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ASSESSMENT OF PROBIOTIC POTENTIAL OF *LACTOBACILLUS* SP. ISOLATED FROM CHEESE AND PREPARATION OF PROBIOTIC ICE-CREAM

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ABSTRACT
Probiotic microorganisms are naturally present in milk and fermented milk products such as different kinds of cheese, yogurt, butter etc. The aim of the present study was isolation and taxonomic determination of lactic acid bacteria (LAB) from cheese (wild type). *Lactobacillus* sp. was isolated from indigenous cheese sample, identified and characterized on the basis of their morphological and biochemical characteristics at genus level. The pure isolated *Lactobacillus* was assessed for various probiotic properties such as tolerance to acidic pH, bile salt, antibiotic susceptibility, antibacterial activity and hemolytic activity. They showed better tolerance to pH 2 and pH 3 and 2 % bile salt; resistant to antibiotics viz. chloramphenicol, oxacillin and vancomycin. It was found active against test bacteria *Escherichia coli* and *Salmonella typhimurium* and found to be non-hemolytic when compared to the positive control strain of *Staphylococcus aureus*. Probiotic ice-cream was prepared, its safety and keeping quality was determined by significant total bacterial count, coliform count and the presence of yeast and moulds.
KEY WORDS: Probiotic, *Lactobacillus*, Cheese, Ice-Cream, Safety.

INTRODUCTION

Probiotics are live microorganisms, which when administered in adequate amounts confer a health benefit on the host[1]. These include bacteria, moulds, yeast; but most probiotics are bacteria of which, LAB are the most common type[2]. These live bacteria can resist the rigors of the human digestive system and that help to improve the gut flora balance[3]. They should be preferably of human origin, non-pathogenic, nontoxic, resistant to gastric acid, adhere to gut epithelial tissue and produce antibacterial substances[4, 5]. It can persist in the gastro-intestinal tract influencing metabolic activities like cholesterol assimilation, lactose activity and vitamin production[6].

LAB which found commonly as resident microflora of the gastro-intestinal and genitor-urinary tract of vertebrates, are considered as the major probiotic organisms[7]. Two genera, *Lactobacillus* and *Bifidobacterium*, have been found to be excellent potential sources of bacterial probiotics[8, 9].

The lactic acid fermentation process has long been known and applied by the humans for making different foodstuff[10]. It plays an essential role in the production of all dairy products and is involved in the production of many other foods and drinks[11].

Recently, increased focus has been given to food as potential vehicles of microorganisms with probiotic properties. The nutritious and therapeutic benefits of probiotic microorganisms have been most extensively investigated in dairy products such as milk, yogurt and cheese[12, 13]. These foods have become an important health care sector in most countries. In the present work, white cheese was taken as a source of probiotic organism. The isolated *Lactobacillus* sp. was used for preparation of probiotics ice-cream.

MATERIALS AND METHODS

Isolation, Identification and Characterization of *Lactobacillus* Sp. From Cheese

The white cheese pickled samples were obtained from local market, homogenized and suspension was prepared. The suspension was diluted in 2% sodium citrate solution (10^{-1}) and further serial dilutions were made by 0.1 % peptone water and plated by spreading 0.1ml higher dilution onto the surface of Rogosa agar. The cultures were incubated anaerobically at 37^0C for 48h. The pure *Lactobacillus* sp. was tested by Gram staining technique, cultivated in MRS broth at 37^0C. The pure *Lactobacillus* isolates were preserved at 4^0C on MRS agar stabs in triplicates after sealing with paraffin wax[14].

The characterization *Lactobacillus* sp. was carried out by gram reaction, spore formation, growth at different temperatures and catalase activity[15, 16]. Gram positive, non-sporforming and catalase negative rods were sub-cultured on MRS agar media at 37°C for 24 h[17].

Assessment of Probiotic Potential of *Lactobacillus* Sp

The main *in vitro* selection criteria for potential probiotic strains are acid and bile resistance activities, indicating the ability of the organism to survive the passage through the gastrointestinal tract[18, 19]. Production of antimicrobial compounds, such as bacteriocins, lactic and acetic acid; and competition for nutrients and adhesion sites may contribute to the control of intestinal pathogens[20, 21]. The following *in vitro* tests with standard methodology are recommended for screening putative probiotic strains[22].

Acid pH Tolerance

The pure *Lactobacillus* isolate was grown in MRS broth at 37°C for 24 h incubation[23]. The cells were harvested and washed twice with sterile phosphate buffer saline (pH 7) by centrifugation at 10,000 rpm for 5 min[24, 25]. The cell pellets were suspended in sterile MRS broth adjusted with a pH 1, 2 and 3 using 1 M HCl[26] and incubated at 37°C. After 2 h, the bacterial suspension was used for plating in

MRS agar media. Acid tolerant *Lactobacillus* sp. was assessed in terms of colony growth i.e. colony forming units (cfu/ml) in plate after 48 h anaerobic incubation[27].
All plates were duplicated and each reading represents the mean of three observations. Percentage of resistance to acidic pH was determined according to the equation, %Resistance = 100 (CFUsample/CFUcontrol) [26, 28].

Bile Salt Tolerance

The bile salt tolerance was assessed by investigating the ability of strains to grow in the presence of bile. The fresh culture of isolated *Lactobacillus* acid tolerant was grown at 37°C for 24 h in MRS broth without bile salt and 100 µl of culture of appropriate dilution were spread on sterile broth was employed in MRS containing 2% bile salts[29,30] and incubated anaerobically at 37°C[31,32]. After 48 h incubation, the growth of bacteria was evaluated to select the bile tolerant *Lactobacillus* sp. Bacterial growth was expressed as colony forming units/ml (cfu/ml). Reported results are means for triplicate samples and survival % of bile-tolerant species was calculated[31].

Antibiotic Susceptibility Test

Antibiotic susceptibility was determined semi quantitatively according to the antimicrobial disc susceptibility tests[32]. MRS agar plates were prepared and 100 µl of isolated active *Lactobacillus* was spread over the plates. Plates were incubated for 2 h at 37°C. Antibiotic discs (Hi-media Mumbai, India) were placed on the above plates and incubated for 24 h at 37°C[33]. The results were expressed in terms of zone of inhibition and reported as sensitive, intermediate or resistant according to the zone size interpretative chart provided with the antibiotics and the recommended standards[33, 34].

Screening of Isolated *Lactobacillus* Sp. For Antibacterial Activity

The antibacterial activity of isolated acid and bile resistant *Lactobacillus* was assessed by using well-diffusion methods. The indicator pathogens used included *Escherichia coli* and *Salmonella typhimurium*[24]. Cell suspensions were centrifuged at 5000 rpm for 15 min. The cell free supernatant was neutralized and used to test the antimicrobial activity assay[35, 36]. The size of the clear zone around the well was measured and the results were reported in millimeter (mm). The antibacterial activities were classified as none (-) and inhibition (+). The experiment was performed in triplicate.

Hemolytic Activity of *Lactobacillus* Sp.

Freshly prepared sterile blood agar plates were streaked with 6 h old *Lactobacillus* culture[23]. Plates were incubated anaerobically at 37°C for 48h after which they were observed for clear zones surrounding colonies (positive reaction for beta haemolysis)[37]. A control plate with *Staphylococcus aureus* was used as positive control.

Preparation of Probiotic Ice-Cream

The standard method for preparation of probiotic ice-cream was the slight modification of the method as suggested by Lokhande *et al*., 2011[38] and Vijayageetha *et al*., 2011[39]. High fat milk of desired quantity was heated at 65°C for 20 min. then required quantities of skim milk powder, stabilizer and sugar were added with constant stirring. The mix was homogenized and pasteurized at 72°C for 10 min., and then probiotic culture was inoculated aseptically when the temperature of the pasteurized mix reached to the desired temperature of inoculation i.e., 35°C-40°C and incubated for 8 h at 37°C. After the completion of incubation the flavor was added, frozen and stored at 0°C.

Safety and Keeping Quality of Probiotic Ice-Cream

The safety and quality of probiotic ice-cream is determined by significant total bacterial count, coliform count and the presence of yeast and moulds. After a week of storage 10 g melted probiotic ice cream was diluted in normal saline and examinations were carried out according to standard procedure of dairy products. The 0.1 ml inoculum of 10^{-4} dilution was placed on the surface of respective medium. For total bacterial count, the MRS agar plates were incubated at 35°C for 48 hours[40]. Coliform count was determined using MacConkey agar. The plates were incubated at 37°C for 24 h[41]. The count of yeast and mould were determined using potato dextrose agar. The plates were incubated at 25°C for 5 days[42].

Table 1: Identification of *Lactobacillus* Sp. From Cheese (Morphological, Cultural and Physiological Characteristics)

Sr. No.	Characters		Observation
1.	Cell morphology		Medium, long rods
2.	Gram staining		Gram +ve
3.	Spore formation		_
4.	Colony morphology		Circular, irregular, glistening appearance (1-2mm)
5.	Catalase activity		_
7.	Growth at different temperatures	15^0	_
		37^0	+++
		45^0	+
		50^0	_
8.	Optimum pH		6.0
9.	Casein hydrolysis		+

Note: +++: maximum growth; +: growth present; -: growth absent

Table 2: Tolerance of *Lactobacillus* Sp. to Acidic pH Values

S.No.	pH Values	Time (Hr.)	Control	Test	Survival Percentage (% ± SD)
1.	$P^H = 1$	1 hr	264 (10^{-4})	---	0 ± 0.0
			264 (10^{-4})	---	
2.	$P^H = 2$	1 hr	203 (10^{-4})	119	61 ± 3.6
			203 (10^{-5})	130	
		2 hr	167 (10^{-5})	78	47 ± 2.2
			167 (10^{-6})	82	
		4 hr	182 (10^{-6})	54	33 ± 3.1
			182 (10^{-6})	70	
3.	$P^H = 3$	1 hr	244(10^{-4})	168	67 ± 4.5
			244 (10^{-4})	159	
		2 hr	159 (10^{-5})	95	58 ± 2.8
			159 (10^{-5})	90	
		4 hr	178 (10^{-6})	77	44 ± 3.0
			178 (10^{-6})	83	

SD; Standard Deviation

Table 3: Showing Antibacterial Activity, Bile Salt Tolerance Of *Lactobacillus* Sp. and Safety Assessment of Probiotic Ice-Cream

Antibacterial Activity	**Response**			
Test Organisms	**Control**		**Test**	
Escherichia coli	_		+	
Salmonella typhimurium	_		+	
Bile Salt Tolerance	274		187	
Survival percentage (% ± SD) after incubation			68 ± 3.5	
Safety Assessment of Probiotic Ice-Cream				
Coliform Test (Dilution 10^{-2})	**Sample A**		**Sample B**	
Cfu/ml	Nil		Nil	
Yeast And Mould Count (Dilution 10^{-2})				
Yeast	3		2	
Mould	4		5	
Total Viable Count	1	3	5	7
cfu/ml	$1.8x10^4$	$1.7x10^4$	$2.0x10^4$	$2.1x10^4$

Note: SD; Standard Deviation, + Indicates Zone Of Inhibition, - Indicates No Zone of Inhibition

Table 4: Testing of *Lactobacillus* Sp. For Antibiotic Resistance

Sr. No.	Antibiotic	Concentration	Response
1.	Ampicillin	10 µg	S
2.	Chloramphenicol	30 µg	R
3.	Ciprofloxacin	5 µg	S
4.	Clindamycin	2 µg	S
5.	Rifampicin	5 µg	S
6.	Gentamycin	5 µg	S
7.	Oxacillin	1 µg	R
8.	Vancomycin	30 µg	R

Where S = Sensitive, R =Resistant

RESULTS AND DISCUSSION

Cheese is a milk product, during its preparation various processes are involved such as acidification, coagulation, cooking, salting, dehydration, molding and pressing, packaging and maturation or storage. Many native microflora (microorganisms naturally occurring in a given region), which acts as probiotics may get affected due to these processes. Hence there is need to monitor the viability and potential of these microorganisms.

Isolation, Identification and Characterization of *Lactobacillus* Sp. From Cheese

Isolated strains were identified based on their morphological and biochemical characteristics at genus level following the criteria and descriptions in the literature[15, 16]. The cultures were examined microscopically and observed that the cells were rod shaped, non-sporforming, circular, irregular and colony showed glistening appearance (1-2 mm) (Table1). Biochemical observations indicated that they were gram-positive, catalase-negative and casein hydrolyzing bacteria with optimum growth at pH 6.0. Growth of the culture at different temperatures showed that maximum growth observed at 37^0C, some growth at 45^0C and no growth was observed at 15^0C and 50^0C (Table1). Hence it was confirmed that the isolated bacterium was *Lactobacillus* sp.

Acid pH and Bile Salt Tolerance

The effect of pH and bile salt on the viability of strains is shown in Table 2 and 3 respectively. Results showed that the isolated *Lactobacillus* was tolerable to pH 2 and pH 3; and exhibited better tolerance to 2 % bile salt; while no tolerance was found to pH 1.

Before reaching the intestinal tract, probiotic bacteria must first survive transit through the stomach where the pH can be as low as 1.5 to 2. The ingested bacteria must be resistant to the enzymes in the oral cavity (e.g., lysozyme), as well as to the environment during the digestion process in the stomach and the intestine (e.g., exposure to bile). The average bile concentration is around 0.3%, and may range up to the extreme[43, 44].

Antibiotic Susceptibility Test

The susceptibility to antibiotics is graded as either R (Resistant) or S (Susceptible) [45]. Inhibition zone diameters were measured in mm in which diameter of ≥21 mm is referred as sensitive and ≤15 mm was resistant[30]. The isolated *Lactobacillus* sp. was found resistant to three antibiotics viz. chloramphenicol, oxacillin and vancomycin whereas sensitive to remaining antibiotics viz. ampicillin, ciprofloxacin, clindamycin, rifampicin and gentamycin (Table 4).

The resistant property of *Lactobacillus* sp. against some antibiotics would be associated with preventive and therapeutic purposes in controlling intestinal infections. It is well known that vancomycin is an antibiotic belongs to glycopeptide antibiotics inhibits the peptidoglycan synthesis which is an important structural component of the bacterial cell wall. Therefore, Gram-positive bacteria, including LAB are especially vulnerable to vancomycin treatment

Screening of Isolated *Lactobacillus* Sp. For Antibacterial Activity

The results indicated that *Lactobacillus* sp. possesses significant antibacterial activity against the indicator organisms *E. coli* and *S. typhimurium* (Table 1). LAB produces antimicrobial substances with activity against gastric and intestinal pathogens and other microbes, or competes for cell surface and mucin binding sites. LAB commonly produces bacteriocins which are peptides with bactericidal activity usually against strains of closely related species[46]. Although bacteriocins may enhance survival of LAB in complex ecological systems interest has focused on prevention of growth of harmful bacteria in the fermentation and preservation of dairy products. In general, the antimicrobial activity of *Lactobacilli* may be due to organic acids, hydrogen peroxide, bacteriocins or other inhibitory substances from metabolites[47].

Hemolytic Activity of *Lactobacillus* Sp.

In hemolytic activity, there was no change in color surrounding the colony on blood agar plate. Therefore isolated *Lactobacillus sp*. found to be non-hemolytic compared to the positive control strain of *S. aureus*.

Safety and Keeping Quality of Probiotic Ice-Cream

In probiotic Ice-cream, coliform count was not observed (Table 3). During storage the counts of *Lactobacillus* sp. remained broadly stable over 7 days and highest count achieved by 7th day. Few colonies of yeast and moulds were observed which are within the acceptable limits[48]. According to a literature, the isolated yeast produces exopolysaccharides and exhibited antioxidative activity[49].

Ice cream can be an attractive vehicle for probiotic intake combining both health-promoting and mood-boosting effects[50]. Monitoring and characterizing the survival of the probiotic bacteria in ice cream after freezing is of high importance. This will allow the acquisition of the probiotic food label and the sales and advertisement certification for the product. The freezing step in the process of making ice cream could cause a reduction in the concentration of the bacterial load indicates that quantification of this reduction is important to maintain the bacterial load within the food industry standards.

In a study on the viability of probiotic *Lactobacillus acidophilus*, it was observed that the rate of survival was better even when inoculated into ice cream and stored at -25˚C for 60 days[51].

Lactobacillus sp. is one of the most important industrial bacteria, being widely used in food production, health improvement, and production of macromolecules, enzymes, and metabolites.

LAB has been used since ancient times in food fermentation processes and preservation. Many health benefits have been claimed for probiotics[52] e.g. improvement of the normal microbiota, prevention of infectious diseases and food allergies, reduction of serum cholesterol, anticarcinogenic activity etc.

CONCLUSION

Results obtained in the present work showed a survivability of *Lactobacillus* sp. Isolated from cheese. The bacterium was tested in high bile salt concentration and acidic pH values, exhibited no hemolytic activity and showed susceptibility to some antibiotics. It has shown antimicrobial activity against *E. coli and S typhimurium*. These characteristics will help isolated *Lactobacillus* to reach the digestive system and contributing balance of intestinal microflora. Probiotic ice-cream so prepared found to be safe for consumption and preservation.

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