In Vitro and In Vivo Inhibition of *Helicobacter pylori* by *Lactobacillus casei* Strain Shirota

D. Sgouras,[1]† P. Maragkoudakis,[2]† K. Petraki,[3]† B. Martinez-Gonzalez,[1] E. Eriotou,[1]
S. Michopoulos,[4] G. Kalantzopoulos,[2] E. Tsakalidou,[2] and A. Mentis[1]*

*Laboratory of Medical Microbiology, Hellenic Pasteur Institute,[1] Laboratory of Dairy Research, Agricultural University of Athens,[2]
Laboratory of Pathology, Hippocration Hospital,[3] and Gastroenterology Clinic, Alexandra Hospital,[4] Athens, Greece*

We studied the potential inhibitory effect of *Lactobacillus casei* strain Shirota (from the fermented milk product Yakult [Yakult Ltd., Tokyo, Japan]) on *Helicobacter pylori* by using (i) in vitro inhibition assays with *H. pylori* SS1 (Sydney strain 1) and nine *H. pylori* clinical isolates and (ii) the in vivo *H. pylori* SS1 mouse model of infection over a period of 9 months. In vitro activity against *H. pylori* SS1 and all of the clinical isolates was observed in the presence of viable *L. casei* strain Shirota cells but not in the cell-free culture supernatant, although there was profound inhibition of urease activity. In vivo experiments were performed by oral administration of *L. casei* strain Shirota in the water supply over a period of 9 months to 6-week-old C57BL/6 mice previously infected with *H. pylori* SS1 (study group; *n* = 25). Appropriate control groups of *H. pylori*-infected but untreated animals (*n* = 25) and uninfected animals given *L. casei* strain Shirota (*n* = 25) also were included in the study. *H. pylori* colonization and development of gastritis were assessed at 1, 2, 3, 6, and 9 months postinfection. A significant reduction in the levels of *H. pylori* colonization was observed in the antrum and body mucosa in vivo in the lactobacillus-treated study group, as assessed by viable cultures, compared to the levels in the *H. pylori*-infected control group. This reduction was accompanied by a significant decline in the associated chronic and active gastric mucosal inflammation observed at each time point throughout the observation period. A trend toward a decrease in the anti-*H. pylori* immunoglobulin G response was measured in the serum of the animals treated with lactobacillus, although this decrease was not significant.

Lactic acid bacteria are widely used in the production of fermented foods and beverages and contribute both to the sensory qualities of the food and to the prevention of spoilage. Moreover, they are present in large numbers in the normal human and animal gastrointestinal floras. In recent decades, much attention has been paid to the health-promoting (probiotic) properties of lactobacilli, as it has been claimed that when administered in adequate amounts, they confer a health benefit on the host (Joint FAO/W. H. O. Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria, Cordoba, Argentina, October 2001). Furthermore, for use in foods, probiotic microorganisms not only should be capable of surviving passage through the digestive tract but also should have the capability to proliferate in the gut (Joint FAO/W. H. O. Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria). Several probiotic lactobacilli have been shown to survive transit through the human gastrointestinal tract and to maintain a balanced intestinal microflora (32). In particular, *Lactobacillus casei* strain Shirota, contained in the commercial fermented milk product Yakult (Yakult Ltd., Tokyo, Japan), has been reported to exhibit antitumor, immunostimulatory, and antimicrobial activities. More specifically, the oral administration of *L. casei*

strain Shirota stimulated a type 1 response (Th1), activated the cellular immune system, and inhibited the incidence of tumors and immunoglobulin E (IgE) production in a murine model (22, 34). Furthermore, *L. casei* strain Shirota orally administered to rats infected with *Listeria monocytogenes* enhanced cellular immunity, as determined by a delayed-type hypersensitivity reaction, thus conferring enhanced resistance against the pathogen (8). Finally, *L. casei* strain Shirota administration before or after an initial challenge dramatically inhibited *Escherichia coli* growth in a murine model of urinary tract infection and decreased the severity of diarrhea resulting from Shiga toxin-producing *E. coli* O157:H7 in infant rabbits (3, 25).

Helicobacter pylori, a spiral gram-negative microaerophilic stomach pathogen that infects over 50% of the population worldwide (10), is the cause of gastritis (4) and peptic ulcers (11) and has been linked to the development of gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (27). Recently, attention has been paid to the interactions between *H. pylori* and probiotic lactobacilli. *H. pylori* colonization was inhibited in *Lactobacillus salivarius*-fed gnotobiotic BALB/c mice, and *H. pylori*-specific antibody titers became marginal, while *H. pylori* colonized in large numbers and caused active gastritis in lactobacillus-free mice (15). In another study, the oral administration of a spent culture supernatant of *L. acidophilus* resulted in the suppression of *H. felis* in a murine infection model (6, 7). In addition, an *L. acidophilus* culture supernatant was effective in vitro and had a partial, long-term suppressive effect on *H. pylori* in humans (24). Finally, in a recent intervention study involving administration of the fermented milk product Yakult containing *L. casei* strain

* Corresponding author. Mailing address: Laboratory of Medical Microbiology, Hellenic Pasteur Institute, Vas. Sophias 127, 115 21 Athens, Greece. Phone: 30210-6478816. Fax: 30210-6423498. E-mail: mentis@mail.pasteur.gr.

† D.S., P.M., and K.P. contributed equally to this work.

Shirota to 14 *H. pylori*-positive subjects, a slight but nonsignificant trend toward a suppressive effect of *L. casei* on *H. pylori* was observed, as assessed by a ^{13}C urea breath test (UBT) (5). With respect to these clinical studies of the effect of probiotics on *H. pylori* stomach infections, probiotic preparations were administered in the form of spent culture supernatants or yogurt, without any indication about the effective probiotic dose. Furthermore, anti-*H. pylori* activity in these studies was assessed with indirect methods, such as the UBT, instead of quantitative *H. pylori* cultures and histopathologic evaluation. Therefore, no definite conclusions can be drawn about the effectiveness of probiotics in *H. pylori*-infected people, and this issue certainly warrants more detailed studies, based on conclusions from animal studies, with respect to the form in which the probiotic is administered, the dosage scheme, the mode of administration, and the duration of the regimen.

The aim of the present work was to assess the potential inhibitory activity of *L. casei* strain Shirota, isolated from the commercial fermented milk product Yakult, on *H. pylori*. The basis for the selection of *L. casei* strain Shirota was its reported activity against gram-negative pathogens, most specifically, *H. pylori*, as well as a range of probiotic properties, such as the ability to survive transit through the stomach and resistance to bile salts (data not shown). In order to study anti-*H. pylori* activity, primarily in vitro assays involving *H. pylori* cultures grown on solid agar as well as in liquid media were used. Furthermore, *L. casei* strain Shirota administered in vivo was evaluated for its ability to reduce colonizing *H. pylori* viable counts and the associated inflammation of the gastric mucosa, as assessed by histopathologic evaluation, in the *H. pylori* SS1 (Sydney strain 1) murine infection model (17). Finally, we studied the long-term effect of its administration to healthy mice, along with its intestinal colonization and its ability to persist in the gut.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *L. casei* strain Shirota was isolated from the fermented milk product Yakult. It was stored in DeMan-Rogosa-Sharpe (MRS) broth (Biokar Diagnostics, Beauvais, France) at −80°C and subcultured twice in MRS broth for 24 h at 30°C prior to experimental use. *L. casei* strain Shirota culture supernatants free of cells, used in the experiments, were derived from fresh overnight (o/n) *L. casei* strain Shirota cultures, centrifuged twice at $17,000 \times g$ for 10 min at 4°C, and filtered through a 0.22-μm-pore-size filter (Millipore, Bedford, Mass.). *L. casei* strain Shirota culture supernatants either were used at their native pH, 4.5, or were adjusted to pH 6.5 with NaOH. MRS controls at pH 4.5 were adjusted by the addition of HCl. As the exact compositions and activities of *L. casei* strain Shirota supernatants would change depending on the growth phase of the cultures, the cultures used were of the same growth phase (late logarithmic), starting from equal initial populations to ensure that the products of metabolism were the same in terms of quality and quantity.

H. pylori clinical strains (HPI19, HPI32, HPI47, HPI66, HPI91, HPI93, HPI101, HPI123, and HPI137) were isolated from patients with gastritis and/or peptic ulcers and stored at −80°C. Early-passage *H. pylori* SS1 (17) was kindly provided by L. Ferrero (Institut Pasteur, Paris, France). All *H. pylori* strains were grown at 37°C for 48 h under microaerophilic conditions on Chalgren-Wilkins agar enriched with 7% (vol/vol) horse blood and 1% (vol/vol) VITOX (CHW agar plates; all reagents were purchased from Oxoid, Basingstoke, United Kingdom) and containing antibiotics (vancomycin at 10 μg/ml, trimethoprim at 10 μg/ml, polymyxin B at 10^4 IU/liter, amphotericin B at 2 μg/ml, nalidixic acid at 10 μg/ml, bacitracin at 30 μg/ml, and fluorocytosine at 5 μg/ml; all antibiotics were obtained from Sigma, St. Louis, Mo.). *H. pylori* liquid cultures were prepared in brain heart infusion broth (BHIB; Oxoid) supplemented with 10% horse serum (Seromed, Berlin, Germany) and 0.25% yeast extract (Sigma) under

the same conditions in a shaking incubator. Unless otherwise stated, for all in vitro and in vivo procedures, we used highly motile bacillary *H. pylori* cells derived from fresh o/n cultures. *E. coli* strain HB101 was cultured in tryptic soy broth (Oxoid) at 37°C for 24 h.

In vitro *H. pylori* inhibition experiments. (i) Well diffusion assay. o/n *H. pylori* cultures were plated on fresh CHW agar plates without antibiotics (10^7 CFU per plate), and wells were drilled into the agar by using sterile Pasteur pipettes. Depending on the experimental design, 50-μl aliquots of fresh o/n *L. casei* strain Shirota cultures, cell-free culture supernatants, or washed cells resuspended in fresh MRS broth were suspended in the agar wells. Plates were incubated for 48 to 72 h under microaerophilic conditions at 37°C, and the diameters of inhibition zones around the wells were measured. Results were expressed as a mean diameter and standard error. Statistical analysis was performed with Fisher's exact test (GraphPad Software). An *E. coli* HB101 culture was used as a negative control for in vitro anti-*H. pylori* activity.

(ii) Liquid culture assay. *H. pylori* SS1 cells (10^8 CFU/ml) suspended in BHIB in the absence of antibiotics were incubated under microaerophilic conditions at 37°C in the presence of a 10% volume of *L. casei* strain Shirota culture supernatant (pH 4.5 or 6.5) or the appropriate MRS medium control. The viability of *H. pylori* at 24 and 48 h was evaluated by determination of viable CFU on CHW agar plates following incubation at 37°C under microaerophilic conditions. Urease activity was determined by a modification of the phenol red method (13). Briefly, 10 μl of *H. pylori* cell suspension was added to 300 μl of urease reaction buffer (20% [wt/vol] urea and 0.012% phenol red in phosphate buffer, with the final pH adjusted to 6.5) on a microtiter plate, and the plate was incubated for 1 h at 37°C. The absorbance at 550 nm was measured with a Sunrise microtiter plate reader (Tekan, Grödig, Austria).

Determination of lactic acid production. Determination of lactate in *L. casei* strain Shirota culture supernatants was performed by high-pressure liquid chromatography (HPLC) analysis (Varian Associates Inc., Palo Alto, Calif.) following $HClO_4$ protein precipitation. A filtered supernatant (0.22-μm-pore-size filter) was injected into an Aminex HPX-87H column (300 mm by 7.8 mm; Bio-Rad, Hercules, Calif.) connected to a refractive index detector (GBC Scientific Equipment Pty Ltd., Dandenong, Victoria, Australia). Elution was performed at 35°C with 5 mM H_2SO_4 at a flow rate of 0.5 ml/min. Concentrations were calculated by using a lactate standard curve.

***H. pylori* infection of C57BL/6 mice.** Specific-pathogen-free 6- to 8-week-old inbred female C57BL/6 mice were obtained from the Central Animal Facility of the Hellenic Pasteur Institute. They were housed according to relevant Greek national legislation, were fed a commercial diet, and were given water ad libitum, except as otherwise stated. A fresh o/n *H. pylori* SS1 culture grown in BHIB was resuspended in phosphate-buffered saline at a concentration of 10^7 CFU/ml. Aliquots (100 μl, 10^6 CFU) were administered to mice under light ether anesthesia via orogastric inoculation with a 0.6-mm polyethylene catheter mounted on a 1-ml syringe, three times within a week, with a 1-day interval between inoculations. Initial *H. pylori* inoculation experiments were performed for calculation of the lowest concentration of inoculated bacteria sufficient to infect 100% of the animals; we repeatedly obtained a concentration as low as 10^4 CFU.

In vivo *H. pylori* inhibition experiments. To test for potential antimicrobial activity of *L. casei* strain Shirota in vivo, cultures grown in MRS broth were administered to *H. pylori* SS1-infected mice during the week following the initial *H. pylori* challenge. The probiotic was used as a daily supplement at 10^8 CFU/ml in the animals' drinking water over a period of 9 months following the initial *H. pylori* infection. Daily water consumption and bacterial viability in the water were monitored. In preliminary experiments, daily water intake was measured at 6.0 ± 0.2 ml, and no difference was recorded between the animal groups used in the study. *L. casei* strain Shirota viable counts in the animals' water were measured repeatedly in previous independent experiments as well as during the animal work described here. A reduction in the *L. casei* strain Shirota population of greater than 0.5 log unit was never recorded (data not shown).

Four groups of animals were included in the study: *H. pylori*-infected mice treated with *L. casei* strain Shirota (*H. pylori*-*L. casei* strain Shirota group; n = 25); *H. pylori*-infected mice left untreated (*H. pylori* SS1 group; n = 25); mice administered *L. casei* strain Shirota only (*L. casei* strain Shirota group; n = 25); and uninfected, untreated mice (n = 25). An *L. casei* strain Shirota supernatant-treated animal group was not included in the study on ethical grounds, as we had no data supporting anti-*H. pylori* activity in the absence of *L. casei* strain Shirota live cells. At time intervals of 1, 2, 3, 6, and 9 months, blood samples were collected, and five animals per group were sacrificed by cervical dislocation and aseptically dissected. The entire stomach was dissected and analyzed for the presence of the administered probiotic and *H. pylori* colonization as well as the associated gastritis. The small and large intestines, including intestinal contents, also were collected and analyzed for the presence of the administered probiotic.

Analysis of the results from the in vivo experiments was performed with respect to *H. pylori* colonization by the Mann-Whitney test (GraphPad) and with respect to the associated gastritis by the Wilcoxon rank sum test due to the ordinal nature of the data (14). Finally, anti-*H. pylori* IgG antibodies were detected in the collected serum samples by an in-house enzyme-linked immunosorbent assay (ELISA). Briefly, 15 μg of *H. pylori* SS1 antigen produced by sonication and subsequent dialysis (SpectraPor; cutoff pore size, 8 kDa) was used to coat 96-well plates. Collected mouse serum samples (diluted 1/50) were incubated on the plates for 24 h at 4°C (primary incubation), and then rabbit anti-mouse IgG (entire molecule)– peroxidase conjugate (Sigma) was used for the secondary incubation (2 h at 37°C). Color was developed by the addition of *o*-phenylene-diamine (Sigma), and the optical density at 492 nm was measured with a Sunrise microtiter plate reader.

Bacterial detection in vivo. *H. pylori* in gastric tissue was detected by quantitative culturing, *H. pylori*-specific PCR, and histopathologic evaluation. For *H. pylori* SS1 quantitative culturing, preweighed half-stomach samples were homogenized in thioglycolate medium (Oxoid), serially diluted in phosphate-buffered saline, and plated on CHW agar plates with antibiotics. The cultures were incubated under microaerophilic conditions at 37°C for up to 6 days. *H. pylori* colonies were visualized on the basis of urease activity (12), and results were expressed as CFU per gram of gastric tissue. For *L. casei* strain Shirota isolation, stomach, intestinal, and fecal samples prepared as described above were plated on MRS agar supplemented with vancomycin (10 μg/ml) and polymyxin B (10 μg/ml) and incubated at 30°C for 48 to 72 h. Colonies were characterized on the basis of morphology, Gram stain, sugar fermentation patterns (API 50CHL kit; BioMerieux, Marcy l'Etoile, France), and species-specific PCR.

PCR conditions. Genomic DNA for the detection by PCR of bacterial strains in tissue samples or bacterial colonies was isolated by the standard phenol-chloroform method (29). For the detection of *H. pylori* by PCR, specific primers for the *ureC* (*glmM*) gene were used (19). For *L. casei* identification, species-specific PCR was performed with primers PrI and CasII for the 16S-23S intergenic spacer region (33).

Histopathologic analysis of gastric tissue samples. Excised stomachs were opened along the lesser curvature, and the longitudinal half was fixed in 10% neutral buffered formalin solution, embedded in paraffin, and processed for histopathologic analysis. Antral, body, and cardioesophageal mucosa samples were examined in the same section. Eleven serial longitudinal 4-μm sections were cut from each specimen; 9 of them were stained with hematoxylin-eosin (H&E) for evaluation of gastric inflammation, and 2 were stained by the May-Grünwald Giemsa stain method for the assessment of *H. pylori* colonization. The bacterial density was scored from 0 to 4, according to the Lausanne criteria (17). The pathologic characteristics of the gastric mucosa were assessed according to the updated Sydney system (9). Histopathologic evaluation was performed with no prior knowledge of the identity of the samples by the histopathologist.

RESULTS

In vitro screening for *H. pylori* inhibition. With the well diffusion assay, the activity of *L. casei* strain Shirota against *H. pylori* SS1 and wild-type clinical isolates ($n = 9$) was assessed. Antimicrobial activity was evident only when *L. casei* strain Shirota cells from o/n cultures (inhibition zones, 12.7 to 15.7 mm) or cells washed and resuspended in fresh MRS medium (inhibition zones, 11.0 to 15.0 mm) were used. No significant difference in anti-*H. pylori* activity was observed between o/n cultures of *L. casei* strain Shirota and cells washed and resuspended in MRS medium. Likewise, no inhibition was observed when the *L. casei* strain Shirota cell-free culture supernatant was used at its native pH 4.5, or at pH 6.5 or when *E. coli* HB101 was used instead of *L. casei* strain Shirota. These results suggest that the presence of living *L. casei* strain Shirota is required for *H. pylori* inhibition.

To elaborate further on the mechanism of in vitro *H. pylori* inhibition, we studied the effect of the *L. casei* strain Shirota supernatant on urease activity and simultaneously measured the effect on *H. pylori* viability in liquid *H. pylori* cultures. The *L. casei* strain Shirota supernatant was tested only at 10% the final incubation volume, because initial experiments involving

higher percentages in MRS medium resulted in reduced viability of *H. pylori*. In the presence of 10% *L. casei* strain Shirota cell-free supernatant at pH 4.5, inhibition of *H. pylori* urease activity (Fig. 1a) and viability (Fig. 1b) was observed. Similar results were obtained with the respective pH 4.5 MRS medium control.

In the above-described experiments, the pH of the incubation medium was 5.1 ± 0.2 due to the acidic pH (4.5) of the *L. casei* strain Shirota supernatant or acidified MRS medium. The concentration of lactic acid in the *L. casei* strain Shirota supernatant-containing incubation medium, determined by HPLC, was about 15 mM. To exclude the effect of a low pH on *H. pylori* urease activity and viability, an *L. casei* strain Shirota cell-free supernatant at pH 6.5, adjusted by the addition of NaOH, also was used. Urease activity was strongly inhibited in these samples (Fig. 1a), although no inhibition of *H. pylori* growth was observed (Fig. 1b). In the same experiments, the presence of a 10% pH 6.5 MRS medium control did not affect the urease activity or viability of *H. pylori* cells.

Inclusion of amoxicillin at 0.12 μg/ml (two times the MIC) as a positive control for *H. pylori* growth inhibition (Fig. 1b) resulted in a concomitant reduction in urease activity (Fig. 1a). Finally, in experiments in which *H. pylori* was incubated for 24 h with DL-lactic acid (at 100, 50, 25, 10, 5, 1, and 0 mM concentrations), over 70% inhibition of urease activity was seen at concentrations of lactic acid higher than 15 mM at 3 and 24 h (data not shown).

Evaluation of in vivo *H. pylori* colonization and associated gastritis in the infected and uninfected control groups. The presence of *H. pylori* and associated gastritis was not observed in any of the uninfected control animals. However, *H. pylori* was successfully detected in the gastric mucosa of mice in the *H. pylori* SS1 group throughout the entire 9-month observation period by viable counts, PCR, and histopathologic evaluation (25 of 25 mice for each of the three methods). *H. pylori* populations isolated from the gastric samples of mice in this group ranged from 3.3×10^4 to 2.6×10^6 CFU/g of gastric tissue (Fig. 2), with no statistical difference over the observation period. In these animals, *H. pylori* was detected on the mucosal surface and in the upper foveolae by histopathologic evaluation. At a higher magnification, the bacteria appeared to be adherent to the gastric epithelium (Fig. 3a). Colonization density was medium to high in the antrum (score, 2 or 3) but much lower in the body (score, 0 or 1) and the cardioesophageal junction. The mice developed chronic active gastritis (Fig. 3b) closely resembling the *H. pylori* gastritis observed in humans. At 1 month postinfection, the associated gastritis was evaluated for grade (presence of chronic inflammatory cells, scored 0 or 1) (Fig. 4a) and activity (neutrophilic infiltration, scored 0 or 2) (Fig. 5a), according to the updated Sydney system. However, in samples collected at 2 months postinfection, more severe gastritis was evident (chronic gastritis score, 1 or 2 [Fig. 4a], and active gastritis score, 1, 2, or 3 [Fig. 5a]). In animals with severe gastritis (grade score, 2; activity score, 3), the formation of lymphoid follicles also was observed (data not shown), as was that of intraluminal crypt neutrophilic abscesses (Fig. 3b). After 2 months postinfection, a gradual attenuation of active gastritis was observed (Fig. 5a), as reported before (20). In the body and cardioesophageal junction, the severity of gastritis was milder (grade score, 0; activity score, 1) (data not
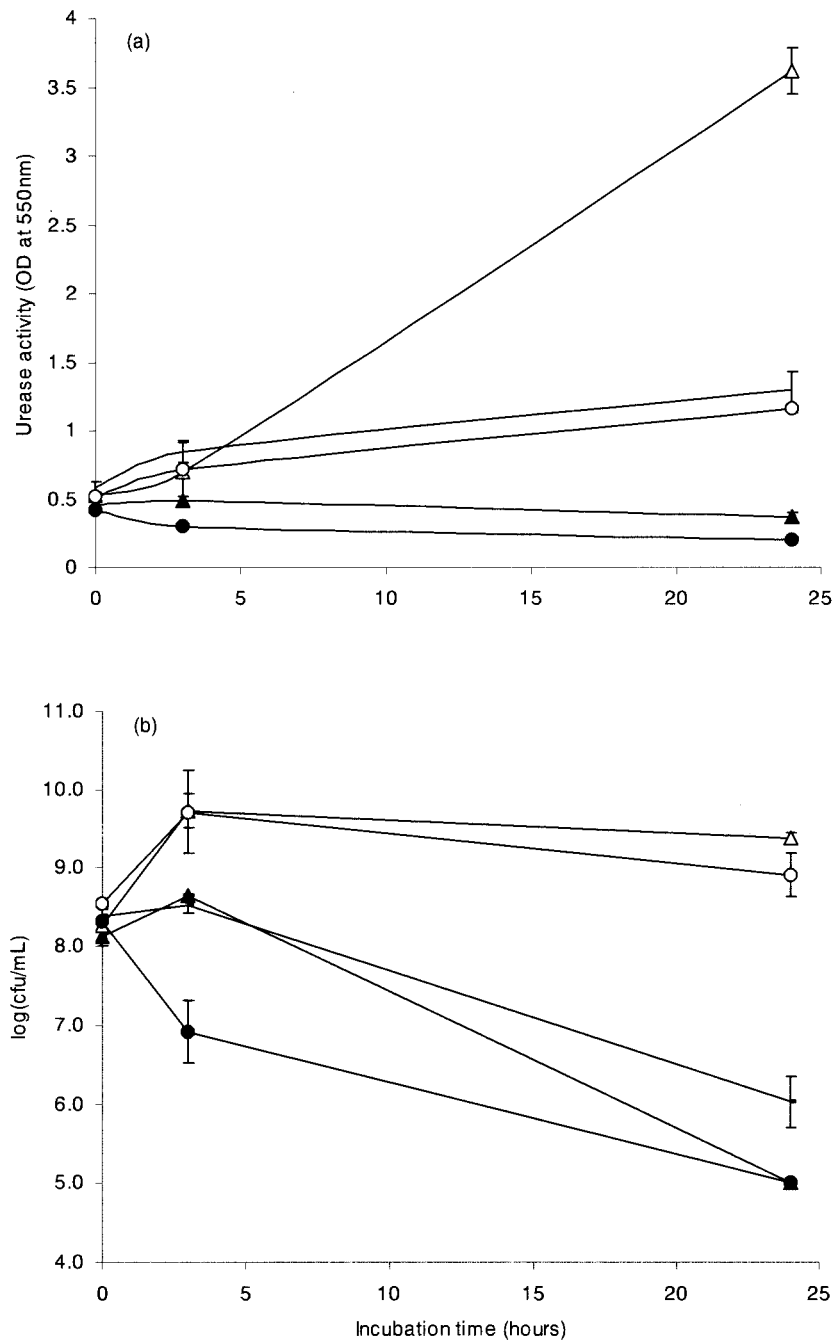
FIG. 1. In vitro effect of *L. casei* strain Shirota on *H. pylori* urease activity and viability. *H. pylori* SS1 cells (10^8 CFU/ml) suspended in BHIB without antibiotics were incubated under microaerophilic conditions at 37°C in the presence of a 10% volume of *L. casei* strain Shirota culture supernatant (pH 4.5 [●] or pH 6.5 [○]) or the appropriate MRS medium control (pH 4.5 [▲] or pH 6.5 [△]). Amoxicillin at 0.12 μg/ml (two times the MIC; solid line with no symbols) was included as a positive control for *H. pylori* growth inhibition. At 0, 3, and 24 h, samples were collected and assayed for urease activity and *H. pylori* viability. (a) Relative urease activity measured by introducing 10-μl *H. pylori* suspensions as described above to 300 μl of urease reaction buffer, incubating the mixtures for 2 h at 37°C, and measuring the optical density (OD) at 550 nm. Points represent the mean and standard error of the mean for three independent experiments. (b) *H. pylori* viability evaluated by determining viable counts on CHW agar plates following incubation at 37°C under microaerophilic conditions. Points represent the mean and standard error of the mean for three independent experiments. Note that there was a significant reduction in the urease activity of *H. pylori* cells incubated with *L. casei* strain Shirota supernatant at pH 6.5 (a), but the cells retained their viability (b). In the presence of 10% *L. casei* strain Shirota supernatant at pH 4.5 and the respective MRS medium at pH 4.5, control inhibition of urease activity and *H. pylori* viability was observed.
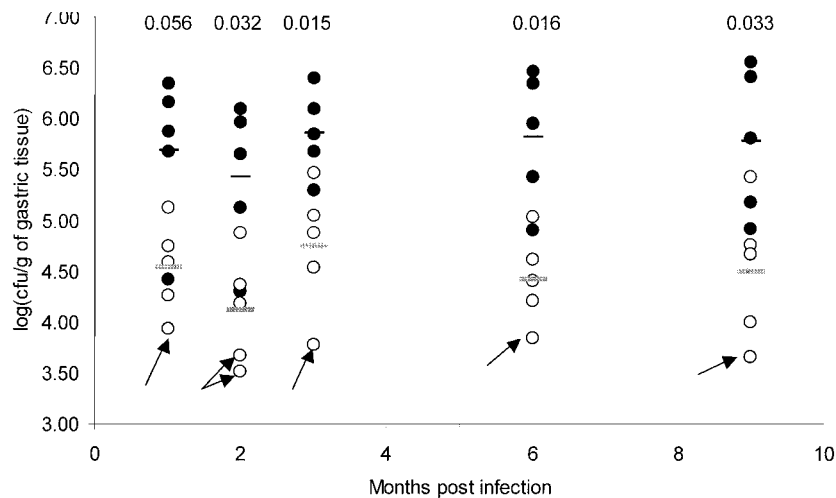
FIG. 2. *H. pylori* colonization of gastric mucosa in the *H. pylori* SS1 group (control group) (●) and the *H. pylori*-*L. casei* strain Shirota group (study group) (○). Each circle represents one animal. Lines represent the respective means in the control group (black lines) and the study group (gray lines). Numbers above each time point represent the *P* values calculated between the two groups at each time point (Mann-Whitney test; significant difference, *P* < 0.05). Arrows identify the six animals in the study group which were found *H. pylori* negative by histopathologic evaluation. Significant differences between the two groups, with respect to the number of colonizing bacteria, were observed at 2, 3, 6, and 9 months postinfection. No significant difference was observed within each group during the observation period.

shown). These results are consistent with the histopathologic scenario presented by the *H. pylori* SS1 mouse infection model (17).

For the *L. casei* strain Shirota group (control mice), given just the probiotic, gram-positive colonies isolated from the feces were initially classified by the API 50CHL test and verified by species-specific PCR. PCR was very effective in discriminating the administered *L. casei* strain Shirota from other lactobacilli, such as one *L. reuteri* and two *L. gasseri* strains also isolated in the normal gut flora of our mice. The administered *L. casei* strain Shirota was isolated at populations of 10^7 to 10^8 CFU/g of wet feces. However, it was not isolated or detected in washed intestinal tissue samples by species-specific PCR. In preliminary experiments, in which we examined the ability of *L. casei* strain Shirota to colonize the gut of healthy animals, we failed to continue isolating it in the feces approximately 20 days after the discontinuation of administration. All of the above results clearly indicate that *L. casei* strain Shirota does not colonize the gut epithelium and therefore will be present in the microflora only for nearly as long as it is administered. Histopathologic examination of gastric samples showed normal mucosa with the absence of gastritis in the antrum (Fig. 3c), body, and cardioesophageal junction.

We monitored weight as an index of the general well-being of the animals and found no significant difference among the groups of mice in the study. Although the presence of *H. pylori* in humans is related to type II gastritis, duodenal ulcer, mucosa-associated lymphoid tissue lymphoma, and gastric adenocarcinoma, there is no indication in the literature of an effect on the weights of *H. pylori*-infected persons or animals in the associated animal models. Finally, monitoring of water intake revealed that the animals consumed equal amounts of untreated water or *L. casei* strain Shirota-containing water. The daily probiotic dose was calculated to be 3×10^7 CFU/g of body weight.

Evaluation of in vivo *H. pylori* colonization and associated gastritis in *H. pylori*-infected animals treated with *L. casei* strain Shirota. For the *H. pylori*-*L. casei* strain Shirota group, *H. pylori* was detected in gastric samples by viable counts and PCR (25 of 25 mice for each of the two methods). Histopathologic evaluation revealed the presence of *H. pylori* in 19 of 25 mice. The difference in *H. pylori* populations (CFU per gram of gastric tissue) between the gastric samples of mice in the study group and those of mice in the control group was significant at 2 months postinfection and throughout the remainder of the 9-month observation period (*P* < 0.05; Mann-Whitney test) (Fig. 2). A significant decrease in chronic (Fig. 4b) and active (Fig. 5b) gastric mucosal inflammation also was observed at each time point throughout the observation period. The lamina propria usually contained a small amount of scattered lymphocytes (score, 0), as they are normally seen in the gastric mucosa despite the presence of *H. pylori* colonization (Fig. 3d). The grade of chronic gastritis was assessed as mild (score, 1) in 9 of 25 animals, because diffuse mild lymphocytic and plasmacytic inflammatory infiltration was observed (Fig. 4b). Mild neutrophilic infiltration of the lamina propria (activity score, 1) was observed in 11 of 25 animals (Fig. 5b). Significant differences in the body and the cardioesophageal mucosa with regard to *H. pylori* colonization and associated gastritis also were observed between the study and control groups (data not shown). *L. casei* strain Shirota was isolated from the intestinal contents throughout the entire administration period. However, albeit repeated attempts, isolation or detection of *L. casei* strain Shirota in washed intestinal tissue samples by PCR was not feasible.

Detection of anti-*H. pylori* IgG antibodies. All animals in the *H. pylori* SS1 group (control group) and the *H. pylori*-*L. casei* strain Shirota group (study group) were found to be positive for the presence of specific anti-*H. pylori* IgG antibodies in the serum samples collected, unlike the uninfected animals (Fig.
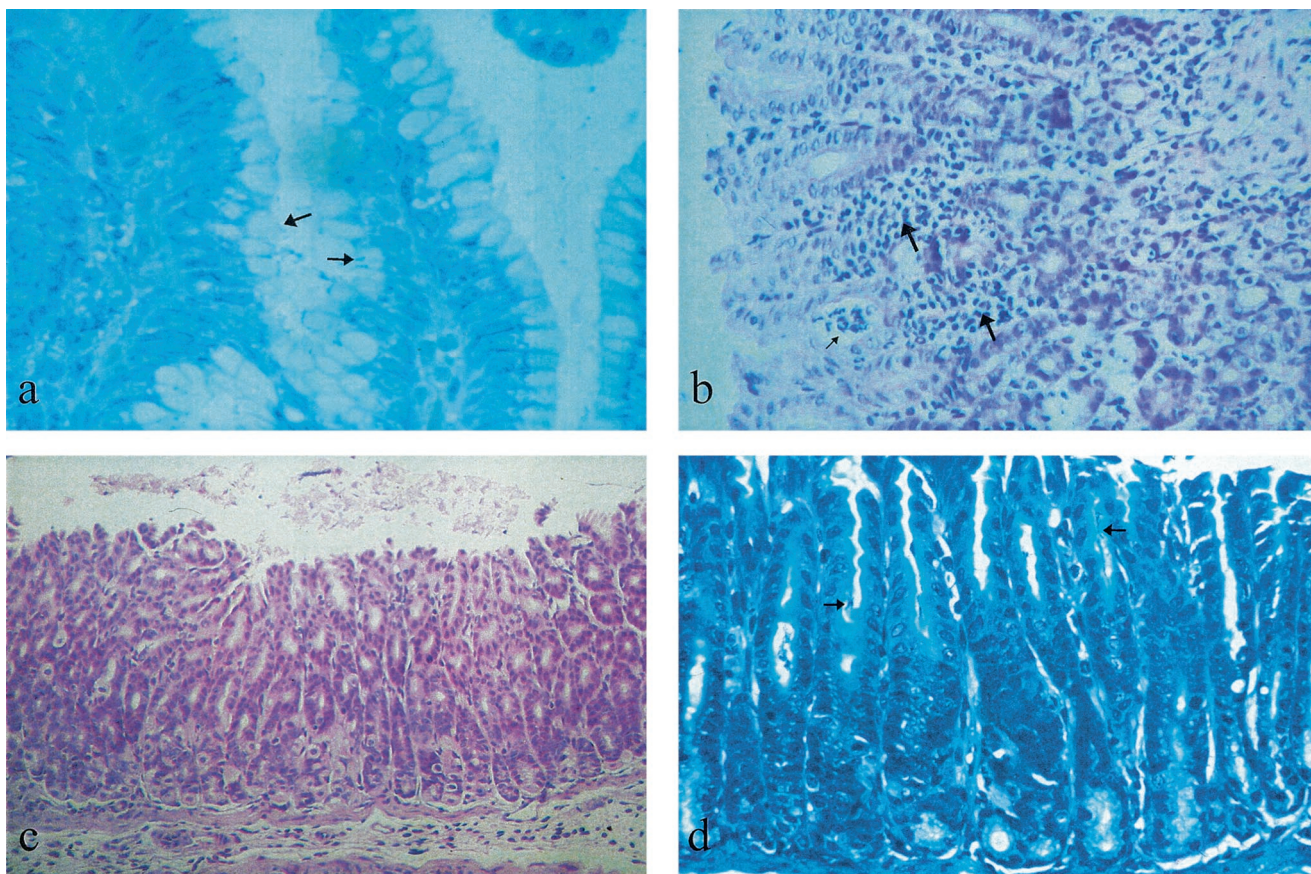
FIG. 3. Histopathologic evaluation of antral gastric samples from *H. pylori* SS1 group mice (a: Giemsa stain, magnification, ×316; b: H&E stain, magnification, ×158), *L. casei* strain Shirota group mice (c: H&E stain, magnification, ×79), and *H. pylori-L. casei* strain Shirota group mice (d: Giemsa stain, magnification, ×158). Note the presence of *H. pylori* in the gastric pits (a, arrows); the presence of chronic active inflammation in the lamina propria (b, large arrows), with the formation of intraluminal abscesses in the gastric pits (b, small arrow); the apparently normal mucosa in the *L. casei* strain Shirota group mice (c); and the absence of gastritis in the presence of *H. pylori* (d, arrows) in the *H. pylori-L. casei* strain Shirota group mice.

6). There were wide variations in the optical densities detected by the ELISA (ranging from 0.765 to 2.450) for serum samples at 6 and 9 months postinfection. A tendency for a reduction in antibody response in the *H. pylori-L. casei* strain Shirota study group was observed, although it was not statistically significant ($P > 0.05$; Mann-Whitney test).

DISCUSSION

In our study, inhibition against *H. pylori* SS1 and the nine wild-type clinical strains was evident with the agar diffusion assay only when there was a direct involvement of viable *L. casei* strain Shirota cells. No anti-*H. pylori* activity was obtained with a cell-free *L. casei* strain Shirota culture supernatant, whether the pH was preserved at 4.5 or adjusted to 6.5, possibly due to the buffering capacity of the medium and the horse blood serum proteins present. Our data are in complete accordance with recent observations in which *L. casei* strain Shirota culture supernatants isolated by plain centrifugation (containing as much as 10^4 CFU of viable bacteria/ml) were active in vitro against *H. pylori* in well diffusion assays; subsequent sterilization through 0.22-μm-pore-size filters abolished the inhib-

itory activity (5). Like us, the authors were unable to produce evidence of bacteriocin activity in the *L. casei* strain Shirota cell-free culture supernatants.

In our assays involving liquid *H. pylori* cultures, the addition of an *L. casei* strain Shirota culture supernatant (pH 4.5) and the respective MRS medium control (pH 4.5) decreased the final incubation pH to 5.1, resulting in the inhibition of urease activity and *H. pylori* viability. It is well documented that *H. pylori* viability in vitro under acid stress in the absence of urea is very low (30). Apart from the resulting low pH, others utilizing in vitro inhibition screening methods have attributed such anti-*H. pylori* activity of lactobacilli to lactic acid production (1, 2). More specifically, in cocultures of *H. pylori* and *L. salivarius*, concentrations of more than 10 mM lactic acid produced by the lactobacilli in the incubation medium strongly inhibited urease activity and *H. pylori* viability (1). When we incubated *H. pylori* with lactic acid concentrations of 100 to 1 mM, we observed over 70% inhibition of urease activity for 15 mM lactic acid within 3 h of incubation. In our liquid culture assays in which only 10% *L. casei* strain Shirota culture supernatant was present, the lactic acid concentration measured by HPLC was in about 15 mM, a concentration capable of induc-
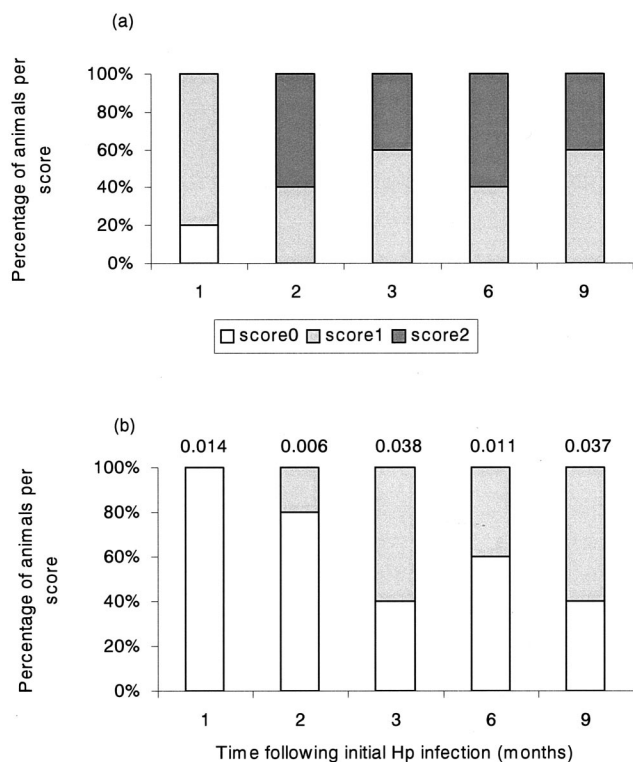
FIG. 4. *H. pylori*-associated chronic gastritis in the antrum. Bars represent the percentages of samples with each histopathologic score (0, 1, or 2) in the *H. pylori* (Hp) SS1 group (control group; five animals per time point) (a) and the *H. pylori*-*L. casei* strain Shirota group (study group; five animals per time point) (b). Numbers above the columns in panel b represent the *P* values calculated between the two groups at each time point, with reference to *H. pylori* chronic gastritis score. Significant differences were observed at all time points during the observation period (Wilcoxon rank sum test).

kinetics of lactic acid production in *L. casei* strain Shirota cultures grown in MRS medium and found lactic acid to be the main product (at concentrations of up to 150 mM). Furthermore, only traces of lipid are present, attributed to the 0.1% Tween detergent in the MRS medium, and *L. casei* strains in general have low proteolytic activity compared to other lactobacilli, such as *L. bulgaricus*. Finally, competition for nutrients can be ruled out, as we did not observe inhibition of *H. pylori* when *E. coli* was used as a positive control for nutrient depletion in cultures.

Since its introduction, the *H. pylori* SS1 mouse infection model has been used extensively in the field of *H. pylori* research, and the conditions for its application have been described in detail (12, 17). We used the particular infection model in which animals were treated with large doses of *L. casei* strain Shirota in a continuous fashion through the water supply. *L. casei* strain Shirota exhibited good viability in water; therefore, we succeeded in giving it to the animals through the normal water supply, in effect alleviating the burden of repeated orogastric administrations (35). Furthermore, this method of administration was superior to mixing freeze-dried

ing the inhibition of *H. pylori* urease activity and viability. Furthermore, when an *L. casei* strain Shirota culture supernatant adjusted to pH 6.5 was used, there was significant inhibition of *H. pylori* urease activity, although no reduction in viable counts was observed over 24 h, possibly due to the absence of acid stress on *H. pylori*. Collectively these results suggest that lactic acid produced by *L. casei* strain Shirota is involved in inhibition of the bacterial urease system. This activity of lactic acid is probably a combination of its inhibitory effect on the bacterial urease system (1) and the reduced ability of *H. pylori* to survive at a low pH in the absence of urea (30). In our experimental design, urease inhibition by lactic acid contained in the *L. casei* strain Shirota culture supernatant can render *H. pylori* incapable of overcoming the low-pH conditions. In the respective MRS medium control (pH 4.5) acidified with HCl, in which the urease system is functioning, small amounts of endogenous urea (23) could sustain viability for the first few hours, but not over the entire 24-h incubation period.

The exact mechanism by which lactic acid inhibits urease activity remains elusive. However, others (5, 7) have observed very little or no effect on urease activity attributed to lactic acid, although direct comparisons with our work are impossible due to the different experimental designs used for the determination of lactic acid. We have studied in detail by HPLC the
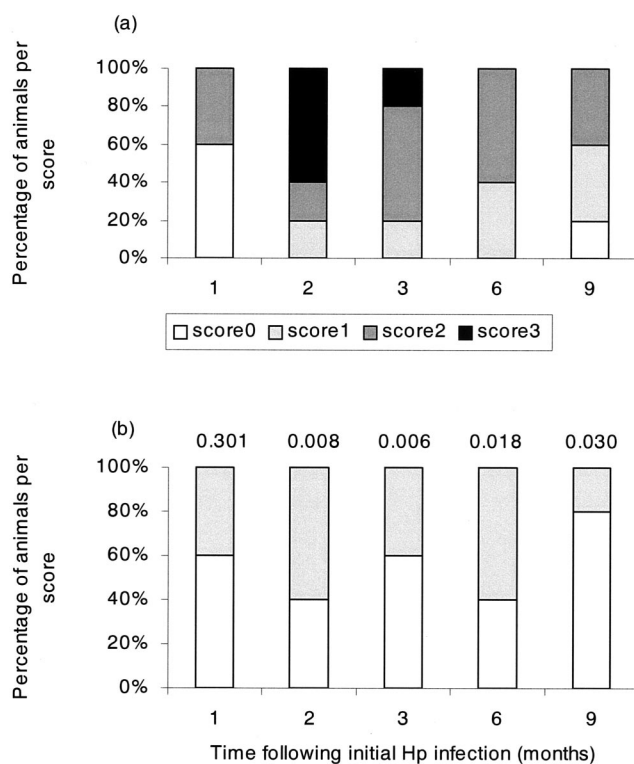

FIG. 5. *H. pylori*-associated active gastritis in the antrum. Bars represent the percentages of samples with each histopathologic score (0, 1, 2, or 3) in the *H. pylori* (Hp) SS1 group (control group; five animals per time point) (a) and the *H. pylori*-*L. casei* strain Shirota group (study group; five animals per time point) (b). Numbers above the columns in panel b represent the *P* values calculated between the two groups at each time point, with reference to *H. pylori* active gastritis score. Significant differences in gastritis activity were observed after month 2 and throughout the entire observation period thereafter (Wilcoxon rank sum test). After month 2, a gradual attenuation of the phenomenon within the *H. pylori* SS1 group was observed, unlike the results for the *H. pylori*-*L. casei* strain Shirota group.
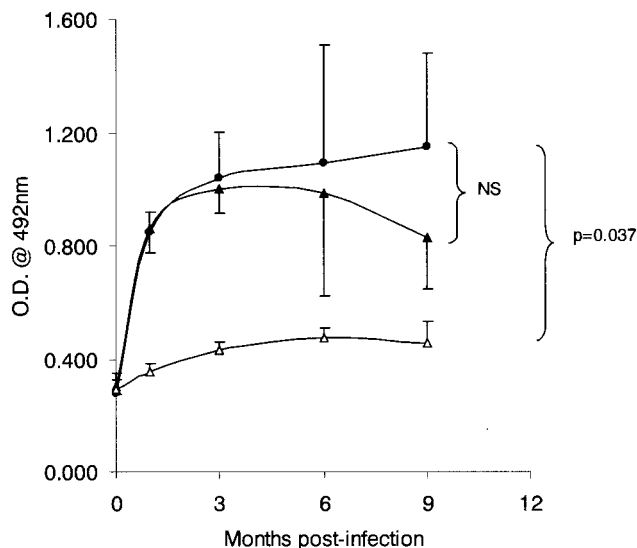
FIG. 6. Total IgG response to *H. pylori* infection in C57BL/6 mice. Serum samples collected at 0, 1, 2, 3, 6, and 9 months postinfection were assayed for anti-IgG *H. pylori* antibodies by an ELISA. Although there was a decrease in the IgG response in the *H. pylori-L. casei* strain Shirota group (study group; five animals per time point; ▲) compared to the results in the *H. pylori* SS1 group (control group; five animals per time point; ●), this decrease was not found to be significant (Mann-Whitney test). Open triangles represent the *L. casei* strain Shirota group (control group; five animals per time point). O.D., optical density; NS, not significant.

Lactobacillus cultures in animal feed, because we could monitor the viability as well as the concentration of the bacteria administered. Finally, it provided a continuous administration of lactobacilli, thus maintaining a constant "therapeutic dose" to the animals. We observed no differences in the drinking habits of the animals due to the presence of lactobacilli, as consumption of water containing lactobacilli was no different from consumption by the control animals, which received only plain water.

Monitoring the kinetics of *L. casei* strain Shirota after ingestion is a formidable task, because of the plethora of microbes present in the normal mouse intestinal microflora. Numerous existing methods encompass classical culture-dependent techniques, with selective or nonselective media, and culture-independent techniques, including microscopy, enzyme metabolite analysis, and molecular sequence analysis (26). We were able to isolate *L. casei* strain Shirota from intestinal contents by using selective media as well as to discriminate it by species-specific PCR from other lactobacilli contained in the normal flora of the mouse. For the *H. pylori-L. casei* strain Shirota group, we observed a significant reduction of *H. pylori* colonization in the gastric mucosa throughout the entire observation period (9 months). In our experiments, *H. pylori* colonization in the range of 10^4 CFU/g was hardly detectable by histopathologic evaluation, an observation made by others as well (31). We therefore based our conclusion regarding *H. pylori* colonization on data obtained by quantitative isolation of the bacterium from dissected gastric samples rather than just by histopathologic evaluation. Such a protocol has been used routinely for the assessment of *H. pylori* viable counts in the SS1 mouse model (12, 17) and has proved very

efficient in measuring the colonization efficiency of *H. pylori* colonization-deficient mutant strains with gene knockouts (23). Also, to the best of our knowledge, there is no literature supporting the formation of biofilms by *H. pylori*. Whether this in vivo suppressive effect of *L. casei* strain Shirota on *H. pylori* is associated with the observed in vitro inhibition of bacterial urease activity, which is essential for the survival of *H. pylori* in the stomach, remains to be further documented. In a similar study, *L. salivarius* was able to suppress *H. pylori* in vivo in an *H. pylori*-infected gnotobiotic murine model (1). The authors were able to measure lactic acid concentrations of between 2.3 and 18.9 μmol/g in the gastric contents and found that such concentrations inhibited *H. pylori* viability and completely abolished urease activity in vitro. In a recent intervention study in which the effect on *H. pylori* colonization was assessed by the UBT, *L. casei* strain Shirota-containing milk administered to *H. pylori*-colonized subjects had a slight but nonsignificant suppressive effect on *H. pylori* (5). In view of the data on urease inhibition by lactic acid, methods more stringent than UBT (i.e., determination of viable counts or histopathologic evaluation) should be used for the evaluation of *H. pylori* colonization in clinical studies involving lactic acid bacteria with a potential for inhibition of urease activity. In our study, the observed significant attenuation in the chronic and acute inflammation of the gastric mucosa, with no to moderate lymphoplasmacytic infiltration in the lamina propria, can be attributed primarily to the reduced *H. pylori* colonization levels in the *L. casei* strain Shirota-treated mice. However, involvement of a systemic mechanism with a possible immunological background cannot be ruled out. Indeed, studies on the immunological effects of orally administered *L. casei* strain Shirota have highlighted its ability to modulate immune responses in the establishment of tumor immunity and the induction of specific antitumor activity (21, 22). In addition, *L. casei* strain Shirota promoted the production of several different cytokines involved in the regulation of host cellular immune responses, resulting in the alteration of lymphocyte susceptibility to apoptosis (16). Finally, *L. casei* strain Shirota was able to increase cellular immunity significantly, as determined by the delayed-type hypersensitivity response to heat-killed *L. monocytogenes*, and therefore to enhance the host response against oral *L. monocytogenes* infection in rats (8). Therefore, the stimulation of specific immunity and nonspecific immunity (16, 18, 25, 28) could be another mechanism by which this particular probiotic exerts its anti-*H. pylori* activity, a hypothesis currently being investigated in our laboratory. A reduction of *H. pylori*-associated gastritis also was reflected by the marked yet not significant reduction in the anti-*H. pylori* IgG response detected in the study group animals over the 9-month observation period. Aiba et al. (1) observed a significant reduction of antibody titers to *H. pylori* in mice dosed with *L. salivarius*, but they did not evaluate *H. pylori*-associated gastritis by histopathologic analysis.

However complex the inhibition mechanism may be, the fact remains that significant suppression of the colonization ability of *H. pylori* and the associated inflammation was evident in *L. casei* strain Shirota-treated animals. To our knowledge, this is the first documented report of *L. casei* strain Shirota administration promoting a decrease in *H. pylori* colonization and a significant attenuation of the associated gastritis in a well-

established animal model of *H. pylori* infection. We believe that our results also could be related to the continuous mode of administration of the probiotic through the water supply, in combination with the high doses given to the animals. We have succeeded in administering doses of lyophilized *L. casei* strain Shirota of higher than 50 mg, a daily dose of approximately 5×10^8 CFU. This daily dose expressed in the human condition amounts to as much as 175 to 200 g of lyophilized *Lactobacillus* culture, assuming an average human weight of 70 kg. It is clear that there is a lack of data on an effective therapeutic dose for probiotics. In comparison to doses used in existing clinical trials evaluating the efficacy of probiotics, this dose is potentially a very high dose sustained over a period of 9 months. It is conceivable that the probiotic effect of lactic acid bacteria would be best manifested if the bacteria were administered at high doses in a continuous fashion; however, such an approach needs to be proven in an appropriate clinical setting.

REFERENCES

1. **Aiba, Y., N. Suzuki, A. M. Kabir, A. Takagi, and Y. Koga.** 1998. Lactic acid-mediated suppression of *Helicobacter pylori* by the oral administration of *Lactobacillus salivarius* as a probiotic in a gnotobiotic murine model. Am. J. Gastroenterol. **93:**2097–2101.
2. **Alakomi, H. L., E. Skytta, M. Saarela, T. Mattila-Sandholm, K. Latva-Kala, and I. M. Helander.** 2000. Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. Appl. Environ. Microbiol. **66:**2001–2005.
3. **Asahara, T., K. Nomoto, M. Watanuki, and T. Yokokura.** 2001. Antimicrobial activity of intraurethrally administered probiotic *Lactobacillus casei* in a murine model of *Escherichia coli* urinary tract infection. Antimicrob. Agents Chemother. **45:**1751–1760.
4. **Blaser, M. J.** 1990. Helicobacter pylori and the pathogenesis of gastroduodenal inflammation. J. Infect. Dis. **161:**626–633.
5. **Cats, A., E. J. Kuipers, M. A. R. Bosschaert, R. G. J. Pot, C. M. J. E Vandenbroucke-Grauls, and J. G. Kusters.** 2003. Effect of frequent consumption of a Lactobacillus casei-containing milk drink in Helicobacter pylori-colonized subjects. Aliment. Pharmacol. Ther. **17:**429–435.
6. **Coconnier, M. H., V. Lievin, M. F. Bernet-Camard, S. Hudault, and A. L. Servin.** 1997. Antibacterial effect of the adhering human *Lactobacillus acidophilus* strain LB. Antimicrob. Agents Chemother. **41:**1046–1052.
7. **Coconnier, M. H., V. Lievin, E. Hemery, and A. L. Servin.** 1998. Antagonistic activity against *Helicobacter* infection in vitro and in vivo by the human *Lactobacillus acidophilus* strain LB. Appl. Environ. Microbiol. **64:**4573–4580.
8. **de Waard, R., J. Garssen, G. C. A. M. Bokken, and J. G. Vos.** 2002. Antagonistic activity of *Lactobacillus casei* strain Shirota against gastrointestinal *Listeria monocytogenes* infection in rats. Int. J. Food Microbiol. **73:**93–100.
9. **Dixon, M. F., R. M. Genta, J. H. Yardley, and P. Correa.** 1996. Classification and grading of gastritis. The updated Sydney system. Am. J. Surg. Pathol. **20:**1161–1181.
10. **Dunn, B. E., H. Cohen, and M. J. Blaser.** 1997. *Helicobacter pylori*. Clin. Microbiol. Rev. **10:**720–741.
11. **Everhart, J. E.** 2000. Recent developments in epidemiology of *Helicobacter pylori*. Gastroenterol. Clin. North Am. **29:**559–578.
12. **Ferrero, R. L., J. M. Thiberge, M. Huerre, and A. Labigne.** 1998. Immune responses of specific-pathogen-free mice to chronic *Helicobacter pylori* (strain SS1) infection. Infect. Immun. **66:**1349–1355.
13. **Hazell, S. L., T. J. Borody, A. Gal, and A. Lee.** 1987. *Campylobacter pyloridis* gastritis. I. Detection of urease as a marker of bacterial colonization and gastritis. Am. J. Gastroenterol. **82:**292–296.
14. **Ilstrup, D. M.** 1990. Statistical methods in microbiology. Clin. Microbiol. Rev. **3:**219–226.
15. **Kabir, A. M., Y. Aiba, A. Takagi, S. Kamiya, T. Miwa, and Y. Koga.** 1997. Prevention of *Helicobacter pylori* infection by lactobacilli in a gnotobiotic murine model. Gut **41:**49–55.
16. **Kirjavainen, P. V., H. S. El-Nezami, S. J. Salminen, J. T. Ahokas, and P. F. Wright.** 1999. The effect of orally administered viable probiotic and dairy lactobacilli on mouse lymphocyte proliferation. FEMS Immunol. Med. Microbiol. **26:**131–135.
17. **Lee, A., J. O'Rourke, M. C. De Ungria, B. Robertson, G. Daskalopoulos, and M. F. Dixon.** 1997. A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain. Gastroenterology **112:**1386–1397.
18. **Link-Amster, H., F. Rochat, K. Y. Saudan, O. Mignot, and J. M. Aeschlimann.** 1994. Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake. FEMS Immunol. Med. Microbiol. **10:**55–63.
19. **Lu, J. J., C. L. Perng, R. Y. Shyu, C. H. Chen, Q. Lou, S. K. Chong, and C. H. Lee.** 1999. Comparison of five PCR methods for detection of *Helicobacter pylori* DNA in gastric tissues. J. Clin. Microbiol. **37:**772–774.
20. **Mahler, M., C. Janke, S. Wagner, and H. J. Hedrich.** 2002. Differential susceptibility of inbred mouse strains to *Helicobacter pylori* infection. Scand. J. Gastroenterol. **37:**267–278.
21. **Matsuzaki, T., and J. Chin.** 2000. Modulating immune responses with probiotic bacteria. Immunol. Cell. Biol. **78:**67–73.
22. **Matsuzaki, T., R. Yamazaki, S. Hashimoto, and T. Yokokura.** 1998. The effect of oral feeding of *Lactobacillus casei* strain Shirota on immunoglobulin E production in mice. J. Dairy Sci. **81:**48–53.
23. **McGee, D. J., F. J. Radcliff, G. L. Mendz, R. L. Ferrero, and H. L. T. Mobley.** 1999. *Helicobacter pylori rocF* is required for arginase activity and acid protection in vitro but is not essential for colonization of mice or for urease activity. J. Bacteriol. **181:**7314–7322.
24. **Michetti, P., G. Dorta, P. H. Wiesel, D. Brassart, E. Verdu, M. Herranz, C. Felley, N. Porta, M. Rouvet, A. L. Blum, and I. Corthesy-Theulaz.** 1999. Effect of whey-based culture supernatant of *Lactobacillus acidophilus* (*johnsonii*) La1 on *Helicobacter pylori* infection in humans. Digestion **60:**203–209.
25. **Ogawa, M., K. Shimizu, K. Nomoto, M. Takahashi, M. Watanuki, R. Tanaka, T. Tanaka, T. Hamabata, S. Yamasaki, and Y. Takeda.** 2001. Protective effect of *Lactobacillus casei* strain Shirota on Shiga toxin-producing *Escherichia coli* O157:H7 infection in infant rabbits. Infect. Immun. **69:**1101–1108.
26. **O'Sullivan, D. J.** 1999. Methods of analysis of intestinal microflora, p. 23–44. *In* G. W. Tannock (ed.), Probiotics. A critical review. Horizon Scientific Press, Norfolk, United Kingdom.
27. **Parsonnet, J., S. Hansen, L. Rodriguez, A. B. Gelb, R. A. Warnke, E. Jellum, N. Orentreich, J. H. Vogelman, and G. D. Friedman.** 1994. *Helicobacter pylori* infection and gastric lymphoma. N. Engl. J. Med. **330:**1267–1271.
28. **Perdigon, G., S. Alvarez, M. Rachid, G. Aguero, and N. Gobbato.** 1995. Immune system stimulation by probiotics. J. Dairy Sci. **78:**1597–1606.
29. **Sambrook, J., E. F. Fritsch, and T. Maniatis (ed.).** 1989. Molecular cloning: a laboratory manual, 2nd ed., p. 9.16. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
30. **Stingl, K., E. M. Uhlemann, G. Deckers-Hebestreit, R. Schmid, E. P. Bakker, and K. Altendorf.** 2001. Prolonged survival and cytoplasmic pH homeostasis of *Helicobacter pylori* at pH 1. Infect. Immun. **69:**1178–1180.
31. **Sutton, P., J. Wilson, and A. Lee.** 2000. Further development of the *Helicobacter pylori* mouse vaccination model. Vaccine **18:**2677–2685.
32. **Tannock, G. W.** 1999. A fresh look at the intestinal microflora, p. 5–14. *In* G. W. Tannock (ed.), Probiotics. A critical review. Horizon Scientific Press, Norfolk, United Kingdom.
33. **Tilsala-Timisjarvi, A., and T. Alatossava.** 1997. Development of oligonucleotide primers for the 16S-23S rRNA intergenic sequences for identifying different dairy and probiotic lactic acid bacteria by PCR. Int. J. Food Microbiol. **35:**49–56.
34. **Yasui, H., K. Shida, T. Matsuzaki, and T. Yokokura.** 1999. Immunomodulatory function of lactic acid bacteria. Antonie Leeuwenhoek **76:**383–389.
35. **Zhou, J. S., Q. Shu, K. J. Rutherfurd, J. Prasad, M. J. Birtles, P. K. Gopal, and H. S. Gill.** 2000. Safety assessment of potential probiotic lactic acid bacterial strains *Lactobacillus rhamnosus* HN001, *Lb. acidophilus* HN017, and *Bifidobacterium lactis* HN019 in BALB/c mice. Int. J. Food Microbiol. **56:**87–96.