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Evaluation of probiotic attributes of Lactobacillus sp. *in vitro*

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ASTRACT

Probiotic when administrated in adequate amount confers health benefits to the host. Literatures have suggested the beneficial effects of probiotics on lifestyle disorders viz. hypercholesterolemia, diabetes and obesity in both humans and animals. Probiotics must survive the harsh conditions of the gastrointestinal tract and colonize, even temporarily, in the colon to confer health benefits. Therefore, in the present study probiotic attributes of Lactobacillus amylovorus MTCC8129 and L. bifermentum MTCC3818 were evaluated in vitro. Different properties viz. acid tolerance, BSH activity, cell surface hydrophobicity, auto-aggregation and antimicrobial activity were studied. Both strains have shown the good growth at pH 2.0 and 3.0 when compared to growth at pH 6.5. However, L. bifermentum MTCC3818 exhibited high acid tolerance compared to L. amylovorus MTCC8129. These two strains of Lactobacillus exhibited the bile salt hydrolase activity, confirmed by the formation of precipitates of sodium deoxycholate on MRS agar plate. This BSH activity is reported to be the one of the causes of cholesterol lowering effects of probiotics in humans and rodents. L. bifermentum MTCC3818 exhibited higher affinity to n-hexadecane (93.97%) and xylene (94.2%) when compare to L. amylovorus MTCC8129 (71.6% with n-hexadecane, 69.33% with xylene). L. amylovorus MTCC8129 exhibited higher auto-aggregation (65.9%) compared to L. bifermentum MTCC3818 (57.24%). Higher cell surface hydrophobicity and auto-aggregation properties are considered essential for the colonization of probiotics on the epithelial cells of intestine. Antimicrobial activity was determined by agar well diffusion assay. Both strains exhibited antimicrobial activity against E. coli MTCC1698, E. faecium MTCC2729, B. cereus MTCC6629 and S. aureus MTCC3160, repectively. L. amylovorus MTCC1829 have shown higher antimicrobial activity than L. bifermentum MTCC3818. These results have confirmed probiotic attributes

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of both Lactobacillus strains. Further in vivo studies are required to evaluate the beneficial effects of these probiotics.

***Keywords**: L. amylovorus MTCC8129, L. bifermentum MTCC3818, acid tolerance, bile salt hydrolase activity, cell surface hydrophobicity, auto-aggregation, antimicrobial activity*

1.0 Introduction

Now-a-days lifestyle disorder viz. obesity, diabetes, insulin resistance, hyperglycemia, hypercholesterolemia and cardiovascular diseases are increasing at epidemic rate in developing as well as developed countries. Although various treatment viz. drugs, surgery are available in the market but these treatment are associated with side effects [1]. Moreover, people fail to comply with treatment regime that require sustained lifestyle changes. So, some natural dietary interventions are required for prevention and treatment of lifestyle disorders. In this regard probiotics are gaining the consumers' attention as functional food ingredient because of their promising health benefits [2]. About $10^{13\text{-}14}$ microorganisms residing in the gut of humans [3]. Some of these beneficial microorganism considered as probiotics. World Health Organization (WHO) and Food and Agriculture Organization (FAO) in 2002 defined probiotics as "live microorganisms, which when administered in adequate amounts confers health benefits on the host." Most of the probiotics belong to *Bifidobacterium* and *Lactobacillus* genera. Some strains of *Enterococcus*, *Lactococcus*, and *Streptococc*us genera, and some strains of *E. coli* and yeasts e.g. *Saccharomyces boulardii* are also considered as probiotics [4].

Multiple mechanism of action of probiotics have been reported in the literature. Some probiotics confers health benefits by conjugated linolenic acid [5], some modulate the gut microbiota [6], some inhibits the fat storage by suppressing LPL activity by increasing intestinal fiaf level [7], some increases the expression of genes related to beta-oxidation and decreasing the expression of lipogenic genes [8], some decreases the inflammatory markers (IL6 and TNF alpha) through decreasing the adipocyte size [9]. However, some probiotics have been reported to decrease the body weight [10] and some probiotics have been reported to have no effect on

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body weight [11]. Therefore, we can say health beneficial effects of probiotics are strain-dependent. However exact mechanism how probiotic works in the body is yet to be elucidated.

To confer health benefits to the host probiotics has to reach the alive and colonize in the colon when consumed orally. They should remain stable in the harsh condition (low pH, bile acids, and digestive enzymes) of the gastrointestinal tract [12]. Attributes like cell surface hydrophobicity, auto-aggregation help the probiotics to adhere to the epithelial cells of intestine. Some probiotics known to secrete mucus-binding protein (adhesion protein), which help probiotics as well as other probiotics to adhere to the epithelia cell of intestine. This colonization prevents the colonization and growth of the pathogenic bacteria viz. *Escherichia coli* spp. and *salmonella* spp. in the intestine of host [12]. Lactic acid bacteria are known to produce different antimicrobial compounds viz. hydrogen peroxide, diacetyl, carbon dioxide, bacteriocins, which inhibits the proliferation of many pathogenic microorganisms in the gastrointestinal tract of human and animal [13].

Bile salt hydrolase activity of some strains of *Lactobacillus* give them ability to lower hypercholesterolemia, and there are many reports indicating the beneficial effects of *Lactobacillus* on humans and rodents [14,15]. *Lactobacillus* known to cause deconjugation of bile acid with the help of their BSH activity. Deconjugated bile acids are known to have less water solubility. This interferes with their enterohepatic reabsorption. This leads to more secretion of bile acids in the faeces. Cholesterol is considered as precursor for the new bile salts. This decreases the pool of cholesterol in the liver and blood of the individuals [16]. All probiotics are not found to be equally effective in lowering the cholesterol level in the body. This statement again supports the strain-dependent effects of probiotics.

Keeping above discussed points in the mind the present study was designed to study the probiotics attributes of *Lactobacillus amylovorus* MTCC8129 and *Lactobacillus bifermentum* MTCC3818 under *in vitro* conditions.

2.0 MATHERALS AND METHODS

The present work was carried out in the laboratory of School of Bioengineering and Biosciences at the Lovely Professional University Phagwara, Punjab.

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2.1 Bactria cultures

L. amylovorus MTCC8129, *L. bifermentum* MTCC3818 and pathogenic bacterial strains (Table 1) were purchased from IMTech, Chandigarh. All bacterial strains were maintained by sub-culturing fortnightly, and stored at 4^0C until used. Before use each bacterial culture was activated in their respective broth at 37 ^{0}C for 18-24 hours.

2.2 Gram staining

Gram staining kit used in this study were product of HiMedia. Purity of cultures were determined by Gram staining as per manufacturer's instructions. Briefly, smear of *Lactobacillus* culture was prepared on clean slide, air dried, and then heat fixed. Slide was flooded with Gram's crystal violet for a minute, then rinsed with water. Smear was covered with Iodine for a minute. Smear was flooded with decolorizer until no blue color was flowing out of the slide. Slide was washed with tap water. In last, smear was flooded with counter strain, safranin for one minute and then washed with tap water, and air dried. Slides were then examined under the microscope with 100x objectives.

2.3 Examination of acid tolerance of probiotic

Acid tolerance was evaluated by using the protocol as described previously [17]. Briefly, *Lactobacillus* cultures were grown in MRS broth (pH 6.5) at 37 ^{0}C for 18-24h for activation. MRS broths adjusted to pH 2.0 and 3.0 were also prepared. pH of these broths was set with the help of 1.0 N HCl. MRS broth of pH 6.5 was considered as positive control. One ml of 18-24 h activated culture was added into MRS broth of different pH (pH 2.0, 3.0 and 6.5). Thereafter, one ml of MRS broth was taken at 0 hour, and serially diluted in saline solution (0.85% of NaCl) (Loba Chemie Pvt. Ltd.) and appropriate dilution was pour plated, and incubated at 37^0 C for 48 h. After incubation at 37 ^{0}C for 30, 60 and 120 minutes, one ml of MRS broth (pH 2.0, 3.0 and 6.5) was again serially diluted, pour plated and incubated at 37 ^{0}C for 48 h. Thereafter colonies were counted.

2.4 Bile salt hydrolase activity of probiotic

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Bile salt hydrolase activity of probiotics was determined by direct plate assay method. Twenty-four hour activated culture was streaked on MRS agar containing 0.5% Sodium deoxycholate (Loba Chemie Pvt. Ltd.). Plates were incubated under anaerobically condition (in anerobic jar containing AnaeroGas Pack and Anaero Indicator tablet) at 37 ^{0}C for 72 hours. Bile salt hydrolase activity of probiotic was confirmed by formation of precipitates on the MRS agar plates due to hydrolysis of bile salts (sodium deoxycholate) [18].

2.5 Determination of cell surface hydrophobicity

Cell surface hydrophobicity was determined by adopting the method as described previously [18]. In this method probiotics ability to adhere to the hydrocarbons was determined. Briefly, probiotic was grown in MRS broth for 18 hours at 37 ^{0}C. Cells were harvested by centrifugation for 20 min at 6000 rpm. Pellet (cells) was washed two times with phosphate urea magnesium buffer (K_2HPO_4 2.22gr, KH_2PO_4 0.726gr, Urea 0.18gr, $MgSO_4$.7H2O 0.02gr in 100ml distilled water, pH 7.4). Pellet was dissolved in phosphate urea magnesium buffer, and OD (A) was set to approx. 0.7 at 600 nm (initial absorbance, A). Three milliliter of probiotic suspension was mixed with 1.0 mL xylene or n-hexadecane, vortexed, and incubated for 10 min at 37 ^{0}C condition for the equilibration of the temperature. This cells suspension was again vortexed, and incubated for one hour at 37 ^{0}C for separation phase. One mL of aqueous phase was taken carefully and OD was measured at 600nm (final absorbance, A_0). The cell surface hydrophobicity percent was calculated by using following formula as:

Cell surface Hydrophobicity (%): Affinity = 100× (A-A_0/A)

A = initial absorbance, A_0 = final absorbance.

2.6 Determination of Cell auto-aggregation

Probiotic was grown in MRS broth for 18-24 h at 37 ^{0}C. Cells were harvested by centrifugation for 20 min at 6000 rpm. The supernatant recovered was collected into another Eppendorf tube for further use (step1). Pellet (cells) was washed twice with phosphate buffer saline (PBS) buffer (NaCl 0.8gr, KCl 0.02gr, Na_2HPO_4 0.144gr, KH_2PO_4 0.024gr in 100ml, pH 7.4). After washing, pellet was dissolved in the same buffer, and the absorbance was set to 0.5 at

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600nm (initial absorbance, A). Again pellet was recovered by centrifugation at 6000 rpm for 20 minutes, and pellet (cells) was re-suspended in supernatant (broth) which was recovered from step 1. The mixture was vortexed, and incubated at 37 ^{0}C for two hours. One mL of from upper layer was taken, and absorbance was measured at 600nm (final absorbance, A_0). Broth was used as reference. Auto-aggregation property of probiotic was expressed using fallowing formula as below: [18].

Auto-aggregation (%): Auto-aggregation (%) affinity = $(A-A_0)/A \times 100$

A = initial absorbance, A_0 = final absorbance

2.7 Determination of antimicrobial activity of probiotic

For determination of antimicrobial activity of *Lactobacillus* agar well diffusion method was adopted. Freshly grown culture (18-24 hours at 37 ^{0}C) was centrifuged at 6000 rpm for 20 min, and cells free broth (supernatant) was collected carefully (step 1). Overnight activated pathogenic strains were mixed with MRS agar. After solidification 5.0 mm diameter wells were prepared with the help of sterilized puncher. Wells were loaded with 100 microliter of cells free supernatant (recovered at step 1). Petri plates were incubated at 37 ^{0}C for 24 -36 hours. Clear zone extended laterally around the well showed antimicrobial activity. Clear zone of inhibition were measured in mm [19].

3.0 RESULTS and DISCUSSION

3.1 Gram staining

Gram staining was done to check the purity of *Lactobacillus* cultures viz. both *L. amylovorus* MTCC8129, *L. bifermentum* MTCC3818. Cultures were found be pure and Gram positive (Figure 1a and 1b).

3.2 Acid tolerance feature of probiotic

The results obtained from three independent experiments, each experiment was done in duplicate. Results are presented mean ± SD. Food remains approx. 2 hours in the stomach,

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therefore growth of probiotics was checked at 30, 60 and 120 min of incubation at 37 ^{0}C at pH 2.0 and 3.0. (Table 2) shows the results of acid tolerance experiment with *L. amylovorus* MTCC8129 and *L. bifermentum* MTCC3818 strains. The selected strains were found to have the ability to survive at low pH (pH 3.0 and pH 2.0) when compared to pH 6.5, which was considered as positive control. The findings of this study showed that *L. amylovorus* MTCC8129 and *L. bifermentum* MTCC3818 could survive at pH 2.0, pH 3.0, even after two hours of incubation at 37 ^{0}C. However, their number decreased gradually (Table 2), after 30 minutes, 60 minutes and 120 minutes of incubation. Surviving rates at pH 2.0 were less compared to the pH 3.0. *L. bifermentum* MTCC3818 ability to grow at pH 2.0 and 3.0 was more as compared to *L. amylovorus* MTCC8129.

Stomach environment is acidic (pH 2-3) due to HCl, and the most of the microorganisms either killed or harmed at this pH (acidic condition of stomach) when they are ingested orally [20]. Probiotics are also like other microorganisms which are taken orally. They should have resistant to low pH, and pass alive through the stomach, finally to reach colon where they get colonized [21]. In addition, probiotic strains should have ability to survive the digestive enzyme of gastrointestinal tract like lysozyme [22]. The resistance of probiotics to low pH even after 120 minutes of incubation at 37 ^{0}C showed their ability to withstand the acidic environment of gastrointestinal tract. This ability is important as it enables the probiotics (taken orally) to survive at the low pH of stomach [21]. Other studies conducted in this area showed that hydrochloric acid (HCl) found in the human stomach denatures the microorganisms' biomolecules like protein, DNA and fatty acid. This low pH inhibits the cell metabolism, and therefore, decreases the lactobacilli viability and growth [23].

3.3 Bile salt hydrolase (BSH) activity of probiotics

BSH activity is considered as genetic marker to select lactobacilli as a probiotic. This activity is vital for those microorganism or bacteria which colonize and grow in the intestine. BSH activity causes the deconjugation of bile salts [24].

In this study, bile salt hydrolase activity of *L. amylovorus* MTCC8129 and *L. bifermentum* MTCC3818 was studied using direct plate assay method. MRS plates were

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containing 0.5% Sodium deoxycolate. Both strains exhibited the bile salt hydrolase activity, confirmed by the formation of precipitation zones in the MRS agar plate. These precipitation zones were formed due to the hydrolysis of Sodium deoxycholate (Table 3).

Most of the probiotic organisms that produce BSH enzyme are gram-positive bacteria except two strains of bacteroides that are gram negative [25]. Lactic acid bacteria like bifidobacteria and lactobacilli known to have bile salt hydrolase genes in their genomes [26]. Intestinal bacteria that produces bile salt hydrolase enzyme converts the conjugated bile salt to de-conjugated bile acid. Human liver produces bile salts from cholesterol while the gall bladder releases them into the small intestinal (duodenum). Gall bladder releases conjugated form of bile salts approx. 500-700 ml per day [24]. Under physiological conditions in humans, bile salt concentration ranges in between 0.3-0.5% [27]. Bile acids when conjugated with taurine or glycine in the liver, they are called conjugated bile salts. Deconjugated bile acid such as chenodeoxycholic acid and cholic acid along with free amino acids (e.g taurine and glycine) are produced by hydrolyzing the amide bond in the conjugated bile salts due to BSH activity [28]. Bile salt hydrolase activity is considered an important activity in decreasing the cholesterol in serum. Deconjugated bile salt are less soluble as compared to conjugated bile salts. Therefore, deconjugated bile salts are less reabsorbed from the lumen of intestine, therefore, bile salts are easily removed from the body through faces [29]. Bile salt hydrolase activity might be essential for the colonization and growth of bacteria in the intestines (Moser and Savage). Therefore, bile salt hydrolase activity is considered as an important attribute for the selection of probiotic [24]. Thus, deconjugation by bile salt hydrolase is also considered an important factor for maintaining the positive gut microflora balance [30].

3.4 Cell surface hydrophobicity

Cell surface hydrophobicity is another vital parameter for the selection of a potential probiotic strains. On reaching the colon probiotic should adhere to the mucosal cells of gastrointestinal tract colonized and protect GIT from pathogenic bacteria. Therefore, *L. amylovorus* MTCC8129 and *L. bifermentum* MTCC3818 ability to bind to hydrocarbons (xylene and n-hexadecane) was examined. Affinity of probiotics to the hydrocarbons viz. n-hexadecane

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and xylene shows their ability to adhere to the mucosal cells of intestine. Cell surface hydrophobicity is considered important for colonization of bacterial cells in the digestive tract of human body [31].

(Table 4) shows the cell surface hydrophobicity(%) results of *Lactobacillus amylovorus* MTCC8129 and *L. bifermentum* MTCC3918. Hydrophobicity with n-hexadecane and xylene was studied, and results indicated the adequate and significant hydrophobicity exhibited by both strains. Both strains showed high affinity for hydrocarbons i.e. xylene and n-hexadecane. Whereas *L. amylovorus* MTCC 8129 exhibited 71.6% and 69.33% affinity with n-hexadecane and xylene, *L. bifermentum* MTCC 3818 exhibited 93.97 % and 94.2% affinity with n-hexadecane and xylene. These results have been obtained from three independent experiments, each experiment was done duplicate.

Zhao *et al.* [32] have studied the cell hydrophobicity of different strains of *L. acidophilus*. *L. acidophilus* LA7 affinity for xylene and n-hexadecane was found to be in the range of 57%-58%. Whereas other strains of *L. acidophilus* exhibited very low affinity to hydrophobicity (2%-5%). Cell surface hydrophobicity and surface charge differ among different strains of probiotics, and the reason might be different cell physiological conditions, composition of media, different expression of surface-associated proteins among different strains [33]. Cell surface hydrophobicity potential of microorganisms is largely dependent upon the presence of proteinaceous compounds, glycol, lipoteichoic acids, and cell surface proteins like mucus binding protein on the surface of microorganism [34]. Cell surface hydrophobicity of *L. acidophilus* NCDC15 was reported to be 74.6±2.6% whereas *L. helveticus* NCDC292 affinity was 36±2.6% with n-hexadecane. With xylene *L. casei* NCDC17 affinity was reported to be 90.95±4.4% while *Lactobacillus helveticus* showed 34.45±2.2% affinity. Their affinity with n-octane was reported to be in the range of 23-35%. Different mechanisms have been proposed to explain the differences in cell surface hydrophobicity of different probiotics; (1) different carbohydrates on their cell surface, (2) source of probiotic, (3) environmental conditions [35].

3.5 Cell auto-aggregation feature of probiotic

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Cell auto-aggregation is also one of the property of probiotic. Auto-aggregation is a kind of aggregation that happens between same microorganisms. Cell auto-aggregation property of *L. amylovorus* MTCC8129 and *L. bifermentum* MTCC3818 was evaluated in vitro, and both strains were found to exhibit the auto-aggregation property (Table 5). Cell auto-aggregation is also considered as essential property for the adherence of probiotic on the intestinal epithelial cells. Probiotic adherence to the epithelial cells also prevents the colonization of pathogen (colonization resistance) [36]. The cellular auto-aggregation of probiotic bacteria has the ability to interact in various ways such as electrostatic interaction, passive force and hydrophobic interaction. In order to adhere to epithelial cells, probiotic must exhibit more than 40% cell auto-aggregation. On the surface of probiotics substances like lectins and polysaccharide are present that give them ability to adhere to the epithelial cell of intestine [37]. Auto-aggregation attribute of Lactobacillus is linked to presence of polysaccharide, proteins and lipoproteins on their cell surface cell [38]. Exopolysaccharides is considered as cause of adhesion of bacteria cells to the epithelial cells of intestine. *L. delbrueckii* ssp. *Bulgaricus* B2, B3, G12 that have shown high auto-aggregation (89-93%) property, have produced high amount of exopolysaccharide whereas strains with less auto-aggregation (45-56%) property, have produced less amount of expopolysaccharide [39].

3.6 Antimicrobial activity of probiotic

Antimicrobial activity is considered as of utmost important attribute for a probiotic. In the present study, *L. amylovorus* MTCC8129 and *L. bifermentum* MTCC3818 were screened for their anti-microbial activities against *E. coli* MTCC1698, *S. aureus* MTCC3160, *B. cereus* MTCC6629 and *E. faecium* MTCC2729 using agar well diffusion method. Both *Lactobacillus* strains exhibited antimicrobial activities against pathogenic bacteria (Table 6).

Genera *Bifidobacterium*, *Streptococcus* and *Lactobacillus* are known to produce several antimicrobial compounds that inhibits the growth of both gram positive and gram negative bacteria residing in human colon [19]. Antimicrobial compound like hydrogen peroxide, organic acid (acetic acid, propionic acid, succinic acid, lactic), bacteriocins and low molecular weight antimicrobial compounds are produced by different strains of probiotics [40]. In addition, some

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produces fatty acids, secondary metabolites, diacetyl, ethanol, reutericycline, formic acid and reuterin to exhibit the antimicrobial activity [41]. Besides producing antimicrobial compounds, probiotics also lowers the pH, and this low pH inhibits the growth of pathogenic bacteria. Colonization resistance is considered as another important activity conferred by probiotics to eliminate the pathogenic bacteria from the gut. *Lactobacillus* antimicrobial activities largely dependent upon the growth conditions (anaerobic or aerobic conditions) [19]. *Lactobacillus acidophilus* obtained from fermented milk have shown antimicrobial activity against pathogenic bacteria like *Salmonella typhimurium* (zone of clearance, 4.3mm±.02), *Listeria monocytogenes* (zone of clearance, 5.0±0.2 mm) and *Enterotoxigenic E. coli* (zone of clearance 4.2±0.4) [42]. *Lactobacillus paraplantarum* isolated from the leaves of tea, exhibited antimicrobial activity against *E.coli* (zone of clearance, 30 mm), *S. aureus* (56mm), *S. typhii* (65mm), *Citrobacter* (60mm) and *E. faecalis* (55mm) [40].

Conclusion

This research work has proved the probiotic attributes of *Lactobacillus bifermentum* MTCC3818 and *Lactobacillus amylovorus* MTCC8129. Both strains exhibited a tolerance to acidic conditions, therefore their survival under acidic conditions of stomach could be expected. Both probiotics exhibited the bile salt hydrolase activity which could be considered beneficial in lowering blood cholesterol. Both have showed good affinity for organic solvents like n-hexadecane and xylene. This affinity is considered important in terms of adherence of probiotics to intestinal mucosa and elimination of pathogenic bacteria from the colon (colonization resistance). Therefore, these strains of *Lactobacillus* like other probiotics might have numerous health benefits in humans and rodents and therefore can be used in the treatment of life-style disorders such as diabetes, obesity, cardio-vascular diseases and hypercholesterolemia. Therefore, further studies are warranted to examine the health benefits of these probiotics in humans and animals.

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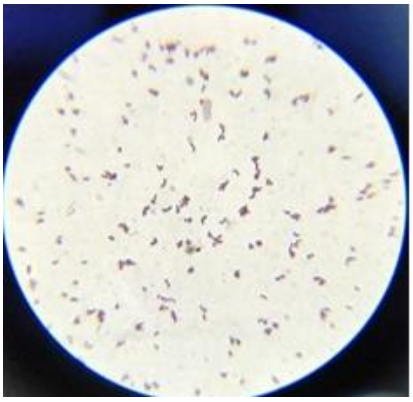

Fig. 1a

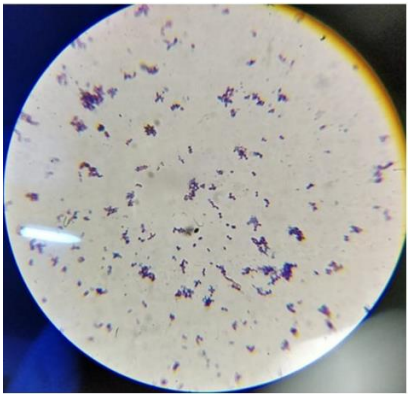

Fig. 1b

Figure 1: Microscopic view of *L. bifermentum* MTCC3818 (a) and *L. amylovorus* MTCC 8129 (b)

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Table 1: bacterial strains that are used in the present study.

Species of bacteria	Gram staining	Strains	Growing condition of culture
Lactobacillus amylovorus	Gram positive	MTCC8129	37 ^{0}C, MRS broth
Lactobacillus bifermentum	Gram positive	MTCC3818	37 ^{0}C, MRS broth
Enterococcus faecium	Gram positive	MTCC2729	37 ^{0}C, nutrient broth
Bacillus cereus	Gram positive	MTCC6629	37 ^{0}C, nutrient broth
Staphylococcus aureus	Gram positive	MTCC3160	37 ^{0}C, nutrient broth
Escherichia coli	Gram negative	MTCC1698	37 ^{0}C, nutrient broth

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Table 2: Growth of *L. amylovorus* MTCC8129 and *L. bifermentum* MTCC3818 in acidic environment at 37 ^{0}C.

Strains	pH 6.5				pH 3.0				pH 2.0			
	Minutes				Minutes				Minutes			
	0	30	60	120	0	30	60	120	0	30	60	120
L. amylovorus MTCC8129	7.37 ± 0.03	7.47 ± 0.008	7.576 ± 0.012	7.765 ± 0.0041	7.5 ± 0.0036	7.35 ± 0.03	7.24 ± 0.006	7.078 ± 0.01	7.49 ± 0.005	7.38 ± 0.018	7.023 ± 0.012	6.92 ± 0.02
L. bifermentum MTCC3818	7.58 ± 0.003	7.61 ± 0.033	7.685 ± 0.0042	7.76 ± 0.0092	7.75 ± 0.0082	7.64 ± 0.0065	7.541 ± 0.004	7.381 ± 0.014	7.76 ± 0.007	7.63 ± 0.085	7.48 ± 0.006	7.32 ± 0.014

Results are presented mean ± SD

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Table 3: Bile salt hydrolase activity of *L. amylovorus* and *L. bifermentum* under anaerobic condition.

	Bile salt deconjugation
Strains	Sodium deoxycholate
L. amylovorus MTCC8129	+*
L. bifermentum MTCC3818	+*

*(+) Positive results indicate the bile salt hydrolase activity in MRS agar plates by precipitation zone.

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Table 4: Cell surface hydrophobicity feature of *L. amylovorus* MTCC8122 *and L. bifermentum* MTCC3818

	Cell surface Hydrophobicity (%) affinity	
Culture	n-hexadecane	Xylene
L. amylovorus MTCC8129	71.6 ± 2.7	69.33 ± 3.04
L. bifermentum MTCC3818	93.97 ± 0.23	94.2 ± 0.5

Results are presented as mean ± SD

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Table 5: cell auto-aggregation property of *L. amylovorus* MTCC8129 and *L. bifermentum* MTCC3818

Probiotic Cultures	Cell auto-aggregation(%) affinity
L. amylovorus MTCC8129	65.9 ± 0.57
L. bifermentum MTCC3818	57.24 ± 2.23

The values exhibited have shown as means ± SD. Results were obtained from three independent experiments, each experiment was done in duplicate.

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Table 6: inhibition zone of *L. amylovorus* MTCC 8129 and *L. bifermentum* MTCC3818 antimicrobial activity against different pathogenic bacteria.

Zone of inhibition (mm)		
Indicator bacteria	*L. amylovorus* MTCC8129	*L. bifermentum* MTCC3818
E. coli MTCC1698	28.5 ± 1.65	21 ± 2.23
B. cereus MTCC6629	20.75 ± 1.9	13 ± 1.87
S. aureus MTCC3160	17 ± 1	14.5 ± 1.25
E. faecium MTCC2729	21 ± 1	15 ± 1

5mm is the diameter of the well. The volumes show the mean ± SD of the four replicate.

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