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Colonization and Immunomodulation by *Lactobacillus reuteri* ATCC 55730 in the Human Gastrointestinal Tract

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***Lactobacillus reuteri* ATCC 55730 is a probiotic (health-promoting) bacterium widely used as a dietary supplement. This study was designed to examine local colonization of the human gastrointestinal mucosa after dietary supplementation with *L. reuteri* ATCC 55730 and to determine subsequent immune responses at the colonized sites. In this open clinical investigation, 10 healthy volunteers and 9 volunteers with ileostomy underwent gastroscopy or ileoscopy and biopsy samples were taken from the stomach, duodenum, or ileum before and after supplementation with 4×10^8 CFU of live *L. reuteri* ATCC 55730 lactobacilli per day for 28 days. Biopsy specimen colonization was analyzed using fluorescence in situ hybridization with a molecular beacon probe, and immune cell populations were determined by immunostaining. Endogenous *L. reuteri* was detected in the stomach of 1 subject and the duodenum of 3 subjects (out of 10 subjects). After *L. reuteri* ATCC 55730 supplementation, the stomachs of 8 and the duodenums of all 10 subjects were colonized. Three ileostomy subjects (of six tested) had endogenous *L. reuteri* at baseline, while all six displayed colonization after *L. reuteri* supplementation. Gastric mucosal histiocyte numbers were reduced and duodenal B-lymphocyte numbers were increased by *L. reuteri* ATCC 55730 administration. Furthermore, *L. reuteri* administration induced a significantly higher amount of CD4-positive T-lymphocytes in the ileal epithelium. Dietary supplementation with the probiotic *L. reuteri* ATCC 55730 induces significant colonization of the stomach, duodenum, and ileum of healthy humans, and this is associated with significant alterations of the immune response in the gastrointestinal mucosa. These responses may be key components of a mechanism by which *L. reuteri* ATCC 55730 exerts its well-documented probiotic effects in humans.**

Lactobacillus reuteri (13) is a heterofermentative bacterium that resides in the gastrointestinal tract of humans and animals (2, 11) and is considered to be one of the few true autochthonous (indigenous) *Lactobacillus* species in humans (14). *L. reuteri* ATCC 55730 (also designated SD2112) has been extensively studied and is widely used as a food additive to improve human gastrointestinal health. Oral administration delivers *L. reuteri* ATCC 55730 to the gastrointestinal tract, leading to shedding of live bacteria in the feces (16, 20). Clinical trials have shown that *L. reuteri* ATCC 55730 administration is safe (20, 21), significantly reduces the incidence and the severity of diarrhea of different origins (16, 17), reduces gastrointestinal illness and infections (G. Asli, A. Alsheikh, and Z. Weizman, Abstr. 36th Annu. Meet. Eur. Soc. Paediatr. Gastroenterol. Hepatol. Nutr., abstr. O041, 2003), and may be useful in the relief of constipation in elderly subjects (12).

Fecal colonization does not, however, provide information on the specific sites of colonization of *L. reuteri* ATCC 55730 along the gastrointestinal tract. Our aim was to document these specific sites and to examine possible alterations in the immune system associated with this colonization.

MATERIALS AND METHODS

Subjects. The investigation had an open design and was performed in two parts: a gastroscopy session investigating the upper gastrointestinal tract and an ileoscopy session investigating the distal small bowel. Healthy adult subjects (>18 years of age) were recruited from the hospital staff at Roskilde County Hospital, Köge, Denmark, while ileostomy adult patients were recruited at the Gastroenterology Department. Subjects all had normal eating habits and gave written informed consent before entering the study. The exclusion criteria were (i) use of antibiotics 2 weeks before or during the study; (ii) the use of probiotics or probiotic foods 3 weeks before or during the study; (iii) ongoing treatment with nonsteroid anti-inflammatory drugs and/or proton pump inhibitors; (iv) regularly treated, severe organic disease; and (v) acute or chronic gastrointestinal illness in an active phase or with persistent mucosal abnormalities.

Study product. Dietary supplementation with *L. reuteri* ATCC 55730 was the same for all subjects in the study. Each subject was asked to take two chewable tablets in the morning and two in the evening every day for 28 days. Each tablet contained 10^8 CFU of *L. reuteri* ATCC 55730 to give a total daily dose of 4×10^8 CFU of *L. reuteri*. Biopsies were performed at baseline (day 0) and again the day after *L. reuteri* supplementation was complete (day 28). The subjects thus acted as their own controls.

Biopsy procedures. In the gastroscopy group, 10 healthy volunteers were studied. A gastro-duodenoscopy was performed following an overnight fast, and biopsy samples were taken from the gastric corpus and antrum and from the third part of the duodenum. Samples of gastric juice were also taken. In the ileoscopy group, nine volunteers with ileostomy following colectomy for ulcerative colitis (three subjects) or Crohn's disease (six subjects) were studied. Two subjects with Crohn's disease had resection of 50 and 150 cm of small intestine. The remaining small bowel was without signs of inflammation in all subjects. Ileoscopies were performed and biopsy specimens were taken within the distal 20 cm of the small bowel.

The same physician took all the biopsy specimens, and each had an average approximate weight of 33 mg. Specimens were either prepared for histological examination or transported in cold phosphate-buffered saline (PBS) to BioGaia Laboratories (Lund, Sweden) for analysis of *Lactobacillus* counts (principally as described below for fecal samples). The time from sampling to analysis was less than 36 h. For histological analysis, biopsy specimens were formalin fixed and embedded in paraffin. Subsequently, 4-μm-thick sections were cut and stained histochemically (with hematoxylin-eosin, van Gieson, periodic acid-Schiff–Alcian, and periodic acid-Schiff–diastase stain) and immunohistochemically. The primary antibodies used were CD20 (B lymphocytes); CD3, CD4, and CD8

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TABLE 1. Recovery of *L. reuteri* in the feces of subjects before and after *L. reuteri* ATCC 55730 supplementation[a]

Session	% Compliance (SD)	CFU of *L. reuteri*/g of fecal material (SD) at day:			
		0	28	42	56
Gastroscopy	87 (7) ($n = 10$)	ND[b] ($n = 10$)	4.0×10^4 (1.1×10^5) ($n = 10$)	1.0×10^3 (5.3×10^2) ($n = 7$)	1.2×10^3 (6.0×10^2) ($n = 5$)
Ileoscopy	97 (8) ($n = 9$)	ND ($n = 7$); 1.0×10^3 (5.3×10^2) ($n = 2$)[c]	ND ($n = 3$); 8.0×10^3 (1.6×10^4) ($n = 6$)[c]	1.0×10^3 (6.1×10^2) ($n = 4$)	6.7×10^2 (4.2×10^2) ($n = 3$)

[a] Fecal or ileostomy output samples were taken from subjects at baseline (day 0) and after the daily administration of *L. reuteri* ATCC 55730 at 4×10^8 CFU/day for 28 days (day 28). Sample collection was optional on days 42 and 56 (i.e., 28 days after dosing of *L. reuteri* was stopped), and in all these samples *L. reuteri* was detected. Compliance was calculated from the reported intake of the subjects and is expressed as the average intake of tablets of each person as a percentage of the total per protocol dose over 28 days. Results are expressed as CFU per gram (wet weight) of fecal material, with standard deviations and number of observations in parentheses.

[b] ND, not detected (<100 CFU/g).

[c] Mean of samples where *L. reuteri* was detected.

(T lymphocytes); CD68 (histiocytes); *Helicobacter pylori*; and Ki-67 (a proliferation marker) and were obtained from DakoCytomation (Glostrup, Denmark). Immunohistochemical staining was performed on a DAKO TechMate 500 immunostainer to obtain uniform staining.

A pathologist evaluated the biopsy specimens. Using histochemical staining, tissue damage was graded on a scale of 0 to 10 according to Madsen et al. (8), with grades representing the numerical sum of four criteria: ulceration of the mucosa, epithelial hyperplasia, and the amounts of mononuclear cells and neutrophil granulocytes in the lamina propria. On the basis of the immunohistochemical staining, the numbers of CD20-, CD3-, CD4-, CD8-, and CD68-positive cells in lamina propria were evaluated semiquantitatively. One section of each biopsy sample was used for each specific stain. Each slide was evaluated and scored in the following way: 1 = no cells detected, 2 = single cells detected, 3 = dispersed cells or aggregations of cells detected, 4 = several adjoining groups of cells detected. The whole biopsy section was evaluated, and cells in as many fields as possible were counted. The mean scores were then calculated for each cell type. In order to confirm the observations, the biopsy slides were reevaluated by the same pathologist after being blinded by an independent person and the overall concordance between the two analyses was calculated.

FISH probe and hybridization procedures. A 16S rRNA-targeted molecular beacon probe for the detection of *L. reuteri* using fluorescence in situ hybridization (FISH) was developed. The probe was 100% matched to the 16S rRNA sequence available for *L. reuteri*. Specificity of the probe was carefully evaluated in a previous study (N. Carbajal and J. Abad, submitted for publication) by analysis of hybridization of the *L. reuteri*-beacon probe with *Lactobacillus oris*, *Lactobacillus fermentum*, and *Lactobacillus rhamnosus*—all of which have 16S rRNA sequences closely related to that of *L. reuteri*. No binding of the probe was observed, and thus a nonspecific reaction with other bacterial species can be excluded. The probe is species but not strain specific, and thus although it identifies *L. reuteri* under the conditions used, it cannot confirm that the strain on the biopsy samples is the *L. reuteri* ATCC 55730 given to the subjects.

Biopsy sections were deparaffinated by baking at 60°C for 1 h followed by rehydration with successive treatments with ethanol (100, 95, 80, 70, and 50%) and finally molecular-biology-grade water. The cells on the sections were lysed by incubation at 37°C for 1 h in a solution containing 25 mM Tris-HCl (pH 7.5), 10 mM EDTA, 600 mM sucrose, lysozyme (2 mg/ml), and mutanolysin (0.2 μl/50-μl mixture) in a humid chamber. After rinsing in PBS for 5 min, the tissue sections were treated with 50 μl of proteinase K in PBS (100 μg/ml) for 20 min at 37°C in a humid chamber. Proteinase K was then inactivated with 0.2% glycine (15 s), and the slides were washed in PBS for 5 min before being dehydrated in ethanol. Fifty microliters of denaturing-hybridization buffer was added [60% formamide, 14% dextran sulfate (60% solution), 7% SSC (pH 7.4) (1× SSC is 1.2 M NaCl plus 0.27 M sodium citrate), probe (2.5 ng/μl; 0.05 mg/ml), and poly(A) (0.5 μg/μl)] at a temperature of 75°C for 90 s. Hybridization was performed at 50°C for 2 to 5 h. The slides were then washed three times in prewarmed (37°C) 50% formaldehyde–4× SSC at 37°C for 5 min, and this was followed by two washes with 1× SSC for 5 min. Slides were examined with a confocal laser scanning system (Leica TCS SP) attached to a Leica DM IRBE microscope and a 100× PL APO 1.4-0.7NA oil immersion objective. Oregon Green-labeled samples were excited using an argon laser at 488 nm with the emission window set to 499 to 570 nm. Images were processed using Leica TCS software and Adobe Photoshop 5.5. The entire biopsy section was scanned at 40× power to detect fluorescence.

Fecal analysis. Stool samples were collected at baseline (day 0) and at the end of the study (day 28). Fecal samples from the ileostomy subjects were taken from the stoma output. Optional stool samples were provided by some of the subjects 14 days (day 42; $n = 11$) and 28 days (day 56; $n = 8$) after cessation of *L. reuteri* intake. Samples (no less than 5 g) were collected into a sterile container and placed immediately in a refrigerator. Within 24 h, 20 ml (1:5, wt/vol) of 0.1% peptone water was added, the sample was homogenized, and aliquots were dispensed into cryo-vials and frozen at −70°C before being shipped (frozen and on dry ice) to the BioGaia Laboratories for analysis of *L. reuteri* content. Thawed samples were diluted and plated on modified Rogosa Sharp plus 2% sodium acetate (MRS-3) agar containing vancomycin (50 mg/liter). Plates were incubated anaerobically at 37°C for 48 h, after which colonies were confirmed as *L. reuteri* using a BioGaia proprietary method (4a).

PCR. DNA from *L. reuteri* ATCC 55730 in the tablets and certain randomly selected fecal isolates from the study (see Results) were analyzed by PCR using Bacterial Barcodes repPRO DNA Fingerprinting kit (BBCI, Houston, Tex.), and the fingerprints were analyzed using Bionumerics software.

Blood analysis. Blood samples were taken at baseline and day 28, and the following were analyzed using standard clinical laboratory methods: hemoglobin, hematocrit, thrombocytes, leukocytes, C-reactive protein, potassium, sodium, creatinine, blood urea, plasma glucose, cholesterol, high-density lipoprotein, low-density lipoprotein, very-low-density lipoprotein, triglycerides, total bilirubin, urate, alanine aminotransferase, alkaline phosphatase, and lactate.

Symptoms. At the baseline evaluation and on day 28, all subjects were asked to score subjective symptoms that they experienced during the previous 7 days. There were eight questions concerning physical discomfort, ability to work, abdominal pain, bowel distension, flatus, and stool consistency. Each subject answered all questions with a score ranking from 1 (no problem or symptom) to 7 (constant problems or symptoms).

Statistical analysis. The Wilcoxon signed-rank test was used to compare symptoms, blood values, and histological differences between baseline and day 28, and a P of <0.05 was considered significant.

Ethical approval. The protocol was approved by the Scientific Ethical Committee for Bornholms, Frederiksborg, Roskilde, Storstrøms, and West Sjaellands counties, Denmark, prior to start and was performed in accordance with the Declaration of Helsinki.

RESULTS

All subjects completed the study, and compliance was high (Table 1). While none of the subjects in the gastroscopy group had detectable *L. reuteri* in the feces at baseline, 28 days of *L. reuteri* tablet intake led to fecal shedding of live bacteria in all 10 subjects (Table 1). Fecal *L. reuteri* persisted at reduced but detectable levels 4 weeks after the end of intake. In the nine ileoscopy subjects, *L. reuteri* could be detected in the ileostomy output of two subjects at baseline and six subjects (including these two) after *L. reuteri* ATCC 55730 intake (Table 1). In the other three subjects fecal *L. reuteri* was not detectable. Again, in those that displayed fecal shedding, *L. reuteri* persisted 4 weeks after the end of intake (Table 1). DNA fingerprint analysis was performed on fecal *L. reuteri* isolates from three subjects on day 28, and all these isolates were found to have 98% similarity to the *L. reuteri* ATCC

TABLE 2. FISH detection of *L. reuteri* in gastric and intestinal biopsy specimens[a]

Subject group	Biopsy specimen	*L. reuteri* detected by:					
		FISH		Biopsy plating		Fecal plating	
		Day 0	Day 28	Day 0	Day 28	Day 0	Day 28
Gastroscopy	Corpus	1 of 10	7 of 10	2 of 10	1 of 10	0 of 10	10 of 10
	Antrum	1 of 10	8 of 10	3 of 10	1 of 10		
	Duodenum	3 of 10	10 of 10	3 of 10	0 of 10		
Ileoscopy	Ileum	3 of 6	6 of 6	4 of 9	1 of 9	2 of 9	6 of 9

[a] Biopsy and fecal or ileostomy output samples were taken from subjects at baseline (day 0) and after the daily administration of *L. reuteri* ATCC 55730 at 4×10^8 CFU/day for 28 days (day 28). *L. reuteri* was detected in biopsy specimens by either FISH or plating using classical methods. Results are expressed as the number of subjects in which *L. reuteri* was detected out of the total number of subjects analyzed. Examples of FISH detection are shown in Fig 1.

55730 strain incorporated in the tablets. Although *L. reuteri* could be detected in a few biopsy samples using plating, there was no consistent detection of *L. reuteri* at 28 days, despite the presence of the bacteria in the feces of the subjects (Table 2). *L. reuteri* could not be detected in any of the samples of ventricle gastric juice.

FISH analysis clearly demonstrated the presence of *L. reuteri* on the gastric, duodenal, and ileal epithelia. A low incidence of endogenous *L. reuteri* in the gastric corpus and antrum at baseline was dramatically elevated by *L. reuteri* ATCC 55730 consumption (Table 2). Similar results were seen in the duodenum (Table 2). In the ileoscopy group, only six subjects were analyzed using the FISH probes due to the lack of available biopsy material. The incidence of endogenous *L. reuteri* at baseline was somewhat higher (50%), increasing to 100% after *L. reuteri* ATCC 55730 administration (Table 2). Examples of typical findings are presented in Fig. 1.

The histology score was zero in all biopsy sections (Table 3) with the exception of one subject who had abnormal histology in the stomach throughout the study due to *H. pylori* infection (not discussed in this report). Further, all biopsy specimens had normal numbers of Ki-67-positive cells (a proliferation marker in the epithelium of the mucosa; data not shown). Thus, there was no evidence of any histological damage to the gastric or intestinal mucosal membrane after the intake of *L. reuteri* ATCC 55730 for 28 days.

Most gastric biopsy samples displayed no detectable B lymphocytes (CD20-positive cells) and overall, there was no significant difference in the occurrence of B-lymphocytes in the stomach due to the administration of *L. reuteri* ATCC 55730 (Table 3). The occurrence of B lymphocytes in the duodenal biopsy specimens was significantly increased by *L. reuteri* ATCC 55730 administration (Table 3), but there were no significant changes in the occurrence of T lymphocytes (CD3-, CD4-, and CD8-positive cells) in the stomach or duodenum biopsy specimens during the study (Table 3). In both the corpus and the antrum epithelia, CD68-positive cells (histiocytes) were significantly reduced by *L. reuteri* ATCC 55730 administration, an effect not seen in the duodenum (Table 3). Concordance between the two investigations (first the not-blinded analysis and second the blinded analysis of histological samples) for the histiocyte observations in the corpus and antrum was only 40 and 65%, respectively, and for the duodenal B (CD20) cells it was only 60%. Thus, there is some uncertainty in these findings. Concordance in the CD3, CD4, and CD8 analyses was, however, much higher (82 to 89%).

The levels of B lymphocytes, CD3- and CD8-positive T lymphocytes, and histiocytes in the ileal epithelium remained unchanged during the study, but there was a significantly higher number of CD4-positive T-lymphocytes in the ileum epithelium after supplementation with *L. reuteri* ATCC 55730 (Table 3). An example typical of these findings is shown in Fig. 2. Concordance between the two investigations of the ileum sections was generally high for all cell types examined, and for the CD4-positive cells concordance was 78%, indicating good reliability in these data.

Blood parameters were normal and no systematic changes were observed following supplementation with *L. reuteri* ATCC 55730 (results not shown). There were no significant differences in symptom scores due to *L. reuteri* supplementation except for a significantly higher flatus score in the gastroscopy group (results not shown).

DISCUSSION

Administration of *L. reuteri* ATCC 55730 at a dose of 4×10^8 CFU/day was well tolerated by both healthy individuals and subjects with an end ileostomy. Although the gastroscopy group did report an increase in flatus, these findings might be explained by a sudden greater awareness of symptoms in this group induced by the questionnaire. The lack of symptom reporting in the ileoscopy subjects, who are accustomed to registering their gastrointestinal symptoms, supports this idea. It should be emphasized that it is difficult to evaluate symptoms in an open study design.

A significant increase in live *L. reuteri* in feces after intake is a traditional indication of colonization (17, 20, 21), and this was also seen in our study. Surprisingly, we could detect *L. reuteri* in the feces of only six of the nine subjects with ileostomy after supplementation with the probiotic. Normally these subjects have smaller amounts of fecal bacteria since transit time is short (with little time for microbial multiplication) while the distribution volume is large. Thus, *L. reuteri* may have been present in their feces but in an undetectable (diluted) amount. The fecal *L. reuteri* recovered in random samples after supplementation was found to be genetically very similar to *L. reuteri* ATCC 55730, providing strong evidence that this was the colonizing strain in our subjects.

Using plating techniques, endogenous *L. reuteri* was detected in the feces of only 2 of the 19 (10.5%) subjects at the start of the study, while using FISH detection on biopsy specimens, baseline colonization was seen in 6 of 16 (37.5%) of the

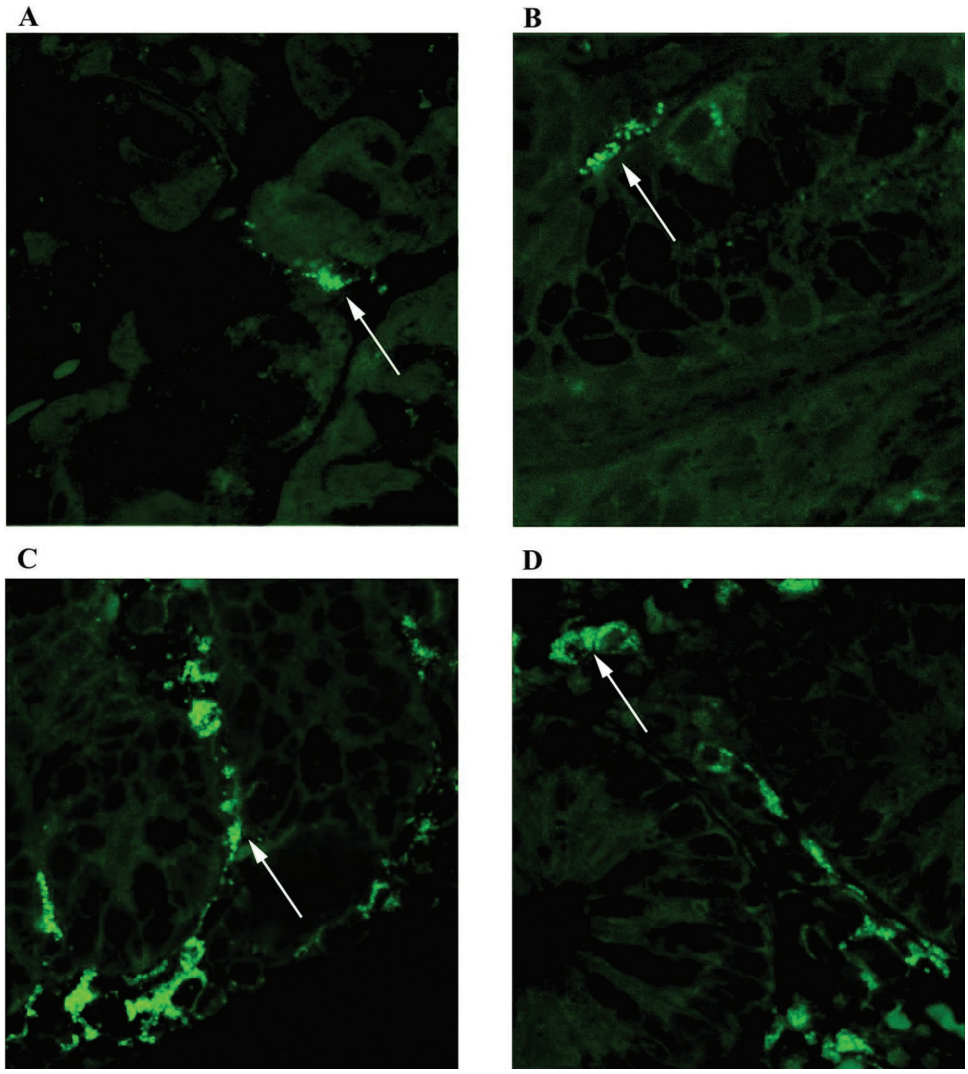

FIG. 1. FISH detection of *L. reuteri* on biopsy specimens from the human gastrointestinal tract. Biopsy specimens were taken from subjects after the daily administration of *L. reuteri* ATCC 55730 at 4×10^8 CFU/day for 28 days. *L. reuteri* was detected using a specific *L. reuteri* FISH molecular beacon probe. The bright green colonies of *L. reuteri* can be clearly seen (examples shown by arrows). Biopsy specimens were taken from the gastric corpus (A), gastric antrum (B), duodenum (C), and ileum (D).

subjects. Thus, not only is the fecal plating method a very poor method to detect endogenous *L. reuteri* levels in the human gastrointestinal tract, but it also gives no information on the site of colonization. Fecal colonization is thus probably best used only as marker of compliance.

Detection of live bacteria in human biopsy specimens is difficult due to the practical issues of obtaining biopsy specimens, performing immediate analyses, and the lack of sensitivity of the plating methods (15), and our experience confirms these difficulties. However, others (6) have successfully studied colonization by lactobacilli of jejunal and rectal biopsy samples in 12 volunteers given a mixture of 19 different strains of lactobacilli (including *L. reuteri* at 5×10^8 CFU/day) for 10 days. *L. reuteri* (strain 108) was found in both the rectal and jejunal biopsy specimens after supplementation.

Our data, obtained by FISH, provide the first clear and direct evidence of colonization of the human stomach, duodenum, and ileum by *L. reuteri*. The appearance of *L. reuteri* lactobacilli at these sites after exogenous delivery of *L. reuteri* ATCC 55730 supports the idea that they come from the supplement. The demonstration that *L. reuteri* colonizes the stomach and duodenum combined with recent data that *L. reuteri* ATCC 55730 is a potent inhibitor of *H. pylori* growth (C. Johnson, H. Jonsson, and S. Roos, Abstr. 16th Int. Workshop Gastrointest. Pathol. Helicobacter, abstr. 16.33, p. 473, 2003) encourages further investigation of this bacteria on *H. pylori* infections at these sites in humans.

Since we generally failed to recover live (culturable) bacteria from the biopsy specimens and since it is true that FISH can detect dead cells, we cannot be sure that the cells detected were living at the time of the biopsy. However, the sensitivity of the technique (using a molecular probe that anneals to bacterial RNA) is high when cells are growing (since the RNA comprises up to 80% of the total nucleic acid) and fades in

TABLE 3. Histological evaluation of gastric and intestinal biopsy specimens[a]

Biopsy specimen	Day 0						Day 28					
	Histology score	Mean score (SD) for:					Histology score	Mean score (SD) for:				
		CD20	CD3	CD4	CD8	CD68		CD20	CD3	CD4	CD8	CD68
Corpus	0[c]	1.2 (0.4)	2.1 (0.3)	2.0 (0)	2.1 (0.3)	1.9 (0.3)	0[c]	1.1 (0.3)	2.0 (0)	2.0 (0)	2.0 (0)	1.4 (0.7)[b]
Antrum	0[c]	1.2 (0.6)	2.1 (0.3)	2.0 (0)	2.1 (0.6)	2.1 (0.3)	0[c]	1.4 (0.7)	2.0 (0)	2.1 (0.3)	2.1 (0.3)	1.7 (0.7)[b]
Duodenum	0	1.4 (0.5)	2.5 (0.7)	2.7 (0.5)	2.6 (0.5)	2.6 (0.5)	0	1.8 (0.4)[b]	2.6 (0.7)	3.0 (0)	2.5 (0.5)	2.6 (0.5)
Ileum	0	2.1 (0.6)	3.2 (0.4)	3.2 (0.4)	2.8 (0.4)	2.8 (0.4)	0	2.0 (0)	2.8 (0.4)	4.0 (0)[b]	3.0 (0)	2.8 (0.4)

[a] Biopsy specimens were taken from subjects at baseline (day 0) and after the daily administration of *L. reuteri* ATCC 55730 at 4×10^8 CFU/day for 28 days (day 28). Histological damage scoring was determined according to the method of Madsen et al. (8), and semiquantitative analysis of B lymphocytes (CD20 positive), T lymphocytes (CD3, CD4, or CD8 positive), and histiocytes (CD68 positive) was performed after specific staining of the sectioned biopsy samples. One biopsy specimen from each subject ($n = 10$ for corpus, antrum, and duodenum; $n = 9$ for ileum) was scored as follows: 1 = no cells detected, 2 = single cells detected, 3 = dispersed cells or aggregations of cells detected, and 4 = several adjoining groups of cells detected. Results are expressed as the means and standard deviations of the scores for each cell type and group.

[b] $P < 0.05$ (Wilcoxon signed-rank test) compared to same cell type on day 0.

[c] One subject had a score of 2 related to an *H. pylori* infection.

dead cells. The *L. reuteri* cells on the biopsy specimens showed a high level of fluorescence, suggesting that they are growing, live cells. Further, there is a clear correlation between the appearance of *L. reuteri* on the biopsy specimens and shedding of live *L. reuteri* in the feces of the subjects after supplementation with the probiotic. Thus, there is no real reason to believe that the *L. reuteri* cells die and then "stick" to the mucosa in transit. Finally, higher magnifications of the FISH images reveal colony-like formations of the stained cells, suggesting growth at the time of biopsy. Thus, it is highly likely that the *L. reuteri* lactobacilli detected on the biopsy samples are snapshots of live and growing bacteria in situ on the mucosa.

New emerging evidence suggests that *L. reuteri* may be able to modulate the immune system in the gastrointestinal tract (4, 7, 8, 18, 19). Our data support such a hypothesis and provide a first firm indication of an in situ effect of *L. reuteri* on the immune system in the human gut. We observed an immune cell response in the mucosal biopsy specimens, with a significant increase in CD4-positive cells in the ileum after the intake of *L. reuteri* ATCC 55730. This observation is in good agreement with earlier observations in poultry (3), where an increased CD4/CD8 ratio in the ileum mucosa was found after intake of *L. reuteri* (poultry strain) in a model where colonization by *Salmonella enterica* serovar Typhimurium is markedly reduced by such supplementation (2, 3). Furthermore, Mao et al. (10) studied methotrexate-induced enterocolitis in rats and found that *L. reuteri* R2LC could increase both ileal and colonic secretory immunoglobulin A levels as well as $CD4^+$- and $CD8^+$-cell populations in the gut lamina propria and that these changes were associated with decreased intestinal permeability, increased mucosal mass, and recovery from enterocolitis (9). It is also worth noting that Ferreira et al. (5) have shown that activated T lymphocytes in the human small intestinal lamina propria are involved in enhancing proliferation of intestinal epithelial cells and that lactobacilli (1), including *L. reuteri* ATCC 55730 (2), have been shown to stimulate ileum mucosal growth in animals. *L. reuteri* is known to be an indigenous species in the human ileum (14), and thus stimulation of T-helper cells by this bacterium may be a central mechanism of symbiosis for improving the health of the host gut and a key mechanism of action for this probiotic.

In conclusion, this study shows colonization of the human gastrointestinal tract by *L. reuteri* ATCC 55730 delivered in a tablet formulation and consequent modulation of local im-

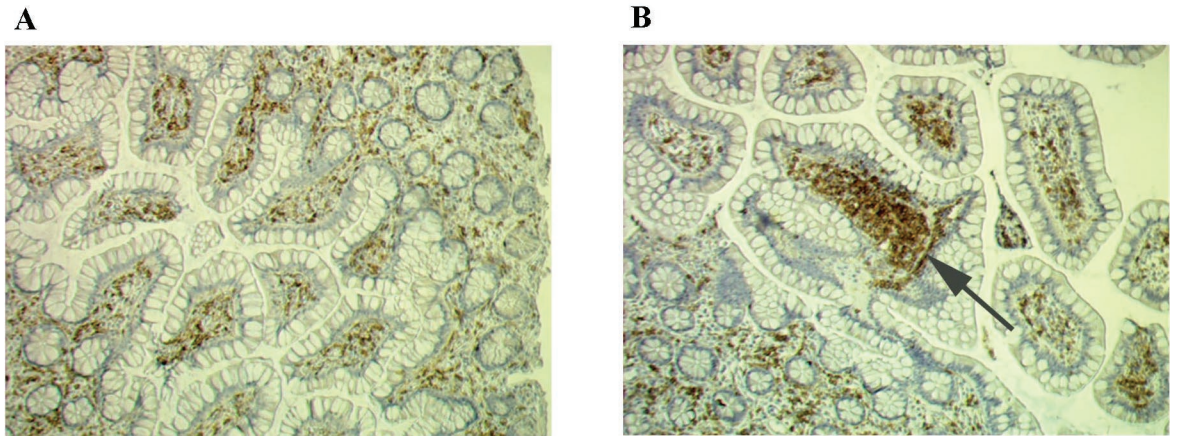

FIG. 2. CD4-positive cells (T lymphocytes) in ileal mucosa before and after *L. reuteri* ATCC 55730 administration. Biopsy specimens were taken from subjects at baseline and after the daily administration of *L. reuteri* ATCC 55730 at 4×10^8 CFU/day for 28 days, and the histological sections were stained for $CD4^+$ cells. (A) Before *L. reuteri* ATCC 55730 administration ("dispersed cells or aggregations of cells"); (B) After *L. reuteri* ATCC 55730 administration ("several adjoining groups of cells" [arrow]).

mune cell populations. It seems likely that this response to exogenous *L. reuteri* may be involved in maintaining gastrointestinal well-being and defense against pathogens in an already-healthy recipient.

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