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## Immunization of Mice with *Lactobacillus casei* Expressing a Beta-Intimin Fragment Reduces Intestinal Colonization by *Citrobacter rodentium*<sup>∇†</sup>

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**Enteropathogenic *Escherichia coli* (EPEC) is a common cause of diarrhea in children from developing countries. Intimate adhesion of the bacteria to intestinal cells occurs via binding of the adhesin intimin to the TIR receptor exposed on cell surfaces. Here, *Lactobacillus casei* expressing a fragment of  $\beta$ -intimin (*L. casei*-Int<sub>cv</sub>) was tested as mucosal vaccines in mice against intestinal colonization with the murine pathogen *Citrobacter rodentium*. Oral or sublingual immunization of C57BL/6 mice with *L. casei*-Int<sub>cv</sub> induced anti-Int<sub>cv</sub> IgA in feces but no IgG in sera. Conversely, anti-Int<sub>cv</sub> IgG was induced in the sera of mice after sublingual immunization with purified Int<sub>cv</sub>. All vaccines were able to decrease *C. rodentium* recovery from feces. However, this reduction was more evident and sustained over time in mice immunized with *L. casei*-Int<sub>cv</sub> by the sublingual route. These mice also displayed an increase in interleukin 6 (IL-6) and gamma interferon (IFN- $\gamma$ ) secretion by spleen cells 10 days after infection. Additionally, oral or sublingual immunization of C3H/HePas mice, which are highly susceptible to *C. rodentium* infection, with *L. casei*-Int<sub>cv</sub> induced anti-Int<sub>cv</sub> antibodies and significantly increased survival after challenge. Immunohistological analysis of colon sections revealed that *C. rodentium* was located in deep fractions of the tissue from C3H/HePas mice immunized with *L. casei* whereas superficial staining was observed in colon sections from mice immunized with *L. casei*-Int<sub>cv</sub>. The results indicate that vaccines composed of *L. casei* expressing intimin may represent a promising approach and that the C3H/HePas infection model with *C. rodentium* can be used to evaluate potential vaccines against EPEC.**

Gastrointestinal infections are among the leading causes of childhood mortality around the world and are responsible for approximately 2 million deaths every year (12). Enteropathogenic *Escherichia coli* (EPEC) is one of the main causes of childhood diarrhea in developing countries and has been considered a worldwide pathogen (7, 18, 31, 59). EPEC has been classified into typical or atypical according to the expression or lack of expression, respectively, of the bundle-forming pilus, encoded by the EAF plasmid (1, 50). Epidemiological studies suggest that the prevalence of atypical EPEC has increased in acute or persistent diarrhea cases in both children and adults (23, 29, 52, 62). EPEC colonizes the intestinal mucosa, causing the characteristic attaching and effacing (A/E) lesions. The formation of the A/E lesion involves a type III secretion system (T3SS), encoded by the LEE pathogenicity island, which delivers a range of effector proteins into host cells. Using this strategy, the pathogen triggers actin polymerization, producing a pedestal-shaped structure to which the bacteria strongly attach through the interaction of the adhesin intimin with the translocated receptor Tir (27, 38). Intimin is a 94-kDa protein inserted into the bacterial outer membranes from typical and

atypical EPEC, enterohemorrhagic *E. coli* (EHEC), and the mouse pathogen *Citrobacter rodentium*. This adhesin is encoded by the *eae* gene and is essential for virulence (28, 48). The N-terminal region is conserved among intimins, whereas the C-terminal portion is highly variable and has been used to classify intimin into 29 types that can be related to tropism for different intestinal tissues (26, 35, 49). Among them, the  $\beta$  subtype is one of the intimins most frequently expressed by EPEC isolates (6, 56, 60). EPEC and EHEC infections can be prevented by breastfeeding (17, 57), and antibodies against intimin are usually observed in the milk of women living in areas of endemicity (58, 70). In addition, these antibodies are able to inhibit the adhesion of EPEC to epithelial cells *in vitro* (15, 19). Several vaccination strategies using intimin have reduced colonization or mortality in animal models of infection with rabbit enteropathogenic *E. coli* (REPEC), EHEC, or *C. rodentium* (4, 30, 36, 39), suggesting that intimin is a good candidate for the formulation of vaccines against these infections. A minimal intimin fragment able to bind to the receptor Tir is composed of the last 190 amino acids, but high-affinity binding was observed only with a larger fragment comprising the last 280 amino acid residues (Int<sub>280</sub>) (8). In fact, subcutaneous immunization of mice with the Int<sub>280</sub> fragment from  $\alpha$ -intimin reduced intestinal colonization by a *C. rodentium* strain expressing an intimin from the same subtype (30). However, analysis of the immunodominant regions inside Int<sub>280</sub> have shown that antibodies against this fragment were directed to 2 regions in the N terminus of Int<sub>280</sub>, more precisely, a

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region located in the first 80 amino acids and a region between amino acids 80 and 130 of Int<sub>280</sub> (3). Moreover, human colostrum samples specifically recognized these fragments, but not the C-terminal region of Int<sub>280</sub>, indicating that natural exposure to intimin-bearing pathogens elicits antibodies that are reactive only to the N-terminal region of Int<sub>280</sub> (3).

The development of mucosal vaccines requires the design of immunogenic but safe vaccines that can prevent infections and reduce the transmission of a given pathogen (9). Mucosal vaccines can offer many advantages, like easy administration, which may contribute to lower costs of mass immunization (37, 55); low risk of individual contamination, as it allows needle-free administration; and the induction of immune responses that are active at the pathogen entry site (47). Previous works have already tested attenuated pathogens, such as *Salmonella enterica* and *Vibrio cholerae*, as live vectors for mucosal delivery of intimin (39–40). A safer alternative to deliver antigens at mucosal sites can be the use of lactic acid bacteria as live vectors. This strategy has been successfully applied with different antigens in animal experimentation (11, 16, 24, 33, 45, 67).

In a previous study, we showed that *Lactobacillus casei* expressing intimin fragments that contained the immunodominant epitopes of Int<sub>280</sub> induced specific antibodies in mice after nasal immunization. These antibodies were able to bind to the EPEC surface and to inhibit bacterial adhesion to epithelial cells *in vitro* (25), confirming the functionality of antibodies against intimin fragments that contain the N-terminal region of Int<sub>280</sub>. In the present work, we analyzed the ability of one of these recombinant bacteria, *L. casei*-Int<sub>cv</sub>, or the pure recombinant intimin fragment (Int<sub>cv</sub>) to induce humoral and cellular immune responses in mice after oral or sublingual (s.l.) immunization. Moreover, the protective potential of these vaccines was analyzed through the challenge of immunized mice with the murine pathogen *C. rodentium*.

## MATERIALS AND METHODS

**Bacterial strains, antigens, and growth conditions.** Recombinant *L. casei* (*L. casei*-Int<sub>cv</sub>) expressing a fragment containing the conserved plus the variable region of  $\beta$ -intimin (Int<sub>cv</sub>; amino acids 363 to 808) was previously described (25). Int<sub>cv</sub> expression in this strain is intracellular, and no protein is observed in the supernatants under the growth conditions used here, as measured by Western blots using anti-Int<sub>cv</sub> antibodies (data not shown). In addition, no reactivity of anti-Int<sub>cv</sub> antibodies with proteins exposed on the *L. casei*-Int<sub>cv</sub> surface was observed (data not shown). The amount of protein expressed was estimated as 70 to 90 ng per 10<sup>9</sup> bacteria (25). *L. casei*-Int<sub>cv</sub> and the control strain bearing the empty expression vector pTINX (*L. casei*) were grown in MRS (Difco, MI) containing 5  $\mu$ g/ml of erythromycin at 37°C without shaking (25). Recombinant Int<sub>cv</sub> was expressed in *E. coli* BL21 (salt induced [SI]) and purified through affinity chromatography, as previously described (25). *C. rodentium* DBS100 (21) was grown in Luria-Bertani broth (LB) (Difco) at 37°C with constant shaking. In colonization experiments, feces samples were homogenized and plated on MacConkey agar (Difco), and the plates were incubated at 37°C for 18 h for *C. rodentium* CFU counting. All bacterial stocks were maintained at –80°C in their respective culture media containing 20% glycerol.

**Immunization of mice.** Female specific-pathogen-free (SPF) C57BL/6 mice (5 to 7 weeks old; 4 or 5 animals per group) were obtained from The Jackson Laboratory (Bar Harbor, ME) or from the animal facility of Universidade Federal de São Paulo (CEDEME). Conventional C3H/HePas mice were obtained from the animal facility of Faculdade de Medicina Veterinária, Universidade de São Paulo. The mice were supplied with food and water *ad libitum*. The experimental procedures were previously approved by the Ethical Committee in Animal Experimentation from Instituto Butantan under license number 588/09 or by the University of California—San Diego (UCSD) Institutional Animal Care and Use Committee. Before starting the immunization protocols, preimmune

sera and feces were collected and analyzed for the presence of antibodies reacting with Int<sub>cv</sub> by enzyme-linked immunosorbent assay (ELISA). Animals presenting positive reactions were not used in the experiments. For immunization with recombinant *L. casei*, bacteria were grown until late stationary phase (optical density at 550 nm [OD<sub>550</sub>], 2.0), collected by centrifugation (16,000  $\times$  g for 6 min at room temperature), washed twice, and suspended in saline to 2  $\times$  10<sup>10</sup> CFU/ml for oral immunization or 10<sup>11</sup> CFU/ml for s.l. immunization. Prior to oral immunization, all mice received 0.2 M sodium bicarbonate in 300  $\mu$ l of saline by oral gavage. Mice were immunized through the oral route on days 0, 1, 2, 14, 15, 16, 28, 29, and 30 with 10<sup>10</sup> CFU of *L. casei*-Int<sub>cv</sub> or *L. casei* in 500  $\mu$ l of saline, using a gavage. Control mice received 500  $\mu$ l of saline. For the s.l. immunizations, mice were anesthetized by intraperitoneal injection with 0.2% xylazine and 1% ketamine in 200  $\mu$ l of saline, and the groups received 10<sup>9</sup> CFU of *L. casei* or *L. casei*-Int<sub>cv</sub> in 10  $\mu$ l of saline on days 0, 14, and 28. An additional group received 15  $\mu$ g of purified Int<sub>cv</sub> on the same days. Control mice received saline. For all immunizations with Int<sub>cv</sub>, the excess of lipopolysaccharide (LPS) was removed from the purified protein by Triton X-114 extraction (5). The final amount of LPS in these preparations was determined by the *Limulus* amoebocyte lysate test as 0.5 endotoxin units/ $\mu$ g protein. The vaccines were delivered with the help of a micropipette under the tongue and directed toward the floor of the mouth (20). Mice were maintained with their heads still and flexed up for 30 min. Subcutaneous immunization was used as a control for the capacity of the Int<sub>cv</sub> fragment to reduce *C. rodentium* colonization in mice (see the supplemental material).

**Measurement of antibodies by ELISA.** Fourteen days after the last immunization, blood was collected from the mice by retroorbital bleeding. Sera were collected after incubation of the samples for 30 min at 37°C, followed by incubation for 10 min at 4°C and centrifugation at 400  $\times$  g for 10 min. Clots were removed, and the sera were stored at –20°C until use. Feces were collected on the same day, weighed, and homogenized at a final concentration of 100 mg/ml in PBS containing 2% nonfat dry milk (in order to avoid nonspecific reactivity in ELISAs) and a protease inhibitor cocktail (Sigma, St. Louis, MO). Samples were centrifuged at 1,500  $\times$  g for 5 min, and the supernatants were stored at –80°C until use. Antibodies were evaluated by ELISA, using *E. coli*-produced recombinant Int<sub>cv</sub> as a coating (10  $\mu$ g/ml) and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG or anti-mouse IgA (Southern Biotech, Birmingham, AL). Standard curves were generated using mouse IgG or IgA (Southern Biotech).

**Detection of IFN- $\gamma$  or IL-6 in supernatants of spleen cell cultures by ELISA.** Spleens were obtained from immunized mice (4 animals per group) before or 10 days after challenge with *C. rodentium* (described below). Cell suspensions were prepared in RPMI medium (Gibco, Carlsbad, CA) as previously described (51), and 5  $\times$  10<sup>6</sup> cells/ml were plated in 48-well plates. The cells were stimulated for 72 h with 5  $\mu$ g/ml of Int<sub>cv</sub> at 37°C in 5% CO<sub>2</sub> (vol/vol). After this period, the supernatants were collected and stored at –80°C for later quantification of gamma interferon (IFN- $\gamma$ ) and interleukin 6 (IL-6). Secretion of IFN- $\gamma$  and IL-6 was evaluated by sandwich ELISA (Peprotech, Rocky Hill, NJ). The results were expressed as the concentrations of cytokines observed in supernatants from stimulated cells after subtraction of the cytokine levels in nonstimulated cultures.

**Infection of mice with *C. rodentium*.** *C. rodentium* was grown in LB at 37°C for 18 h, and the culture was diluted 50 $\times$  in fresh medium. Bacteria were incubated at 37°C, and the optical density was followed until the culture reached an OD<sub>600</sub> of 0.8. The bacteria were centrifuged, washed, and suspended at a concentration of 2.5  $\times$  10<sup>9</sup> CFU/ml in saline. C57BL/6 mice were infected 15 days after the last immunization with 200  $\mu$ l of the bacterial suspension (5  $\times$  10<sup>8</sup> CFU) by oral gavage. Fecal pellets were collected individually on days 4, 7, and 10 after challenge; weighed; and homogenized in 500  $\mu$ l of sterile saline. Serial dilutions of the fecal homogenates were plated onto MacConkey agar (Difco), and the CFU were determined after incubation for 18 h at 37°C. The minimal limit of detection was 100 CFU/g of feces. Infection of C3H/HePas mice (7 to 13 mice per group) was performed in the same way, and survival was recorded for 14 days. *C. rodentium* colonies were distinguished by morphology. Representative colonies in each experiment were submitted to PCR analysis using specific primers for  $\beta$ -intimin amplification. The results confirmed the identity of the *C. rodentium* colonies.

**Immunohistological analysis.** Nine days after *C. rodentium* infection, immunized C3H/HePas mice were euthanized and the colons were removed. This time point was chosen because clear differences between the states of health of mice from immunized and control groups were observed. The colons were opened longitudinally and prepared as Swiss rolls before fixation. The fixed tissues were embedded in paraffin, and 5- $\mu$ m sections were prepared. The sections were deparaffinized and incubated with 0.3% H<sub>2</sub>O<sub>2</sub> in PBS for 20 min at room temperature to inactivate endogenous peroxidase. Samples were blocked with

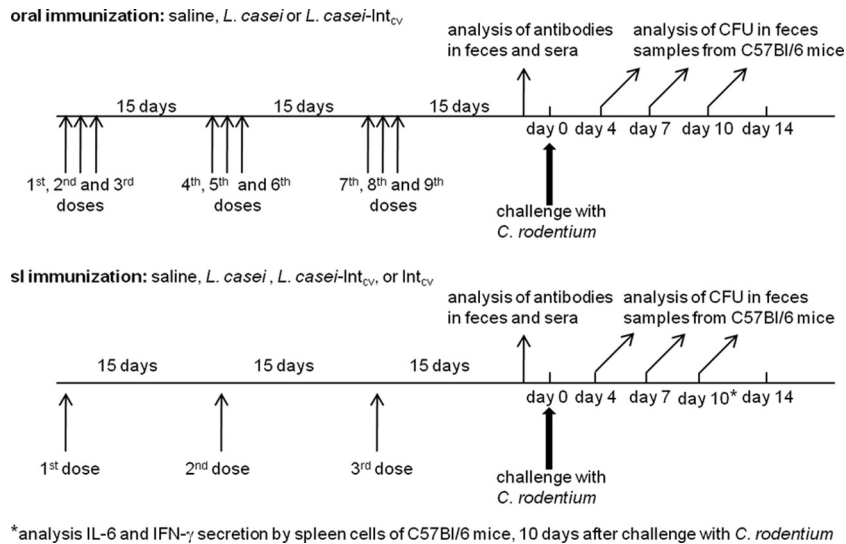


FIG. 1. Immunization and challenge of C57BL/6 and C3H/HePas mice with *C. rodentium*. Oral immunization was performed using a gavage. Mice received saline, *L. casei* ( $10^{10}$  CFU), or *L. casei*-Int<sub>cv</sub> ( $10^{10}$  CFU) on three consecutive days at 15-day intervals, totaling 9 doses. S.I. immunization was performed in three doses of saline, *L. casei* ( $10^9$  CFU), *L. casei*-Int<sub>cv</sub> ( $10^9$  CFU), or Int<sub>cv</sub> (15 μg). Fifteen days after the last immunization, the mice were challenged with  $5 \times 10^8$  CFU of *C. rodentium* by the oral route, and colonization or survival was followed in C57BL/6 or C3H/HePas mice, respectively. Anti-Int<sub>cv</sub> antibodies were measured in blood and feces before challenge or in feces at different periods after challenge. Secretion of cytokines by spleen cells collected at the endpoint of the colonization experiment (10 days after challenge) was measured in C57BL/6 mice immunized by the s.i. route.

PBS containing 2% bovine serum albumin (BSA) for 30 min at room temperature and incubated for 2 h in the same buffer containing a 1:5,000 dilution of polyclonal rabbit anti-intimin IgG-enriched fraction (41, 46). Sections were washed three times with PBS and incubated for 1 h at room temperature with biotin-labeled goat anti-rabbit IgG (Sigma) diluted in PBS-2% BSA. The samples were washed again and incubated for 30 min with ExtrAvidin-Peroxidase (Sigma). The sections were developed with 3,3'-diaminobenzidine and H<sub>2</sub>O<sub>2</sub> in 0.1 M Tris-HCl, pH 6.8, and counterstained with Gill's hematoxylin (Sigma). Slides were analyzed with the help of a light microscope (DMLB; Leica), and photographs were taken at magnifications of  $\times 10$  and  $\times 40$ .

**Statistical analysis.** Differences in survival times were analyzed with Kaplan-Meier survival curves. Differences in antibody concentrations and *C. rodentium* loads in the feces were analyzed by the Mann-Whitney *U* test. Differences in cytokine secretion by spleen cells were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's test. In all cases, a *P* value of  $\leq 0.05$  was considered significantly different.

RESULTS

**Induction of antibodies by immunization of mice with recombinant *L. casei* or Int<sub>cv</sub>.** Before starting the immunization experiments, we determined the permanency of the recombinant *L. casei* and *L. casei*-Int<sub>cv</sub> in the intestinal mucosa of C57BL/6 mice by determining the recovery of erythromycin-resistant colonies in feces. Recombinant *L. casei* or *L. casei*-Int<sub>cv</sub> could be recovered from feces for up to 48 h after a single oral inoculation, and after that point, no erythromycin-resistant colonies were observed. No differences were observed between the inoculation of *L. casei* or *L. casei*-Int<sub>cv</sub> (data not shown). C57BL/6 mice were then immunized through the oral or s.i. route with *L. casei*-Int<sub>cv</sub>, or through the s.i. route with the recombinant Int<sub>cv</sub> fragment, according to the protocols described and the scheme presented in Fig. 1. As controls, mice were immunized with *L. casei* or with saline. The levels of anti-Int<sub>cv</sub> IgA detected in feces collected 14 days after the last immunization are shown on Fig. 2A and B. Both oral and s.i.

immunization of mice with *L. casei*-Int<sub>cv</sub> induced significantly higher levels of anti-Int<sub>cv</sub> IgA in feces than in those of mice that received saline or *L. casei* (for oral immunization, *P* = 0.01 or *P* = 0.02 when mice that received *L. casei*-Int<sub>cv</sub> were compared with mice that received saline or *L. casei*, respectively; for s.i. immunization, *P* = 0.01 or *P* = 0.002 when mice that received *L. casei*-Int<sub>cv</sub> were compared with mice that received saline or *L. casei*, respectively). On the other hand, no induction of anti-Int<sub>cv</sub> IgA was observed in feces from mice immunized with Int<sub>cv</sub> by the s.i. route (Fig. 2B). Conversely, evaluation of anti-Int<sub>cv</sub> IgG in the sera (Fig. 2C and D) of mice immunized with the different formulations revealed that only the s.i. vaccination with Int<sub>cv</sub> induced significantly higher concentrations of these antibodies than in mice that received saline or *L. casei*, respectively). No systemic induction of anti-Int<sub>cv</sub> IgG was observed in the sera of mice immunized with *L. casei*-Int<sub>cv</sub> by the oral (Fig. 2C) or s.i. (Fig. 2D) route.

**Decrease in *C. rodentium* colonization by immunization of mice with *L. casei*-Int<sub>cv</sub> and Int<sub>cv</sub>.** Before testing the effectiveness of *L. casei*-based vaccines against the murine model of *C. rodentium* infection, we decided to validate the use of the Int<sub>cv</sub> fragment as an antigen against *C. rodentium* colonization. In a previous work, Ghaem-Maghami and collaborators (30) showed that subcutaneous immunization of mice with a fragment of  $\alpha$ -intimin containing the 280 carboxy-terminal amino acids of the protein (Int 280 $\alpha$ ) induced specific immune responses and significantly reduced colon colonization by a *C. rodentium* strain that expresses  $\alpha$ -intimin. We thus performed experiments of subcutaneous immunization of C57BL/6 mice with the Int<sub>cv</sub> fragment. The immunization induced significantly higher concentrations of anti-Int<sub>cv</sub> IgA in feces (*P* = 0.01), as well as anti-Int<sub>cv</sub> IgG in sera (*P* = 0.0001), than the inoculation of saline (see Fig. S1A and B in the supplemental

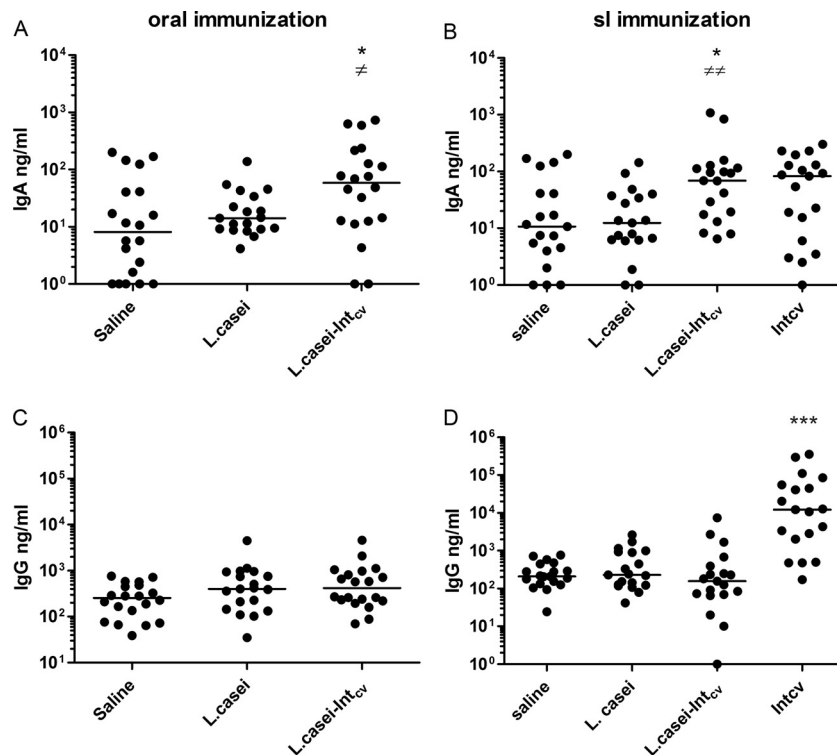


FIG. 2. Induction of antibodies by sublingual or oral immunization of C57BL/6 mice with *L. casei*-Int<sub>cv</sub> or Int<sub>cv</sub>. (A and C) Anti-Int<sub>cv</sub> IgA in feces (A) and anti-Int<sub>cv</sub> IgG in sera (C) from mice that received the formulations through the oral route. (B and D) Anti-Int<sub>cv</sub> IgA in feces (B) and anti-Int<sub>cv</sub> IgG in sera (D) from mice that received the formulations through the s.l. route. The circles represent individual concentrations, and the lines represent the medians of the groups. The levels of antibodies were determined by ELISA using standard concentration curves. The asterisks indicate data that were significantly different from those observed in mice inoculated with saline: \*,  $P = 0.01$ , and \*\*\*,  $P = 0.0001$ . # indicates data that were significantly different from those observed in mice inoculated with *L. casei*: #,  $P = 0.02$ , and #=,  $P = 0.002$ . Statistical analyses were performed by the Mann-Whitney  $U$  test. The results represent four experiments.

material). Evaluation of *C. rodentium* challenge in immunized mice revealed that significantly lower levels of bacteria were recovered from mouse feces than were observed in mice inoculated with saline. This reduction was observed at all points evaluated, which were on days 4 ( $P = 0.002$ ), 7 ( $P = 0.008$ ), and 10 ( $P = 0.0004$ ) (see Fig. S2A, B, and C, respectively, in the supplemental material). The levels of *C. rodentium* recovery from the feces of all C57BL/6 mice (both control and immunized groups) were drastically reduced after the 10th day postinfection, impairing the analysis of the effects induced by the vaccines. Therefore, this was used as the endpoint in all subsequent experiments.

Groups of mice inoculated with saline or immunized with *L. casei* or *L. casei*-Int<sub>cv</sub> through the oral or s.l. route were challenged with *C. rodentium* 15 days after the last immunizations. Feces were collected at the determined intervals, and CFU were evaluated after plating the samples onto MacConkey agar. Oral immunization of mice with *L. casei*-Int<sub>cv</sub> led to a significant reduction in *C. rodentium* recovery from feces on days 4 and 7 after challenge, compared with the control groups, which received saline ( $P = 0.0001$  on day 4 and  $P = 0.0003$  on day 7) or *L. casei* ( $P = 0.0002$  on day 4 and  $P = 0.0001$  on day 7) (Fig. 3A and B). However, 10 days after challenge, the reduction observed was less evident, and the recovery of *C. rodentium* at this point was only significantly different from that observed in mice that received saline ( $P = 0.01$ ) (Fig. 3C).

After this point, all mice displayed a marked reduction in *C. rodentium* loads in feces and recovered from infection (data not shown). Similarly, s.l. immunization of mice with *L. casei*-Int<sub>cv</sub> led to a significant decrease in *C. rodentium* recovery from feces on days 4 ( $P = 0.008$  compared with mice that received saline) and 7 ( $P = 0.001$  and  $P = 0.007$  compared with mice that received saline or *L. casei*, respectively) (Fig. 3D and E). Moreover, mice inoculated with *L. casei*-Int<sub>cv</sub> through the s.l. route still displayed a significant reduction in fecal loads of *C. rodentium* compared with mice inoculated with saline ( $P = 0.004$ ) or *L. casei* ( $P = 0.005$ ) 10 days after the challenge (Fig. 3F). Notably, in at least 4 animals from the group immunized with *L. casei*-Int<sub>cv</sub>, *C. rodentium* recovery was below the limit of detection throughout the experiment (Fig. 3D to F). Sublingual immunization of mice with the recombinant fragment Int<sub>cv</sub> induced a significant reduction in *C. rodentium* recovery from feces, compared with mice inoculated with saline, only at day 4 after infection ( $P = 0.006$ ) (Fig. 3D). However, after this time point, the CFU levels were not statistically different from those observed in mice inoculated with saline (Fig. 3E and F). As observed in the experiments performed with oral immunization, all mice recovered from infection after day 10, displaying a marked reduction in *C. rodentium* colonization (data not shown).

**Immune responses after *C. rodentium* challenge in mice immunized through the s.l. route.** The *L. casei*-Int<sub>cv</sub> vaccine pro-

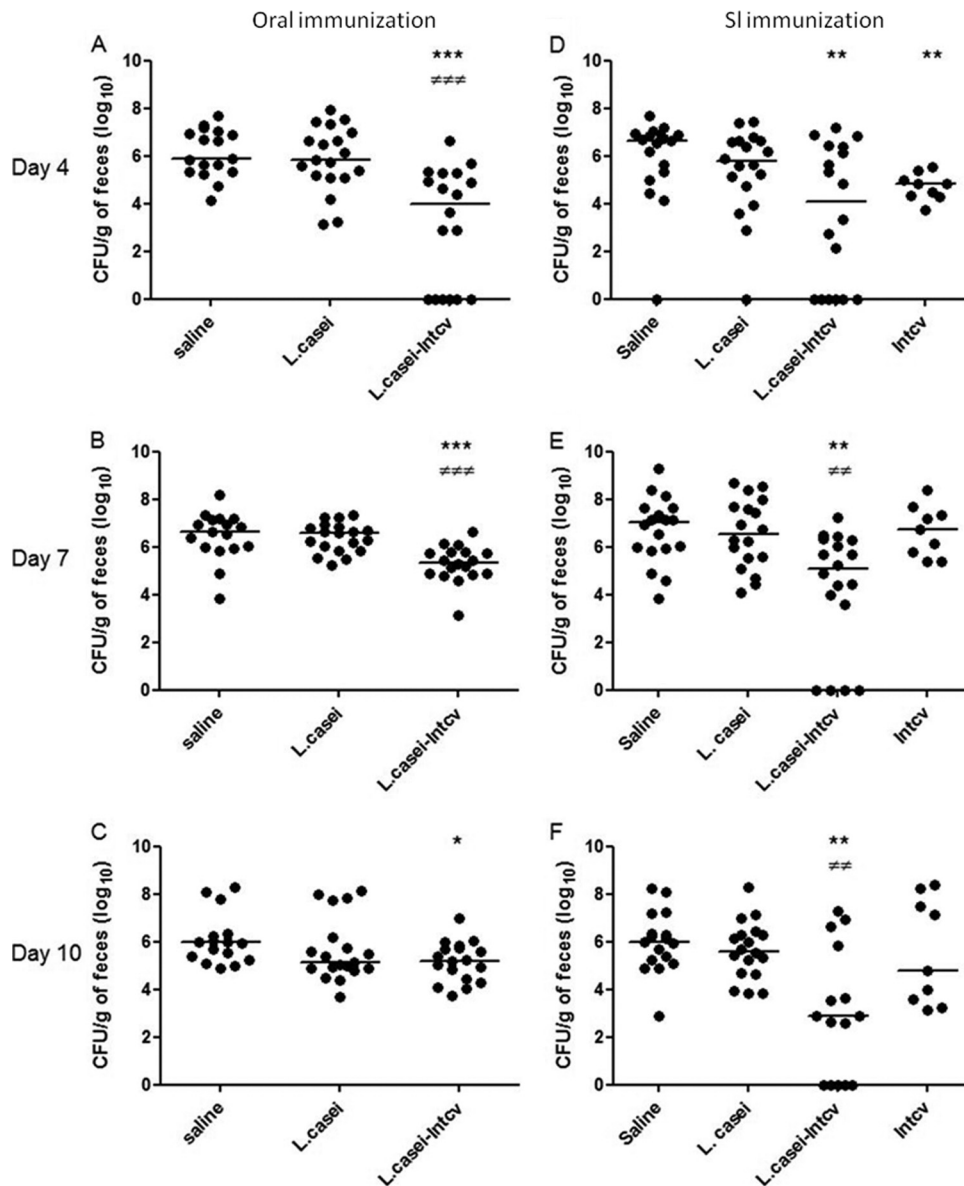


FIG. 3. Recovery of *C. rodentium* in feces of C57BL/6 immunized mice. (A, B, and C) Mice immunized with the different formulations through the oral route. (D, E, and F) Mice immunized with the different formulations through the s.l. route. Two weeks after the last administration, the groups were challenged with *C. rodentium*. Feces samples were collected on days 4 (A and D), 7 (B and E), and 10 (C and F) after challenge and plated for *C. rodentium* CFU counting. Zero represents no CFU detection, and the minimal limit of detection was 100 CFU/g of feces. The asterisks indicate data that were significantly different from those observed in mice inoculated with saline: \*\*\*,  $P = 0.0001$  (day 4) or  $0.0003$  (day 7), and \*,  $P = 0.01$  (day 10) for oral immunization; \*\*,  $P = 0.008$  and  $0.006$  (day 4, for *L. casei*-Int<sub>cv</sub> and Int<sub>cv</sub>, respectively); \*\*,  $P = 0.001$  (day 7) and  $0.004$  (day 10) for s.l. immunization. ≠ indicates data that were significantly different from those observed in mice inoculated with *L. casei*: ≠≠≠,  $P = 0.0002$  (day 4) or  $0.0001$  (day 7) for oral immunization; ≠≠,  $P = 0.007$  (day 7) or  $0.005$  (day 10) for sublingual immunization. Statistical analyses were performed by the Mann-Whitney *U* test. The results represent at least two experiments.

duced the best effect on the reduction of *C. rodentium* colonization in the C57BL/6 mouse model. Therefore, we decided to use these conditions to investigate the immune responses in immunized mice elicited after challenge with *C. rodentium*. The profile of anti-Int<sub>cv</sub> IgA responses in feces of mice immunized with *L. casei*-Int<sub>cv</sub> through the s.l. route at different time points after infection with *C. rodentium* is shown in Table 1. Significantly higher levels of anti-Int<sub>cv</sub> IgA in feces were observed in mice immunized with *L. casei*-Int<sub>cv</sub> before challenge

( $P = 0.02$  and  $P = 0.01$  compared with mice inoculated with saline or *L. casei*, respectively) and 12 h ( $P = 0.02$  compared with mice inoculated with saline or *L. casei*), as well as 4 days ( $P = 0.02$  compared with mice inoculated with saline or *L. casei*), after challenge (Table 1). Anti-Int<sub>cv</sub> IgA detected in feces from mice immunized with *L. casei*-Int<sub>cv</sub> through the s.l. route reached a mean concentration of 132 ng/ml 12 h after challenge, slightly higher than the mean concentration detected before challenge (101 ng/ml). Although a decrease in

TABLE 1. Anti-Int<sub>cv</sub> IgA concentrations and recovery of *C. rodentium* in feces from C57BL/6 mice immunized by the s.l. route<sup>i</sup>

Immunization	IgA (ng/ml) before challenge	12 h		Day 4		Day 10	
		IgA (ng/ml)	CFU/g of feces (log <sub>10</sub> )	IgA (ng/ml)	CFU/g of feces (log <sub>10</sub> )	IgA (ng/ml)	CFU/g of feces (log <sub>10</sub> )
Saline	8 ± 11	15 ± 17	5.33 ± 0.4	21 ± 13	9.18 ± 0.32	111 ± 69	6.69 ± 0.37
<i>L. casei</i>	10 ± 8	14 ± 13	5.78 ± 0.7	16 ± 18	8.82 ± 0.7	26 ± 40	6.2 ± 0.83
<i>L. casei</i> -Int <sub>cv</sub>	101 ± 55 <sup>a,c</sup>	132 ± 29 <sup>a,f</sup>	4.72 ± 0.63 <sup>g</sup>	70 ± 22 <sup>b,h</sup>	7.7 ± 0.6 <sup>c</sup>	24 ± 28 <sup>b</sup>	5.6 ± 0.59 <sup>d</sup>

<sup>a,b,c,d</sup> Significantly different from saline ( $P = 0.02$ ,  $P = 0.03$ ,  $P = 0.001$ , and  $P = 0.01$ , respectively).

<sup>e,f,g,h</sup> Significantly different from *L. casei*; ( $P = 0.01$ ,  $P = 0.02$ ,  $P = 0.05$ , and  $P = 0.03$ , respectively).

<sup>i</sup> Statistical analyses were performed by the Mann-Whitney *U* test.

anti-Int<sub>cv</sub> IgA detection was observed 4 days after challenge, the concentrations were still significantly higher in samples collected from mice immunized with *L. casei*-Int<sub>cv</sub> than in those from mice immunized with *L. casei* or saline. Further reduction in detection of anti-Int<sub>cv</sub> IgA was observed after that, reaching a mean concentration of 24 ng/ml 10 days after challenge. Conversely, anti-Int<sub>cv</sub> IgA concentrations in mice inoculated with saline reached a mean value of 111 ng/ml only 10 days after infection (Table 1). Significantly lower levels of *C. rodentium* could be recovered from the feces of mice immunized with *L. casei*-Int<sub>cv</sub> than from mice inoculated with saline or *L. casei*. This reduction was statistically significant at 12 h after infection compared with mice immunized with *L. casei* ( $P = 0.05$ ) and was statistically significant on days 4 ( $P = 0.03$ ) and 10 ( $P = 0.01$ ) after infection compared with mice inoculated with saline (Table 1).

Besides the induction of antibodies, some cytokines, such as IL-6 and IFN- $\gamma$ , have been reported to participate in the control of infections with A/E pathogens (21, 34, 43, 63). Therefore, we decided to analyze the possible induction of these cytokines by the *L. casei*-Int<sub>cv</sub> s.l. vaccine. Secretion of IL-6 and IFN- $\gamma$  was evaluated in spleen cells collected from mice immunized with *L. casei*-Int<sub>cv</sub> and stimulated *in vitro* with Int<sub>cv</sub>. However, due to the variability observed among animals, the results were inconclusive (data not shown). We then decided to analyze the secretion of these cytokines by spleen cells col-

lected from immunized mice 10 days after challenge with *C. rodentium* (the endpoint of the colonization experiment), and stimulated *in vitro* with Int<sub>cv</sub>. Cells from mice immunized with the *L. casei*-Int<sub>cv</sub> vaccine through the s.l. route showed a significant increase in IL-6 secretion 10 days after *C. rodentium* infection compared with cells from mice inoculated with *L. casei* ( $P < 0.01$ ) or saline ( $P < 0.001$ ) (Fig. 4A). Moreover, the levels of IL-6 were also significantly higher than the levels observed in cells obtained from animals immunized with Int<sub>cv</sub> ( $P < 0.05$ ). On the other hand, the levels of IL-6 secretion by cells collected from mice immunized with Int<sub>cv</sub>, were not statistically different from the levels secreted by cells collected from mice that received saline. Secretion of IFN- $\gamma$  by spleen cells was also evaluated in mice immunized through the s.l. route 10 days after challenge with *C. rodentium*. Once again, the highest levels of secretion were observed in cells from mice immunized with *L. casei*-Int<sub>cv</sub>, and the results were significantly different from those observed in cells from mice immunized with *L. casei* ( $P < 0.05$ ) or inoculated with saline ( $P < 0.05$ ) (Fig. 4B). Although higher levels of IFN- $\gamma$  secretion were also observed in cells collected from mice immunized with the Int<sub>cv</sub> fragment through the s.l. route, the results were not statistically different from the levels secreted by cells from animals that received saline (Fig. 4B).

***L. casei*-Int<sub>cv</sub> vaccine extends the survival time of C3H/HePas mice after *C. rodentium* challenge.** The effect of *L.*

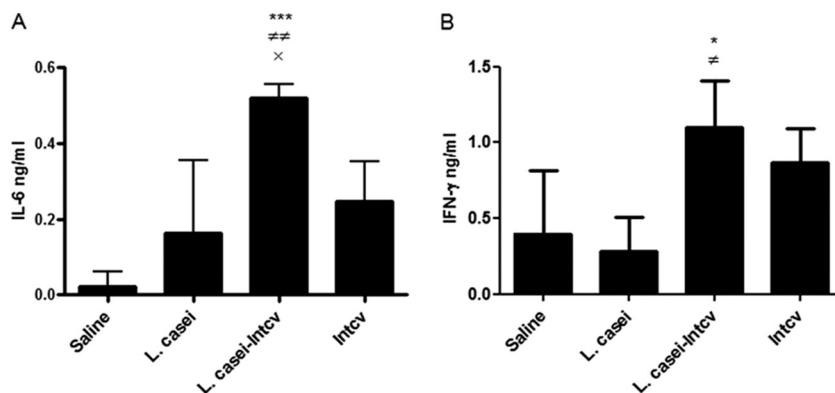


FIG. 4. Secretion of cytokines by spleen cells induced by s.l. immunization of C57BL/6 mice with *L. casei*-Int<sub>cv</sub>. Spleen cells were isolated from immunized mice 10 days after challenge with *C. rodentium* and were stimulated *in vitro* with Int<sub>cv</sub> for 72 h. Secretion of IL-6 (A) and IFN- $\gamma$  (B) in supernatants was detected through sandwich ELISA. The bars represent the means of the groups, with standard deviations, after subtracting the levels of cytokines in nonstimulated cells. The asterisks indicate data that were significantly different from those observed in mice inoculated with saline: \*,  $P < 0.05$ , and \*\*\*,  $P < 0.001$ .  $\neq$  indicates data that were significantly different from those observed in mice inoculated with *L. casei*:  $\neq$ ,  $P < 0.05$ , and  $\neq\neq$ ,  $P < 0.01$ .  $\times$  indicates data that were significantly different from those observed in mice immunized with Int<sub>cv</sub>:  $P < 0.05$ . Statistical analyses were performed by Tukey's test.

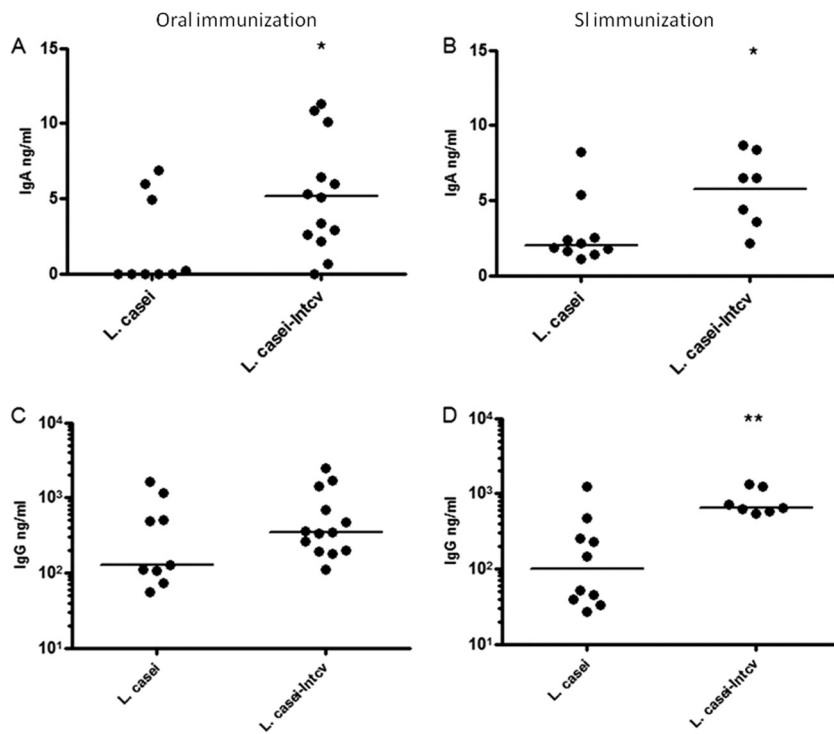


FIG. 5. Induction of antibodies by oral or sublingual immunization of C3H/HePas mice with recombinant *L. casei*. (A and C) Anti-Int<sub>cv</sub> IgA in feces (A) and anti Int<sub>cv</sub> IgG in sera (C) from mice that received the formulations through the oral route. (B and D) Anti-Int<sub>cv</sub> IgA in feces (B) and anti-Int<sub>cv</sub> IgG in sera (D) from mice that received the formulations through the s.l. route. The circles represent individual concentrations, and the lines represent the medians of the groups. The levels of antibodies were determined by ELISA using standard concentration curves. The asterisks indicate data that were significantly different from those observed in mice immunized with *L. casei*. \*,  $P = 0.04$  and  $0.02$  for orally and s.l. immunized groups, respectively; \*\*,  $P = 0.003$ . Statistical analyses were performed by the Mann-Whitney  $U$  test. The results represent two experiments.

*casei*-Int<sub>cv</sub> vaccination through both oral and s.l. routes was evaluated in a C3H-derived mouse strain (C3H/HePas) that is highly susceptible to *C. rodentium* infection (64). The permanency of *L. casei* and *L. casei*-Int<sub>cv</sub> in the intestinal mucosa of C3H/HePas mice was determined as described for C57BL/6 mice, and the results showed that *L. casei* and *L. casei*-Int<sub>cv</sub> could be recovered from feces for up to 24 h after a single inoculation. After this time point, no erythromycin-resistant *L. casei* colonies were observed in feces and no differences were observed between the two *L. casei* strains (data not shown). Immunization of mice with *L. casei*-Int<sub>cv</sub> by the oral route induced significantly higher concentrations of anti-Int<sub>cv</sub> IgA in feces than the immunization of mice with *L. casei* ( $P = 0.04$ ) (Fig. 5A). However, the concentrations of anti-Int<sub>cv</sub> IgG observed in sera from mice immunized with these formulations were not statistically different (Fig. 5C). Sublingual immunization of C3H/HePas mice with *L. casei*-Int<sub>cv</sub> instead induced significantly higher levels of both anti-Int<sub>cv</sub> IgA in feces ( $P = 0.02$ ) (Fig. 5B) and IgG in sera ( $P = 0.003$ ) (Fig. 5D) than in mice immunized with *L. casei*. C3H/HePas mice immunized with both formulations by the oral or the s.l. route were challenged with *C. rodentium*, and survival was followed for 14 days. The group that received *L. casei*-Int<sub>cv</sub> by the oral route showed a significant increase in mean survival time after challenge compared with the group immunized with *L. casei* ( $P = 0.0001$ ) (Fig. 6A). Mice immunized by the oral route with *L. casei* died between days 8 and 10 after infection (no mice

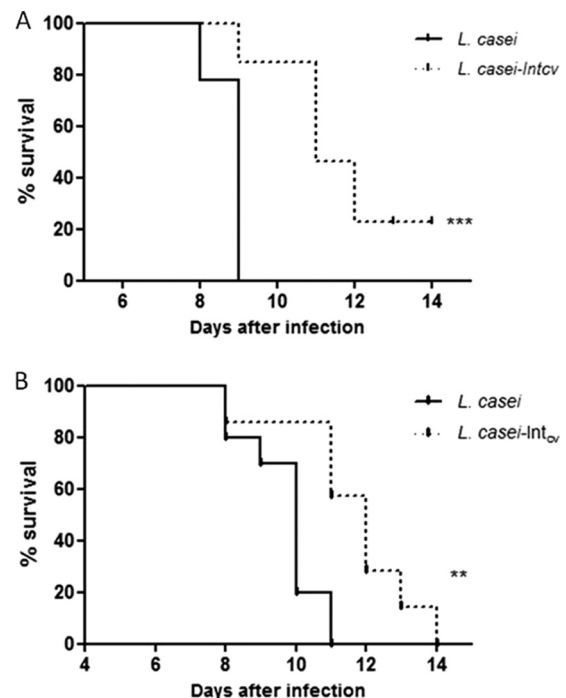


FIG. 6. Survival of immunized C3H/HePas mice after *C. rodentium* challenge. Mice immunized with *L. casei* (solid lines) or *L. casei*-Int<sub>cv</sub> (dotted lines) by the oral (A) or s.l. (B) route were infected with *C. rodentium*. Survival was followed for 14 days. \*\*\*,  $P = 0.0001$ ; \*\*,  $P = 0.007$ , using Kaplan-Meier survival curve analysis.



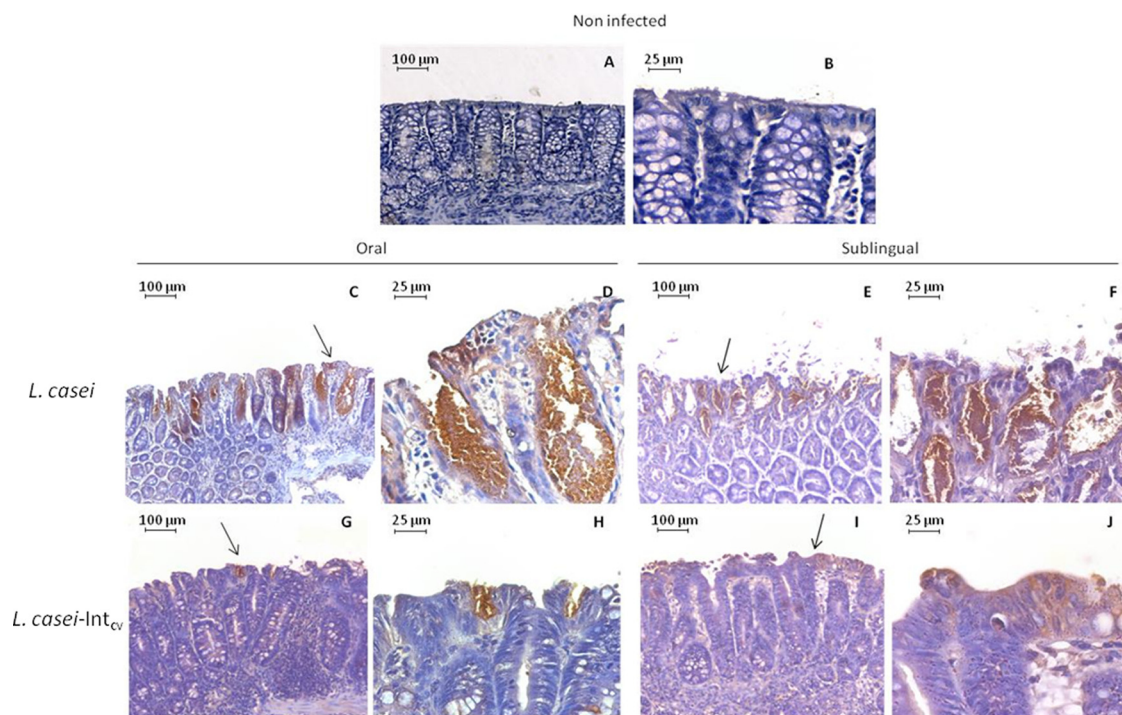


FIG. 7. Mucosal damage and *C. rodentium* localization in colon sections of C3H/HePas mice immunized with recombinant *L. casei*. Colon segments from C3H/HePas mice immunized with *L. casei* or *L. casei-Int<sub>cv</sub>* were collected 9 days after challenge. Paraffin sections were submitted to immunohistological analysis for *C. rodentium* staining, using a rabbit polyclonal anti-intimin IgG-enriched fraction. (A and B) Noninfected control mice. Mice immunized with *L. casei* (C and D) or *L. casei-Int<sub>cv</sub>* (G and H) through the oral route. Mice immunized with *L. casei* (E and F) or *L. casei-Int<sub>cv</sub>* (I and J) through the s.l. route. The photographs in panels A, C, E, G, and I were taken at a magnification of  $\times 10$ ; the photographs in panels B, D, F, H, and J were taken at a magnification of  $\times 40$ . All photographs were processed equally for each panel. The arrows indicate the amplified regions.

survived from a total of 9 mice), while most mice immunized with *L. casei-Int<sub>cv</sub>* died between days 11 and 12 after infection. At day 14 after infection, three mice (from a total of 13 mice) remained alive, and none of them displayed any sign of disease. Similarly, immunization of mice with *L. casei-Int<sub>cv</sub>* through the s.l. route significantly extended the mean survival time of the group compared with the immunization of mice with *L. casei* ( $P = 0.007$ ). Mice that received *L. casei* through the s.l. route died between days 8 and 11 (no mice survived from a total of 10 mice), while the majority of animals from the *L. casei-Int<sub>cv</sub>* group died between days 11 and 14 (no mice survived from a total of 7 mice) (Fig. 6B). However, in this case, no mice survived the challenge. Sections of the colons from mice immunized through both the oral and s.l. routes with *L. casei* and *L. casei-Int<sub>cv</sub>* were prepared 9 days after infection and were stained for the detection of *C. rodentium*. At this time point, the differences observed between the animals from the *L. casei* or the *L. casei-Int<sub>cv</sub>* group regarding the signs of disease were more evident. Colon sections collected from all infected mice were positive for *C. rodentium* staining (Fig. 7C to J). However, damage to the intestinal epithelium was clearly in an advanced stage in mice immunized with *L. casei* by the oral and s.l. routes 9 days after challenge. At this stage, *C. rodentium* staining was observed in deep regions of the crypts (Fig. 7C to F). Conversely, mice immunized with *L. casei-Int<sub>cv</sub>* displayed a more preserved structure of the intestinal epithelium, and *C. rodentium* staining was observed in superficial layers (Fig. 7G to J). No specific staining was observed in uninfected mice used as controls (Fig. 7A and B).

## DISCUSSION

Oral vaccines against gastrointestinal infections usually require a delivery system able to protect the antigen from the aggressive environment found at the mucosa. Several strategies are designed to optimize antigen presentation and the induction of immune responses, which may improve protection. Lactic acid bacteria can resist passage through the gastrointestinal tract, are usually safe when administered through mucosal routes, and have the potential to express heterologous antigens. All these characteristics make them attractive alternatives for the development of mucosal live vaccines. *L. casei* strains expressing several heterologous antigens have been tested as vaccines in animal models, with promising results (2, 16, 24, 32, 42, 54). In a previous work, we engineered *L. casei* to express the Int<sub>cv</sub> fragment from  $\beta$ -intimin (*L. casei-Int<sub>cv</sub>*). Nasal immunization of mice with the recombinant *L. casei* elicited antibodies that were able to bind to the EPEC surface and inhibited bacterial adhesion to epithelial cells *in vitro* (25). The efficacy of subcutaneous immunization of mice with the Int<sub>cv</sub> fragment against colonization with the murine pathogen *C. rodentium* was shown in the present work, confirming the protective potential of such a fragment *in vivo*. *L. casei-Int<sub>cv</sub>* was therefore tested here as live oral and s.l. vaccines in mice against infections with *C. rodentium*. The s.l. route has been used for many years as an effective way to deliver low-molecular-weight drugs (71) and small immunogenic peptides, eliciting systemic responses (10). The maintenance of the formulation in the s.l. mucosa prior to swallowing allows antigen

uptake by dendritic cells that are present in the thin s.l. epithelium (53). Modulation of the immune system after administration of different lactobacillus species by the s.l. route in mice has been described, especially for animal models of allergy (44, 65, 66). However, the use of this route for vaccination, using recombinant lactic acid bacteria expressing heterologous antigens, has been less explored (22).

Oral and s.l. immunization of mice with *L. casei*-Int<sub>cv</sub> did not induce significantly higher concentrations of anti-Int<sub>cv</sub> IgG in sera than controls. Still, vaccinated mice displayed significantly higher concentrations of anti-Int<sub>cv</sub> IgA in feces. Very few studies in the literature report the induction of anti-intimin IgA in gut mucosal sites, especially after mucosal immunization, and this effect usually requires the use of potent adjuvants or attenuated pathogens (14, 40). Here, we have shown that *L. casei*-Int<sub>cv</sub> vaccine formulations are capable of inducing specific IgA responses in the gut mucosa, the site of infection for pathogens that cause A/E lesions, without the use of additional adjuvants. In the present work, we used an *L. casei* strain expressing the Int<sub>cv</sub> fragment in the intracellular compartment. Different works have tested the efficacy of lactobacilli as vaccine vectors expressing heterologous antigens at different locations (67). After a comparison of *Lactobacillus plantarum* expressing the C fragment of the tetanus toxin at different cellular locations (intracellular, secreted, or cell surface exposed), Reveneau and collaborators concluded that the immunization of mice by the oral or intranasal route with the strain that expressed the protein intracellularly was the best way to induce specific systemic and mucosal antibodies (61). In our hands, the intracellular expression of Int<sub>cv</sub> was more efficient for inducing antibodies in mice than strains expressing the same antigen exposed on the cell surface. This may be related to undesirable processing of the antigen attached to the cell wall, resulting in the release of a great part of the heterologous protein into the growth medium (unpublished data from our group). In the case of the *L. casei*-Int<sub>cv</sub> strain, capture of the bacteria by antigen-presenting cells at mucosal surfaces should occur, resulting in the presentation of Int<sub>cv</sub> peptides by major histocompatibility complex (MHC) class II molecules and the induction of antibody-producing cells. In the context of the common mucosal immune system (CMIS), secretion of antibodies at distant effector sites, as well as the induction of systemic antibodies (as occurs after sublingual immunization, for example), takes place. Challenge of immunized mice showed that the *L. casei*-Int<sub>cv</sub> oral or s.l. vaccine significantly decreased intestinal colonization by *C. rodentium*. This effect was more evident in mice that received *L. casei*-Int<sub>cv</sub> through the s.l. route, as this group showed a decrease in *C. rodentium* recovery from feces from the 4th day after infection until the 10th day after challenge. In this work, we have not evaluated bacterial colonization in intestinal tissues of C57BL/6 mice, such as the colon or cecum, because this would hamper the analysis of colonization kinetics in each animal. In previous works, Wiles and collaborators used bioluminescence imaging to follow *C. rodentium* (using bioluminescent strains that derive from the DBS100 strain) infection of C57BL/6 naïve mice and observed a pattern very similar to what we have observed in the present work. According to their results, the highest *in vivo* signal of bacterial infection could be detected on day 8 postinfection. This was accompanied by a plateau in bacterial

shedding from stools. Bioluminescence started to decrease on day 10 postinfection, and a very low signal was observed in only one animal on day 15 postinfection (69). Moreover, a tight correlation between bioluminescence and CFU counts obtained from stools was observed (68). These results confirm that bacterial recovery from feces reflects the burden of *C. rodentium* infection in C57BL/6 mice and support our analysis.

In a previous work, Maaser and collaborators (43) showed that clearance of *C. rodentium* colonization by nonimmunized mice is dependent on the presence of IgG against the bacteria. Administration of sera from mice that recovered from *C. rodentium* colonization protected naïve mice against infection. However, when IgG was depleted from these sera, protection was no longer observed (43). Sublingual immunization of mice with the purified Int<sub>cv</sub> fragment induced significantly higher concentrations of anti-Int<sub>cv</sub> IgG in sera but no anti-Int<sub>cv</sub> IgA in feces. The vaccine significantly reduced *C. rodentium* recovery from feces on the fourth day after challenge. However, this reduction was not maintained in the following days of collection compared with mice inoculated with saline. It is possible that the levels of IgG induced were not high enough to maintain low levels of colonization. When the same fragment was inoculated through the subcutaneous route, anti-Int<sub>cv</sub> IgG concentrations in sera were around 10 to 100 times higher than that observed in mice immunized through the s.l. route. Similar amounts of anti-Int<sub>cv</sub> IgG1 and IgG2a were observed in both immunizations (with IgG1/IgG2a ratios of 2.5 and 1.3 for the subcutaneous and s.l. immunization, respectively), indicating a balanced Th1/Th2 profile. However, only subcutaneous immunization with Int<sub>cv</sub> elicited significantly higher concentrations of anti-Int<sub>cv</sub> IgA. Thus, higher levels of total anti-Int<sub>cv</sub> IgG in sera and the induction of significant levels of anti-Int<sub>cv</sub> IgA in feces may have contributed to the reduction in *C. rodentium* recovery from feces in subcutaneously immunized mice throughout the experiment (see the supplemental material). On the other hand, in the *L. casei* system, protection elicited by *L. casei*-Int<sub>cv</sub> vaccines was observed even under conditions where no significant induction of anti-Int<sub>cv</sub> IgG in sera was detected. In addition, no anti-Int<sub>cv</sub> IgG was detected in mouse feces (data not shown). These data imply that other mechanisms are involved in the protection elicited by *L. casei*-Int<sub>cv</sub> oral and s.l. vaccines. The kinetics of IgA induction and *C. rodentium* CFU recovery from feces suggests that the localized anti-Int<sub>cv</sub> IgA response induced by *L. casei*-Int<sub>cv</sub> s.l. vaccine may have contributed to the lower number of bacteria observed after the early phases of infection by preventing or reducing initial attachment of the bacteria to the epithelial gut. Detection of these antibodies was progressively less after infection, and one possible explanation for this observation may be related to the consumption or the turnover of antibodies after infection. Interestingly, in the group of mice inoculated with saline, anti-Int<sub>cv</sub> IgA in feces could be detected 10 days after challenge with *C. rodentium*, a time point that precedes the clearance of the bacteria in our model. Other groups have described the induction of mucosal and systemic antibodies against *C. rodentium* antigens (including intimin) in naïve mice, and this was followed by bacterial clearance (13, 30). Despite these observations, we were not able to determine a significant negative correlation between *C. rodentium* recovery from feces and the concentrations of anti-Int<sub>cv</sub> IgA. Similar results were

reported by Khare and colleagues (40), who showed that an oral vaccine based on attenuated *Salmonella enterica* expressing intimin was able to induce IgA in feces and to reduce *E. coli* O157:H7 shedding in cattle. In their study, the magnitude of bacterial shedding also was not negatively correlated with the levels of anti-intimin IgA (40).

Dann and collaborators have demonstrated that the IL-6 cytokine plays an important role in intestinal defenses against A/E pathogens (21). IL-6-deficient mice failed to control *C. rodentium* intestinal colonization and displayed an increase in the apoptosis of epithelial colon cells with subsequent ulcerations after infection. Higher mortality rates were observed in these mice than in wild-type mice (21). Infection of human epithelial colon cells with EPEC strains activates the NF- $\kappa$ B transcription factor, a key element in the regulation of *il-6* gene transcription (63). Together, these results suggest that the mechanisms of IL-6 expression in bacterial intestinal infections work similarly in humans and mice and play an important role in protection against these pathogens. In our experiments, a significant increase in IL-6 secretion by spleen cells 10 days after challenge was observed only in C57BL/6 mice immunized with *L. casei*-Int<sub>cv</sub> through the s.l. route, the group which showed the best reduction in *C. rodentium* colonization. In addition, secretion of IFN- $\gamma$  by spleen cells was also significantly higher in this group of mice than in control groups. This cytokine was also described as a key factor for protection against these pathogens, and its expression was increased in cells from colons of mice infected with *C. rodentium* (34, 43).

Immunization of C3H/HePas, a mouse strain highly susceptible to *C. rodentium* infection, with both oral and s.l. *L. casei*-Int<sub>cv</sub> vaccines significantly extended the survival of these mice after challenge. The s.l. vaccine induced significantly higher levels of anti-Int<sub>cv</sub> IgG in sera and IgA in the feces of these mice, whereas the oral vaccine induced only slightly higher concentrations of anti-Int<sub>cv</sub> IgG in sera and significantly higher levels of anti-Int<sub>cv</sub> IgA in feces. *C. rodentium* infection causes inflammation in colon mucosa (43). In C3H mice, this effect is particularly severe, with more prominent submucosal edema, epithelial damage, crypt hyperplasia, and goblet cell depletion (64). Analysis of colon sections obtained from C3H/HePas mice immunized by both routes 9 days after challenge revealed that mice that received the *L. casei*-Int<sub>cv</sub> vaccines showed less epithelial damage and ulceration at this time point than *L. casei*-vaccinated mice. *C. rodentium* staining was localized more superficially in the intestinal epithelia from mice immunized with *L. casei*-Int<sub>cv</sub>, whereas bacterial staining was observed in deep regions of intestinal crypts from mice immunized with *L. casei*. However, later on, the severity of erosions and the epithelial damage in *L. casei*-Int<sub>cv</sub>-vaccinated mice probably reached the levels observed in colon sections of *L. casei*-vaccinated animals, since almost all mice succumbed to infection.

In summary, we investigated the protective potential of *L. casei*-Int<sub>cv</sub> by two mucosal routes against the murine pathogen *C. rodentium*. Our results suggest that *L. casei*-Int<sub>cv</sub> formulations could represent a promising strategy for the development of mucosal vaccines against A/E pathogens. In particular, the s.l. route of administration induced specific responses (IgA, IgG, and secretion of IL-6 and IFN- $\gamma$ ) that were shown by other groups to be important for protection against these pathogens (21, 34, 43, 63). The s.l. *L. casei*-Int<sub>cv</sub> vaccine

showed good protection levels against mouse models of *C. rodentium* infection with fewer immunization doses. Together, the present results indicate that the development of mucosal vaccines based on *L. casei* expressing intimin is a worthwhile strategy against pathogens that cause A/E lesions.

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