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Original Research Article

Deciphering Beneficial Health Promoting Properties of Lactic Acid Bacteria Isolated From Infant Gut Microbiome

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Abstract

Aim and Objective: To evaluate the probiotic potenties along with safety of probiotics within infant faecal origin. ***Methods and Results:*** The present study isolates lactic acid bacteria from infant feces were characterized and identified by 16S rRNA sequencing. On evaluation of *in vitro* probiotic capability, the test isolates exhibited *in vitro* probiotic potency, antagonistic property and better cell surface properties. The Bile salt hydrolase activity, cholesterol assimilation, antioxidant activity and angiotensin converting enzyme inhibitory action of the test isolates proves their health conferring potency. Standard protocols were used for the estimation of lymphocyte proliferation rate, phagocytosis rate, cytokine response and faecal enzyme levels. Animal experiment using different doses of *L. plantarum* (MBTU-S1B) substantiated its immunotolerance against inhibitory effects of supraoptimal concentration of both T and B cell mitogens with significant reduction on β-Glucosidase and Nitroreductase activity with $p< 0.05$, while contrasting effect was noticed for β-Glucuronidase. Findings of the *in vivo* study proved higher dose of probiotic would not provide any additional benefit on host body. ***Conclusions:*** The test isolates possesed *in vitro* probiotic potency and the *in vivo* study with MBTU- S1B proved their applicability in nutraceutical and pharmaceutical industries. Significance and Impact of Study: To conflict disease outbreaks by improving general immunity via probiotics.

Keywords: Gut microbiome,cholesterol assimilation, antioxidant, antihypertensive, immunomodulation, dose-response curve.

INTRODUCTION

Probiotics (Greek: for life) are defined as mono or mixed cultures of live microbes that, when applied to animal or human, possess beneficial effect on health of the host (Shehata *et al.,* 2016). The escalated consumer demand for natural non-drug approaches to alleviate health has fuelled up the consumption of products containing beneficial microbes. In this context, over the last few decades, researchers has given increased attention on envisaging the health benefits of novel strains isolated from intestinal microbiota since it is considered to be the largest microbiome reservoir explored so far. Lactic acid bacteria (LAB) and *Bifidobacterium* cover a significant proportion of gastrointestinal (GI) probiotic population and they are the most common microbiota with claimed probiotic properties .However, these beneficial microbiota and their metabolites play a crucial role in the establishment and maintenance of general homeostasis of the body through the gut-brain axis.

Probiotics are known to ascribe various health benefits to the host such as alleviation of lactose intolerance, bile salt hydrolase activity, antioxidant activity, antihypertension and recently proved to play a pivotal role in psychological aspects like emotional and behaviour well being of humans (McKean *et al*., 2017; Nakamura *et al*., 1995; Helland *et al*., 2004; Parvez *et al*., 2006) More over dietary /non dietary elements (stress) and environmental factors may alter the complexity and metabolic activity of gut microbial po0pulation (Su and Mitchell 2007). In addition, growing evidence emphasis that gut microbiota interacts with xenobiotics such as persistent foodborne chemicals and this interplay is of great concern since its chronic exposure may progressively result in the induction of a low grade inflammatory status in gastro intestinal tract (GI) of host that could ward off these

beneficial microbes subsequently (Conlon and Bird 2015). Along side, majority of dietary origin chemicals are either T or B cell mutagens with potent immunosuppressive and oncogenic activity. Meanwhile,studies have also disclosed that these microbes are capable of harmonizing both the innate and adaptive immune reactions via immunoregulation and immunostimulation mechanisms and thereby can elicit prophylactic and therapeutic effects on the host (Yan F, 2014). Surviviability and tolerance of LAB against varying doses of dietary carcinogens is of great importance since they play a crucial role in degradation of these dietary carcinogens and reduction in occurrence of GI dysbiosis such as colo rectal cancer by depleting the specific activity of microbial faecal enzymes that convert procarcinogens to carcinogens. Natural food products with immune boosting properties and devoid of detrimental effects would be useful for people with altered immune status and having auto immune disorders.Hence the current study was carried out to isolate, identify and characterize *Lactobacillus* species with potential health promoting activities from the feces of healthy, exclusively breastfed infants. Furthermore,continuously increasing scientific knowledge empowering the beneficial aspects of these microbiota has resulted in a spurious inflow of food products especially the dairy products incorporated with these micro organisms to the market. While the probiotic dose contained in such products are still a matter of debate and the information regarding minimal effective concentration is still inadequate, the current study also apprehended to explore the variations in dose-response curve employing different probiotic doses avail in the market using suitable animals such as Balb/c.

MATERIALS AND METHODS

Isolation of LAB

Fresh faecal samples were collected exclusively from vaginally delivered breast fed human infants of age (1-10 day old). The samples were inoculated onto selective MRS (de Man Rogosa Sharpe) medium with lithium chloride, neomycin sulphate and nalidixic acid (Khalil *et al.,* 2007). All plates were incubated at 37°C for 48 h in CO_2 incubator (GalaxyBiotech) supplied with 5% CO_2 and sufficient moisture.

Biochemical characterization

The isolates were Gram-stained and examined for bacterial morphology, motility by hanging drop method, catalase, oxidase test and milk coagulation (Honey and Keerthi 2018).

Molecular Identification

Genomic DNA was isolated and used as template for *16S rDNA*amplification. Primers used for the amplification of part of 16S rDNA were Forward primer- 27 F 51 AGAGTTTGATCMTGGCTCAG31 Reverse primer- 1492 R 51 AAGGAGGTGWTCCARCC31. The PCR reaction was performed based on the method of Zhang *et al.,* (2016).

In vitro probiotic characterization

Tolerance to gastric conditions

Tolerance to different acidic pH (2,3,4) and bile (0.2 and 0.4% (w/v) of bile) were examined according to Khalil *et al.,* (2007) respectively. Gastric juice was prepared with 0.3% pepsin, 0.5% NaCl at pH 2 and 3 and the tolerance was determined according to Charteris *et al.,* (1998) with slight modifications.

Antagonistic action towards pathogens

Antibacterial activity of the isolates against the pathogens viz., *Salmonella typhi* (MTCC 734), *Salmonella paratyphi* A (MTCC 735) and *Vibrio cholera* (MTCC 3906) were studied by agar diffusion test (Herrerosa *et al.,* 2005).

Cell Surface properties

Hydrophobicity

Hydrophobicity of test isolates were performed by using xylene - a nonpolar solvent and chloroform an apolar solvent (Vinderola *et al.,* 2011).Cultures were harvested by centrifugation at 12 000×g for 5 min at 5^0C,washed and resuspended in 50 mM K_2HPO_4 (pH 6.5) buffer. Turbidity adjusted bacterial suspension (3ml) was put in contact with 0.6 mL of solvent and vortexed for 120 s.The aqueous phase obtained was removed and the absorbance at 560 nm was measured. The decrease in the absorbance of the aqueous phase was taken as hydrophobicity percentage (H%) and was calculated using the the formula H%=[(A0–A)/A0]×100, where A0 and A are the absorbance before and after extraction with solvent, respectively.

Auto aggregation and Co-aggregation

Auto aggregation and Co-aggregation were evaluated according to 12 and 13 respectively. Turbidity adjusted 4 mL of cell suspension was incubated at room temperature for 5h. 0.1 mL of the upper suspension was mixed with 3.9 mL of phosphate buffered saline (PBS) and absorbance was measured at 600 nm. Auto-aggregation (%) =1–(At/A0)×100, where At denotes the absorbance at time t, and A0 the absorbance at t=0.

For estimation of co-aggregation, equal amount of turbidity adjusted cell suspension and pathogenviz., *Salmonella typhi,* MTCC 734 were incubated for 5h and absorbance was examined at 600 nm. Percentage of co-aggregation expressed as percentage reduction was determined using the equation:Co−aggregation (%)= (Ax+Ay)A(x+y)/2Ax+Ay/2×100, where Ax and Ay be the absorbance of the test strains and pathogen in the control tubes, respectively and A (x+y) is that of the mixed bacterial suspensions (test strain and pathogen) for co-aggregation.

Health Promoting attributes

Bile salt hydrolase activity and Cholesterol assimilation capability

Test isolates were spotted on MRS agar plates supplemented with 0.5% sodium salt of taurodeoxycholic acid (TDCA), followed by anaerobic incubation at 37° C for 72 h and observed for precipitation zone around colonies (Begley and Gahan 2006).

Cholesterol assimilation was determined using freshly prepared MRS broth containing 0.2% sodium thioglycholate,1% glucose supplemented with oxgall at two different bile concentrations viz., 0.2 and 0.4% to mimic approximate levels in the intestinal tract followed by water soluble cholesterol (poly oxyethyl-cholesteryl- sebacate) at a final concentration of 1 mg/ml. The isolates were inoculated at 1% level and incubated anaerobically at 37 ^{0}C for 24 h. After incubation, the cell pellet were discarded following centrifugation at 9000xg for 15 min and cholesterol in the spent broth was determined by O-phthalaldehyde method. 1ml of cell- free broth (1 mL) was added to 1 mL of 33 % (w/ v) of KOH and 2 mL of absolute ethanol, mixed for 1 min, then heated at 37 ^{0}C for 15 min. 2 mL of distilled water and 3 mL of hexane were added and mixed well for 1 min. 1mL of hexane layer was transferred to a fresh tube and kept in water bath at 65 ^{0}C to evaporate the hexane vapours. The residue was dissolved in 2 mL of O-phthalaldehyde reagent. After complete mixing, 0.5 mL of concentrated sulphuric acid was added and mixed for 1 min. The absorbance was observed at 550 nm (Thermofischer scientific varioskan flash multimode reader (3001-1885), Vantaa, Finland) after 10 min of incubation (Kimoto and Okamoto 2002).

β - Galactosidase activity

Qualitative test using 2-Nitrophenyl β-Dgalactopyranoside (ONPG) test disks.

Quantitative test were performed according to Gheytanchi *et al.,* (2010). The isolates were inoculated in MRS- lactose broth and incubated for 24 hrs at 37° C. The cell suspension was permeabilized with Toluene/Acetone (1:9) and analyzed for β-Galactosidase activity using Ortho Nitro Phenyl β-D Galactopyranoside.

ACE inhibitory assay

The ACE inhibitory activity was determined by the method of Cheung and Cushman (1971) with some modifications. The test isolates were inoculated to reconstituted skim milk and incubated for 24-48 h. The fermented milk thus obtained was centrifuged at 4000 rpm for 15 min at 4ºC.The supernatant was collected and the pH was subsequently adjusted to 8.3 using 10M NaOH. The suspension was centrifuged for 5 min at 14000 x g 4ºC and the resultant supernatant was used for further analysis. Thehippuryl –histidine –leucine (Hip- His -Leu) was dissolved in 0.1 M Sodium borate buffer (pH 8.3) containing 0.3M NaCl and 200µl of the solution was mixed with 80µl of supernatant and then preincubated for 3 min at 37ºC. The reaction was initiated by the addition of 20µl of the inhibitory solution [ACE-rabbit lung (0.1unit/ml)] followed by incubation at 37ºC for 60 min.The reaction was stopped by adding 250µl of 1N HCl and mixed with 1.7 ml of ethyl acetate.The mixture was centrifuged (4000 rpm) and 1.4 ml of the organic phase was transferred to a fresh tube.The ethyl acetate was evaporated to dryness on a water bath at 100ºC for 30 min. Then the residue containing hippuric acid was dissolved in 1ml of deionized water. The absorbance of the solution was measured spectrophotometrically at 228 nm against water as blank. The extent of inhibition was calculated as follows: ACE inhibitor activity (%) = (B-A) / (B-C) x 100. Where, A= optical density in the presence of ACE and ACE inhibitory component,B= optical density without ACE inhibitory component and C= optical density without ACE.

Free radical scavenging activity

The isolates were grown overnight in MRS and after centrifugation the cells were subjected to different process such as seperating the cell free supernatant (CFS) for the generation of intact cells and CFS independantly, enzymatic degradation by using lysozyme, temperature disintegration by placing at 100°C and ultrasound sonication. DPPH radical scavenging capacity of the test isolates were verified using the method of Brand Williams *et al*., (1995) (Brand-Williams *et al*., 1995). 60 µmoll^{-1} of methanolic solution of DPPH was freshly prepared. 1ml freshly prepared DPPH solution and turbidiy adjusted 25µl of an overnight culture of the test isolates were incubated in dark at 37^{0}C for 45 min. The mixture was centrifuged after incubation and the scavenging activity of test isolates (S) were examined spectrophotometrically by a decrease in absorbance at 517 nm against 1.15g KCL l^{-1}solution as blank (B).

% of scavenging activity = 1-[$A_{(S)}$/ $A_{(B)}$] x 100

In vivo evaluation of MBTU-S1B (accession no: MN 883893)

Animal model and probiotic treatment

6–8 week old (female,18-20g) BALB/c mice were raised and housed in the Animal Research Facility (ARF) of Rajiv Gandhi Center for Biotechnology. They were randomly allocated into thirteen groups of five animals and were given free acess to sterilized diet and water for thirty days. The selected *Lactobacillus* (MBTU-S1B) was grown in selective de Man Rogosa Sharpe (MRS) medium at 37ºC for 24-48 hrs followed by centrifugation at 4000 rpm for 5 min. The turbidity of cell pellet was adjusted using PBS of pH 7.4. The test group were fed daily once at a dose rate of 1.0×10^9 CFU/ml (low dose) or 5.0×10^9 CFU/ml (high dose), respectively and control group was treated with PBS solution of pH 7.4.

Animals from low dose and high dose group were sacrificed on days 10, 20 and 30 for the following experiments to be performed.

Evaluation of immune organ indices

Immune organs such as spleen and thymus were aseptically removed and weighed immediately following sacrifice. The indices were expressed as percentage using the following equation:

Spleen or Thymus index = [weight of thymus or spleen (g) / bodyweight (g)] ×100.

Determination of lymphocyte proliferation using T cell and B cell mitogens

Lymphocyte proliferation rate was assessed by the method prescribed by Ren *et al*., (2015). 50 µl of spleenocytes at a cell density of 5.0×10^5 cells/well were seeded in 96 wells in the presence of T cell mitogen concanavalin A (ConA, 2.5,5,10 µg/ml) for detecting T cell proliferation and Lipopolysaccharides (LPS, 12.5,25,50 µg/ml) for B cell proliferation at suboptimal, optimal and supraoptimalconcentration respectively. Spleenocytes with RPMI 1640 medium alone was considered as the negative control. The cell suspension was incubated at 37 °C under 5% CO_2 for 72 h and after co-incubation, 10 µl WST-1 solution (5 mg/ml) were added to individual well followed by incubation for 3 hr. 150 µl dimethyl sulfoxide were added to each well and the absorbance was measured at 570 nm using TECAN infinite 200PRO, Switzerland. Proliferation rate was expressed as stimulation index using the following equation

Stimulation index (SI) = Absorbance of stimulated cells /Absorbance of negative control.

In-vivo and In-vitro pinocytosis by peritoneal macrophages

Pinocytosis rate was estimated using Ren et al. (2015). The peritoneal lavage fluid was centrifuged at 2000 rpm for 10 min at 4ºC and the pellet was resuspended in RPMI-1640 medium. 200 µl of 3×10^5cells/ml were seeded on 96 well plate followed by overnight culture at 37 °C under 5% CO_2. The supernatant was discarded and 150 µl of 0.072% neutral red were added to each well and cultured for 0.5 hr at 37ºC. Supernatant was discarded and subsequent to dual wash with PBS buffer, 150 µl of acetic acid: alcohol, 1:1 v/v as lysis solution were added and incubated overnight at 4°C. Absorbance at 570 nm was taken using TECAN infinite for the evaluation of *in-vivo* pinocytosis index. For *in-vitro* analysis, co-suspension of 1x 10^8 cells/ml of peritoneal cells and *Escherchia coli* ATCC was plated on nutrient agar at 37^0C, for 24 h. *E.coli* alone on nutrient agar plate was treated as control and *in-vitro* pinocytosis rate was expressed as bactericidal activity (%) = Control (CFU/ml) – Test (CFU/ml) / Control (CFU/ml)*100

CFU-Colony Forming Units

Quantitation of cytokines and intestinal IgA

Fresh blood (0.5-1ml) was collected via cardiac puncture for serum separation. It was kept standing for 20 min at 37 °C followed by cold centrifugation at 2000 rpm for 10 min at 4^0C. Quantitative evaluation of different cytokines (TNF-α, IL-6 and IL-10) in serum was determined using multiplex bead assay kits (BD biosciences, USA), according to manufacturer's protocols. The secretory IgA values were estimated by ELISA (G-Biosciences, USA) using the supernatant of homogenised colon solution subjected to cold centrifugation at 8000 rpm for 15 min.

Assessment of delayed hypersensitivity reaction

Probiotic impact on delayed hypersensitivity reaction was analysed using carrageenan induced paw edema by subplantar injection using 1% carrageenan in PBS on the eleventh day of probiotic treatment. Animals without any probiotic treatment injected with carrageenan solution and PBS at pH 7.4 was taken as positive and negative control respectively. The acute inflammation induced was measured using verniercaliper after 24 h of administration and the percentage inhibition of delayed hypersensitivity reaction was expressed by the following equation by Solanki *et al*., (2015).

% of inhibition = $(V_t - V_0)$ Control − $(V_t - V_0)$ Treated/ $(V_t - V_0)$ Control × 100

V_t-paw thickness after 6 hr, V_0-paw thickness before administration

Faecal Enzyme Analysis

Fresh faecal samples were collected by gentle rectal palpation on a day before sacrifice (9^{th}, 19^{th} and 29^{th} day). Samples were suspended in cold pre reduced 0.1 M potassium phosphate buffer (pH 7.0) for β-glucuronidase and β-glucosidase assay and in 0.1 M Tris-HCl buffer (pH 7.0) for nitroreductase assay. The faecal suspension was subjected to homogenization, disruption via sonication for 5 min (30 on and off cycle) at 4^0C and cold centrifugation at 500xg for 20 min. The supernatant was used for further analysis and the different specific enzyme activity was determined by using the method described by Verma and Shukla (2013). The total faecal protein concentration was determined by Lowry's method.

β-Glucuronidase

1 ml of the reaction mixture containing a final concentration of 0.01 M potassium phosphate buffer, 1 mM phenolphthalein-β-glucuronide, 0.1 M EDTA and 0.1 ml of sample was run at 37^0C for 15-20 min. 5 ml 0.1 M glycine buffer (pH 10.2) containing 0.1 M NaCl was added to stop the enzymatic reaction and the absorbance was recorded at 540 nm using TECAN infinite. The amount of phenolphthalein released by enzymatic action was compared with standard

phenolphthalein curve and the activity was expressed as microgram of phenolphthalein formed per minute per milligram of faecal protein.

β-Glucosidase

0.2 ml of faecal supernatant was added to 0.8 ml reaction mixture containing a final concentration of 0.2 M phosphate-buffered saline and 1 mM p-nitro phenyl β-pyranoside. The reaction was stopped by the addition of 4 ml of 0.1 M NaOH after incubation at 37°C for 60 min. Absorbance at 420 nm was compared with standard curve of p-nitrophenol and the activity was expressed as micrograms of p-nitrophenol per minute per milligram of faecal protein.

Nitroreductase

500 µl of reaction mixture containing a final concentration of 0.40 mM m-nitrobenzoic acid, 0.5 mM NADPH, 0.1M Tris-HCl buffer and 80 µl of the sample was run at 30^{0}C for 1 h. 300 µl of 1.2 N HCl was added to stop the enzymatic reaction. The amount of m-aminobenzoic acid produced was measured at 540 nm and compared with standard curve of m-aminobenzoic acid. The activity was expressed as microgram of m-aminobenzoic acid formed per hour per milligram of fecal protein.

Statistical Analysis

Statistical analysis of the data obtained was performed by conducting One way analysis of variance by Tukey's test and Student *t*-test (two-tailed). Significance difference taken at p< 0.01- 0.05, values were expressed as mean ± SE (Standard Error of the Mean). All statistical analyses were performed using the software Graph Pad Prism 5.

RESULTS

Isolation and identification of LAB

Based on molecular characterization studies, majority of the selected test isolates were identified as either *Lactobacillus plantarum*(eg:MBTU- S1B (MN 883893) MBTU-S1A (MN 883892)or *Enterococcus faecium*(MBTU- S2C (MN 883895), MBTU- S2a (MN 883897), MBTU-5A(MN 883888) and the details were deposited in Gen Bank under the afore mentioned accession numbers.

In vitro probiotic characterization

Tolerance to gastro intestinal conditions

The main selection criteria of probiotics is based on their potential to survive the harsh environmental conditions provided by GI tract. The isolates lost their survivability at pH 2, shown moderate survivability at pH 3 and better at pH 4 (Table 1).

Bile salt tolerance is a prerequisite for the metabolic activity and colonization of bacteria in the small intestine of the host (Shehata *et al*,. 2016). The test strains showed significant survivability at 0.2 and 0.4% of bile concentrations (Table 1). All the strains exhibited better survivability at a higher concentration of 0.4%.

Tolerance to simulated gastric juice was studied in order to determine transit tolerance (Table 1) MBTU-S1A and MBTU-S2c possessed survivability even at a pH of 2. Most of the studies stated that resistance at pH 3 was set as standards for acid tolerance of probiotic culture (Shehata *et al*., 2016).

Table 1: Tolerance to gastro intestinal conditions: (A)Acid Tolerance at pH 2, 3 and 4 by LAB isolates (Log CFU/mL).(B) Bile Tolerance at 0.2 and 0.4 % by LAB isolates (Log CFU/mL) and (C) Tolerance to simulated gastric juice by LAB isolates (Log CFU/mL). Results were expressed as mean ± Standard error (SE). Values in the column with different superscripts are significantly different, where p < 0.05

		(A) Acid Tolerance								(B) Bile Tolerance						(C) Tolerance to simulated gastric juice					
		pH 2		pH 3		pH 4		pH 6.5		0.2%		0.4%		Control		pH 2		pH 3		pH 7	
SI No	Isolate	0 h	3h	0 h	3 h	0 h	3 h	0 h	3 h	0 h	3 h	0 h	3 h	0 h	3 h	0 h	3 h	0 h	3 h	0 h	3 h
1	MBTU-S1B	7.14±0.019^{a}	-	6.13±0.012^{a}	1.71±0.13^{a}	7.305±0.02^{a}	3.96±0.038^{a}	7.26±0.007^{a}	7.299±0.01^{e}	7.05±0.02^{a}	6.69±0.03^{a}	7.19±0.006^{ab}	5.3±0.07^{a}	7.2±0.007^{a}	8.28±0.005^{a}	7.2±0.022^{a}	-	7.25±0.015^{ab}	3.21±0.08^{a}	7.23±0.019^{a}	7.35±0.016^{ca}
2	MBTU- S1A	7.01±0.025^{ae}	-	7.26±0.005^{b}	2.73±0.4^{b}	7.26±0.01^{a}	5.84±0.02^{b}	7.06±0.003^{b}	7.39±0.03^{ae}	7.1±0.02^{b}	6.26±0.07^{b}	7.26±0.005^{ae}	5.8±0.07^{b}	7.26±0.004^{b}	8.35±0.02^{a}	7.06±0.025^{ab}	1.06±0.07^{a}	7.2±0.008^{a}	3.31±0.72^{b}	7.15±0.022^{a}	7.37±0.04^{c}

3	MBTU-S2C	7.02 ± 0.023^{be}	-	7.28 ± 0.005^{eb}	3.9 ± 0.2^{c}	7.3 ± 0.01^{a}	7.27 ± 0.02^{c}	7.33 ± 0.003^{c}	7.46 ± 0.003^{b}	7.09 ± 0.04^{eb}	6.81 ± 0.03^{ad}	7.28 ± 0.005^{ce}	6.9 ± 0.006^{cd}	7.33 ± 0.003^{c}	8.05 ± 0.007^{b}	7.12 ± 0.06^{a}	0.2 ± 0.1^{b}	7.28 ± 0.005^{ab}	3.99 ± 0.24^{c}	7.23 ± 0.021^{a}	7.42 ± 0.02^{da}
4	MBTU-S2a	7.02 ± 0.003^{ceb}	-	7.28 ± 0.005^{dbc}	5.31 ± 0.02^{d}	7.31 ± 0.008^{b}	7.25 ± 0.02^{dc}	7.33 ± 0.003^{dc}	7.46 ± 0.005^{cb}	7.3 ± 0.01^{d}	7.28 ± 0.01^{c}	7.17 ± 0.005^{adb}	7.05 ± 0.02^{d}	7.3 ± 0.003^{dc}	8.4 ± 0.005^{cb}	7.03 ± 0.015^{b}	-	$7.20.01^{b}$	3.65 ± 0.02^{abc}	7.36 ± 0.009^{b}	7.45 ± 0.002^{bd}
5	MBTU-5A	6.99 ± 0.01^{deb}	-	7.26 ± 0.004^{ebcd}	1.74 ± 0.016^{a}	7.25 ± 0.01^{a}	5.8 ± 0.4^{eb}	7.11 ± 0.06^{e}	7.4 ± 0.02^{abcd}	7.04 ± 0.01^{ac}	6.9 ± 0.03^{d}	7.1 ± 0.06^{b}	6.28 ± 0.08^{e}	7.11 ± 0.06^{e}	7.9 ± 0.2^{a}	7.09 ± 0.01^{a}	-	7.22 ± 0.012^{ab}	2.6 ± 0.3^{a}	7.14 ± 0.052^{a}	7.42 ± 0.02^{bca}

Antagonistic action against pathogens

In the current study, the test strains exhibited significant antimicrobial activity depicted as zone of inhibition against the indicator strains *Vibriocholerae,Salmonella typhi and Salmonella paratyphi* (Figure 1 and Table 2). Among the test isolates MBTU-S1B possessed highest inhibition against the tested pathogens.

Table 2: Antibacterial activity of pathogens against pathogens

No.	Isolate Code	*S.typhi*	*S.paratyphi*	*V. cholerae*
		Zone diameter in milli meter (mm)		
1	MBTU-S1B	15.1±0.1	16.4±0.4	15.5±0.28
2	MBTU-S1A	13±0.28	12.1±0.16	13.3±0.3
3	MBTU-S2C	15±1.04	11.5±0.28	12±1.15
4	MBTU-S2a	14.1±0.4	14.8±0.16	15.1±0.16
5	MBTU-5A	13±0.5	11.3±0.3	13±0.57

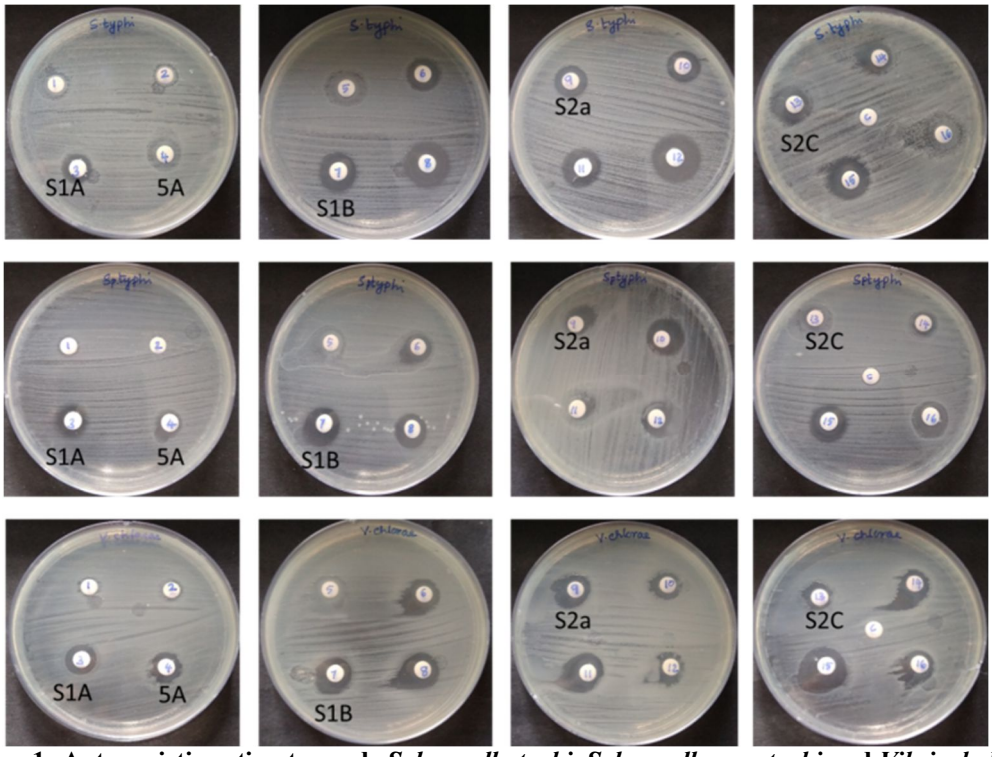

Figure 1: Antagonistic action towards *Salmonella typhi, Salmonella paratyphi* and *Vibriocholerae*

Cell surface properties

Hydrophobicity, Auto-aggregation, and Co-aggregation

The test isolates were evaluated for their cell surface properties using hydrocarbon xylene. All the isolates possessed efficient hydrophobicity and the highest hydrophobicity was retained by the isolate MBTU-S1B of 73.2±2.8 (Table 3). Bacterial aggregation between microorganisms of the same strain (autoaggregation) or between genetically different strains (co-aggregation) is of considerable importance in several ecological niches, especially in the human gut, where probiotics are to be active. In the present study, all the test isolates showed auto aggregation ability within the range of 96.5±0.05 to 98.03±0.03 % (Table 3).Co-aggregation ability of probiotic strain helps in excluding the pathogens prior to the proper adhesion. The test isolates demonstrated co-aggregation against *Salmonella typhi*. (Table 3) and the maximum co-aggregation was observed for MBTU-S2C (65.1±1.9) and MBTU- S1B (65.03±0.01).

Table 3: Cell surface properties of fecal isolates. Results were expressed as mean ± SE. Values in the column with different superscripts are significantly different, where p < 0.05

SI No.	Isolate	Hydrophobicity(%)	Auto aggregation(%)	Co aggregation(%)
1	MBTU- S1B	73.2 ± 2.8^{a}	98.03 ± 0.03^{a}	65.03 ± 0.019^{a}
2	MBTU- S1A	19.63 ± 2.9^{b}	97.34 ± 0.17^{b}	58.1 ± 1.9^{a}
3	MBTU-S2C	58.3 ± 1.193^{c}	97.3 ± 0.011^{b}	65.1 ± 1.9^{a}
4	MBTU-S2a	57.9 ± 0.4^{c}	96.5 ± 0.05^{d}	62.6 ± 3.05^{a}
5	MBTU-5A	58.3 ± 1.02^{c}	97.45 ± 0.15^{b}	49.7 ± 4.8^{a}

Health Promoting Capabilities

Bile salt hydrolase activity and cholesterol assimilation capability

Isolates capable of producing BSH activity generated fine precipitation around the colonies (Figure 2).The results of current study revealed that test isolates assimilates cholesterol under gastro intestinal condition (Table 4) and the maximum cholesterol removel was observed for MBTU-S1B(23.85 %) from the growth medium supplemented with 0.2% bile salt and MBTU-S2a removed 4.54% of cholesterol in the presence of 0.4% bile salt.

Table 4: Cholesterol assimilation capability of test isolates. Results were expressed as mean ± SE. Values in the column with different superscripts are significantly different, where p < 0.05

SI No.	Isolate	Cholesterol removed(%)		Bile Salt Hydrolase activity
		0.2% Bile salt	0.4% Bile salt	
1	MBTU- S1B	23.85 ± 0.17^{a}	1.71 ± 0.36^{a}	-
2	MBTU- S1A	5.26 ± 0.27^{b}	1.19 ± 0.3^{a}	-
3	MBTU-S2C	6 ± 1.08^{b}	1.69 ± 0.41^{a}	+
4	MBTU-S2a	11.96 ± 0.8^{c}	4.54 ± 1.67^{a}	+
5	MBTU-5A	1.83 ± 0.3^{d}	1.03 ± 0.19^{a}	+

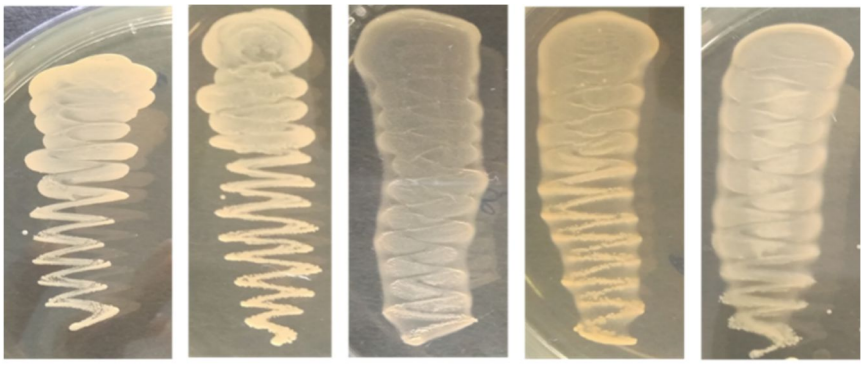

Figure 2: BSH activity of the test isolates. Fine precipitation was observed around the colonies of BSH producers

βGalactosidase activity

In the qualitative test, β galactosidase producing strains exhibited yellow color while in the quantitative method, β –galactosidase enzyme activity of the test isolates were in the range of 1.86± 0.12 to 23.7±0.15 (MillerUnits/ml). Among the testisolates, MBTU- 5A possessed highest β –galactosidase enzyme activity (Figure 3).

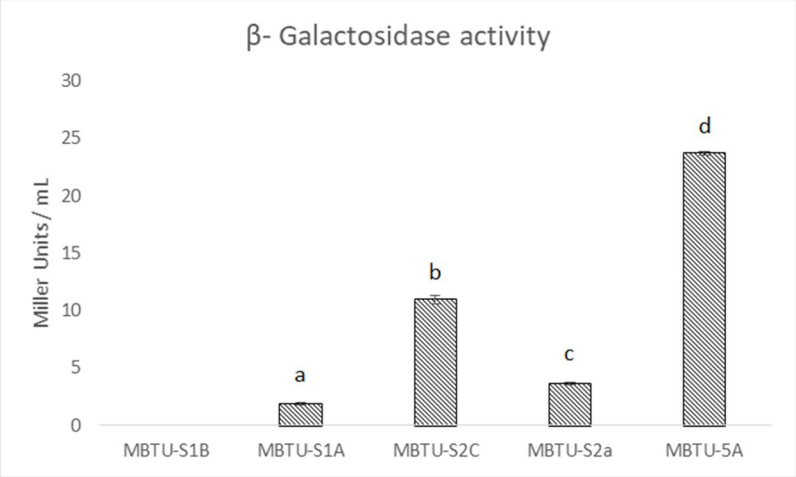

Figure 3: β –galactosidase activity of fecal isolates. Results were expressed as mean ± SE. Values with different superscripts are significantly different, where p < 0.05

ACE inhibitory Activity

ACE inhibitory activity is the most common strategy followed in the selection of antihypertensive hydrolysates or peptides derived from milk proteins. Test isolates generated varying levels of inhibitory activity and the details were furnished in Figure 4.

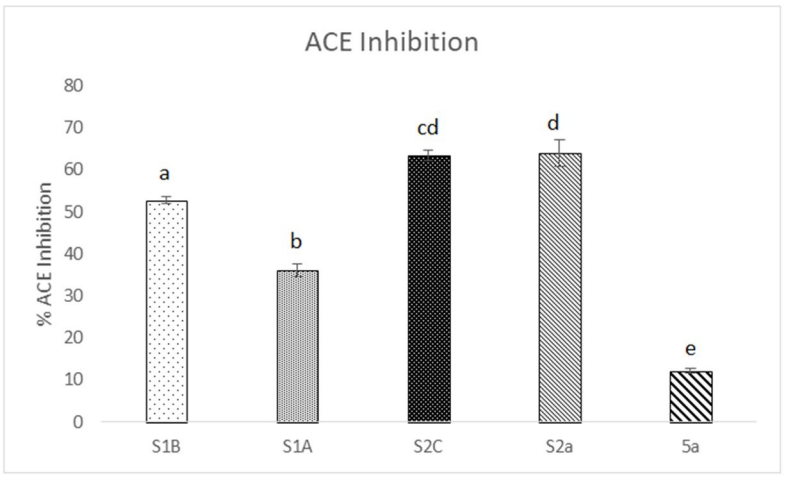

Figure 4: ACE inhibitory activity of fecal isolates. Results were expressed as mean ± SE. Values with different superscripts are significantly different, where p < 0.05

Antioxidant Assay

The DPPH radical is a stable free radical, which has been widely used for estimating the free radical scavenging activities of antioxidants. The scavenging activity expressed by the test isolates were depicted in table 5. The maximum antioxidant activity was observed for cell free supernatant followed by inact cells, sonicated, heat treated and the least activity retained by the lysozyme treated. Among the test isolates, MBTU-S1B possessed the highest antioxidant activity with CFS and after heat treatment. The maximum antioxidant activity was determined in intact cells of MBTU-5A, in lysozyme treated MBTU-S2a and with sonicated cells of MBTU-S2C.

Table 5: Scavenging activity offecalisolates.Results were expressed as mean ± SE. Values in the column with different superscripts are significantly different, where p < 0.05

SI No.	Isolate	Scavenging activity(%)				
		CFS	Intact cells	Lysozyme treated	Sonicated	Heat treated at 100°C
1	MBTU- S1B	43.7± 2.5^{b}	35.29± 6.4^{ab}	20.8± 1.6^{ab}	31.4± 1.64^{b}	36.7±1.97^{b}
2	MBTU- S1A	32.1± 2.01^{a}	17.65± 0.45^{a}	15.1± 0.65^{a}	24.73± 0.81^{a}	11.22±1.46^{a}
3	MBTU-S2C	41.03± 2.08^{ab}	30.16± 2.4^{a}	12.3± 2.2^{ac}	35.06± 1.54^{b}	6.18±0.86^{a}
4	MBTU-S2a	21.6± 1.97^{a}	18.5± 3.13^{a}	25.11± 3.5^{b}	12.24± 1.41^{d}	17.8±1.6^{ac}
5	MBTU-5A	38.9± 2.7^{ab}	37.2± 4.4^{b}	3.7± 0.73^{c}	24.01± 0.85^{a}	23.30± 2.9^{c}

In vivo evaluation of MBTU-S1B (acession no: MN 883893)

In vivo evaluation of probiotic traits was performed in the BALB/c micemodel by using the isolate MBTU-S1B, which was shown to display efficient *in vitro* probiotic potency. The isolate MBTU-S1B was selected for the *in vivo* study since it possessed efficient antioxidant activity, ACE inhibition, cholesterol lowering potential, excellent cell surface properties for sustaining in the GI tract of host and spectacular antagonism against pathogens.

Immune organ indices and lymphocyte proliferation

Compared with control, the immune organ index of test groups (both low and high dose) didn't show any significant difference. Spleen index of all groups on first, second and third sacrifices were found to be 2 ± 0.165, 3 ±0.241 and 3.5 ±0.289 respectively with $p< 0.05$. 2.5-2.8 ± 0.254 were the thymus index of all group on subsequent sacrifices. The *ex-vivo* lymphocyte proliferation using T cell and B cell mitogen demonstrated tolerance against increase in concentration of both mitogens (Figure 5). Basal lymphoproliferation remained unaltered in negative control. The test groups especially the lower dose withstand supraoptimal concentration of mitogens with better resistance to ConA compared to LPS with $p<0.05$-0.01. The low dose test group maintained enhanced tolerance against suboptimal concentration of T cell mitogen upto second sacrifice (20th day sacrifice) however; the third sacrifice results revealed reduction and generated values with par to the control group. The stimulation index of three groups to LPS at different points of sacrifice was in between 0.3-0.5 and they yielded satisfactory tolerance to supraoptimal concentration of LPS without much effect on lymphoproliferation. Contradict to the response against T cell mitogen, low and high dose test groups denoted more or less similar activity against B cell mitogen.

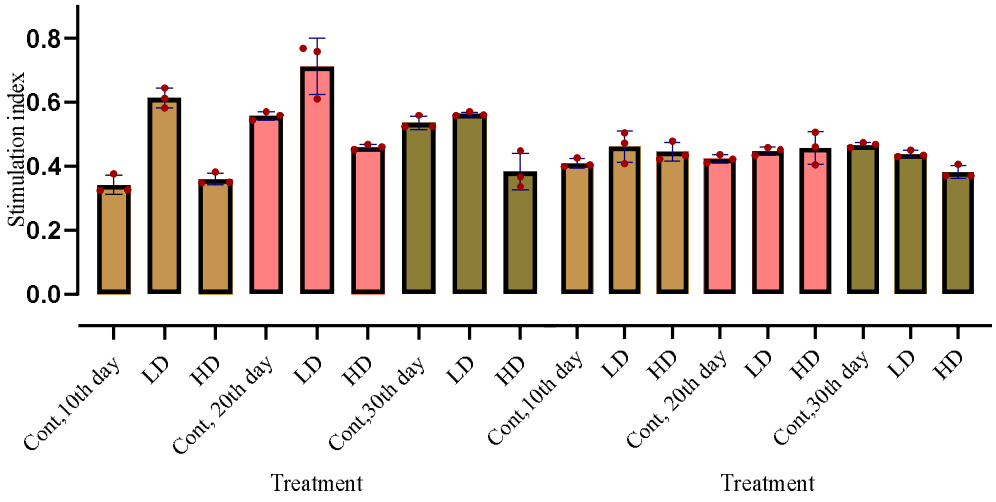

Figure 5: Depicts lymphoproliferation and tolerance against sub optimal, optimal and supraoptimal concentration of mutagen a) against T cell mutagen b) against B cell mutagen. The results expressed as mean ± SE with $p<0.05$-0.01. Cont-control, LD-Low dose, HD-high dose

Non specific cellular immunity

The probiotic treatment resulted significant difference ($p<0.01$) in *in-vivo* phagocytic activity while compared with control group (Figure 6). The *in-vitro* bactericidal activity of three groups including control developed 28-30 ± 0.254, 32-34 ± 0.44 and 17-20 ± 0.496 with $p<0.01$ at three subsequent sacrifices. Unlike to lymphoproliferation assay, the pinocytosis rate under *in-vivo* assay was not reduced after third sacrifice i.e., 20 days posts probiotic supplementation. Moreover, it showed drastic reduction under *in-vitro* conditions.

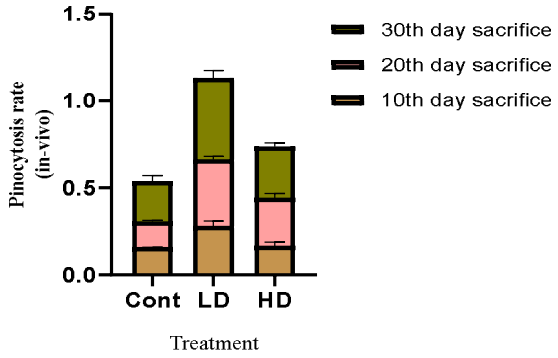

Figure 6: Estimation of *in-vivo* phagocytic activity.The results expressed as mean ± SE with $p<0.01$. Cont-control, LD-Low dose, HD-high dose

Cytokine and IgA profile, evaluation of delayed hypersensitivity reaction

The T cell response was examined based on concentrations of IL-6, IL-10 and TNF α. Except for IL-10, no significant expression was noted for IL-6 and TNF α on subsequent sacrifices. Furthermore, IL-10 generated augmented expression on first sacrifice with $p<0.05$ (Control- 52.2 ± 0.785 pg/ml, Low dose- 69 ± 0.847 pg/ml, High dose- 58.69 ± 0.68 pg/ml) and on subsequent sacrifices the expression was reduced to the basal value of 6-8 ± 0.58 pg/ml with $p< 0.01$.The secretory IgA concentration was determined by ELISA. On day 10; 11 ± 0.689, 19± 0.44, 16± 0.44 were detected for control, low and high dose respectively. The IgA profile (expressed as ng/ml) was elevated on subsequent sacrifices (33 ± 0.254, 35 ± 0.847, 36 ± 0.541 on day 20; 40 ± 0.287, 44 ± 0.381, 42 ± 0.549 on day 30) with significant difference between $p<0.05$-0.01. Carrageenan solution produced progressive edema in hind paw of all tested groups. Low and high dose test group didn't exhibit any remarkable variation on percentage of inhibition (32 ± 0.09) while compared with positive (32 ± 0.26) and negative control group (31 ± 0.18) with $p<0.05$

Faecal enzyme activity

The impact of probiotic therapy on faecal enzyme activity was depicted on Table 6. β-Glucuronidase activity was found to be elevated while compared with control except for high dose at second and third sacrifices. In contrast, β-Glucosidase and Nitroreductase activity was reduced while compared with control on subsequent sacrifices with $p<0.05$.

Table 6: Effect of test isolate on specific faecal enzyme activity at three subsequent sacrifices. The results expressed as mean ± SE with $p<0.05$.Cont-control, LD-Low dose, HD-high dose, NS-non significant

Treatment	β-Glucuronidase(µg/min/mg of fecal protein)			β-Glucosidase(µg/min/mg of fecal protein)			Nitroreductase(µg/min/mg of fecal protein)		
	9th	19th	29th	9th	19th	29th	9th	19th	29th
Cont	0.603± 0.275	0.504± 0.464	0.587± 0.769	0.058± 0.432	0.057± 0.292	0.054± 0.649	0.066± 0.73	0.066± 0.482	0.07± 0.737
LD	0.669 ±0.264*	0.628± 0.353*	0.545± 0.159NS	0.054± 0.534*	0.055± 0.189*	0.049± 0.795*	0.050± 0.189*	0.06± 0.323*	0.052± 0.594*
HD	0.710± 0.359*	0.429± 0.259*	0.471± 0.186*	0.051± 0.189*	0.057± 0.157*	0.051± 0.354*	0.054± 0.575*	0.052± 0.470*	0.050± 752NS

DISCUSSION

The current study focussed on isolation and evaluation of health promoting attributes of LAB from human infant feces,since human gut microbiota is considered as the prime reservoir of probiotic diversity.The isolates used in current study were either *Enterocoocus* or *Lactobacillus* species.The GI tract of newborn rapidly gets colonized by facultative anaerobes including *E. faecium* and are colonized from the first day of birth. The mode of delivery could influence the microbial colonization in the GI tract of infants. Most of the *E. faecium* were observed in infants, they were born vaginally (Zhang *et al.,* 2016). In the present study, the tested strains possessed acid tolerance only at pH 3 and 4. The result indicated that the strains had the capability to survive in the acidic condition of stomach, during their passage to intestine. When the pH increases, there were gradual increase in the growth rate was observed. Acid tolerance of bacteria is important not only for withstanding gastric stresses, but also a prerequisite for their use as dietary adjuncts and enables strains to survive for longer period of time in high acid carrier food without larger reduction in humans (Shehata *et al.,* 2016). As probiotics are usually administrated orally, they must have the ability to survive passage through the stomach and small intestine. All the test isolates possessed better survivability at 0.2 and 0.4% of bile salts. Tolerance to bile salts is a prerequisite for colonization and metabolic activityof bacteria in small intestine of the host and contributes in balancing the

intestinal microflora (Shehata *et al.,* 2016). In the current study, test isolates were able to survive simulated gastric juice at pH 3 after 3 h of incubation, similar findings were previously reported for *L.plantarum* (Honey and Keerthi, 2018). Antagonistic action towards pathogen is a desirable character for probiotic bacteria (Kos et al. 2003). In the present study antibacterial activity of the fecalisolates against the pathogens were verypromising. The inhibitory action may be due to acidity imparted by organic acid present in the supernatant or there is an inhibitory agent (like bacteriocins) whoseactivity is optimum at this acidic pH (Raghavan *et al.*, 2013).

Evaluation of cell surface properties gives an information on the structural properties like aggregation and adhesion ability of probiotic strain (Charteris et al. 1998). Cell surface properties of the test isolates revealed that they were more hydrophobic in nature and more hydrophobicity was retained by the strain of MBTU-S1B, 73.2 ± 2.8 %. The presence of (glyco-) proteinaceous material was indicated by hydrophobicity (Kos *et al*., 2003) and the presence of polysaccharide components attributes the hydrophilicity (Collado *et al*., 2007). Hydrophobicity of the probiotic strains aids in cell-to-cell attachment and intestinal mucosal adherence further boost the barrier function to avert the intestinal leakage and pathogenicity (Ouwehand *et al*., 2001). Cell surface components like proteins, carbohydrates and lipoteichoicacid are involved in cell aggregation and interaction (Boris et al.1997). Hydrophobic quality of test strains were further established by their auto-aggregation ability. Among the test strains MBTU-S1B showed maximum auto-aggregation. Co-aggregation express the cell to cell adherence between genetically different strains .Among the test isolates, MBTU-S1B and MBTU-S2C co-aggregated *S.typhi* efficiently (65.03±0.019 and 65.1±1.9 respectively), allowing them to form a barrier that blocks pathogenic colonization (Boris *et al*., 1997).

BSH activity is a desirable property for a probiotic to be used as a dietary adjuvant, as it aids them to survive the intestinal stress (De Smet *et al*., 1995). The test isolates MBTU-S2C, MBTU- S2a and MBTU- 5A were found to be capable of producing BSH. Bacteria producing bile salt hydrolase have the ability to deconjugate bile salts, play a pivtal role in gut microflora equilibrium and reduction in plasma cholesterol level (Begley *et al*., 2006; Vinderola *et al*., 2008). In both developed and developing countries, hypercholesterolemia is considered as a major risk factor for the development of coronary heart disease. Studies prved that *Lactobacillus* sp. reduce plasma cholesterol level through several mechanisms including bile salt deconjugation (Sieladie *et al*., 2011). Findings of current study revealed that test isolates exhibited cholesterol reduction property as well as BSH activity. Moreover, a negative correlation was also observed between cholesterol reduction by the test strain and concentration of bile salt in the medium. The cholesterol reduction property along with bile salt hydrolysate activity is an added advantage and this points out its utility as starter culture in dairy as well as functional food supplement industry .

β-galactosidase production is one among the well established probiotic effects of LAB identified till date (Charteris *et al*., 1998). The enzyme is widely used in dairy industry and is produced by most of the *Lactobacilli*. The enzyme hydrolyzes lactose in milk into glucose and galactose, which can be absorbed across the intestinal epithelium (Vasiljevic T, 2001). The test isolates of MBTU-S1A, S2C, S2a8 and 5A produced the enzyme β galactosidase with a value of 93-96 Miller units activity. Vinderola and Reinheimer (2003) observed that the value of β galactosidase activity was ranged from 0-2053 Miller units among various probiotic strains of *L. delbruecki* subsp. *bulgaricus.* The symptoms of lactose intolerance can be reduced by eliminating lactose from the diet or by the use of supplemental β galactosidase enzyme replacement (Vasiljevic T, 2001). WHO guidelines considers βgalactosidase production as a mandatory criteria for an ideal probiotic. Hence the discovery of new strains with enhanced β galactosidase activity has gained more attention on infant feed industry.

In the present study, the test isolates also proved their ability to inhibit ACE. Nakamura *et al*., (1995) reported that hypertension can be mediated through inhibition of ACE which plays an important role in the regulation of peripheral blood pressure. More over ACE inhibitory peptide production is a strain dependent characteristic and it also rely on the source of milk (Nedelcheva *et al*., 2010). Significant free radical scavenging activity was observed for test isolates, indicating their potent antioxidant pursuit. The enhanced antioxidant activity was observed with CFS because the CFS serves as a crude collection of metabolites produced by the test isolates, followed by intact cells (the exopolysaacharides componets of cells exerts scavenging property (Seo *et al*., 2015), sonicated, heat treated and the least activity retained by the lysozyme treated. Current research in the antioxidant properties of LAB has shown that, metabolic products reduce the risk of ROS accumulation through food ingestion, and also degrade superoxide anion and hydrogen peroxide ions (Liu *et al*., 2011). Nedelcheva *et al*., (2010) suggested that probiotic bacteria with antioxidant activity in foods increase their biological activity, quality, as well as their shelf life.

In vivo study was carried out with MBTU-S1B to elucidate the dose dependent relationship of probiotics with their health benefitting attributes such as immunomodulation and faecal enzyme activity. Probiotic doses used in the current study were selected based on the routine human consumption levels. The lower test dose corresponds to the average daily intake

of LAB via cheese, yogurt etc and the higher test dose was accomplished by consuming commercial therapeutic probiotic supplements.

The immune organ index is generally used to judge the immunoregulatory aspect of probiotics. In the present study, the probiotic treatment had no significant impact on organ index. The inclusion of sub optimal, optimal and supra optimal concentration of T cell and B cell mitogen enabled the exploration of probiotic treatments on the mitogen concentration-response curve. Ample variation exists among LAB strains with reference to immunomodulating activity. The increase in concentration from suboptimal to supraoptimal may result in a switch over from mitogenicity to cytotoxicity (OM., 1995). All the test groups survived the inhibitory effects of supraoptimal concentration of both mitogens with more emphasis on T cell mitogen. The T cell tolerance and proliferation appeared to be a transient phenomenon that started to gradually subside after second sacrifice. T cell reacts to Con A binding by producing a burst of free radicals which if extended might result in cytotoxic changes similar to apoptosis. The T cell immunomodulatory results from current study revealed that higher dose of probiotic treatment had no significant impact on neither immune tolerance nor proliferation. Moreover, all the test groups developed and maintained almost uniform immunotolerance with slight proliferation to supraoptimal concentration of LPS. LPS play a pivotal role in Gram negative cytotoxic shock and sepsis. Hence, it may be worthwhile to explore the prophylactic potential of probiotic treatments on sepsis as it could augment the resistance of lymphocytes to endotoxins. Besides specific immunity, we also investigated peritoneal macrophage phagocytosis as it is the primary line of host defence against pathogen attack. Low dose probiotic treatment exerted better response under both conditions and this is indicative of its up regulating activity on nonspecific immune responses by release of reactive intermediates from macrophages.

The most interesting aspect of immune modulation by probiotic treatment is through its impact on cytokine production. In the current study, probiotic treatment had no impact on the selected cytokines except IL-10. Furthermore, the rise was noted as a transient phenomenon that existed only during the probiotic treatment. IL-10, an anti inflammatory cytokine plays a pivotal role in regulating immune responses via its control over macrophage and dendritic cell activity. IL-10 inducers would be benefitted in auto immune diseases, allergies and infectious bowel diseases since they are effective in restricting excessive immune responses that could be otherwise detrimental to host life. The effect of probiotic treatment on cytokine profile is strain dependant. This could be the reason for the absence of elevation on TNF-α and IL-6 values, which are considered as one of the essential component of the cell mediated immune mechanism. Furthermore, the test isolate irrespective of the dose generated significant activity on T cell mediated delayed hypersensitivity reactions. Mucosal immunity with more emphasis to secretory IgA constitutes the major defence mechanism in GI tract against invading pathogens. The probiotic treatment resulted in amelioration of secretory IgA level and moreover, the rise was found to be persistent throughout the study.

Several studies suggested that consumption of probiotics had a profound effect on lowering the incidence rate of colorectal cancer. LAB execute this by either degradation of the dietary carcinogens or by reducing the specific activity of microbial faecal enzymes that convert procarcinogens to carcinogens and this attribute was proved to be strain specific (Saarela *et al*., 2003). Bacterial β-glucuronidase and β-glucosidase plays a crucial role in induction of colorectal cancer due to its broad substrate specificity and hydrolysis of various glucuronides (Verma A, 2013). Bacterial Nitroreductase converts nitroso- and *N*-hydroxy dietary compounds to carcinogenic aromatic amines (Haberer *et al*., 2003) . Concluded from the current study, the test isolate proved to cause significant reduction on β-Glucosidase and Nitroreductase specific activity while contrasting effect was noticed for β-Glucuronidase. Taken together, the results of the current study substantiate that higher dose of probiotic would not provide any additional benefit on host body.

Besides maintaining gastrointestinal health, probiotics are now being explored as vehicles for drug/vaccine delivery due to their close coalition with host immunity. Probiotic administration prior to or concurrent with vaccination augment humeral antibody and B cell responses and protect the mucosa from invading pathogen via its direct interaction with innate immune system. In addition, Generally Regarded as Safe Status (GRAS) enables probiotic to act as adjuvant in vaccine/drug distribution system (Abdo *et al*., 2019) . Hence the immune modulating response of the test isolate of the current study has to be exploited further for its diverse implications. The current investigation had certain limitations, eventhough certain strains showed better probiotic efficiency but moreover on considering the overall parameters the study had selected only one strain (*L.plantarum* MBTU-S1B) for the animal study because of its superior *in vitro* probiotic potency.

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Conflicts of Interest: None

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