Modulation of intestinal barrier function to ameliorate *Salmonella* infection in mice by oral administration of fermented milks produced with *Lactobacillus plantarum* MTCC 5690 – a probiotic strain of Indian gut origin

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Probiotic *Lactobacillus plantarum* MTCC 5690, a probiotic strain of Indian gut origin, and milk formulations produced with the same were explored in this study as biotherapeutics by evaluating their functional efficacy against *Salmonella* infection in mice. The efficacy of milk formulations (fermented/unfermented) of MTCC 5690 for enhancement of intestinal barrier function was determined by monitoring the permeability and histopathology of the intestine. Infected mice fed with probiotic Dahi, fermented probiotic drink and sweetened fermented probiotic drink maintained the health and integrity of the intestinal epithelium as compared to those fed with PBS, milk, unfermented probiotic milk and Dahi. Our relative expression data revealed that the changes caused by MTCC 5690 in intestinal barrier function components were established through modulation of the key regulatory receptors Toll-like receptor 2 and Toll-like receptor 4. The results suggest that fermented milks of MTCC 5690 could enhance the defences of the intestinal barrier in enteric infection condition and, therefore, can be explored as a dietary-based strategy to reduce *Salmonella* infection in the human gut.

INTRODUCTION

The intestinal mucosa is a selective physical barrier for translocation and processing of dietary nutrients, and at the same time it also prevents the potential of foreign antigens, harmful enterotoxins and luminal micro-organisms including pathogens to gain access to the internal milieu of the murine gut. In addition, the intestinal lumen also harbours various aerobic and anaerobic microbial species, referred to as the commensal gut microbiota. The intestinal barrier function (IBF) comprises the mucosal layer, secretory antimicrobial components and the trans-epithelial electrical resistance of intestinal epithelial cells (IECs), wherein the structural part of the IBF is regulated by transmembrane tight junction (TJ) proteins (occludin and claudin) which bind to the actin cytoskeleton with the help of cytoplasmic zonula occludin proteins. Additionally, there are some ‘pattern recognition receptors’ such as Toll-like receptors (TLRs) on IECs, which recognize several ‘microbial-associated molecular patterns’ present on the luminal microbes. Binding of patterns on the receptors differentially regulates physiological barrier functions of the intestine by triggering diverse signalling pathways and alters the secretion of mucin, antimicrobial components and secretory IgA via modulation of the expression of pro- and anti-inflammatory cytokines from enterocytes (Voltan *et al.*, 2007).

High-risk pathogens like *Salmonella*, when gaining access to the gut from external sources including the environment and infected food, interact with the intestinal epithelium and modify epithelial barrier function to enhance their penetration across the epithelium and thereby breach the host defence barrier to initiate the onset of illness in the host (Patel & McCormick, 2014). Modulation of the mucosal

Abbreviations: FD, fermented probiotic drink; FITC-Dx, FITC--dextran; GHS, general health score; IBF, intestinal barrier function; IEC, intestinal epithelial cell; LAB, lactic acid bacteria; mCRAMP, mouse cathelicidin-related antimicrobial peptide; MRS, de Man–Rogosa–Sharpe; MUC2, mucin 2; NDRI, National Dairy Research Institute; PCA, principal component analysis; PD, probiotic Dahi; PM, probiotic milk; PRR, pattern recognition receptor; SFD, sweet fermented probiotic drink; SNF, solid not fat; TJ, tight junction; TLR, Toll-like receptor; XLD, xylose lysine deoxycholate; ZO-1, zonula occludens-1.

Two supplementary figures and four supplementary tables are available with the online Supplementary Material.
barrier is triggered by a cascade of cellular events leading to rearrangement in TJ proteins, downregulation of secretion of antimicrobial peptides (Berkes et al., 2003; Vora et al., 2004) that eventually accounts for intrusion into the epithelial barrier by the pathogen, and translocation to other vital organs (Everest et al., 2001). Salmonella-induced illness is endemic in developing countries, and emergence of antibiotic resistance in the pathogen is a common phenomenon, thereby making it a challenge to effectively contain this infectious disease (Chau et al., 2007). In this situation, a non-antibiotic approach to the prevention of this foodborne pathogen seems to be a better option from the human health perspective. Therefore probiotic bacteria, in particular Lactobacillus and Bifidobacterium spp., are now being explored and found to be effective in protecting the intestinal epithelium from pathogen-induced inflammation and infections by virtue of their tolerance, besides their expression of target pathogen-attenuating mechanisms (Ohland & MacNaughton, 2010; Abdel-Daim et al., 2013; Ballal et al., 2015). Hence, probiotic intervention can be considered as a cost-effective, safe and novel dietary strategy that can be explored to alleviate Salmonella infection, initially by establishing anti-Salmonella efficacy in animal models before replicating the same in the target human population.

The food matrix used to deliver probiotics can also affect the functional efficacy of the organism (Vinderola et al., 2011). However, the impact of the physicochemical conditions of different types of unfermented and fermented milk formulations on the functionality of probiotic strains against enteropathogenic infections has seldom been taken into consideration when evaluating their efficacy. Moreover, it is also unclear whether the functionality of a probiotic would be changed if the probiotic strain is added in the presence of lactic starter culture or is used as the sole fermentative agent in milk (Bosgan et al., 2014). Since Salmonella Typhimurium infection in mice has some similarities to human typhoid fever, which enters the body via disruption of the intestinal barrier, the present study was undertaken to investigate the effect of traditional Indian dairy-based unfermented and fermented milk-based probiotic foods, prepared with an indigenous probiotic strain MTCC 5690 of Indian gut origin, on the IBF and health-promoting functions in the Salmonella Typhimurium-infected mouse model.

**METHODS**

**Bacterial strains and culture conditions.** Lactobacillus plantarum MTCC 5690 (previously known as L. plantarum Lp91) was the laboratory isolate of Indian gut origin [isolated in the Molecular Biology Unit, National Dairy Research Institute (NDRI), India, and deposited at the Microbial Type Culture Collection and GeneBank (MTCC), Institute of Microbial Technology, Chandigarh, India] whose identity by 16S rRNA and whole-genome sequencing, probiotic attributes and therapeutic and health-promoting potentials was established previously in our laboratory as per FAO/WHO (2001) guidelines (Duury et al., 2012, 2014; Grover et al., 2013; Sudhakaran et al., 2013). Streptococcus thermophilus, a Dahi starter culture, was procured from the National Collection of Dairy Cultures, NDRI, Karnal, India. Salmonella enterica subsp. enterica serovar Typhimurium LT2 ATCC 700720 (LT2) was obtained as a gift from the National Institute of Cholera and Enteric Diseases, Kolkata, India. The purity of the aforementioned bacterial cultures was ascertained prior to use by Gram staining and microscopic examination based on typical cell morphology. The Lactobacillus and Salmonella were propagated and maintained in de Man–Rogosa–Sharpe (MRS) broth (HiMedia) and brain–heart infusion (BHI) broth (HiMedia), respectively, at 37 °C for 18 h, while S. thermophilus was propagated in M17 broth (HiMedia) at 37 °C for 6 h. The active cultures of Lactobacillus and Streptococcus were maintained in skim milk [10% solid not fat (SNF), stored at 4 °C], and all three bacterial cultures were also preserved as 20% glycerol stocks at −70 °C.

**Preparation of unfermented probiotic suspensions/formulations.** The active cultures of MTCC 5690 and LT2 in their log phase were harvested (8000 g, 10 min, 4 °C), washed with sterilized PBS and resuspended in fresh PBS to obtain 1 × 10^8 c.f.u. ml^−1 (MTCC 5690) and 1 × 10^7 c.f.u. ml^−1 (LT2) cell numbers in the final respective suspensions. For the preparation of unfermented probiotic milk (PM) suspension, harvested MTCC 5690 cells were suspended in sterile skim milk [prepared by reconstitution of 10% (w/v) spray dried skim milk powder in distilled water and pasteurized] per se. The final concentration of MTCC 5690 in PM was 1 × 10^9 c.f.u. ml^−1, and the fresh preparation was immediately stored under refrigerated conditions prior to use.

**Preparation of fermented dairy products.** The inoculum of MTCC 5690 was prepared as a concentrated biomass to reach the desired count of 1 × 10^6 c.f.u. ml^−1 of the probiotic strain in the final product after 10 h of fermentation. For biomass preparation, MTCC 5690 was initially inoculated in 5 ml of MRS broth and incubated at 37 °C for 18 h. After incubation, 5 ml active culture was inoculated in 100 ml of MRS broth and again incubated at 37 °C for 18 h for scale-up of microbial density. Cells were harvested and washed with PBS and used as inoculum for 1% standardized milk (using pooled mixed milk from cow and buffalo with 4.5% fat, 10%–11% SNF, pasteurized at 90 °C for 15 min) and fermented at 37 °C for 6 h until pH reached 4.3±0.1. For the preparation of the starter culture inoculum, S. thermophilus was inoculated in 10 ml of reconstituted skim milk (10%), incubated at 37 °C for 6 h and kept at 4 °C prior to use. Dahi, a popular Indian fermented milk used regularly as an integral part of the diet across the country, was prepared from standardized milk after inoculating only with 1% S. thermophilus followed by incubation for 6 h at 37 °C. Probiotic Dahi (PD) was prepared by inoculating standardized milk with 1% starter and 10% w/v concentrated biomass of MTCC 5690 followed by incubation for 6 h at 37 °C. For the preparation of fermented probiotic drink (FD) and sweetened FD (SFD), reconstituted skim milk (10% and 13% SNF content, respectively) was inoculated with a concentrated biomass of MTCC 5690 (10%, v/v, 1 × 10^8 c.f.u. ml^−1 MTCC 5690 in 10% inoculum) and incubated at 37 °C for 10 h and/or until pH reached 4.3±0.1 (Table 1), followed by blending the set curd into an FD. The SFD was prepared by blending of fermented reconstituted skim milk with sugar (10%, w/v), while FD was also the same but without the added sugar. Hence, both drinks looked like another very popular traditional fermented drink, Lassi, prepared from Dahi. After incubation, PD, FD and SFD contained 7.4 × 10^9, 5.4 × 10^9 and 5.0 × 10^9 c.f.u. ml^−1 of MTCC 5690, respectively, and the final concentrations of S. thermophilus in Dahi and PD were recorded as 1.6 × 10^7 and 1.58 × 10^7 c.f.u. ml^−1, respectively. All products were stored at 7 °C until further use.

**Experimental animals.** Swiss Albino male mice (7–8 weeks old, 25 ±2 g) were obtained from the small animal house at the Indian Council of Agricultural Research–NDRI. Animals were maintained in accordance with guidelines for the care and use of laboratory animals of the National Institute of Nutrition, India. All animal experiments were approved by the institutional animal ethics committee (registration no. 1703/GO(ac)/13/CP/CPCSEA Dt. 3/7/2013). Animals were acclimatized for...
Table 1. Detailed profile of preparations of different dairy products

<table>
<thead>
<tr>
<th>Type of milk product</th>
<th>Base matrix</th>
<th>Inoculum</th>
<th>Incubation period (h)</th>
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<tbody>
<tr>
<td>PM</td>
<td>Reconstituted skim milk</td>
<td>MTCC 5690</td>
<td>No incubation</td>
</tr>
<tr>
<td>(unfermented)</td>
<td></td>
<td>(1×10⁹ c.f.u. ml⁻¹)</td>
<td></td>
</tr>
<tr>
<td>Dahi</td>
<td>Standardized milk</td>
<td>1 % <em>Streptococcus thermophilus</em></td>
<td>6</td>
</tr>
<tr>
<td>PD</td>
<td>Standardized milk</td>
<td>1 % <em>Streptococcus thermophilus</em> + 10 % MTCC 5690</td>
<td>6</td>
</tr>
<tr>
<td>FD</td>
<td>Reconstituted skim milk</td>
<td>10 % MTCC 5690</td>
<td>10</td>
</tr>
<tr>
<td>SFD</td>
<td>Reconstituted skim milk=10 % sugar</td>
<td>10 % MTCC 5690</td>
<td>10</td>
</tr>
</tbody>
</table>

2 to 3 days prior to the start of experiments and were fed with normal diet chow and water ad libitum.

Experimental design. In order to determine the potential mechanism of probiotic mode of action involved in the protection and reinforcement of IBF against LT2 infection, respective groups were continuously administered separately with probiotic strain per se and its unfermented and fermented milk formulations, respectively. Each group was further divided into control (non-infected) and *Salmonella*-infected disease groups. Each animal received its respective treatment once daily by oral gavage of 500 μl formulation during the 14-day course of the experiment, as per the published protocol (Gill et al., 2001).

The outline of the experimental design with appropriate controls and disease groups of mice under normal and diseased conditions is illustrated in Fig. 1, from which it is clear that, under these two broad groups, eight individual treatment categories of animal groups were included and were designated thus: PBS (group receiving no probiotic administration but rather 500 μl of sterile PBS by oral gavage); MTCC 5690 (gavaged with MTCC 5690 suspension in PBS); milk (gavaged with 500 μl of sterile reconstituted skim milk only); unfermented PM (by addition of 1×10⁹ c.f.u. of MTCC 5690 in standardized milk); and Dahi, PD, FD and SFD groups. The control groups received the respective treatment for 14 days, but the diseased groups received respective treatment for 7 days followed by a single dose of LT2 (200 μl of 1×10⁷ c.f.u. ml⁻¹) on the seventh day, and then respective treatments were continued for an additional period of 7 days post-infection. A period 7 days post-infection was chosen based on similar published reports (Esvaran & Conway, 2012). At the end of the experiment (Day 14), animals were euthanized by exsanguination of the abdominal aorta under deep ether anaesthesia. The intestine (ileum) was carefully dissected and luminal contents washed with nuclease-free water, snap-frozen in liquid nitrogen and stored at −80 °C. Part of the intestine was also preserved in 10% formalin for histopathological studies.

Clinical manifestations in infected mice. *Salmonella*-challenged groups were critically monitored daily for their health-related behaviour, using the general health score (GHS) index (Gill et al., 2001) on a scale of 1 to 5 as described in Table 2. Other clinical characteristics relating to health were also assessed, including average weight gain or loss per mouse by weighing the animals on alternative days over the 7-day period post-infection.

Enumeration of total lactic acid bacteria (LAB), faecal *Lactobacillus* and *Salmonella Typhimurium*. In order to enumerate faecal lactobacilli, MTCC 5690 and LT2, faecal samples were collected on alternate days. Freshly collected faecal matter (100 mg) was homogenized in sterile saline (10 ml) and diluted serially with the same diluent. For enumeration of total lactobacilli including MTCC 5690 and LT2, plating was done on MRS agar, MRS agar supplemented with 10 μg ml⁻¹ ciprofloxacin and xylose lysine deoxycholate (XLD) (HiMedia) agar plates, respectively. Plates were incubated at 37 C for 24 to 48 h, and results were expressed as log₁₀ c.f.u. g⁻¹ wet weight of faeces. The selectivity of MRS+ciprofloxacin for lactobacilli has been checked in our laboratory by random amplified polymorphic DNA profiling of randomly selected colonies grown on agar plates (Sudhakaran et al., 2013).

Translocation of *Salmonella* to the spleen and liver. The spleen and liver were removed aseptically from the killed experimental mice, weighed and homogenized individually in 10 ml sterile PBS. Tissue suspension from each sample was serially diluted and plated on XLD agar. Bacterial colonies exhibiting characteristic black precipitation with a red zone, typical of *Salmonella*, were enumerated after 24 h incubation at 37 °C.

Measurement of intestinal permeability. Intestinal epithelial permeability from lumen to blood was determined by FITC–dextran (FITC-Dx; 4000 Da, Sigma-Aldrich) transport from lumen to blood as described previously by Cani et al. (2009). Briefly, mice were subjected to fasting overnight, and FITC-Dx (500 mg kg⁻¹) solution was administered by oral gavage. After 4 h, mice were euthanized, blood was collected in heparin-coated tubes and plasma was obtained by centrifugation of blood samples at 1000 g at 4 °C for 5 min. The FITC-Dx concentration was measured with a fluorescence spectrophotometer (Synergy H1 hybrid reader; BioTek) at an excitation wavelength of 485 nm and emission wavelength of 535 nm. A standard curve for FITC-Dx was prepared in non-treated plasma to determine the level of FITC-Dx in the samples.

Expression of the key genes associated with IBF. Total RNA was isolated from the ileum using TRI Reagent (Sigma-Aldrich), and the same was quantified by measuring absorbance at 260 nm (Synergy H1 hybrid reader; BioTek); the quality of RNA was checked by agarose gel electrophoresis, and the purity was also ascertained by 260/280 nm absorption ratio. All RNA samples were treated with DNase I (Thermo Fisher Scientific) to remove any trace of genomic DNA. The cDNA was synthesized, using a RevertAid first-strand cDNA synthesis kit (Thermo Fisher Scientific), from RNA as per the protocol given in the instruction manual. Quantitative real-time PCR was performed in a Roche LightCycler 480 using SYBR Green as the intercalating dye (Roche Molecular Biochemical) following the protocol given in the instruction manual. The primer sequences of reporter and barrier function genes, namely mouse β-defensin-2, mouse cathelicidin-related antimicrobial peptide (mCRAMP), mucin 2 (MUC2), occludin, claudin, zonula occludens-1 (ZO-1), TLR2 and TLR4, along with the housekeeping reference gene (glyceraldehyde 3-phosphate dehydrogenase) used for expression analysis, were selected from previously published reports (Table S1, available in the online Supplementary Material). The efficiencies of primers were calculated by preparing standard curves for the target/test genes along with the reference gene from the given slopes plotted with the help of LightCycler software. The corresponding real-time PCR efficiency (E) of primers ranged from 95 to 99% (Table S1). The specificity of primers was also checked by melt curve analysis and analysis of the size of the amplified product using gel electrophoresis (Fig. S1). Comparison of intestinal barrier gene expression was carried out using the Relative Expression
Statistical Tool software (2009; Qiagen) to determine the respective fold increase or decrease in the level of expression.

Histopathology. Ileal tissue excised from each test animal was fixed in 10 % neutral buffered formalin and processed for paraffin embedding. Sections of 5 µm thickness were obtained using a Coslab RS-305 microtome (Coslab), and these were mounted on glass slides. Sections were stained with haematoxylin and eosin and examined microscopeally by a pathologist who was also blinded to the experiment.

Statistical analysis. All the results obtained from the outcome of this study are presented as the mean of three independent observations along with the SD of the mean. Statistical significance was determined by one-way ANOVA followed by Newman–Keuls, Dunnett’s and Bonferroni’s multiple comparison post hoc tests using Prism 5.0 software (GraphPad Software). The level of significance was set a priori at P<0.05. To examine the relationship between the GHS and different parameters related to IBF of all mouse treatment groups, principal component analysis (PCA) was performed using XLSTAT 7.5.2 software (Addinsoft).

RESULTS

Behavioural and health parameters of mice groups during post-infection period

The Salmonella-infected, PBS-treated mice (positive control) demonstrated visual clinical symptoms of infection and exhibited low health index compared to the negative (non-infected, PBS treated) control group, whose health index remained fairly high throughout the study (Fig. 2).

However, in comparison to PBS-, Dahi- and milk-fed groups, treatment with PD, FD, SFD, PM and MTCC 5690 resulted in overall good health scores. Over the 14 days of the experiment, an average gain of 8 % in body weight was recorded for negative control groups, whereas a loss of 6 % body weight was recorded for Salmonella-challenged groups (Fig. 3).

Microbiological analysis of faecal samples

PBS-fed (both negative non-infected and positive infected control) groups showed 6.4±0.28 to 7.13±0.61 log_{10} c.f.u. g^{-1} total LAB count on MRS agar in the faecal samples during the experimental period. However, total LAB in groups treated with MTCC 5690, PM, PD, FD and SFD was significantly higher and was found to be more than 8 log_{10} c.f.u. g^{-1}. This increase in total LAB count by more than one to two log counts is possibly attributed to successful colonization of MTCC 5690 in the mouse gut (Table S2). This can be further corroborated from the enumeration of total lactobacillus counts including MTCC 5690 (on MRS + ciprofloxacin) from the faecal samples of both control and diseased groups, which revealed that MTCC 5690 became established in the gut at levels of 4 to 6 log c.f.u. g^{-1} and remained at this high

Table 2. GHS index [adapted from Gill et al. (2001)]

<table>
<thead>
<tr>
<th>Criteria of the health index</th>
<th>Score</th>
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<tbody>
<tr>
<td>Mouse bright eyed and alert, has a smooth coat with a sheen, responds to a stimulus, shows interest in its environment</td>
<td>5</td>
</tr>
<tr>
<td>Fur slightly ruffled, a loss of sheen to the coat, mouse remains alert and active</td>
<td>4</td>
</tr>
<tr>
<td>Fur noticeably ruffled, parts of coat form clumps, less interested in environment outside of cage</td>
<td>3</td>
</tr>
<tr>
<td>Mouse hunched over and lethargic, little interest shown in environment, fur clumped with signs of hyperventilating when handled</td>
<td>2</td>
</tr>
<tr>
<td>Mouse non-reactive to stimulus, fur has a bottle-brush appearance, mouse hunched over, preferring to sleep; cold to touch.</td>
<td>1</td>
</tr>
</tbody>
</table>
level until completion of the study (Table S3). However, no significant difference was recovered in faecal levels of total lactobacilli, including MTCC 5690, between diseased groups and their respective control groups \((P>0.05)\).

The number of \textit{Salmonella} shed in the faecal content of all groups was surprisingly found to be 3 to 4 log c.f.u. g\(^{-1}\) faecal content up to Day 10 post-infection, which indicated the persistence of a high level of \textit{Salmonella} in the intestine (Fig. 4 and Table S4). However, contrary to this, flushing out of \textit{Salmonella} was significantly increased \((P<0.05)\) (>5 log c.f.u. g\(^{-1}\) of faeces) in FD and SFD groups on Day 14.

Contrary to this, daily administration of MTCC 5690, milk, PM and Dahi did not result into any significant effect on shedding of \textit{Salmonella} from the intestinal lumen (Fig. 4 and Table S4). It was further noted that no lactobacillus colony was recorded on XLD agar plates from faecal samples of negative control groups, thereby revealing the specificity of XLD agar toward \textit{Salmonella}. Similarly, the lack of growth on MRS plates containing antibiotic as selective agent in mice not treated with MTCCC 5690 also demonstrated the specificity of this medium toward lactobacilli, including MTCC 5690.

**Translocation of \textit{Salmonella} to vital organs**

The results obtained from the study demonstrated that prefeeding (7 days) of mice with FD and SFD prior to \textit{Salmonella} challenge could reduce the mean pathogen burden by more than one and a half log\(_{10}\) count in the spleen \((P<0.01)\) and liver \((P<0.05)\) as compared to the PBS-fed \textit{Salmonella}-challenged group (Table 3). On the other hand, no significant difference was recorded in all other treatment groups. However, none of the uninfected mice shed any \textit{Salmonella} in the spleen and liver, as \textit{Salmonella} counts in the tissue samples were nil.

**Efficacy of probiotic formulations against intestinal permeability in mice infected with \textit{Salmonella}**

In order to assess the stage and severity of infection in mice, we examined the integrity of the intestinal membrane using an indirect method for assessment of gut leakiness by measuring the level of FITC-Dx in plasma. The findings of the bacterial translocation experiment revealed that PM-, PD-, FD- and SFD-fed groups recorded relatively lower increase in the level of plasma FITC-Dx, while PBS- and Dahi-fed \((P<0.001)\) groups followed by milk-fed \((P<0.01)\) groups recorded a significant increase in the level of FITC-Dx in plasma (Fig. 5). Similarly, histopathological analysis of the small intestine had shown that changes induced by salmonellosis were considerably reduced in groups before and after being dosed with PD and fermented drink (FD and SFD) preparations, which demonstrated that treatment with fermented milk preparations produced with MTCC 5690 had ameliorated the acute innate response of the host mucosal immune system evoked by \textit{Salmonella} to induce inflammation. However, feeding with MTCC 5690, milk, PM and Dahi did not exhibit any suppressive effect on early infiltration of neutrophils and deformation of villi after infection with \textit{Salmonella}. Interestingly, regularity of the epithelial layer was maintained in MTCC 5690-, milk- and PM-fed mice, thereby suggesting a relatively mild level of protection against \textit{Salmonella}-induced deterioration in these groups (Fig. S2).

**PCA of clinical symptoms**

Our results were also subjected to PCA to detect any correlation among different treatment groups and various parameters of barrier function (Fig. 6). From PCA, it was revealed that the MTCC 5690, PM, PD and SFD groups behaved more or less similar to each other, virtually
exhibiting an identical kind of pattern of health index and comparatively higher *Salmonella* counts in faeces. Moreover, these groups demonstrated relatively lower intestinal permeability and exhibited lower translocation of *Salmonella*. Similarly, milk and Dahi groups were linked by their comparable pattern for all measured parameters. However, the PBS group was quite different with regard to all the parameters under consideration, showing the highest intestinal permeability, presence of pathogen in the liver and spleen and the lowest health index (GHS, 2). The PCA plot illustrates that, among all treatment groups, the health status of FD- and SFD-fed groups, followed by the PD-fed group, was significantly better than that of the groups fed with unfermented PM and MTCC 5690 alone.

**Relative expression of genes associated with IBF in mice**

The relative expression of specific genes associated with IBF induced with pre- and post-treatment of MTCC 5690 and its unfermented and fermented dairy preparations in the *Salmonella*-infected/non-infected mouse model was determined at the transcriptional level using the quantitative real-time PCR method. The expression of occludin,
claudin, ZO-1, β-defensin-2 and MUC2 was significantly downregulated in the positive control group (PBS + Salmonella) relative to negative control (PBS only). Contrary to this, TLR4 expression was significantly increased in the positive control group compared to that of the negative control group. However, no significant difference was recorded in the expression of TLR2 and mCRAMP between positive and negative controls. It was noted that PM-, PD-, FD- and SFD-fed Salmonella-challenged groups significantly (P<0.001) upregulated the relative expression of occludin by 3.05±0.70, 3.98±0.75, 3.21±1.05 and 5.59 ±0.64-fold, respectively, whereas expression of claudin-1 was significantly (P<0.001) upregulated in the Salmonella-challenged group fed with MTCC 5690 (5.47±1.12-fold), PD (7.49±0.74-fold), FD (8.96±1.55-fold) and SFD (8.95 ±1.38-fold) in comparison to the negative control group. A similar trend was also recorded with respect to the expression of intracellular TJ protein ZO-1, which was also moderately (P<0.01) stimulated in the Salmonella-challenged groups fed with PM (1.57±0.26-fold), Dahi (1.57±0.26-fold), PD (1.73±0.22-fold), FD (2.28±0.90-fold; P<0.001) and SFD (1.70±0.16-fold) relative to negative control. On the other hand, the expression of a key antimicrobial component of secretory barrier function, i.e. β-defensin-2, was enhanced significantly (P<0.001) in mice fed with PD (13.87±4.24-fold) and SFD (16.70 ±4.82-fold), whereas mCRAMP was significantly (P<0.001) upregulated in SFD-fed control (15.57±1.87-fold) and Salmonella-challenged groups fed with SFD (15.64±3.22-fold; P<0.001) and PD (12.39±5.74-fold; P<0.05), respectively, relative to negative control. A similar pattern was recorded with regard to expression of MUC2 in the aforementioned groups (Fig. 7).

Since the potential mechanism behind reinforcement of the mucosal barrier is expected to involve the activation of key pattern recognition receptors such as TLR2 and TLR4 signalling pathways in probiotic-treated mice, we also looked at the expression of these TLRs. Our results in this regard demonstrated a significant increase in TLR2 expression by 3.46±0.76, 4.38±2.23 and 3.22±0.73-fold in the groups subjected to continuous treatment with PD, FD and SFD, respectively, compared to that of negative control. However, mice challenged with Salmonella when fed with PM, PD, FD and SFD resulted in significant (P<0.001) downregulation in TLR4 expression (Fig. 7).
DISCUSSION

In our daily life, our body is constantly exposed to infection by different kinds of food pathogens posing serious risks to our health. Among these, high-risk gastrointestinal pathogens like *Salmonella* in particular are of considerable human health concern because the oral route is a very common and easy target for entry into the host by these organisms. However, the consumption of probiotics has been found to be an effective strategy on account of their inherent ability to suppress gastrointestinal pathogens, including *Salmonella*, during infection (Rolfe, 2009). In this context, the current focus of much of attention across the world is to explore dietary interventions based on the use of probiotics to minimize the risk of pathogens in the human population. Adequate knowledge with regard to the underlying mechanisms of the preventive effects of probiotics against pathogens is extremely vital in establishing their efficacy, by providing sufficient scientific evidence in support of prospective health claims. Moreover, it is now well recognized that the specific functional efficacy of probiotics is highly strain specific. Each strain has its own unique properties, and hence it is not necessarily so that the results obtained from one strain can be extrapolated to another. Similarly, the origin of probiotic strains from the gut of the native population may be highly critical in the optimal expression of their prophylactic/therapeutic functions in the local target population from where the strains were isolated, as they were evolved under different gut conditions along with the normal commensal microbiota of the local gut relative to their Western counterparts due to different food habits. The same holds good for Western strains originating from Western subjects, as these would be more effective in the Western population due to the conditioning effect. However, this does not mean that probiotic strains originating from heterologous hosts will be totally ineffective in the local population, although they will not be as effective as in their own homologous target population in quantifiable terms. Hence, an attempt was made in this study to explore one of the potential probiotic strains, i.e. *L. plantarum* MTCC 5690 of Indian gut origin, extensively investigated in our laboratory previously, and its traditional Indian fermented milk formulations with regard to ameliorating the problem of *Salmonella* infection in a murine model through modulating mucosal barrier function. Since traditional fermented milks have been an integral part of the Indian diet since time immemorial, the main focus of this study was to explore these as the ideal matrix to deliver indigenous probiotic strains in the gut for management of high-risk pathogens in the target Indian population. This is the first study carried out in this direction with the long-term objective of controlling *Salmonella* infection in the vast Indian population suffering from this high-risk disease, through intervention in traditional fermented milk formulations enriched with MTCC 5690 after establishing their functional efficacy initially in animal models and after extrapolating their efficacy clinically in the target Indian population. Dietary formulation of probiotics, specifically fermented milks containing probiotic LAB, is the most common type of supplementation included in the normal diet of different ethnic populations (Sanders & Marco, 2010) and is considered as an effective means of combating gastrointestinal disorders, including *Salmonella* infection.

It has previously been reported by different investigators that probiotics can prevent generalized infection of *Salmonella* in the host (Castillo *et al.*, 2011; Zacarias *et al.*, 2014). In our study, relatively high health index scores were recorded in groups fed with PD, FD and SFD prepared with MTCC 5690 post-*Salmonella* infection. Our results clearly showed a moderate decrease in severity of the infection in these groups relative to the control group. Our results in this regard are in close agreement with the findings of a previous study (Silva *et al.*, 1999; Esvaran & Conway, 2012), which also demonstrated that the severity of disease induced by infection with *Salmonella* Typhimurium was significantly reduced with probiotic fermentation milk treatment in comparison to a control group. Silva *et al.* (1999) examined the efficacy of a fermented ‘bifidus milk’ containing *Bifidobacterium bifidum* against *Salmonella enterica* serovar Typhimurium infection in both gnotobiotic and conventional mice. Our own findings in this regard are in agreement with the outcome of the aforementioned studies, which also observed a higher survival rate in groups receiving bifidus milk than control groups, although the probiotic types used in the two studies were different.

To decipher the plausible mechanism behind the activity of probiotic, in our study, we evaluated the transition of *Salmonella* in the presence of MTCC 5690 into the digestive tract, because colonization in the intestine is the very first step for the pathogen or probiotic to confer its effects on the host. The suppression of pathogen adhesion in the presence of probiotic has been widely reported by different groups (Kim *et al.*, 2013; Chapman *et al.*, 2014). The anti-adhesive potential of MTCC 5690 against *Escherichia coli* O157:H7 on immobilized collagen has also been demonstrated previously in our laboratory (Yadav *et al.*, 2013). The results obtained from the present study further indicated increased shedding of *Salmonella* in the faeces of probiotic-treated groups relative to the PBS control group, which is consistent with the findings of a previous report (Kim *et al.*, 2013). The validity of our findings can be further substantiated by the outcome of a study undertaken by de Moreno de LeBlanc *et al.* (2008), who also reported the elimination of pathogens from the gastrointestinal tract when animals were fed fermented milk products. The removal of pathogens from the intestine could be attributed to pathogen exclusion and the immunomodulatory activity of probiotics. In a similar study, Medici *et al.* (2005) reported that administration of fermented milk containing *Lactobacillus delbrueckii* subsp. *bulgaricus* increased the level of phagocytosis and the number of IgA+ cells in the small intestine of *enteroinvasive E. coli*-infected mice. However, despite hostile conditions present in the gut, the possibility of pathogen overload in the murine gut cannot be ruled out.
Fig. 7. Relative expression of IBF genes in control and diseased groups. The level of expression among different groups was compared using Bonferroni's multiple comparison test. Values significantly differing from the PBS+S group, *P<0.05, **P<0.01 and ***P<0.001. PBS+S, PBS-fed Salmonella-challenged group; MTCC 5690, MTCC 5690 control; MTCC 5690 +S, MTCC 5690 with Salmonella; M, milk; MS, milk with Salmonella; PMS, PM with Salmonella; D, Dahi; DS, Dahi with Salmonella; PDS, PD with Salmonella; FDS, FD with Salmonella; SFDS, SFD with Salmonella.
Since increased shedding was not observed in control groups, it could be speculated that increased shedding of Salmonella in the treatment groups is directly linked to the enhanced competitive binding capacity and immunomodulatory activity of MTCC 5690 in the gut.

It was further noted in the study that the exclusion results are inversely related to the outcome of bacterial translocation, which suggests that treatment with MTCC 5690-fermented formulations decreased the pathogen load of the spleen and liver 1.3-fold relative to pathogen levels in these organs of the respective untreated control groups. These results clearly indicate that treatment with probiotic strains decreased the establishment of pathogens and their translocation to other organs. Our results with regard to pathogen translocation in the presence of probiotic strains are in close agreement with the outcome of a previous study (Zacarias et al., 2014), which also recorded reduced bacterial translocation from the intestine to the spleen in mice fed with fermented milk products. The reduction in translocation rate of the pathogen in the presence of probiotics as recorded in our study may be attributed to stimulation of the host immune response in the form of increased anti-Salmonella immunoglobulin in both the gut and circulatory system, which reduced the survivability of pathogen within host tissues as reported by Gill et al. (2001).

It is well recognized that systemic infection caused by Salmonella can be characterized by the loss of intestinal integrity that facilitates the movement of pathogens and toxins from the lumen to various tissues. The assembly of TJ proteins, as stated above, played a key role in establishing reinforcement of epithelial barrier function. In our study, it was noted that probiotic treatment led to upregulation of TJ protein expression. These results are inversely related to the permeability results, where we recorded a significant decrease in the permeability of FITC-Dx following Salmonella insult relative to PBS treatment.

Furthermore, the expression of TJ proteins was found to be much higher in Salmonella-treated probiotic groups relative to non-Salmonella-insulted animals. Our results in this regard are consistent with those from other studies that also reported reinforcement of the gut barrier following treatment with probiotics (Mennigen et al., 2009). For instance, protection against gut permeability with increased ZO-1 expression was reported in mice subjected to probiotic (E. coli Nissle 1917) treatment (Ukena et al., 2007). The increase in gut barrier function by upregulation of the expression of TJ proteins was reported as the key mechanism of probiotic action (Liu et al., 2011). Other components of barrier function, such as secreted mucus mainly composed of MUC2, provide the first line of defence against bacterial (enteric) pathogens and various toxins. We recorded an increase in expression of MUC2 in MTCC 5690-treated groups, which indicated an improved defence mechanism against Salmonella. Hence, the findings of our study clearly suggest a correlation between the paracellular permeability of FITC-Dx and the expression level of TJ proteins, which could offer new insights in understanding the mechanism behind reinforcement of the mucosal barrier by probiotics.

In addition to TJ proteins, two surface components of IECs, i.e., TLR2 and TLR4, have also been reported to be potentially involved in conferring probiotic-related effects on the host. It is noted from previous studies (Uluwischewa et al., 2011) that epithelial resistance may be enhanced by activation of TLR2 by probiotic bacteria and their fermented biogenic components. In fact, probiotics showed the ability to suppress the deleterious inflammatory response generated by excessive stimulation of pathogen-associated molecular patterns (Vinderola et al., 2005). Several studies have also described the capability of probiotics to modulate the expression of pro-inflammatory cytokine by triggering the expression of TLR4 against Salmonella infection (Bermudez-Brito et al., 2012; Castillo et al., 2012). Our results in this regard have demonstrated downregulation of TLR4 expression in MTCC 5690-fermented product-fed animals, as reported in the aforementioned studies, thereby further substantiating the validity of the outcome of our study. We also recorded a significant induction of antimicrobial components (mouse β-defensin-2 and mCRAMP) against Salmonella infection in the presence of probiotics, which is also in accordance with similar findings from some earlier studies (Trebičavský & Splichal, 2006) that described the modulatory effect of probiotics on the biochemistry of barrier function through activation of different host cell cytokines.

To the best of our knowledge, our study is the first to compare the functionality of an indigenous probiotic strain in both unfermented and fermented milk matrices. Furthermore, the fermented milks of MTCC 5690 prepared in conjunction with and without the Dahi starter culture (S. thermophilus) were also compared to check the effects of lactic starter culture on probiotic efficacy. The findings of our study clearly indicate that all fermented milk formulations of MTCC 5690 were relatively more efficient in countering the infection and pathogenicity of Salmonella in mice. However, non-fermented milks provided protection to the probiotic during their transition through the gastrointestinal tract but did not improve probiotic efficacy against pathogen infection. It was also observed that the presence of lactic starter culture did not affect or change the functionality of MTCC 5690 in PD. The role of specific bioactive metabolites produced by probiotic strains during the preparation of fermented milks in suppressing Salmonella infection in the murine gut cannot be ruled out. These could be the same or different in both types of fermented milk preparations. Attempts are now being made in our laboratory to identify a specific metabolite that can directly influence the aforementioned functions related to suppression of Salmonella infection in treated groups, so that the precise mechanism of probiotic mode of action in fermented milk preparations can be established from a scientific perspective. Hence, it can be concluded that traditional fermented milk formulations such as PD, probiotic

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drinks and sweetened probiotic drinks developed with the probiotic L. plantarum MTCC 5690 can serve as ideal carriers of MTCC 5690 for the alleviation of Salmonella infections by reinforcing mucosal barrier function in the gut.

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REFERENCES


