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Probiotic Screening of Lactobacilli Isolates from Uttapam Batter Fermented Supplementing with *Piper betle* L. Leaves

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Abstract

The *in vitro* screening of lactobacilli isolates from uttapam batter, fermented supplementing with *Piper betle* L. leaves, was performed in order to select potent isolates for probiotic use. Their resistance to simulated gastric and intestinal juices as well as their adhesion to epithelial intestinal HCT-15 and vaginal HeLa cell lines were assessed and also evaluated for their immunomodulatory ability in *ex vivo* condition. Isolates were able to adhere as well as reduce pathogen adhesion to monolayer cell lines' surfaces and reduced production of pro-inflammatory cytokine TNF-α in LPS treated PBMCs, while enhanced that of anti-inflammatory cytokine IL-10. The isolates exhibited properties of auto-aggregation, co-aggregation, hydrophobicity, bile salt hydrolase activity and strong antimicrobial activity against pathogenic bacteria. Thus, the different lactobacilli isolates displayed potent probiotic and immunomodulatory properties among which AJ7 and AJ82 had a great potential and may have applications in fermented foods as immunomodulatory probiotic additives.

Keywords

Acid Tolerance, Adhesion, Immune Modulation, Lactobacilli, Probiotic

1. Introduction

The multidrug resistance of common pathogens to several traditional antibiotics has been a serious worldwide problem which has directed to the discovery of new substitutes and consequently there is an increasing concentration towards the usage of probiotic bacteria for the management of various bacterial and fungal infections [1]

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[2] of digestive tract in humans and animals [3]. Probiotics are live microorganisms that diminish the progression of harmful microbes and encourage well balance of microbiota in the gastrointestinal system [4]. Lactic acid bacteria (LAB) are thought to be dominant in the small intestine [5] and are known to produce lactic acid, hydrogen peroxide, carbon dioxide, diacetyl, bacteriocins [6] [7] which contribute to destroying harmful pathogens *in situ*. LAB are able to interact with the immune system [8] [9] and could act as bio-therapeutic agent in inflammatory bowel disease [9], irritable bowel syndrome [10], travelers' diarrhoea [11], antibiotic associated diarrhoea [12], enteric diseases [13], bacterial infections in oral cavity and vaginosis [14]. In recent years, probiotics gained significance because of their roles in gastrointestinal and immunological aids as well as there has been an increased arrival of probiotic products in the world market [15]. A probiotic microorganism must resist gastric and intestinal juices, adhere mucosal surfaces of intestine, and, additionally, suitable traits could be the production of antimicrobial substances, cholesterol reducing property, and the production of β-galactosidase [16] [17].

During our study on the effect of *Piper betle* L. leaves on fermentation of Uttapam (traditional south Indian fermented food) batter, lactobacilli with potent antimicrobial activity profile were isolated, characterized and identified [18]. The objective of this study was to evaluate these lactobacilli isolates from uttapam batter fermented supplementing with *Piper betle* L. leaves for probiotic properties *in vitro* and immunomodulatory activities *ex vivo* as per WHO/FAO and ICMR/DBT guidelines [16] [17].

2. Materials and Methods

2.1. Microorganisms and Chemicals

The following *Lactobacillus pentosus* and *Weissella confusa* isolates used in this study to evaluate their probiotic potential were already characterized using 16S rRNA gene sequence analysis and their sequences were submitted to GenBank [18]: *Lb. pentosus* AJ7 (JX683265), *Lb. pentosus* AJ9 (JX683266), *Lb. pentosus* AJ15 (JX683267), Lb. *pentosus* AJ82 (JX683270), *Lb. pentosus* AJ94 (JX683271), *Lb. pentosus* AJ99 (JX683272), *Lb. pentosus* AJ112 (JX683273), *Lb. pentosus* AJ116 (JX683274), *Lb. pentosus* AJ125 (KC533686), *W. confusa* AJ53 (KC533685), *W. confusa* AJ67 (JX683268) *W. confusa* AJ79 (JX683269). The indicator strains *Listeria monocytogenes* MTCC657, *Staphylococcus aureus* subsp. *aureus* MTCC737, *Lactobacillus rhamnosus* MTCC1408, *Lactobacillus plantarum* 6160 were procured from MTCC (Microbial Type Culture Collections), Institute of Microbial Technology, Chandigarh, India. The microbial culture media and analytical grade chemicals were procured from HiMedia, (Mumbai, India) and Animal cell culture medium and Histopaque from Sigma-Aldrich, (USA).

2.2. Effect of pH and Bile on Growth of Lactobacilli

The effect of pH and bile on the growth of lactobacilli was studied by growing the isolates in the de Mann Rogosa & Sharpe (MRS) broth adjusted to different pH (3.0, 3.5 and 7.0) using 2 mol·L^{-1} HCl or NaOH and MRS broth supplemented with 0.05%, 0.1%, 0.3% (w/v) of bile and MRS broth without bile at pH 6.5 with initial inoculum of overnight culture of isolates to obtain the final optical density (OD) 0.2 at 600 nm, incubated at 37˚C. The OD readings were taken every hour for eight hour then at twenty-fourth hour in a sterile flat bottom ninety-six well microtitre plate using 200 microliters of culture at 600 nm using ELISA reader (Molecular Devices) [19].

2.3. Resistance to Simulated Gastric Fluid and Intestinal Fluid

The effect of gastric juice on the survival of the isolates was checked at 0, 2 and 4 h because the time from entrance to release from the stomach is 3 - 4 h and similarly, for the intestinal juice also the survivability of isolates was checked at 0, 2 and 4 h [20]. The isolates were propagated overnight in MRS broth, cells were harvested by centrifugation and 10^9 - 10^{10} CFU mL^{-1} were suspended in artificial gastric fluid (NaCl, 0.72 g·L^{-1}; KCl, 0.05 g·L^{-1}; $NaHCO_3$ g·L^{-1}; pepsin, 0.3 g·L^{-1}) adjusted to pH 2.0, 2.5 and 3.0 and incubated for 0, 2 and 4 h. The intestinal juice resistance was studied by exposing the isolates (10^9 - 10^{10} CFU mL^{-1}) to artificial intestinal fluid (0.1% w/v pancreatin and 0.3% w/v bile salts, pH 8.0) for 0, 2 and 4 h [20] [21]. Samples from all trials plated in MRS agar to count colony forming units. The sterile saline (0.85% w/v NaCl) adjusted to pH 7.0 was used as control.

2.4. Bile Salt Hydrolase

The bile salt hydrolase (BSH) activity of LAB isolates was checked by streaking the fresh culture of LAB on BSH medium consisting of MRS Agar supplemented with 0.5% (w/v) sodium salt of TDCA (taurodeoxycholic acid) and 0.37 $g \cdot L^{-1}$ $CaCl_2$. The plates were incubated in an anaerobic jar at 37˚C for 36 - 48 h. Precipitation zones around colonies were considered as positive test for the BSH activity [22].

2.5. Antibiotic Susceptibility Test

The antibiotic susceptibility of the lactobacilli isolates was checked using antibiotic octadiscs (HiMedia, Mumbai, India) towards various antibiotics such as aminoglycosides, penicillins, tetracyclines, cephalosporins, inhibitors of urinary tract antiseptics, nucleic acid synthesis and cytoplasmic membrane functions [23]. The antibiotic discs were placed on the MRS agar media seeded with the LAB isolates and incubated anaerobically at 37˚C for overnight. The susceptibility was recorded by measuring diameter of inhibition zones around the discs.

2.6. Auto-Aggregation

For the aggregation between same cells (auto-aggregation), LAB isolates were grown in MRS broth for overnight at 37˚C followed by harvesting (centrifugation, 10,000 g), washing and re-suspension of pellet in sterile PBS (pH 7.0) and adjusted to 1OD at 600 nm. The cell suspension left for 1 h at room temperature followed by centrifugation at 300 g for 2 min at 20˚C and its OD was read at 600 nm. Auto-aggregation was determined using the following equation.

$$\%\ \text{Auto-aggregation} = \left[\frac{\left(OD_0 - OD_{60}\right)}{OD_0}\right] \times 100$$

OD_0 and OD_{60} refers to the initial OD and OD of the supernatant determined after 60 min, respectively [19].

2.7. Co-Aggregation

The co-aggregation (aggregation between genetically different microorganisms) of LAB isolates was performed with the indicator strains *Lactobacillus plantarum* MTCC 6160 and *Lactobacillus rhamnosus* MTCC 1408 which were grown in 10 mL of MRS broth and *Listeria monocytogenes* MTCC 657 and *Staphylococcusaureus* subsp. *aureus* MTCC 737 grown in 10 mL of Tryptic soya broth at 37˚C [19]. Briefly, the cells were harvested by centrifugation (10,000 g) after overnight incubation, washed and resuspended in sterile PBS and adjusted to 1 OD at 600 nm. One mL of each cell suspension (*i.e.*, indicator strain and LAB strain to be evaluated for probiotic potential) was transferred to a tube and OD recorded at 600 nm. The mixed cell suspension left for 1h at room temperature followed by centrifugation at 300 g for 2 min at 20˚C and its OD was read at 600 nm. The co-aggregation was determined using the following equation.

$$\%\ \text{Co-aggregation} = \left[\frac{\left(OD_{tot} - OD_s\right)}{OD_{tot}}\right] \times 100$$

OD_{tot} and OD_S refers to the initial *OD* taken immediately after the relevant strains mixed and *OD* of the supernatant after 60 min respectively.

2.8. Hydrophobicity

The hydrophobicity assay is an index to measure the ability of isolates to adhere the mucosal surfaces of epithelial lining [23]. The LAB isolates were grown in MRS broth for overnight at 37˚C followed by harvesting, washing and re-suspension of pellet cells in sterile PBS (pH 7.0) and adjusted to *OD* of 1 at 600 nm. A sample of 1.5 ml LAB suspension was mixed to 1.5 ml of n-hexadecane by vortex and incubated 30 min at room temperature for the separation of aqueous and organic phases. The *OD* of aqueous phase was read at 600 nm [22]. The hydrophobicity percentage was calculated as follows

$$\%\ \text{Hydrophobicity} = \left[\left(OD1 - OD2\right)/OD1\right] \times 100\%$$

where *OD*1 refers to the initial *OD* of the cell suspension and *OD*2 refers to the *OD* of the cell suspension in aqueous phase after incubation of 30 min.

2.9. LAB Adhesion and Inhibition of Pathogen Adhesion to Cell Lines

A probiotic strain must adhere the intestinal mucosal surfaces to act *in situ* and provide gut health benefits. To evaluate the adhesion ability of isolates to intestinal mucosa, epithelial intestinal HCT-15 cell line was chosen [24]. The adhesion of LAB isolates and inhibition of pathogen adhesion to HCT-15was evaluated. Additionally, it is recognized that the application of probiotics can go beyond gut health and it can be used as suppository for vaginal health and thereby may reduce the risk of urinary tract infections, bacterial vaginosis and yeast vaginosis by balancing the microflora [25]-[27]. To study the adhesion ability of isolates at vaginal mucosa, vaginal HeLa cell line was chosen [26] [27]. Both the cell lines were maintained in the DMEM medium supplemented with 10% of fetal bovine serum (FBS), 2 5 mM HEPES, 50 μg·mL^{-1} penicillin, 50 μg·mL^{-1}gentamicin, 50 μg·mL^{-1} streptomycin and 1.25 μg·mL^{-1} amphotericin-B. The cell lines were used for assay when reached to 90% to 95% confluences. For the LAB adhesion and inhibition of pathogen adhesion to the cell lines, monolayers of cell lines were maintained in the six well tissue culture plates (Tarson, India) and the LAB isolates and pathogen were suspended in serum and antibiotic free DMEM medium. The monolayers of cell lines were washed with the PBS thrice to remove antibiotics followed by the addition of LAB suspensions (10^{10} - 10^{11} CFU mL^{-1}) and kept for 90 min at 37˚C [20]. After incubation, wells were washed gently with PBS thrice and the non-adherent recovered LAB cells were plated over MRS agar. The adhesion of LAB isolates were calculated as the amount of adhered bacterial cells, compared to the initial amount of LAB cells added to the cell lines with the following formula,

Amount of adherent LAB cells = log (A) − log (B)

Where A is the No. of viable LAB cells added to the well for adhesion and B is the No. of viable LAB cells non-adhered to the cell lines in the well.

The culture wells, subsequent to incubation with LAB isolates and removing the non-adhered bacterial cells with PBS wash, were fixed with 2% formaldehyde followed by the staining with 0.1% acridine orange dye and observed under fluorescent microscope. For the pathogen (*Listeria monocytogenes* MTCC 657) exclusion assay, the monolayers were first treated with the LAB isolates (5×10^{10} - 10^{11} CFU mL^{-1}) for 90 min, washed with PBS to remove non-adhered LAB followed by addition of *Listeria* cells (2×10^{10} CFU mL^{-1}) suspended in serum and antibiotic free medium and incubated for 60 min [20]. The excluded *Listeria* cells were recovered by washing with the PBS and were plated on *Listeria* selective agar [28]. The amount of excluded *Listeria* cells were calculated as the amount of *Listeria* cells recovered after incubation compared to initial amount added to the cell lines with the following formula,

Amount of excluded *Listeria* cells = log (*X*) − log (*Y*)

where *X* is the no. of viable *Listeria* cells added to the well for adhesion and *Y* is the no. of viable *Listeria* cells adhered to the cell lines in the well.

2.10. Isolation of Human Peripheral Blood Mononuclear Cells (hPBMCs) and Induction of Cytokine Release

The hPBMCs were isolated from the blood of healthy human donor. Briefly, 3.0 mL of Histopaque 1077 (Sigma Aldrich, USA) was placed in a 15 mL centrifuge tube and 3.0 mL of heparinized whole blood was layered on the top followed by centrifugation (400 g, 30 min at room temperature). The upper plasma layer was removed and discarded. The cells separated at the interface was taken up by aspiration and washed with PBS. The cell viability test was determined by trypan blue exclusion and the cell density was adjusted to 10^{7} cells mL^{-1} in RPMI-1640 medium supplemented with 10% autologous serum, 50 μg·mL^{-1} penicillin, 50 μg·mL^{-1} gentamicin, 50 μg·mL^{-1} streptomycin and 1.25 μg·mL^{-1} amphotericin-B and incubated at 37˚C for overnight in a suspension flask. The hPBMCs were incubated with the LPS (1.0 μg·mL^{-1}) for 30 min before exposed to LAB isolates. Fresh bacterial suspensions of LAB isolates (10^{8} CFU mL^{-1}) were added to hPBMCs (10^{7} cells mL^{-1}) resulting in bacteria-to-cell ratio 10:1 and co-incubated for 24 h in antibiotic and serum free RPMI-1640 medium at 37˚C in a 12-well tissue culture plates [29]. After incubation the supernatants were collected and used immediately for estimation of cytokines. Because, Lipopolysaccharide (LPS) provokes host cells to produce pro-inflammatory mediators including TNF-*α*, IL-1*α*, IL-6 by activating several types of transcription factors, LPS stimulated

hPBMCs culture was used to study the anti-inflammatory potential of probiotic strains for reduction in TNF-α. The anti-inflammatory cytokine IL-10 was also measured in the supernatant of hPBMCs. The assays for both the pro-inflammatory (TNF-α) and anti-inflammatory (IL-10) cytokines were performed twice independently in duplicates [29]. TNF-α and IL-10 cytokines were measured using anti-human TNF-α and anti-human IL-10 monoclonal antibodies respectively by sandwich ELISA kit (Diasource) as per manufacturer's recommendations.

2.11. Inhibition of *Listeria* by Lactobacilli (LAB) Isolates *in Situ*

In situ inhibition of *Listeria* by LAB isolates was studied by co-culturing both LAB and *Listeria* for different time intervals and subsequently their CFU were calculated. The LAB isolates were grown in nutrient broth till the OD reached 0.8 at 600 nm. Overnight culture of *Listeria monocytogenes* MTCC657 grown in nutrient broth was added to the LAB culture and incubated at 30˚C under shaking condition (180 rpm) in a shaker incubator. The CFU in the co-culture were calculated for LAB and *Listeria* by selectively plating over MRS agar and *Listeria* selective agar respectively at 0, 3, 6, and 9 h of intervals. A control LAB and control *Listeria* culture were also processed in similar way.

3. Results

3.1. Resistance to Simulated Gastric Fluid and Intestinal Fluid

The *Lb. pentosus* isolates were growing in the MRS broth containing 0.3% of bile (normal concentration in the intestine of healthy man) [4] [30] whereas no growth was observed in case of *W. confusa* isolates AJ53, AJ67 and AJ79 (**Table 1**) and were excluded for the further studies. Their resistance to simulated gastric and intestinal juices is shown in **Table 2**. Based on the growth profile in acidic conditions and presence of bile salts (**Table 1**) and survivability in the simulated gastric and intestinal juices (**Table 2**), six isolates AJ7, AJ9, AJ15, AJ82, AJ99 and AJ112 were chosen for the cell line adhesion studies.

3.2. Bile Salt Hydrolase Activity

The isolates AJ9, AJ99 and AJ116 were BSH negative, while others were positive (**Table 3**).

Table 1. Effect of pH and bile on growth of lactobacilli isolates.

Isolates	pH		Bile %			
	3.0	3.5	7.0	0.05	0.10	0.30
AJ7	++	+++	++++	++++	+++	++
AJ9	+	++	++++	++++	+++	++
AJ15	++	+++	++++	++++	+++	++
AJ53	−	−	++++	++	+	−
AJ67	−	−	++++	++	+	−
AJ79	−	−	++++	++	+	−
AJ82	++	+++	++++	++++	+++	++
AJ94	+	++	++++	++++	+++	++
AJ99	++	+++	++++	++++	+++	++
AJ112	+	++	++++	++++	+++	++
AJ116	+	++	++++	++++	+++	++
AJ125	+	++	++++	++++	+++	++

Legends: −, no growth; +, very weak growth, ++, weak growth, +++, good growth, ++++, luxurious growth.

Table 2. Survivability of lactobacilli isolates in simulated gastric and intestinal juices.

Isolates	Conditions	log CFU/mL		
		0	2	4 h incubation
AJ7	Control (pH 7.0)	10.0 ± 0.09	9.9 ± 0.12	9.9 ± 0.04
	Gastric juice (pH 2.0)	9.7 ± 0.04	7.1 ± 0.03	5.2 ± 0.02
	Gastric juice (pH 2.5)	9.9 ± 0.11	7.3 ± 0.14	5.8 ± 0.02
	Gastric juice (pH 3.0)	10.0 ± 0.07	7.7 ± 0.13	6.3 ± 0.13
	Intestinal juice (pH 8.0)	10.4 ± 0.05	10.1 ± 0.11	9.6 ± 0.05
AJ9	Control (pH 7.0)	10.2 ± 0.02	9.9 ± 0.17	9.8 ± 0.06
	Gastric juice (pH 2.0)	9.7 ± 0.07	5.5 ± 0.14	4.8 ± 0.23
	Gastric juice (pH 2.5)	9.7 ± 0.34	5.9 ± 0.09	5.7 ± 0.01
	Gastric juice (pH 3.0)	10.3 ± 0.26	6.0 ± 0.04	5.7 ± 0.40
	Intestinal juice (pH 8.0)	10.3 ± 0.15	9.9 ± 0.14	9.8 ± 0.04
AJ15	Control (pH 7.0)	10.2 ± 0.06	10.1 ± 0.03	9.6 ± 0.13
	Gastric juice (pH 2.0)	9.7 ± 0.05	5.5 ± 0.07	4.8 ± 0.19
	Gastric juice (pH 2.5)	9.8 ± 0.07	6.3 ± 0.28	5.5 ± 0.12
	Gastric juice (pH 3.0)	10.3 ± 0.13	6.3 ± 0.28	6.2 ± 0.16
	Intestinal juice (pH 8.0)	10.2 ± 0.09	10.0 ± 0.21	9.6 ± 0.21
AJ82	Control (pH 7.0)	10.1 ± 0.02	10.1 ± 0.12	9.8 ± 0.03
	Gastric juice (pH 2.0)	8.8 ± 0.04	3.2 ± 0.02	2.0 ± 0.08
	Gastric juice (pH 2.5)	9.3 ± 0.07	4.8 ± 0.03	4.0 ± 0.04
	Gastric juice (pH 3.0)	9.3 ± 0.04	5.4 ± 0.06	4.2 ± 0.05
	Intestinal juice (pH 8.0)	10.1 ± 0.09	10.1 ± 0.12	9.6 ± 0.06
AJ94	Control (pH 7.0)	10.2 ± 0.04	10.0 ± 0.05	9.8 ± 0.21
	Gastric juice (pH 2.0)	7.1 ± 0.05	0.0	0.0
	Gastric juice (pH 2.5)	9.4 ± 0.62	5.1 ± 0.18	0.0
	Gastric juice (pH 3.0)	10.1 ± .21	5.3 ± 0.04	0.0
	Intestinal juice (pH 8.0)	10.1 ± 0.03	10.0 ± 0.09	9.7 ± 0.15
AJ99	Control (pH 7.0)	10.5 ± 0.12	10.1 ± 0.08	9.8 ± 0.38
	Gastric juice (pH 2.0)	10.2 ± 0.05	3.5 ± 0.05	0.0
	Gastric juice (pH 2.5)	10.1 ± 0.04	4.0 ± 0.04	0.0
	Gastric juice (pH 3.0)	10.4 ± 0.07	8.9 ± 0.03	6.4 ± 0.04
	Intestinal juice (pH 8.0)	10.4 ± 0.04	10.2 ± 0.24	10.1 ± 0.32
AJ112	Control (pH 7.0)	10.1 ± 0.04	10.1 ± 0.03	10.0 ± 0.06
	Gastric juice (pH 2.0)	7.5 ± 0.09	3.5 ± 0.07	0.00
	Gastric juice (pH 2.5)	9.9 ± 0.07	4.5 ± 0.04	2.0 ± 0.07
	Gastric juice (pH 3.0)	10.9 ± 0.08	6.2 ± 0.08	5.9 ± 0.08

Continued

	Intestinal juice (pH 8.0)	10.3 ± 0.12	10.1 ± 0.07	10.0 ± 0.07
AJ116	Control (pH 7.0)	10.1 ± 0.03	10.0 ± 0.02	9.8 ± 0.06
	Gastric juice (pH 2.0)	7.3 ± 0.14	3.2 ± 0.08	0.00
	Gastric juice (pH 2.5)	9.7 ± 0.02	4.3 ± 0.04	0.00
	Gastric juice (pH 3.0)	10.3 ± 0.05	6.6 ± 0.02	4.4 ± 0.01
	Intestinal juice (pH 8.0)	10.4 ± 0.01	10.2 ± .09	10.0 ± 0.14
AJ125	Control (pH 7.0)	10.1 ± 0.11	10.1 ± 0.09	9.7 ± 0.07
	Gastric juice (pH 2.0)	5.4 ± 0.02	0.00	0.00
	Gastric juice (pH 2.5)	9.4 ± 0.10	2.3 ± 0.04	0.00
	Gastric juice (pH 3.0)	10.4 ± 0.08	3.8 ± 0.09	0.00
	Intestinal juice (pH 8.0)	9.7 ± 0.02	9.5 ± 0.09	9.3 ± 0.02

Table 3. Bile salt hydrolase, hydrophobicity, auto-aggregation and co-aggregation and cell line adhesion assays.

Characteristics	**LAB Strains**								
	AJ7	**AJ9**	**AJ15**	**AJ82**	**AJ94**	**AJ99**	**AJ112**	**AJ116**	**AJ125**
Bile salt hydrolase	+	−	+	+	+	−	+	−	+
Hydrophobicity (%)	65	22	19	90	89	78	80	77	21
Autoaggregation (%)	62	45	34	41	46	63	45	40	47
Co-aggregation (%) with									
Staphylococcus aureus	53	52	57	50	52	55	57	49	47
Listeria monocytogenes	66	63	47	62	62	60	62	46	46
Lactobacillus plantarum	42	41	31	50	42	39	38	35	33
Lactobacillus rhamnosus	48	26	24	45	43	47	45	48	57
Cell adhesion*									
HCT-15	2.05	0.46	0	2.25	ND	2.3	1.46	ND	ND
HeLa	3.83	0.26	0.7	3.19	ND	4.64	4.47	ND	ND
Listeria exclusion**									
HCT-15	9.83	6.83	0	9.74	ND	9.89	9.02	ND	ND
HeLa	10.07	8.88	8.89	10.12	ND	9.98	10.12	ND	ND

The values are mean of two independent experiment performed in duplicates, Legends: +, positive; −, negative; ND, not determined, *calculated by formula: amount of adhered LAB cells = log (*A*) – log (*B*), where *A* is the number of viable LAB cells added to the well and *B* is the number of viable non-adhered LAB cells, **calculated by formula: amount of excluded *Listeria* by LAB cells = log (*X*) – log (*Y*), where *X* is the number of viable *Listeria* cells added to the well and *Y* is the number of viable adhered *Listeria* cells.

3.3. Antibiotic Susceptibility

The LAB isolates were found to be susceptible to most of the antibiotics screened (**Table S1**). All lactobacilli isolates were sensitive to penicillin, ampicillin, erythromycin, ciprofloxacin, gentamicin, lincomycin, chloramphenicol, streptomycin, amikacin, cephaloridine and tetracycline. However, all the isolates were resistant to colistin.

3.4. Auto-Aggregation, Co-Aggregation and Hydrophobicity

The auto-aggregation between lactobacilli isolates varied from 34% to 63%, while co-aggregation between LAB and non-LAB strains varied from lowest 24% to highest 57% (**Table 3**). The hydrophobicity of LAB isolates

were ranged from least 19% (AJ15) to maximum 90% (AJ82) (**Table 3**).

3.5. LAB Adhesion and Inhibition of Pathogen Adhesion to Epithelial Cell Lines

The ability of six selected isolates to adhere to epithelial cell lines and pathogen exclusion is shown in **Table 3**. The isolateAJ15 neither adhered to intestinal HCT-15 cell line and nor inhibited the adhesion of *Listeria* pathogen, however, strains AJ7, AJ9, AJ82, AJ99 and AJ112 were able to adhere to both the epithelial cell lines HCT-15 and HeLa (**Figure 1(A)** & **Figure 1(B)**) considerably better or similar percentage than other lactobacilli strains evaluated in literature [26] as well as inhibited the adhesion of *Listeria* cells greatly (**Table 3**).

3.6. Immunomodulation

The production of cytokines TNF-α and IL-10 by four selected LAB isolates (AJ7, AJ82, AJ99 and AJ112) was used as an index of immunomodulation (**Table 4**). The TNF-α induction was higher in control sample of LPS treated hPBMCs than the hPBMCs incubated with LAB strains. However, there was weak induction of TNF-α in the case of hPBMCs incubated with both the LPS and LAB together (**Table 4**). It indicates that LAB isolates AJ7, AJ82, AJ99 and AJ112 were able to reduce induction of pro-inflammatory cytokine TNF-α in LPS treated hPBMCs. The stimulation of anti-inflammatory cytokine IL-10 was observed in case of hPBMCs incubated with both the LPS and LAB strains independently and together. The induction of IL-10 in hPBMCs incubated with the LPS was weak in comparison to the hPBMCs incubated with LAB and hPBMCs incubated with LPS and LAB strains both (**Table 4**). The results show that these isolates AJ7, AJ82, AJ99 and AJ112 induce low TNF-α and high IL-10.

3.7. *In Situ* Inhibition of *Listeria*

The two potent isolates AJ7 and AJ82 displayed a very good antagonistic activity against *Listeria monocytogenes* MTCC657 in nutrient broth when co-cultured (**Figure 2(A)** & **Figure 2(B)**). The isolates AJ7 and AJ82 competitively inhibited *Listeria*. The individual control cultures of both the LAB and *Listeria* had grown exponentially whereas the number of viable *Listeria* cells when co-cultured with LAB isolates were dramatically decreased to zero at 9 h of co-incubation probably owing to the effects of various antimicrobial substances released by LAB [6]. On the other hand, the LAB isolates co-cultured with *Listeria* grew similar to control LAB culture.

4. Discussions

One of the essential properties of a probiotic is inhibition of the growth of pathogenic microorganisms. In the present study, the LAB isolates screened for probiotic had inhibited the growth of different pathogens owing to the production of various antimicrobial substances [6] [18]. In the earlier study these LAB isolates were found to

Table 4. Induction of cytokines TNF-α and IL-10.

Sample	TNF-α (pg/mL)	IL-10 (pg/mL)
hPBMCs + RPMI (blank)	0	0
hPBMC + RPMI + LPS	145.7 ± 6.28	168.2 ± 16.05
hPBMC + RPMI + LPS + AJ7	68.8 ± 4.58	268.8 ± 4.50
hPBMC + RPMI + LPS + AJ82	62.7 ± 2.80	292.4 ± 9.50
hPBMC + RPMI + LPS + AJ99	68.4 ± 8.69	242.0 ± 3.50
hPBMC + RPMI + LPS + AJ112	71.8 ± 4.58	242.8 ± 19.50
hPBMC + RPMI + AJ7	32.5 ± 3.87	189.7 ± 5.50
hPBMC + RPMI + AJ82	21.3 ± 0.70	239.1 ± 11.50
hPBMC + RPMI + AJ99	31.6 ± 3.80	196.9 ± 2.45
hPBMC + RPMI + AJ112	34.4 ± 2.00	208.7 ± 13.75

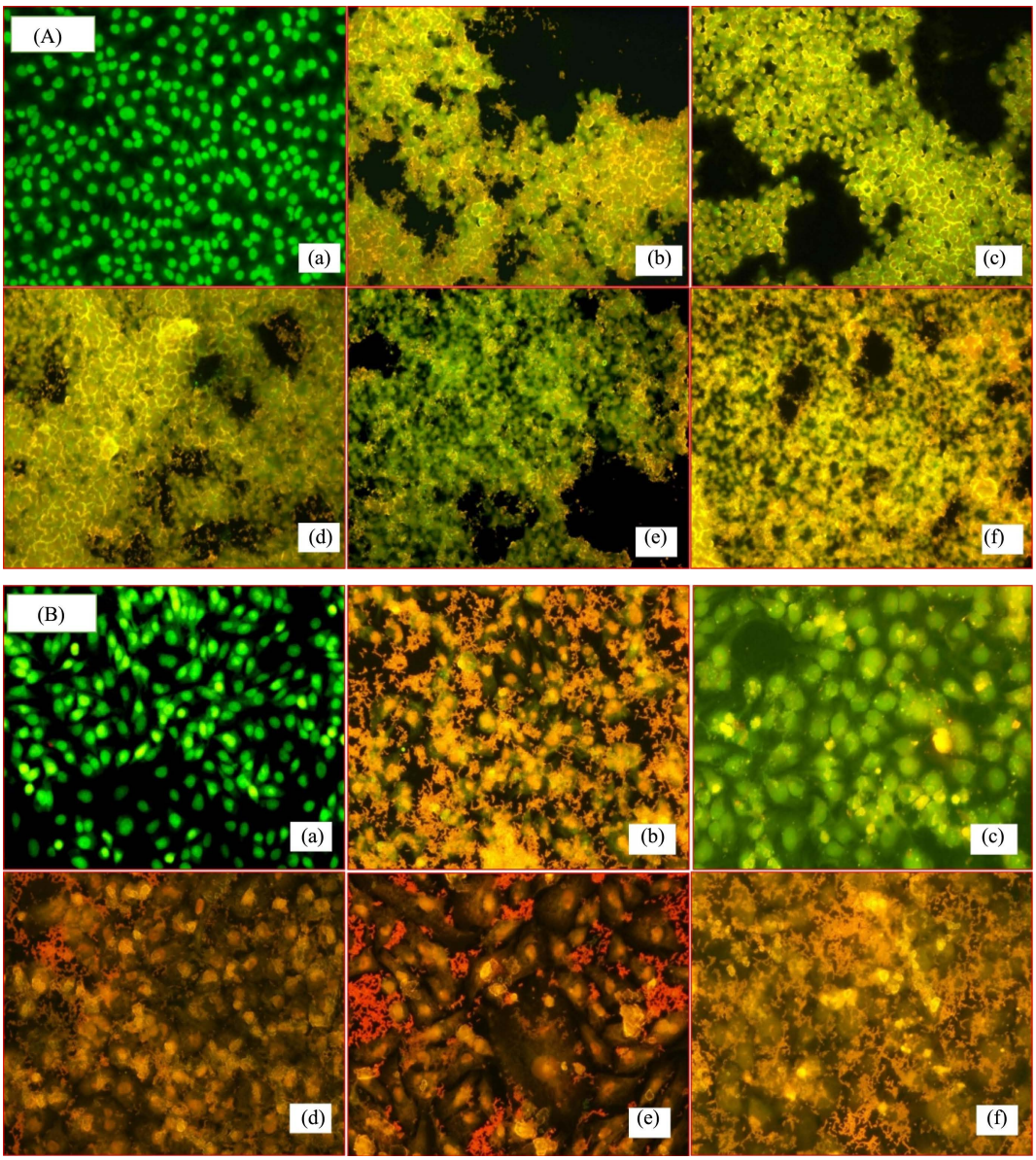

Figure 1. (A) Adhesion of LAB isolates on HCT-15 cell line: the control is only HCT-15 with no LAB isolate (a) Control, (b) *Lb. pentosus* AJ7, (c) *Lb. pentosus* AJ15, (d) *Lb. pentosus* AJ82, (e) *Lb. pentosus* AJ99, (f) *Lb. pentosus* AJ112; (B) adhesion of LAB isolates on HeLa cell line: the control is HeLa cell line alone without LAB isolate (a) Control, (B) *Lb. pentosus* AJ7, (c) *Lb. pentosus* AJ15, (d) *Lb. pentosus* AJ82, (e) *Lb. pentosus* AJ99, (f) *Lb. pentosus* AJ112.

be bacteriocinogenic [18]. However, the LAB isolates did not pose much threat to other LAB strains *Lactobacillus rhamnosus* MTCC1408 and *Enterococcus faecalis* MTCC439.

The probiotic strain intended for oral delivery has to travel through the GI tract of the host and therefore it must resist the physiological conditions of the gut such as acidic pH of gastric fluid and presence of bile salts in order to arrive at the action site. The LAB strains in the present study were resistant to acidic pH of artificial gastric fluid and bile concentration of artificial intestinal fluid. The LAB isolates were able to grow at the concentration of intestinal bile in human GIT (0.3% w/v) as well. WHO/FAO recommends a probiotic strain with an ability to hydrolyze bile salts as the BSH positive microorganisms are known to remove cholesterol [31]. The BSH positive isolates of this study may offer a promising use as anti-cholesterolaemic agents for functional foods. Another requirement for a probiotic is that it should not carry transmissible genes for antibiotic resistance

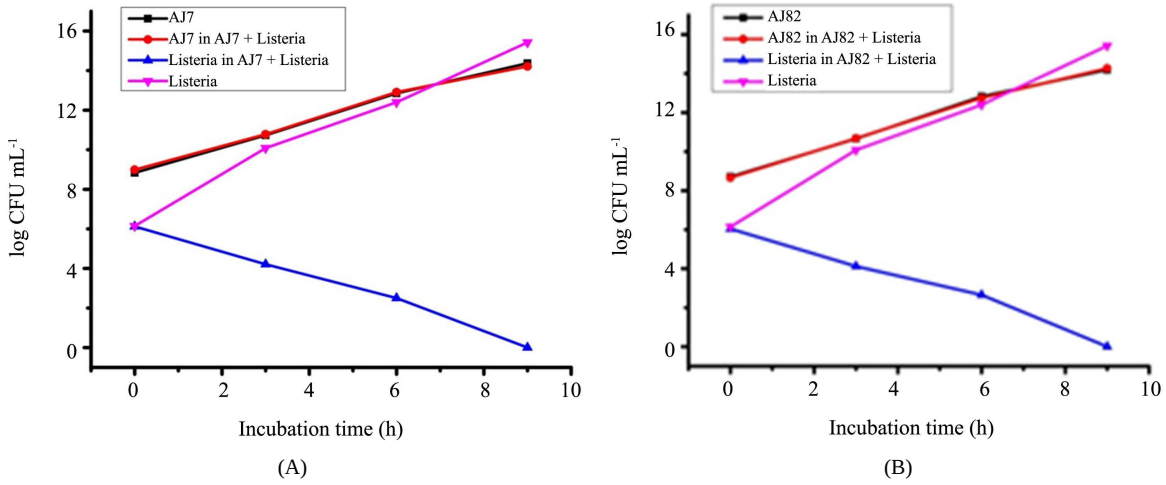

Figure 2. *In situ* Inhibition of *Listeria* by LAB isolates (A) *Lb. pentosus* AJ7 and (B) AJ82.

[29] otherwise undesirable microbes in the gut may acquire resistance via horizontal gene transfer. Since the screened LAB isolates were susceptible to most of the antibiotics resistance transfer cannot happen, however, this necessitates frequent administration of these organisms incorporated foods or the organisms per se in large quantity.

All the LAB isolates had shown high auto-aggregation and co-aggregation characteristics. The aggregation between the same LAB isolates aids in colonization thereby preventing pathogen adhesion in the intestinal mucosa [20] and the co-aggregation of LAB and pathogens enables the LAB to find the pathogen in a close proximity and eliminate pathogen by releasing antimicrobial substances [20]. The antimicrobial substances released by aLAB isolate should inhibit the co-aggregating pathogenic microbe partner otherwise both may co-exist and form mixed culture biofilm. Conversely, a LAB strain co-aggregating with other LAB strain or other normal flora should not antagonistic to each other. In this study, all the LAB isolates had shown high level of hydrophobicity (except AJ9, AJ15 & AJ125). An isolate with higher percentage of hydrophobicity would have strong adhesion characteristics. The adhesion ability of a probiotic strain to the epithelial mucosal surfaces is pivotal for its colonization and *in situ* antagonism against pathogens subsequently [23]. The LAB isolates adhered to cellline effectively which appears to be a *sine quo non* for desirable probiotic benefit. It is reported, cell wall composition profile may influence the adhesion of LAB cells to the epithelial cells [4] and the variability observed in the adhesion profile of LAB isolates may be attributed to their variable cell wall composition profile [32]. The adhered LAB isolates to the epithelial cells serve as physical barrier to non-desirable agents. Therefore, the isolates with good adhesion and pathogen exclusion profile (AJ7, AJ82, AJ99, and AJ112) for both the cell lines HCT-15 and HeLa can be applicable to both the anatomic locations, GIT as well as vagina for potential biotherapeutic effects subsequent to their in vivo evaluation.

Probiotics may also provide health benefits via immune modulations. Microorganisms including·Lactobacilli are able to cross the intestinal mucous layer and can penetrate the gut wall either through epithelial layer or Peyer's patches. Subsequently, the microbial components interact with the immune cells and induce the production of cytokines, chemokines and other innate effectors [33]. The four isolates screened (**Table 4**) were able to reduce the induction of pro-inflammatory cytokine (TNF-α) and enhanced that of anti-inflammatory cytokine (IL-10).

The immunomodulation ability of a microorganism is believed to be strain specific [34] which varies from one strain to another. The induction of cytokines TNF-α and IL-10 by LAB strains was more or less similar to the other reports [29] [34]. Thus these isolates can be used as anti-inflammatory strains. A probiotic has to act *in situ* to inhibit the undesirable microorganisms. The two selected isolates AJ7 and AJ82 had inhibited *Listeria* strain *in situ* when co-cultured in nutrient broth owing to the effects of antimicrobial substances [18].

5. Conclusion

The uttapam batter fermented supplementing with the *Piper betle* L. leaves may offer a promising source for

probiotic isolation. The different isolates were resistant to acidic pH, able to adhere human epithelial cell lines, antagonistic to different food borne pathogens and immunomodulatory. Our results had shown that the two Lactobacillus pentosus isolates AJ7 and AJ82, with anti-inflammatory effects, possessed the properties to be used as potential probiotic additives to foods subsequent to their in vivo screening.

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Supplementary Table

Table S1. Antibiotic susceptibility of the lactobacilli isolates.

		Lactobacilli isolates								
Antibiotic	**Amount**	**AJ7**	**AJ9**	**AJ15**	**AJ82**	**AJ94**	**AJ99**	**AJ112**	**AJ116**	**AJ125**
Cefuroxime	30 mg	R	S	S	R	R	R	R	R	R
Cephalexin	30 mg	S	S	S	S	R	S	S	S	R
Cephradine	30 mg	S	S	S	S	R	S	R	S	S
Cloxacillin	5 mg	S	S	S	S	S	S	R	S	S
Co-trimazine	25 mg	S	S	S	S	S	S	S	S	S
Co-trimoxazole	25 mg	S	S	S	S	S	S	S	S	S
Mecillinam	33 mg	R	R	S	R	R	S	R	S	R
Nalidixic acid	30 mg	R	R	R	R	R	R	R	S	R
Nitrofurantoin	300 mg	S	S	S	S	S	S	S	S	S
Norfloxacin	10 mg	S	R	S	S	R	S	S	S	R

Legends: R, resistant; S, sensitive. All the isolates were sensitive to penicillin-G, tetracycline, lincomycin, gentamicin erythromycin, ciprofloxacin, chloramphenicol, streptomycin, amikacin, cephaloridine and ampicillin while resistant to colistin.