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ISOLATION AND CHARACTERIZATION OF PROBIOTIC LACTIC ACID BACTERIA FROM MILK AND CURD SAMPLES

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ABSTRACT

Probiotic bacteria are dietary supplements of live microorganisms thought to be healthy for the host organisms. In this study, lactic acid bacteria were isolated from 2 different curd and milk sample. A total of 20 strains were studied for their characterization, from that 2 potential strains *Lactobacillus acidophilus* and *Lactobacillus casei* were identified. The potential strains were studied by curd production, antibacterial and bacteriocidal activity also their antibiotic susceptibility. The quality of curd was analyzed by qualitatively and quantitatively by nutrient evaluation (CHO, protein, ash, lipid, *etc.*). The antibiotic resistance of potential strain was studied using Vancomycin, gentamycin, chloromphenicol, ciprofloxicine and cefataximide. PB1, PB3 shows the good resistance activity to the antibiotics. The curd produced by those strains has good quality and quantity of nutrients. Also the total viable count in curd was performed; it showed 13 - 15 X 10^{-7} (CFU) cells per ml of curd.

KEY WORDS: **Lactic acid bacteria, *Lactobacillus* species, curd production, bacteriocin production**

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INTRODUCTION

Probiotics control intestinal pathogens by production of antibacterial compounds, including lactic acid and acetic acid and antibiotic like substances, competition of nutrients and adhesion sites and decreased enzyme activity, increased antibody levels and increased macrophage activity[1, 2, 3]. Lactic acid bacteria are a group of Gram-positive, non-spore forming, cocci or rod shaped, Catalase-negative and fastidious organisms, considered as 'Generally Recognized as Safe' (GRAS) organisms [4]. Probiotic strains of *Lactobacillus* and *Bifidobacterium* generally recognized as non-pathogenic and increasingly used in dairy production[5], especially used as starter organism for fermentation. Curd is a popular fermented milk product in India consumed in almost every household[6]. It is prepared from buffalo milk (6-8%) fat, cow milk (3.5- 4.5%) of fat or standardized milk (4.5% fat). Curd accounts for around 90% of the total cultured milk products produced in India. Among the various fermented milk products, the international market for curd and chilled desserts has been benefited due to considerable growth toward healthy food and much new product development. Demand for curd has increased considerably in the last decade [7], owing to curd's nutritional and therapeutic benefits [8]. The antimicrobial activity of lactic acid bacilli may be due to the production of a number of antimicrobial substances such as lactic acid, hydrogen peroxide, diacetyl and bacteriocins[9]. The antagonistic property of curd having *Lactobacillus* is attributed to the lowered pH, the undissociated acids and the production of other primary and secondary anti-microbial metabolites such as bacteriocin or bacteriocin like compounds[10]. Bacteriocin producing strains of Lactic acid bacteria (LAB) used as bio-preservatives [11]. The objective of the study is to screen and isolate the lactic acid bacteria as probiotics from the fermented products, selection of best potential strains.

MATERIALS AND METHODS

Isolation and identification of strains

The microbial strains were isolated from branded curd samples (Cavin's (n= 15) and Ananya (n= 12)). Thoroughly stirred 1 g of each sample were taken aseptically and subjected to 10-fold dilution, 0.1 ml of diluted sample was inoculated on MRS agar plates under anaerobic condition and incubated at 32^0C for 48 hours[12]. The different morphological colonies were isolated and pure cultures were maintained in MRS agar slant at 4^0C. Gram staining, cell morphology, biochemical test, sugar fermentation, growth at different temperatures and sodium chloride tolerance were performed for all isolated strains [13, 14]. The strains were identified based on Bergey's manual of determinative bacteriology[15].

Antimicrobial and bacteriocin activity

The LAB strains were screened for antimicrobial activity against *Staphylococcus sp., Pseudomonas sp., Klebisiella sp., Escherichia coli and Proteus sp*., by agar diffusion method[7]. The cell free neutralized supernatants of LAB isolates were screened for bacteriocin activity by agar spot method[16]. For the detection of antibacterial activity of the strains of *Lactobacillus* spp., MRS containing only 0.2% glucose was used. The test was performed as per the method described by Ozlem Erdogrul and Feryal Erbulur [12].

Determination of antibiotic resistance of the isolates

In this study, 6 antibiotic discs (Vancomycin, Gentamycin, Chloramphencol, Cefodizime, and Ciprofloxacin) were used to determine the antibiotic susceptibility of isolated *Lactobacilli* strains. The bacterial cell on MRS agar slant was mixed with saline. Cell suspensions (0.5 on the Mcfarland scale) were inoculated to Muller-Hinton Agar (MHA) plates [12], after few minutes all the

antibiotic disc were placed aseptically on the MHA plate and the plates were incubated at 37^0C for 24 hours.

Preparation of curd by isolated strain

The standardized milk was collected from local market, boiled at 90°C for 15 minutes and poured into sterile glass jars (100 ml). The probiotic curd was prepared by inoculating the isolated strains [17]. The control samples were prepared by inoculating milk with 10^7 CFU/ml of strains. The preparation of curd was kept for incubation at 45°C for 7 hours.

Nutrition analysis of the curd

The prepared curd was qualitatively analyzed by titratable acidity. 2g of sample was weighed and mixed with 10ml of hot distilled water. The contents were titrated with 0.1N sodium hydroxide solution in the presence of 0.5% phenolphthalein indicator. TA was calculated as the percentage of lactic acid in the product[18]. For quantitative analysis, the carbohydrate was quantified by Anthrone method[19] and the total protein content was estimated by Lowry's method using Bovine serum albumin as standard [20], Calcium by the method of Clark & Collip [21], [22] and β-galactosidase activity by ONPG test.

Enumeration of viable cells in the Curd

1 ml of each curd was transferred aseptically into 9 ml sterile peptone water, mixed thoroughly and serially diluted (10-fold) using 9 ml peptone water blanks. 0.1 ml of diluted sample was inoculated on MRS agar plate. After incubation, the total *Lactobacilli* counts were enumerated on MRS agar using colony counter.

RESULTS

Isolation and identification of strains

In this study, 20 strains were isolated from the 2 different brands of curd and milk samples. They were named as PB1, PB2, PB3 up to PB20. The twenty strains were subjected to Gram staining and motility test, Catalase, Oxidase, sugar fermentation, temperature and salt tolerant test (Table 1). From results two lactic acid strains such as *Lactobacillus casei* (PB1, PB16 & PB18) and *Lactobacillus acidophilus* (PB2) were identified.

Table 1
Microscopical, cultural and biochemical characterization of isolated strains

Strain	Gram staining	Motility	Catalase	Oxidase	Glucose	Lactose	Mannose	Maltose	Ribose	Temperature (^{0}C)		Salt tolerant (%)				
										15	45	3	5	7	9	11
PB1	G +ve Bacilli	NM	-	-	-	+	+++	-	+++	+	+	+	+	+	+	+
PB2	G +ve Bacilli	NM	-	-	+++	+++	+++	-	-	-	+	+	-	-	-	-
PB3	G +ve Bacilli	NM	-	-	+	++	+++	-	+++	+	+	+	+	+	+	+
PB4	G +ve Bacilli	NM	+	-	-	-	++	-	-	+	+	+	+	-	-	-
PB5	G +ve Bacilli	NM	-	-	-	-	+++	-	-	+	+	+	+	+	-	-
PB6	G +ve Bacilli	NM	+	-	+++	++	++	-	-	+	+	+	+	+	-	-
PB7	G +ve Bacilli	NM	+	-	+++	-	+	-	-	-	-	+	+	-	-	-
PB8	G +ve Bacilli	NM	-	-	+++	++	+	++	-	+	+	+	+	+	-	-
PB9	G +ve	NM	-	-	++	-	-	-	-	+	+	+	+	+	-	-

	Bacilli				+											
PB10	G +ve Bacilli	NM	-	-	-	-	++	++	-	-	-	+	+	-	-	-
PB11	G +ve Bacilli	NM	+	-	-	-	++	+++	-	-	-	+	+	+	-	-
PB12	G +ve Bacilli	NM	+	-	-	-	-	-	-	-	-	+	+	+	+	+
PB13	G +ve Bacilli	NM	+	-	-	-	++	-	-	-	-	+	+	+	+	+
PB14	G +ve Bacilli	NM	+	+	-	-	++	-	-	-	-	+	+	+	+	-
PB15	G +ve Bacilli	NM	-	+	+	++ +	++	++	-	-	-	+	+	+	_	-
PB16	G +ve Bacilli	NM	-	-	++ +	++	++	+	+++	+	+	+	+	+	_	-
PB17	G +ve Bacilli	NM	-	-	++ +	++	+++	+	+++	+	+	+	+	+	_	-
PB18	G +ve Bacilli	NM	-	-	++ +	++	+	+++	+++	+	+	+	+	+	-	-
PB19	G +ve Bacilli	NM	+	-	++ +	++	+++	++	-	-	-	+	+	+	+	-
PB20	G +ve bacilli	NM	+	+	++ +	++	+++	+++	-	-	-	+	+	+	+	-

'NM' Non motile; ('+') Positive; ('-') Negative [Catalase and Oxidase]
('+') Poor activity; ('++') Moderate activity; ('+++') Good activity; ('-') No activity [sugar fermentation]
('+') Growth; ('-') No growth [temperature and salt tolerant]

Antimicrobial and Bacteriocin activity

All the 20 isolates were analyzed for antimicrobial and bacteriocin activity against selected bacterial pathogens. In antibacterial activity, the pathogen *Staphylococcus* sp., showed sensitive to PB5, PB7, PB15, PB16, PB17, PB18 and PB20; *Pseudomonas sp.,* showed sensitive to PB2, PB8, PB9 and PB16; *Klebisiella sp.,* showed sensitive to PB1, PB3, PB13, PB14, PB19 and PB20. Incase of bacteriocin, most of the strains showed good bacteriocin activity on test organisms (Table 2).

Table 2
Antimicrobial and bacteriocin activity of isolated strains

Isolates	Antimicrobial activity				Bacteriocin production				
	Staphylococcus sp.,	*Pseudomonas sp.,*	*Klebisiella sp.,*	*Proteus sp.,*	*Staphylococcus sp.*	*Klebisiella sp.,*	*Bacillus sp.,*	*Pseudomonas sp.,*	*E. coli*
PB1	R	R	S	S	-	-	-	-	+
PB2	R	S	R	R	-	-	-	-	-
PB3	R	R	S	S	-	-	-	-	+
PB4	R	R	R	R	-	-	-	-	-
PB5	S	R	R	R	+	+	+	-	+
PB6	R	R	R	R	+	-	-	-	-
PB7	S	R	R	R	-	-	+	+	-
PB8	R	S	R	R	-	-	+	+	-
PB9	R	S	R	R	-	+	-	-	-
PB10	R	R	R	R	-	+	-	-	-
PB11	R	R	R	R	-	+	-	-	-
PB12	R	R	R	R	-	-	-	-	-
PB13	R	R	S	S	-	-	-	-	+
PB14	R	R	S	R	-	-	-	-	-
PB15	S	R	R	R	+	-	-	-	-
PB16	S	S	R	R	-	-	+	+	+

PB17	S	R	R	R	-	-	+	+	+
PB18	S	R	R	R	+	-	+	-	+
PB19	R	R	S	R	-	-	-	-	-
PB20	S	R	S	R	+	+	-	-	-

'S' sensitive; 'R' Resistance; '+' good activity; '-'poor activity

Determination of antibiotic resistance of the isolates

The antibiotic resistances of the isolates were studied using antibiotic discs. The behavior of each isolate to different antibiotics in terms of resistant and sensitive. Among the strains PB1, PB3 shows the good resistance activity to the antibiotics has been shown in table 3.

Table 3
Antibiotic resistance of the isolates

Isolates	Vancomycin	Gentamycin	Chloramphenicol	Ciprofloxicine
PB1	R	R	R	R
PB2	R	R	R	S
PB3	R	R	R	R
PB9	R	S	R	R
PB16	R	R	S	R
PB17	R	R	S	R
PB18	R	R	S	R

R- resistant, S- sensitive

Nutrition analysis of the curd

The titratable acidity of the curd was accessed by the disappearance of pink color and the observed pH values. Carbohydrate, protein, crude ash, lactose, calcium content and β-galactosidase activity were estimated (Table 4). The curd produced by isolated strains showed carbohydrate content ranges from 8500 - 17500 mg, protein content ranges from 3100 - 5400 mg and Calcium content ranges from 840 - 164 mg.

Table 4
Nutritional analysis of curd produced by isolated strains

Isolates	Qualitative		Quantitative analysis			
	TA Values	pH values	CHO (mg/100g)	Protein (mg/100g)	Calcium (mg/100g)	β-galactosidase activity
PB1	3.33	5.0	10000	5000	132	Positive
PB2	4.4	5.0	17500	5400	163	Positive
PB3	1.7	4.5	17500	5200	164	Positive
PB16	1.54	4.0	10000	3500	84	Positive
PB17	1.25	4.2	13000	4800	159	Positive
PB18	2.7	4.0	8500	3100	130	Positive

TA- Titratable acidity

Enumeration of viable cells

The total number of probiotic organisms in curd was determined by serial dilution method. In this study, the curd prepared by PB1 and PB2 has the viable count range between 13 - 15×10^{-7} CFU/ml.

DISCUSSION

Milk, curd and other milk products are involved as food substances with Indian society. Right from the day of Lord Krishna to the modern age, milk, and thousands of milk-products like curd, cream, butter, paneer, sandesh, rassogolla, ksheer; sreekhand *etc*., are used commonly and regularly. Curd is a very essential and regular food item to Indian's with reference to south India[23]. The curd used by Indian peoples was generally prepared by themselves or industrial one. The present study was carried out for knowing the necessity of the probiotics, its role in curd production and also the beneficial effects. We have selected twenty (20) isolates from milk and curd samples; all are subjected to physiological, cultural and biochemical studies. Of the 20 isolates, the two important lactic acid probiotic strains *Lactobacillus casei* and *Lactobacillus acidophilus* were identified and used for curd production. This study supported by previous workers, Shafei *et al.,* screened and characterizes 100 strains of bacteriocin-producing lactic acid bacteria from traditional fermented foods [24], and Mallesha *et al*., identified 16 lactic acid bacteria from fermented products [25].

All the strains were subjected to antibacterial, bacteriocin activity against *Staphylococcus aureus, Bacillus sp., Klebsiella Sp., Pseudomonas sp., Proteus sp.,* and *Escherichia coli*. The inhibition activity against the tested bacterial pathogens indicating a relatively narrow antimicrobial spectrum of probiotic lactic acid bacilli, this finding supported with the earlier reports of Aslim *et al*, *Lactobacillus* isolates obtained from Turkish dairy products have antimicrobials activity against *Staphylococcus aureus* and *Escherichia coli* [26]. The curd produced by identified probiotic strain *Lactobacillus casei* and *Lactobacillus acidophilus* was showed good nutritional value like carbohydrate (8500 – 17500 mg/100g) (Normal = 15700 mg), protein content (3100 – 5400 mg/100g) (Normal = 5100 mg) and Calcium (84 - 164 mg/ 100g) (Normal = 160 mg). The viable count (13 - 15×10^{-7} CFU) of the produced curd shows the potential probiotic load. In previous study, it was ranged between 1.0×10^{-7} to 9.4×10^{-7} CFU/ml [27]. The high incidence of microbial load (Lactic acid bacteria) in curd sample is known for their ability to produce antibacterial substances such as organic acids, hydrogen peroxide and bacteriocins[28]. From the above strains PB2 and PB3 shows the higher nutritional values than the normal values, these strains may be used as best probiotic or as bio-preservative for the curd production in commercial scale.

CONCLUSION

Probiotic bacteria in curd or other milk products could be a very good choice for value added food products. The probiotic lactic acid bacterial strains particularly *Lactobacillus casei* and *Lactobacillus acidophilus* with viable count have the ability to produce good quality of curd and it can inhibit pathogenic bacterial mass. It has been observed throughout the present research work. The curd produced by the identified strains showed good nutritional value. Hence, this study strongly supports the knowledge about the selection of inoculums for curd preparation especially on probiotic properties to promote people health.

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