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Antibacterial activity of Bacterocin producing *Lactobacillus* sp., isolated from traditional milk products

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ABSTRACT

Lactic acid bacteria commonly used as a natural food preservative to improve the food safety and stability. These organisms produce certain antimicrobial substance such as bacteriocins. The present study was focused on isolation and characterization of bacteriocin producing L*actobacillus* sp*.,* from a traditional milk product such as Curd, Cheese, Butter, Milkpeda and Ghee. The isolates were identified, based on characteristics of the strains of *Lactobacillus* sp*.,* as present in Bergey's manual of determinative bacteriology, the metabolite bacteriocin was extracted from the isolated *Lactobacillus* LBC and the antibacterial activity was evaluated against bacterial pathogens. The bacteriocin producing *Lactobacillus* LBC exhibited the highest zone of inhibition (15 mm) against *Staphylococcus aureus*. This study revealed the possibility of using bacteriocin as food biopreservative to control food spoilage and pathogenic bacteria.

Introduction

Lactic acid bacteria, particularly those belonging to beneficial and non-pathogenic genera (*Lactobacillus, Lactococcus, Streptococcus, Pediococcus* and *Leuconostoc*) are widely used in food industry. Among lactic acid bacteria; *Lactobacilli* are the most important group and are gaining increasing attention in food fermentation industry because of their potential biotechnological interest. This organism prevents the growth of pathogenic bacteria in different ecosystems by production of antimicrobial substance such as organic acids, hydrogen peroxide and bacteriocins. (Reid *et al.,* 2001). Bacteriocins are small proteins with bactericidal or bacteriostatic activity. (Klaenhammer *et al*., 1988).

The bacteriocin-producing *Lactobacillus* may be used as protective culture to improve the microbial safety of foods (Olasupo *et al.,* 1997) The antagonistic effects of bacteriocins against food spoilage (Leroy *et al*, 2003), which is usually achieved by inhibition of *Pseudomonas, Staphylococcus aureus, Salmonella* sp. and *Listeria monocytogenes* (Chiang *et al*., 2000) and they have great potential as biopreservatives for food (Gravesen *et al.,* 2004).

There is a growing consumer demand for processed dairy products containing no chemical preservatives, leading to indigenous studies in the field of screening bacteriocin as food preservatives. However, most of these indigenous research studies have always attempted to access the effectiveness of bacteriocin-producing lactic acid bacteria strains that were isolated from certain traditional dairy products. This present study was conducted to evaluate the antibacterial activity of bacteriocin producing *Lactobacillus* sp. isolated from traditional milk products.

Material and Methods

Collection of dairy products

Five different dairy products *viz.*, Curd cheese, Milk peda, Ghee and Butter were collected from local market in Pondicherry.

Viable Microbial Count

Lactobacilli Counts were determined on MRS agar with glucose a source of energy. Appropriate dilutions were plated on MRS agar and incubated aerobically at 37°C for 48hrs. Yeasts and moulds were enumerated by surface plating on Potato Dextrose agar and incubated aerobically at room temperature for 3days Coliform counts were enumerated by using MacConkey agar. The Characteristic colonies were counted by dilution factor and expressed as colony forming units per milliliter (cfu mL^{-1}).

Test Organisms

The Test organisms used were *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Salmonella* sp. and *Shigella dysenteriea.* All the cultures were maintained in the refrigerator at 4°C.

Isolation and identification of bacteriocin producing *Lactobacillus* sp

The bacteriocin producers from naturally traditional milk products were isolated by Pour plate technique as per the conventional method using MRS agar, and the plates were incubated at 37°C for 24hrs, the typical colonies were purified and identified. The isolates were differentiated on the basis of their morphological, cultural and biochemical characteristics.

Screening of lactic acid bacteria for antimicrobial activity

An overnight culture of each isolate grown in MRS broth at 37 °C was standardized to an optical density of 0.5 at a wavelength of 600 nm. (spectrophotometer). One percent of standardized culture was used to inoculate MRS broth. After incubation at 37°C for 24 hrs, cells were removed by centrifugation at 10,000 rpm for 15 min. The pH of one portion of supernatant was adjusted to 7.0 and filtered through 0.22 µm membranes. The filtrates of both pH and non-pH adjusted were used to evaluate antimicrobial activity using agar well diffusion

method. Positive results were recorded when the zone of inhibition of at least 1 mm around the wells was observed.

Extraction of bacteriocin

The *Lactobacillus* isolates were propagated each in 250ml MRS broth (pH 6.8) for extraction of bacteriocin, a culture supernatants were obtained by centrifuging (6.000 rpm for 30min at 4°C).The cell free solution was precipitated with ammonium sulphate (40% saturation). The mixture was rotated for 2hrs at 4°C and later centrifuged at (10.000 rpm for 20 min). The precipitates were obtained and resupended in 10ml of 0.05M potassium phosphate buffer (pH 7.0).

Detection of antimicrobial activity by agar well diffusion method

An agar well diffusion method as described by Klaenhammer was used with some modifications. An overnight culture of pathogens including were *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Salmonella* sp. and *Shigella dysenteriea* grown in TSB medium at 30 °C was diluted to a turbidity equivalent to that of a 3.0 McFarland standard with a sterilized 0.85% NaCl solution. A lawn of an indicator strain was made by spreading the cell suspension over the surface of BHI plates with a cotton swab. The plates were allowed to dry and a sterile cork borer of diameter 7.0 mm was used to cut uniform wells in the agar plates. Each well was filled with 70 µl of filter sterilized supernatant obtained from culture grown in MRS medium. All the assays were carried out in triplicate. After incubation at 37°C for 24 hrs, the diameter (mm) of the inhibition zone around the well was measured.

Results

In this study of *Lactobacillus* sps, isolated from different milk products such as Curd, Cheese, Milk peda, Butter and Ghee were characterized and identified. Their antimicrobial activity was evaluated against different food borne pathogens. The total microbial populations of different milk products were enumerated and the results in Table 1. The highest microbial population was found in the curd sample, which recorded a *Lactobacillus* population of 4.5 ±0.05 and yeast population of 1.8 ± 0.02. This was closely followed by the cheese sample, which recorded *Lactobacillus* population of 3.9 ±.10 and yeast population of 1.1±0.14. The least *Lactobacillus* population was recorded in the Butter sample 2.5 ±0.05. However, the yeast populations were found to be below not detectable level in butter samples. Based on the morphological, physiological and biochemical studies in Table 2. All the five isolates (LBC, LBL, LBB, LBH, and LBF) were found to be gram positive, catalase-negative, non spore-forming and rods shaped. The optimum temperature of the isolates varied from 37-40°C; pH ranged from 6.0-6.8. Three of the isolates i.e., LBL, LBB and LBH were found to belong to homofermentative group while the other two isolates i.e., LBC and LBF were found to belong to heterofermentative group. The five isolates were characterized based on the carbohydrate utilization and the results are presented in Table 3. All the five isolates i.e., LBC and LBH were found to be positive for utilization of glucose, lactose, maltose.galactose and fructose. While none of the two isolates were able to utilize D-xylose. The extracts of five isolated of *Lactobacillus* gave zones of inhibition against the indicator food pathogenic tested. The table 4 gives the results of inhibition; pathogenic strains inhibited are *Escherichia coli, Staphylococcus aureus, Bacillus cereus, Salmonella typhi* and *Shigella dysenteriea*. The diameters of inhibition are included between 9mm to 15mm. The highest diameter of 15mm inhibition is obtained with the extract of LBC on *Staphylococcus aureus,* as for the smallest diameter is obtained with extract of LBH on the same pathogen *Staphylococcus aureus*.

Discussion

Lactobacilli have been used for many centuries in food fermentation process. These *Lactobacilli* are a diverse group of genera, which can be characterized as gram-positive, catalase negative, non-sporulating, non-pigmented bacteria (Axelson *et al.,* 1993). Lactic acid bacteria (LAB), particularly those belonging to beneficial and non-pathogenic bacteria (*Lactococus, Lactobacillus, Leuconostoc, Oenococcus* and *Streptococcus*) have traditionally been used in the food industry. They also play an essential role in the dairy industry due to the tremendous level of human consumption of several important fermented products, mainly cheese and acidified or fermented milks (Farkye, 2004).

A detailed study on the total microbial population of different milk products studied revealed that the highest bacterial population was found in the curd (dahi) sample. This was closely followed by the cheese sample. While least microbial population was recorded in the butter sample. The incidence of different *Lactobacillus* sp., *i.e., Lactobacillus bulgaricus* in yogurt (Gilliland, 1990), *Lactobacillus. casei* in cheese (Aran, 1998), *Lactobacillus. helveticus* in butter (Robinson *et al.,* 1981) *Lactobacillus lactis* and *Lactobacillus fermentum* in sour cream and other milk products (Gobbeetti and Dicagno, 2002) has been reported earlier.

The results of our present study are in line with the earlier findings of Riadh Al-Tahiri (2005). They reported a high incidence of microbial load in Curd (dahi) sample, when compared with other dairy products like Milk peda and cheese. Lactic acid bacteria are known for their ability to produce antibacterial substances such as organic acids, hydrogen peroxide and bacteriocins (Tagg *et al.,* 1976; Kanmar *et al.,* 1995). Bacteriocins are bactericidal agents as claimed by Klaenhammer (1993), hence they may be used as probiotic or as biopreservative.

The inhibition zones of between 0.5-13.0 mm in diameter by the Bacteriocin –Producing *Lactobacillus* strains against the indicator organisms as reported by Enan *et al* .,(1999) however can only be classified as being between non-inhibition and moderate inhibition, indicating a relatively narrow antimicrobial spectrum, this finding may be supported with the earlier reports of (Aslim *et al* 2005), that all the *Lactobacillus* isolates obtained from Turkish dairy products have antimicrobials activity against *Staphylococcus aureus* and *Escherichia coli*. In the present study an attempt was made to find out the antibacterial property of *Lactobacillus* against certain bacteria *viz., Staphylococcus aureus, Bacillus cereus, Escherichia coli, Salmonella typhi* and *Shigella dysenteriae*.

Table - 1: Microbial viable counts log 10cfu g-1 and pH of milk products

S.No	Microbial Count	Total Microbial Population				
		Curd	Cheese	Butter	Milk peda	Ghee
1	*Lactobacilli*	4.5±0.05	3.9±0.10	2.5±0.05	3.0±0.15	3.5±0.05
2	Yeast	1.8±0.02	1.1±0.14	ND*	1.2±0.16	1.1±0.38
3	Coliform	3.8±0.05	3.1±0.15	2.6±0.09	2.8±0.02	3.2±0.28
4	pH	6.8±0.15	6.8±0.15	6.8±0.15	6.8±0.15	6.8±0.15

*=Below detectable level of $1x10^2$ cfu/ml

Values are mean ± SD of three replicates from one representative experiment. Within a column different letters after values indicate that there is a significant difference at P value of 0.05, as determined by one way analysis of variance followed by a post hoc test.

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Table - 2: Morphological and Physiological characterization of the *Lactobacillus* isolates

S. No.	Characterization	LBC	LBL	LBB	LBH	LBF
1.	Gram strain	+	+	+	+	+
2.	Cell morphology	rod	rod	rod	rod	rod
3.	Size	1.2 × 2 □m	2.9 × 0.2 □m	2.7 × 0.1 □m	1.2 × 2 □m	0.5 × 0.9 □m
4.	Catalase	-	-	-	-	-
5.	Gas from glucose	-	-	-	-	-
6.	Mode of fermentation	Hetero	Homo	Homo	Homo	Hetero
7.	Optimal pH for growth	7 ± 0.2	7 ± 0.1	6.8 ± 0.1	7 ± 0.2	6.9 ± 0.3
8.	Optimal growth temperature	37°C	37°C	37°C	38°C	40°C

+ = Positive
□ = Negative
Values are mean ± SD of three replicates from one representative experiment. Within a column different letters after values indicate that there is a significant difference at P value of 0.05, as determined by one way analysis of variance followed by a post hoc test.

Table 3. Isolates based on characterization of carbohydrate utilization from different *Lactobacillus* sp.

S. No.	Sugar	LBC	LBL	LBB	LBH	LBF
1.	Glucose	+	+	+	+	+
2.	Maltose	+	+	-	***	+
3.	Lactose	+	+	+	+	+
4.	Galactose	+	*	-	+	+
5.	Fructose	+	+	+	*	+
6.	Mannitol	-	-	-	-	-
7.	Sucrose	+	+	-	-	+
8.	D-xylose	-	-	-	-	-

Studied at an optimum temperature of 28 ± 2°C.
+ = Good growth
□ = No growth
*** = Poor growth

Table 4. Evaluation of Lactobacillus isolates or its antimicrobial activity against different food borne pathogens

S. No.	Strain	Diameter of inhibition zone				
		S. aureus	*B. cereus*	*E. coli*	*S. typhi*	*S. dysenterinea*
1.	LBC	15.00 ± 0.20	-	-	10.75 ± 0.2	9.50 ± 0.50
2.	LBL	-	9.00 ± 0.25	14.00 ± 0.5	13.00 ± 1.0	-
3.	LBB	9.80 ± 0.20	-	11.00±1.2	10.75±0.8	10.00 ± 0.50
4.	LBH	14.00 ± 0.50	-	10.75 ± 0.25	-	12.50 ± 0.50
5.	LBF	-	11.25 ± 0.25	13.70 ± 0.10	-	9.75 ± 0.25

*Includes 7 mm diameter, the size of the disc
8 – 10 mm = Moderate inhibition zone
11 – 14 = Strong inhibition zone
15 – 16 mm = very strong inhibition zone
□ = No inhibition growth
Values are mean ± SD of three replicates from one representative experiment. Within a column different letters after values indicate that there is a significant difference at P value of 0.05, as determined by one way analysis of variance followed by a post hoc test.

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