Evidence for Hypocholesterolemic Effect of *Lactobacillus reuteri* in Hypercholesterolemic Mice

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ABSTRACT

Swiss Albino mice were fed a diet enriched with fat to produce hypercholesterolemia. The further administration of *Lactobacillus reuteri* CRL 1098 (10^4 cells/d) to hypercholesterolemic mice for 7 d decreased total cholesterol by 38%, producing serum cholesterol concentrations similar to that of the control group (67.4 mg/ml). This low dose of *L. reuteri* caused a 40% reduction in triglycerides and a 20% increase in the ratio of high density lipoprotein to low density lipoprotein without bacterial translocation of the native microflora into the spleen and liver. These data suggest that *L. reuteri* CRL 1098 is an effective hypocholesterolemic adjuvant at a low cell concentration for mice.

(**Key words**: *Lactobacillus reuteri*, hypocholesterolemia, probiotic)

Abbreviation key: **HC** = hypercholesterolemic, **HDL** = high density lipoprotein, **LDL** = low density lipoprotein.

INTRODUCTION

It has been recognized for many years that elevated serum cholesterol concentration is a risk factor associated with atherosclerosis and coronary heart disease, the latter being a major cause of death in Western countries (1). Numerous drugs that lower cholesterol, including the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors and drugs that increase the net excretion of bile acids, have been used to treat hypocholesterolemic (**HC**) individuals (22). However, the undesirable side effects of these compounds have caused concerns about their therapeutic use (6).

In addition to these therapeutic resources, the ingestion of probiotic lactic acid bacteria possibly would be a more natural method to decrease serum cholesterol concentrations in humans. Several studies (14, 16) report a decrease in serum cholesterol during the consumption of large doses (680 to 5000 ml/d) of fermented dairy products, but those results cannot be extrapolated to more realistic conditions of consumption. Massey (15) showed that, initially, yogurt consumption significantly reduced cholesterol by 10 to 12% in human adult males, but, 2 wk later, concentrations returned to the control values even with continued yogurt consumption. Similar conflicting results were obtained with experimental animals that were fed with milk and its fermented products (10, 13). Rao et al. (18) reported an HC effect in rats fed milk that had been fermented by *Streptococcus thermophilus*; Rodas et al. (20) found a similar effect in HC pigs that were fed with *Lactobacillus acidophilus.*

Previous in vitro assays suggest that *Lactobacillus reuteri* also might influence serum cholesterol concentrations (25). The present study investigated the effect of *L. reuteri* CRL 1098 on total cholesterol, triglycerides, and the ratio of high density lipoproteins (**HDL**) to low density lipoproteins (**LDL**) in the serum of mice previously fed with a diet that had been enriched with fat. *Lactobacillus reuteri* was chosen because it is a native inhabitant of the gastrointestinal tract of human and animals (5).

MATERIALS AND METHODS

Microorganism and Culture Conditions

The strain *L. reuteri* CRL 1098 used in this study was obtained from the culture collection of CERELA (San Miguel de Tucumán, Argentina). The microorganism was cultured in MRS broth (4) at 37°C for 16 h; the cells were harvested by centrifugation (6000 × *g* for 10 min) and washed three times with a sterile

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saline solution. The cells were then resuspended in sterile 10% NDM.

Mice and the Hypercholesterolemic Animal Model

Swiss Albino mice weighing 25 g were obtained from the randomly numbered closed colony kept at CERELA. The mice were split into two groups. Each experimental group consisted of 10 mice housed individually and maintained on a cycle of 12 h of light and 12 h of dark.

As a preliminary treatment to produce hypercholesterolemia, the treated mice were fed a diet based on 10% (wt/vol) sterile NDM supplemented with 10% cream for 7 or 15 consecutive d. The control and the HC groups received a solid conventional diet (rodent chow: 32% protein, 5% fat, 2% fiber, and 60% nitrogen-free extract). The total fat content of the diet was 17.6% for HC group and 6.7% for the control group. After 1 and 2 wk of feeding, blood samples of 10 mice per group were drawn from the retroorbital venous plexus for determination of serum total cholesterol, HDL cholesterol, LDL cholesterol, and serum triglycerides. The cholesterol and triglycerides concentrations were determined enzymatically using an enzymatic reagent kit (Sigma Chemical Co., St. Louis, MO).

Feeding Procedure for Testing Bacterial Translocation

The control group was fed viable cells of *L. reuteri* CRL 1098 that were suspended in 5 ml of sterile 10% NDM at the following concentrations: 10^4, 10^6, 10^7, and 10^8 cells/d per mouse. The cell suspension was administered at 20% (vol/vol) in the drinking water during 2 d for the doses of 10^6 to 10^8 cells/d per mouse and during 2, 5, and 7 d for the doses of 10^4 cells/d per mouse.

Mice were killed by cervical dislocation on d 2 after they had been fed *L. reuteri* of 10^6 to 10^8 cells/d per mouse and on d 2, 5, and 7 for the dose of 10^4 cells/d per mouse. The spleen and liver were removed under aseptic conditions, weighed, and homogenized. Serial dilutions of the organs were plated in the following agarized media: LBS broth for the enumeration of lactobacilli, MacConkey for enterobacteria, and blood-supplemented brain-heart infusion broth for anaerobes. Plates were incubated in anaerobic jars (Oxoid System; Oxoid, Basingstoke, Haunts, England) for 48 h at 37°C. The colony-forming units per gram of organ tissue were counted after that time.

TABLE 1. Diets fed to the different groups for 1 wk.

Group	Diet
Control	Chow and water
HC[1]	Chow and milk cream-enriched NDM
HC with *Lactobacillus reuteri*	Chow and milk cream-enriched NDM Chow and *L. reuteri*
HC without *L. reuteri*	Chow and milk cream-enriched NDM Chow and water

[1]Hypercholesterolemic.

Feeding Procedure for Evaluating *L. reuteri* Effects

The HC group was fed for 7 d with *L. reuteri* at a concentration of 10^4 cells/d per mouse. The viable cells were suspended in NDM as before and administered at 20% (vol/vol) in the drinking water. The control group was composed of HC mice that had received 20% (vol/vol) NDM in the drinking water (HC without *L. reuteri*). The serum lipids were determined as mentioned previously.

The composition of the diets is given in Table 1.

Statistical Analysis

The following variables were taken into consideration: total cholesterol, ratio of HDL to LDL, and concentration of triglycerides. The means per mouse of each of these variables, as well as the differences among them for each treatment, were measured by ANOVA. The results obtained for each treatment group were evaluated in pairs by the honestly significant difference test of Tukey. The cluster analysis of the different groups was performed by K means. Calculations were made with the software SYSTAT (23).

RESULTS

HC Model

The fat-enriched diet fed to the HC group increased the total cholesterol by 1.9 times, HDL cholesterol by 2.1 times, LDL cholesterol by 1.6 times, and triglycerides by 1.1 times compared with the values of the control group. The increase in lipid concentration was significant ($P < 0.001$), but no differences ($P > 0.05$) were obtained in the weight of spleen and liver in relation to the body weight among the groups throughout the study. A similar increase in lipids was found after 7 and 15 d of the enriched diet. Therefore, the first period was chosen for further assays. Table 2

TABLE 2. Lipid profile of control (n = 10) and hypercholesterolemic (HC; n = 10) groups.

	Control		HC	
	$\bar{X}$	SD	$\bar{X}$	SD
Total cholesterol, mg/dl	67.4[b]	6.9	128.4[a]	2.1
Triglycerides, mg/dl	85.7[b]	14.5	154.5[a]	19.7
HDL:LDL[1]	1.5[a]	0.2	1.2[b]	0.1

[a,b]Means within a row with no common superscripts differ ($P < 0.05$).

[1]HDL = High density lipoprotein; LDL = low density lipoprotein.

shows the values obtained for 7 d of dietary treatment.

Bacterial Translocation

To select the maximun dose of *L. reuteri* that does not induce side effects, the translocation of the normal intestinal microflora was determined in the control group. The results obtained are shown in Table 3. The colonization of the liver and spleen occurred after 2 d of dietary treatment of 10^6 to 10^8 cells/d per mouse, and no translocation was found after 7 d of dietary treatment at the concentration of 10^4 cells/d per mouse. Results were similar for the HC group (data not shown).

Effect of *L. reuteri* on Hypercholesterolemia

Table 4 shows that the 7-d dietary treatment of the HC group with *L. reuteri* decreased total cholesterol by 38% ($P < 0.001$); the values obtained for the HC group that received milk in the drinking water without *L. reuteri* remained unchanged. A 20% increase in the ratio of HDL to LDL was also obtained for the HC group treated with the lactobacilli. These data probably indicate a lower amount of cholesterol bound to the LDL. A 40% reduction in triglycerides occurred after *L. reuteri* administration; this value was slightly lower than that of the control group (Table 2).

Statistical Analysis

The cluster analysis of the total cholesterol, the ratio of HDL to LDL, and the triglycerides was performed for each mouse of each feeding group. These results determined the distribution of the mice into four groups, which was performed by the K means procedure (23). This distribution coincided with the feeding groups previously established (Table 1). Thus, group 1 included HC mice that received the *L. reuteri* treatment, group 2 corresponded to the control group, group 3 was the HC group that was not fed *L. reuteri*, and group 4 was the HC group that was fed *L. reuteri*.

Euclidian differences among these groups are presented in Table 5. The data clearly show the close relationship that exists between groups 1 and 2 on the basis of the slight difference (1.1) between them. In contrast, both groups are separated from groups 3 and 4 by 1.3 to 1.7.

DISCUSSION

The development of any therapy, even those using biological products, requires the methodological research of the potential side effects. Lactic acid bacteria are considered to be GRAS (Generally Recognized as Safe) organisms, and some probiotic strains have been proposed as an alternative therapy for the treatment of gastrointestinal disorders (17). However, there are exceptional cases of local and systemic infections under conditions that could favor

TABLE 3. Effect of *Lactobacillus reuteri* in the diet on the translocation of intestinal microflora in mice.[1]

		Lactobacilli				Enterobacteria				Anaerobes			
Dose	Treatment	Liver		Spleen		Liver		Spleen		Liver		Spleen	
(cell/d per mouse)	(d)	($\log_{10}$ cfu/g)											
		$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
1×10^8	2	4.2	0.7	3.4	0.2	3.4	0.6	2.9	0.6	3.9	0.5	3.5	0.2
1×10^7	2	4.0	0.6	ND		3.5	0.6	ND		3.7	0.5	3.0	0.2
1×10^6	2	2.8	0.6	ND		3.9	0.5	ND		3.9	0.5	2.9	0.2
1×10^4	2, 5, 7	ND[2]		ND		ND		ND		ND		ND	

[1]n = 10 per group.

[2]Not detected.

translocation of lactic acid bacteria from the gut lumen into the blood (3).

The dose of *L. reuteri* CRL 1098 that we found to be effective in decreasing serum concentrations of lipids without producing side effects (i.e, translocation) is the lowest one reported in the literature for such purpose. Rodas et al. (20) observed a hypocholesterolemic effect in pigs that were fed large doses (about 10^{12} cells/d) of *L. acidophilus*, which was many times higher than the 10^4 cells/d used in our study. Grunewald (9) found similar effects in rats that were fed large amounts of fermented dairy products, but Gilliland and Walker (8) reported no probiotic effect in a study using human subjects. These controversial results may be partially explained by the use of unsuitable probiotic bacteria, which possibly justified the use of high doses (10^6 to 10^8 viable cells) to ensure the probiotic effect in the host (14).

The results obtained in this study confirmed previous in vitro assays (25) and also showed that *L. reuteri* CRL 1098 is an effective hypocholesterolemic adjuvant at very low doses (10^4 cells/d). Based on these data, the cells appear to be able to overcome the gastrointestinal barrier and to adapt themselves to the environmental conditions prevailing in the gut.

In our experimental model, using mice fed an HC diet (HC group), the serum cholesterol concentration was increased 1.9-fold. The further administration of *L. reuteri* CRL 1098 for 7 d produced a 38% decrease in total cholesterol, reaching the normal values without side effects such as bacterial translocation.

The HC mice fed *L. reuteri* CRL 1098 had a ratio of HDL to LDL that was 20% higher than that of the control group, indicating that a greater amount of cholesterol was bound to HDL, which would reduce the risk for atheroma (2, 21).

The triglyceride values obtained for the HC group after the dietary treatment with *L. reuteri* were lower than those of the control group. The structure of the liver as well as the ratio of liver weight to body weight were not significantly different among the groups. These results indicate no induction of fatty degeneration in liver. Thus, the hypolipemic effect of *L. reuteri* CRL 1098 may not be due to a redistribution of lipids from plasma to liver but rather to a lower intestinal absorption of lipids or a higher lipid catabolism.

TABLE 4. Effect of *Lactobacillus reuteri* on total cholesterol, triglycerides, and ratio of high density lipoproteins (HDL) to low density lipoproteins (LDL) in the hypercholesterolemic (HC; n = 20) group.

	HC			
	With *L. reuteri*		Without *L. reuteri*	
	$\bar{X}$	SD	$\bar{X}$	SD
Total cholesterol, mg/dl	80[b]	5.0	126.5[a]	5.5
Triglycerides, mg/dl	73.8[b]	9.9	129.1[a]	11.0
HDL:LDL	1.8[a]	0.2	1.3[b]	0.08

[a,b]Means within a row with no common superscripts differ ($P < 0.05$).

TABLE 5. Euclidian distance between the treatment groups.[1]

Group	1	2	3	4
1	0.00	1.18	1.55	1.63
2	1.18	0.00	1.34	1.73
3	1.55	1.34	0.00	0.99
4	1.63	1.73	0.99	0.00

[1]Group 1 = Hypercholesterolemic (HC) group treated with *Lactobacillus reuteri*, 2 = control group, 3 = HC group without *L. reuteri*, and 4 = HC group. Groups were classified according to the K means procedure.

The precise mechanism of the cholesterol-lowering activity of lactic acid bacteria has been difficult to explain. For *L. reuteri* CRL 1098, the hypocholesterolemic effect seems to be related to the hydrolase activity of bile salts of the cells. Based on in vitro data (24, 25), we assumed that *L. reuteri* CRL 1098 might deconjugate about half of the bile salt conjugates that are excreted into the small intestine during its residence, which would increase the fecal excretion of deconjugated bile salts (12). To maintain bile salt homeostasis, the bile acids have to be newly synthesized in the liver, thus increasing the demand for cholesterol as a precursor for bile acids (11, 26).

The alteration in bile salt metabolism through enhanced hydrolase activity might also affect cholesterol more directly by influencing its solubility and intestinal absorption (1, 19). Based on the 1 to 2 rule (7), which states that a 1% reduction in the serum cholesterol causes a 2% lowering of the risk for coronary heart disease, a significant positive effect for patients suffering from elevated cholesterol might be obtained by ingesting lactobacilli to improve hydrolase activity of bile salts.

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