EVALUATION OF PROBIOTIC PROPERTIES OF ISOLATED LACTIC ACID BACTERIA AND THEIR ASSESSMENT FOR SYNBIOTIC FORMULATION

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Abstract
Background: In the present studies lactic acid bacteria were isolated i.e. Lactococcus lactis sub-species cremoris from curd and Pediococcus pentosaus from germinating wheat. The purpose of this study is to evaluate the probiotic properties of lactic acid bacteria and to formulate the synbiotic using various prebiotics. The prebiotic assay was designed to measure the growth of lactic acid bacteria in presence of different amounts of prebiotic supplements. The formulated synbiotic may be used as neutraceutical agent.
Material and Methods: Probiotic properties of isolated bacteria were determined using tests such as acid and bile tolerance, cell surface hydrophobicity, cell adhesion and antimicrobial potential. Prebiotic assay was measured in terms of colony forming units (cfu/ml) on MRS agar plates. Different range of pH ie. 1.5, 3 and 7 with different incubation times were considered in acid tolerance test. In bile tolerance assay, wide ranges of bile concentration were tested. Cell adhesion assay was done using Caco-2 and HT-29 cells. Antimicrobial assay was performed by agar well diffusion method. Probiotic characteristics of these isolated bacteria were compared with known Lactobacillus acidophilus (La) and Lactobacillus plantarum (Lp).
Results & Conclusion: The growth of lactic acid bacteria supported by prebiotics, as a carbon source could be considered for various synbiotic formulation. Isolated strains of lactic acid bacteria passed in acid and bile tolerance tests. Lactococcus lactic showed good cell adhesive properties. All the lactic acid bacteria showed potential antimicrobial properties.

Key words: Prebiotic, Probiotic, Synbiotic, Acid & Bile tolerance, Cell adhesion assay

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INTRODUCTION

The application of microbes in human health has been getting attention since last two decades.
(Maria G. Dominguez-Bello and Martin J. Blaser, 2008). The interest in the area of probiotics was intensified by the scientific evidences of these in different diseases. (Wilhelm H. Holzapfel and Ulrich Schillinger, 2001). Although probiotics have a long history in human diet, the information on specific probiotic strains – disease interaction, actual dosage of probiotics, mechanism of action at molecular levels are the topics of upcoming research. According to WHO/FAO, Probiotics are generally defined as live microorganisms which, when administered in adequate amount, confers health benefit to the host. (Bhupinder Singh Sekhon and Saloni Jairath, 2010, Parisa Shokryazdan et al 2014) Most probiotic microorganisms belong to two major family i.e.. Lactic Acid Bacteria (LAB) and Bifidobacteria groups. Strains of *Enterococcus* sp., *Bacillus, Pediococcus, Clostridium butyricum*and and some yeast like *Saccharomyces boulardii* have also been found as suitable probiotic candidates. (Asa Ljungh and Torkel Wadstrom, *Carlos Ricardo Soccol et at 2010.*)
Prebiotics are like the complimentary growth supplements for probiotic microbes. The prebiotics are defined as 'non-digestible food substances that benefit the host organisms by selectively stimulating the externally administered probiotics or bacteria which are already present in the intestine. (Włodzimierz

Grajek et at 2005, Corliss A O'Bryan et al 2013) All currently known prebiotics are carbohydrates. The most widely studied and used prebiotics till now are inulin, fructo-oligosaccharides, galacto-oligosaccharides and lactulose. (Corliss A O'Bryan et al 2013, Julien Grimouda, et al 2010, E. Rurangwa 2008, Ping Sua et al 2007.)

A synbiotic is a combination of a prebiotic and probiotic, in which the prebiotics increases the population and function of the probiotic it, is paired with and the mixture of the two, may augment synergistic effect. Examples like *Bifidobacteria* and fructo oligosaccharides (FOS), *Lactobacillus rhamnosus* GG and Inulin. (Bhupinder Singh Sekhon and Saloni Jairath, 2010)

Therefore, the present studies include various prebiotics which supports the growth of probiotic organisms, which may lead to make the best synbiotic combination, along with the evaluation of probiotic properties of lactic acid bacteria.

MATERIALS AND METHODS

1. Prebiotic and Probiotic source

In this study, Inulin, Fructo-oligosaccharides [FOS] (Hi-Media, India) and Lactulose (TIS, Japan) were used as prebiotics. *Lactobacillus plantarum* (Lp) NCIM 2912, *Lactobacillus acidophilus* (La) NCIM 2285 were procured from National Centre for Industrial Microorganisms (NCIM), NCL, Pune, India and used as standard probiotics at our lab. The other two lactic acid bacteria used were *Lactococcus lactis sp.cremoris* (Ll) and *Pediococcus pentosaus* (Pp) which were isolated from curd and fermented wheat respectively and identified at Department of Microbiology, KEM hospital, Pune, India.

2. Preparation of bacterial cell suspension

All the strains of lactic acid bacteria were grown in MRS (deMan, Rogosa and Sharp broth; HiMedia, India) broth aerobically for 18-24 hrs at 37^0 C. After the incubation period, cells were harvested by centrifugation (2500 rpm, 15 mins). Pellets were washed twice in sterile normal saline, re-suspended in 10 ml sterile normal saline and kept at 2-8^0 C until further use. The bacterial suspensions, for all the organisms, were freshly prepared on the day of experiments.

3. Prebiotic Assay

Prebiotics (Inulin, FOS and Lactulose) consumption by lactic acid bacteria was evaluated by Total Viable Count (TVC) on MRS agar plates. Briefly, 100 ml MRS broth was prepared without glucose and Inulin was added in the broth at final concentration of 1 % as the sole carbon source. Similarly 100 ml glucose free MRS broth was prepared with 2 % inulin as the sole carbon source. The medium was sterilized by autoclaving at 121^0 C at 15 lb for 10 minutes. After sterilization of the medium, 1ml of overnight culture (1 X 10^9 cfu/ml) of each probiotic (individually) lactic acid bacteria was added aseptically in the medium and kept for incubation at 37^0 C for 18-24 hrs aerobically. After incubation period, growth was observed in the form of turbidity and measured the optical density (OD) at 600 nm using a spectrophotometer (UV-1201 Shimadzu, Japan). For TVC, 1 ml from the incubated tube was plated on MRS agar plates till the dilution 10^{-10} (glucose free MRS agar supplemented with inulin) and plates were incubated aerobically in inverted position at 37^0 C for 48 hrs. After the incubation period colonies were counted and recorded as cfu/ml. Same procedure was applied for other prebiotics FOS and Lactulose. These were compared with glucose (1% & 2%) as a standard carbon source. The experiments were carried out in triplicates.

4. Acid tolerance test

The protocol used to assess the viability of the cells under acidic stress was adapted from Sahadeva, R.P.K et al 2011. Acidic conditions were simulated by using different pH range such as 1.5, 3 and 7 as a control. In addition to that, three incubation periods of 0 hr., 1.5 hrs. and 3 hrs.were used. In short, 10 ml MRS broth was prepared and pH was adjusted to 1.5, 3 using 1M HCl and 7 as a control before autoclaving in three different individual sets. 1 ml of overnight culture of each lactic acid bacteria (1X10^9 cfu/ml) was inoculated aseptically into MRS broth having pH 1.5, 3

and 7 tubes and mixed thoroughly. Initially at 0 time point, 1 ml of the sample from each tube was removed aseptically for TVC. The appropriate serial dilutions was carried out and plated on MRS agar plates. These plates were incubated aerobically at 37^0 C for 48 hrs. Each assay was performed in triplicates.

After 1.5 hrs and 3 hrs of incubation 1 ml sample was removed aseptically and TVC was performed on MRS agar plates and plates were incubated. The same procedure was repeated for pH 3 and pH 7. Acid tolerance was measured by comparing the colony forming units on MRS agar plates after 48 hours. **(**Mehmet Tokatl et al 2015**)**

5. Bile tolerance test

For bile tolerance test, the procedure was modified, as suggested by Sahadeva, R.P.K et al 2011 to check effect of bile on the growth of lactic acid bacteria. MRS broth was prepared having different bile concentrations between 0.3 % to 2% and 0% bile was kept as a control. Bile tolerance test was initiated at the end of 3 hours of acid pre-treatment (pH 1.5 and pH 3). Centrifugation was carried out at 2500 rpm for 10 minutes. The supernatant was discarded and the pellets were washed with normal saline (0.85%) of pH 7.2. Centrifugation was repeated and the pellets were re-suspended in normal saline. 1 ml of this culture was inoculated in 9 ml MRS broth having different bile concentrations (0%, 0.3%, 0.5%, 0.7%, 1% & 2%). Tubes were incubated aerobically at 37^0 C for 24 hrs. After incubation period, 1 ml was pipetted out from each tube and serial dilutions were prepared up to 10^{-8}. Appropriate dilutions were taken and plated on MRS agar plates. All the plates were incubated aerobically at 37^0 C for 48 hours and colonies were counted and recorded as cfu/ml. **(**Mehmet Tokatl et al 2015**)**

6. Cell surface hydrophobicity

Cell surface hydrophobicity of isolated lactic acid bacteria and procured strains was determined by microbial adhesion to hydrocarbons (MATH) method described by (Raj Kumar Duary et al 2011) using hexadecane and toluene as solvents.

The overnight culture of lactic acid bacteria (each culture individually) were grown in MRS broth at 37^0 C /24 hrs. The cells were collected by centrifugation and washed the pellets with sterile normal saline twice. The initial absorbance (A0) was recorded at 600 nm and adjusted to 0.70±0.02. 5 ml of the same suspension was taken in clean and dry sterile tube and 1 ml of hexadecane added into the tube. It was vortexed for 2-3 mins. The tube was kept for 1 hr. incubation at 37^0C to allow for phase separation. The lower aqueous phase was carefully removed and absorbance (A1) was recorded at 600 nm.

The same protocol was followed using toluene instead of hexadecane and percentage Hydrophobicity (% H) was measured based on the following formulae.

$$\% H = (A0-A1)/ A0 \times 100$$

Where,

A0 = Initial OD600, A1 = Final OD600

The expriment was repeated twice and the average % hydrophobicity of each strain calculated.

7. Cell adhesion assay

The human adenocarcinoma cell lines Caco-2 and HT-29 for adhesion assay were procured from National Center for Cell Sciences (NCCS), Pune, India. Caco-2 cells were cultured and maintained in Dulbecco's Modified Essential Medium (DMEM, Sigma, USA) supplemented with deactivated 10% Fetal Bovine Serum (FBS, Sigma) whereas HT-29 cells grown in Minimal Essential Medium (MEM, Sigma) with deactivated 10 % FBS. The cell lines were grown in T-25 cm^2 tissue culture flask (Corning) and incubated at 37^0 C and 5 % CO_2/ 95 % air environment.

The protocol of Raj Kumar Duary et al 2011 was modified and used for cell adhesion assay for both cell lines. The assay was carried out in 12 well tissue culture plate (corning). Sterile glass cover slip was placed aseptically into each well. Cells were counted on neubauer chamber and final cell density of 1 X 10^5 cells/ml was seeded into each well of 12 well plate. The plate was kept for incubation in CO_2 incubator (5 % CO_2, 37^0 C). Media was changed (as per respective cell lines) on every alternate day. After reaching the confluency of the cells, adhesion assay was initiated. The

spent medium was removed completely 24 hrs before the adhesion assay and plain medium lacking antibiotics was added into each well. After 24 hrs.incubation, cells were washed with plain medium (without sera and antibiotics). After that 2 ml of plain medium (without sera and antibiotics) were added into each well and incubated in CO_2 incubator (5 % CO_2, 37^0 C) for 30 minutes. Meanwhile, overnight cultures of each lactic acid bacteria (individually) were grown in MRS broth and harvested lactic acid bacterial cells were washed with sterile normal saline and re-suspended in plain medium (without sera and antibiotics). After 30 minutes, each lactic acid bacterial culture ($1X10^9$ cfu/ml), suspended in plain medium, and added to 12 well tissue culture plate, as per grid. The plate was again incubated at 37^0C for 2 hrs. After the incubation period, spent medium was removed and cover slip, on which assay was performed, washed five times with plain medium. Cover slip was removed carefully and placed on clean and dry glass slide. After fixing the slide with methanol, Giemsa staining was carried out. The cells were observed under oil immersion microscope (100 X) and 20 random microscopic fields were observed for adherence of lactic acid bacteria to cells and adhesion score was recorded as per the criteria mentioned. Same protocol was followed for both the cell lines and each lactic acid bacterial strains.

8. Antimicrobial assay

Agar well diffusion method was used to carry out antimicrobial assay. Non-pathogenic strains of *E.coli, Pseudomonas aerogenosa, Salmonella typhi, Staphylococcus aureus* and *Bacillus subtillis* were used as test bacteria. *Aspergillus niger* and *Candia albicans* were used as test fungal culture. 24 hrs old cultures of bacterial test organisms were enriched in nutrient broth whereas fungal test organisms were grown in potato dextrose broth (72-96 hrs.). 1% of test organisms were taken and nutrient agar plates for bacteria and potato dextrose agar plates for fungi were prepared and plates were left to solidify at room temperature. After solidification of plates, 6 mm diameter well was punched in the agar plate using sterile cork borer. Meanwhile, isolated and procured lactic acid bacterial strains were cultured overnight in MRS broth and centrifuged (2500 rpm for 10 mins) to get supernatant. The supernatants were passed through filters to avoid the action of hydrogen peroxide and lactic acid. 50 µl from each, were taken to fill the wells separately. After filling all the wells, plates were kept for 30 minutes at room temperature for diffusion and then incubated aerobically at 37^0 C/24 hrs. (bacterial plates) and 28-30^0 C/72-96 hrs. (fungal plates). After incubation period, diameter of the zone of inhibition in each wells were measured in mm. The zone of inhibition was scored as follows: as the diameter of the well is 6 mm, 6mm equals no inhibition (-), diameter between 0 and 3 mm (weak, +), diameter between 3 and 6mm (good, ++) and diameter larger than 6 mm (strong, +++). The assay was performed in triplicates. Ciprofloxacin and Gentamicin were used as standard antibacterial and antifungal drugs respectively. (Xiaodong.Pan et al 2009)

RESULTS AND DISCUSSION

1. Initial count of Lactic Acid Bacteria

All the lactic acid bacterial strains *Lactobacillus acidophilus* (La), *Lactobacillus plantarum* (Lp), *Lactococcus lactis sp.cremoris* (Ll) and *Pediococcus pentosaus* (Pp) have showed much more growth than minimum requirement criteria ($1 X 10^6$ CFU/ml) of WHO/FAO (Table 1). As all four lactic acid bacteria met the acceptance criteria, as per WHO/FAO, these all can be considered as a good source of probiotics.

2. Prebiotic Assay

The consumption of prebiotics by lactic acid bacteria was checked by prebiotic assay in terms of total viable count and optical density of broth. The experiment was conducted in carbohydrate free MRS broth and different percentage of Inulin, FOS and Lactulose along with glucose (as positive control). The results were expressed as average value of three samples ± SD of $\log_{10}$ cfu/ml as shown in Table 2 Figure 1 indicates the viability of lactic

acid bacteria in presence of prebiotics at 600 nm.

All the three prebiotics Inulin, FOS and Lactulose at 1% and 2% concentrations supported the growth of lactic acid bacteria and fulfilled the minimum requirement criteria set by WHO/FAO (1 X 10^6 CFU/ml).

The lactic acid bacteria were able to use the selected prebiotic as sole carbon source and showed comparable growth with glucose. *Lactococcus lactis* and *Pediococcus pentosaus* with 1 % Inulin and 2% Inulin respectively showed better growth behavior which is comparable to standard probiotic organisms and could be best option for synbiotic formulation.

Table 1: Initial count of Lactic acid bacteria measured as TVC (log_{10} CFU/ml) on MRS agar plates under aerobic condition at 37^0 C after 48 hrs.incubation.

TVC (log_{10} CFU/ml)	
Lactobacillus acidophilus (La)	10.17 ± 0.09
Lactobacillus plantarum (Lp)	10.45 ± 0.08
Lactococcus lactis (Ll)	10.5 ± 0.20
Pediococcus pentosaus (Pp)	10.46 ± 0.24

Results were expressed as Mean ± standard deviation (SD); n=3

Table 2: Lactic acid bacterial growth as TVC (log_{10} CFU/ml) under aerobic condition at 37^0 C after 48 hrs.incubation.

LAB	**1 % Inulin**	**1 % FOS**	**1 % Lactulose**	**1 % Glucose**	**2 % Inulin**	**2 % FOS**	**2 % Lactulose**	**2 % Glucose**
La	7.88 ± 0.17	7.2 ± 0.17	7 ± 0.0	8.89 ± 0.14	8.56 ± 0.31	7.93 ± 0.20	8.29 ± 0.40	9.27 ± 0.22
Lp	8.32 ± 0.25	7.61 ± 0.39	7.5 ± 0.63	8.91 ± 0.17	8.78 ± 0.16	8.07 ± 0.021	8.29 ± 0.30	9.4 ± 0.74
Ll	7.87 ± 0.51	8.24 ± 0.27	7.77 ± 0.74	9.17 ± 0.26	8.39 ± 0.27	8.52 ± 0.66	8.52 ± 0.24	9.54 ± 0.47
Pp	7.98 ± 0.33	7.75 ± 0.39	8.15 ± 0.46	8.98 ± 0.07	8.53 ± 0.21	8.26 ± 0.10	8.6 ± 0.52	9.36 ± 0.39

All values are expressed as Mean ± standard deviation (SD); n=3

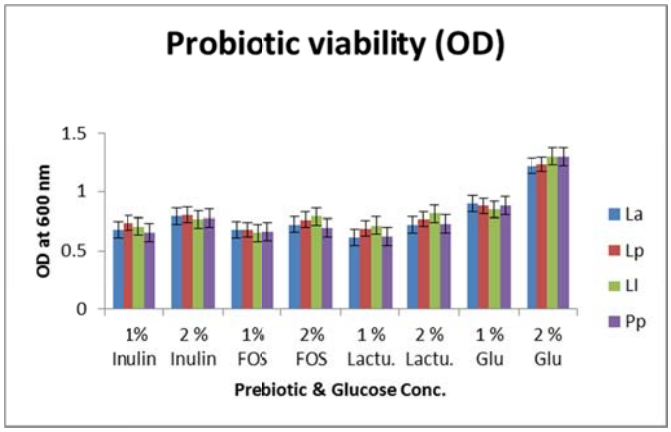

Figure 1: Optical Density at 600 nm

3. Acid Tolerance

The most important criteria for any lactic acid bacteria to be a good source of probiotic are tolerance to acidic conditions. It is known that pH of stomach acid is 1.5 to 3.5. Good probiotic organisms should withstand at least pH 3.0 (Sahadev et al) to execute the beneficial effect, the lactobacilli should resist the stressful conditions of the stomach and upper intestine that contain bile. (Xiaodong Pan et al)

In the present study it was observed that viable count of all the four strains of lactic acid bacteria was decreased when they were incubated with pH 1.5 and pH 3.0 (Table 3). At pH 3 and pH 7, both the bacterial strains showed comparable growth pattern with standard probiotic strain.

4. Bile Tolerance

Resistance to bile salts is another important selection criteria for probiotics since the small intestine and colon contain relatively high concentrations of bile salts which are toxic for living cells. (Mehmet Tokatl et al**)**

It was reported that the different species of *Lactobacillus* showed significant variations in relation to their bile salt tolerance. (Sahadev et al)

In this study, all the studied lactic acid bacterial strains showed significant growth inhibition till 1 % bile concentration ($p<0.001$) at both pH 1.5 and pH 3.0 acid-pre-treatment.

At pH 3 and 1% bile concentration, Ll and Pp showed 4 X 10^6 and 2.1 X 10^6 cfu/ml which was comparable to standard Lp probiotic, however La showed better growth potential against all. (Table 4.1 & 4.2)

Optical density was measured at 600 nm. The viability of all lactic acid bacteria decreased as the bile concentrations increased. (Fig.2) However even at pH 1.5 and bile concentration 0.3 % both La and Lp showed better growth as compared to Ll and Pp. Similar growth pattern observed at pH 3 and bile concentration 0.3 %.

Table 3. Viable count of lactic acid bacteria at pH 1.5, 3.0 and 7.0 at 0, 1.5 and 3 hrs.

TVC (log_{10} CFU/ml)				
LAB	**pH Con.**	**0 hr.**	**1.5 hrs.**	**3 hrs.**
Ll	**1.5**	6.79 ± 0.39	-	-
	3	7.48 ± 0.59	7.26 ± 0.37	6.54 ± 0.34
	7	9.29 ± 0.37	9.63 ± 0.39	9.25 ± 0.62
Pp	**1.5**	6.56 ± 0.31	-	-
	3	4.67 ± 0.31	6.64 ± 0.58	6.23 ± 0.40
	7	9.04 ± 0.24	9.1 ± 0.69	9.53 ± 0.46
La	**1.5**	6.19 ± 0.16	-	-
	3	8.16 ± 0.19	7.46 ± 0.06	4.66 ± 0.04
	7	9.8 ± 0.045	9.71 ± 0.24	9.48 ± 0.49
Lp	**1.5**	6.69 ± 0.60	1.63 ± 0.42	-
	3	8.08 ± 0.56	7.6 ± 0.56	6.84 ± 0.78
	7	9.34 ± 0.3	9.21 ± 0.78	9.52 ± 0.45

All values are expressed as Mean ± standard deviation (SD); n=3

Table 4.1: Lactic acid bacteria growth as cfu/ml at different bile concentrations for pH 1.5 after 3 hrs. acid-pre-treatment

pH 1.5	**Bile conc.**			
LAB	**0.3**	**0.5**	**0.7**	**1**
Ll	8 X 10^6	1 X 10^6	1.2 X 10^6	0.4 X 10^6
Pp	13 X 10^6	3 X 10^6	0.6 X 10^6	0.1 X 10^6
La	3 X 10^6	3 X 10^6	0.1 X 10^6	0.13 X 10^6
Lp	10 X 10^6	2 X 10^6	3 X 10^6	0.5 X 10^6

Table 4.2: Lactic acid bacteria growth as cfu/ml at different bile concentrations for pH 3.0 after 3 hrs. acid-pre-treatment

pH 3.0	**Bile conc.**			
LAB	**0.3**	**0.5**	**0.7**	**1**
Ll	90 X 10^6	60 X 10^6	10 X 10^6	4 X 10^6
Pp	40 X 10^6	20 X 10^6	8 X 10^6	2.1 X 10^6
La	40 X 10^6	10 X 10^6	15 X 10^6	8 X 10^6
Lp	110 X 10^6	40 X 10^6	19 X 10^6	11 X 10^6

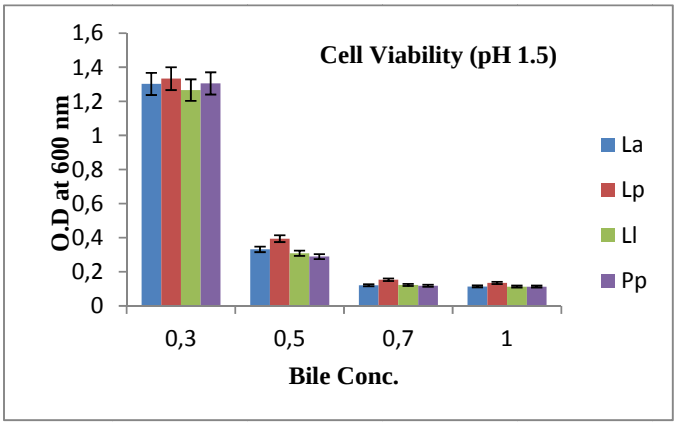

Figure 2: Optical Density at 600 nm

5. Cell Surface Hydrophobicity

The surface properties, like auto aggregation and hydrophobicity, are used as a measurement directly related to ability to adhere to cell monolayers (Xiaodong Pan et al)

The results showed that *Pediococcus pentosaus* (Pp) has better hydrophobicity i.e. 87.46 % and 87.7 % with hexadecane and toluene respectively whereas *Lactococcus lactis* (Ll) showed comparable hydrophobicity with standard probiotics (Table 5).

Table 5: % Cell surface Hydrophobicity by MATH method

	% Cell surface Hydrophobicity	
LAB	**Hexadecane**	**Toluene**
Ll	83.31 ± 0.15	85.12 ± 0.09
Pp	87.46 ± 0.31	87.75 ± 0.22
La	86.52 ± 0.23	86.38 ± 0.39
Lp	84.41 ± 0.41	83.71 ± 0.64

All values are expressed as Mean ± standard deviation (SD); n=3

6. Cell Adhesion Assay

All the lactic acid bacteria were tested for their cell adhesion property using Caco-2 and HT-29 cells as per the method described.(Raj Kumar Duary et al 2011) The direct microscopic count were recorded for adhesion of bacteria to the cells. Twenty random microscopic fields (100X) were measured to calculate adhesive property of bacteria to cells. The organisms were categorized as follows: non-adhesive (≤ 40 bacteria/cell), adhesive (41-100 bacteria/cell) and strongly adhesive group (≥ 100 bacteria/cell).

Lactococcus lactic showed very strong adhesion to Caco-2 cells and better adhesion to HT-29 cells. *Pediococcus pentosaus* also showed adhesion properties to Caco-2 and HT-29 cells which were comparable to *Lactobacillus acidophilus* (La). *Lactobacillus plantarum* (Lp) also found to be strongly adhesive to Caco-2 and HT-29 cells. (Table 6) (Fig. 3.1, 3.2)

Table 6: Cell Adhesion Score

Cell line	La	Lp	Ll	Pp
Caco-2	Adhesive	Strongly Adhesive	Strongly Adhesive	Adhesive
HT-29	Adhesive	Strongly Adhesive	Adhesive	Adhesive

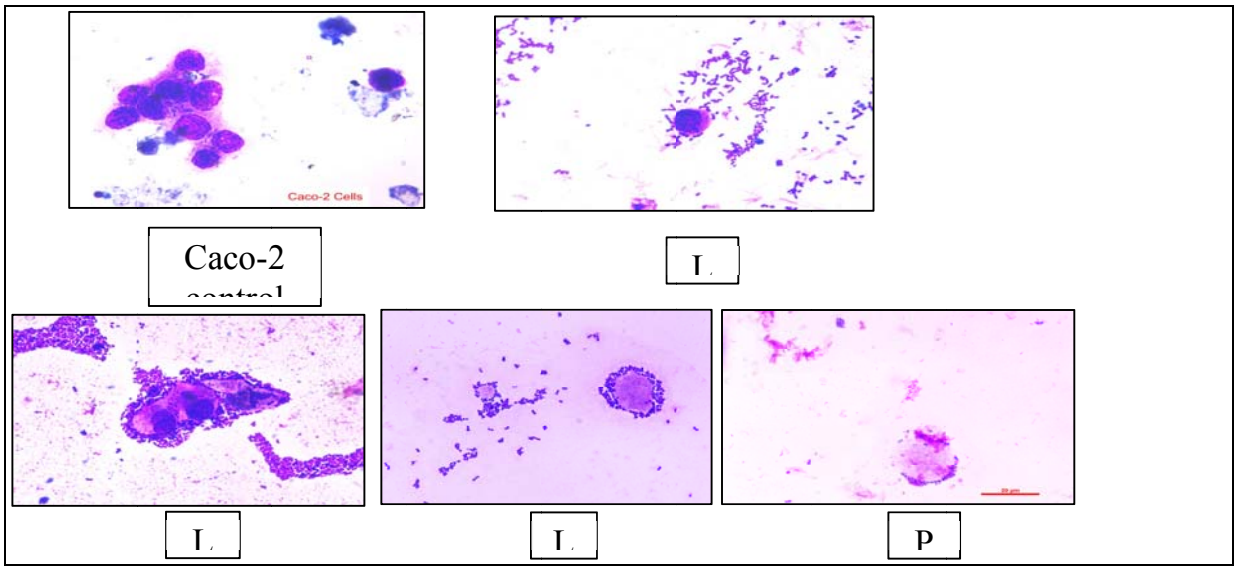

Figure 3.1 Cell adhesion of Lactic acid bacteria to Caco-2 cells

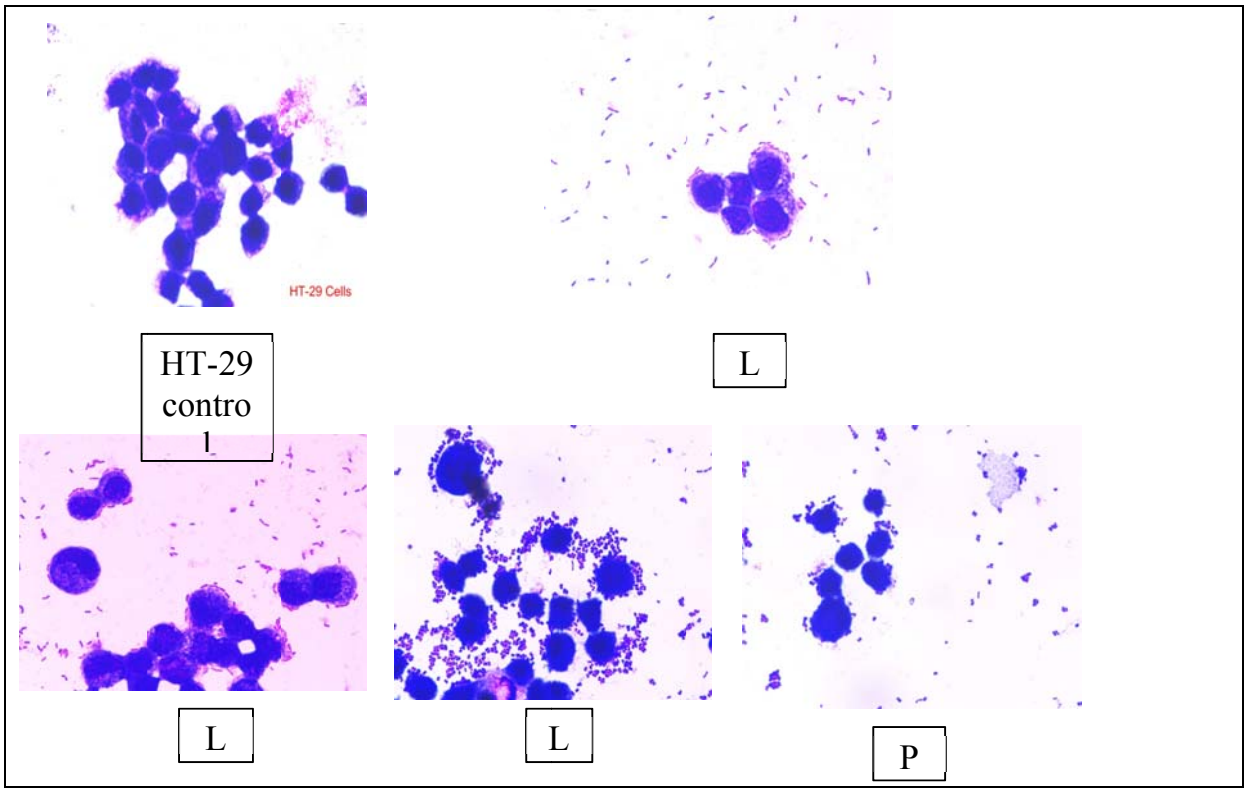

Figure 3.2 Cell adhesion of Lactic acid bacteria to HT-29 cells

Table 7: Antimicrobial activity of Lactic acid bacteria (zone of inhibition in mm)

Strains	*E.coli*	*Pseudomonas*	*Salmonella*	*S.aureus*	*Bacillius*	*Candida*	*A.niger*
La	9.3	12	11.3	7.6	8.6	-	6
Lp	8	8.3	12	8	11	-	4
Ll	7.8	8	9.6	8	8	-	3
Pp	7.3	8.3	8.5	7.5	7.6	-	5
Consortia	10.67	11	10.67	8.6	9.9	-	5
Ciprofloxacin	21.3	22.5	29.3	24	28.3	-	-
Gentamicin	-	-	-	-	-	16	9

All values are expressed as Mean ± standard deviation (SD); n=3

7. Antimicrobial assay

The antimicrobial activity of lactic acid bacteria was measured by agar well diffusion assay (Xiaodong Pan et al) and expressed as zone of inhibition in mm. (6 mm is subtracted from all the results) Table 3.7.

The supernatant of all the strains of lactic acid bacteria showed good antimicrobial property, as almost all lactic acid bacteria showed inhibition more than 6 mm. Apart from alone organism, consortia of all the strains also checked and it showed slightly better inhibitory action against test bacteria.

DISCUSSION

In recent times, synbiotics have been shown to be more effective than prebiotics or probiotics alone, in improving the quality of life in patients suffering from ulcerative colitis (Fathia Bahri et al 2014), in colorectal cancer prevention or in very general positive regulation of the microbiota (Juhein Grimouda et al 2010).

We have investigated the probiotic properties of isolated lactic acid bacteria i.e. *Lactococcus lactis* and *Pediococcus pentosaus* as well as procured strains of lactic acid bacteria in terms of survival at low pH, at different bile concentrations, their adherence to human enterocytes such as Caco-2 and HT-29 cells, cell surface hydrophobicity and antimicrobial activity.

Any ideal probiotic strains much express high tolerance to acid and bile environment (Sahadev et al). In the present studies we found that both the strains i.e. *Lactococcus lactis* (isolated from curd) and *Pediococcus pentosaus* (isolated from germinating wheat) showed good acid and bile tolerance at pH 3 and at 0.3 % bile concentration. Standard probiotic strains also showed similar growth pattern. It was suggested that surface hydrophobicity of probiotics directly relates with its cell adhesion properties. In our studies though we did find higher cell surface hydrophobicity of *Pediococcus pentosaus* with hexadecane and toluene, it did not reflects into higher cell adhesive strength as compared to other probiotic strains.

Probiotic bacteria produce different types of metabolites like organic acids such as lactic and acetic acids, bacteriocins ,reuterin, proteinaceous compounds and cyclic dipeptides which are responsible to inhibit the growth of numbers of pathogenic organisms. (Bhupinder Singh Sekhon and Saloni Jairath, 2010, Shabana Maqsood et al 2013) Antimicrobial activity against enteric pathogens is a desirable property of probiotics and gives the potential for their use in the treatment or prevention of enteric infections. (E. Likotrafitia, 2016)

In our studies we observed comparable antimicrobial activity against *E.coli, Pseudomonas, Salmonella, S.aureus, Bacillus* and *A.niger* with *Lactococcus lactis* and *Pediococcus pentosaus* and other standard probiotic strains. Consortia of all the strains showed slightly better antimicrobial activity.

In conclusion our results showed that out of all possible combinations *Lactococcus lactis* and *Pediococcus pentosaus* with 1 % inulin and 2% inulin respectively showed better growth

pattern and would be best option for synbiotic formulation.

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REFERENCES

[1] Asa Ljungh and Torkel Wadstrom, Lactic Acid Bacteria as Probiotics, Curr. Issues Intestinal Microbiol. 7: 73–90.

[2] Bhupinder Singh Sekhon and SaloniJairath, 2010. Prebiotics, probiotics and synbiotics: an overview, Journal of Pharmacy Education Research Vol.1 13-36.

[3] Bojana Bogovi Matija, Mojca Narat and Metoda Zoric, *2003.* Adhesion of Two *Lactobacillus gasseri* Probiotic Strains on Caco-2 Cells, Food Technology, Biotechnology, *41* (1) 83–88

[4] Carlos Ricardo Soccol, Luciana Porto de Souza Vandenberghe, Michele RigonSpier,Adriane Bianchi Pedroni Medeiros, Caroline TiemiYamaguishi,Juliano De Dea Lindner, Ashok Pandey and VaneteThomaz-Soccol, *2010.* The Potential of Probiotics: A Review, *Food Technol. Biotechnol. 48* (4) 413–434.

[5] Corliss A O'Bryan, Dolar Pak, Philip G Crandall, Sun Ok Lee and Steven C Ricke, 2013. The Role of Prebiotics and Probiotics in Human Health, Journal of Probiotics and Health 1:2 1-8.

[6] E. Rurangwa, J.L. Laranja, R. Van Houdt, Y. Delaedt, Z. Geraylou, T. Van de Wiele, J. Van Loo,V. Van Craeyveld, C.M. Courtin, J.A. Delcour and F. Ollevier, 2008. Selected nondigestible carbohydrates and prebiotics support the growth of probiotic fish bacteria mono-cultures in vitro, Journal of Applied Microbiology, 932–940, ISSN 1364-5072.

[7] E. Likotrafitia, K. M. Tuohyb, G. R. Gibsonc and R. A. Rastall, 2016. Antimicrobial activity of selected synbiotics targeted for the elderly against pathogenic Escherichia coli strains, INTERNATIONAL JOURNAL OF FOOD SCIENCES AND NUTRITION

[8] Fathia Bahri, Annick Lejeune, Robin Dubois-Dauphin, ThamiElmejdoub, AbderrahmaneBoulahrouf and Philippe Thonart, 2014. Characterization of *Lactobacillus* strains isolated from Algerian children faeces for their probiotic properties, African Journal of Microbiology Research 8(3) 297-303.

[9] J.M. Laparra and Y. Sanz, 2009. Comparison of in vitro models to study bacterial adhesion to the intestinal epithelium, Letters in Applied Microbiology 49 695–701

[10] Julien Grimouda,b, Henri Durand b, Céline Courtina,b, Pierre Monsanc, Françoise OuarnéVassiliaTheodoroue, Christine Roques, 2010. In vitro screening of probiotic lactic acid bacteria and prebiotic glucooligosaccharides to select effective synbiotics, Clinical Microbiology, *Anaerobe* pp. 493-500. ISSN 1075-9964.

[11] Kontham Kulangara Varsha & Sulochana Priya & Leena Devendra & Kesavan Madhavan Nampoothiri, 2014. Control of Spoilage Fungi by Protective Lactic Acid Bacteria Displaying Probiotic Properties, Appl Biochem Biotechnol.

[12] Maria G. Dominguez-Bello and Martin J. Blaser, 2008. Do you have a probiotic in your future? *Microbes Infect* 10(9): 1072–1076.

[13] Marelize Botes, Ben Loos, Carol A. van Reenen and Leon M. T. Dicks, 2008. Adhesion of the probiotic strains Enterococcus mundtii ST4SA and Lactobacillus plantarum 423 to Caco-2 cells under conditions simulating the intestinal tract, and in the presence of antibiotics and anti-inflammatory medicaments, Arch Microbiol, 190:573–584.

[14] Mehmet Tokatl, GokGen Gulgor, Simel Balder ElmacJ, Nurdan Arslankoz EGleyen, and Filiz Ozcelik, 2015. *In Vitro* Properties of Potential Probiotic Indigenous Lactic Acid Bacteria Originating from Traditional Pickles, BioMed Research International, Volume 2015, Article ID 315819.

[15] Neerja Hajela, G. Balakrish Nair, B.S. Ramakrishna & N.K. Ganguly, 2014. Probiotic foods: Can their increasing use in India amelioratethe burden of chronic lifestyle disorders? Indian Journal of Medical Research 139 19-26.

[16] Parisa Shokryazdan, Chin Chin Sieo, Ramasamy Kalavathy, Juan Boo Liang, Noorjahan Banu Alitheen, Mohammad FaselehJahromi, and Yin Wan Ho, 2014. Probiotic Potential of *Lactobacillus* Strains with Antimicrobial Activity against Some Human Pathogenic Strains BioMed Research International 1-16.

[17] Ping Sua, Anders Henrikssona, b, Hazel Mitchel, 2007. Selected prebiotics support the growth of probiotic mono-cultures in vitro, Anaerobe 13 134–139.

[18] Pooja N. Thakkar, H.A.Modi, B.K. Dabhi, J.B.Prajapati, 2015. Evaluation of probiotic potential of Lactic acid bacteria isolated from South Indian special Dosa batter, International Journal of Pharmaceutical Science and Health Care, Issue 5, Vol. 3.

[19] Raj Kumar Duary, Yudhishthir Singh Rajput, Virender Kumar Batish & Sunita Grover, 2011. Assessing the adhesion of putative indigenous probiotic lactobacilli to human colonic epithelial

cells, Indian Journal of Medical Research 134,664-671.

[20] Sahadeva, R.P.K., Leong, S.F., Chua, K. H., Tan, C.H., Chan, H.Y., Tong, E.V., Wong, S.Y.W. and Chan, H.K., 2011. Survival of commercial probiotic strains to pH and bile, *International Food Research Journal 18(4): 1515-1522.*

[21] Shabana Maqsood, Faria Hasan, Tariq Masud, Shehla Sammi and Syed Muhammad Saqlain Naqvi, 2013. *In vitro* pre-selection criteria for probiotic *Lactobacillus acidophilus* TS1 isolated from fermented milk product, Dahi, Malaysian Journal of Microbiology, Vol 9(4), 326-330.

[22] Sunita Grover, Hogarehalli Mallapa Rashmi, Anil Kumar Srivastava and Virender Kumar Batish, 2012. Probiotics for human health –new innovations and emerging trends, Gut Pathogens 2012, 4:15.

[23] Wilhelm H. Holzapfel, Ulrich Schillinger, 2001. Food Research International 35 109–116.

[24] Włodzimierz Grajek, Anna Olejnik and Anna Sip, 2005. Probiotics, prebiotics and antioxidants as functional foods, Acta Biochimica Polonica, Vol. 52 No. 3/2005, 665–671.

[25] Xiaodong Pan, Fenqin Chen, TianxingWua, Honggang Tang, Zhanyu Zhao, 2009. The acid, bile tolerance and antimicrobial property of Lactobacillus acidophilus NIT, Food Control 20 598–602.

[26] Zhechko Dimitrov, Irina Gotova and Elena Chorbadjiyska, 2014. In vitro characterization of the adhesive factors of selected probiotics to Caco-2 epitheliumcell line, Biotechnology & Biotechnological Equipment, Vol. 28, No. 6, 1079-1083.