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ORIGINAL PAPER

Lactobacillus rhamnosus GG antagonizes *Giardia intestinalis* induced oxidative stress and intestinal disaccharidases: an experimental study

Nisha Goyal · Praveen Rishi · Geeta Shukla

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Abstract The present study describes the in vivo modulatory potential of *Lactobacillus rhamnosus* GG (LGG), an effective probiotic, in *Giardia intestinalis*-infected BALB/c mice. Experimentally, it was observed that oral administration of lactobacilli prior or simultaneous with *Giardia* trophozoites to mice, efficiently ($p < 0.05$) reduced both the severity and duration of giardiasis. More specifically, probiotics fed, *Giardia*-infected mice, showed a significant increase in the levels of antioxidants [reduced glutathione (GSH) and superoxide dismutase (SOD)] and intestinal disaccharidases [sucrase and lactase] and decreased levels of oxidants in the small intestine, in comparison with *Giardia*-infected mice. Histopathological findings also revealed almost normal cellular morphology of the small intestine in probiotic-fed *Giardia*-infected mice compared with fused enterocytes, villous atrophy and increased infiltration of lymphocytes in *Giardia*-infected mice. The results of the present study has shed new light on the antioxidative properties of *L*GG in *Giardia* mediated tissue injury, thereby suggesting that the effects of probiotic *L*GG are biologically plausible and could be used as an alternative microbial interference therapy.

Keywords Giardiasis · Probiotic · Lipid peroxidation · Antioxidants · Mucosal disaccharidases · Microbial interference therapy (MIT)

Abbreviations

*L*GG	Lactobacillus *rhamnosus* GG
GSH	Reduced glutathione
SOD	Superoxide dismutase
ALP	Alkaline phosphatase
PBS	Phosphate buffered saline

N. Goyal · P. Rishi · G. Shukla (✉)
Department of Microbiology, Panjab University, Chandigarh 160014, India
e-mail: geeta_shukla@pu.ac.in

Introduction

Giardia intestinalis is a microaerophilic, flagellated, intestinal protist, causing waterborne diarrheal disease, giardiasis, which mainly affects school children, malnourished, hypogammaglobulineamic individuals and immunocompromised hosts. The prevalent rates of giardiasis range from 2 to 7 % in developed countries and 20–30 % in most developing countries (Ratanapo et al. 2008; Mastronicola et al. 2011). The transmission of this infection occurs through contaminated food, water and the oral fecal route (Pérez et al. 2001). *Giardia* trophozoites mainly colonize the proximal jejunum by adhering to the brush border of enterocytes. Giardiasis can be asymptomatic or symptomatic; characterized with mild, self limiting illness or chronic diarrhea with or without illness (Katelaris et al. 1995; Buret 2007). A diffused loss of microvillus brush border with or without villus atrophy is responsible for intestinal disaccharidase insufficiency and malabsorption of electrolytes and nutrients, mainly carbohydrates, fats and water leading to diarrheal symptoms (Khanna et al. 1988; Buret 2007). In addition, it has been found that inflamed intestines are deficient in antioxidants, making them more susceptible to tissue damage (Lih-Brody et al. 1996; Kruidenier et al. 2003).

The preferred choice for the treatment of giardiasis is antibiotics such as nitroimidazoles and nitrofurans. However, due to emergence of drug-resistant strains, low compliance, recurrence of infection and other adverse effects, the interest of scientists has tilted towards the use of safe,

effective and inexpensive bio interventions (Gardner and Hill 2001; Lemee et al. 2000). Probiotics have emerged as a potential form of live drug for gastrointestinal diseases and their administration for disease alleviation is known as microbial interference therapy. Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit to the host" (Heyman and Menard 2002; Bergonzelli et al. 2005). The interactions between probiotic and inflamed intestinal mucosa have been best highlighted in animal models of inflammatory bowel diseases. Since giardiasis too leads to inflammation of the small intestine in humans, experimental giardiasis is a useful model to study the underlying mechanism of pathogenicity, the host-parasite relationships and the effect of treatment (Carmen et al. 2011). Earlier, we have found that various probiotic strains have the potential to reduce both the duration and severity of murine giardiasis, however of these, *L. rhamnosus* GG was found to be the most effective probiotic (Shukla et al. 2008, 2010a, b; Shukla and Sidhu 2011, Goyal et al. 2011). To date, no studies have assessed the antioxidative effect of *L*GG on intestinal parasitosis, particularly giardiasis. Therefore, the present study was specifically addressed to delineate the antioxidative property of *L*GG in murine giardiasis.

Methods

Parasite and culture conditions

Giardia intestinalis trophozoites (Portland strain I) were grown axenically in TYI-S-33 medium supplemented with horse serum and antibiotic solution (streptomycin 4 μg/100 ml, penicillin 2,000 units/100 ml and gentamycin 1,000 units/100 ml), and the pH adjusted to 6.9 prior to filter-sterilization using 0.22 μm Seitz filters. Briefly, actively growing trophozoites (48–72 h old culture) were chilled in ice for 15 min followed by centrifugation at 200×*g* for 10 min, washed three times and the pellet finally re-suspended in phosphate buffered saline pH 7.2 (Gillin and Diamond 1981).

Bacterial strains, preparation and inoculation

Lactobacillus rhamnosus GG was grown in de Man-Rogosa-Sharpe (MRS) broth and was maintained on MRS-agar slants by regular sub culturing at 15 day intervals. For experimental use, *L*GG grown in MRS broth for 18 h was sedimented by cold centrifugation at 1,200×*g* for 10 min, washed three times and the pellet finally suspended in phosphate buffered saline, pH 7.2 (PBS). The animals were fed with 1×10^9 lactobacilli/0.1 ml via oro-gastric gavages (Goyal et al. 2011).

Animals

BALB/c mice of either sex, aged 5–6 weeks old (18–20 g) were obtained from Central Animal House, Panjab University, Chandigarh, India. These were housed under standard conditions of light and dark cycle. Animals were fed with standard pellet diet (Hindustan Liver Products Limited, Kolkata, India) and were given water ad libitum. Animals were screened for *Giardia* infections via stool examination for three consecutive days. Only *Giardia*-free mice were used for the study. Care and use of animals were in accordance with the guidelines of the institutional animal ethical committee.

Experimental design

Animals were divided into five groups. *Group I (control, n = 18)*: Mice were fed orally with single dose of 0.1 ml PBS via oro-gastric gavages daily for 25 days. *Group II (probiotic, n = 18):* These mice were fed orally with single dose of probiotic (1×10^9 lactobacilli/0.1 ml) daily up to 25 days. *Group III (Giardia–infected, n = 24):* Normal mice were challenged orally with single dose of *Giardia* trophozoites (1×10^6/0.1 ml). *Group IV (L.rhamnosus GG-Giardia, n = 24):* Animals were fed orally with single dose of probiotic (1×10^9 lactobacilli/0.1 ml) daily for 7 days. On day 8, single challenge dose of *Giardia* trophozoites (1×10^6 trophozoites/0.1 ml) was given orally along with single dose of probiotic. However, single dose of probiotic administration was continued for further 25 days. *Group V* (*Giardia-L.rhamnosus GG, n = 24*): Mice belonging to this group were challenged orally with single dose of *Giardia* trophozoites (1×10^6/0.1 ml) and simultaneously with single dose of probiotic (1×10^9 lactobacilli/0.1 ml). Thereafter, the probiotic feeding was done daily up to 25 days.

Follow-up of the animals

Following the respective treatments, animals belonging to different groups, were monitored for the establishment of *Giardia* infection by counting cysts in mice feces on every alternate day. Animals from each group were sacrificed in batches of six on day 3–5 (establishment phase), 7–9 (acute phase) 20–22 (decline phase) post infection (PI) respectively, to monitor various biochemical parameters in the small intestine such as lipid peroxidation (MDA) levels, antioxidant levels (superoxide dismutase [SOD], reduced glutathione [GSH],) intestinal alkaline phosphatase (ALP) and disaccharidases (maltase, sucrase, lactase) *vis- a- vis* histopathological alterations in the small intestine.

Giardia cysts in feces

Briefly, mouse fecal pellets were collected for 2 h by keeping each mouse in separate cage. One gram of freshly voided fecal samples was suspended in 10 ml of formol saline and mixed with a pestle and mortar mixer. Cyst count was performed in iodine-stained stool samples using hemocytometer and counts were expressed as cysts/ml (Shukla et al. 2008).

Preparation of brush border membrane (BBM)

Brush border membrane was isolated and purified from the small intestinal tissue by method of Kessler et al. (1978). Briefly, mice were sacrificed via cervical dislocation, intestines were removed, washed, homogenized (5 % W/V) in Tris- Mannitol buffer (pH 7.2) and filtered through muslin cloth. An aliquot of the filtrate was used for protein, alkaline phosphatase estimations and labelled as crude BBM. Brush border membrane was further purified with addition of 10 mM $CaCl_2$ to the filtrate with constant stirring for 15 min at 37 °C. Mixture was cold centrifuged at 800×*g* for 25 min and supernatant re-centrifuged at 7,000×*g* for 30 min. The sediment was re-suspended in 2 ml of 50 mM sodium maleate buffer (pH 6.8), labeled as purified BBM and was used for estimation of alkaline phosphatase and disaccharidase. Purity of membrane was evaluated by comparing the alkaline phosphatase activities both in crude BBM and purified BBM preparation.

Estimation of intestinal alkaline phosphatase and disaccharidases

Assay of alkaline phosphatase

Alkaline phosphatase in the intestinal homogenate was estimated by using *p*-nitrophenyl phosphate as substrate by the method of Bergmeyer (1963). Briefly, 0.5 ml buffered substrate (0.1 M glycine- NaOH buffer with 5.5 mM *p*-nitrophenyl phosphate, pH 10.5) was added to the test tubes and kept at 37 °C for 5 min for equilibration followed by addition of 0.1 ml of sample (BBM). Reaction was stopped by adding 5 ml of 0.1 M NaOH. Absorbance was read at 420 nm and results were expressed as micromoles *p*-nitrophenol formed per milligram protein.

Assay of disaccharidases

Disaccharidases (sucrase, maltase and lactase) in the BBM were assayed by measuring the D-glucose liberated from the respective sugars using glucose oxidase–peroxidase system of Dahlquist (1964). Substrates (sucrose, maltose and lactose, 0.15 M) were prepared in 50 mM sodium maleate buffer, pH 6.8. Reaction mixture containing 0.1 ml of respective substrate and 0.3 ml sodium maleate buffer was taken in separate test tubes and kept at 37 °C for 5 min for equilibration. To each tube, 0.1 ml of sample was added and kept at 37 °C for 30 min for sucrase, maltase and 1 h for lactase. The amount of glucose liberated from the respective sugars was estimated by addition of 2.5 ml of glucose oxidase peroxidase system (Reckon GOPPAP kit, Vadodara, India) by keeping at 37 °C for 1 h. The optical density was measured at 500 nm. Enzyme activity was expressed as units/mg of protein, where one enzyme unit is defined as the amount of enzyme, which transformed 1 μmol of substrate to product per minute under standard assay conditions.

Preparation of tissue homogenate and post mitochondrial supernatant (PMS)

After sacrificing mice, the small intestines were removed and the intestinal homogenates were prepared in PBS using mechanically driven Teflon-fitted Potter Elvjheim type homogenizer under ice cold conditions for 40–45 s. Post-mitochondrial supernatant (PMS) was prepared by cold centrifugation of intestinal homogenates at 400×*g* for 10 min. The pellet was discarded and supernatant was again centrifuged at 2,200×*g* for 10 min and supernatant was labeled as PMS. Protein concentration both in the homogenate and PMS was estimated by the Lowry method.

Estimation of lipid peroxidation (Malondialdehyde) levels

The quantitative measurement of lipid peroxidation was performed according to Wills (1966). A 0.5 ml of intestinal homogenate and 0.5 ml of Tris–HCl buffer (0.1 M, pH 7.4) was mixed and kept at 37 °C for 2 h. Following incubation 1 ml of 10 % (w/v) chilled trichloroacetic acid was added and the mixture was centrifuged at 100×*g* for 10 min. One millilitre of supernatant was mixed with 1 ml of 0.67 % (w/v) thiobarbituric acid and the mixture was kept in aboiling water bath for 10 min, tubes were cooled and 1 ml of distilled water was added. Absorbance was measured at 532 nm. Results were expressed as millimoles of malondialdehyde (MDA) per milligram of protein using molar extinction coefficient of chromophore (1.56×10^5 M^{-1} cm^{-1}).

Estimation of reduced glutathione (GSH)

Reduced glutathione in the small intestine was estimated by the method of Ellman (1959). One millilitre of intestinal homogenate was precipitated with 1 ml of 4 % sulfosalicylic acid and cold digested at 4 °C for 1 h. The sample was cold centrifuged at 1,200×*g* for 15 min. To 1 ml of

supernatant 2.7 ml of phosphate buffer (0.1 M, pH8) and 0.2 ml of 5, 5-dithiobis (2-nitrobenzoic acid [DTNB]) were added. The yellow color developed was read immediately at 412 nm using a spectrophotometer. The results were calculated using the molar extinction coefficient of the chromophore (1.36×10^4 M^{-1} cm $^{-1}$) and expressed as nanomoles of GSH per milligram of protein.

Estimation of superoxide dismutase (SOD)

SOD activity in post-mitochondrial supernatant (PMS) was assayed as per Kono (1978). The reaction was initiated by addition of 0.5 ml of hydroxylamine hydrochloride to the reaction mixture containing 2.0 ml nitroblue tetrazolium (NBT) and 0.1 ml PMS. SOD activity was expressed as units of SOD per milligram of protein where one unit activity is defined as the amount of SOD required to inhibit the rate of reduction of nitro blue tetrazolium (NBT) by 50 %.

Histopathology

Mice were sacrificed via cervical dislocation and the proximal jejunum was removed, fixed in 10 % buffered formalin, dehydrated in different grades of alcohol i.e. 70, 80, 90 % and absolute alcohol for 30 min, 40 min and 1 h respectively and finally washed with xylene for 1 h. Dehydrated tissues were embedded in molten paraffin wax and thin sections of tissues were cut, mounted on clean glass microscopic slides and stained with haematoxylin and eosin stain (H&E stain). The slides were blot-dried, mounted with Distyrene Plasticizer Xylene (DPX) and were examined by light microscopy.

Statistical analysis

Results were expressed as mean ± SD. Inter-group variation was assessed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Statistical significance was calculated at $p < 0.05$.

Results

Giardia cycle

It was found that oral feeding of probiotic *LGG* either before or simultaneously with *Giardia* infection led to significantly ($p < 0.05$) decreased cyst counts at each point of observation (Group IV and V) and became *Giardia*-free by day 13 PI, in comparison with day 25 PI of *Giardia*-infected mice (Group III, Fig. 1).

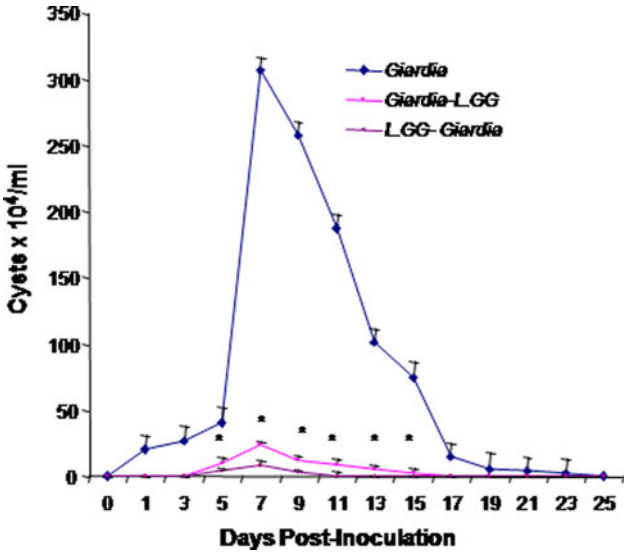

Fig. 1 *Giardia* cysts in feces. Values are mean ± SD, *$p < 0.05$ versus *Giardia*- infected

Alkaline phosphatase and diasaccharidases

The activities of intestinal enzymes (alkaline phosphatase, sucrase and lactase) in the brush border membrane of *Giardia*-infected mice (Group III) declined significantly ($p < 0.05$) during the course of infection compared with control animals (Group I, Figs. 2, 3, 4). Interestingly, oral supplementation of probiotic *LGG* to *Giardia*-infected mice (Group IV and V) resulted in significantly ($p < 0.05$) increased activities of alkaline phosphatase, sucrase and lactase at each point of observation compared with *Giardia*-infected mice (Group III, Figs. 2, 3, 4). However, maltase activity did not show significant change between the groups during course of *Giardia* infection (Fig. 5).

Lipid peroxidation

Lipid peroxidation measured in terms of MDA levels was found to be significantly higher ($p < 0.05$) in *Giardia*-infected mice (Group III) during the course of infection compared with control mice (Group I, Fig. 6). However, probiotic-fed *Giardia*-infected animals (Group IV and V) had significantly ($p < 0.05$) decreased levels of lipid peroxidation compared with *Giardia*-infected mice (Fig. 6).

Antioxidant levels

Giardia-infected mice (Group III) displayed a significant ($p < 0.05$) decrease in the levels of antioxidants SOD and GSH compared with control mice (Group I, Figs. 7, 8). Interestingly, oral administration of probiotic to *Giardia*-

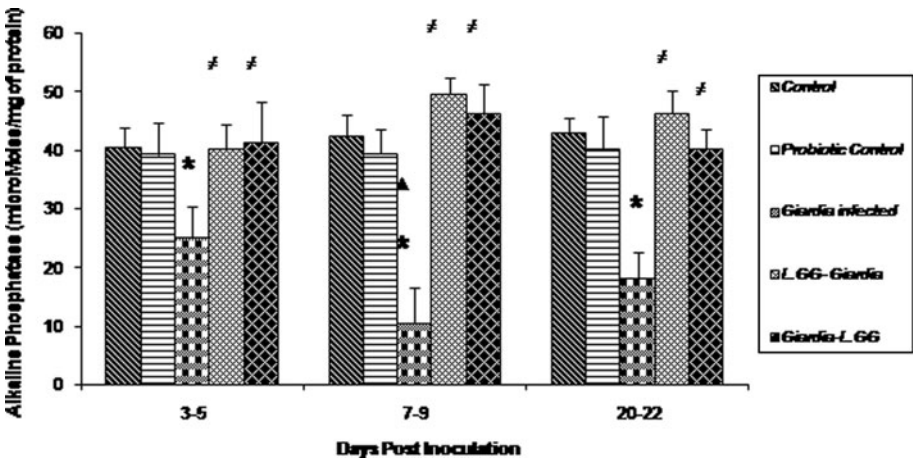

Fig. 2 Alkaline phosphatase activity in the small intestine of mice belonging to different groups. Values are mean ± SD, $*p < 0.05$ versus Control, $\neq p < 0.05$ versus *Giardia*- infected, *filled triangle* $p < 0.05$ versus *Giardia*-infected day 3–5

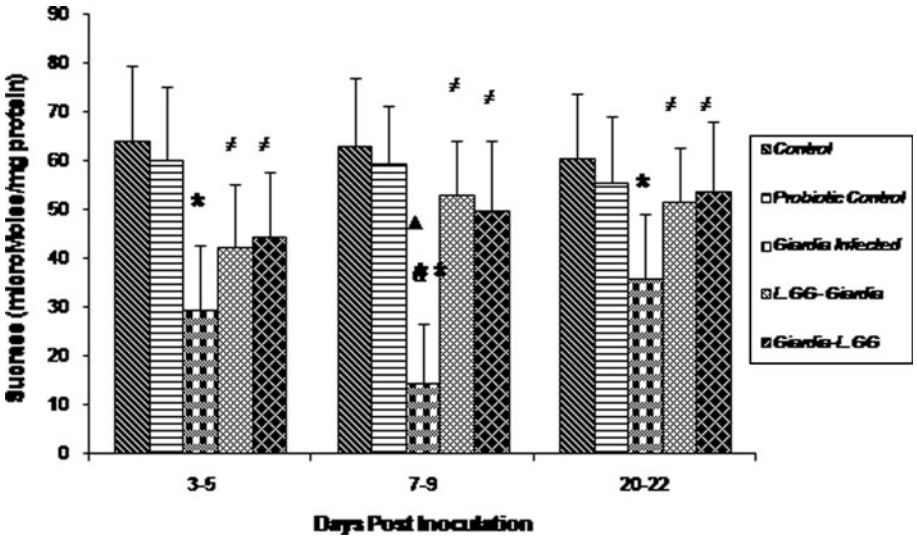

Fig. 3 Sucrase activity in the small intestine of mice belonging to different groups. Values are mean ± SD, $*p < 0.05$ versus Control, $\neq p < 0.05$ versus *Giardia*- infected, *filled triangle* $p < 0.05$ versus *Giardia*-infected day 3–5

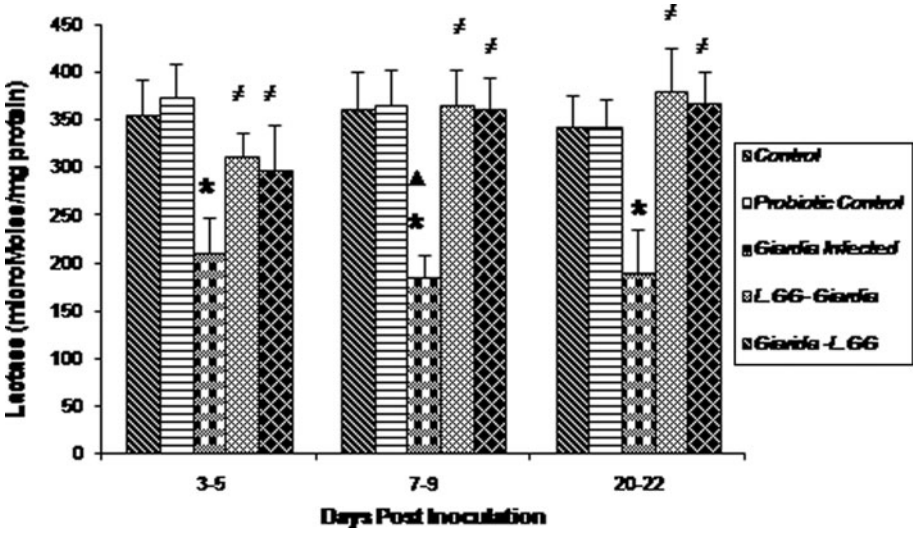

Fig. 4 Lactase activity in the small intestine of mice belonging to different groups. Values are mean ± SD, $*p < 0.05$ versus Control, $\neq p < 0.05$ v/s *Giardia*- infected, *filled triangle* $p < 0.05$ versus *Giardia*-infected day 3–5

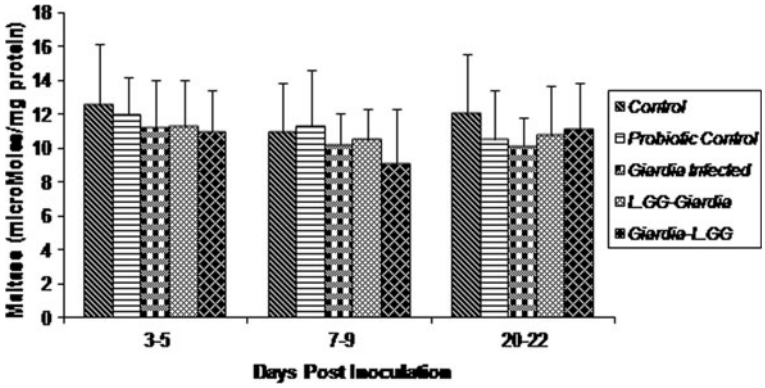

Fig. 5 Maltase activity in the small intestine of mice belonging to different groups. Values are mean ± SD

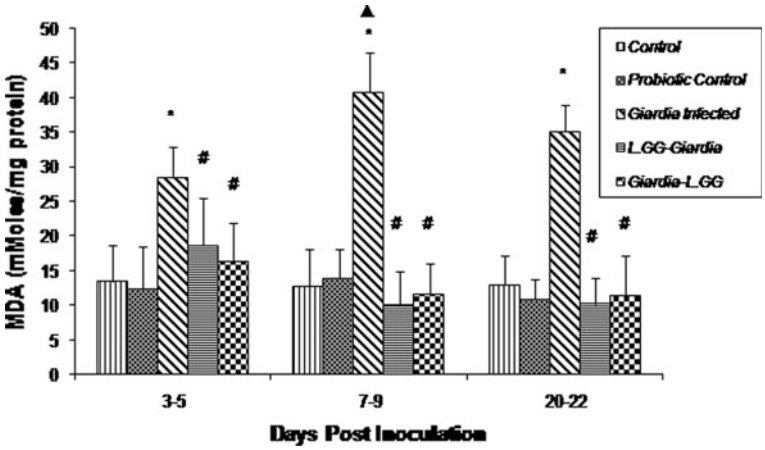

Fig. 6 MDA levels in the small intestine of mice belonging to different groups. Values are mean ± SD, *$p < 0.05$ versus Control, # $p < 0.05$ v/s *Giardia, filled triangle* $p < 0.05$ versus *Giardia-* infected mice day 3–5

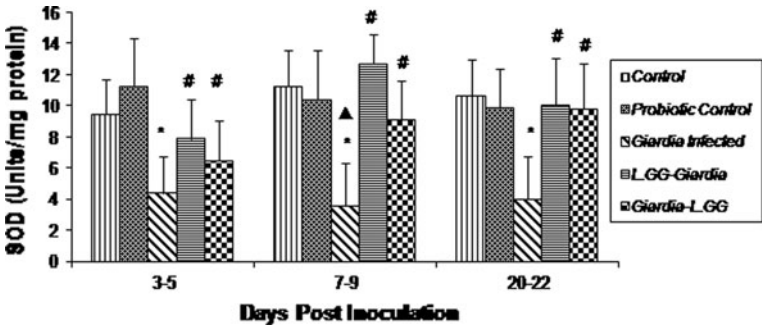

Fig. 7 SOD levels in the small intestine of mice belonging to different groups. Values are mean ± SD, *$p < 0.05$ versus Control, # $p < 0.05$ versus *Giardia-* infected, *filled triangle* $p < 0.05$ versus *Giardia-* infected day 3–5

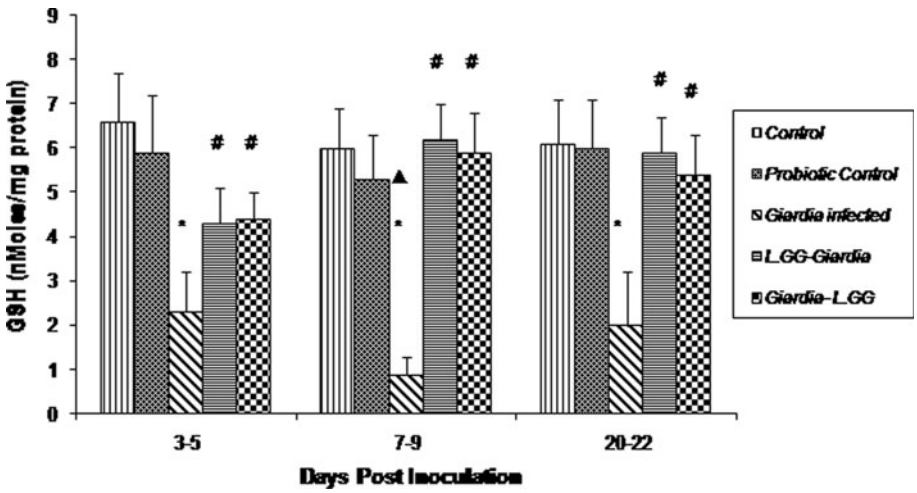

Fig. 8 GSH levels in the small intestine of mice belonging to different groups. Values are mean ± SD, *$p < 0.05$ versus Control, # $p < 0.05$ v/s *Giardia-* infected, *filled triangle* $p < 0.05$ versus *Giardia-* infected day 3–5

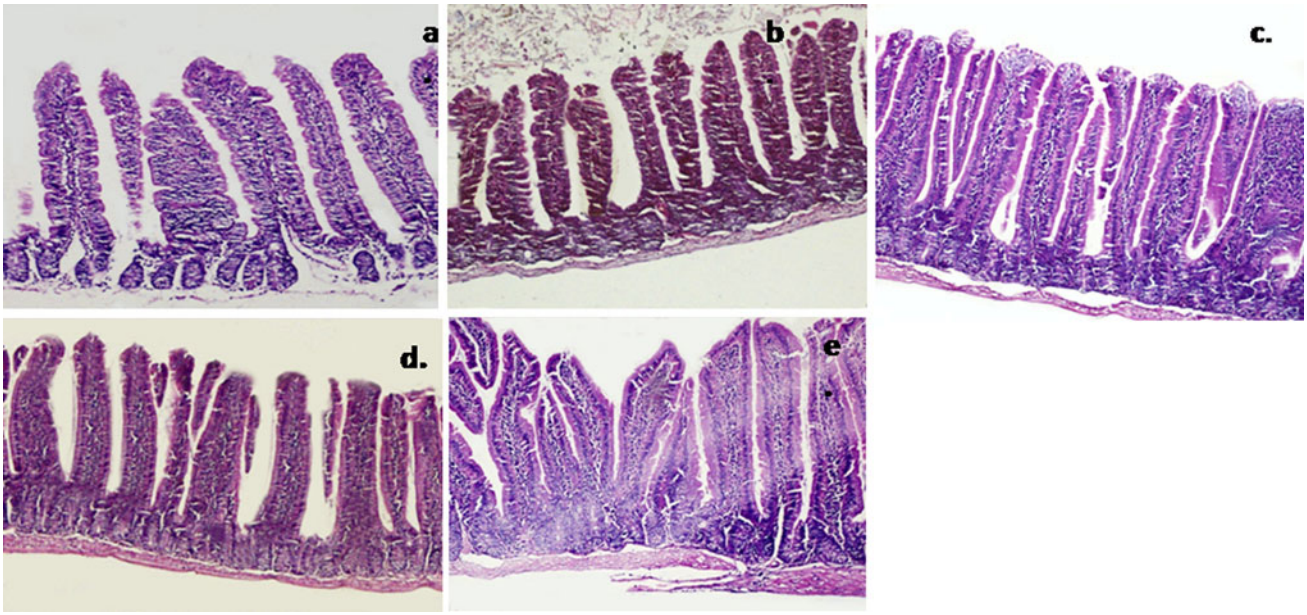

Fig. 9 Photomicrograph of the small intestine: (**a**) Control mice showing intact mucosal epithelial lining, basal crypts and normal villi; (**b**) Mice fed with probiotic showing normal intestinal morphology; (**c**) *Giardia*- infected mice on day 9 PI showing damaged microvilli and mild ileitis, increased lymphonuclear cell infiltration in lamina propria; (**d**) *L*.GG-*Giardia* mice on day 9 PI showing almost the normal architecture of brush border membrane, villi, and crypts; (**e**) *Giardia*-*L*.GG mice on day 9 PI showing infiltration of lymphocytes and almost normal structure of brush border membrane and crypts (H&E stain 100x)

infected mice (Group IV and V) led to a significant ($p < 0.05$) increase in the levels of SOD and GSH compared with *Giardia*-infected mice during the course of infection (Figs. 7, 8).

Histopathology

Giardia intestinalis infection had a profound effect on the morphological and cellular alterations in the small intestinal mucosa as these mice showed villous atrophy, fused enterocytes, swollen villi and increased infiltration of lymphocytes in the lamina propria, compared with the intact brush border membrane, basal crypts and normal villi in control and probiotic fed mice (Fig. 9a–c). Interestingly, mice fed with probiotic, either prior or simultaneously with *Giardia* infection, had almost normal morphometry of jejunal villous enterocytes (Fig. 9d, e).

Discussion

The importance of probiotics has been recognized due to their fermentative ability, nutritional and health benefits that help in toning up the intestinal environment. Therefore, for a healthy life, a good probiotic supplement containing millions of live bacteria, to boost and replenish the levels of health-promoting microorganisms in the digestive tract should be employed (Bergonzelli et al. 2005). The main thrust of the present study was to monitor the underlying protective potentials offered by LGG in murine giardiasis with respect to intestinal disaccharidases *vis-a-vis* oxidative stress.

It is very well documented that an individual's susceptibility to infections depends upon the composition of intestinal microbiota that interfere with the colonization of pathogens. In the present study, colonization of *Giardia* trophozoites was reduced in the intestines of probiotic-fed mice, in contrast to *Giardia*-infected mice. The present observation suggests that lactobacilli not only survive the gastrointestinal passage but are also able to colonize the gut enterocytes and help in balancing the gastrointestinal microbiota and these observations are in concurrence with earlier observations (Humen et al. 2005; Shukla et al. 2008 Shukla and Sidhu 2011).

The well acknowledged fact is that *G. intestinalis* induces structural and functional derangement of the small intestine leading to damaged brush border microvilli and impaired activities of brush border membrane enzymes (Khanna et al. 1988; Céu Sousa et al. 2001. Alkaline phosphatase, lactase and sucrase, the biomarkers for intestinal damage, are expressed predominantly in the upper villus, whereas maltase activity is present towards the base of villous (James et al. 1987). Interestingly, it was observed that probiotic supplementation to *Giardia*-infected mice further prevented the brush border injury in the small intestine by significantly enhancing the specific activities of brush border enzymes (alkaline phosphatase, sucrase and

lactase) and subscribes with the previous studies (Humen et al. 2005; Southcott et al. 2008). Southcott et al. (2008) have reported increased activity of brush border enzymes namely sucrase and lactase in methotrexate-induced rat mucositis, following supplementation with sheep yoghourt containing *Lactobacillus bulgaricus* and *Streptococcus thermophilus* whereas Humen et al. (2005) found only increased sucrase activity in *Lactobacillus jonsonii* la1-treated rats.

The development of tissue injury and the outcome of the disease depend on the fine balance between the generation of toxic radicals and tissue antioxidant status. Also mammalian cells are well equipped with antioxidant systems to combat the radical-mediated tissue damage (Windrow et al. 1993). Data described here shows decreased MDA levels in the small intestine of probiotic fed "*Giardia*-infected" mice, reflecting reduced lipid peroxidation thereby further preventing any intestinal tissue damage. Archibald and Fridovich (1981) have also observed that lactic acid bacteria (LAB) can deal with oxygen radicals by either SOD or higher internal Mn^{2+} concentration. It is also believed that during transit through the gastrointestinal tract, lactobacilli may release their intracellular antioxidative constituents that may reduce tissue injury and thereby protect the host (Lin et al. 1998). We too have found that supplementation of LGG to *Giardia*-infected mice has resulted into enhanced levels of antioxidants SOD and GSH and are in accordance with the earlier studies (Peran et al. 2007, Han et al. 2006). These researchers have reported that supplementation of *Lactobacillus fermentum* alone or *Lactobacillus plantarum* and *Lactococcus lactis* together have anti-inflammatory effects in rat colitis mainly by producing SOD or GSH (Han et al. 2006).

Histopathological findings also showed almost normal cellular morphology of the small intestine in probiotic-fed *Giardia*-infected mice compared with fused enterocytes, villous atrophy and increased infiltration of lymphocytes in *Giardia*- infected mice. This again shows the colonizing ability of probiotics that inhibited the adherence of *Giardia* trophozoites to the enterocytes, thereby preventing mucosal damage, and is in accordance with the earlier studies (Shukla et al. 2008, 2010a).

The novel observations made in this study highlight the underlying protective mechanisms conferred by the effective probiotic LGG in murine giardiasis that appears to be multifactorial, like better colonization of the enterocytes, thereby modifying the intestinal microbiota, gut morphology, restoring the impaired activities of brush border membrane enzymes, scavenging the oxygen free radicals and releasing antioxidants in the small intestine.

This is the first study that provides scientific evidence for the in vivo effect of *L*GG in modulating the pathophysiology of murine giardiasis and suggests that it can be extended as a safe and a cost effective alternative, for the widespread intestinal diseases however, this needs to be clinically correlated.

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