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Lactobacillus johnsonii La1 Antagonizes *Giardia intestinalis* In Vivo

Martín A. Humen,[1] Graciela L. De Antoni,[1,2] Jalil Benyacoub,[3] María E. Costas,[4] Marta I. Cardozo,[4] Leonora Kozubsky,[4] Kim-Yen Saudan,[3] Angele Boenzli-Bruand,[3] Stephanie Blum,[3] Eduardo J. Schiffrin,[3] and Pablo F. Pérez[1,2]*

Centro de Investigación y Desarrollo en Criotecnología de Alimentos,[1] and Cátedra de Microbiología[2] and Cátedra de Parasitología,[4] Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina, and Nestlé Research Center, Vers-Chez-les-Blanc, Lausanne, Switzerland[3]

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This study describes the in vivo activity of *Lactobacillus johnsonii* La1 (NCC533) in *Giardia intestinalis*-infected gerbils (*Meriones unguiculatus*). Daily administration of lactobacilli in the drinking water from 7 days before inoculation with *Giardia* trophozoites efficiently prevented *G. intestinalis* strain WB clone C6 from infecting gerbils. More specifically, shedding of fecal *Giardia* antigens (GSA65 protein) was diminished in the La1-treated group, and resolution of infection was observed by 21 days postinoculation. Histology and analysis of enzymatic markers of microvillus membrane integrity revealed that probiotic administration also protected against parasite-induced mucosal damage. In addition, a cellular response to *Giardia* antigens was stimulated in spleen cells from La1-treated gerbils. Results show for the first time the antigiardial effect of probiotic lactobacilli in vivo and provide further insight into the antagonistic properties of lactic acid bacteria against protozoa involved in intestinal infections.

Giardia intestinalis, a flagellated protozoan, is the etiological agent of giardiasis, an intestinal infection which causes diarrhea and malabsorption in humans and animals (19, 22). This parasite has a biphasic developmental cycle with two morphologically different forms: cysts and trophozoites. The latter is the vegetative form of the parasite which is responsible for infection and pathological manifestations (19). Infection follows the ingestion of cysts which then give rise to trophozoites after passage through the stomach and the upper intestinal tract.

Giardia is generally treated with antibiotics (e.g., nitroimidazoles and nitrofurans). However, clinical failures, occurrence of resistant strains, and side effects of anti-*Giardia* drugs (21, 26, 30, 44) have encouraged research on alternative therapeutic strategies which have included the use of plant extracts and products derived from bees (12, 21, 25). However, since probiotic microorganisms provide health benefits to the host by antagonizing pathogens and modulating both innate and acquired immunity at local and systemic levels (13, 23, 24, 33, 40), we wondered if such organisms could also be used in the treatment of parasitic infections. Certainly, colonization of the intestine by *Giardia* strongly depends on the intestinal microflora (43). Furthermore, in vitro studies show that some probiotic lactobacilli (e.g., *Lactobacillus johnsonii* La1) are able to interfere with the cell cycle of *G. intestinalis* (36). However, since this antigiardial effect does not seem to be a general property of lactobacilli, further research is required to select probiotic strains which can be used in the prevention and treatment of such an infection. For this reason, we used an in vivo model of giardiasis (*Meriones unguiculatus*) to gain further insight into the antigiardial properties of *L. johnsonii* La1 (NCC533; Nestec Culture Collection). The protocol was approved by the Nestlé Research Center and state ethical committees.

Frozen suspensions of *L. johnsonii* (hereafter La1) were thawed and reactivated in MRS broth before the study. The bacteria were then subcultured in MRS broth for 16 h at 37°C and harvested by centrifugation at 10,000 × *g* for 10 min. Concentrated suspensions were frozen at −80°C with 10% (vol/vol) glycerol until required. After thawing, bacterial counts were determined by plating serial dilutions on MRS agar.

Trophozoites of clone C6 of the strain WB of *G. intestinalis*, kindly provided by Hugo Luján, University of Córdoba, Córdoba, Argentina, were grown in Keister's modified TYI-S-33 medium (28) as previously described (36) and then frozen in liquid nitrogen until required.

Suspensions of La1 were prepared daily by diluting thawed concentrated suspensions in mineral water. Young adult Mongolian gerbils (*Meriones unguiculatus*) ingested bacterial suspensions ad libitum in their drinking water each day beginning 7 days prior to inoculation with *Giardia* trophozoites. Based on the daily water intake and the bacterial concentration in drinking water, the daily bacterial intake was estimated to be 10^8 CFU per animal. Control animals received mineral water instead of the bacterial suspensions throughout the course of the study. Seven days after probiotic treatment had begun, frozen trophozoites were thawed in a water bath at 37°C, and a volume of 200 μl (5×10^5 trophozoites) was administered by gavage to each animal in the two treatment groups. At various time points thereafter, animals were killed by cervical dislocation, and the duodenal contents were analyzed for the presence of trophozoites. To this end, 5-cm-long segments of the small

* Corresponding author. Mailing address: CIDCA, Universidad Nacional de La Plata, Calle 47 y 116 (CC 553), 1900 La Plata, Argentina. Phone: 54 221 424 92 87. Fax: 54 221 425 48 53. E-mail: pfp@nahuel.biol.unlp.edu.ar.

intestine were excised at a distance of 11 cm from the pylorus. These samples were then placed in 2 ml of ice-cold culture medium, opened longitudinally, and vortexed. Enumeration of trophozoites was done by using a hemocytometer with a detection limit of 800 trophozoites/cm.

In order to determine fecal giardial antigens, five fecal pellets (0.27 [±0.05] g [wet weight]) per gerbil were homogenized in 0.5 ml of extraction buffer (50 mM EDTA, 100 μg of soybean trypsin inhibitor [Sigma, St. Louis, Mo.]/ml in phosphate-buffered saline), vortexed for 2 min, and centrifuged at 13,000 × *g* for 15 min. Detection of GSA65 antigen in the supernatants (1) was performed by using a ProSpecT kit according to the instructions of the manufacturer (Alexon-Trend). Cyst determination was performed microscopically on samples stored in 10% formaldehyde.

For histological analysis, 1 cm of the small intestine collected 10 cm from the pylorus was fixed in Bouin fixative and dehydrated in a graded series of ethanol. Paraffin-embedded sections of 5 μm were rehydrated and stained with hematoxylin and eosin.

Intestinal sucrase activity was measured in 5-cm-long sections of the small intestine which were collected 5 cm below the pylorus. The intestinal samples were placed in ice-cold phosphate-buffered saline and flushed with 50 ml of ice-cold distilled water using a syringe. The segments were then opened longitudinally, and the mucosa was collected by scraping with a glass microscope slide. Scrapings were stored at −20°C until analysis. Intestinal disaccharidase activity was determined as previously reported (16). Protein concentrations were determined using the Bradford assay, and the results are expressed as arbitrary units per gram of protein (3).

To determine cell proliferation indices, spleens were placed in 2 ml of complete RPMI 1640 medium (10% fetal calf serum, 1% nonessential amino acids), homogenized through a cell strainer (70 μm; BD Falcon, Bedford, Mass.), and centrifuged at 250 × *g* for 5 min. The pellets were then rapidly lysed with 1 ml of sterile distilled water and centrifuged at 250 × *g* for 5 min. Cells were resuspended in complete RPMI 1640 medium at a concentration of 10^6 cells/ml before testing cell proliferation in the presence of the T-cell mitogen concanavalin A (ConA) at a concentration of 3 μg/ml or the B-cell mitogen pokeweed mitogen (PWM) at a concentration of 1 μg/ml (both from Sigma). Stimulation in the presence of *Giardia* antigens was also evaluated. These membrane antigens were extracted as reported previously (17) and used at a concentration of 10 μg/ml to stimulate the cells.

Spleen cells (10^5 cells/well) were incubated with complete RPMI 1640 medium (control) supplemented with either ConA, PWM, or *Giardia* antigen in a final volume of 200 μl of medium. After incubation at 37°C for 72 h, the cells were pulse labeled with 1.5 μCi of [^{3}H]thymidine (Amersham Pharmacia Biotech, Dübendorf, Switzerland) for a further 16 h. The cells were then harvested on nitrocellulose filters (Packard Biosciences B.V., Groningen, The Netherlands), and the incorporation of [^{3}H]thymidine was measured by scintillation counting (TopCount; Packard Biosciences B.V.). Stimulation indices (SI) were determined as the severalfold increase in titrated thymidine incorporation in stimulated cells compared to that of nonstimulated cells.

Fisher's two-tailed exact statistical analysis was used to compare infection rates, and the two-tailed *t* test with equal variances was used to compare mucosal sucrase activity and cell proliferation indices of the two groups (Software InfoStat, 2004; Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Cordoba, Argentina).

TABLE 1. Infection of gerbils at different time points after inoculation with *G. intestinalis* WB clone C6[a]

Days after infection with *Giardia*	Placebo-treated gerbils		La1-treated gerbils	
	No. of infected animals[b]	Log intensity[c]	No. of infected animals[b]	Log intensity[c]
7	10/12	4.63 ± 1.14	3/11 ($P = 0.01$)	3.81 ± 0.88
14	6/14	5.07 ± 0.93	0/12 ($P = 0.02$)	ND
21	3/6	4.81 ± 1.68	0/6 ($P = 0.09$)	ND

[a] Gerbils received either La1 or placebo daily from 7 days before inoculation with the trophozoites of *G. intestinalis* WB clone C6 (5×10^5 trophozoites per animal). Results are from four independent experiments.

[b] Number of gerbils with detectable parasites in the small intestine/total number of gerbils studied. The statistical significance of the infection rate of La1-treated animals compared to that of controls was calculated using Fisher's exact test. *P* values are shown in parentheses.

[c] Averages ± standard deviations of the log of parasites per infected gerbil. ND, no parasites were detected (<800 trophozoites/cm).

As shown in Table 1, there was a peak in the *Giardia* infection rate 7 days postinoculation. At this time point, 10 of the 12 gerbils (83%) in the placebo group and 3 out of 11 gerbils in the La1-treated group had trophozoites in their small intestine ($P = 0.01$). By day 14, the infection was resolved in the La1-treated group, but 6 out of 14 gerbils in the placebo group were still infected (Table 1) ($P = 0.02$). Of these gerbils, three were still infected 21 days after inoculation. Although probiotic treatment reduced the infection rate, it had no significant effect on the intensity of infection (Table 1).

Fecal shedding of GSA65 antigen followed different kinetics from that of live trophozoites in the small intestine. In the placebo group, the number of GSA65-positive gerbils progressively increased from day 7 to day 21 post-*Giardia* inoculation (Fig. 1). In contrast, the single gerbil positive for GSA65 antigen in the La1-treated group at day 14 was no longer positive at day 21 postinoculation (Fig. 1).

G. intestinalis infection in gerbils has a profound effect on the structure and function of the intestinal mucosa (Fig. 2). Villus shortening and hypercellularity of the lamina propria due to increased numbers of mononuclear, polymorphonuclear, and eosinophilic cells were observed in control animals. In contrast, no morphological damage of the gut mucosa was observed in La1-treated animals. These findings correlated well with the difference in mucosal sucrase activity observed in the two groups. While the intestines of La1-treated animals maintained an enzymatic activity of approximately 200 arbitrary units/g of protein throughout the study, the intestines of control animals had decreasing enzymatic activity over time (Fig. 3). Differences between the two groups were statistically significant at both 14 and 21 days postinoculation ($P < 0.05$).

No differences in spleen cell proliferation were observed between the two treatment groups 7 days after inoculation with *Giardia* trophozoites (data not shown). However, at day 14, splenocytes of La1-treated animals showed a greater response to ConA (placebo group SI, 7.8 ± 2.2; La1-treated group SI, 22.8 ± 11.6 [$P = 0.09$]), to PWM, and to *Giardia* antigen

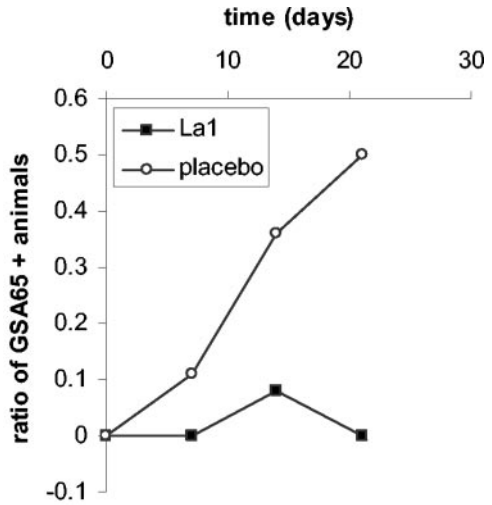

FIG. 1. Shedding of fecal GSA65 antigen. Gerbils received either La1 (■) or placebo (○) daily from 7 days before inoculation with the trophozoites of *G. intestinalis* WB clone C6 (5×10^5 trophozoites per animal). Values represent the ratio between GSA65-positive (GSA65 +) gerbils and the total number of gerbils at various times after *Giardia* infection. The number of animals per group was 18, 12, and 5 at 7, 14, and 21 days post-*Giardia* infection, respectively.

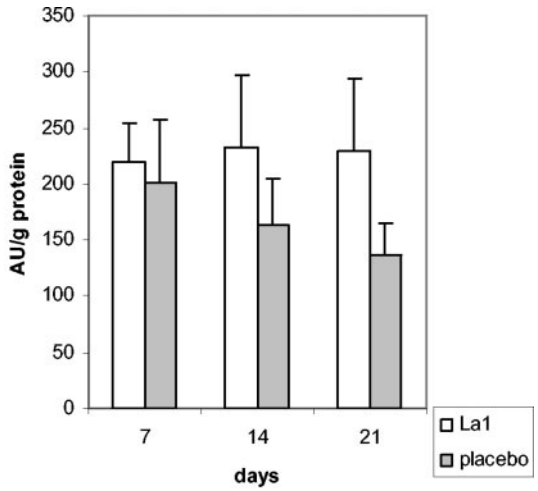

FIG. 3. Intestinal sucrase activity. Gerbils received either La1 or placebo daily from 7 days before inoculation with the trophozoites of *G. intestinalis* WB clone C6 (5×10^5 trophozoites per animal). Values (arbitrary units [AU] per gram of protein) represent averages from six gerbils. Error bars indicate standard deviations. Differences between La1 and placebo groups are statistically significant ($P = 0.05$ and $P = 0.009$ at 14 and 21 days, respectively).

(placebo group SI, 1.2 ± 0.2; La1-treated group SI, 3.7 ± 0.8 [$P = 0.006$]) than those of control animals. At 21 days post-*Giardia* infection, a significantly greater response to *Giardia* antigen (placebo group SI, 0.9 ± 0.2; La1-treated group SI, 3.6 ± 0.9 [$P = 0.007$]) and to the B-cell mitogen PWM (placebo group SI, 2.1 ± 0.6; La1-treated group SI, 3.6 ± 0.6 [$P = 0.03$]) was observed, but there was no longer a difference in the cellular response to ConA between the two groups (data not shown). Research on the efficacy of probiotic bacteria against

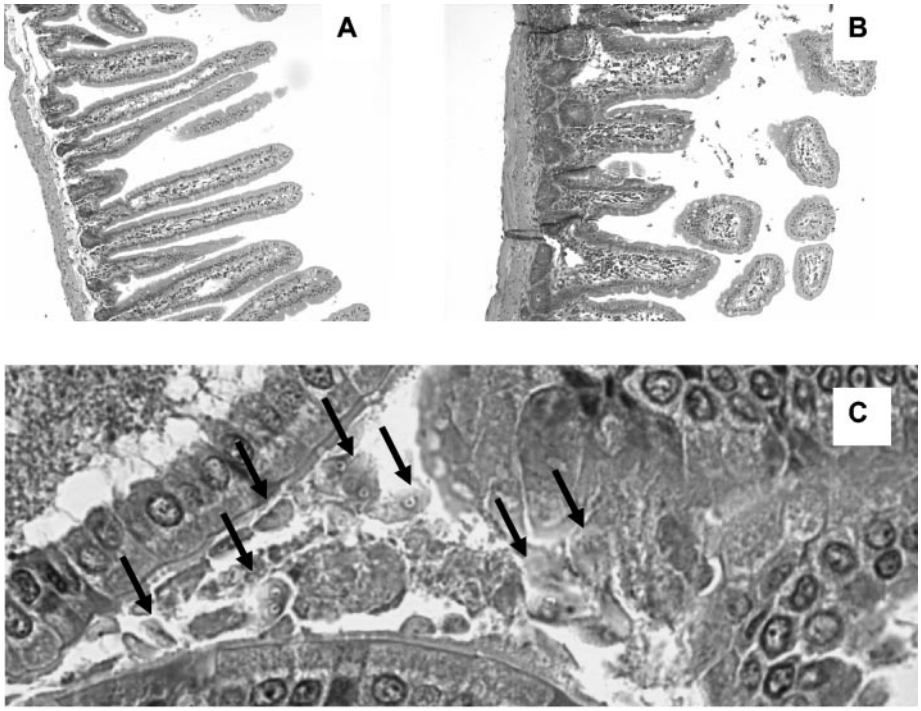

FIG. 2. Histological sections of small intestine. Gerbils received either La1 (A) or placebo (B) daily from 7 days before inoculation with the trophozoites of *G. intestinalis* WB clone C6 (5×10^5 trophozoites per animal). Intestinal segments were collected 7 days post-*Giardia* infection. Sections were stained with hematoxylin and eosin and observed at ×100 original magnification. (C) Further magnification of the intestine from a control animal. Arrows indicate the presence of trophozoites.

intestinal infections has focused primarily on protection against bacterial pathogens. The present in vivo study consolidates recent reports describing the capacity of some lactobacilli of human and canine origin to antagonize *Giardia* infection which interferes with the growth and cell cycle of *G. intestinalis* in vitro (36). Mongolian gerbils (*Meriones unguiculatus*) are a good animal model for studying *Giardia* infection with strains of human origin (3, 4, 11). Their susceptibility to giardiasis has been attributed to a deficient antibody response to specific *Giardia* antigens (34). We have observed that gerbils are readily infected with trophozoites administered by gavage and begin to eliminate cysts in their feces 1 week after inoculation (data not shown). As a consequence of this infection, the animals have reduced disaccharidase activity in their intestinal mucosa.

The presence of viable trophozoites in the small intestine is a recognized marker of *Giardia* infection (37, 42). We found that administration of *L. johnsonii* La1 dramatically reduces the proportion of gerbils with active trophozoites in the gut and leads to resolution of infection within 14 days. In contrast, viable trophozoites were consistently observed in the intestines of the placebo group at all the time points studied (Table 1). Furthermore, we have found a time-dependent increase in the proportion of GSA65-positive gerbils in this group (Fig. 1). Since GSA65 antigen is present on both trophozoites and cysts (38), it is important that the increased proportion of GSA65-positive gerbils with time may reflect changes in the numbers of both trophozoites and cysts reaching the distal small intestine. Certainly, differences in the kinetics of both GSA65 shedding and trophozoite load in the small intestine are related to the distribution of trophozoites and cysts along the oradcaudad axis (29).

Progression of *Giardia* infection in gerbils correlates with a progressive impairment in intestinal sucrase activity (3), which may account for the diminished nutrient absorption and failure to thrive associated with giardiasis (11, 19). However, administration of *L. johnsonii* La1 protects against the impaired sucrase activity and the histological changes found in infected animals. (Fig. 2). It is noteworthy that cysts were eliminated in only a few of the gerbils in the placebo group (data not shown). This is an important observation because cyst production is the only means by which the parasite is known to spread. This observation is also in agreement with previous studies in vitro which have demonstrated that extracellular factors from La1 arrest *G. intestinalis* in the G_1 phase of the cell cycle (36). According to Bernander et al. (6), only trophozoites in G_2 phase progress to the cyst form.

The protective effect of probiotics against intestinal pathogens has been ascribed to many factors including antagonism by extracellular factors (8, 15, 31), interference with pathogen-enterocyte interactions (7, 9), and modulation of the immune response (5, 23, 39). Not only innate defense mechanisms but both acquired humoral and cell-mediated immune responses protect against *Giardia* infection (2, 18, 20, 27, 28, 42). Here, we suggest that La1 treatment reinforces the immune response against *Giardia* in inoculated gerbils. More specifically, we show an increased splenocyte response to a T-cell mitogen and to *Giardia* antigens at 14 days postinfection and an increased response to a B-cell mitogen and to *Giardia* antigens at 21 days postinfection. Even though selection of trophozoites bearing different variant surface proteins in control and La1-treated groups could not be ruled out (35), our results suggest a priming of the immune system by La1 that leads to clearance of both trophozoites and *Giardia* antigens from the intestines (Table 1 and Fig. 1). It is possible that this reflects the development of a memory response following probiotic administration.

Although the mechanisms underlying the virulence of *G. intestinalis* remain poorly understood, both direct interaction with enterocytes and secretion of soluble mediators are thought to contribute to the pathogenesis (14). The immune response of the host and inflammatory status of the intestinal mucosa are also key determinants (41). It is therefore interesting that a lower cellular infiltration was observed in the intestinal villi of La1-treated animals than that of controls (Fig. 2). This result indicates the absence of inflammatory processes in the intestine of La1-treated gerbils.

In the present study, it is unlikely that metabolic products in the bacterial suspensions contribute to the protective effects observed. Although lactic acid is the main product of La1 metabolism, only traces of this organic acid were detected in the bacterial suspensions administered (data not shown). Thus, the antigiardial effect of *L. johnsonii* La1 in vivo is probably due to the production of inhibitory substances in situ and/or to the modulation of the immune response of the host. Certainly, previously published results have shown that *L. johnsonii* La1 mediates its anti-*Giardia* effect through the production of soluble mediators (36) and that it is able to modulate the immune response (10, 32, 39).

To summarize, we report for the first time an in vivo effect of a probiotic lactobacillus against *G. intestinalis*. Our results provide scientific evidence that the applications of probiotic organisms can be extended to include alternative strategies in the prevention of a widespread parasitic infection.

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