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Protective role of lactobacilli in *Shigella dysenteriae* 1–induced diarrhea in rats

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Abstract

Objective: Studies on lactic acid bacteria exemplify their use against various enteropathogens in vitro. Nevertheless, in vivo effects of *Lactobacillus* during *Shigella* infection have not been evaluated. The present study evaluated the effect of *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* on neutrophil infiltration and lipid peroxidation during *Shigella dysenteriae* 1–induced diarrhea in rats.

Methods: The rats were divided into eight groups ($n = 6$ in each group). Induced rats received single oral dose of *S. dysenteriae* (12×10^8 colony-forming units [cfu]/mL). Treated rats received *L. rhamnosus* (1×10^7 cfu/mL) or *L. acidophilus* (1×10^7 cfu/mL) orally for 4 d, alone or in combination, followed by *Shigella* administration. At the end of the experimental period, animals were sacrificed and the assay of the activity of alkaline phosphatase, myeloperoxidase, and antioxidants and the estimation of lipid peroxides were performed. Activity staining of superoxide dismutase and catalase was done in addition to gelatin zymography for matrix metalloproteinase (MMP; MMP-2 and MMP-9) activity. A portion of the intestinal tissue was fixed in 10% formalin for histologic studies.

Results: Administration of *S. dysenteriae* 1 alone resulted in increased levels of myeloperoxidase, lipid peroxidation, alkaline phosphatase, and the expression of MMP-2 and MMP-9 with concomitant decrease in the antioxidant levels. Pretreatment with the combination of *L. rhamnosus* (1×10^7 cfu/mL) and *L. acidophilus* (1×10^7 cfu/mL) significantly attenuated these changes when compared with the diseased group. Histologic observations were in correlation with biochemical parameters.

Conclusion: *Lactobacillus rhamnosus* plus *L. acidophilus* offered better protection when compared with individual treatment with these strains during *Shigella* infection.

Keywords: *Shigella* infection; Probiotics; *Lactobacillus rhamnosus*; *Lactobacillus acidophilus*; Diarrhea

Introduction

Shigellosis is a highly contagious enteric bacterial infection characterized by fever, diarrhea, and bloody mucoid stools [1]. Among *Shigella* species, *Shigella dysenteriae* 1 has been associated with epidemic outbreaks of bacillary dysentery that pose major public health problems in developing countries [2] and is particularly fatal to young children [3]. The frequency of strains with resistance to multiple antibiotics such as ampicillin, trimethoprim-sulfamethoxazole, streptomycin, chloramphenicol, and tetracycline is a cause of growing concern [4]. *Shigella*-induced diarrhea is specific to humans but can also occur in a few simian species. An inoculum containing as few as 10 to 100 bacteria can induce diarrhea in humans [5]. Kamgang et al. [6] established a rat model of *S. dysenteriae* type 1–induced diarrhea by giving orally an inoculum of 12×10^8 *S. dysenteriae* type 1, which resulted in dysenteric diarrhea. This animal model is very useful in understanding the pathogenesis of *Shigella* in vivo and to evaluate the efficacy of various pharmacologic agents, drugs, or medicinal plants [6,7].

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The intestinal epithelium is a highly polarized structure and plays an important role as a protective barrier against luminal threats. Intestinal epithelial cells constitutively express several Toll-like receptors. Toll-like receptors are transmembrane receptors that serve as the pattern-recognition receptors of the mucosal innate immune system. They play a key role in the activation of immune cells in response to various bacterial products. These receptors seem to function as a major link between innate and adaptive cellular immune gene responses in various mammalian cell systems. Under normal conditions, the apical surface not only encounters microbial threats but also supports the presence of numerous commensals. Conversely, the basolateral surface interfaces the underlying immune system and provides mucosal protection by serving as a substrate for resident and emerging cells. The basolateral membrane is most likely free of lipopolysaccharide because the intestinal epithelium is normally impenetrable to most bacteria. But *Shigella* has evolved ways to penetrate the epithelium and gain access to the basolateral surface. The presence of such intact lipopolysaccharides results in an inflammatory response and an influx of polymorphonuclear neutrophils (PMNs) [8]. PMNs release a complex assortment of agents that can destroy normal cells and dissolve connective tissue. Myeloperoxidase (MPO), which is an essential enzyme for normal PMN function, is released as a response to various stimulatory substances [9]. *Shigella* infection also stimulates macrophage cells to release interleukin-1β, which may initiate or potentiate the host inflammatory response [10]. The proinflammatory cytokines activate inflammatory cells such as neutrophils, macrophages or monocytes, platelets, and mast cells, which release large amounts of toxic reactive oxygen metabolites, which cause cellular injury by several mechanisms including the peroxidation of membrane lipids and the oxidative damage of proteins and DNA [11].

The microflora that inhabits the human intestinal tract is part of an extremely complex ecological system. These micro-organisms interact not only with each other but also with their host. Among intestinal microflora, lactic acid bacteria play significant roles in the gut ecosystem. Lactic acid bacteria are considered beneficial micro-organisms and have been widely used as dietary adjuncts in cultured dairy foods and other fermented products. The origin of lactic acid bacteria may come from human and animal intestines or from naturally fermented foods. The potential benefits of lactic acid bacteria for human health include improvement of lactose intolerance, prevention of intestinal infection, reduction of serum cholesterol, stimulation of the immune system, anticarcinogenic action, and antioxidative effects [12]. It is more and more recognized that this resident microflora plays an important role in inhibiting gut colonization by the incoming pathogens [13,14]. Among lactic acid bacteria, *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* have attracted a lot of attention for their beneficial effects in human health.

Centuries of use of these lactic acid–producing bacteria in the food industry and lack of significant adverse effects with most strains currently in use are reassuring. A recent review identified 143 human clinical trials of probiotics between 1961 and 1998, involving more than 7500 subjects, with no adverse events reported [15]. Members of the genera Lactococcus and Lactobacillus are most commonly given generally-recognized-as-safe status [16].

Lactobacillus acidophilus (ATCC 4356), a human isolate, is widely used as a dietary adjunct in various cultured dairy products [12]. *Lactobacillus rhamnosus* has been reported to interact with intestinal epithelium and prevent the internalization of enteropathogenic bacteria such as enterohemorrhagic *Escherichia coli* [17]. Prevention of internalization of pathogens will eventually block the inflammatory response of the host. Rodent animal species infected by pathogenic strains of *E. coli* have been used to investigate the in vivo activity of lactic acid bacteria. *Lactobacillus rhamnosus* strain HN001 produces a protective effect in mice infected with *E. coli* O157:H7 [18]. Hence, it was of interest to investigate the effect of *Lactobacillus* in *S. dysenteriae* type 1 infection in vivo in rat.

A previous report has suggested that probiotic strains may complement each other and even work synergistically and has pointed out that investigation of the effects of combinations of probiotic microorganisms is worth testing in vivo [19]. Combinations of specific probiotic strains have been reported to enhance adherence of these bacteria to the intestinal mucus in a synergistic manner in vitro [20]. A protective role of a synergistic interaction of these strains has been reported during *Salmonella* infection [21]. However, whether *L. rhamnosus* and *L. acidophilus* exhibit a synergistic interaction to protect the intestine during *S. dysenteriae* type 1–induced diarrhea has not been previously studied.

Hence, the present study evaluated the effect of *L. rhamnosus* and *L. acidophilus*, alone or in combination, on the inflammatory response during *S. dysenteriae* 1–induced diarrhea in rats using biochemical and histologic approaches.

Materials and methods

Bacterial strains

The strain used in this study, *S. dysenteriae* type 1, was isolated from the stool of a patient with dysentery and was provided by the Department of Microbiology, Christian Medical College, Vellore, India. *Shigella dysenteriae* 1 was routinely grown in Luria-Bertani broth at 37°C under aerobic conditions. *Shigella dysenteriae* 1 was maintained in nutrient agar slants, stored in a refrigerator, and subcultured for every 15 d. The two lactobacilli studied were *L. rhamnosus* (MTCC 1408/ATCC 7469) and *L. acidophilus* (MTCC 447/ATCC 4356), which were obtained from the Institute of Microbial Technology, Chandigarh, India. These lactobacilli were routinely grown in deMan-Ragosa-Sharpe broth at 37°C under

microaerophilic conditions. Lactobacilli were maintained in deMan-Ragosa-Sharpe agar slants, stored in the refrigerator, and subcultured for every 15 d. Bacteria were grown in their respective broths for 18 to 20 h before use.

Animals

Adult male albino rats of Wistar strain, weighing approximately 120–140 g, were obtained from Tamilnadu Veterinary and Animal Science University, Chennai, India. They were acclimatized to animal house conditions and fed a commercial pellet rat chow (Hindustan Lever Ltd., Bangalore, India) and water ad libitum. The study was conducted according to the ethical norms approved by the Ministry of Social Justices and Empowerment, Government of India and by the animal ethics committee guidelines of our institution.

Experimental design

The rats were divided into 8 groups ($n = 6$ in each group): group 1, control; group 2, *S. dysenteriae* 1 induced; group 3, *L. rhamnosus* alone; group 4, *L. acidophilus* alone; group 5, *L. rhamnosus* + *L. acidophilus*; group 6, *L. rhamnosus* + *S. dysenteriae* 1; group 7, *L. acidophilus* + *S. dysenteriae 1*; group 8, *L. rhamnosus* + *L. acidophilus* + *S. dysenteriae* 1. A dosage of 1×10^7 colony-forming units [cfu]/mL of *L. rhamnosus* or *L. acidophilus* was administered orally [22] for 4 d alone or in combination to the rats. This particular dosage was fixed based on the protection offered by *L. rhamnosus* and *L. acidophilus* against *Shigella* infection after trying out different doses (1×10^6 cfu/mL, 1×10^7 cfu/mL, 1×10^8 cfu/mL, 1×10^9 cfu/mL) for 2, 4, 6, and 8 d before *S. dysenteriae 1* induction. After pretreatment of rats with *Lactobacillus* for 4 d, 12×10^8 cfu of *S. dysenteriae* 1 was orally administered to rats. This dosage provoked diarrhea within 24 h, which was also observed by Kamgang et al. [6]. Hence, on the second day after induction, animals were sacrificed; the intestine was removed, slit longitudinally, weighed, and rinsed with cold 0.9% NaCl. Mucosae were scraped off, homogenized in 10 mM sodium phosphate buffer at pH 7.4 (1:10 w/v), and centrifuged at 3000*g* for 10 min at 4°C. The supernatant was used for the estimation of protein [23], assay of alkaline phosphatase (ALP) [24], MPO [25], superoxide dismutase (SOD) [26], catalase (CAT) [27], and reduced glutathione (GSH) [28] and for activity staining of SOD [29] and CAT [30].

Assay of ALP

Disodium phenyl phosphate was the substrate used for ALP estimation. The assay mixture consisted of supernatant, disodium phenyl phosphate (0.01 M), $MgCl_2$ (0.1 M), carbonate-bicarbonate buffer (0.1 M, pH 10), Folin-Ciocalteu (diluted 1:2 with water), and Na_2CO_3 (15%). The color developed was read at 640 nm [24].

Assay of MPO

Myeloperoxidase activity was measured by using 3,3′,5,5′-tetramethylbenzidine as a substrate. The assay mixture consisted of supernatant, tetramethylbenzidine (1.6 mM) dissolved in dimethyl sulfoxide, and H_2O_2 (3.0 mM) diluted in 80 mM phosphate buffer, pH 5.4. Enzyme activity was assessed spectrophotometrically at 630 nm [25].

Assay of GSH

Supernatant was precipitated with 10% trichloroacetic acid and the precipitate was removed by centrifugation. To an aliquot of the resulting supernatant, phosphate buffer and 5,5′dithiobis-(2-nitro) (0.6 mM benzoic acid) reagent was added and the color developed was read at 420 nm [28].

Assay of SOD

Supernatant was added to tubes containing a mixture of ethanol and chloroform (chilled in ice) and centrifuged; 0.6 mM of ethylene-diaminetetra-acetic acid solution and carbonate-bicarbonate buffer (0.1 M, pH 10.2) was added to the resulting supernatant. The reaction was initiated by the addition of epinephrine (1.8 mM) and the increase in absorbance at 480 nm was measured [26].

Assay of CAT

For this assay, 0.05 M phosphate buffer (pH 7.0) was added to the supernatant and the enzyme reaction was started by the addition of H_2O_2 (0.03 M in phosphate buffer) solution. The decrease in absorbance was measured at 240 nm at 30-s intervals for 3 min [27].

The intestinal tissue was washed in ice-cold isotonic saline, homogenized in 1 M Tris buffer (1: 10 w/v), and centrifuged at 3000*g* for 10 min at 4°C. The supernatant was used for the estimation of levels of lipid peroxides [31] and was subjected to gelatin zymography [32] for matrix metalloproteinase-2 (MMP-2) and MMP-9 activities.

Assay of lipid peroxide

The reaction mixture consisted of supernatant, sodium dodecylsulfate (8.1%), 20% acetic acid (pH 3.5) and thiobarbituric acid (0.8%), 1,1,3,3, tetra ethoxy propane malondialdehyde bis (diethyl acetate), and an *n*-butanol/pyridine mixture (15:1 v/v). The tubes were shaken vigorously and centrifuged at 3000*g* for 10 min, the organic layer was taken, and its absorbance at 532 nm was measured [31].

Histologic studies

A portion of the tissue was fixed in 10% buffered neutral formalin solution for histologic studies. After fixation, tissues were embedded in paraffin wax, and solid sections

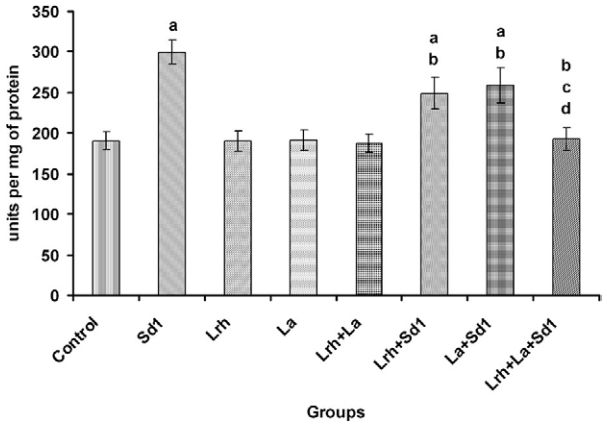

Fig. 1. Effect of Lrh and La pretreatment on the activity of alkaline phosphatase in the intestinal mucosa of control and experimental groups of rats. Results are expressed as mean ± SD ($n = 6$). Activity is expressed as units per milligram of protein. [a]Significance ($P < 0.05$) versus group 1 (control); [b]significance ($P < 0.05$) versus group 2 (Sd1); [c]significance ($P < 0.05$) versus group 6 (Lrh + Sd1); [d]significance ($P < 0.05$) versus group 7 (La + Sd1). La, *Lactobacillus acidophilus*; Lrh, *Lactobacillus rhamnosus*; Sd1, *Shigella dysenteriae* type 1 induced.

were cut at 5 μm, stained with hematoxylin and eosin, and viewed under a light microscope for histologic changes.

Shigella dysenteriae *1 density in feces*

For this determination, 0.5 g of diarrheal feces was homogenized using sterile saline, and further serial dilutions were made in sterile saline. Five hundred microliters of each dilution tube was spread over the surface of a *Shigella-Salmonella* agar plate in a glass spreader. Plates were then incubated at 37°C for 18–20 h and the colonies were counted. Biochemical tests of the colonies were performed using a *Himedia enterobactericeae* kit.

Statistical analysis

All grouped data were evaluated with SPSS 10 (SPSS, Inc., Chicago, IL, USA). Hypothesis testing methods included one-way analysis of variance followed by least significant difference testing. $P < 0.05$ was considered statistically significant. All results were expressed as mean ± SD for six animals in each group. *Shigella dysenteriae* 1 density was $\log_{10}$-transformed before analysis of means.

Results

ALP activity

Intestinal ALP is a membrane-bound enzyme, is present in large amounts in the intestine, and is reported to be involved in fat absorption in the intestine [33]. It is a marker enzyme that increases during colonic injury [34]. Figure 1 shows the level of ALP in the control and experimental groups of rats. A marked elevation ($P < 0.05$) in the activity of ALP was observed in group 2 (*S. dysenteriae* 1 infected) rats. Rats pretreated with a combination of *L. rhamnosus* and *L. acidophilus* (group 8) showed a significant decrease in the activity of ALP when compared with group 6 (*L. rhamnosus* + *S. dysenteriae* 1), group 7 (*L. acidophilus* + *S. dysenteriae* 1), and group 2 (*S. dysenteriae* 1 infected) rats.

MPO activity

MPO is a marker of PMN accumulation [35] and inflammation [36]. Figure 2 shows the level of MPO in the control and experimental groups of rats. A marked elevation ($P < 0.05$) in the activity of MPO was observed in group 2 (*S. dysenteriae* 1 infected) rats. Rats pretreated with a combination of *L. rhamnosus* and *L. acidophilus* showed a significant decrease in the activity of MPO when compared with group 6 (*L. rhamnosus* + *S. dysenteriae* 1), group 7 (*L. acidophilus* + *S. dysenteriae* 1), and group 2 (*S. dysenteriae* 1 infected) rats.

Lipid peroxides

Figure 3 shows the level of lipid peroxides in the intestinal tissue of the control and experimental group of rats. A maximum induction of lipid peroxides was observed in group 2 (*S. dysenteriae* 1 infected) rats. This distorted change was significantly decreased ($P < 0.05$) in group 8 rats pretreated with *L. rhamnosus* and *L. acidophilus* when compared with group 6 (*L. rhamnosus* + *S. dysenteriae* 1),

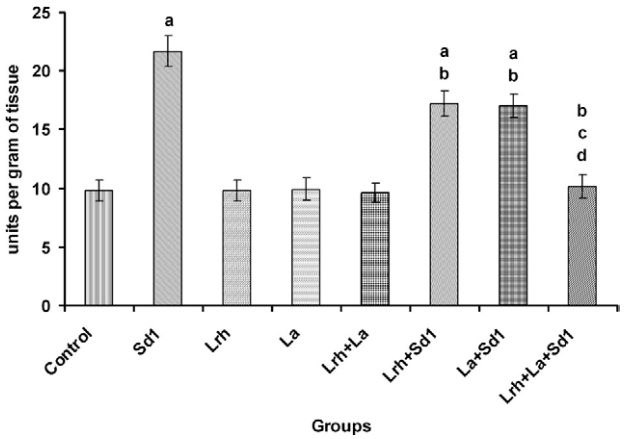

Fig. 2. Effect of Lrh and La pretreatment on the activity of myeloperoxidase in the intestinal mucosa of control and experimental groups of rats. Results are expressed as mean ± SD ($n = 6$). Activity is expressed as units per gram of intestinal tissue. [a]Significance ($P < 0.05$) versus group 1 (control); [b]significance ($P < 0.05$) versus group 2 (Sd1); [c]significance ($P < 0.05$) versus group 6 (Lrh + Sd1); [d]significance ($P < 0.05$) versus group 7 (La + Sd1). La, *Lactobacillus acidophilus*; Lrh, *Lactobacillus rhamnosus*; Sd1, *Shigella dysenteriae* type 1 induced.

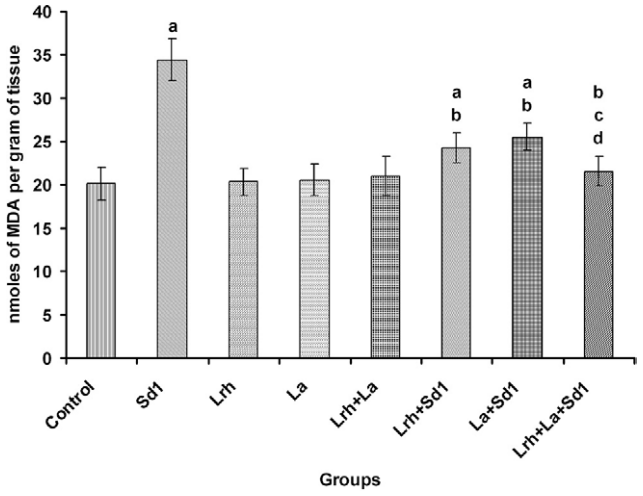

Fig. 3. Effect of Lrh and La pretreatment on levels of lipid peroxide in control and experimental groups of rats. Results are expressed as mean ± SD ($n = 6$). MDA levels are expressed as nanomoles per gram of intestinal tissue. [a]Significance ($P < 0.05$) versus group 1 (control); [b]significance ($P < 0.05$) versus group 2 (Sd1); [c]significance ($P < 0.05$) versus group 6 (Lrh + Sd1); [d]significance ($P < 0.05$) versus group 7 (La + Sd1). La, *Lactobacillus acidophilus*; Lrh, *Lactobacillus rhamnosus*; MDA, malondialdehyde; Sd1, *Shigella dysenteriae* type 1 induced.

group 7 (*L. acidophilus* + *S. dysenteriae* 1), and group 2 (*S. dysenteriae* 1 infected) rats.

Antioxidants

Table 1 lists levels of antioxidants (GSH, SOD, and CAT) in the intestinal mucosa of the control and experimental group of rats. A significant decrease in levels of GSH, SOD, and CAT was observed in group 2 (*S. dysenteriae* 1 induced) rats when compared with group 1 (control) rats. Pretreatment with a combination of *L. rhamnosus* and *L. acidophilus* significantly ($P < 0.05$) prevented these alterations and restored the altered levels to near normal when compared with group 6 (*L. rhamnosus* + *S. dysenteriae* 1), group 7 (*L. acidophilus* + *S. dysenteriae* 1), and group 2 (*S. dysenteriae* 1 infected) rats.

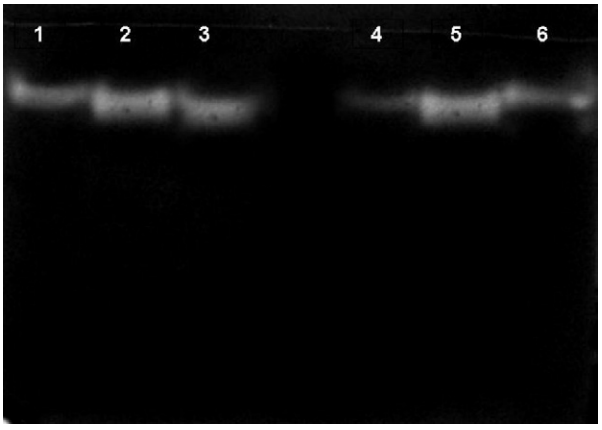

Fig. 4. Activity staining of catalase. Lane 1, *Lactobacillus acidophilus* + *Shigella dysenteriae* 1 (group 7); lane 2, *Lactobacillus rhamnosus* + *L. acidophilus* + *S. dysenteriae* 1 (group 8); lane 3, *L. rhamnosus* + *L. acidophilus* (group 5); lane 4, *S. dysenteriae* 1 induced (group 2); lane 5, control (group 1); lane 6, *L. rhamnosus* + *S. dysenteriae* 1 (group 6). Forty micrograms of protein was loaded in each well.

Figure 4 shows the activity staining of CAT. A marked decrease in the activity staining of CAT was observed in group 2 (*S. dysenteriae* 1 induced) rats (lane 4) when compared with group 1 (control) rats (lane 5). Pretreatment with a combination of *L. rhamnosus* and *L. acidophilus* significantly prevented these alterations and restored the altered levels to near normal (lane 2) when compared with group 6 (*L. rhamnosus* + *S. dysenteriae* 1, lane 6), group 7 (*L. acidophilus* + *S. dysenteriae* 1, lane 1), and group 2 (*S. dysenteriae* 1 infected, lane 4) rats.

Figure 5 shows the activity staining of SOD. A marked decrease in the activity staining of SOD was observed in group 2 (*S. dysenteriae* 1 induced, lane 2) rats when compared with group 1 (control) rats (lane 1). Pretreatment with

Table 1
Effect of *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* pretreatment on levels of GSH, SOD, and CAT in control and experimental groups of rats*

Group	CAT (U/mg protein)	SOD (U/mg protein)	GSH (μmol/g intestinal tissue)
1. Control	171.66 ± 15.52	274.83 ± 21.99	2.55 ± 0.21
2. *Shigella dysenteriae* type 1 induced	106.66 ± 10.63†	151.00 ± 10.31†	0.90 ± 0.14†
3. *Lactobacillus rhamnosus* alone	173.66 ± 11.60	280.50 ± 17.42	2.55 ± 0.21
4. *Lactobacillus acidophilus* alone	173.66 ± 11.60	279.16 ± 19.19	2.58 ± 0.15
5. *L. rhamnosus* + *L. acidophilus*	173.83 ± 15.63	277.16 ± 23.11	2.62 ± 0.20
6. *L. rhamnosus* + *S. dysenteriae* type 1	135.33 ± 13.41†‡	194.00 ± 16.67†‡	1.45 ± 0.18†‡
7. *L. acidophilus* + *S. dysenteriae* type 1	125.83 ± 10.72†‡	177.33 ± 15.88†‡	1.47 ± 0.20†‡
8. *L. rhamnosus* + *L. acidophilus* + *S. dysenteriae* type 1	169.00 ± 11.84‡§‖	272.33 ± 15.85‡§‖	2.52 ± 0.56‡§‖

CAT, catalase; GSH, reduced glutathione; SOD, superoxide dismutase
* Results are expressed as mean ± SD ($n = 6$).
† Significance ($P < 0.05$) versus group 1 (control).
‡ Significance ($P < 0.05$) versus group 2 (*S. dysenteriae* type 1 induced).
§ Significance ($P < 0.05$) versus group 6 (*L. rhamnosus* + *S. dysenteriae* type 1).
‖ Significance ($P < 0.05$) versus group 7 (*L. acidophilus* + *S. dysenteriae* type 1).

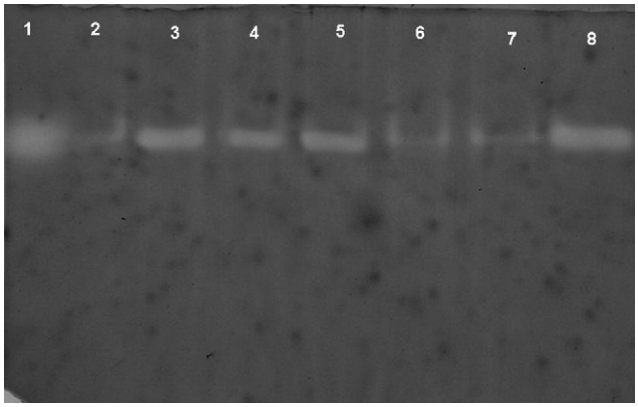

Fig. 5. Activity staining of superoxide dismutase. Lane 1, control (group 1); lane 2, *Shigella dysenteriae* 1 induced (group 2); lane 3: *Lactobacillus acidophilus* alone (group 3); lane 4, *Lactobacillus rhamnosus* alone (group 4); lane 5, *L. rhamnosus* + *L. acidophilus* (group 5); lane 6, *L. rhamnosus* + *S. dysenteriae* 1 (group 6); lane 7, *L. acidophilus* + *S. dysenteriae* 1 (group 7); lane 8, *L. acidophilus* + *L. rhamnosus* + *S. dysenteriae* 1 (group 8). Forty micrograms of protein was loaded in each well.

a combination of *L. rhamnosus* and *L. acidophilus* significantly prevented these alterations and restored the altered levels to near normal (lane 8) when compared with group 6 (*L. rhamnosus* + *S. dysenteriae* 1, lane 6), group 7 (*L. acidophilus* + *S. dysenteriae* 1, lane 7), and group 2 (*S. dysenteriae* 1 infected, lane 2) rats.

Gelatin zymography

Figure 6 shows the gelatin zymographic pattern of MMP-2 and MMP-9 activities in the intestine of the control and experimental groups of rats. Levels of MMP-9, a marker of intestinal inflammation, was found to be highly expressed (lane 7) in group 2 (*S. dysenteriae 1* induced) rats

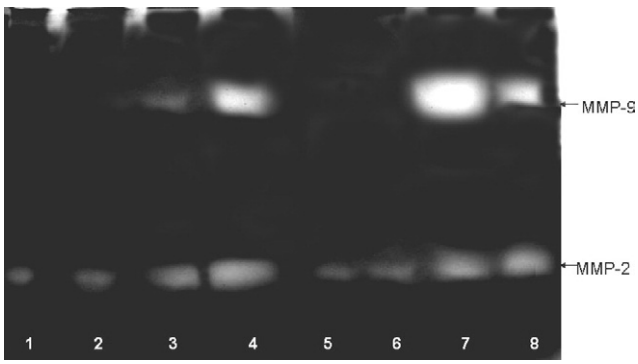

Fig. 6. Effect of *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* pretreatment on levels of matrix metalloproteinase-2 and matrix metalloproteinase-9 in control and experimental groups of rats. Lane 1, control (group 1); lane 2, *L. acidophilus* alone (group 4); lane 3, *L. rhamnosus* + *L. acidophilus* + *Shigella dysenteriae* 1 (group 8); lane 4, *L. rhamnosus* + *S. dysenteriae* 1 (group 6); lane 5, *L. rhamnosus* alone (group 3); lane 6, *L. rhamnosus* + *L. acidophilus* (group 5); lane 7, *S. dysenteriae* 1 induced (group 2); lane 8, *L. acidophilus* + *S. dysenteriae* 1 (group 7). Forty micrograms of protein was loaded in each well.

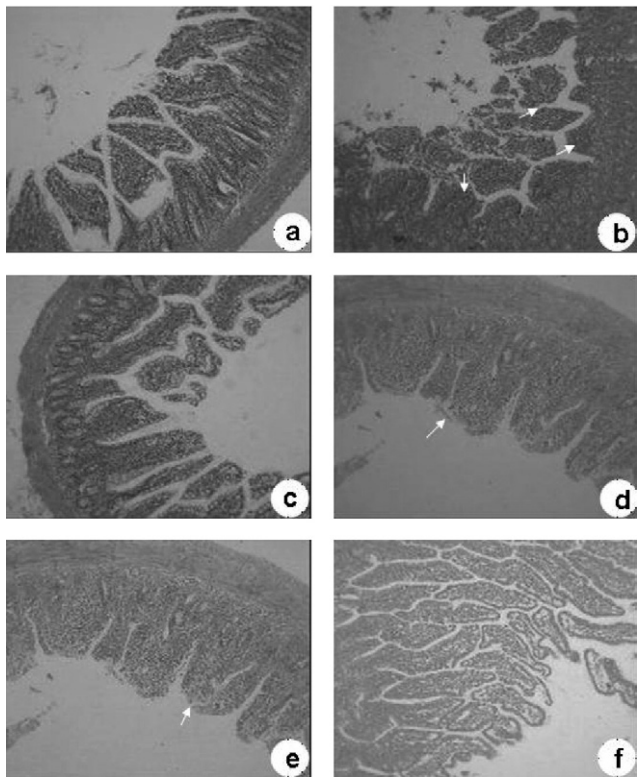

Fig. 7. Histologic studies of control and experimental groups of rats. (a) Section of intestine from a control rat showing normal architecture. (b) Intense neutrophil infiltration and epithelial desquamation (arrows) in the tissue of *Shigella dysenteriae* 1–induced rats. (c) Normal intestinal architecture in a rat administered *Lactobacillus acidophilus* and *Lactobacillus rhamnosus*. (d) Maintained villus/crypt ratio with minimal exudate (arrow) in the intestine of *L. rhamnosus* + *S. dysenteriae* 1 administered rats. (e) Focal villi broadening (arrow) in the intestine of *L. acidophilus* + *S. dysenteriae* 1 administered rats. (f) Normal intestinal architecture in *L. acidophilus* + *L. rhamnosus* + *S. dysenteriae* 1 rats.

when compared with group 1 (control) rats (lane 1). Pretreatment with a combination of *L. rhamnosus* and *L. acidophilus* (group 8) prevented this alteration and expression of MMP-9 was minimal (lane 3) when compared with group 6 (*L. rhamnosus* + *S. dysenteriae* 1, lane 4), group 7 (*L. acidophilus* + *S. dysenteriae* 1, lane 8), and group 2 (*S. dysenteriae* 1 infected, lane 7) rats.

Histologic examination of intestinal tissue

The following observations were made in the intestines of the control and experimental groups. Figure 7a shows the normal architecture of the intestine. The rats that received *S. dysenteriae* 1 showed damage to the intestinal tissue as evidenced by pathologic changes in the architecture of the intestine, namely ulceration, epithelial desquamation, and intense infiltration of PMNs (Fig. 7b). Rats administered with *L. rhamnosus* and *L. acidophilus* showed normal architecture (Fig. 7c). *Lactobacillus rhamnosus* pretreated rats with *S. dysenteriae* 1 administration showed minimal exu-

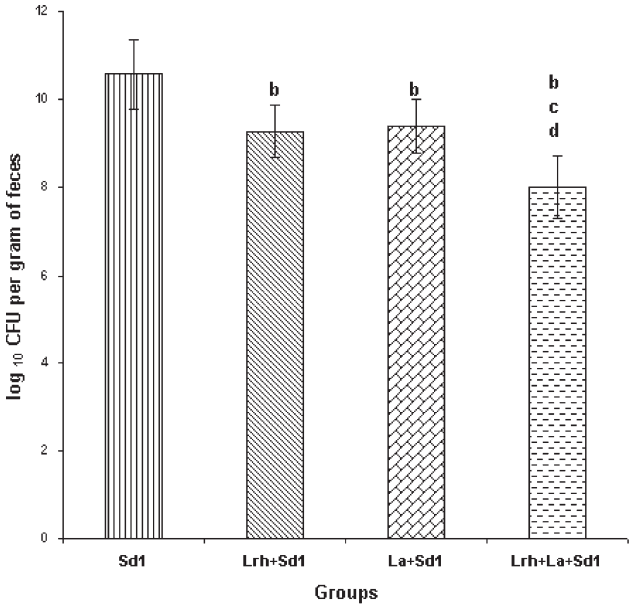

Fig. 8. Sd1 density in feces. [b]Significance ($P < 0.05$) versus group 2 (Sd1); [c]significance ($P < 0.05$) versus group 6 (Lrh + Sd1); [d]significance ($P < 0.05$) versus group 7 (La + Sd1). CFU, colony-forming units; La, *Lactobacillus acidophilus*; Lrh, *Lactobacillus rhamnosus*; Sd1, *Shigella dysenteriae* type 1 induced.

date and maintained a villus/crypt ratio (Fig. 7d). *Lactobacillus acidophilus* pretreated rats with *S. dysenteriae* 1 administration showed occasional PMNs and focal villi broadening without any ulceration and exudates (Fig. 7e). Combined pretreatment with *L. rhamnosus* and *L. acidophilus* showed better protection as observed by the absence of any adverse pathologic changes in the intestine (Fig. 7f) of rats pretreated with *L. rhamnosus* and *L. acidophilus* and administered *S. dysenteriae* 1.

Shigella dysenteriae 1 density in feces

Figure 8 shows the number of *Shigella* in the feces. A significant decrease in the number of bacteria was observed in group 6 (*L. rhamnosus* + *S. dysenteriae* 1) and group 7 (*L. acidophilus* + *S. dysenteriae* 1) when compared with group 2 (*S. dysenteriae 1* induced). Pretreatment with a combination of *L. rhamnosus* and *L. acidophilus* resulted in a significant decrease ($P < 0.05$) in the number of bacteria in feces when compared with group 6 (*L. rhamnosus* + *S. dysenteriae* 1), group 7 (*L. acidophilus* + *S. dysenteriae* 1), and group 2 (*S. dysenteriae* 1 infected) rats.

Discussion

Intestinal epithelium is an important factor of the gut mucosal barrier and participates in innate immunity. It has been clearly documented that *Shigella* infection leads to dramatic recruitment of PMNs from peripheral blood to the infection site [37]. Activated neutrophils secrete enzymes (e.g., MPO, elastase, and proteases) and liberate oxygen radicals [38,39].

Myeloperoxidase is an enzyme that is found predominantly in the azurophilic granules of PMNs. Because tissue MPO activity correlates significantly with the number of PMNs determined histochemically, it is frequently used to estimate tissue PMN accumulation in inflamed tissues [35] and serves as a marker of inflammation and tissue injury [36]. MPO activity is used as a quantitative index of inflammation and neutrophil infiltration in tissues [40]. In the present study, the presence of elevated MPO activity in intestinal tissues indicates the PMN infiltration in rat intestinal tissue infected with *Shigella* alone. In the case of pretreatment with *L. rhamnosus* and *L. acidophilus*, MPO levels were comparable to those in control rats, indicating the attenuation of PMN influx. The PMN influx inhibitory potential of *L. rhamnosus* was found to be more when compared with *L. acidophilus.* This may be due to the excellent adherence potential of *L. rhamnosus* to the epithelial cells [17]. Such an efficient binding may prevent the pathogen from interacting with the potential binding sites. *Lactobacillus acidophilus* has been reported to inhibit the adhesion and invasion of *Salmonella typhimurium*, enteropathogenic *Escherichia coli*, *Yersinia pseudotuberculosis*, and *Listeria monocytogenes* through steric hindrance [41]. Thus, the combination of *L. rhamnosus* and *L. acidophilus* significantly attenuated the MPO activity in the intestinal mucosa when compared with the individual treatment groups, suggesting an attenuation of PMN influx.

Matrix metalloproteinase are divided into four groups: collagenases, stromelysins, gelatinases, and membrane-type MMPs. Gelatinase comprises two members: MMP-2 (gelatinase-A), a 72-kDA proteinase that is normally present in tissue, and MMP-9 (gelatinase-B), a 92-kDA proteinase that is expressed in inflamed tissue. MMP-9 is mainly synthesized by inflammatory cells, particularly PMNs [42] and is stored in the secondary and tertiary granules of neutrophils for rapid release into the inflammatory sites [43]. It is also reported that in the presence of PMN-derived MPO, reactive oxygen metabolites can generate hypochlorous acid (HOCl) and initiate the deactivation of antiproteases and activation of latent proteases, which cause tissue damage [44]. MMP-9 expression was found to be increased in the case of *S. dysenteriae* 1–infected rats due to the PMN influx caused by *Shigella* infection. In the case of pretreatment with the combination of *L. rhamnosus* and *L. acidophilus*, PMN influx was attenuated, hence the observed decrease in the MMP-9 in group 8 when compared with group 2 (*S. dysenteriae* 1 infected) rats.

Experimental and clinical studies have shown that any harmful tissue event, including the endotoxins, is perceived by macrophages and monocytes, which in turn secrete several proinflammatory cytokines such as tumor necrosis factor-α, interleukin-1, and interleukin-6. Cytokines then activate inflammatory cells (neutrophils, macrophages/monocytes, platelets, and mastocytes) by releasing large

amounts of toxic oxygen and nitrogen species, proteases, arachidonic acid metabolites, etc., which cause cellular injury by several mechanisms including peroxidation of membrane lipids and oxidative damage of proteins and DNA [45–47]. Peroxidation of lipids is thus an important pathogenic event in shigellosis.

Several mechanisms of lipid peroxidation have been postulated. The free radical chain reaction proposed by Farmer et al. [48] is the most widely accepted. According to their theory, free radicals, other reactive oxygen species, and toxic products produced by the oxidation process can attack and damage biological molecules. The molecules attacked by free radicals also produce free radicals. The consequences of a free radical chain reaction can result in serious damage to living organisms. Intact cells and intracellular cell-free extracts of *L. acidophilus* have been shown to have a very good antioxidative effect [49] on inhibiting linoleic acid peroxidation and scavenging the 1, 1 diphenyl-2-picryl hydrazl radical. *Lactobacillus acidophilus* also has been found to protect intestinal cell line 407 cells from the cytotoxicity of 4-nitroquinoline-N-oxide (a mutagen and carcinogen), which causes DNA oxidative damage [12].

Observations have suggested that reactive oxygen species (ROS) play a role in the recruitment of neutrophils into injured tissues, but activated neutrophils are also a potential source of ROS [9]. A growing body of evidence indicates that ROS, such as peroxide anion, hydrogen peroxide (H_2O_2), and hypochlorous acid, are not merely byproducts of the inflammatory process but are actually involved in its pathogenesis. To regulate overall ROS levels, the intestinal mucosa possesses a complex assortment of antioxidant systems, of which the SODs are the initial enzymes, converting superoxide anion to H_2O_2 [50]. SOD and CAT are antiperoxidative enzymes that protect the cellular constituents against oxidative damage. A significant decrease in the activities of SOD and CAT with a concomitant increase in lipid peroxide was observed in group 2 (*S. dysenteriae* 1 induced) rats.

Significant decreases in levels of the antioxidant enzymes SOD and CAT may have been due to the consumption of antioxidants by enhanced radical reactions [51]. In addition, the efficacy of the antioxidant defense system may be impaired during inflammation, partly as a result of autooxidation [34]. Metabolites of *L. acidophilus* and *Bifidobacterium* have been shown to inhibit ileal ulcer formation and lipid peroxidation in rats treated with a non-steroidal anti-inflammatory drug, 5-bromo-2-(4-fluorophenyl)-3-(4-methylsulfonylphenyl) thiophene [52,53]. *Lactobacillus acidophilus* has significant antioxidative activity as assessed by its free radical scavenging activity [54,55]. The intact cells and intracellular cell-free extracts of intestinal lactic acid bacteria *Bifidobacterium longum* (ATCC 15708) and *L. acidophilus* (ATCC 4356) demonstrated high antioxidative activity and efficiently inhibited linoleic acid peroxidation [54] and plasma lipid peroxidation [12].

Lactobacillus acidophilus was found to be efficient in inhibiting the colonization of ingested *Shigella sonnei* to the liver and spleen in an animal model [56]. Lactic acid is a byproduct of *Lactobacillus* strains. Lactic acid, in addition to its antimicrobial effect due to its lowering of pH, functions as a permeabilizer of the outer membrane of gram-negative bacteria [57].

Lactobacillus rhamnosus (ATCC 7469) used in this study is equivalent to *Lactobacillus casei* (Orla-Jensen). In a previous study the protective effect of milk fermented with *L. acidophilus* and/or *L. casei* in mice challenged with *Salmonella typhimurium* was investigated. Only pretreatment with multistrain (combination of both strains) fermented milk was effective in preventing colonization of *S. typhimurium*, whereas individual treatment with these strains did not [58]. In our study, we also observed that the combination of *L. rhamnosus* and *L. acidophilus* offered better protection to the intestine during *Shigella* infection when compared with the individual treatment groups. Pretreatment with the combination of *L. rhamnosus* and *L. acidophilus* significantly attenuated the pathologic features and offered protection that was evident from biochemical and histologic studies and bacterial analysis of feces.

In conclusion, our study shows that the combination of *L. rhamnosus* and *L. acidophilus* proved to be more effective in reducing neutrophil infiltration and significantly counteracted oxidative stress during diarrhea induced by *S. dysenteriae* 1 in rats.

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