Probiotic Characteristics and Anti-inflammatory Properties of an Indian Indigenous *Lactobacillus plantarum* against Pathogenic *Salmonella* Typhimurium

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Running title: Probiotic Characteristics and Anti-inflammatory Properties of *L plantarum*

Abstract

Probiotic should be well characterized for any preventive and therapeutic application. Studies have reported that the population specific indigenous probiotics are more effective. Huge number of probiotics are available globally but indigenous Indian probiotic candidates are limited. Therefore, a study was conducted to evaluate probiotic characteristics and anti-inflammatory properties of an Indian indigenous *Lactobacillus plantarum* (Lp1) – (NCBI accession no- MN386242) against gastrointestinal (GI) pathogen *Salmonella* Typhimurium (ST) using Caco-2 cell lines. Anti-inflammatory properties were assessed by evaluating the expression of Immunoglobulins (IgA, IgG) receptors, Toll Like Receptors (TLR2, TLR4) and cytokine (IL10, IL-8, IL17 and IL23) using flowcytometry and Real Time-PCR. Lp1 showed good adhesion and ability to survive in high acidic and bile conditions of the gut. Lp1 demonstrated antagonistic potential against GI pathogens and modulated pro and anti-inflammatory cytokines expression significantly. Observed result indicated that this indigenous Lp1 is a good probiotic candidate with anti-inflammatory potential and can be evaluated further clinically for the treatment or prevention of salmonellosis and other GI inflammatory disorders.

Keywords: Probiotic, *Lactobacillus*, *Cytokines*, *Salmonella* Typhimurium

Introduction

The human microbiome that acquired during and after birth is a major determinant of human health ¹ and play an important role in the human physiology, development, immunity and tolerance ². The concept of microbial intervention through probiotics ³ in human body for health benefits has gained significant importance in the last few decades ². Probiotics have been shown to alter the gastrointestinal microflora, prevent translocation and overgrowth of enteric pathogens. The probiotics also improves the immune function, reduces lipid and cholesterol level and exhibits anti-allergic, anticancer and anti-oxidative effects ⁴. Therefore, they are used to prevent and treat various lifestyle and gastrointestinal (GI) diseases but the health effects of probiotic are strain-specific. An effective probiotic strain should be safe, have originated from human, able to adhere and colonize the human gut, should resist degradation by hydrochloric acid, bile, pancreatic enzymes, have reduced intestinal permeability, should be able to antagonize pathogenic bacteria ⁵⁻⁷. Probiotics are among the potential alternatives to combat the global challenge of antimicrobial resistance (AMR).

Lactobacilli are the potential probiotic candidate because of their beneficial and protective effects on health and generally regarded as safe (GRAS) status. *L. plantarum* is considered as one of the most significant *Lactobacilli* because of its distinctive probiotic properties ⁸ and its antagonistic activity against intestinal pathogens ^{9,10}. *L. plantarum* attenuates intestinal inflammation ¹¹, modulates chronic inflammation and promotes human health. A symbiotic combination of *L. plantarum* effectively treated sepsis and lower respiratory tract infections in infants ¹². Immuno-modulatory effects of probiotics mediate through toll like receptors (TLRs) which recognizes microbe-associated molecular patterns (MAMPS) of the bacterial cells and trigger the host immune system. TLRs are involved in the modulation for intestinal inflammation and can activate signalling that results into cytokines production ¹³.

Several studies have reported the significant differences in the gut microbiome of geographical diverse population due to the difference in diets and lifestyles and that support the use of locally sourced probiotics to achieve best health benefits ¹⁴. Despite the fact that the probiotic market is blooming across the globe, the indigenous Indian probiotic strains are limited. Therefore, it is important to isolate and evaluate indigenous Indian probiotic candidates with proven targeted functional properties and thus, a study was conducted to isolate and evaluate probiotic and anti-inflammatory properties of an indigenous *L. plantarum* (Lp1) against GI pathogen *Salmonella enterica* serovar Typhimurium.

Material and Methods

The study was carried out at All India Institute of Medical Sciences (AIIMS), New Delhi, India with ethical approval (ref no: IECS/T-13401-04-2015). Healthy subjects were enrolled in the study as per inclusion and exclusion criterion. A written consent was obtained from each enrolled subject to participate in the study.

Collection of samples, isolation and purification of *Lactobacilli*: Fresh stool samples were taken from the subject and processed for *Lactobacillus* isolation. A loop full of sample was inoculated into De Mann Rogosa and Sharpe (MRS) medium and incubated anaerobically for 48h at 37°C for isolation of *Lactobacillus*. Only gram positive, non-spore forming, catalase negative isolates from MRS plates were further identified using API 50 CHL (Biomeruex, USA). Molecular identification of *Lactobacillus* was done by genus specific 16S rRNA PCR¹⁵. The identification was confirmed by amplification and sequencing of 1.5 kb region of 16 rRNA and the indigenous *Lactobacillus* strain used for this study was identified as *Lactobacillus plantarum* and named as Lp1 for in house use. The sequences of the strain Lp1 were submitted to NCBI gene bank (Accession no: MN386242).

Screening of Probiotic Properties of *Lactobacillus* Isolates

- **Acid tolerance:** Sterile MRS Broth was adjusted to pH 2.0, pH3.0, and pH 6.4 (as control) using 1.0 M HCl. Test tubes containing 9 ml of MRS broth at each pH were inoculated with 1.0 ml (1×10^9 CFU/ml) of Lp1 cultures and incubated at 37°C for 3 hours. After incubation, 1.0 ml of culture was serially diluted using 0.85 % saline solution. The viable colonies was counted by plate count method¹⁶. The known probiotic strain (*L. plantarum* - ATCC-202195) was used as a control.
- **Bile tolerance:** Bile tolerance was estimated on MRS broth supplemented with 0% (control), 0.5 % and 1 % bile salts by standard method¹⁶. Each experiment was done in triplicate.
- **Bile Salt Hydrolase Activity:** Bile salt hydrolase (*Bsh*) activity was assayed by direct plate method using sodium taurodeoxycholate (TDCA)¹⁷. Each experiment was performed in triplicate.
- **Antibiotic Susceptibility:** Antibiotic susceptibility of Lp1 was performed by disc diffusion method against commonly used antibiotics including -gentamicin, vancomycin, chloramphenicol, clindamycin, ampicillin, erythromycin and penicillin as per standard methods¹⁸. The result of the disc diffusion test was interpreted by according to CLSI 2015.

The zone of inhibition around the antibiotic less than or equal to 14 is considered as resistant, zone of inhibition between 15 to 19 are intermediate and zone of inhibition more than and equal to 20 are sensitive.

- **Antagonistic Activity:** Antagonistic activity of Lp1 was tested against, *Salmonella* Typhimurium, by well diffusion assay ¹⁹. The diameter of the clear zone excluding the diameter of 6mm well was scored as no inhibition – (negative), diameter 0 - 3 mm (weak); diameter 3-6 mm – (good) and diameter > 6 mm – (strong). Each experiment was performed in triplicate.
- **Cell culture.** Human colorectal adenocarcinoma cell line (Caco-2 cells) was procured from National Centre for Cell Sciences, Pune, India and maintained in an incubator at 37°C, 5% CO₂, in Dulbecco's modified Eagle's minimal essential medium (DMEM, high clone, USA) (high glucose) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Hyclone, USA), 10 mg/ml streptomycin, and 10 U/ml penicillin. The Caco-2 cells (1X10⁵ cells/well) were allowed to attach and grow in 6 and 12 well culture plates (Corning). The culture medium was changed every alternate day for 15 to 20 days until the cells were fully differentiation. Subsequently, these Caco-2 cells were used in different experiments conducted in this study.
- **Adhesion assay:** Adhesion to intestinal cell line was evaluated on Caco-2 cell line using standard method ²⁰. The results were expressed as percent adhesion and the ratio between adherent bacteria and initial inoculums of bacteria ²⁰.
- **Cell surface hydrophobicity:** The cell surface hydrophobicity was evaluated by the microbial adhesion to hydrocarbons (MATH) using xylene. The surface hydrophobicity (%) was calculated using absorption at 600nm at 0h (A₀) and 1h (A₁) using the formula described previously {H % = (1 - A₁/A₀) X 100} ¹⁶.
- **Cell Auto-Aggregation:** The auto-aggregation assay was performed as per method described by Ren et al., 2014 and percentage was expressed as (1-A₁/A₀) × 100 ^{16,21}.
- **Co-Aggregation of Lactobacilli and S Typhimurium:** Equal quantity of (1 mL) Lp1 and S Typhimurium (pathogenic strain) were thoroughly mixed and incubated at 37°C without agitation. The absorbance of the mixtures was taken at A_{600nm} at different time intervals (0 or 5 h). Absorbance was recorded for the mixture and for the suspensions individually. Coaggregation was calculated as follows: $\{[(A_{ST} + A_{Lp1})/2 - A_{mix}]/(A_{ST} + A_{Lp1})/2\} \times 100$, where A_{ST} and A_{Lp1} represent A_{600nm} of the separate suspensions of S Typhimurium and *L. plantarum* Lp1 in control tubes and A_{mix} represents the absorbance of the mixed bacterial suspension at 5 h ^{22,23}.

- **Fibronectin-binding protein genes (*fbp*) and mucus-binding protein (*mub*) gene:** The presence of *fbp* and *mub* were detected by reported¹⁷ and designed species-specific primers. The primers sequences used in the study are given in the Table 1.

Table1: List of primers used in Polymerase Chain Reaction (PCR)

Primer (5'-3')		Product size (bp)	Annealing temp.	Reference
LbLMA-1R 16S rRNA (<i>Lactobacillus</i> genus specific)	CTCAAAACTAAACA AAGTTTC	250	55°C	15
LbLMA-R-161 16S rRNA	CTTGTACACACCGC CCGTTCA			
fD1 (16s rRNA universal primer)	AGAGTTTGATCCTG GCTCAG	1,500	60 °C	24
rP 2	ACGGCTACCTTGTT ACGACTT			
LpMubD1F/R MUB-domain of Mub protein	ATGCGTATCGGACG GCTGATAC	425, 1050	55°C	17
LpMubD1R MUB-domain of Mub protein	AACAGCCTCAAAAC GACCAGTC			
Lp FBPF Fibronectin binding protein	GTCCTTTGATGGTTT ATTACCC	1500	50 °C	17
Lp FBPR Fibronectin binding protein	AGAAGTATGCGGCG AGATTCGC			

Immuno-modulatory effect of *L. plantarum* (Lp1) on Caco-2 cell line

Challenge with *Salmonella* Typhimurium (ST) (clinical isolate) along with Lp1 strain:

Caco-2 cells (1X 10⁵ cells / mL) were seeded in 12-well plates for 15-20 days and confluent monolayer was used for the experiment. The cells were incubated with antibiotic free medium 1day before the experiment. The cells were washed and co-incubated with 1 ml of Lp1 suspension (1X10⁹ CFU/ml in antibiotic-free DMEM), *S* Typhimurium (1X10⁸ cfu /ml) and both. After incubation for 4 h at 37°C under 5% CO₂, the bacteria were removed and the cells were washed thoroughly with sterile PBS and used for further experiments using standard protocol²⁵.

Immunoglobulin (IgA & IgG) expression: The expression of IgA and IgG on the surface of Caco-2 cells was measured by standard flowcytometry method²⁶.

TLRs and cytokines profiling of the treated Caco-2 cells: TLRs and cytokine expressions were studied at mRNA level by reported method²⁷ using quantitative real-time polymerase chain reaction (qRT-PCR). The RNA extraction of the cells was carried out using Trizol. The RNA was treated with DNase enzyme to remove any genomic DNA contamination. The

quality and quantity of RNA was checked using Nanodrop. The synthesis of c-DNA from mRNA was done using c-DNA kit (BioRad, USA). The relative expression of TLR-2, TLR-4, and IL10, IL8, IL17 and IL23 mRNA was quantified by qRT-PCR using the SYBR Green PCR Master Mix and ABI-Step one plus Detection System (Applied Biosystems, USA). The PCR mixture consists of 12.5 µL of Maxima SYBR Green Mastermix (thermo scientific), 0.5 µL of each primer and 5 µL of c DNA (0.5 µg/L) as the template. PCR water was used to adjust the final volume to 25 ml. The initial denaturation at 95°C for 1 minute was followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at suitable temperature (Table 2) for 30 seconds, and extension at 72°C for 40 seconds. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, housekeeping gene) was amplified as the internal standard ²⁵. The following equation was used to analyse the data:

$$\text{Relative expression of mRNA} = 2^{-\Delta\Delta Ct} \text{ (Where } Ct = \text{threshold cycle, } \Delta Ct = Ct_{\text{target gene}} - Ct_{\text{GAPDH}}, \Delta\Delta Ct = \Delta Ct_{\text{treated}} - \Delta Ct_{\text{untreated}})^{28}$$

Statistical analysis: All of the tests were performed in triplicates and the results were presented as mean ± SD. All the statistical analysis was performed using SPSS Version 16. The Kruskal-Wallis test, followed by post hoc LSD test, were used to determine statistical significance.

Table 2: List of primers used in RT- qPCR for relative quantification of following genes

Primer (5'-3')		Product size	Annealing temp.	Temp (°C)	Reference
IL 8 forward	TCTGGACCCCAAGGAAAC	183bp	58°C	78.3 °C	Designed for this study
IL8 reverse	CATCTGGCAACCCTACAACA				
IL10 forward	AAA GAA GGC ATG CAC AGC TC	132bp	60 °C	90 °C	²⁹
IL 10 reverse	AAG CAT GTT AGG CAG GTT GC				
IL17 forward	GGCTGGAGAAGATACTGGTGT	171bp	58 °C	84.6 °C	Designed for this study
IL 17 reverse	TTAGTCCGAAATGAGGCTGTCT				
IL 23 forward	CTGAGGGAACCAAACCAGAG	166bp	58 °C	83.04 °C	Designed for this study
IL 23 reverse	GCAGCAACAGCAGCATTACA				
GAPDH forward	CAACGACCACTTTGTCAAGC	140bp	58 °C	82.8 °C	²⁹
GAPDH reverse	TTCCTCTTGTGCTCTTGCTG				

TLR2 forward	GATGCCTACTGGGTGGAGAA	159bp	58 °C	79.2 °C	Designed for this study
TLR2 reverse	GACGGAAATGGGAGAAGTCC				
TLR4 forward	CGGTCCTCAGTGTGCTTGTA	220bp	58 °C	82 °C	Designed for this study
TLR4 reverse	TCCTTACCCAGTCCTCATCC				

Results and Discussion

Lactobacilli are generally regarded as safe (GRAS) and the most common probiotic candidates because of their beneficial and protective effects on health ³⁰. *L. plantarum* is one of the important *Lactobacilli* due to its distinctive probiotic properties ⁸ and antagonistic activity against intestinal pathogens ^{9,10}. *L. plantarum* attenuates intestinal inflammation ¹¹, and promotes human health ^{31,32}. A symbiotic combination of *L. plantarum* effectively treated sepsis and lower respiratory tract infections in infants ¹². The aim of the present study was to evaluate the probiotic potential and anti-infective properties of *Lactobacilli* isolated from healthy Indians. For the selection of potential probiotic strain, species identification, evaluation of physio-chemical and functional properties of the strain are prerequisite (FAO/WHO2002). There are many phenotypic and genotypic tools available for the identification of *Lactobacillus* species including 16S rRNA gene sequencing ¹⁰.

In this study a total of 10 *Lactobacilli* were isolated from faecal samples of Indian healthy subjects. Strains were identified by phenotypic and genotypic methods and evaluated for their probiotic properties and the isolates were identified as *Lactobacilli* by 16S rRNA genus specific PCR. One strain which demonstrated good probiotic properties and effective against ST among others was confirmed by sequencing of 1.5 kb region of 16 rRNA and identified as *Lactobacillus plantarum* and named as Lp1 for in-house use. BLAST match of 16S rDNA sequences of the Lp1 strain showed 95% homology with NCBI database and the sequences were submitted to NCBI (Gene bank accession no: MK723996). Carbohydrate fermentation of Lp1 was tested by API 50CH which showed the hetero-fermentative nature of this strain and that was in agreement with another study ¹⁰. Lp1 utilised L-arabinose, ribose, D-glucose, D-galactose, D-mannose, D-fructose, lactose, mannitol, sorbitol, rhamnose, methyl- α D-Mannopyranoside, N-acetyl-glucosamine, arbutine, amygdaline, esculin, salicine, cellobiose, maltose, melezitose, melibiose, gentiobiose, saccharose, raffinose, trehalose and D-turanose Xylose, but methyl- β D-xylopyranoside, methyl- α D-glucopyranoside variably.

An effective probiotic strain should survive in the upper digestive tract to reach the large intestine and to be colonized in gut so that it can improve intestinal function and maintain the integrity of the lining of the intestines ³³. Therefore, acid and bile salt tolerance are important selection criteria for a probiotic strain and these properties are strain specific ^{17,25}. Bile salts play a vital role in the immune mechanism of the gut which is associated with carbohydrate fermentation, glycosidase activity ³⁴, exo-polysaccharide production ³², facilitates adhesion of probiotics to mucin and prevents adhesion of pathogens ³². In the present study *Lactobacilli* Lp1 showed best survival (8.7 ± 0.1 Log cfu/ml) rate at pH 2 for 3 h similar to the control strain (*L. plantarum* ATCC-202195, 8.8 ± 0.04 Log cfu/ml). Other isolated *Lactobacilli* Lb3, Ldel4, Lb2, Lb5, Lp2 and Ldel1 showed poor survival at pH 2 with significant decrease in their viability ($p < 0.05$) compared to the control strain. Lp1 showed good survival (≥ 8 Log CFU/ml) similar to control strain (8.83 ± 0.2 Log cfu/ml) at 1% concentration of bile salt which was more than the physiological range (0.3 to 0.5%) of human bile concentration (Mainville et al., 2005). Lp1 was tested positive for production of bile salt hydrolase (*bsh*) enzyme and was able to de conjugate the bile salt TDCA and precipitates forming a halo around the colony.

Table 3: Probiotic properties of Lactobacilli isolated from healthy human

SL. no	Isolate	Identification (API)	Initial (Log CFU/ml)	pH 3 (Log CFU/ml)	pH 2 (Log CFU/ml)	0.5% Bile (Log CFU/ml)	1% Bile (Log CFU/ml)	BSH activity
1	<i>L. plantarum</i> - ATCC-202195	Control	9 ± 0.05	9 ± 0.2	8.8 ± 0.63	8.9 ± 0.87	8.8 ± 0.34	+
2	Lp1	<i>L. plantarum</i>	9.1 ± 0.3	8.8 ± 0.02	8.8 ± 0.3	8.6 ± 0.08	8.7 ± 0.67	+
3	Lb1	<i>L. brevis</i>	9.2 ± 0.03	8.2 ± 0.09	8 ± 0.05	8.3 ± 0.64	8 ± 0.07	-
4	Ldel1	<i>L. delbrurkii</i>	9.05 ± 0.2	NG	NG	7.6 ± 0.09	4 ± 0.88	-
5	Lb2	<i>L. brevis</i>	9.0 ± 0.5	8.6 ± 0.62	8.6 ± 0.72	8.7 ± 0.03	8 ± 0.59	-
6	Lb3	<i>L. brevis</i>	8.8 ± 0.4	8.9 ± 0.23	8.7 ± 1.92	8 ± 0.73	8 ± 0.07	-
7	Ldel2	<i>L. delbrurkii</i>	8.9 ± 0.3	7 ± 0.72	5 ± 0.67	7.2 ± 0.57	7.1 ± 0.08	-
8	Lb4	<i>L. brevis</i>	9.5 ± 0.73	8.9 ± 0.09	8.7 ± 0.62	8.5 ± 0.33	8.5 ± 0.93	-
9	Lb5	<i>L. brevis</i>	9.06 ± 0.2	7.6 ± 0.02	NG	6.7 ± 0.06	NG	-
10	Ldel3	<i>L. delbrurkii</i>	8.9 ± 0.03	6 ± 0.08	NG	7.2 ± 0.32	NG	-
11	Lp2	<i>L. plantarum</i>	9.1 ± 0.1	8 ± 0.82	7.4 ± 0.21	NG	NG	-

Values are means of 3 replicates $\pm$ SD.

*Significant difference ($P < 0.05$) among all *Lactobacillus* strains within the same column

Adhesion is another important characteristic of probiotics which prevent their elimination by peristaltic movement of intestine, helps in the competitive exclusion of enteropathogens ²², stimulates immune system¹³ and promote antagonistic activity against enteropathogens ²⁵. Lp1 exhibited very good (14%) adhesion to Caco-2 cells. The adhesion of Lp1

was in the higher side of the range of adhesion (4 -14%) reported for other *Lactobacilli* strains tested in other studies ^{17,25}. Lp1 showed the presence of *mub* and *fbp* genes involved in adhesion of bacteria to epithelial cells and that further supports the adhesion potential of the Lp1. Lp1 exhibited good hydrophobicity (43%) and auto-aggregation of 38%. Lp1 also exhibited good coaggregation with S Typhimurium (27.23±1.57%) which was statistically significant (p value= 0.032)

Table 4: Probiotic properties of Lactobacilli isolated from healthy human

	Isolate	Identification (API)	Initial (Log CFU/ml)	Adhesion (Log CFU/ml)	Hydro-Phobicity (%)	Auto-aggregation (%)	Co-aggregation (%)
1	<i>L. plantarum</i> ATCC-202195	Control	9±1.2	7.9±0.04	40.5±1.1	32±1.3	25±1.03
2	Lp1	<i>L. plantarum</i>	9.4±0.82	8.1±0.32	43.6±0.91	38.7±0.87	27.23±1.57
3	Lb1	<i>L. brevis</i>	8.7±2.1	5.1±0.63	2.4±3.1	30.8±0.06	23±0.93
4	Ldel1	<i>L. delbrurkii</i>	9.05±1.6	NG	5.2±0.92	21±0.63	11.04±0.64
5	Lb2	<i>L. brevis</i>	9.0±0.08	5.7±0.08	13±0.07	18.7±0.22	18.03±1.03
6	Lb3	<i>L. brevis</i>	9.2±0.07	4.1±0.09	1.3±0.72	32.08±2.1	21±0.32
7	Ldel2	<i>L. delbrurkii</i>	8.9±0.09	NG	11±0.59	18±1.09	9.32±2.1
8	Lb4	<i>L. brevis</i>	9.2±0.91	3.3±0.24	26.06±1.9	25.9±0.82	24.42±1.1
9	Lb5	<i>L. brevis</i>	8.6±2.1	NG	12±1.5	8.63±0.99	10.2±.92
10	Ldel3	<i>L. delbrurkii</i>	8.9±1.2	NG	13±0.39	16.93±0.03	12.84±0.43
11	Lp2	<i>L. plantarum</i>	9.01±0.3	NG	18±1.92	8.63±0.73	8.1±0.09

The knowledge of the antibiotic resistant phenotypes is important and the resistance genes should not be present in the Lactobacilli. The *Lactobacilli* commonly exhibits intrinsic resistance to various classes of antibiotics, which can be advantageous for their use in the prevention and treatment of various gastrointestinal infections. AST of the Lp1 showed that the strain was sensitive to common antibiotics including penicillin, erythromycin, imipenem, clindamycin, Chloramphenicol, ciprofloxacin, amikacin, amoxiclav and gentamycin. Lp1 was found resistant to metronidazole and vancomycin. Previous studies reported that *Lactobacilli* are commonly resistant to vancomycin and metronidazole ^{35,36}. Studies reported that Lactobacilli exhibit high resistance to aminoglycosides ^{35,37}, variable resistance to chloramphenicol and erythromycin ³⁸⁻⁴¹ and generally susceptible to cephalosporins ^{40,42}.

Salmonella Typhimurium (ST) is a gram negative enteric pathogen ⁴³ which enters human gut through consumption of contaminated food or drink. An increase in antibiotic resistance strains of ST raised the concern and developed the need to find alternative prophylactic and therapeutic agents to control this important gastrointestinal pathogen ⁴⁴⁻⁴⁶. *S.*

Typhimurium adheres to the host GI epithelial cells, invades and disrupts the GI epithelial integrity and activates host innate immune response through cell signal pathways such as Toll Like Receptors, TLRs¹⁴ or nucleotide-binding oligomerization domain-(NOD)-like receptors (NLRs) of gastrointestinal epithelium identify pathogen associated molecular patterns (PAMPs) on ST leading to inflammation of the gut⁴⁷. Isolated *Lactobacilli* in our study exhibit broad range antagonistic activity against both gram negative and gram-positive pathogens. Lp1 inhibited all the target organisms exhibited strong antagonistic activity against *S. Typhimurium* (5.6 ± 0.14). Other *Lactobacilli* showed less inhibition against the target organisms as compared to Lp1. Observed results indicated that Lp1 is a good probiotic candidate, which may be tested and used to treat targeted infections and antibiotic resistance. Other studies also reported that *L. plantarum* found to inhibit *S. Typhimurium*²¹, sepsis and lower respiratory tract infections in infants¹².

Secretory IgA is present in high concentrations in the lumen of the intestine. There it plays a pivotal role in trapping pathogenic bacteria in the mucus layer, thereby preventing infection⁴⁸. Lp1 significantly increases the expression of IgA receptors in Caco-2 cells when co-stimulated with *S. Typhimurium* as compared to control (untreated Caco-2 cells). The Lp1 increases the expression of IgA receptors to 20.92 ± 1.44 % and 23.90 ± 0.97 % respectively when treated alone and co-stimulated with *S. Typhimurium* and the expression was significant ($p=0.0035$), as compared to control. While the expression of IgA receptors in control (untreated Caco-2 cells) was 9.44 ± 2.01 %. IgA reduces the motility and virulence of pathogenic microorganism such as *Salmonella* by altering the expression of flagellin that compromises its motility⁴⁹. IgA shields epithelial cells against the harmful effects of bacterial toxins by neutralising them^{50,51}. The Lp1 increases the expression of IgG receptors to 1.77 ± 0.35 % and 0.99 ± 0.23 respectively when treated alone and co-stimulated with *S. Typhimurium* although the increase was not statistically significant as compared to the control (0.74 ± 0.25 %). Other studies also reported that IgG are generally present in very low concentration in the GI lumen, imparts immunity to the host against infection⁵².

Lp1 up-regulated the expression of TLR2 by 8.21 ± 0.95 folds as compared to control when treated alone. Co-stimulation of Lp1 with *S. Typhimurium* up-regulated the expression of TLR2 to 7.68 ± 0.72 folds as compared to control. Lp1 up-regulated the expression of TLR4 by 1.52 ± 0.26 folds while *S. Typhimurium* stimulated cells, up-regulated the expression of TLR4 by 13.35 ± 0.70 folds. Lp1 altered the expression of TLR4 by 24.99 ± 0.55 folds when co stimulated with *S. Typhimurium* which indicated Lp1 was able to increase TLR4 expression significantly ($p=0.00065$). Studies have reported that TLR 2 is activated by probiotic bacteria

and their fermented biogenic components⁵². A study showed that there was significant increase in TLR2 expression in *S Typhimurium* infected mice when treated with *L plantarum* MTCC5690⁵³.

Lp1 was able to increase expression of IL-10 (anti-inflammatory cytokine) significantly ($p=0.001$) and decrease the expression of IL-8, IL-17 and IL-23 (pro-inflammatory cytokines) significantly ($p=0.03$, 0.00043 , 0.0043) even in challenged environment induced by *S. Typhimurium* ($P < 0.001$). The anti-inflammatory potential of Lp1, which was in agreement with the reports that probiotics may protect intestinal epithelial cells from entero-pathogens by inhibiting IL-8 production⁵⁴. IL23 are important for Th17 cell expansion, stabilization, and conditioning for a fully inflammatory cell phenotype^{55,56}. The cytokine IL-23 is primarily associated with host innate immune responses against Salmonella. It induces the production of IL-17 and IL-22, which results in the prompt response against the pathogens⁵⁷. A study showed that *L plantarum* ZS2058 strongly promotes the IL-23/IL-17 axis whereas LGG significantly reduced the TNF- α levels in the colon while maintaining IL-10⁵⁸. Our findings were consistent with other studies^{25,59}. *S Typhimurium* infection upregulated the expression of IL23 and IL17 which plays an important role in the inflammatory response of the intestine⁵⁸.

Figure 2: a) IgA and IgG expression on Caco-2 cell line by *L. plantarum* (Lp1); b) TLR2 and TLR4 expressions on Caco-2 cell line by Lp1; c) expressions of anti and pro inflammatory cytokines on Caco-2 cell line by Lp1

Clinical salmonellosis commonly treated with extended-spectrum antibiotics which may lead to antibiotic resistance and gut dysbiosis. The emergence of anti-microbial resistance (AMR) is a global issue that makes the treatment challenging for various infections including *S Typhimurium*. An increase in MDR *S Typhimurium* necessitates an urgent alternative approach. Probiotics are a promising substitute to antibiotics in various gastrointestinal diseases including *S Typhimurium* infection⁶⁰. They are able to directly inhibit growth of *S Typhimurium* by competing for nutrient sources, producing antimicrobial peptide, colonization resistance, increasing epithelial tight junction and increasing the expression and production of both SIgA and anti-inflammatory cytokine against *S Typhimurium*^{61,62}. The observed results indicated that Lp1 have antagonistic and anti-inflammatory potential and can be evaluated for the management of GI inflammatory disorders and *S Typhimurium* infection.

Conclusion

Lactobacillus plantarum (Lp1) possess good probiotic properties which showed good survival in high acidic and bile conditions of the human gut without losing the viability. Lp1 indicated significant antagonistic effect against GI pathogens *S. Typhimurium* and showed promising immuno-modulatory potential by inhibiting the expression of pro-inflammatory cytokines and enhancing the expression of anti-inflammatory cytokines even in the challenged environment with *S. Typhimurium* induced Caco-2 cell line. The strain also demonstrated immunogenic potential by inducing significant up regulation of TLR-2 and IgA receptors expression even in challenged Caco-2 cells induced by *S. Typhimurium*. Therefore, the findings of the study strongly support that the Lp1 is a good probiotic candidate which can be evaluated further for the prevention and treatment of *S Typhimurium* infection, and GI inflammatory conditions.

Conflict of interest: All the authors declared no conflict of interest

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