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Ability of *Lactobacillus* GR-1 and RC-14 to Stimulate Host Defences and Reduce Gut Translocation and Infectivity of *Salmonella typhimurium*

Gregor Reid*,,***†, Duane Charbonneau****, Silvia Gonzalez*****, Gillian Gardiner*, Julie Erb****, Russ Poehner**** and Andrew W. Bruce***

**Lawson Health Research Institute, and Departments of **Microbiology and Immunology, and ***Surgery, The University of Western Ontario, London, Ontario, Canada*
*****Procter & Gamble Healthcare Research Centre, Mason, Ohio, USA*
******Centro de Referencias para Lactobacilos (CERELA), Tucuman, Argentina*

Abstract

Gastrointestinal infections kill over two million people each year, and pathogen contamination of livestock causes many cases of food poisoning. Two candidate intestinal probiotic strains, *L. rhamnosus* GR-1 and *L. fermentum* RC-14 were found to inhibit the growth of *Salmonella typhi, Shigella dysenteriae, E. coli* O157:H7, *Listeria monocytogenes, L. innocua, Enterococcus faecalis,* and *Bacteroides fragilis.* In a series of mouse experiments, *L. rhamnosus* GR-1 and *L. rhamnosus* GG protected against *S. typhimurium* infection and translocation to the liver and spleen, reduced mortality and induced intestinal phagocytic and immunoglobulin responses. In a second series of experiments, the combination of *L. rhamnosus* GR-1 and *L. fermentum* RC-14 was superior to *L. rhamnosus* GG and placebo in protecting the mice from the lethal effect of salmonella. In summary, the use of combinations of probiotic lactobacilli as dietary supplements or foods could be considered for people at high risk of salmonella intestinal infection. Given the post-infection complications that can arise, such natural methods warrant further exploration especially given the increasing problem of antibiotic resistance and the lack of alternative measures available to many developing countries.

Key words: lactobacilli, probiotics, intestine, immune response, pathogen inhibition

INTRODUCTION

The World Health Organization reported 2.2 million deaths and 73 million disability adjusted life years lost due to diarrhea in 1998. The death toll has since risen to above 3 million people (1). This impact on human health along with the increased problem of multi-drug resistant bacteria makes it crucial to identify practical, inexpensive treatments for the maintenance of intestinal health (2). *Salmonella typhimurium* is one of the main causes of gastrointestinal disease and is a common food contaminant especially in chickens reared in large livestock plants. Salmonellosis can be fatal. Most often the disease causes severe sickness, but it can also trigger complications such as reactive and inflammatory arthritis, irritable bowel syndrome and inflammatory bowel disease. Recently, multi-drug resistant strains have emerged making it more important that preventive measures be found (3,4).

Probiotics ('Live microorganisms which when administered in adequate amounts confer a health benefit on the host') (5) especially *Lactobacillus*, have been shown to prevent and treat diarrheal diseases (5-10). A number of strains with either documented clinical effectiveness or published mechanisms of anti-infective activity are available for intestinal use, for example *Lactobacillus rhamnosus* GG (Valio, Finland) (7-9). However, direct comparisons have not been made between strains, and it is not clear if a single probiotic isolate is optimal for *in vivo* effects. Given the recent evidence that *L. rhamnosus* GR-1 and *L. fermentum* RC-14 can colonize the intestine and vagina following oral administration (11-14), the present study was designed to examine whether these strains were able to inhibit growth of intestinal pathogens, and reduce the risk of *S. typhimurium* infection and death in two animal models.

MATERIALS AND METHODS

Bacterial strains

A number of commercially available probiotic strains were used to screen for anti-pathogenic properties: *Lactobacillus rhamnosus* GG (Valio, Helsinki, Finland), *L. casei* Shirota (Yakult, Tokyo, Japan), *L. plantarum* 299V (Probi, Lund, Sweden), *L. casei* DN-114 001 (Dannon, Paris,

†Corresponding author. E-mail: gregor@uwo.ca
Phone: 1-519-646-6100 x65256, Fax: 1-519-646-6110

France), *L. johnsonii* LJ1 (Nestle, Vevey, Switzerland), *L. rhamnosus* GR-1, *L. fermentum* RC-14, and two freshly isolated intestinal organisms, *L. crispatus* PTL 37 and *L. acidophilus* PTL 19. These organisms were grown in MRS broth in anaerobic conditions at 37°C for 1～2 days. The lactobacilli were tested for their ability to kill the following pathogens: type strains of *Salmonella typhimurium, S. typhi, Shigella dysenteriae, Listeria monocyogenes, L. innocua, Escherichia coli* O157:H7, and *Bacteroides fragilis.* These were grown in enriched media overnight at 37°C.

Inhibition of pathogen growth

An agar overlay technique was used as described previously (15), whereby *L. rhamnosus* GR-1 or *L. fermentum* RC-14 were grown as single spot inocula at 37°C in a bottom layer of agar, a top layer was then added with pathogens (*S. typhi, S. dysenteriae, L. monocyogenes, L. innocua, E. coli* O157:H7 and *B. fragilis*), and zones of inhibition around the lactobacilli spots were noted after 24～48 hours.

Animal studies I

Female Swiss albino mice, each weighing 25 to 30 g, were obtained from a random-bred colony kept at CERELA, Tucuman, Argentina. The animals were housed in plastic cages and kept at room temperature. Each experimental group consisted of 25～30 mice (5～6 for each different period). *Lactobacillus* GG, GR-1 and RC-14 were cultured overnight at 37°C in MRS broth, harvested by centrifugation at 5000 rpm for 10 min, and washed three times with sterile saline solution. The infective microorganism *Salmonella typhimurium* (as used previously (16)), was subcultured then grown in brain-heart infusion (BHI) medium (Difco Laboratories, Detroit, MI, USA) for 6 h at 37°C.

Mice were housed individually and fed daily with 10^7 lactobacilli suspended in sterile 10% non-fat milk (NFM) for 2, 5 or 7 consecutive days. Controls were given sterile 10% NFM. All animals were fed *ad libitum* with a conventional diet.

The mice were challenged at the end of each feeding period with $20 \times DL_{50}$ (2×10^7 CFU) *S. typhimurium* using an oral catheter. Animals were sacrificed by cervical dislocation on the second and seventh days post-challenge, and the liver and spleen were removed aseptically, homogenized, and plated on MacConkey agar for 48 h at 37°C. The organisms were identified by standard biochemical tests, and results were expressed as log of CFU per gram of organ.

Phagocytosis assay

Mice were fed daily with *Lactobacillus* GG, GR-1 or RC-14 (10^7 CFU/mouse) for 2, 5 and 7 consecutive days, and controls were given sterile 10% NFM. The animals were sacrificed and the peritoneal fluid was collected with 5 mL of RPMI-1640 (GIBCO) after gentle abdominal massage. A phagocytosis assay was performed according to Perdigon et al. (17): nonviable *Streptomyces cerevisiae* (5×10^7 cells/mL) previously opsonized with mouse autologous serum, was added to 10^6 macrophages/mL and incubated 15 min at 37°C. The phagocytosis rate was measured by counting 200 cells using a light microscope.

Immunoglobulin A (IgA) from intestinal fluid (IF)

An enzyme-linked immunosorbent assay (ELISA) technique was developed to measure total IgA. Each plate was coated with 200 μL of an appropriated dilution in a sodium carbonate-bicarbonate buffer (pH 9.6) of goat anti-mouse: IgA (alfa-chain specific, Sigma Chemical Co., St Louis, Mo, USA). After overnight incubation at 4°C, the plates were washed five times with PBS containing 0.05% (v/v) Tween 20 (PBS-T). Nonspecific protein binding sites were blocked with PBS containing 5% nonfat dry milk for 30 min at room temperature. After addition of 200 μL portions of the appropriate dilutions of the samples with PBS-T, the plates were incubated for 60 min at 37°C. After the plates were washed five times with PBS-T, peroxidase-conjugated goat anti-mouse IgA, was diluted 1:1000 in PBS-T and 200 μL added, incubated at 37°C for 60 min and washed five times with PBS-T. Plates were poured with 200 μL of a substrate solution [3-3', 5-5'-tetramethylbenzidine (Sigma Chemical Co.)] in citrate-phosphate buffer (pH 5, containing 0.05% H_2O_2), incubated for 30 min at room temperature and the reaction was stopped by the addition of 50 μL of 2 N H_2SO_4. Readings were carried out at 493 nm and the antibody concentration in each unknown sample was expressed as OD/200 μL of sample.

All animal data were expressed as the means ± standard deviation (SD). Significance was assessed with Student's t-test.

Animal studies II

A second series of animal studies were performed in an independent laboratory (Procter & Gamble) to verify the first series and to determine the outcome in a mortality model of *L. rhamnosus* GG versus the combination of *L. rhamnosus* GR-1 and *L. fermentum* RC-14. C3H/HeN mice, each weighing 20 to 25 g, were obtained from Charles River Laboratories Wilmington, Massachusetts. The animals were housed in plastic cages and kept at room temperature. A total of 30 mice were utilized in this study. Animals were divided into three equal sized treatment groups. Prior to treatment baseline stool samples were collected from a subset of animals and processed for mi-

crobiology analysis. The animals were fasted for 24 hours before bacterial dosing. A gastric probe was utilized for oral dosing. At the initiation of the study, one group of animals were treated with placebo (MRS broth), another with *L. rhamnosus* GR-1 and *L. fermentum* RC-14 and the third group received *Lactobacillus* GG. All probiotic strains were dosed in 0.2 mL volumes at concentrations of 10^9 viable organisms. Following the initial probiotic treatment the 30 animals were given a single challenge inoculation of 0.2 mL of *Salmonella typhimurium* UK1 ($\sim 1 \times 10^9$ CFU/mL) using a gastric probe.

Daily administration of probiotic and placebo treatments was continued for the next 7 consecutive days. Feces were collected on days 1, 4, 7 and 11 and the fecal pellets were dispersed into Dental Transport Medium (Anaerobe Systems) using glass beads and vortexing. Bacteria were propagated and enumerated on selective media. *Salmonella* were propagated on SS agar and the lactobacilli on MRS agar containing 200 ppm rifampicin. All countable colonies of bacteria were enumerated by hand using a colony counter (Bel-Art products, Pequannock NJ).

A mortality end point was analyzed using pairwise treatment comparisons for lactobacilli versus placebo under nonparametric SAS protocols. For the microbial counts, the raw numbers were converted to log numbers prior to analysis using a nonparametric SAS procedure.

RESULTS

In vitro inhibition studies

Both *L. rhamnosus* GR-1 and *L. fermentum* RC-14 inhibited the growth of intestinal pathogens, including *Salmonella* and *E. coli* O157:H7 (Table 1).

Animal study I

Feeding mice for two days with 10^7 cells/day *L. rhamnosus* GG and GR-1, not *L. fermentum* RC-14, decreased liver and spleen infection with *S. typhimurium*: on the second and seventh day post-challenge the salmonella counts were significantly reduced ($p<0.05$) as illustrated for the liver (Fig. 1). Mice treated with *L. rhamnosus* GG during 2 and 5 days, showed a significant increase ($p < 0.05$) in phagocytic activity (Table 2). Oral administration of *L. rhamnosus* GR-1 during 2, 5 and 7 days enhanced phagocytic activity. A peak was reached on the seventh day of feeding, with values two times higher than the control. Treatment with *L. fermentum* RC-14 induced a significant increase ($p<0.05$) in phagocytic activity on the second day of administration, however on the 3rd and 5th day the values were similar to controls. The administration of GR-1, for 2, 5 and 7 days induced a significant enhancement ($p<0.05$) of the IgA levels in intestinal fluid, with a peak in the second day of feeding (Table 3). Mice treated with GG for 2 days, showed a significant increase of IgA levels, however no significant change occurred with RC-14.

Animal study II

All the animals in the placebo group died on average by day 18.8; all the animals dying in the GG group within 16.6 days; but only 6/10 animals died in the GR-1/RC-14 group and at a later timepoint of 25.4 days ($p>0.001$ compared to placebo and GG groups). Necropsies of the

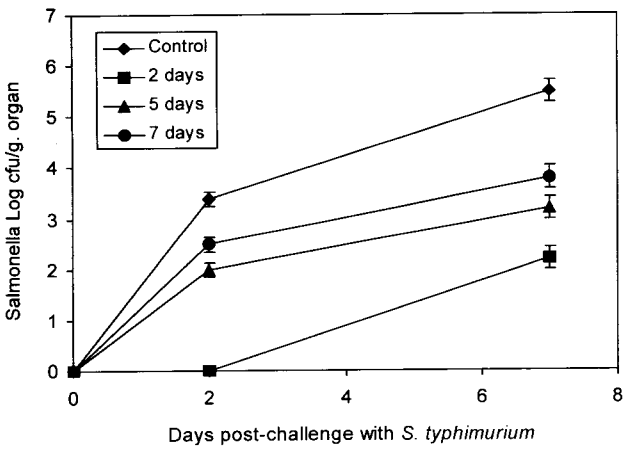

Fig. 1. This graph shows the viable counts of *S. typhimurium* in the liver of mice pretreated For 2, 5 and 7 days with 10^7 *L. rhamnosus* GR-1 per day then challenged with 2×10^7 *S. typhimurium*. Two days treatment with GR-1 resulted in no liver colonization at 2 days and significantly reduced numbers at day 7.

Table 1. Growth inhibition of intestinal and urogenital pathogens: zone of inhibition (mm) for *L. rhamnosus* GR1, *L. fermentum* RC-14, *L. rhamnosus* GG, *L. casei* Shirota, *L. johnsonei* LJ1, *L. casei* DN-114 001, and *L. plantarum* 299V

Strain	GR-1	RC-14	GG	Shirota	LJ-1	DN114001	299V
S. typhi	40.7	58.21	54.72	44.3	NT	NT	NT
L. monocytogenes	31.03	36.11	31.26	26.92	33.47	22.73	27.46
E. coli 0157:H7	31.4	32.27	32.54	28.04	22.09	21.02	24.2
Sh. dysenteriae	26.6	55.24	26.5	19.58	NT	17.24	20.5
E. faecalis	23.76	26.2	25.03	19.58	0	18.9	20.67
L. innocua	30.15	23.19	24.04	22.65	35.51	13.11	18.22
B. fragilis	48.5	51.92	45.53	43.14	37.23	45.16	56.35

NT = not tested; 0 = no inhibition detected; all values are mean of four experiments; standard deviation within 15% for three repeats.

Table 2. Phagocytic activity of peritoneal macrophages of mice feeding with 10^7 cell/day/mice of *Lactobacillus* GG, GR-1 and RC-14

Days of feeding	Phagocytic activity (%)		
	GG	RC-14	GR-1
2 days	62.1±0.3*	44.9±1.5*	51.2±0.4*
5 days	58.9±0.2*	22.2±1.3	47.9±0.6*
7 days	28.0±0.8	39.1±1.5	75.8±1.5*

Mice were fed with *Lactobacillus* GG, GR-1 or RC-14 during 2, 5, and 7 days. At the end of each treatment, the phagocytic activity was determined in peritoneal macrophages. Results are expressed as the means±SD. Control value without treatment was: 32.1±1.8. *Represent significant differences with $p<0.05$ compared to control.

Table 3. Immunoglobulin A levels in intestinal fluid of mice feeding with 10^7 cell/day/mice of *Lactobacillus* GG, GR-1 and RC-14

Days of feeding	IgA levels (OD/200 μL of intestinal fluid)		
	GG	RC-14	GR-1
2 days	4.21±0.29*	2.00±0.22	4.93±0.43*
5 days	2.61±0.35	2.06±0.03	4.70±0.21*
7 days	2.74±0.27	2.95±0.31	3.35±0.22*

Mice were fed with GG, GR-1 and RC-14 during 2, 5, and 7 days. At the end of each treatment, the IgA levels were determined by ELISA test in intestinal fluid. Results are expressed as the means±SD. Control value without treatment: 2.18±0.24. *Represent significant differences compared to control.

deceased animals and microbiology evaluation of the internal organs revealed the presence of *S. typhimurium* in the stomach, liver, intestinal tract, lung, kidney and spleen. No salmonella were detected in the organs of animals not inoculated with *S. typhimurium* UK1. Also, *S. typhimurium* UK1 was detected in the organs of mice 4 days after probiotic treatment was removed (data not shown).

The microbiology studies of the feces showed no statistical difference in the number of salmonella present in the GG group (Log 6.5) versus the GR-1/RC-14 group (Log 6.2) and placebo (Log 6.1) (Fig. 2). The highest rate of recovery of viable *Lactobacillus* occurred in the GR-1/RC-14 treated animals.

DISCUSSION

The use of probiotic bacteria, such as lactobacilli and bifidobateria (18,19) have the potential to reduce the effects of salmonella infection. The present study demonstrated that urogenital probiotic strains, *L. rhamnosus* GR-1 and *L. fermentum* RC-14 also have excellent potential to confer health benefits to the intestine. The inhibition of pathogen growth demonstrated *in vitro* is important for

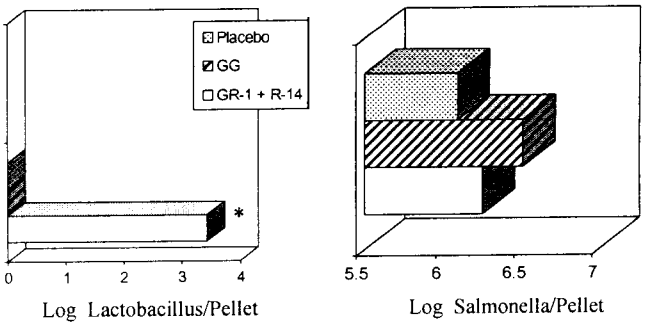

Fig. 2. Fecal recovery of *Lactobacillus* and *Salmonella*. *Significant nonparametric SAS procedure $p \leq 0.10$ between treatment groups.

strains that reduce the risk of infection, and improve recovery from diarrhea (7,11,20). The inhibition is not due to hydrogen peroxide, as RC-14 produces it, while GG does so weakly and GR-1, Shirota and DN-114 001 do not produce it (11). Of note, RC-14 had the highest inhibitory zones against salmonella, *Listeria monocytogenes, Shigella dysenteriae* and *Enterococcus faecalis*. Yet, in the first series of animal experiments it did not have as good an effect on salmonella as *Lactobacillus* GG and GR-1. This would indicate that growth inhibition is not sufficient to prevent infection *in vivo*, and other factors likely including modulation of the immune response play a role in the process. The apparent priming of phagocytic cells by GR-1 and GG could be of more importance in certain hosts, while the inhibitory effects of RC-14 more critical in others. Thus, use of more than one probiotic strain could be advantageous in treating a wider population.

Ingestion of *L. rhamnosus* GR-1 plus *L. fermentum* RC-14 does not lead to immunoglobulin or cytokine changes beyond normal values in humans, indicating that they do not invoke a peripheral inflammatory response (13). However, the present animal studies showed that an intestinal mucosal immune response took place and may have contributed to reduced infectivity of *S. typhimurium*, thereby supporting the belief that certain lactobacilli can boost the host's immune response (21). This potentially represents a major advancement in treatment strategy for the gut, by overcoming the two main limitations of drug therapy: their suppression of the host response and lack of appreciation for the contribution made by gut pathogens in the disease process; and their lack of specificity in that they affect both the mucosal and systemic host responses inducing unwanted side-effects (21). Given the added inhibitory effect of the lactobacilli on pathogen growth, and the anti-adhesive effects expressed by GR-1 and RC-14 (22,23), a good case can be made for these strains as intestinal probiotics.

In terms of sIgA levels, strains GR-1 and GG elevate these within two days, while the concentration was highest for RC-14 after 7 days. One can only speculate the rea-

sons for this difference. The *L. rhamnosus* strains may be more immunogenic at the viable count level that was delivered, while the *L. fermentum* strain may have to multiply to reach a higher number capable of invocating a response. By day 5, the sIgA concentration in the GR-1 and GG treated animals fell back to control levels.

The recovery of viable lactobacilli in the stool of survivors in the mortality model and the longer mean survival time supported the proposed role of the double strain combination as interventional therapy for gastroenteritis. A previous human study has confirmed that these strains survive intestinal passage (13). Interestingly, in terms of salmonella numbers in the stool, no significant differences were observed between the treatment groups. Thus, the differences in survival among the treatment groups are not related to an overall reduction in pathogen levels within the gastrointestinal tract. This suggests that the critical anti-infective effect is taking place at the gut epithelium and could for example involve blocking of translocation. The mechanism of action remains to be determined, but one could speculate that some form of cell-to-cell communication is taking place between the lactobacilli and pathogens, or the lactobacilli and host cells (for example switching on mucus production (24) or modulating the immune response). Such signaling could diminish the overall virulence properties of the pathogen rendering it incapable of becoming invasive. Further research is needed to test this hypothesis.

These studies indicate that ingestion of two probiotic organisms, *L. rhamnosus* GR-1 and *L. fermentum* RC-14 can act as therapeutic prophylactic agents against gastrointestinal pathogens. Given the post-infection complications that can arise following foodborne salmonella infection, particularly reactive arthritis and irritable bowel syndrome, the potential protection afforded by certain lactobacilli strains warrant further exploration. Ingestion of these organisms as dietary supplements or foods could be an inexpensive and practical means to benefit large numbers of people in many developing countries (2,9).

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