological, physiological and histopathological variables, according to the guidelines provided by the World Health Organization (2005) (11). We report here the evaluation of colonization, autoimmunity and toxicity of *L. lactis*-Mx10 vaccine and of the wild type lactococcal vector (*L. lactis*-WT) in mice.

MATERIALS AND METHODS

Strains, media and culture conditions

All vaccine strains were constructed using the NICE genetic system (MoBiTech, Gottingen, Germany) according to previously published protocols (1, 2). Briefly, portions of the N-terminal domains of M proteins of 41 to 50 amino acids were PCR amplified from *S. pyogenes* of the 10 M types selected using primers with restriction enzyme sites. Epitopes responsible for autoimmune diseases are localized in the B domain and some regions of the A and C domains (12). The sequences were analyzed to verify the absence of sequences resembling human proteins and therefore likely to elicit autoreactive antibodies. To achieve surface display of the M protein, the hypervariable peptide was fused to 148 amino acids of the C-terminal membrane anchoring domain of M6 protein. The resulting fusion fragment was ligated to the linearized vector pNZ8149 (NICE System) and introduced into *L. lactis* strain NZ3900 (NICE System) through electroporation. Transformed clones were selected in M17-lactose-bromocresol purple agar plates. Selection of transformants was based on auxotrophy for lactose utilization: *lacF* gene (necessary for using lactose as sole carbon source) was deleted from *L. lactis* NZ3900; it is provided in plasmid pNZ8149 strain. *L. lactis*-WT control strain was obtained after transformation of *L. lactis* NZ3900 with the empty plasmid pNZ8149. The presence of the plasmid bearing the insert was verified through PCR. For protein expression, recombinant *L. lactis* strains were grown at 30°C in M17-lactose broth supplemented with 20 ng/mL nisin. Three hours after addition of nisin, the cultures were centrifuged and pellets sonicated. The resulting crude extracts were centrifuged and analyzed through 12.5% SDS-PAGE. All of the vaccine strains expressed a protein of the expected size.

Colonization evaluation

Groups of four to five female Balb/c and Rag-1–deficient mice (6 to 8-weeks-old) were intranasally immunized with 10^9 CFU of streptomycin-resistant *L. lactis*-WT or *L. lactis*-Mx2 on Day 0. Mx2 is composed of two streptomycin-resistant vaccine strains: M6 and M77. Spontaneous streptomycin-resistant mutants of *L. lactis*-WT, M6 and M77 vaccine strains were generated by spreading 100 μL of overnight cultures in M17-lactose agar plates supplemented with streptomycin 100 μg/mL. For preparation of *L. lactis*-WT vaccine doses, 1 mL of freshly induced culture (with 20 ng/mL nisin) was centrifuged and resuspended in 20 μL of PBS; that amount was used to immunize one mouse. For *L. lactis*-Mx2, the immunization dose was prepared by mixing 2.5 mL of each M type, centrifuged and resuspended in 100 μL of PBS; that amount was used to immunize five

mice. On Days 1, 7, 14, 28, 42 and 98, mouth washes were performed by pipetting 50 μL PBS five times up and down into the mouths of the mice and then plated on M17-lactose-bromocresol purple agar supplemented with 100 μg/mL streptomycin. On Day 98, mice were killed humanely and tissues (lungs, trachea and nose) were harvested, homogenized in PBS and plated on M17-lactose-bromocresol purple agar supplemented with streptomycin. Vaccine strains are capable of using lactose as their sole source of carbon. This generates intense yellow colonies and indicates that vaccine strains have conserved their plasmids for heterologous M protein expression. All plates were incubated for 48 hr at 30°C and then colonies were identified by MALDI-TOF MS (Bruker Daltonik, Bremen, Germany) and counted. The colonies were identified according to the database provided by the manufacturer: MALDI Biotyper library v4.0 5.627 MSP (Bruker Daltonik) using the MALDI Biotyper 3.1 software package (Bruker Daltonik) with default settings. Identification scores of $\geq$2.0 indicate species-level identification (13).

Evaluation of vaccine toxicity

All animal experiments were performed according to protocols approved by the Ethics and Biosafety Committee of the Pontifical Catholic University of Chile. Groups of four to five female BALB/c mice (6 to 8 weeks-old) were intranasally immunized with 10^9 CFU of *L. lactis*-Mx10 vaccine on Days 0, 14 and 28. For *L. lactis*-WT vaccine dose preparation, 1 mL of freshly induced culture (with 20 ng/mL nisin) was centrifuged and resuspended in 20 μL of PBS; this amount was used to immunize each mouse. For the *L. lactis*-Mx10 vaccine, each immunization dose was composed of a mixture of 10 different M types (M1 to M77) and was prepared by mixing 0.5 mL of each M type, centrifuging and resuspending in 100 μL of PBS; this amount was used

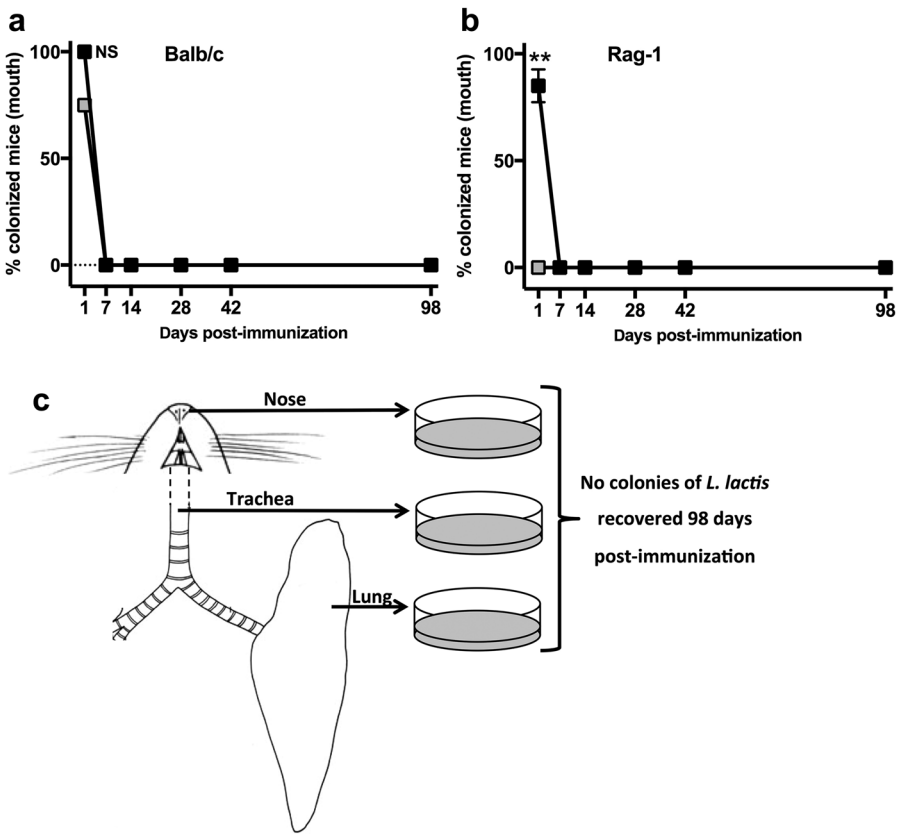

Fig. 1. Evaluation of colonization capacity of L. lactis vaccine. (a) Percentage of colonized Balb/c mice, (b) Percentage of colonized Rag-1 mice 1, 7, 14, 28, 42 and 98 days after intranasal immunization performed on day 0. (c) Diagram of sources of cultures (minced nose, trachea and lungs) performed after mice were sacrificed on day 98. Each data point represents the mean ± SE of two independent experiments with measurements of six to eight mice in each group. Black squares: *L. lactis*-Mx2; grey squares: *L. lactis*-WT. $^{**}P < 0.005$, by Student's t-test between values of mice immunized with *L. lactis*-Mx2 and *L. lactis*-WT. NS, not significant.

to immunize five mice. Unimmunized mice received 20 μL of PBS. The mice were weighed daily for one week after each immunization. Blood samples were obtained from all animals on Days 2, 31 and 42. Complete blood cell counts with leukocyte differential counts were determined by using a Unicel DxH 800 System (Beckman Coulter, Fullerton, CA, USA). Blood samples used for serum analysis were incubated for 1 hr at 37°C and centrifuged for 5 min at 5000 g. Chemical analysis of serum was performed on a Roche Cobas 8000 system (C702 and ISE modules; Indianapolis, IN, USA). On Day 42, the mice were killed humanely and tissues (lungs, liver, nose and cervical lymph nodes) harvested. All tissues were fixed in 10% paraformaldehyde solution for 48 hr at 4°C and then embedded in paraffin. Tissue sections of 5 μm were cut and mounted onto glass slides for hematoxylin and eosin staining.

Autoimmune reactivity assessment

Immune mice sera were tested for the presence of heart, kidney and brain cross-reactive antibodies by indirect immunofluorescence tests using heart (NBP2-46893), kidney (NBP1-78269) and brain (NBP2-50617) normal human formalin-fixed, paraffin-embedded tissue slides (Novus Biologicals, Littleton, CO, USA). Five micrometer

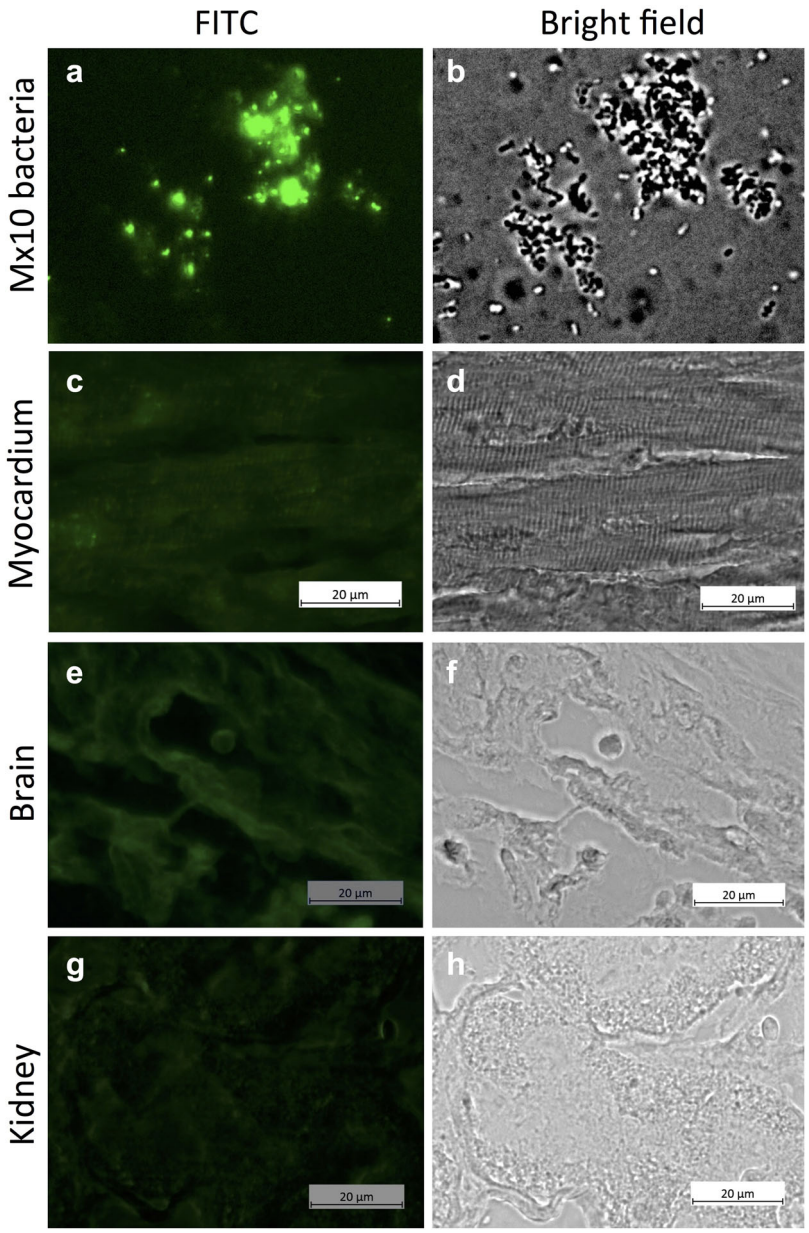

Fig. 2. Fluorescence microscopy analysis of tissue cross-reactive antibodies in the sera of mice immunized with *L.lactis*-Mx10 vaccine. In (a, c, e, g) fluorescence micrographs for FITC. In (b, d, f, h) brightfield of each fluorescence micrograph. In (a, b) *L. lactis*-Mx10 vaccine suspension was evaluated against sera of mice immunized with L. lactis-Mx10. Sera of *L. lactis*-Mx10-immunized mice was used to treat human myocardium (c and d), brain (e and f), and kidney (g and h). Antibody binding was detected with anti-IgG FITC-coupled antibody. More than 10 different fields were analyzed and no fluorescence was observed. Representative micrographs are shown.

sections were deparaffinized by two passages through Neo-Clear (Merck, Darmstadt, Germany) for 10 min each. Sections were hydrated by sequential incubations in ethanol 100% (twice), 95% and 70% for 2 min each. The samples were then washed in deionized water and incubated for 5 min with 0.2% pepsin solution (Dako, Santa Clara, CA, USA) at room temperature for antigen retrieval. A drop of mice immune sera (diluted 1:4 with PBS) was applied to tissue slides, incubated for 30 min at room temperature, and then washed three times with PBS. The slides were similarly treated with a 1:50 dilution of fluorescein goat anti-mouse lgG (Invitrogen, Carlsbad, CA, USA), incubated for 1 hr at room temperature in the dark and then mounted with ProLong Diamond Antifade Mountant (Invitrogen) and a coverslip. The tissue slides were examined under a Zeiss fluorescence microscope (Oberkochen, Germany).

RESULTS

Mice cleared the vaccine from their oropharynx without receiving antibiotics or T/B lymphocytes

THE Colonization capacity of *L. lactis* vaccine was evaluated in Balb/c and Rag-1 mice. Rag-1 mice are deficient in T and B lymphocytes because of a mutation in the *rag-1* gene, which impairs V(D)J recombination (14). After intranasal administration of 109 CFU of vaccine strains *L. lactis*-M6 and *L. lactis*-M77 (*L. lactis*-Mx2), or *L. lactis*-WT vaccine, oropharyngeal washes were performed 1, 7, 14, 28, 42 and 98 days after immunization. Unimmunized mice were administered PBS intranasally. Cultures of oropharyngeal washes of PBS-administered mice were negative for *L. lactis*, indicating that this bacterium was not present in these mice. *L. lactis* was recovered from oropharyngeal washes of Balb/c and Rag-1 mice 24 hr after immunization with L. lactis-Mx2, as determined by MALDI-TOF MS identification (Fig. 1a,b). Surprisingly, *L. lactis* was recovered from Balb/c mice immunized with L. lactis-WT, but not from similarly vaccinated Rag-1 mice. *L. lactis* was not recovered in any of the subsequent cultures made on days 7, 14, 28, 42 or 98 from Balb/c or Rag-1 mice. *L. lactis* was not recovered from cultures of minced nose, trachea, or lungs on Day 98 after immunization (Fig. 1c). These results indicate that even mice devoid of T and B lymphocytes are able to clear *L. lactis* from their respiratory tracts without receiving antibiotics.

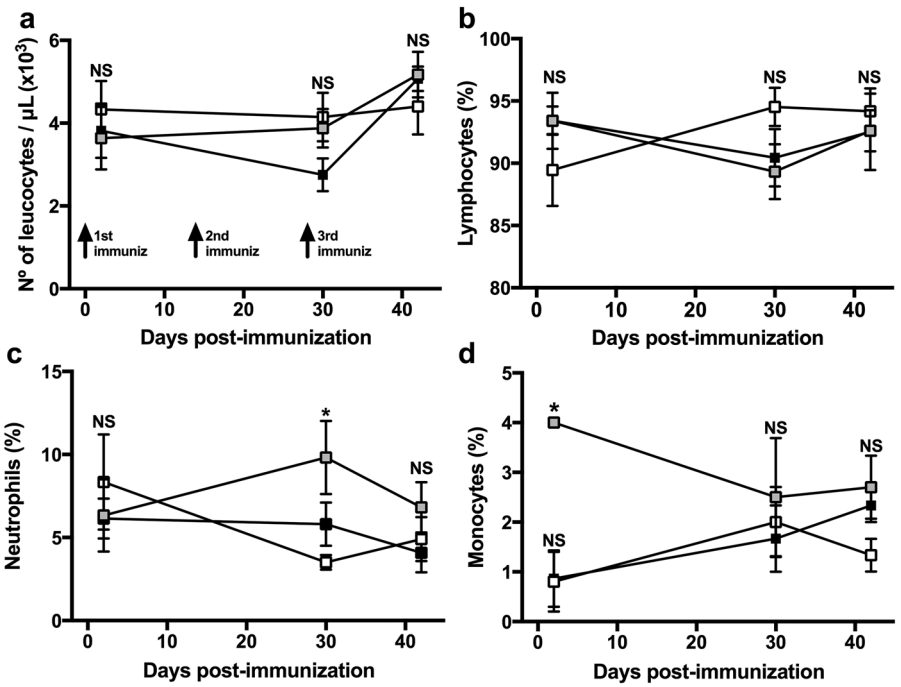

Fig. 3. Leucocyte counts of immunized mice. Each data point represents the mean ± SE of two or three independent experiments with samples from six to eight mice in each group. Immunization of mice on Days 0, 14 and 28 is shown by arrows in (a). Blood samples were obtained on Days 2, 30 and 42 and results are shown as square data points. Black squares: *L. lactis*-Mx10; grey squares: *L. lactis*-WT; white squares: unimmunized. $^{*}P < 0.05$ by Student's *t*-test (c) between values of mice immunized with *L. lactis*-WT and unimmunized mice and (d) between values of *L. lactis*-WT and *L. lactis*-Mx10 and unimmunized mice. NS, not significant.

No autoimmune reactions against human tissues were detected in sera from *L. lactis*-Mx10-immunized mice

Sera of mice immunized with *L. lactis*-Mx10 were tested against human myocardium, kidney and brain tissue. The presence of bound antibodies was detected with an anti-IgG antibody coupled to FITC under a fluorescence microscope. As a positive control, sera of mice immunized with *L. lactis*-Mx10 were tested against a suspension of *L. lactis*-Mx10 bacteria; it was observed that most bacteria were fluorescent green (Fig. 2a,b). In contrast, the immune sera did not react against human myocardium (Fig. 2c,d), brain (Fig. 2e,f) or kidney tissue (Fig. 2g,h).

L. lactis-Mx10 vaccine has no detectable toxicity in mice according to a preliminary evaluation

Mice were intranasally immunized every 14 days (total of three doses) with *L. lactis*-Mx10 (10^9 CFU), *L. lactis*-WT or not immunized (PBS). Blood samples were obtained on Days 2, 30 and 42. Total white blood cell, lymphocyte, monocyte and neutrophil counts varied only slightly between *L. lactis*-Mx10 and unimmunized mice (Fig. 3a–d). However, neutrophil and monocyte counts were significantly higher in *L. lactis*-WT-immunized mice on Days 30 and 2, respectively (Fig. 3c,d). The values obtained for unimmunized mice were in their normal ranges (15, 16).

Biochemical analysis of sera showed no significant variations in creatine kinase, potassium or sodium concentrations between the three groups of animals analyzed (Fig. 4b–d). However, the albumin/globulin ratio was greater in *L. lactis*-Mx10-immunized mice than in *L. lactis*-WT and unimmunized mice (Fig. 4a).

On Day 42, nose, cervical lymph nodes, liver and lungs were removed and stained with hematoxylin and eosin for histopathological analysis. Lymph nodes of *L. lactis*-WT-immunized mice were significantly larger than those of *L. lactis*-Mx10 or unimmunized mice (Fig. 5a–d). Liver tissue was normal in all groups of mice (Fig. 5e–g). Lung tissue was significantly more strongly infiltrated with inflammatory cells in *L. lactis-WT* than in *L. lactis*-Mx10 or unimmunized mice (Fig. 5h–k). Because nasal epithelium is the portal of entry of this vaccine, its integrity was investigated. As

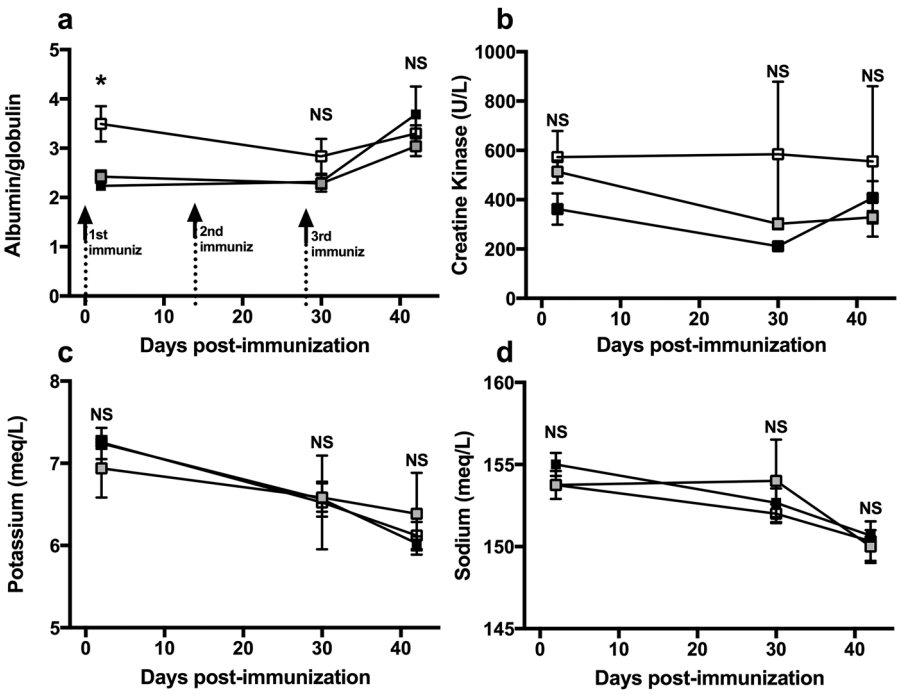

Fig. 4. Biochemical analysis of serum of immunized mice. Immunization of mice on Days 0, 14 and 28 is shown by arrows in (a). Each data point represents the mean ± SE of two or three independent experiments with blood samples from six to eight mice in each group. Blood samples were obtained on Days 2, 30 and 42 and results are shown as square data points: Black squares: *L. lactis*-Mx10; grey squares: *L. lactis*-WT; white squares: unimmunized. $^*P < 0.05$ by Student's *t*-test between values of unimmunized mice and mice immunized with *L. lactis*-WT and *L. lactis*-Mx10. NS, not significant.

shown in Figure 5l–n, the nasal epithelial surface was intact in *L. lactis*-Mx10 (Fig. 5m) and in unimmunized mice (Fig. 5l), as demonstrated by the normal structure of ciliated respiratory epithelium. However, the epithelium was sparsely disrupted and covered by a mucus layer in *L. lactis*-WT-immunized mice (Fig. 5l).

Body weight was monitored throughout the 42 days of the experiment. The weights of mice immunized with *L. lactis*-Mx10 and *L. lactis*-WT decreased significantly during the 2–6 days after every immunization by around 10% and 5% respectively (Fig. 6). The decrease was maximal 2 days after immunization. Both groups of mice recovered their normal weights by 6 days after immunization.

DISCUSSION

We only detected *L. lactis* vector 24 hr after immunization, indicating that it was efficiently cleared without administration of antibiotics by both immunocompetent and immunodeficient mice. This is very desirable in terms of safety. However, this trait could constitute a limitation for a bacterial vector used for immunization because rapid clearance may leave insufficient time to mount an optimal immune response. In this context, *Streptococcus gordonii* is known to be a persistent colonizer of the oral cavity and it could be considered a promising bacterial vector capable of inducing a strong immune response. In fact, clinical tolerance and

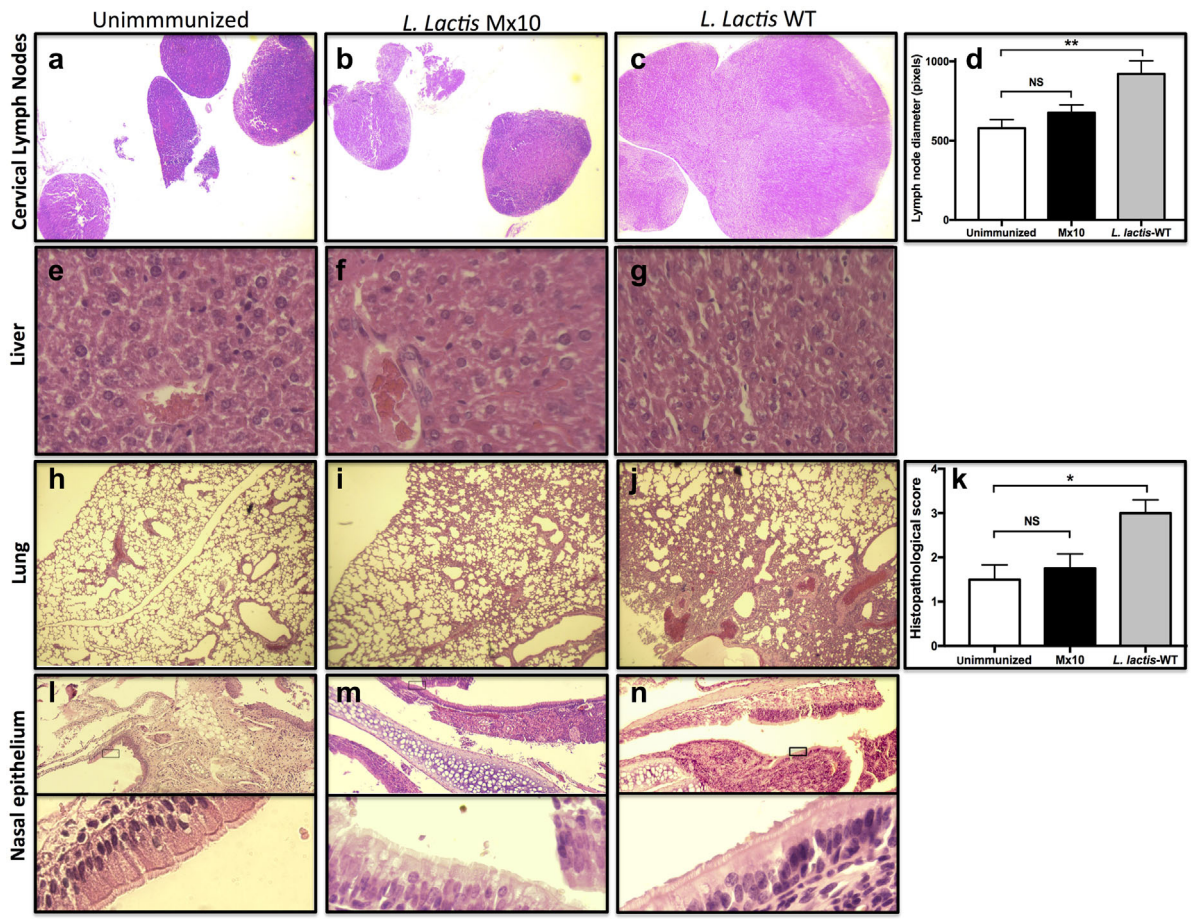

Fig. 5. Histopathological analysis of tissue sections. Tissue sections were stained with hematoxylin and eosin and examined microscopically at ×40 (cervical lymph nodes and lung), ×400 (liver), ×100 and ×1000 (nasal epithelium). Several micrographs were assessed; representative ones are shown. (d) The diameters of three or four lymph nodes per mouse were determined in pixels; mean diameters per group. Bars represents the mean ± SE of two independent experiments with measurements of nodes from six to eight mice in each group. (k) The degree of lung damage in each experimental group was blind-scored by three independent observers using the following criteria: 0, no cell infiltration; 1, minimal cell infiltration; 2, slight cell infiltration; 3, moderate cell infiltration; and 4, severe cell infiltration. The presence of hyaline nasal cartilage indicates that nose epithelium is shown. The lower panels of (l–n) are magnifications (×1000) of the inlets indicated by rectangles in the upper panels (×100) . $^{*}P < 0.05$; $^{**}P < 0.005$ determined by one-way anova corrected for multiple comparisons using Tukey test. NS, not significant.

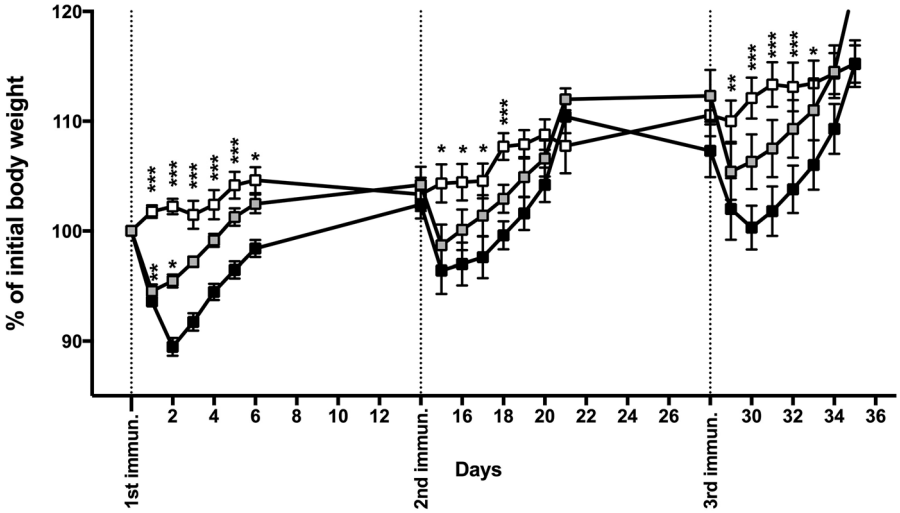

Fig. 6. Weight loss during the immunization period. Percentage of initial body weight was determined for *L. lactis*-Mx10 (black squares); *L. lactis*-WT (grey squares) and unimmunized mice (white squares). Each data point represents the mean ± SE of three independent experiments with measurements of six to 12 mice in each group. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***} < 0.0005$ determined by Student's *t*-test between unimmunized and *L. lactis*-Mx10 values (asterisks above white squares) and between unimmunized and *L. lactis*- (asterisks above grey squares).

colonization has been evaluated with a WT strain of *S. gordonii* administered intranasally to humans (17). Bacteria were detected in ∼20% of volunteers 7 days after inoculation. However, this persistence could increase the risk of transmission to susceptible individuals. More importantly, it could increase the risk of low level antigenic stimulation leading to immunologic tolerance or production of low-avidity antibodies (18, 19). Thus, the poor colonization ability of *L. lactis* would be advantageous from the safety point of view for a bacterial vector to be used with a potential vaccine. The appropriate balance between safety and duration of colonization requires clarification by future clinical evaluations of vaccine safety and immunogenicity.

An altered albumin/globulin ratio can be attributable to liver dysfunction. However, histopathological examination showed no alterations in liver architecture. One of the most affected variables in *L. lactis*-Mx10 and *L. lactis*-WT-immunized mice was body weight, which decreased significantly 2 days after vaccine administration. During assessment of *S. gordonii* in humans, Kotloff and coworkers (17) reported appetite and energy loss in 6% and 13% of individuals respectively. They also reported nasal congestion and burning sensations in up to 40% of individuals. There was no evidence of systemic or local toxicity in the *L. lactis*-Mx10-immunized mice. In contrast, there was some evidence of toxicity in *L. lactis*-WT-immunized mice; namely, increased numbers of neutrophils and monocytes, increased size of lymph nodes, augmented pulmonary infiltration and altered nose epithelium. These histopathological alterations may have been associated with nasal irritation and congestion, resulting in loss of appetite. These observations were surprising given that *L. lactis* has a GRAS status (Generally Recognized as Safe) according to the United States Food and Drug Administration. Moreover, Kato and coworkers (20) have demonstrated a lack of adverse effects in humans after excessive oral intake of a heat-killed probiotic *L. lactis* strain. It is worth mentioning that, unlike in the current study, these authors evaluated oral intake and used heat killed bacteria. We believe that our findings may be the result of using a mouse model; *L. lactis* is not recognized as normal microbiota of mice but is found in human microbiota (21). Therefore, immune responses to the WT vector may be amplified in mice. It is not at all clear why the *L. lactis*-Mx10 vaccine strains were apparently less toxic than the WT strain. One possible explanation is that the surface expression of M protein fragments masks recognition of WT surface antigens that otherwise activate the innate immune cascade. Interestingly, the greater reactogenicity of the *L. lactis*-WT strain could explain our previous findings of partial protection from infection observed in *L. lactis*-WT-immunized mice (2), which may be attributable to them having stronger innate immune responses than in *L. lactis*-Mx10-immunized mice.

The results obtained of this study provide initial preclinical data on *L. lactis*-Mx10 group A streptococcal vaccine. Further investigation of whole animal toxicity and autoimmune complications is required prior to initiating clinical trials to test the safety and immunogenicity of the vaccine in humans.

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DISCLOSURE

The authors declare they have no conflict of interest to declare.

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