



## Remediation of intramacrophageal *Shigella dysenteriae* type 1 by probiotic lactobacilli isolated from human infants' stool samples

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**Background & objectives:** *Shigella dysenteriae* is one of the most virulent pathogens causing bacillary dysentery and is responsible for high mortality in infants. To reduce the load of antibiotic therapy for treating shigellosis, this study was carried out to assess the *ex vivo* effect of novel probiotic lactobacilli, isolated from infant's stool samples, on killing *S. dysenteriae* type 1 residing in the rat macrophages.

**Methods:** Stool samples from infants were collected, processed for the isolation of lactobacilli and screened for the probiotic attributes (acid tolerance, bile tolerance, ability to adhere intestinal cells and anti-*S. dysenteriae* activity). The effect of cell-free supernatant of lactobacilli on *Shigella*-infected macrophages in terms of phagocytic ability, extent of lipid peroxidation, nitrite, superoxide dismutase and glutathione levels was evaluated.

**Results:** Based on the probiotic attributes, three lactobacilli were isolated from the stool samples of infants. Using classical and molecular tools, these isolates were identified as *Lactobacillus pentosus*, *L. Paraplantarum* and *L. rhamnosus*. All the three lactobacilli had the ability to kill intramacrophage *S. dysenteriae* type 1. The anti-*Shigella* activity of the probiotic lactobacilli was attributed to increased antioxidative ability and decreased free radical production by the infected macrophages.

**Interpretation & conclusions:** Probiotic cocktail of *L. pentosus*, *L. paraplantarum* and *L. rhamnosus* showed *ex vivo* killing of *S. dysenteriae* residing inside the rat macrophages significantly. This cocktail has the potential to be used as a natural alternative for treating *S. dysenteriae* infection, especially in infants, however, further studies need to be done to confirm these finding *in vivo*.

**Key words** Infant - *Lactobacillus paraplantarum* - *Lactobacillus pentosus* - *Lactobacillus rhamnosus* - macrophage - probiotic - *Shigella dysenteriae* - stool

*Shigella* being facultative intracellular pathogen, having prominent specificity for primate hosts mainly humans, is responsible for acute gastrointestinal infection named shigellosis<sup>1</sup>. Genus *Shigella* consists of four species namely *S. boydii*, *S. dysenteriae*, *S. flexneri* and *S. sonnei*. Amongst these four species, *S. dysenteriae* and *S. flexneri* are prominently found in

developing world that can lead to deadly epidemics<sup>2</sup>. Moreover, *S. dysenteriae* type 1 attracts special attention due to its imperative property to produce potent enterotoxin - Shiga toxin that contributes to high attack rate, high case fatality rate and serious complications<sup>1</sup>. The situation becomes more serious because of lack of vaccine counterparts against *S. dysenteriae* type 1

and emergence of drug resistance to commonly used fluoroquinolones and aminoglycosides which was found to be more common in *S. dysenteriae* as compared to *S. flexneri*<sup>3</sup>.

One of the natural alternative ways to combat *S. dysenteriae* type 1-mediated infections is the use of probiotics<sup>4</sup>. Probiotics are live microorganisms that, when administered in adequate amounts, provide health benefits to the host<sup>5</sup>. In the past decade, the role of probiotics in controlling bacterial infections is on the rise<sup>5</sup>. However, only a limited number of reports on the use of probiotics for the control of *S. dysenteriae* are available. Only two reports<sup>4,6</sup> have discussed mechanism behind lactobacilli-mediated killing of *Shigella* to a certain extent.

The present study was thus undertaken to isolate lactobacilli from stool samples of infants and check their probiotic and anti-*Shigella* activity. The main focus was on the treatment of *S. dysenteriae*-infected rat macrophages with probiotic lactobacilli isolated from infants' stool.

### Material & Methods

This study was carried out in the department of Microbial Biotechnology, Panjab University, Chandigarh, India, from October 2011 to October 2013. Fresh stool samples were collected from healthy, breastfed infants (1-3 months old) attending vaccine clinic in the department of Pediatrics, Government Medical College, Chandigarh, and were subsequently processed. These samples were serially diluted in phosphate-buffered saline (pH 7.0) (HiMedia, Mumbai) and pour plated on de Mann Roger Sharpe-Bromocresol purple (MRS-BCP) (HiMedia) agar plates. Following incubation, the acid-producing colonies obtained on the plates were Gram stained and biochemically tested. These were subjected to *Lactobacillus* genus-specific polymerase chain reaction (PCR) with specific primers (Lacto F, Lacto R) for 16S-23S rRNA intergenic fragment of *Lactobacillus* genus<sup>7</sup>.

*Anti-S. dysenteriae* activity of lactobacilli isolates by agar well diffusion: Lactobacilli isolates were screened for anti-*Shigella* activity against *S. dysenteriae* type 1 (provided by the department of Microbiology, Postgraduate Institute of Medical Education and Research, Chandigarh). Anti-*Shigella* activity was checked by performing agar well diffusion assay<sup>8</sup> on tryptone glucose extract agar plates (HiMedia).

*Probiotic attributes of anti-S. dysenteriae* lactobacilli: Anti-*Shigella* lactobacilli were further screened for

acid (pH 2.0) tolerance for two hours and bile salts (oxgall, 1.5%) (HiMedia) tolerance for four hours, respectively<sup>9</sup>. Hydrophobicity index of isolates was calculated with xylene, higher the hydrophobicity of isolates greater is their adherence to intestinal cells<sup>9</sup>. Lactobacilli isolates showing hydrophobicity more than 30 per cent were subjected to adherence assay on cultured Caco-2 cells as bacterial cultures showing mean adherence to xylene more than 30 per cent were considered as hydrophobic<sup>10</sup>. The adhesion scores were calculated based on the number of bacteria enumerated from 20 microscopic fields. Percentage adhesion of lactobacilli isolates was calculated according to the following Formula<sup>11</sup>:

$$\% \text{ Adhesion} = \frac{\text{Number of bacteria counted after adherence assay}}{\text{Number of bacteria added initially}} \times 100$$

Biocompatibility amongst the selected isolates was checked on MRS agar plates. Isolates were streaked in line at a distance of 1 mm, perpendicular to each other on MRS agar plates and incubated for 24 h. After incubation, MRS agar plates were checked for growth of lactobacilli isolates.

*Molecular identification of isolated anti-Shigella probiotic lactobacilli*: Lactobacilli isolates showing probiotic attributes were subjected to molecular identification by performing 16S rRNA gene sequencing with primers 27f, 685r, 926f and 1492r<sup>12</sup>. BigDye terminator cycle sequencing kit (Applied Biosystems, USA) was used to sequence purified 16S rRNA gene fragment in 3130X Genetic Analyzer (Applied Biosystems). Sequence data obtained were analyzed by DNA sequences assembling software SEQUENCHER™ 4.10.1 (Gene Codes Corporation, Michigan, USA). Related sequences were determined from nucleotide database of National Centre of Biotechnological Information (NCBI). Alignment of all acquired and related sequences was done with Clustal W software (EMBL-EBL, <http://www.ebi.ac.uk/Tools/msa/clustalw2/>) using neighbour-joining method in accordance with MEGA4 and Kimura 2-parameter model ([www.megasoftware.net](http://www.megasoftware.net)) to construct phylogenetic tree. 16S rRNA gene sequences of isolated lactobacilli RT16-2, RT4-54 and RT27-17 were submitted to Bankit NCBI under GenBank accession numbers KJ802480, KJ802481 and KJ802482.

*Ex vivo study of lactobacilli (individually and cocktail) against S. dysenteriae type 1:* The anti-*Shigella* probiotic lactobacilli selected were grown for 48 h in MRS broth. The culture obtained was centrifuged at 2190×g for 10 min (Sigma 2-16 K, Newtown, Shropshire, UK) and lactobacilli cell-free supernatant (LCFS) was collected and filter sterilized using 0.22 µm filters. LCFS was analyzed for the presence of bacteriocin, lactic acid and hydrogen peroxide. Lactic acid content was determined in CFS supernatant<sup>13</sup>. Amount of hydrogen peroxide produced in CFS was also estimated<sup>14</sup>. CFS was treated with 1N sodium hydroxide till LCFS attained pH 7.0 and catalase (1 mg/ml) (HiMedia) for 60 min to eliminate anti-*Shigella* effects of lactic acid and hydrogen peroxide and treated supernatant was designated as S1. Further, CFS was subjected to protein digestion with enzymes pepsin, trypsin, chymotrypsin and renin (HiMedia) at a concentration of 1 mg/ml each for 60 min at 37°C to assure the presence of bacteriocin and designated as CFS S2. Treated LCFS (S1 and S2) was subjected to agar well diffusion assay to check for anti-*Shigella* activity.

*Extraction of peritoneal macrophages from rat:* Healthy Wistar rats (200-250 g) were procured from Central Animal Facility of Panjab University, Chandigarh. Rat peritoneal macrophages were isolated in accordance with method described by Chanana *et al*<sup>15</sup>. The study protocol was approved by the Institutional Animal Ethics Committee.

*Intracellular killing of S. dysenteriae type 1 by macrophages:* Rat peritoneal macrophages were infected with *S. dysenteriae* at 1:100 multiplicity of infection. Macrophage suspension (10<sup>6</sup> cells/ml), normal rat serum and infected *S. dysenteriae* suspension (10<sup>8</sup> cfu/ml) were mixed by vortexing and incubated for two hours at 37°C in five per cent CO<sub>2</sub> incubator (4141, Forma™ series water jacketed, Thermo Scientific, USA). The suspension was further treated with CFS of lactobacilli individually, CFS cocktail of two lactobacilli and CFS cocktail of all the three lactobacilli taken together<sup>16</sup> as mentioned in Table I.

*Effect of LCFS (individually and cocktail) on macrophage functions:* The macrophage suspensions were incubated for 18 h at 37°C in CO<sub>2</sub> incubator and lysed with lysis buffer (20 mM Tris hydrochloride, 150 mM sodium chloride, 1 mM EDTA, 1 % triton-X-100 and 1 mM PMSF; HiMedia) in the ratio of 1:1 (v/v) and incubated for 20 min at 4°C. This reaction mixture

**Table I.** *Ex vivo* experimental groups of *Shigella dysenteriae*-infected macrophages treated with *Lactobacillus* cell free supernatant (LCFS) to check macrophage functions

Treatment
UM (500 µl)
IM (500 µl) + normal saline (200 µl)
IM (500 µl) + L1 (200 µl)
IM (500 µl) + L2 (200 µl)
IM (500 µl) + L3 (200 µl)
IM (500 µl) + L1, L2 cocktail (100 µl each)
IM (500 µl) + L2, L3 cocktail (100 µl each)
IM (500 µl) + L1, L3 cocktail (100 µl each)
IM (500 µl) + L1, L2 and L3 cocktail (66.6 µl each)
UM, untreated macrophage; IM, infected macrophages ( <i>S. dysenteriae</i> infection); L1, LCFS of <i>Lactobacillus pentosus</i> (RT16-2); L2, LCFS of <i>L. paraplantarum</i> (RT4-54); L3, LCFS of <i>L. rhamnosus</i> (RT27-17)

was centrifuged at 101×g for 15 min and supernatant obtained was collected for assaying macrophage functions.

*Estimation of lipid peroxidation (LPO):* Quantitative assessment of lipid peroxidation (LPO) in macrophage culture supernatant was carried out<sup>17</sup>. Protein content was determined by Bradford method<sup>18</sup>. Results were expressed in nanomoles of malondialdehyde (MDA) per milligramme of protein, using chromophore molar extinction coefficient as 1.56×10<sup>5</sup> M<sup>-1</sup>cm<sup>-1</sup>.

*Measurement of nitrite concentration:* Nitrite [as an indicator of nitric oxide (NO)] content in macrophage culture supernatant was determined<sup>19</sup>. Quantification of nitrite was done through a standard curve of sodium nitrite (HiMedia).

*Measurement of superoxide dismutase (SOD) activity:* Method described by Kono<sup>20</sup> was used to assay the levels of superoxide dismutase (SOD) in macrophage culture supernatant. Activity of SOD was expressed as units of SOD per milligram of protein in culture supernatant.

*Reduced glutathione (GSH) levels:* Glutathione (GSH) levels in macrophage culture supernatant were determined<sup>21</sup>. GSH levels were expressed in micromoles of GSH per milligramme of protein applying the molar extinction coefficient for chromophore as 13600/ M/cm.

**Statistical analysis:** Treatment group variations in comparison to positive and negative controls were determined by student *t* test using GraphPad statistical software (GraphPad Prism 4 software, La Jolla, USA).

### Results

Based on the colony morphology (flat, creamish in colour, 2-3 mm colony, convex, raised circular form with entire margin) on MRS-BCP agar plates, 112 putative lactobacilli were isolated from 50 stool samples of infants. On further characterization involving Gram character (Gram-positive), shape (rods) and biochemical tests (catalase negative, triple sugar iron test showing acid production in slant and butt and lactose fermentation), 102 isolates were found to belong to the genus *Lactobacillus*. Using PCR involving amplification of 250 base pair (bp) *Lactobacillus* genus-specific 16S-23S RNA gene fragment, 99 isolates were confirmed as lactobacilli. The complete identification of the three selected probiotic lactobacilli was carried out on the basis of 16S rRNA gene sequencing. The organisms were identified as *L. pentosus*, *L. paraplantarum* and *L. rhamnosus* (GenBank accession numbers KJ802480, KJ802481 and KJ802482). The phylogenetic tree showing genetic relatedness amongst these isolates showed *L. pentosus* and *L. paraplantarum* closely related to each other and relatively distinct from *L. rhamnosus*.

**Anti-*S. dysenteriae* type 1 activity and probiotic attributes:** Only 26 of 99 isolates exhibited significant anti-*S. dysenteriae* activity (zone of inhibition  $\geq 5$  mm). The anti-*Shigella* lactobacilli were evaluated for probiotic attributes *i.e.*, acid tolerance, bile tolerance, hydrophobicity and adherence to Caco-2 cells. Based on the results obtained, three anti-*Shigella* lactobacilli, namely, *L. pentosus* (RT4-54), *L. paraplantarum* (RT16-2) and *L. rhamnosus* (RT27-17) were selected for further studies. These showed 100 per cent bile tolerance at 1.5 per cent concentration of oxgall, high acid tolerance (92-96%) at pH 2.0, substantial hydrophobicity (41-63%) and adherence (15-26%) on Caco-2 cells (Table II). In addition, selected lactobacilli were completely biocompatible to each other.

**Presence of anti-*Shigella* factors in LCFS:** The maximum production of lactic acid was shown by *L. rhamnosus* (176 $\pm$ 12.16 mg/ml) followed by *L. pentosus* (122.6 $\pm$ 14.1 mg/ml) and *L. paraplantarum* (95 $\pm$ 13.22 mg/ml). However, H<sub>2</sub>O<sub>2</sub> production was maximum in case of *L. paraplantarum* (89.3 $\pm$ 4.04  $\mu$ g/ml), followed by *L. rhamnosus* (71.33 $\pm$ 10  $\mu$ g/ml) and *L. pentosus* (63 $\pm$ 8.1  $\mu$ g/ml). The treatment of LCFS of three probiotic lactobacilli with various proteases (pepsin, trypsin, chymotrypsin and renin) did not result in a change in the anti-*Shigella* activity as compared to the untreated LCFS, thereby suggesting a little role of the bacteriocins in anti-*Shigella* activity (Table III).

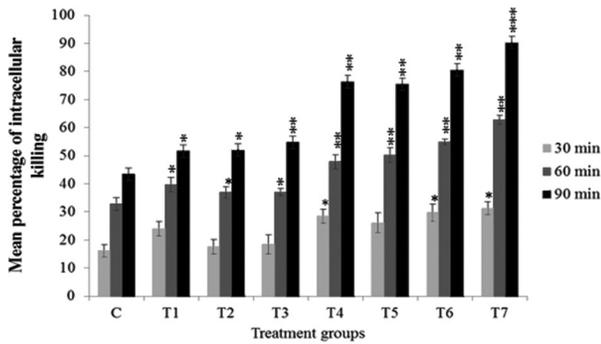
**Table II.** Probiotic attributes of isolated anti-*Shigella* lactobacilli

Isolated lactobacilli	Acid tolerance (% survivability)	Bile tolerance (% survivability)	Hydrophobicity index	Adhesion score	Adhesion (%) to Caco-2 cells
<i>Lactobacillus pentosus</i> (RT16-2)	92 $\pm$ 4	100	41 $\pm$ 2	500 $\pm$ 11	22.6 $\pm$ 2.2
<i>L. paraplantarum</i> (RT4-54)	94 $\pm$ 3	100	45 $\pm$ 5	463 $\pm$ 15	16.06 $\pm$ 1.6
<i>L. rhamnosus</i> (RT27-17)	96 $\pm$ 3	100	63 $\pm$ 2	1540 $\pm$ 20	25.4 $\pm$ 2.5

**Table III.** Anti-*Shigella* activity of treated *Lactobacillus* cell-free supernatant (LCFS) determined by agar well diffusion assay

LCFS treatment	Zone of inhibition (mm) by LCFS of RT16-2	Zone of inhibition (mm) by LCFS of RT4-54	Zone of inhibition (mm) by LCFS of RT27-17
C	12 $\pm$ 2	9 $\pm$ 2	12 $\pm$ 2
S1	1 $\pm$ 0.5	0	0
S2.1	11 $\pm$ 1	8 $\pm$ 2	12 $\pm$ 2
S2.2	10 $\pm$ 2	7 $\pm$ 1	12 $\pm$ 2
S2.3	10 $\pm$ 1.2	7.5 $\pm$ 1.5	12 $\pm$ 2
S2.4	10 $\pm$ 1.2	8 $\pm$ 1.5	12 $\pm$ 1

Values are expressed as mean $\pm$ SD (n=3). C, untreated LCFS; S1, LCFS treated with NaOH and catalase; S2.1, LCFS treated with pepsin; S2.2, LCFS treated with trypsin; S2.3, LCFS treated with chymotrypsin; S2.4, LCFS treated with renin

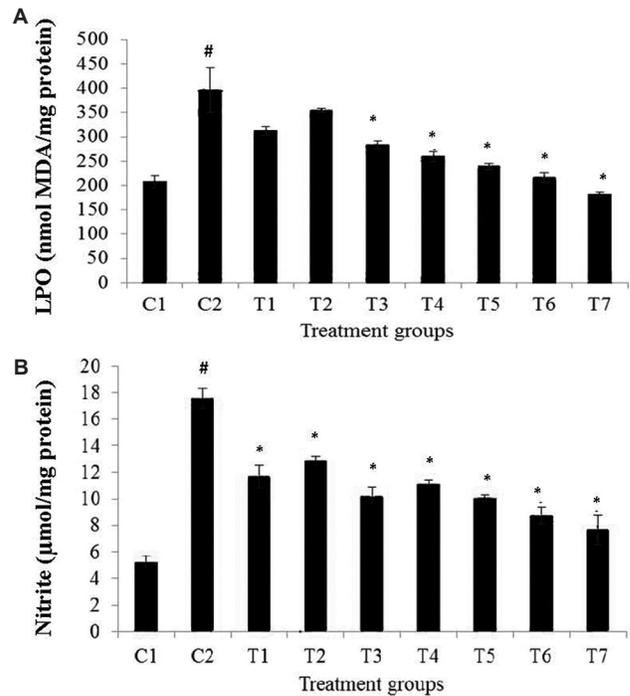


**Fig. 1.** Effect of lactobacilli cell-free supernatant (LCFS) of probiotic lactobacilli on mean percentage of intracellular killing of *Shigella dysenteriae* type 1 by rat peritoneal macrophages. Values are expressed as mean±standard deviation of three individual values.  $P < 0.05$ ,  $** < 0.01$ ,  $*** < 0.001$  compared to killing of *S. dysenteriae* by untreated macrophages. C, control; T, treatment group.

*Effect of LCFS on intracellular killing of S. dysenteriae type 1:* The results of the intracellular killing of *S. dysenteriae* type 1 in rat peritoneal macrophages with LCFS of probiotic lactobacilli are mentioned in Fig. 1. The range of *Shigella* killing using LCFS of individual lactobacilli was found to be 50-55 per cent ( $P < 0.05$ ) whereas intracellular killing of *Shigella* in the control group was  $43.4 \pm 2.16$  per cent. In another set of experiments, in which LCFS of two of the three was administered to the macrophages, the intracellular killing of *Shigella* increased up to 75-80 per cent ( $P < 0.01$ ). However, the maximum killing of *Shigella* ( $90.16 \pm 2.55\%$ ,  $P < 0.01$ ) was observed using a cocktail of all the three organisms.

*Estimation of lipid peroxidation (LPO) and nitrite (NO):* To determine the level of damage to macrophage cell membrane during infection, the magnitude of LPO was measured in terms of level of MDA released by infected macrophage. The challenge group C2 (comprising macrophages and *S. dysenteriae*) showed  $279.667 \pm 22.81$  nanomoles of MDA/mg protein, which was significantly ( $P < 0.05$ ) elevated in comparison to control group C1 (comprising only macrophages), which showed  $79 \pm 3.6$  nanomoles of MDA/mg protein. The mean reduction in MDA levels was highest in probiotic cocktail group T7, comprising all the three probiotic lactobacilli (51%,  $P < 0.01$ ), followed by LCFS cocktail groups (T4-T6) of two lactobacilli (27-40%,  $P < 0.01$ ) and LCFS groups (T1-T3) of individual *Lactobacillus* (15-27%), respectively (Fig. 2A).

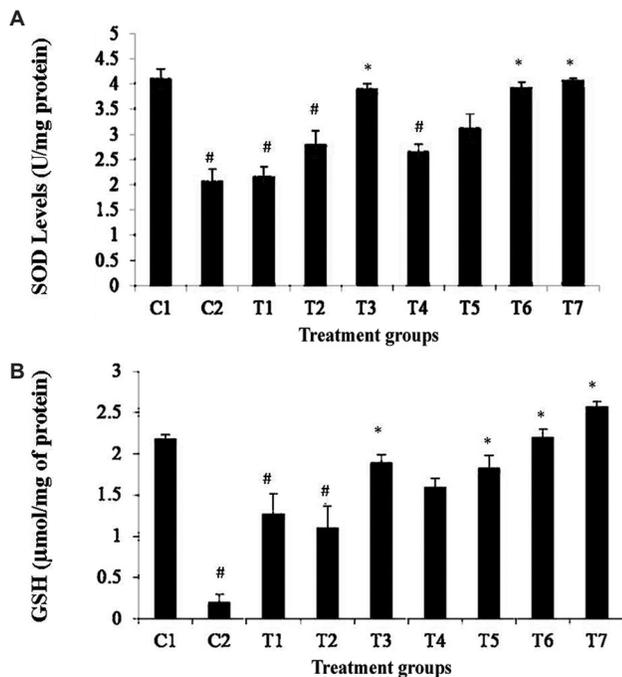
The levels of NO, an indicator of free radical mediated impairment, were determined by estimating



**Fig. 2.** (A) Estimation of lipid peroxidation (LPO). Outcome on the lipid peroxidation levels (in terms of MDA levels) of rat peritoneal macrophages infected with *Shigella dysenteriae* type 1 in the presence of lactobacilli cell-free supernatant (LCFS) of probiotic lactobacilli. (B) Estimation of nitrite levels. Effect of LCFS of probiotic lactobacilli on nitrite levels produced in macrophage infected with *S. dysenteriae* type 1. Values are expressed as mean±standard deviation of three individual values. # $P < 0.05$  compared to negative control group (non infected control group 1, C1), \* $P < 0.01$  compared to challenge group (*Shigella* infected control group 2, C2). MDA, malondialdehyde.

the amount of nitrite present in the infected macrophages. As shown in Fig. 2B, decreased levels of nitrite were observed when infected macrophages were treated with LCFS of probiotic lactobacilli. Challenge group C2, composed of macrophage and *S. dysenteriae* showed  $19.47 \pm 1.50$  µmol of nitrite/mg protein. LCFS cocktail group T7, composed of all the three lactobacilli showed highest and most significant reduction in levels of nitrite (75%,  $P < 0.01$ ), followed by LCFS cocktail groups (T4-T6) of two lactobacilli (67-73%,  $P < 0.01$ ) and LCFS treatment groups (T1-T3) of each lactobacilli (56-63%,  $P < 0.01$ ) taken individually.

*Superoxide dismutase (SOD) estimation:* The levels of SOD under different treatment groups are shown in Fig. 3A. A significant reduction was observed in levels of SOD in challenge group C2 comprising macrophage and *S. dysenteriae* ( $2.06 \pm 0.251$  units of SOD/mg protein,  $P < 0.05$ ), in comparison to



**Fig. 3.** (A) Estimation of levels of superoxide dismutase (SOD). Effect of lactobacilli cell-free supernatant (LCFS) of probiotic lactobacilli on SOD levels of macrophage; (B) Estimation of levels of reduced glutathione (GSH): Effect of LCFS of probiotic lactobacilli on GSH levels in macrophages. Values are expressed as mean±standard deviation of three individual values. # $P<0.05$  compared to negative control group (C1), \* $P<0.01$  compared to challenge group (C2).

control group C1, comprising only macrophages ( $4.1 \pm 0.2$  units of SOD/ mg protein). The levels of SOD were found to be highest and significantly increased (49%,  $P<0.01$ ) when LCFS cocktail of all the three lactobacilli was employed as a treatment. However, the SOD levels (28-49%,  $P<0.01$  for T6) were relatively low, with treatment groups containing LCFS cocktail of two lactobacilli. No significant increase in levels of SOD was observed on treating infected macrophages with LCFS of *L. pentosus* and *L. paraplantarum*, whereas when treated with *L. rhamnosus* showed a significant increase (48%,  $P<0.05$ ), respectively.

**Glutathione (GSH) estimation:** Levels of GSH in normal macrophages present in control group C1 was found to be  $2.133 \pm 0.23$   $\mu\text{mol}$  GSH/mg protein, whereas when macrophages were infected with *S. dysenteriae* as in challenge group C2, GSH levels were significantly reduced to  $0.5 \pm 0.1$   $\mu\text{mol}$  GSH/mg protein ( $P<0.05$ ). As with various treatment measures, the maximum increase in the level of protein GSH was observed with LCFS cocktail of all the three lactobacilli T7 (75%,  $P<0.01$ ),

followed by treatment groups T4-T6 employing LCFS of two lactobacilli (38-72%,  $P<0.01$ ). The GSH levels in treatment groups of individual *Lactobacillus* were not significantly increased except with *L. rhamnosus* treatment (66%,  $P<0.01$ ) (Fig. 3B).

## Discussion

*S. dysenteriae* is one of the few bacterial pathogens which not only prevents its phagocytosis by macrophages but also effectively survives in them<sup>1,22</sup>. The present study indicated that this process could be reversed by treating *Shigella*-infected macrophages with LCFS of three lactobacilli (*L. rhamnosus*, *L. pentosus* and *L. paraplantarum*) which were isolated from infants' stool samples. The percentage of killing of cells of *S. dysenteriae* residing in rat peritoneal macrophages varied depending on the type of LCFS used. Maximum killing of *S. dysenteriae* was observed using LCFS of *L. rhamnosus*, followed by *L. pentosus* and *L. paraplantarum*. However, the killing effect of *S. dysenteriae* was far more pronounced when cocktail of three lactobacilli was used. The maximum killing of *S. dysenteriae* was resulted due to the synergistic effect of probiotic LCFS cocktail. The antimicrobial activity of lactobacilli has been attributed to the presence of bacteriocin, lactic acid and  $\text{H}_2\text{O}_2$  in the culture filtrates<sup>23</sup>. The organisms isolated in this study produced lactic acid and  $\text{H}_2\text{O}_2$ , but not bacteriocin. Our findings were in agreement with others who also showed the prominent role of antimicrobial effect of lactic acid and  $\text{H}_2\text{O}_2$  on bacterial pathogens such as *Escherichia coli* and *Salmonella enterica*<sup>24,25</sup>. Presence of other molecules such as aldehydes and smaller peptides may also be responsible for antimicrobial activity.

There is substantial evidence that *S. dysenteriae* infection results in apoptosis of macrophages<sup>26</sup> which harbour this bacterial pathogen. The macrophage cell death is attributed to the excessive production of reactive oxygen species and reactive nitrogen intermediates<sup>26</sup>. In the present study, probiotic treatment of infected macrophages led to a decrease in the level of LPO and nitrite production. *L. rhamnosus* was most effective to combat free radical-mediated damage in infected macrophages followed by *L. pentosus* and *L. paraplantarum*. Although the three lactobacilli reduced levels of LPO and nitrite, the cocktail of three lactobacilli was most effective probably due to their synergistic effect.

The extent of infection depends on the cell's antioxidant status that scavenges the free radicals

generated in response to infection<sup>27</sup>. Probiotics have been reported to enhance antioxidative ability of macrophages infected with bacterial pathogens such as *S. typhimurium*<sup>28,29</sup>. The potential of probiotics to alter antioxidant status of *S. dysenteriae*-infected macrophages still remains unexplored. In the current study, lactobacilli isolates restored the levels of SOD and GSH in infected macrophages, hence protecting infected macrophages from free radical-mediated toxicity and apoptosis.

In conclusion, probiotic cocktail of *L. pentosus*, *L. paraplantarum* and *L. rhamnosus* showed anti-*Shigella* activity against *S. dysenteriae* type 1 residing inside the rat macrophages. This cocktail has the potential to be used as a natural alternative for treating *S. dysenteriae* infection, especially in infants for whom stringent antibiotic treatment is not recommended. Further studies need to be done to confirm these finding *in vivo*.

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**Conflicts of Interest:** None.

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