

# Probiotic *Lactobacillus rhamnosus* GG inhibits the adhesion of *Giardia intestinalis* to murine enterocytes: An *in vitro* study

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## ABSTRACT

**Background and Objectives:** Giardiasis mainly affects young children causing diarrhea, malnutrition and growth retardation. Due to adverse effects of antiprotozoal treatment such as low compliance with drug therapy, reinfestation, occurrence of resistant strains, headache and metallic taste, the use of natural live bacteriotherapy has been studied. The study was designed to investigate *in vitro* the colonizing ability of probiotic *Lactobacillus rhamnosus* GG (LGG) *viz-a-viz* its ability to inhibit the adherence of *Giardia* trophozoites to murine enterocytes under conditions simulating the intestinal environment. **Materials and methods:** Murine enterocytes were harvested and incubated with *Giardia* trophozoites either prior or simultaneously with probiotic LGG to assess the adhesion using scanning electron microscopy. **Results:** It was observed that 15% of *Giardia* trophozoites adhered to enterocytes at 37°C in Hank's Balanced Salt Solution, after 1h of incubation. However, co-incubation of murine enterocytes with probiotic LGG either 30 min prior or simultaneously with *Giardia* trophozoites led to 23-27% reduction in the adherence of *Giardia* trophozoites compared with 46% adherence in the absence of LGG. Further, scanning electron microscopy also showed *in vitro* inhibition of *Giardia* trophozoites to murine enterocytes due to probiotic supplementation. **Interpretations and conclusion:** The data suggest the colonizing ability of probiotic LGG to murine enterocytes that modulates murine giardiasis mainly by displacing the *Giardia* trophozoites.

**Keywords:** Giardiasis, mouse enterocytes, probiotic, scanning electron microscopy

## INTRODUCTION

*Giardia intestinalis* is a flagellated protist causing diarrheal disease in humans worldwide.<sup>[1,2]</sup> Pathophysiology of giardiasis is due to the adherence of *Giardia* trophozoites to epithelial surface of the small intestine resulting in a series of events that initiate the onset of diarrhea.<sup>[3,4]</sup> Besides the routine anti-*Giardial* drugs, live lactic acid bacteria have been employed *in*

*vivo* as an alternative bio-intervention to control the duration and severity of *Giardia* infection by their ability to antagonize the adherence of trophozoite to enterocytes.<sup>[5,6]</sup>

Probiotic lactobacilli are the part of complex bacterial communities of gastro-intestinal tract (GIT) and are able to survive extreme intestinal conditions. More specifically, adhesive properties of probiotics and their ability to produce antimicrobial substances active against a variety of bacteria, including *Escherichia coli*, *Salmonella* species, *Clostridium* species, *Streptococcus* species, and *Bacteroides* species have made them a part of our daily life management.<sup>[7,8]</sup> To this end, we have established a mouse model to assess the modulatory potentials of *Lactobacillus rhamnosus* GG (LGG) in ameliorating murine giardiasis and have reported that

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among the various probiotics, LGG was the most effective one in reducing both the duration and severity of murine giardiasis.<sup>[9]</sup> Further, it was observed that the probiotic LGG modulated murine giardiasis due to its antioxidative and immunomodulatory properties.<sup>[10]</sup> Thus, the aim of the present study was to assess *in vitro* the adherence and colonizing ability of LGG *viz-a-viz* its inhibitory effect on the adherence of *Giardia* trophozoites to murine enterocytes.

## MATERIALS AND METHODS

### Parasite

*G. intestinalis* trophozoites (Portland strain 1) were grown axenically in TYI-S-33 medium supplemented with antibiotic solution (streptomycin 4 ug/100ml, penicillin 2000 units/100ml and gentamycin 1000 units/100ml) and horse serum, pH 6.9, adjusted prior to filter sterilization using 0.22 µm seitz filter. Actively growing trophozoites (48-72 h old culture) were centrifuged at 200 g for 10min, after chilling the tubes in ice for 15 min, washed thrice with phosphate buffer saline (PBS) pH-7.2 and finally re-suspended in PBS.<sup>[5]</sup>

### Probiotic

*Lactobacillus rhamnosus* GG (MTCC (1408) procured from Institute of Microbial Technology, Chandigarh was grown in de Man Rogosa Sharpe (MRS) broth and maintained on MRS agar slants by regular sub-culturing at 15 days interval. For experimental use, LGG grown in MRS broth for 18 h was sedimented by cold centrifugation at 1200 g for 10 min, washed thrice and finally re-suspended in PBS.

### Animals

BALB/c mice aged 5-6 weeks old (18-20g) of either sex were obtained from Central Animal House, Panjab University, Chandigarh, India. The mice were housed under standard conditions of light and dark cycle, fed with standard pellet diet (Hindustan Liver Products Limited, Kolkata, India) and were given water *ad libitum*. Only *Giardia*-free mice were employed. Care and use of animals was in accordance with the guidelines of the Institutional Animal Ethical Committee.

### Isolation and characterization of murine enterocytes

After sacrificing the animals either by retro-orbital bleeding or by cervical dislocation, intestinal epithelial

cells were isolated as per the Weiser method with minor modifications.<sup>[11]</sup> A segment of small intestine (3 cm) was everted on glass rod, tied and rinsed with 0.9% cold NaCl. The rod was kept in a conical tube containing 10 ml solution A (1.5 mM KCl, 96 mM NaCl, 27 mM sodium citrate, 8 mM KH<sub>2</sub>PO<sub>4</sub>, 5.6 mM Na<sub>2</sub>HPO<sub>4</sub>) for 30min at 37°C and then transferred to another tube with 10ml solution B (277 mM trisodium citrate, 0.5 mM dithiothreitol, 55 mM sorbitol, 44 mM sucrose) followed by gentle shaking for 30 sec, centrifuged at 500g for 10 min at 4°C. Enterocytes were washed, suspended in Hanks Balanced Salt Solution (HBSS) and cell viability was monitored with trypan blue exclusion staining method.

The morphology of isolated murine enterocytes was observed under both light and phase contrast microscopes and purity was monitored by alkaline phosphatase assay as per Bergmeyer.<sup>[12]</sup> Cells were disrupted by sonication for 5 sec at 8Kc/seconds (Soniprep-150MSE) and centrifuged at 12000g for 5min at 4°C. 100µl of cell extract was added to 500 µl buffered substrate (0.1M Glycine-NaOH buffer with 5.5 mM *p*-nitrophenyl phosphate, pH 10.5) and kept at 37°C for 5 min for equilibration. Reaction was stopped by adding 5 ml of 0.1N NaOH. Absorbance was read at 420 nm and results were expressed as µmoles of DNP released per mg of protein.

### *Giardia*-murine enterocytes adherence assay

The adhesion study was performed as per Céu Sousa *et al.*<sup>[13]</sup> Equal amount (1x10<sup>5</sup> cells/ml in HSCB Hepes saline buffer comprising of 10 mM Hepes saline buffer (pH 7.2) containing 2 mmol/l CaCl<sub>2</sub>) of *Giardia* trophozoites and epithelial cell suspension were mixed in tissue culture plate (Laxbro), incubated for 1h at 37°C in CO<sub>2</sub> incubator. Unadhered trophozoites were removed by gently pipetting followed by washing with PBS. Adhered trophozoites were sedimented by centrifugation at 200 g for 5 min and were counted. Percentage of adhered trophozoites to enterocytes was calculated by determining the ratio of adhered trophozoites/total *Giardia* trophozoites seeded.

### Effect of various physicochemical factors on adherence

#### Effect of time

To investigate the ability of *Giardia* trophozoites to

adhere to the mouse enterocytes, adherence assay was performed at time intervals ranging from 30 min to 3h of incubation as per Céu Sousa *et al.*<sup>[13]</sup> Briefly, equal amount ( $10^5$  cells/ml in HSCB) of *Giardia* trophozoites and murine enterocytes were mixed in tissue culture plate and incubated at 37°C in CO<sub>2</sub> incubator for different time intervals.

#### Effect of bile salt and pH

The adhesion study was performed as per Céu Sousa *et al.*<sup>[13]</sup> both in the presence and absence of bile salt (0.03% sodium taurocholate) as well as by varying the pH of HSCB to 5.5, 7.8 and 9.2 either with 1N HCl or 1N NaOH.

#### LGG-*Giardia*-Murine enterocytes adherence assay

The inhibitory effect of LGG on the adherence of *Giardia* trophozoites to murine enterocytes was studied by co-incubating the trophozoites, enterocytes and probiotic LGG as per Céu Sousa *et al.*<sup>[13]</sup> In one set of experiments, enterocytes were pretreated for 30 min with LGG before adding *Giardia* trophozoites to the assay mixture. In the second set of experiments LGG, *Giardia* trophozoites and enterocytes were added together to the assay mixture.

#### Scanning Electron Microscopy

Interactions of *Giardia* trophozoites, LGG and murine enterocytes were monitored by Scanning Electron Microscope (JEOL, JEM 1600 Model, Japan). Respective cells were incubated on coverslips for 1 h in HSCB. Specimens were fixed with 2.5% glutaraldehyde for 2 h and dehydrated in 50, 70, 80, 90 and 100% ethanol for 10, 15, 15, 20, 30 min respectively. Finally, the samples were desiccated, mounted on aluminium stubs, coated with gold-palladium at a thickness of 200Å and were observed by Scanning Electron Microscope.

#### Statistics

All experiments were carried out in triplicates. Results were expressed as mean  $\pm$  SD of three independent experiments. Differences between the observations were evaluated by a two tailed Student's t-test.

## RESULTS

**Effect of time on adherence:** It was observed that the

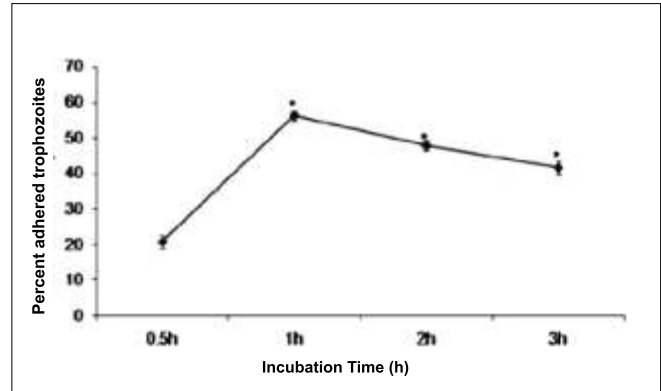


Fig.1: Effect of time on the adherence of *Giardia* trophozoites to murine enterocytes. Values are mean  $\pm$  SD of three independent experiments.

adherence increased significantly from 0.5 h to 1 h and was maximum ( $56 \pm 1.3\%$ ) after 1h of incubation, compared with  $20.8 \pm 1.9\%$  of adhered trophozoites after 0.5 h and thereafter reached a plateau (Fig.1). Thus, for further *in vitro* experiments, 1 h was set as the standard incubation time

**Effect of bile salt and pH:** It was observed  $50 \pm 1.3\%$  of *Giardia* trophozoites adhered to murine enterocyte in the presence of bile salt (0.3% sodium taurocholate) compared with  $53.8 \pm 1.1\%$  in its absence. The percentage of adhered *Giardia* trophozoites was  $38.4 \pm 1.1$ ,  $51.9 \pm 1.0$  and  $42.1 \pm 1.1$  at pH 5.5, 7.8 and 9.2 respectively. However, the adherence of *Giardia* trophozoites to murine enterocytes was most significant ( $p < 0.05$ ) at pH 7.8 as compared to pH 5.5 and 9.2 respectively (Fig.2).

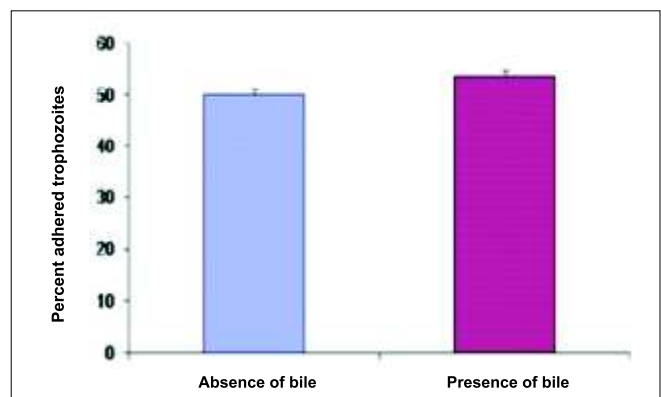


Fig. 2: Effect of pH on the adherence of *Giardia* trophozoites to murine enterocytes. Values are mean  $\pm$  SD of three independent experiments.

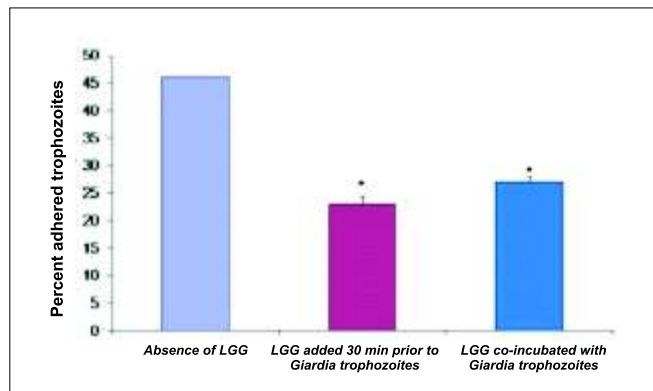


Fig. 3: Effect of probiotic LGG on the adherence of *Giardia* trophozoites to murine enterocytes. Values are mean  $\pm$  SD of three independent experiments.

#### LGG-*Giardia*-enterocytes adherence assay

The treatment of mouse enterocytes either 30min prior or simultaneously with trophozoites significantly ( $p < 0.05$ ) decreased the adherence of *Giardia* trophozoites (23 $\pm$ 1.0 and 27 $\pm$ 1.0% respectively) compared with 46 $\pm$ 1.3% adherence without LGG treatment (Fig.3).

#### Scanning Electron Microscopy

*In vitro* interactions of *Giardia* trophozoites and LGG with murine enterocytes were also confirmed by SEM. The *Giardia* trophozoites and lactobacilli were found

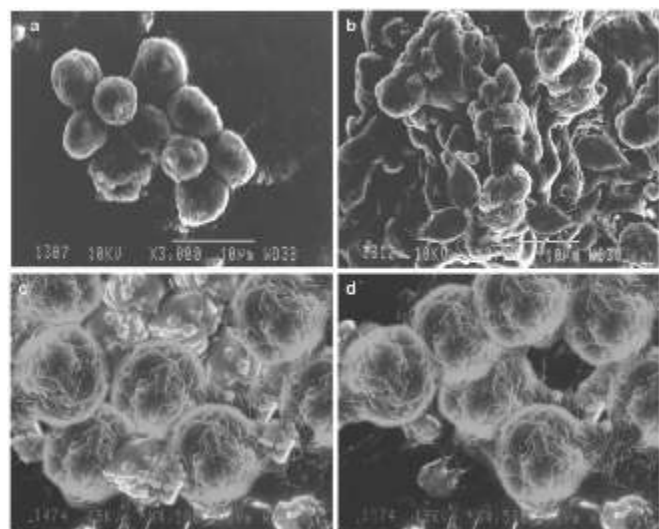


Fig. 4: Scanning electron micrograph showing:(a) normal spherical isolated murine enterocytes (3,000X); (b) adhered *Giardia* tropho-zoites to murine enterocytes (3,300X);(c) colonization of enterocytes with rod shaped LGG (4,500X);(d) displaced *Giardia* topophozoites (4,500X).

adhered to the murine enterocytes as observed under SEM (Fig. 4a, b and c). More specifically, treatment of enterocytes with the LGG either 30 min prior or simultaneously with *Giardia* trophozoites led to displacement of the *Giardia* trophozoites (Fig. 4d).

#### DISCUSSION

In giardiasis, there is a close association of *Giardia* trophozoites with the brush border membrane of small intestine resulting in intestinal lesions, malabsorption and diarrhea.<sup>[3]</sup> Probiotics have the ability to adhere to intestinal mucosa due to which these are generally regarded as safe “GRAS” and are much in consideration for intestinal infections.<sup>[14]</sup>

In the present study it was found that both the *Giardia* trophozoites and probiotic LGG adhered to the isolated mouse enterocytes and the adherence of *Giardia* trophozoites was maximum at pH 7.8 after 1 h of incubation in HBSS at 37°C in the presence of 0.3% bile salt. This observation is in accordance with earlier studies where rat intestinal epithelial cells and human intestinal cell lines were also employed as models to assess the adherence of *Giardia* trophozoites *in vitro*.<sup>[15,16]</sup> Further, it was observed that co-incubation of mouse enterocytes with LGG either 30 min prior or simultaneously with *Giardia* trophozoites led to significant decrease in the number of adhered trophozoites. However, there could also be the display of various specific adhesions and other surface determinants by LGG that may be involved in the interaction with intestinal epithelial cells as suggested by earlier studies.<sup>[17-19]</sup> Moreover, the present observation is in concordance with Collodo<sup>[8]</sup> who has shown that treatment of pig intestinal mucus with *Bifidobacterium lactis* Bb12 and LGG, alone or in combination, significantly reduced the adhesion of pathogens viz. *Salmonella*, *Clostridium* and *E.coli*. Based on these findings, it appears that the probiotic LGG has the potential to displace the *Giardia* trophozoites due to its ability to adhere and colonize with murine enterocytes.

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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