Lactobacilli inhibit Shigella dysenteriae 1 induced pro-inflammatory response and cytotoxicity in host cells via impediment of Shigella-host interactions

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1. Introduction

Shigellosis, a major form of bacillary dysentery, is caused by infection with *Shigella* organisms. Shigellosis is a global human health problem. Four species of *Shigella*, i.e., *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii* and *Shigella sonnei* are able to cause the disease. *S. dysenteriae* Type 1 (Sd1) produces severe disease and may be associated with life-threatening complications [1]. In developing countries and in less developed countries, *Shigella* dysentery is endemic and causes an estimated 163 million illness episodes annually and more than one million deaths [2]. It is mostly a paediatric infectious disease with 69% of all diarrhoeal episodes and 61% of all *Shigella*-related deaths involving children younger than 5 years of age [3]. Travellers from developed to developing regions and soldiers serving under field conditions are also at an increased risk of shigellosis. In the United States, 10,000–15,000 cases of shigellosis are reported each year [4].

The symptoms of shigellosis include diarrhoea and/or dysentery with frequent mucoid bloody stools, abdominal cramps and tenesmus. *Shigella* spp. cause dysentery by invading the colonic mucosa. *Shigella* bacteria multiply within colonic epithelial cells, cause cell death and spread laterally to infect and kill adjacent epithelial cells, causing mucosal ulceration, inflammation and bleeding. Transmission usually occurs via contaminated food and water or through person-to-person contact. Laboratory diagnosis is made by culturing the stool samples using selective/differential agar media. Antimicrobial agents are the mainstay of therapy of all cases of shigellosis. Due to the global emergence of drug resistance, the choice of antimicrobial agents for treating shigellosis is limited. Although a single dose of norfloxacin or ciprofloxacin has been shown to be effective, they are currently less effective against Sd1 infection. Currently, no vaccines against *Shigella* infection exist. Both live and subunit parenteral vaccine candidates are under development [1].

Enteroi invasive bacteria stimulate mucosal inflammation that results in severe tissue destruction. The acute recto-colitis that follows epithelial invasion by *Shigella* is a paradigm of this process [5]. Polymorphonuclear neutrophils (PMNs) are massively recruited to the infected epithelial lining through which they translocate causing rupture of the epithelial barrier that in turn facilitates further bacterial invasion [6]. A central theme in the study of *Shigella*...
pathogenesis is to understand the cross-talk between bacteria and intestinal epithelial cells that leads to mucosal inflammation. Infected epithelial cells are major players in the inflammatory process, both as sentinels achieving bacterial sensing and as effectors producing mediators, particularly cytokines and chemokines, which initiate and orchestrate mucosal inflammation [7].

It is generally believed that some of the resident gastrointestinal bacterial flora represented by lactobacilli are protective in case of pathogenic infections. Pharmaceutical products made from such beneficial bacteria have been clinically employed to ameliorate diarrhoeal symptoms through stabilizing gut microflora. For example, Lactobacillus GG was protective in acute infantile diarrhoea and pseudomembranous post-antibiotic colitis in humans [8]. Various enteropathogenic bacteria must find a way to compete with the resident flora and interact directly with the epithelial cells for their pathogenesis. Lactobacilli and bifidobacteria are natural components of the colonic microbiota and they may play a key role in preventing interactions of the pathogen with the host cell. We have recently reported that a combination of Lactobacillus rhamnosus and Lactobacillus acidophilus prevented the neutrophil infiltration, lipid peroxidation and matrix metalloproteinase induction during Sd1 infection in vivo [9]; however, the mechanism of protective action including the possible prevention of adherence and or internalization of Shigella by lactobacilli during Shigella infection remains unexplored. Hence it was of interest to investigate the effect of L. rhamnosus or L. acidophilus or both on Shigella adherence and internalization, host cell viability, inflammatory response and the mechanism of action of lactobacilli during Shigella infection. Caco-2 cell line was used in the present study. It is an intestinal cell line which was originally isolated from a human colon adenocarcinoma [10]. Caco-2 cell line spontaneously differentiates under standard culture conditions and is widely used as an in vitro model of the human intestine.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The Sd 1 strain used in this study was isolated from the stool of a dysentery patient and was provided by the Department of Microbiology, Christian Medical College, Vellore, India. The bacterium was grown in Luria-Bertani broth at 37 °C for 18–20 h before use. The two lactobacilli studied are L. rhamnosus (MTCC 1408/ATCC 7469) and L. acidophilus (MTCC 447/ATCC 4356) obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India. These bacteria were grown in MRS broth at 37 °C for 18–20 h before use.

2.2. Cell line growth condition

Caco-2 cells were grown in DMEM containing L-glutamine, supplemented with 10% FCS and maintained on 2% DMEM at 37 °C in the presence of 95% air and 5% CO₂. On the day of confluence in 90 mm tissue culture flasks, the cells were trypsinized followed by trypsin blue exclusion assay to determine the viable cell count.

2.3. Comparison of time points of the addition of lactobacilli

Various time points of addition of lactobacilli during Shigella infection were investigated. Lactobacilli were added simultaneously or 1 h earlier or 1 h after Shigella addition to Caco-2. After Shigella infection, cells were incubated for 5 h, washed twice with DMEM medium followed by gentamicin (100 µg/mL) treatment for 1 h to kill the extra cellular bacteria. Thorough washing with gentamicin-free DMEM medium was done followed by PBS wash for three times. The cells were lysed with 0.5% Triton-X 100 in PBS. Aliquots of cell lysate were plated to Salmonella-Shigella agar for the culture of Shigella.

2.4. Experimental design

Uninfected cells served as control (group 1) and cells infected with Shigella without the addition of lactobacilli served as group 2. Cells incubated for 1 h with L. rhamnosus (Lr) or L. acidophilus (La) at the multiplicity of infection (MoI) of 100, followed by the addition of S. dysenteriae 1 (Sd1) at the same MoI for 5 h served as group 3 (Lr pretreatment followed by Sd1 infection) and group 4 (La pretreatment followed by Sd1 infection), respectively. Group 5 cells were incubated with Lr + La without Shigella infection. Cells incubated with a combination of L. rhamnosus and L. acidophilus followed by Shigella infection served as group 6 (Lr + La pretreatment followed by Sd1 infection).

2.5. Effect of lactobacilli on the adherence and internalization of Shigella

Two 6-well plates of confluent Caco-2 cells consisting of the six experimental groups were processed in parallel to enumerate the adherent and internalized Shigella. The experiment was repeated three times and the average value was recorded.

2.5.1. Enumeration of internalized Shigella

Following infection at 37 °C for 5 h, the cells of the first 6-well plates was washed twice with DMEM medium and then treated with gentamicin (100 µg/mL) for 1 h to kill the extra cellular bacteria. Thorough washing with gentamicin-free DMEM medium was done followed by PBS wash for three times. The cells were lysed with 0.5% Triton-X 100 in PBS. Aliquots of cell lysate were plated to Salmonella-Shigella agar and incubated overnight at 37 °C for the enumeration of internalized Shigella.

Number of internalised Shigella was denoted as A.

2.5.2. Enumeration of intra cellular and adherent Shigella

After the infection period, the cells in the second 6-well plates was washed twice with gentamicin-free DMEM medium to remove unadhered bacteria. The cells were lysed with 0.5% Triton-X 100 in PBS. Aliquots of cell lysate were plated to Salmonella-Shigella agar and incubated overnight at 37 °C.

Number of internalised and adherent Shigella was denoted as B.

2.5.3. Enumeration of adherent Shigella

Number of internalised Shigella was subtracted from the number of intracellular and adherent bacteria to give the number of adhered bacteria alone.

Adherent bacteria = B – A

2.6. Effect of various treatments of Lactobacillus on its ability to inhibit adherence and or internalization of Shigella to Caco-2 cells

To obtain killed lactobacilli, PBS suspensions of lactobacilli were heated at 95 °C for 20 min or treated with gentamicin (200 µg/mL) for 60 min. After subsequent washing, killed Lactobacillus was added 1 h before the addition of Shigella to the infection experiments. Lactobacilli culture supernatant was used as the conditioned medium after centrifugation at 5000 × g for 30 min. Killed lactobacilli or culture supernatant was added to the confluent Caco-2 and incubated for 1 h followed by Shigella infection. After the infection period, cells were processed accordingly as described above to enumerate the number of adherent and internalized Shigella.
2. TNF-α/H9251

Oligonucleotide primers used for RT-PCR.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Gene product</th>
<th>Primer pair</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>IL-8</td>
<td>Forward: 5′ AAACCACTGGAAAGCCTAT 3′&lt;br&gt;Reverse: 5′ GCCAGTTTGAAGTCTCATG 3′</td>
<td>117 bp</td>
</tr>
<tr>
<td>2.</td>
<td>TNF-α</td>
<td>Forward: 5′ CGAGAAGGAGATGCCCAG 3′&lt;br&gt;Reverse: 3′ CCTTGTTCTTGAGAGAC 5′</td>
<td>653 bp</td>
</tr>
<tr>
<td>3.</td>
<td>β-Actin</td>
<td>Forward: 5′ TGACGGGTTCACTGCTGCCATCTA 3′&lt;br&gt;Reverse: 3′ CTGAGGCATTGGCCTGAGCATGAGGG 5′</td>
<td>530 bp</td>
</tr>
</tbody>
</table>

2.7. MTT cytotoxicity assay

This assay was performed to assess the viability of Caco-2 cells after the infection period.[11] The Millipore filtered solution of MTT (3-(4,5-dimethyl thiazole 2 yl)-2,5-diphenyl tetrazolium bromide) was prepared in PBS and added to each well at a concentration of 500 μg/mL, incubated for 18 h at 37 °C and 5% CO2 atmosphere. Then the MTT solution was aspirated, and the reduced formazan dye was suspended in DMSO. The purple colour was read at 540 nm and 690 nm. The correction value was calculated by subtracting the OD690 from OD540.

The percentage of cell viability was calculated by

\[
\text{% Cell viability} = \frac{\text{Correction value of treated cells}}{\text{Correction value of cell control}} \times 100
\]

2.8. Immunoblot for heat shock protein (HSP-70)

Confluent Caco-2 cells belonging to the six experimental groups were incubated at 37 °C for the infection period. At the end of the infection, medium was removed and cells were washed with gentamicin (0.5 mg/mL) to kill extra cellular bacteria. Thorough washing with gentamicin-free DMEM medium was done followed by PBS wash for three times. The cells were lysed with 0.5% Triton-X 100 in PBS. Protein content was estimated using Lowry's method and equal concentration (40 μg/mL) of the cell lysate was subjected to 10% SDS-polyacrylamide gel electrophoresis. The proteins of Caco-2 cell lysate were transferred after gel electrophoresis to a nitrocellulose membrane and probed with HSP-70 antibody (1:1000 dilution, Santa Cruz, USA).

2.9. Total RNA isolation

Confluent Caco-2 cells belonging to the six experimental groups were incubated at 37 °C for the infection period. At the end of the infection, medium was removed and cells were washed with gentamicin (0.5 mg/mL) to kill extra cellular bacteria. Thorough washing with gentamicin-free DMEM medium was done followed by PBS wash for three times. The infected monolayers were subsequently lysed with Trizol reagent. The cell lysate was used for the isolation of total RNA using an acidic phenol–chloroform extraction procedure[12] and precipitation with 100% isopropanol. Total RNA was washed twice with 70% ethanol, dried, solubilised in autoclaved RNase-free water (DEPC treated). RNA was quantified by absorbance at OD260/280. Only samples with a ratio of 1.8–2.0 were claved RNase-free water (DEPC treated). RNA was washed twice with 70% ethanol, dried, solubilised in autoclaved RNase-free water (DEPC treated). RNA was quantified by absorbance at OD260/280. Only samples with a ratio of 1.8–2.0 were claved RNase-free water (DEPC treated). RNA was washed twice with 70% ethanol, dried, solubilised in autoclaved RNase-free water (DEPC treated). RNA was quantified by absorbance at OD260/280. Only samples with a ratio of 1.8–2.0 were claved RNase-free water (DEPC treated). RNA was washed twice with 70% ethanol, dried, solubilised in autoclaved RNase-free water (DEPC treated). RNA was quantified by absorbance at OD260/280. Only samples with a ratio of 1.8–2.0 were claved RNase-free water (DEPC treated). RNA was washed twice with 70% ethanol, dried, solubilised in autoclaved RNase-free water (DEPC treated). RNA was quantified by absorbance at OD260/280. Only samples with a ratio of 1.8–2.0 were claved RNase-free water (DEPC treated). RNA was washed twice with 70% ethanol, dried, solubilised in autoclaved RNase-free water (DEPC treated). RNA was quantified by absorbance at OD260/280. Only samples with a ratio of 1.8–2.0 were claved RNase-free water (DEPC treated). RNA was washed twice with 70% ethanol, dried, solubilised in autoclaved RNase-free water (DEPC treated). RNA was quantified by absorbance at OD260/280. Only samples with a ratio of 1.8–2.0 were claved RNase-free water (DEPC treated). RNA was washed twice with 70% ethanol, dried, solubilised in autoclaved RNase-free water (DEPC treated). RNA was quantified by absorbance at OD260/280. Only samples with a ratio of 1.8–2.0 were claved RNase-free water (DEPC treated). RNA was washed twice with 70% ethanol, dried, solubilised in autoclaved RNase-free water (DEPC treated). RNA was quantified by absorbance at OD260/280. Only samples with a ratio of 1.8–2.0 were claved RNase-free water (DEPC treated). RNA was washed twice with 70% ethanol, dried, solubilised in autoclaved RNase-free water (DEPC treated). RNA was quantified by absorbance at OD260/280. Only samples with a ratio of 1.8–2.0 were claved RNase-free water (DEPC treated). RNA was washed twice with 70% ethanol, dried, solubilised in autoclaved RNase-free water (DEPC treated). RNA was quantified by absorbance at OD260/280. Only samples with a ratio of 1.8–2.0 were claved RNase-free water (DEPC treated). RNA was washed twice with 70% ethanol, dried, solubilised in autoclaved RNase-free water (DEPC treated). RNA was quantified by absorbance at OD260/280. Only samples with a ratio of 1.8–2.0 were claved RNase-free water (DEPC treated).

2.10. Reverse transcription-polymerase chain reaction

Total RNA (1 μg) was subjected to RT-PCR with reverse transcription for 60 min at 42 °C followed by PCR in a total volume of 25 μL. The PCR conditions were as follows for all genes: an initial denaturation step at 95 °C for 15 min and then cycling through denaturation at 95 °C for 3 min; annealing at 56 °C for 3 min, extension at 72 °C for 1 min for 30 cycles. Table 1 shows the primer sequences and product size for each gene. The optimal number of cycles needed to quantify the mRNA for each gene was determined by preliminary experiments using total RNA from control cells. The RT-PCR products were subjected to agarose gel electrophoresis and detected by ethidium bromide staining.

2.11. Statistical methods

All the data were evaluated with SPSS/10 software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant difference (L.S.D.) test. p values of less than 0.05 were considered to indicate statistical significance. All the results were expressed as mean ± S.D.

3. Results

3.1. Comparison of time points of the addition of lactobacilli on Shigella internalization

Fig. 1 shows the effect of various time points of addition of L. rhamnosus and L. acidophilus respectively, on Shigella internalization. Addition of lactobacilli after 1 h following Shigella infection was less effective in inhibiting Shigella internalization when compared with simultaneous addition of Shigella and lactobacilli. Addition of lactobacilli 1 h prior to the addition of Shigella resulted in significant (p < 0.05) reduction in Shigella internalization.

3.2. Effect of lactobacilli pretreatment on adherence of Shigella to Caco-2

Adherence of Shigella was inhibited to a significant extent in the individual pretreatment groups when compared to Shigella alone infected cells. In cells pretreated with a combination of both L. rhamnosus and L. acidophilus, adherence of Shigella was significantly (p < 0.05) less when compared to the Shigella infected and individual pretreatment groups (Fig. 2A).

3.3. Effect of lactobacilli pretreatment on internalization of Shigella in Caco-2 cells

Shigella internalization was inhibited to a significant amount in the individual pretreatment groups when compared to Shigella alone infected cells. In cells pretreated with a combination of both L. rhamnosus and L. acidophilus, Shigella internalization was significantly (p < 0.05) less when compared to the Shigella alone infected and individual pretreatment groups (Fig. 2B).

3.4. Effect of various treatments of lactobacilli on adherence of Shigella and internalization

Fig. 3A and B present the effect of various treatments of lactobacilli on Shigella adherence and internalization, respectively. Only live whole lactobacilli inhibited the Shigella adherence and
internalization when compared to heat killed, gentamycin treated lactobacilli and lactobacilli CFCS (cell-free culture supernatant).

3.5. Cell viability test using MTT

Fig. 4 shows the cell viability of the control and experimental groups after the infection period. Cytotoxic effect in (group 2) Sd1 infected cells was very high compared to that of control. During individual pretreatments, cytotoxicity was significantly reduced and in the combination pretreatment (L. rhamnosus + L. acidophilus followed by Sd1 infection) cytotoxicity was minimal comparable to that of control cells.

3.6. Immunoblot analysis of heat shock protein (HSP-70) expression

Expression of HSP-70 was increased slightly in Sd1 infected group 2 cells (Fig. 5, lane 2) when compared to control group 1 cells (Lane 1). In L. rhamnosus + L. acidophilus pretreatment followed by Sd1 infection (Lane 6) the expression of HSP-70 was...
Fig. 3. (A and B) Effect of various treatments of lactobacilli on its ability to inhibit Shigella adherence and internalization in Caco-2 cells. ‘a’, ‘c’, ‘d’, and ‘e’ indicate statistical significance ($p < 0.05$) when compared to Sd1 alone, L. rh + L. a (both heat killed) + Sd1, L. rh + L. a (both gentamicin killed) + Sd1, and L. rh CFCS + L. a CFCS + Sd1, respectively. Values indicated are mean ± S.D. of three independent experiments.

higher when compared to the infected and individual pretreatment groups.

3.7. RT-PCR for pro-inflammatory cytokines (TNF-α and IL-8)

Tumour necrosis factor (TNF-α) gene expression was increased in Sd1 infected cells (Fig. 6A, lane 2) when compared to uninfected control cells (Fig. 6A, lane 1). Cells pretreated with a combination of L. rhamnosus and L. acidophilus followed by Sd1 infection had minimal expression of TNF-α (Fig. 6A, lane 6). Interleukin (IL)-8 gene expression was observed in Sd1 infected cells (Fig. 6B, lane 2). Cells pretreated with a combination of L. rhamnosus and L. acidophilus followed by Sd1 infection had a very minimal expression of IL-8 (Fig. 6B, lane 6).

Equal loading of the sample was confirmed by β-actin expression in control and experimental group of cells (Fig. 6C).

3.8. Discussion

In the present study, adherence and internalization of Shigella was inhibited significantly in cells pretreated with a combination of L. rhamnosus and L. acidophilus. Direct contact between Shigella and host cell is necessary for an efficient entry and expression of virulent secretory proteins which are involved in the pathogenesis [13]. Lactobacilli can inhibit such direct contact with host cell by competitive exclusion and by co-aggregating with Shigella thus inhibiting the adherence resulting in reduced number of internalized Shigella. This could be the reason for the observed significant
The authors are grateful to the Head of the Department of Medical Microbiology, Christian Medical College (CMC), Vellore, India which can stimulate intestinal secretion [16]. Cells pretreated with a combination of L. rhamnosus and L. acidophilus followed by Sd1 infection showed increased expression of IL-8, substantiating the attenuation of inflammatory response during combination pretreatment. Lactobacilli have been shown to stimulate the immune system and regulate cytokine production. They suppress synthesis of IL-8, transforming growth factor (TGF-β) and TNF-α by the intestinal epithelial cells [17,18].

It has also been reported that heat shock proteins might play an essential role through their ability to interfere with cytokine production in intestinal epithelial cells [19]. We observed that cells pretreated with a combination of L. rhamnosus and L. acidophilus followed by Sd1 infection showed increased expression of HSP-70. This is in accordance with an earlier study, which reported that intravenous administration of cultivation products of lactobacilli induced the expression of a protective protein HSP-70 in the rat heart that subsequently protects the heart against ischaemia and repuffusion tachyarrhythmia [20]. Since heat shock proteins suppress the synthesis of inflammatory cytokines like IL-8 [19] and lactobacilli induce these proteins in the rat heart [20], it is suggested that lactobacilli also exert a beneficial effect upon the gut following oral uptake [21].

In vitro models of the probiotic applications of lactic acid bacteria suggest several pathways of protective effect against various infections which can be grouped into two major categories. The first one is dependent on the reduction of the pathogen viability. Of those, one example is by the acidification with lactic acid as observed in L. casei against EHEC growth [22]. Another is by the secreted non-acidic products as exemplified by L. acidophilus which inhibited the growth of Salmonella typhimurium, EHEC, EPEC, Shigella [23]. In the case of L. rhamnosus, notably a combination of both products was involved [24]. The second major category is through the interference of pathogen adhesion and invasion, either directly or indirectly, on the receptors. Lactobacillus reuteri and Lactobacillus crispatus competed with Salmonella typhimurium, Enterococcus faecalis and ETEC directly on the receptor of the host cell while L. acidophilus inhibited the adhesion and invasion of Salmonella typhimurium, EPEC, V. parahaemolyticus and Listeria monocytogenes through steric hindrances [25]. Lactobacilli have also been reported to inhibit IL-8 production by intestinal epithelial cells and some are implicated in the treatment and prevention of such intestinal diseases [26,27]. But the mechanism of protection offered by lactobacilli during Sd1 infection and the link between suppression of IL-8 and alleviation offered by lactobacilli has not been established. In the present study we have observed the suppression of IL-8 coupled with induction of molecular chaperone HSP-70 in the host cells in conjunction with host cells remaining viable. A previous report indicates that lactobacilli can induce HSP-70 that is known to inhibit IL-8 production by intestinal epithelial cells [19,20]. HSP-70 could protect the host cells against any viable intracellular bacteria and/or the pro-inflammatory cytokines they produce [28].

In conclusion, it has been clearly demonstrated in the present study that pretreatment with a combination of L. rhamnosus and L. acidophilus resulted in inhibition of Shigella adherence and internalization into the host cells coupled with the attenuation of expression of inflammatory cytokines and cytotoxicity in host cells corroborating the synergistic protective potential of the combination in preventing Sd1 cross-talk with the host.

**Conflict of interest statement**

There is no conflict of interest regarding this manuscript.

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