Induction of a Humoral Immune Response following an *Escherichia coli* O157:H7 Infection with an Immunomodulatory Peptidic Fraction Derived from *Lactobacillus helveticus*-Fermented Milk

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Numerous beneficial effects have been attributed to probiotic lactic acid bacteria (LAB), such as the stimulation of the immune system, the prevention of enteric infections by enteropathogens, and the regression of immunodependent tumors. It has been shown that biologically active metabolites released during fermentation, in particular biopeptides, could act as immunomodulatory agents. However, no studies have been conducted to evaluate the implication of these bioactive peptides in the induction of a protective immune response against enteric infections. The present study aimed to evaluate the possible immunomodulatory and anti-infectious effects of a peptidic fraction released in milk fermented by *Lactobacillus helveticus*. The immune response in the mucosa-associated lymphoid tissue was monitored following an administration of the potentially bioactive peptidic fraction. The total immunoglobulin A (IgA) immune response was evaluated after an *Escherichia coli* O157:H7 infection in a BALB/c murine model. Immunohistochemical and enzyme-linked immunosorbent assays revealed an increase in the number of IgA-secreting B lymphocytes in the intestinal lamina propria and an enhanced total secretory and systemic IgA response. Cytokine profiling also revealed stimulation of a Th2 response in mice fed the peptidic fraction, whereas infected controls demonstrated a proinflammatory Th1 response. These results indicate that bioactive peptides released during fermentation by LAB could contribute to the known immunomodulatory effects of probiotic bacteria.

Since its first documented outbreak in 1982 (62), enterohemorrhagic *Escherichia coli* O157:H7 has been recognized as an emerging foodborne pathogen. Although various pathogenic serotypes exist (43), *E. coli* O157:H7 has been the most frequently isolated in North America (50). Pathogenesis of *E. coli* O157:H7 is linked to numerous virulent factors (24), leading to pathological conditions such as hemorrhagic colitis, hemolytic uremic syndrome, thrombotic thrombocytopenic purpura, acute renal failure, and even death (6). *E. coli* O157:H7 is considered a worldwide threat not only because of its increasing incidence and low infectious dose, but also due to the severity of clinical presentation and complications during treatment, particularly with the controversial role of antibiotics (12). Recent studies have explored alternative therapeutic strategies, such as the use of probiotic lactic acid bacteria (LAB).

In accordance with Metchnikoff’s theory of the prolongation of life by lactic acid bacteria in yogurt (39), probiotic LAB have shown numerous strain-dependent beneficial roles in the protection of host organisms against a wide variety of enteropathogens, including *Salmonella enterica* serovar Typhimurium (18), *Clostridium difficile* (2), *Listeria monocytogenes* (11), and even *E. coli* O157:H7 (20, 49, 65). Terms such as colonization resistance (68), competitive exclusion (29), and immunomodulation (53–58, 69) have been used to describe mechanisms in which live bacteria could prevent bacterial infections.

Milk fermented by LAB have previously been shown to enhance both specific and nonspecific immune responses. Though most related studies focus on the administration of live bacteria, there is a lack of recognition of the possible immunomodulatory role of the bioactive peptides or other compounds released in the culture medium during fermentation with LAB. Indeed, many beneficial effects have been attributed to bioactive peptides derived from milk, including opiate activity, antimicrobial activity, antihypertension, antithrombotic activity, and immunomodulation (8, 35, 37, 64).

Cell-free supernatants have been used to study the possible role of bioactive compounds released during milk fermentation. Laffineur et al. (22) reported that cell-free supernatants of *Lactobacillus helveticus*-fermented β-casein-enriched medium modulated lymphocyte proliferation in vitro. In parallel, Ng and Griffiths (47) used cultured macrophages to demonstrate that cell-free supernatants of *L. helveticus*-fermented milks exhibit higher interleukin-6 (IL-6) production than with lipopolysaccharide alone. More recently, peptidic fractions of cell-free supernatants of *L. helveticus*-fermented milks have been shown to significantly reduce fibrosarcoma in vivo (25). However, cell-free supernatants of *L. helveticus*-fermented milks have not yet been implicated in the prevention or attenuation of bacterial infections in vivo. In the present study, a BALB/c murine model was used to examine the role of an *L. helveticus*-fermented milk supernatant fraction on the total immunoglobulin A (IgA) response following an *E. coli* O157:H7 infection in a BALB/c murine model. Immunohistochemical and enzyme-linked immunosorbent assays revealed an increase in the number of IgA-secreting B lymphocytes in the intestinal lamina propria and an enhanced total secretory and systemic IgA response. Cytokine profiling also revealed stimulation of a Th2 response in mice fed the peptidic fraction, whereas infected controls demonstrated a proinflammatory Th1 response. These results indicate that bioactive peptides released during fermentation by LAB could contribute to the known immunomodulatory effects of probiotic bacteria.

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O157:H7 infection. Immunohistochemical and double antibody sandwich–enzyme-linked immunosorbent assay (DAS-ELISA) techniques have demonstrated that a cell-free peptidic fraction of *L. helveticus*-fermented milk could enhance both the total humoral and systemic IgA responses.

**MATERIALS AND METHODS**

**Bacterial strains.** *L. helveticus* R389 (33) was maintained in BBL MRS broth medium for lactobacilli (Becton Dickinson, Cockeysville, Md.) and grown to stationary phase at 37°C for 17 h. Lactobacillus growth was determined by counting CFU after plating serial dilutions on MRS agar (Becton Dickinson) and incubation at 37°C for 48 h. Enterohemorrhagic *E. coli* O157:H7 (ATCC 35150) was grown with agitation (100 rpm) in Difco tryptic soy broth (Difco Laboratories, Detroit, Mich.) at 37°C for 7 h using 2% of an overnight culture and resuspended in sterile phosphate-buffered saline (PBS) to the desired concentration of 10^{10} CFU/ml.

**Milk fermentation.** Milk fermentation was achieved by methods described by LeBlanc et al., (25). Nonfat, dried, low-heat-grade, non-vitamin A- and D-added milk (Dairytown Products Ltd., Sussex, New Brunswick, Canada) was rehydrated (125 g [wt/vol]) and then autoclaved at 121°C for 15 min (Samko Vertical Labo autoclave; NB Scientific, Eden, N.J.). The milk was inoculated (2% [vol/vol]) with an overnight culture of *L. helveticus* R389 containing 10^6 to 10^7 CFU/ml and incubated at 37°C for 24 h. The inoculum was then added (2% [vol/vol]) to 2 liters of rehydrated milk (12% [wt/vol]) to start the milk fermentation. Fermentation was achieved using a Bioflow 3000 Biofermentor (NB Scientific) at 37°C with an agitation rate of 100 rpm and CO2 spurring (10 lb/in^2; 0.2 liters/min); pH was buffered to 6.00 by automatic addition of 8 M NaOH as required. Samples were obtained under sterile conditions after 0, 6, 12, and 24 h to verify *L. helveticus* growth. The extent of milk protein proteolysis was evaluated using the o-phthalaldehyde method developed by Church et al. (7) (data not shown).

**Size-exclusion HPLC.** Protein-peptide fractions were obtained by size-exclusion high-performance liquid chromatography (HPLC) with an HP1100 HPLC system (Agilent Technologies). Milk samples were prepared prior to HPLC injection by centrifugation at 6,000 g for 10 min at 4°C and stored at 4°C for later use. Quantification of protein-peptide fractions was evaluated with the Bradford assay (3) (Bio-Rad Laboratories, Hercules, Calif.).

**RP-HPLC.** To confirm the liberation of numerous peptides in fraction II, reverse-phase HPLC (RP-HPLC) was performed as previously described (33) using a 3.5-μm Zorbax SB-C18 column (4.6 by 150 mm; Agilent Technologies). Briefly, the column was equilibrated with solvent A (0.115% trifluoroacetic acid) at a flow rate of 1 ml/min, and peptides were eluted with solvent B (60% acetonitrile in 0.1% trifluoroacetic acid) as follows: 0 to 30 min, 0 to 60% B; 30 to 35 min, 60 to 100% B; 35 to 42 min, 100 to 0% B. As a control, nonfermented milk was analyzed under same conditions. Eluted peptides were monitored at 214 nm.

**Immune system stimulation studies.** The immunomodulatory effect of the peptidic fraction was determined using the in vivo model developed by Perdigon et al. (54). All animal procedures were performed or supervised by qualified technicians following standard operation methods with respect to the Canadian Council on Animal Care and the Comité de protection des animaux de l’Université Laval (www.ssp.ulaval.ca/Da). Throughout the experiment, animals were fed ad libitum water and standard rodent chow (Charles River Laboratories, Wilmington, Mass.), and feed and litter were replaced daily. All experiments were performed under controlled conditions (temperature [21 ± 2°C], humidity, and a 12-h light-dark cycle). After an acclimatization period of 48 h, 100 g female BALB/c mice (Charles River Laboratories) weighing 18 to 20 g (6- to 8-week-old) were assigned randomly to groups corresponding to three different periods of infection (7, 5, or 2 days pre-infection [groups A, B, and C, respectively]). Each group was also divided in five subgroups corresponding to the day of sacrifice postinfection (days 2, 5, 7, and 10). Infected controls did not receive the peptidic fraction preinfection (group D). To assess the effect of the peptidic fraction on the day of the infection, the noninfected control group received the feedings for 7, 5, and 2 days preinfection and were sacrificed on day 0 (group E). Noninfected controls also consisted of mice that had not received the peptidic fraction pre-infection and that were sacrificed on days 2, 5, 7, and 10 postinfection. Figure 1 presents a summary of the animal model.

**Mice experiments.** Mice were fed by gavage, using 20-gauge by 38-mm malleable animal feeding needles (Poper & Sons, Inc.), 50 μg of the peptidic fraction II/day (25) (or sterile water for the control groups) for the duration of the feeding period (2, 5, or 7 days). After feeding periods, mice were challenged intragastrically with 0.5 ml of *E. coli* O157:H7/ml. Groups D and E represent the infected and noninfected controls, respectively. Analysis was performed on days 0, 2, 5, 7, and 10 postinfection.

**FIG. 1.** Schematic representation of the animal model. Mice were fed peptidic fraction II for 7 (A), 5 (B), and 2 (C) days preinfection, as described in the time chart. On day zero, groups A to D were challenged with 10^{10} CFU of *E. coli* O157:H7/ml. Groups D and E represent the infected and noninfected controls, respectively. Analysis was performed on days 0, 2, 5, 7, and 10 postinfection.
30 min and observed by using a Hund H600 fluorescence light microscope. Results were expressed as the number of fluorescent cells counted in 10 fields of vision at 1,000× magnification.

**DAS-ELISA.** Total IgA antibodies were detected by standard DAS-ELISA. Briefly, affinity-purified monoclonal goat anti-IgA (α-chain specific) was added at 1.25 μg/well in 0.05 M carbonate-bicarbonate buffer (pH 9.6) to Costar 96-well, U-bottomed, high-binding polystyrene microplates (Corning Inc.) and incubated at 37°C for 1 h. The plates were then washed three times using an ELx805 Auto plate washer (Bio-Tek Instruments, Inc., Winooski, Vt.) with PBS containing 0.05% Tween 20 (PBS-T) and blocked for 1 h at 25°C with 0.5% nonfat dry milk in PBS. Plates were washed five times with PBS-T and incubated for 2 h at 37°C with either 50 μl of standard kappa IgA or 50-μl samples of intestinal fluid or serum (diluted 1/4,000 and 1/20,000 in blocking solution, respectively), both of which were added in triplicate. Plates were washed seven times with PBS-T and incubated in the presence of horseradish peroxidase-conjugated anti-IgA-specific antibodies at 1.25 μg/well for 1 h at 37°C. Plates were again washed seven times, and 100 μl of trimethylbenzidine reagent containing peroxide (BD Biosciences, Mississauga, Ontario, Canada) was added to each well. Reactions were terminated with 100 μl of H₂SO₄ (2 N) with gentle shaking. The optical density was read at 450 nm by using a μQuant automatic microplate reader (Bio-Tek Instruments). For the IgA-specific DAS-ELISA, all antibodies, standards, and buffers were purchased from Sigma Chemical Co.

**Cytokine production.** Serum IL-4 and gamma interferon (IFN-γ) levels were evaluated with mouse OptEIA ELISA sets (BD Biosciences, San Diego, Calif.) following the instructions provided by the manufacturer.

**Statistical analysis.** Results were expressed as the mean ± standard deviation, and data were subjected to analysis of variance with SPSS software version 11.5 (SPSS Inc., Chicago, Ill.). Tukey’s multiple-range test was used to compare the least square means of all treatments. A P value of <0.05 was considered significantly different.

**RESULTS**

**Milk fermentation.** Size-exclusion and RP-HPLC elution profiles confirmed the proteolysis of milk proteins during fermentation by *L. helveticus* (Fig. 2).
As observed previously (25), Fig. 2B revealed the appearance of small oligopeptides and peptides ranging from 2 to 10 kDa derived from larger milk proteins (compare Fig. 2A and B). RP-HPLC analysis indicated that the peptidic fraction used in this study (Fig. 2B) was effectively composed of several peptides that were not observed in the control (Fig. 2C and D).

**Postchallenge body weight.** Initial body weights were subtracted from subsequent values. It is noteworthy that no differences in initial mean body weight were detected (17.70 ± 1.03, 19.13 ± 0.62, 19.38 ± 0.82, 19.22 ± 1.46, and 18.11 ± 0.18 g for the groups fed for 2, 5 and 7 days preinfection and the infected and noninfected controls, respectively). Following challenge with *E. coli* O157:H7, the infected control showed a significant loss in body weight, particularly on days 1 and 2 postinfection (Fig. 3).

There were no significant differences observed between mice receiving any of the peptide regimens (2, 5, and 7 days preinfection) and the noninfected control; therefore, all regimens of peptide feeding prevented the weight loss caused by the *E. coli* O157:H7 challenge.

**Effect of the peptidic fraction on the number of IgA⁺ B cells in the intestinal lamina propria.** As shown in Fig. 4, the peptidic fraction significantly altered the B-lymphocyte response to *E. coli* O157:H7 infection after all feeding periods (days 2, 5, and 7).

The infected control did not differ in the number of IgA⁺-producing B lymphocytes compared to the noninfected control (83 ± 4 B cells/10 fields of vision at a magnification of ×1,000) on days 0 and 2 postinfection (84 ± 4 and 84 ± 5, respectively); nonetheless, on the 5th, 7th, and 10th days postinfection, numbers reached significant levels (88 ± 3, 89 ± 4, and 108 ± 6, respectively). In parallel with infected controls, a 2-day feeding period with the peptidic fraction (preinfection) demonstrated a significant increase in the number of IgA⁺ B cells in the intestinal lamina propria on days 0 (103 ± 4) and 2 (95 ± 4) postinfection; however, numbers dropped after 5, 7, and 10 days (91 ± 3, 90 ± 3, and 114 ± 5, respectively). A 5-day feeding preinfection showed an increase in the number of IgA⁺ B lymphocytes to significant levels on the 7th and 10th days postinfection (97 ± 4 and 132 ± 5, respectively); however, no significant changes were observed 2 or 5 days following infection (80 ± 4 and 89 ± 4, respectively). Moreover, on day 0, a significant decrease (70 ± 5) was also observed. For all days following *E. coli* O157:H7 challenge (0, 2, 5, 7, and 10), the 7-day feeding period induced a strong increase in the number of IgA⁺ B cells (100 ± 4, 98 ± 4, 99 ± 3, 130 ± 5, and 170 ± 8, respectively).

**Effect of the peptidic fraction on total intestinal IgA secretion.** In parallel with the immunofluorescence assay, an IgA-specific DAS-ELISA confirmed that the altered B-lymphocyte response to *E. coli* O157:H7 infection was caused by the peptidic fraction derived from *L. helveticus*-fermented milk. In comparison to the number of IgA⁺ B cells in the intestinal lamina propria (Fig. 4), similar patterns were observed in IgA content in the intestinal fluid (Fig. 5).

In comparison to the noninfected control (5.84 ± 0.68 µg/ml), the infected control did not differ in IgA secretion on days 0 and 2 postinfection (5.83 ± 0.46 and 5.24 ± 0.13 µg/ml, respectively); nonetheless, on the 5th, 7th, and 10th days postinfection, IgA production reached significant levels (6.51 ± 1.04, 8.01 ± 1.35, and 8.70 ± 0.14 µg/ml, respectively). In comparison to the infected controls, a 2-day feeding period with the peptidic fraction (preinfection) led to a significant increase in IgA production on days 0 (11.14 ± 0.58 µg/ml), 2

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**FIG. 3.** Mean (± standard deviation) weight gain for mice challenged with *E. coli* O157:H7 (*n* = 4). Results are expressed as the cumulative weight change relative to day zero weight.
(9.52 ± 0.46 µg/ml), and 5 (9.49 ± 0.41 µg/ml) postinfection; however, the antibody response did not significantly differ from that in the infected control after 7 and 10 days (9.27 ± 0.55 and 8.85 ± 0.18 µg/ml, respectively). Although IgA levels showed a significant increase on days 5, 7, and 10 postinfection (10.50 ± 0.57, 11.80 ± 0.51, and 11.12 ± 0.68 µg/ml, respectively), a significant decrease in total IgA was observed on day 0 (2.85 ± 1.26 µg/ml) and values were not significantly different on day 2 (6.87 ± 1.26). On the other hand, for all days of analysis postinfection (0, 2, 5, 7, and 10), a strong IgA response was observed, with the results for the 7-day feeding period reaching concentrations of 6.97 ± 0.52, 6.99 ± 0.41, 12.46 ± 0.72, 15.19 ± 0.96, and 15.14 ± 0.85 µg/ml, respectively. The results seem to confirm those observed in the immunofluorescence study (compare Fig. 4 and 5).

**Effect of the peptidic fraction on total serum IgA.** In order to determine if the peptidic fraction increased the systemic immune response to *E. coli* O157:H7, total serum IgA was evaluated by DAS-ELISA (Fig. 6).

In comparison with the noninfected control (11.70 ± 0.22 µg/ml), the infected control did not differ in total serum IgA on days 0, 2, and 5 postinfection (11.27 ± 0.34, 12.61 ± 3.83, and 13.62 ± 2.80 µg/ml, respectively); however, IgA levels differed significantly on the 7th and 10th days postinfection (19.07 ± 1.30 and 18.67 ± 0.49 µg/ml, respectively). A 2-day feeding period with the peptidic fraction preinfection showed no ap-
parent differences in total serum IgA (12.27 ± 0.86, 15.58 ± 3.22, 13.11 ± 2.12, 16.29 ± 1.82, and 17.38 ± 1.00 μg/ml, respectively) relative to the response in infected controls. A 5-day gavage preinfection did not significantly alter serum IgA on days 0 (12.02 ± 1.01 μg/ml) and 2 (13.98 ± 4.47 μg/ml) postinfection; however, this group demonstrated increases on days 5, 7, and 10 postinfection (21.12 ± 0.23, 26.90 ± 0.69, and 22.18 ± 0.88 μg/ml, respectively). For all days of sampling, the group previously fed the peptidic fraction for 7 days showed a significant increase in serum IgA (17.21 ± 1.31, 17.29 ± 1.16, 25.73 ± 2.47, 32.76 ± 1.06, and 30.72 ± 4.72 μg/ml, respectively).

Cytokine production. In order to determine possible mechanisms implicated in the enhanced IgA immune response to E. coli O157:H7, serum IL-4 and IFN-γ levels were determined by DAS-ELISA (Fig. 7).

As illustrated in Fig. 7A, a remarkable increase in IL-4 was observed throughout the experiment for the group that had received a 7-day gavage of the peptidic fraction preinfection. Interestingly, the IL-4 production was also significantly elevated on the day of challenge compared to responses in the other groups. A 5-day feeding period resulted in significantly increased IL-4 production on days 2, 5, 7, and 10 postinfection; however, IL-4 levels did not differ from those in other groups on day 0. The group that received a 2-day feeding showed a slight increase in IL-4 production on day 2 postinfection, but IL-4 concentrations did not differ from those in the noninfected controls on days 5, 7, and 10 postinfection. No apparent differences were observed between the infected and noninfected controls.

As shown in Fig. 7B, increased IFN-γ levels were observed in the infected controls, particularly on day 2 postinfection. Although IFN-γ levels in this group decreased on days 5, 7, and 10 postinfection, they remained significantly higher than in other groups. The group fed the peptidic fraction for 2 days preinfection showed a slight increase in IFN-γ production during infection. However, IFN-γ levels were significantly lower than those of the infected control. A 5-day feeding period preinfection demonstrated high levels of IFN-γ on day 0 but dropped to statistically insignificant amounts for the remainder of the experiment. Remarkably, the 7-day feeding period showed no significant difference from the noninfected control on all days of analysis.

DISCUSSION

This study dealt with the effects of peptides derived from fermented milk on the enhancement of the immune response following an E. coli O157:H7 challenge. The immunopotentiating effect of these peptides was analyzed in vivo by oral administration to BALB/c mice during 2, 5, and 7 days prior to infection with E. coli O157:H7. The peptidic fraction (Fig. 2B) resulted from extensive proteolysis by L. helveticus and was been isolated by size-exclusion HPLC. LAB such as L. helveticus possess proteases and peptidases that permit milk protein degradation (33, 37), yet their specific activities differ markedly.
between bacterial species (16) and strains (14, 15, 23, 41, 72). Consequently, different peptide profiles may be released (34). A better hydrolysis of milk proteins by LAB can increase the yield of bioactive peptides (15, 33, 34, 36, 41). Immunologically active peptides have been obtained from both the casein and whey fractions of human and bovine milk (8, 10, 17, 30, 71).

Size-exclusion and RP-HPLC profiles illustrated that milk proteins were effectively proteolysed by *L. helveticus* (Fig. 2). Fermentation of milk by *L. helveticus* increased the yield of small protein-derived compounds, shown by the appearance of novel peaks after milk fermentation that were not present beforehand. This is consistent with the observations made by LeBlanc et al. (25) and Matar et al. (33). In comparison with other LAB, *L. helveticus* is known to be a highly proteolytic bacterium (41). Previous studies have shown that cell-free supernatants of *L. helveticus*-fermented casein hydrolysates could induce immunomodulatory responses that were not observed in media supernatants or by the bacterium itself (47). It should also be noted that previous results showed that a nonproteolytic mutant of *L. helveticus* did not demonstrate immunomodulatory responses, even when cultured in milk (36). Since the proteolysis increases during fermentation, it has been postulated that the immunomodulatory effect could be attributed to peptides released during fermentation (25, 33). It should also be noted that the peptidic fraction used in our study has previously been shown to be immunomodulatory (25). This fraction is highly enriched with peptides (from 2 to 10 kDa) resulting from protein degradation, and their concentration (0.8 to 3.0 mg/ml) exceeds that of any secondary metabolites or bacterial components whose concentrations in the final product are minute in comparison to that of the peptides. The peptide profiles for the fraction mentioned above and a non-fermented control are reported in the RP-HPLC chromatogram (Fig. 2C and D).

Recent theories suggest that bacterial cleavage of peptide bonds within milk proteins could promote the unfolding of these molecules, facilitating the release of bioactive peptides (34). Supporting this theory, Sütas et al. (66) demonstrated the liberation of immunomodulatory peptides in *Lactobacillus casei* GG-fermented milk that were not released with the digestive enzymes pepsin and trypsin. Other protein hydrolysates have been shown to have an activity towards immune cells. Migliore-Samour et al. (40) have isolated biologically active casein peptides implicated in immunomodulation. Sütas et al. (66) have demonstrated that the proteolysis of \( \kappa \)-caseins by *Lactobacillus rhamnosus* GG, combined with pepsin and trypsin hydrolysis, could stimulate lymphocyte responses in vitro. Recently, Low et al. (30) reported that when BALB/c mice were fed a whey protein concentrate, they produced elevated levels of specific intestinal tract and serum antibodies against orally and parenterally administered antigens. Peptides from *L. helveticus*-fermented milk have also been shown to increase the number of IgA-secreting B cells and decrease the growth of fibrosarcomas (25). In the present study, a peptidic fraction derived from *L. helveticus*-fermented milk supernatants was evaluated for its capacity to prime immune system parameters in BALB/c mice following an *E. coli* O157:H7 challenge.

First, total IgA levels in the intestinal fluid and serum were

![FIG. 6. Effect of consumption of a peptidic fraction derived from *L. helveticus*-fermented milk on total serum IgA response of mice infected with *E. coli* O157:H7. Values are means for \( n = 4 \) (± standard deviations), and significance compared to the values of the infected control is shown (*, \( P < 0.05 \); **, \( P < 0.01 \)).](http://cvl.asm.org/Downloaded from http://cvl.asm.org/)
analyzed to better assess the effect of the peptidic fraction on the induction of an IgA antiadherence barrier against *E. coli* O157:H7, a concept referred to as immune exclusion. The immunofluorescence assay and IgA-specific DAS-ELISA revealed that various feeding durations with the peptidic fraction modulated not only the number of IgA-producing B lymphocytes in the intestinal lamina propria following *E. coli* O157:H7 challenge (Fig. 4), but also enhanced the secretory (Fig. 5) and the systemic (Fig. 6) immune responses. Indeed, mice fed the peptidic fraction for 2 days preinfection demonstrated a slight increase in the number of IgA⁺ B cells in the intestinal lamina propria on days 0 and 2 postinfection. However, the 2-day feeding period seemed insufficient to enhance the local and systemic IgA response after 5, 7, and 10 days. A 5-day or, to a higher extent, a 7-day feeding showed a strong increase in the number of IgA⁺ B lymphocytes and total intestinal and serum...
IgA, particularly on the 7th and 10th days postinfection. These results suggest that peptides derived from *L. helveticus*-fermented milk are capable of enhancing total secretory and systemic IgA responses following an *E. coli* O157:H7 challenge. Interestingly, a 5-day feeding period demonstrated a significant decrease in IgA⁺ B cells and total intestinal IgA on day zero postinfection. Although this observation could be attributed to oral tolerance (70), the low antibody production could also be associated with a transient Th1 cytokine profile (discussed below).

The concept of biologically active peptides has evoked much interest in the past years. Numerous authors have reported that immunomodulatory peptides can produce local effects on the gastrointestinal tract and stimulate immunocompetent cells through the gut-associated lymphoid tissue (4, 5, 30, 33–35, 38, 45, 54–56, 58). Unlike epithelial enterocytes that exclude peptides and macromolecules with antigenic potential, specialized mucosal epithelial cells (M cells), located in the follicle-associated epithelium of Peyer’s patches, transport luminal antigen across the intestinal barrier (21, 46). The apical surface of M cells lacks the brush border microvilli and possesses a large endocytic domain which enables them to uptake and release luminal antigen on their basolateral surface, where there is a complex network of antigen-presenting cells and Th CD4⁺ lymphocytes capable of stimulating the differentiation of underlying B cells into Ig-producing plasma cells (44). The specific humoral (secretory) mucosal immune response is mediated through an increase of IgA-producing B cells and secretory IgA (sIgA) synthesis (48). Since sIgA is considered the major immunological barrier against enteropathogens, an increase in total sIgA could prevent adherence and the colonization by enteropathogens such as *E. coli* O157:H7 (33, 48, 56).

Protective effects of LAB against numerous enteropathogenic bacteria have been reported in several studies (2, 11, 18, 20, 49, 55, 65). Perdigon et al. (52) demonstrated that *L. casei* decreased the infection caused by *S. enterica* serovar Typhimurium by enhancing the secretion of specific IgA. Link-Amster et al. (28) demonstrated production of specific IgA against *S. enterica* serovar Typhimurium in the serum of volunteers who had previously consumed *Lactobacillus acidophilus*. LAB have also demonstrated beneficial effects against *E. coli* O157:H7 infections. For example, Ogawa et al. (49) demonstrated that *L. casei* strain Shirota enhances the local immune response to *E. coli* O157:H7 and decreases the production of its toxins. Paton et al. (51) have demonstrated that antibodies specific to Shiga toxin-producing *E. coli* could inhibit pathogen adherence in vitro. There is also evidence that vaccination against *E. coli* O157:H7 could be successful by increasing the humoral response to virulent factors, such as the translocated intimin receptor (Tir) (27). Since *E. coli* O157:H7 preferentially binds itself to M cells in Peyer’s patches (13, 61), mechanisms that induce an increase of sIgA might be sufficient to prevent or attenuate the infection. Indeed, Conlan and Perry (9) correlated the presence and persistence of serum and fecal anti-O157 IgA to an enhanced resistance to *E. coli* O157:H7 in BALB/c mice.

Interestingly, the immune response to probiotics and probiotic-derived products is strain dependent. Indeed, enhancement of the immune system by orally administered LAB cannot be generalized for genera, or even species. Perdigon et al. (55) have reported that *L. casei* and *Lactobacillus plantarum* interact with Peyer’s patches; *L. rhamnosus*, *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, and *Lactococcus lactis* interact with epithelial cells of the small intestine or in Peyer’s patches; and *L. acidophilus* interacts mainly with epithelial cells of the large intestine. These results suggest that the different immunostimulatory action of LAB could result from strain-specific interactions in the intestinal tract (55, 58). Immune system modulation might also be strongly dependent on the lactobacillus species, or on the metabolites they produce. Thus, species differing in their metabolic activities, such as proteolysis, could release peptides that may (or may not) interact with immune cells to induce distinct immunomodulatory response.

LAB have been known to influence cellular and humoral responses through modulation of the Th1/Th2 balance (31, 56). The classic Th1 cytokine IFN-γ is a potent multifunctional proinflammatory cytokine that triggers innate immune responses, such as phagocytosis and antimicrobial activity, whereas the predominant Th2 cytokine IL-4 regulates a number of events, including antibody production (19). Both IL-4 and IFN-γ generally exist in an antagonistic relationship and have been considered the pivotal cytokines in the induction of Th1 and Th2 responses, respectively (19, 31, 42, 56). In contrast to the strong IFN-γ levels in the infected control (Fig. 7B), the 7-day and, to a lesser extent, the 5-day feeding of the peptidic fraction preinfection led to a remarkable IL-4 increase throughout the experiment (Fig. 7A). These results indicate that the peptidic fraction favors a Th2 response and can be correlated to a high antibody response. In contrast, the infected control displayed high IFN-γ levels with a maximum value on day 2 postinfection. Interestingly, this proinflammatory response could be correlated to the drastic weight loss observed in Fig. 3. Asahara et al. (1) illustrated that *E. coli* O157:H7 proliferated in the intestine of mice within 24 h of infection, and infected mice showed a dramatic decrease in body weight starting at 2 days postchallenge. In our study, weight loss was only observed in the infected control. Mice fed the peptidic regimen were shown to have an enhanced intestinal IgA response even before infection, suggesting that immune modulation by the peptidic fraction could involve homeostasis between Th1 and Th2 activity (31, 56). In mice, this phenomenon is reflected in specific cytokine patterns (31, 56). Since the Th2 response is linked to antibody production and the Th1 response reflects an inflammatory response (19, 56), it is possible that the enhance IgA production could influence the ability of *E. coli* O157:H7 to colonize the intestinal epithelium. However, bacterial clearance studies were not undertaken to confirm this hypothesis. It was also noted that the 2-day feeding demonstrated only an IgA increase in the intestine (Fig. 4 and 5), but it did not show an increase in serum IgA (Fig. 6) or serum IL-4 (Fig. 7). Nonetheless, this local IgA stimulation was sufficient to prevent the weight loss associated with the infection. Overall, these results suggest that the peptidic fraction derived from *L. helveticus*-fermented milk enhances the humoral immune response following an *E. coli* O157:H7 challenge by favoring a Th2 cytokine profile and reducing the proinflammatory Th1 response.

Although LAB have been shown to influence cytokine profiles, little attention has been paid to the modulation of cyto-
kine production following protein hydrolysis by LAB. Sütas et al. (67) indicated that caseins could up-regulate IL-4 and IFN-γ production, whereas L. rhamnosus GG-degraded caseins down-regulated IL-4 production with no effect on IFN-γ. Pessi et al. (59) reported that L. rhamnosus GG-degraded caseins suppressed T-cell activation through down-regulation of IL-2 synthesis and protein kinase C activity. In contrast, this study demonstrated a predominant effect on Th2 cells after biopeptide ingestion. These opposing effects could be attributed to the liberation of distinct peptides released during proteolysis with LAB. It is generally accepted that orally administered LAB produce peptides in vivo (32, 60); therefore, immunomodulatory peptides released from dietary proteins could account for the strain-specific probiotic effects. It is clear that the effects of probiotics are complex and multifactorial and result from different modes of interaction with the immune system. Immune cells, such as macrophages and lymphocytes, possess specific receptors that can interact with cell wall components of bacteria, such as the peptidoglycan and lipopolysaccharides. LAB-derived peptides might also interact with such receptors or selectively influence the immune system through different immunomodulating mechanisms. In all, LAB-derived peptides could greatly contribute to the known probiotic effects of immunomodulation.

This study represents the first documented evidence that peptides derived from L. helveticus-fermented milk can enhance the total IgA humoral and systemic immune response following an E. coli O157:H7 challenge. An increase in total IgA can be beneficial to prevent the entry or colonization of enteropathogens, a concept referred to as immune exclusion. However, subsequent studies will need to address the specific quantification of antipeptide and anti-E. coli IgA responses in relation to bacterial clearance. Isolation and purification of the peptide(s) and dose-dependent relationships will undoubtedly reveal the role of immunomodulatory peptides derived from dietary proteins and their suitability for prevention or attenuation of lethal bacterial infections.

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