Oral Immunization of Mice with Lactic Acid Bacteria Producing Helicobacter pylori Urease B Subunit Partially Protects against Challenge with Helicobacter felis

Blaise Cortheés,1 Soledad Boris,2,a Patrick Isler,3 Corinne Grangette,3 and Annick Mercenier1,a

1R&D Laboratory of the Division of Immunology and Allergy and 2Division of Gastroenterology, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland; 3Laboratory of Bacteriology of Ecosystems, Institut Pasteur de Lille, Lille, France

Background. The development of an efficacious vaccine against infection with Helicobacter pylori, the causative agent of chronic gastritis, peptic ulcer disease, and gastric adenocarcinoma, remains a challenge. Since the use of mucosal adjuvants is limited in human application, we have evaluated the potential of recombinant Lactobacillus strains producing H. pylori urease B (UreB) subunit to deliver this antigen to the gastrointestinal tract.

Methods. Mice were injected orally 3 times with a triple dose of recombinant Lactobacillus plantarum NCIMB8826, the recombinant isogenic cell-wall mutant (alr/H11002 MD007 strain) expressing UreB, or a mixture of recombinant UreB and cholera toxin (rUreB/CT) as a control. Urease-specific seric immunoglobulin (Ig) G and IgA were measured by use of an enzyme-linked immunosorbent assay. After challenge with Helicobacter felis, stomach infection was examined by use of the rapid urease test and by polymerase chain reaction detection of Helicobacter genomic DNA.

Results. Intragastric immunization with both recombinant Lactobacillus strains and rUreB/CT elicited UreB-specific antibodies. After challenge, reduction of H. felis load in the stomachs of mice was observed only after immunization with the recombinant mutant strain MD007 or with rUreB/CT.

Conclusions. This is the first report of successful induction of partial protection against H. felis with a mucosal prime-boost regimen in which recombinant Lactobacillus strains were used as antigen-delivery vehicles.

Helicobacter pylori is recognized as a human-specific gastric pathogen that colonizes the stomachs of at least half of the world’s population. Most infected individuals are asymptomatic, although, for a significant number, infection is associated with the development of duodenal and gastric ulcers and gastric cancers [1]. That natural immunity appears to be inadequate for clearing the infection questions the feasibility of producing an effective H. pylori vaccine [2]. Intragastric immunization with H. pylori virulence factors in combination with mucosal adjuvants, such as bacterial toxins, has been proven to eradicate the pathogen [3–6] and to prevent reinfection [7] in animal models. Notably, the urease B (UreB) subunit of urease, which exhibits substantial cross-reactivity between Helicobacter strains and species, has been extensively used as a protecting antigen in combination with cholera toxin (CT) or Escherichia coli heat-labile toxin [8, 9]. However, toxin-based adjuvants lead to numerous adverse effects, which preclude their applicability in humans [10]. Other approaches based on the use of attenuated Salmonella strains [11, 12] or nanoparticles [13] triggered a val-

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Present affiliations: Area Microbiologia, Departamento de Biología Funcional, Facultad de Medicina, Universidad de Oviedo, Oviedo, Spain (S.B.); Nutrition and Health Department, Nestlé Research Center, Vers-chaz-les-Blanc, Lausanne, Switzerland (A.M.).

Reprints or correspondence: Dr. Annick Mercenier, Nutrition and Health Dept., Nestlé Research Center, PO Box 44, 1000 Lausanne 28, Switzerland (annick.mercenier@rdls.nestle.com).

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uitable degree of protection in mouse models, yet efficacy in humans remains to be established. A key issue is the nature of the immune response required to eliminate Helicobacter infection; ambiguities remain with regard to the dispensability of antibody responses [14–16]. Antigen delivery should be designed to stimulate mucosal immunity with, possibly, a Th2 bias in the absence of proinflammatory adverse effects, because the early induction of a Th1 response causes damage in the host and is ineffective at clearing infection in humans [17].

Prevention of attachment of the pathogen and subsequent colonization at mucosal surfaces is best achieved when immunity is triggered after local stimulation. Different delivery systems satisfying this requirement are currently under development, with various avenues being explored for oral administration [18]. One such delivery system is based on live bacterial vectors, including nonpathogenic, noninvasive lactic acid bacteria (LAB) strains (which are generally recognized as safe) [19]. These carriers do not induce pronounced proinflammatory responses [19, 20], which renders them best suited for immunocompromised subjects, infants, and elderly individuals [21]. In the present study, the Lactobacillus plantarum NCIMB8826 strain was chosen for its capacity to resist passage of pTCP3 plasmid by PCR [27] and cloned into a derivative of pGIT032 [28] pre-cursor for the bacterial cytoplasm.

Structural stability of the recombinant pMEC142 plasmid in vitro. The 8826/pMEC142 strain was subcultured in selective medium (MRS broth supplemented with 5 μg/mL erythromycin). Ten successive cultures were performed by inoculating 10 mL of fresh medium with 10 μL of the previous culture. Bacterial cells from cultures after 1, 5, and 10 passages were recovered and subjected to immunoblot analysis, as described below. Plasmid pMEC142 was extracted from 2 independent colonies originating from passages 1, 5, or 10 and served as a source of DNA for PCR and restriction analyses.

Western-blot assay. A total of 1 × 10^7 recombinant bacteria (L. plantarum and E. coli) were lysed in reducing SDS-containing buffer [31], separated by PAGE, and transferred onto blotting membrane. After blocking for 1 h at 37°C with PBS-
injected intragastrically with cfu of recombinant approved by the local veterinary office. Groups of 10 mice were housed in microisolator cages, with free access to water and chow, as specific pathogen–free female BALB/c mice were housed in 3 times, at 2-week intervals. Serum samples were recovered 7 days after the first and second booster injections.

Antigens. Recombinant UreB was obtained from E. coli, as described elsewhere [5]. CT was purchased from Calbiochem. Recombinant urease holoenzyme [7] or H. felis lysate was used to determine specific seric IgG and IgA antibody titers by use of an ELISA.

Systemic immunization. Eight-week-old female C57/B6 mice (Charles River Laboratories) were immunized by subcutaneous (sc) administration of UreB-producing L. plantarum 8826/pMEC142 or E. coli M15/pMEC142. Mice (groups of 5) received a single dose of 1 × 10⁹ cfu of each recombinant strain 3 times, at 2-week intervals. Serum samples were recovered 7 days after the first and second booster injections.

Intragastric immunization and infection with H. felis. Specific pathogen–free female BALB/c mice were housed in microisolator cages, with free access to water and chow, as approved by the local veterinary office. Groups of 10 mice were injected intragastrically with 1 × 10⁷ cfu of recombinant Lactobacillus strains in 200 µL of gavage buffer 3 times for 3 consecutive days, at 3-week intervals (figure 1). Control mice were injected with MRS broth alone or with rUreB/CT (50 µg/10 µg). Feces were collected to examine the presence of recombinant strains and their capacity to maintain production of urease. Twelve days after the last immunization, mice were infected with 5 × 10⁷ freshly cultured H. felis by orogastric intubation under light anesthesia with isofluran (Baxter); this guarantees >90% infection in unvaccinated mice [7]. Mice were killed 2 weeks later, and stomachs were collected to examine the degree of protection.

Measurement of serum antibodies. Maxisorp immunoplates (Nunc) were coated overnight at 4°C with 0.5 µg of recombinant urease holoenzyme or 1 µg of H. felis lysate [34]. Mouse serum dilutions in Tris-buffered saline–T:0.5% SM were incubated for 2 h at 37°C. Urease-specific antibodies were detected by use of either rabbit anti-IgG or goat anti-IgA (chain specific) coupled with HRP (Sigma). Antibody dilutions yielding absorbance values 2-fold higher than those yielded by the preimmune serum were used to establish end-point titers.

Quantitative analysis of H. felis colonization. In method 1, the presence of H. felis in gastric tissue was assessed by use of RUT (Jatrox-test; Procter & Gamble) [5]. The cut-off value of RUT used to discriminate between infection and cure corresponded to the mean ± 2 SDs of the absorbance values obtained with gastric tissues from naive mice. In method 2, 20 mg of stomach sample was cut into small pieces and homogenized in 180 µL of ATL buffer (Qiagen). After digestion with 1.5 mg/mL proteinase K for 30 min at 55°C, 200 µL of AL buffer (Qiagen) was added. The mixture was incubated for 10 min at 70°C, 200 µL of 100% ethanol was added, and the precipitate was cleared by centrifugation. The lysate was extracted twice with phenol-chloroform (1:1), and the DNA was precipitated with 2.5 vol of ethanol before washing, drying, and final resuspension in water. A specific pair of primers for H. felis flaB (5′-TTCGATTGGTCCTACAGGCTCAGA-3′ [sense] and 5′-TTCTTGTGGATGACATTGACCAACGCA-3′ [anti-sense]) was combined with 1 µg of DNA template, and real-time PCR amplification was performed as described by Stoico et al. [35]. Standards were obtained accordingly, allowing for quantification of bacteria per microgram of gastric tissue [35].

Cytokine/chemokine expression in stomach samples. Total stomach RNA was extracted and reverse transcribed, as described elsewhere [36]. The cDNA was amplified by PCR with primers specific for tumor necrosis factor (TNF)–α, monocyte chemoattractant protein (MCP)–1, and RANTES. The samples were subjected to 40 PCR cycles, consisting of denaturation

Figure 1. Experimental setting for the prophylactic immunization of mice with recombinant Lactobacillus strains (upper part of the time line). Priming and boosting injections were given at 3-week intervals. Control immunization was performed with recombinant urease B (rUreB) combined with cholera toxin (CT) (lower part of the time line). UreB-specific antibodies were measured before immunization, after the second booster injection, and after infection with Helicobacter felis at the time of killing of the mice. Presence of H. felis was examined by rapid urease test and real-time polymerase chain reaction (RT-PCR) analyses of genomic DNA recovered from the stomachs of mice.
E. coli in the NCIMB8826 strain with a yield close to that produced in
illustrating that full-length UreB can be produced intracellularly
purified rUreB and that produced from the pMEC142 plasmid
lanes 1–3 intensity was observed in all 3 lanes (figure 2,
were assayed after 1, 5, and 10 passages (Ps),
L. plantarum lane 2, Escherichia coli
nant UreB; and immunodetection.
was assessed by use of whole bacterial lysates, separation by SDS-PAGE,
UreB production level and structural stability of the
L. plantarum
RESULTS
strains in vitro.
production by the 8826/pMEC142 strain and compared
m of Student's t test. was considered to be statistically
3 independent clones) assayed after 1, 5, and 10 passages (Ps),
8826/pMEC142; lane 3, L. plantarum
basis that produced by positive controls consisting of 5 µg of
E. coli strain M15. By immunoblot analysis, a band of similar
and extension (for 1 min at 72°C). Visualization and quantification
of PCR products were performed as described elsewhere [37],
and results were expressed as ratios to the groups given gavage
buffer by use of glyceraldehyde 3-phosphate dehydrogenase
and results were expressed as ratios to the groups given gavage
buffer by use of glyceraldehyde 3-phosphate dehydrogenase
(GAPDH), for standardization between samples.
Statistical analysis. Antibody titers and H. felis copy
numbers are reported as means ± SDs and were compared by use
of Student’s t test. RUT values were compared by use of the
Mann-Whitney U test. P < .05 was considered to be statistically
significant for differences between groups.

RESULTS
UreB production level and structural stability of the L. plantarum strains in vitro. We first evaluated the amount of UreB produced in vitro by the 8826/pMEC142 strain and compared it with that produced by positive controls consisting of 5 µg of purified rUreB and that produced from the pMEC142 plasmid in E. coli strain M15. By immunoblot analysis, a band of similar intensity was observed in all 3 lanes (figure 2, lanes 1–3), demonstrating that full-length UreB can be produced intracellularly in the NCIMB8826 strain with a yield close to that produced in E. coli (≥ 5 µg/1× 10^9 bacteria). As illustrated in figure 2, similar levels of UreB were observed by immunodetection along a time course covering 10 successive culture passages in selective medium, arguing for structural stability of the expression plasmid. PCR analysis of plasmid pMEC142 extracted from 2 independent clones confirmed that the plasmid was not rearranged during subculturing of the strain (data not shown). The segregational stability of the recombinant strains was examined by inoculating them at a 1:400 dilution in nonselective medium. After ~25
generations, >20% of the cells reaching late stationary phase retained the plasmid (data not shown).

Evaluation of the immunogenicity of the recombinant 8826/pMEC142 strain. Before conducting intragastric immunization, we first examined whether the amount of UreB produced by the model strain 8826/pMEC142 was sufficient to trigger a measurable antibody response when administered sc to mice. Injection of 1× 10^7 cfu resulted in UreB-specific IgG titers well above background levels obtained with sham-injected mice (figure 3). Specific antibody titers were not improved by the concomitant addition of alum as an adjuvant (data not shown). Mice injected with recombinant E. coli producing UreB exhibited 5-fold higher titers (figure 3), which is consistent with the higher intrinsic adjuvanticity of this expression host.

Intragastric immunization with recombinant L. plantarum strains and IgA and IgG responses. As outlined in figure 1, intragastric administration of recombinant L. plantarum strains was performed 3 times on 3 consecutive days. For practical reasons, priming and boosting injections were done with bacteria from the same culture batch kept in gavage buffer for 2 days at 4°C. No viability loss was observed for the recombinant wt strains after 48 h of storage, yet viable counts were reduced by 1 log for the MD007 strain at 48 h (data not shown). Notably, the relative abundance of UreB in bacterial lysates did not decrease during storage and was equivalent to 10 µg of rUreB (figure 4A). Previous experiments have shown that the survival capacities of the NCIMB8826 and MD007 strains in the mouse

Figure 2. Production of urease B (UreB) and plasmid structural stability in the Lactobacillus plantarum NCIMB8826 strain. Production of UreB was assessed by use of whole bacterial lysates, separation by SDS-PAGE, and immunodetection. Lane 1, Five micrograms of purified H. pylori recombinant UreB; lane 2, Escherichia coli strain M15 carrying vector pMEC142; lane 3, L. plantarum 8826/pMEC142; lanes 4–9, UreB production by 8826/pMEC142 (2 independent clones) assayed after 1, 5, and 10 passages (Ps), respectively. The asterisk indicates UreB-specific signals.

Figure 3. Capacity of Lactobacillus plantarum 8826/pMEC142 to deliver urease B (UreB) in vivo. Mice were injected subcutaneously, and serum IgG antibody titer specific for UreB were established by end-point dilutions (reciprocal of the last dilution giving an optical density higher than 2 times the background) after the first and second booster injections. Negative and positive controls included PBS and Escherichia coli carrying vector pMEC142, respectively.
Recombinant LAB Protect against H. pylori Infection

Figure 4. A, Western-blot analysis of urease B (UreB) production by the Lactobacillus plantarum 8826/pMEC142 and MD007/pMEC142 strains. A total of 100 µg of bacterial lysate was loaded per lane; 5 and 10 µg of purified recombinant UreB (rUreB) were loaded alongside to permit quantification. Days 1, 2, and 3 indicate the UreB content of cell lysates from bacteria administered at days 1, 2, and 3 of the first booster injection. Molecular weight markers are shown on the left side of the panel. B, Western-blot analysis of UreB production by 8826/pMEC142 and MD007/pMEC142 recovered from mouse feces, performed as described in panel A (1 representative colony of 10). C, Determination of anti-UreB–specific IgA and IgG responses after immunization. Serum titers were established by end-point dilutions after immunization (gray bars) and after infection with Helicobacter felis (black bars). Data were averaged from 5 mice randomly picked per group and are expressed as means ± SDs. * , by comparison with gavage buffer; ** , by comparison with gavage buffer. 8826/pMEC142, L. plantarum NCIMB8826 producing UreB; 8826/pTG2247, recombinant L. plantarum NCIMB8826 not producing UreB; gavage buffer, negative control; MD007/pMEC142, alr− MD007 mutant producing UreB; rUreB/CT, a mixture of rUreB and cholera toxin serving as positive control.

gastrointestinal (GI) tract were equivalent (data not shown). We further verified that colonies recovered from feces continued to produce UreB (figure 4B).

The production of specific seric IgA and IgG antibodies indicated that mucosal delivery of the antigen was successful [38]. We thus evaluated production of these classes of antibodies to assess whether UreB delivered by L. plantarum was efficiently recognized in the GI tract. Mice that received the 8826/pMEC142 or MD007/pMEC142 strain or rUreB/CT intragastrically produced significant (P < .01–.001) levels of UreB-specific IgA, compared with preimmune mice (figure 4C, gray bars). Infection with H. felis did not induce production of more antibody during the 2 weeks preceding euthanasia of the mice (figure 4C, black bars). Levels of specific IgA barely exceeded background levels in groups receiving the 8826/pTG2247 strain or gavage buffer. A similar response pattern was seen when production of UreB-specific IgG was assessed. We thus explored whether the onset of immune responses by recombinant LAB would lead to protection in a mouse model of H. felis infection.

Protection against H. felis infection in mice. Protection was evaluated 2 weeks after infection of immunized mice with H. felis, by use of RUT. The stomachs of mice given the L. plantarum MD007/pMEC142 mutant strain showed slower color development, compared with the stomachs of mice given the wt 8826/pMEC142 strain. This correlated with end-point values measured at 3 h, which showed a significant difference (P < .05) in the case of only MD007/pMEC142 (figure 5A). No reduction in H. felis load in the stomachs of mice was observed upon intragastric administration of the 8826/pTG2247 control strain or gavage buffer. As a validation of the immunization and
Figure 5. Evaluation of protection against Helicobacter felis infection. 
A, Collection of half stomachs from mice, which were assayed by use of the rapid urease test, 2 weeks after infection, as described in Material and Methods. The presence of \textit{H. felis} in gastric tissues was assessed by urease activity measured photometrically at an optical density (OD) of 550 nm after a 3-h incubation. Abbreviations for lane content are the same as those given in figure 4B. Statistical treatment of the data was conducted by use of the Mann-Whitney U test ( ). B, Quantitative polymerase chain reaction for \textit{H. felis} infection, reported as the no. of bacteria (as a function of gene copy no.) per micrograms of gastric tissue. Results are means ± SDs (n = 6).

Infection studies, local inflammation in the stomach antrum is accompanied by production of MCP-1, RANTES, and TNF-\( \alpha \) [39]. Since quantitative levels of protein cannot be determined from stomach samples, we evaluated possible changes in the gastric mucosal production of these chemokines/cytokine by use of real-time PCR (figure 6A). Although immunization of mice with 8826/pTG2247 or 8826/pMEC142 yielded mRNA levels comparable to those found in mice given gavage buffer, a decrease in expression of MCP-1, RANTES, and TNF-\( \alpha \) was measured in mice immunized with MD007/pMEC142 or rUreB/CT (figure 6B). These findings are consistent with the fact that prevention of attachment or clearance of \textit{Helicobacter} species reduces local inflammation mediated by chemokines released by epithelial cells upon contact with the bacterium [33]. Together, these data demonstrate that partial protection against challenge with \textit{H. felis} in mice can be obtained by use of \textit{Lactobacillus} strains as live vaccine vehicles.

DISCUSSION

The rationale behind the development of dietary LAB as a live mucosal delivery system is that they have been used from time immemorial in the preparation of fermented foods and feeds and, thus, have been consumed worldwide by humans and animals. Moreover, specific LAB strains have been shown to exert beneficial health—that is, probiotic—effects [40, 41] and to be particularly adapted to immunization by the oral route, since they are quite acid resistant. One such strain is \textit{L. plantarum} NCIMB8826, an isolate of human origin that has been shown to establish itself in the mouse intestine and persist for ∼1 week [22], which supports the notion that \textit{L. plantarum} is able to replicate in the mouse intestine. This strain has also been reported to present appropriate pharmacokinetic properties for use in humans [23].

In the present study, we investigated whether the intrinsic characteristics of \textit{L. plantarum} NCIMB8826 would turn it into an adequate vector to deliver the \textit{H. pylori} UreB antigen prophylactically via the intragastric route. The NCIMB8826 strain was previously shown to behave as a well-performing vaccine.
Recombinant LAB protect against H. pylori infection

A

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<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>TNF-α</td>
<td>5'-GGCAGGCTCTATGTGAGATCTTGC-3'</td>
<td>307</td>
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<tr>
<td>MCP-1</td>
<td>5'-CTCTTCTCCACCAACATGCGG-3'</td>
<td>297</td>
</tr>
<tr>
<td>RANTES</td>
<td>5'-GACGATCCAAAGAGTGGTGAAGC-3'</td>
<td>286</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-GTCCACCAACCTGTTCTTCTG-3'</td>
<td>210</td>
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Figure 6. A, Sequences of primers used for real-time polymerase chain reaction (RT-PCR) amplification: tumor necrosis factor (TNF-α), monocyte chemoattractant protein (MCP)-1, RANTES, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). B, Relative mRNA expression, determined by RT-PCR, in the stomachs of immunized mice and in those of control mice. Ratios were obtained by dividing the mean intensities of 6 individual samples in each immunization group with the mean level of expression measured in mice given gavage buffer. Quantification of fluorescent DNA signals in agarose gels was performed by use of the software Quantify1D (Advanced American Biotechnology).

vehicle by different mucosal routes by use of TTFC as antigen [24, 42, 43]. Cell-wall mutants of this carrier that exhibited improved antigen-delivery capacities have recently been described [26]. We therefore used both the wt and the alr- MD007 mutant strains in intragastric immunization experiments. Our data provide evidence that the recombinant strains that we constructed produced the antigen in microgram quantities in vitro. This indicates that, at the time of administration, antigen doses equivalent to those of purified rUreB/CT [5] were given to mice. Recombinant bacteria recovered from feces of immunized mice showed a preserved capacity to produce UreB, suggesting that sustained delivery of antigen occurs in vivo. We demonstrated that, although similar IgG and IgA antibody levels were obtained, the MD007/pMEC142 strain promoted a more pronounced reduction in H. felis load after challenge than did its wt counterpart. This was accompanied by a reduced expression of proinflammatory chemokines/chemokine involved in Helicobacter infection. The wt L. plantarum NCIMB8826 strain did not exhibit intrinsic protective properties in mice challenged with H. felis, as was demonstrated by the absence of an effect induced by the 8826/ pTG2247 control strain. However, it is worth mentioning that probiotic-based interventions against H. pylori infections by use of different species of LAB have shown partial beneficial effects that deserve further examination [44, 45].

A variety of antigens have been produced in different LAB strains, belonging mostly to the Lactococcus and Lactobacillus genera [21]. Although seric or mucosal antibody responses have been measured, protection studies against the targeted pathogen were rarely performed or turned out to be unsuccessful [46, 47], with the notable exception of protective immunity demonstrated by use of the potent immunogen TTFC [48]. The ability of LAB carriers to induce protective immune responses depends on sufficient antigen delivery in vivo and might be limited by their poor adjuvant properties. Optimization of LAB carriers requires selecting or defining the most-suitable LAB vehicle, the mode of antigen presentation (cytoplasmic, secreted, or cell-surface exposed), and the immunization regimen (route, dose, or timing). In this respect, a recombinant Lactococcus lactis strain producing cytoplasmic UreB was shown to be unable to induce protection against H. pylori in a mouse model [47]. Notably, the reported experimental setting led to very weak antibody responses. The authors concluded that the adjuvant effect of L. lactis was insufficient to trigger robust immune responses when used to deliver a weak immunogen, yet poor persistence of the strain in the GI tract might be considered too.

It is worth noting that, in mucosal immunization against Helicobacter species, the recombinant alr- MD007 mutant turned out to be superior to CpG immunostimulatory oligodeoxynucleotides used as adjuvant [49], although it did not perform as well as CT. Our data fully corroborate the results obtained by use of the potent TTFC antigen and, thus, mark the alr- MD007 mutant as a substantially improved delivery system that is also applicable to weak antigens. This likely results from enhanced in vivo release of antigen or improved antigen presentation to antigen-presenting cells; the 8826 and MD007 strains exhibit very similar persistence capacity in the mouse GI tract (data not shown). To the best of our knowledge, this is the first demonstration of the successful induction of partial mucosal protection against H. felis in mice by use of recombinant LAB as live antigen carriers. Coexpression of immunoregulatory cytokines [50, 51], combined with improved LAB vehicles, represents a valuable step toward further improvement of the prototype strains described in the present study.
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