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Screening of Bacteriocin Producing *Lacto Bacillus* species from Cow Milk and its Antibacterial Potential against Foodborne Pathogens

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Abstract

Lactobacilli are well known for their probiotic properties and are playing vital role in human nutrition. The present study was aimed to isolate potential *lactobacilli* with probiotic characteristics from raw cow milk. Totally 10 isolates were obtained from 30 milk samples, identified and analysed for some probiotic properties like acid and bile tolerance, antibiotic resistance, and antibacterial activity against some food borne pathogens such as *Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Bacillus cereus* and *Salmonella typhi*. Among 10 isolates, 7 isolates showed acid (up to pH 2.0) and bile (2.0%) tolerance. Interestingly 5 isolates exhibited resistance towards all the antibiotics tested and inhibited the foodborne pathogens used in this study. These attributes confirmed that the isolated *lactobacillus* species, viz., LC-II, LC-III, LD-I, LA-I and LA-III) could be used as potential probiotic to control foodborne pathogens.

Keywords: Cow Milk, *Lactobacillus* sp, *S. aureus*, *E. coli*, An-tibacterial activity.

1 Introduction

Competition for food and space is one of the major factors that determine which organism succeed and dominates in a particular region. Organisms, which grow faster with available nutrients, will make use of that environmental condition will survive as the best. These microbes mostly change the environment by producing metabolic by products thereby securing and making themselves prevalent in that habitat. They may produce antagonistic compounds like antibiotics or bacteriocin, which make up the competitors more difficult to be established. The bacteriocins are inhibitory substances of proteinaceous nature, production of which is lethal and the action of which is limited to closely relate specific receptor sites. Bacteriocins are ribosomally synthesized bacteriostatic or bactericidal proteins and peptides which are produced by a number of gram positive bacteria [1]. Bacteriocins are defined as bacterially produced, small, heat-stable peptides that are active against other bacteria and to which the producer possesses a specific immunity mechanism [2]. Although bacteriocins can be produced by LAB have received increased attention over the past decades because they are considered to be "generally recognized as safe" microorganisms [3]. The concept of probiotics progressed around 1900, when Elie Metchnikoff hypothesized that the long and healthy lives of Bulgarian peasant were the outcome of their consumption of fermented milk products [4]. Members of the genera *Lactobacillus, Bifidobacterium* and *Streptococcus* are the most common probiotics used in commercial fermented and non fermented dairy products today [5]. Among LAB, *Lactobacillus* is the largest genus, comprising more than 150 species [6]. The species *Lactobacillus plantarum* can adapt to various niches due to its ability to ferment a wide range of carbohydrates [7].

Lactobacilli are an extremely important group of probiotic bacteria inhibit undesirable microflora in gut and create a healthy equilibrium between beneficial and intestinal pathogens. *Lactobacillus* species produce several types of antibacterial substances which different with respect to their chemical nature and mode of action. Apart from the lytic enzymes like lystota P^H in and lysozyme there exist two main groups 1. Bacteriocins and low molecular weight antibiotic like substances.Restricted uses of antibiotic and alternate method to overcome this problem are needed. Bacteriocin, especially those that are effective against pathogens have potential.

2 Experimental

2.1 Collection of the sample:

The raw cow milk was collected from different places *viz*., Vasanthapuram, Pellakuppam, Gidangal, Pattanam and Ural in and around Tindivam. After collection the samples were stored in refrigerator under aseptic conditions and used for further analysis.

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2.2 Enumeration of total Lactic acid bacteria (LAB) from cow milk

The milk sample were serially diluted and estimated by standard plate count method (spread plate and pour plate) in De Man, Rogosa and Sharpe Agar (MRS agar). One ml of sample (cow milk) were serially diluted sample were poured (1ml) on the sterile plate. After, solidification of media then the plate was incubated at 370C for 48 hours. After the incubation the plates were observed for appear-ance of colonies and counted them. At the same way spread plate method was also preceded by using 0.1 ml samples. Total number of Lactic acid bacterial population per ml = Average number of colonies in × Dilution factor. The results were expressed as colony forming unit (CFU) per ml of sample. Four distinct LAB colonies were isolates and puri-fied.

2.3 Isolation and identification of *Lactobacillus*

The milk samples were serially diluted upto 10-5 and were added to pour plated on to MRS agar medium. The plates were incubated at 370C for 24-48 hours. After 48 hours typical colonies were purified by quadrant streak on air dried MRS plates. Purified bacterial cultures were streaked in MRS slants and stored in a refrigerator at 40C. The purified bacterial culture isolates were transferred to MRS broth and incubated at 370C for 24 hours and they were subjected to various identification procedure.

2.4 Biochemical test

2.4.1 Indole test

A loopful of the isolated culture was inoculated into the tryptone broth. The Kovac's reagent was added after the in-cubation periods of 24-48 hours to the broth and shake gently. Development of red colour ring in the top layer of the tube was considered as positive result.

2.4.2 Methyl Red reduction test

The MR broth was prepared and inoculated with loopful of the isolated culture and the tubes were incubated for 24 hours at 37ºC. The red colour was formed when the Methyl Red indicator was added is an evidence of positive result. Whereas there is no colour change indicates a negative re-sult.

2.4.3 Citrate utilization test

Simmon citrate agar slant was streaked with isolated culture and incubated at 300C for 24 hours. Color change from green to deep blue indicates positive result. The result was noted.

2.4.4 Catalase Test:

Hydrogen peroxide (3%) solution was prepared and few drops from this solution were placed on a loopful of organ-isms on a clean glass slide. If any effervescence occurs, the organisms are considered as catalase positive.

2.5 Carbohydrate fermentation test:

The MRS broth was prepared in 100 ml quantity with different carbon sources such as fructose, lactose, glucose, sucrose, maltose and mannitol transferred to test tubes with durham's tube and sterilized. The loopful of the test culture was inoculated to the broth and incubated color change from red to yellow indicates the acid production. Gas production is seen with the help of Durham's tube. If the medium remains unchanged then the culture is a non-fermentative. Acid with gas or without gas reveals that the culture is fermentative.

2.6 Detection of antibacterial activities (Tambekar, 2010):

The antibacterial activities of isolated *Lactobacillus* species were determined by modifying the disc diffusion method [8,9]. Sterile blotting paper discs were dipped into isolated 48h *Lactobacillus* sp. culture broth and then placed on solidified Nutrient agar seeded with 3hours old culture of test clinical pathogens, which included *Escherichia coli, Sta*ph*ylococcus aureus*, *Bacillus subtilis, Bacillus cereus* and *Salmonella ty*phi. The plates kept at 4°C for 1 hour for diffu-sion and then incubated at 37°C for 24 hours and zones of growth inhibition measured in mm.

2.7 Acid and bile salt tolerance of *Lactobacillus* isolates

Isolated *Lactobacillus* sp. were inoculated into MRS medium of varying pH, i.e. pH 2.0-5.0, as well as broth with varying concentrations of bile salt (0.5- 2.0%), and incubat-ed at 37°C for 48 hours. Then 0.1mL inoculums transferred to MRS agar by pour plate method and incubated at 370C for 48 hours. The growth of *Lactobacillus* sp. on MRS agar plate was used to designate isolates as acid or bile salt tol-erant.

2.8 Preparation of crude Bacteriocin

The *Lactobacillus* sp was inoculated into 100 ml of MRS broth in 250 ml conical flask. After the incubation culture was centrifuged at 12,000 rpm for 30 minutes at 40C. The supernatant fluids of bacteriocin producing cultures were harvested and filtered through a membrane filter and pre-cipitated with 85% saturated Ammonium per sulphate and the solution was stand overnight at 40C. The precipitate was dissolved in Phosphate buffer and boiled for 15 minutes and it was utilized as crude bacteriocin. Antibacte-rial activity of crude bacteriocin against food pathogen by agar well diffusion

Antimicrobial activity of *Lactobacillus* isolates against some the food borne pathogenic bacteria were determined by agar well diffusion method. The *Lactobacillus* isolates were grown in MRS broth at 350C for 24hours. After incu-bation the broth were centrifuged at 5000×rpm for 10 minutes and the cells were separated out. Collect the su-pernatant was used as crude bacteriocin.

Muller Hinton agar plates are prepared and swabbed with food borne pathogens like *Sta*ph*ylococcus aureus*, *Strepto-coccus faecails*, *Salmonella ty*phi, *Escherichia coli* and *Pseu-domonas aeruginosa*. In each plate four wells were cut and load various volume (50µl, 100 µl, 150 µl) of crude bacteri-ocin in first three wells, the last one well loaded with steri-lized distilled water act as a control. The plates were kept for incubation at 37ºC for 24 hours. After incubation the zone formation will be developed area of the zone were measured [10].

3 Results and Discussion

3.1 Isolation and identification of *Lactobacillus* sp isolates from milk.

The isolates obtained from milk samples were purified and subjected to various identification procedures *Viz.,* Morphological, cultural and biochemical reactions. Microscopic examination of culture broth revealed the cells to be smooth round colonies, rod shaped, non-motile and gram positive [11-13]. It seemed the isolates could ferment the lactose and galactose. The biochemical profiles showed some variations in reaction pattern among the isolates. Totally about 5 strains were isolated among these, the isolates confirmed closely related to *Lactobacillus* from available in-formation in Bergey's manual of systematic bacteriology. Microscopic, biochemical and the Physiological examina-tion of the test isolates were represented in Table 1.

3.2 Detection of antibacterial activities of *Lactobacillus* isolates

The antibacterial activities of isolated *Lactobacillus* species were determined by modifying the disc diffusion method and the results were presented in Table 2 and shown in Fig 1. The results showed all LB isolates exhibit strong antibacterial activity, especially Gram negative bacteria are more susceptible when compare with Gram positive bacteria. Among the five LB isolates, LB-IV showed highest antibacterial activity against all clinical pathogens especially *E. coli* (18mm) followed by *S. typhi* (16 mm). The lowest antibacterial activity was recorded by LB-III [14].

3.3 Acid and bile salt tolerance of *Lactobacillus* isolates

The results of acid and bile salt tolerance of probiotic *Lactobacillus* isolates were presented in Figs 2 and 3.

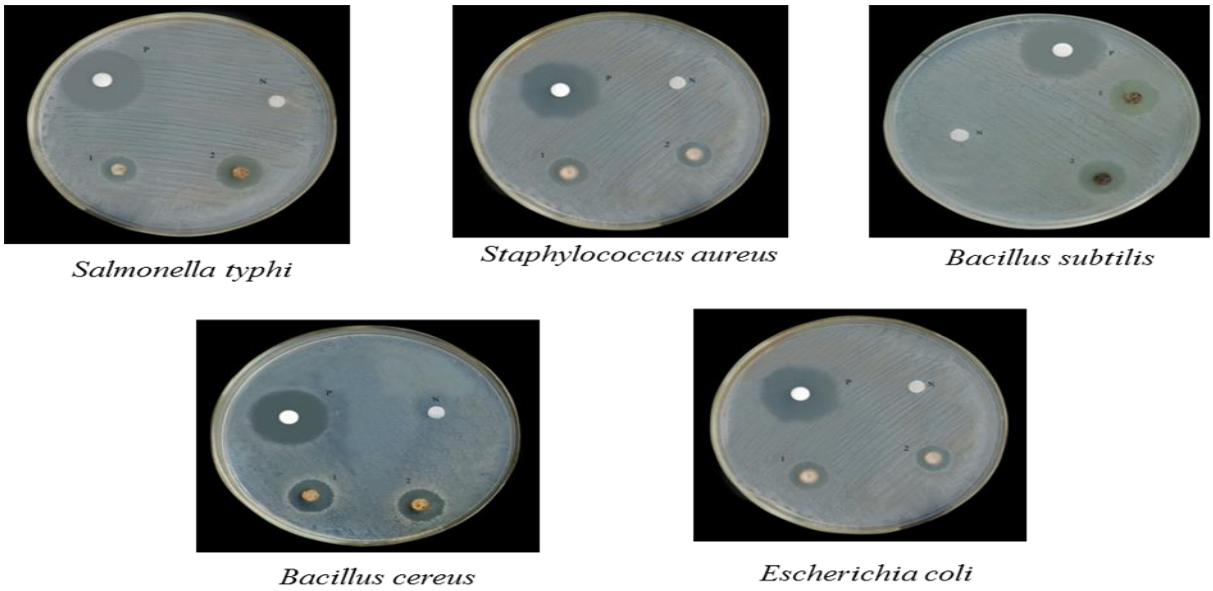

Fig1. Antibacterial activities of *lactobacillus* isolates against food borne pathogen

Table 1.Microscopic, biochemical and the Physiological examination of the test isolates

Isolates	Gram staining	Morphology	Motility	CHO fermentation					Biochemical test					
				Fructose	Lactose	Glucose	Sucrose	Maltose	Manitol	Indole test	Methyl red test	VP test	Citrate test	Catasslase test
LB-I	+	R	NM	+	+	+	+	+	+	-	-	-	-	+
LB-II	+	R	NM	+	+	+	+	+	+	-	-	-	-	+
LB-III	+	R	NM	+	-	+	+	+	+	-	-	-	-	+
LB-IV	+	R	NM	+	+	+	+	+	+	-	-	-	-	+
LB-V	+	R	NM	+	+	+	+	-	+	-	-	-	-	+

Among the five isolates tested, LB-IV showed tolerance up to (pH 7.0) followed by LB-I, LB-V, LB-II and LB-III. Interestingly, Isolate LB-IV also showed better bile salt tolerance (2.5%) followed by Isolate LB-I, Isolate LB-V (2.0%), Isolate LB-II (1.5%) and Isolate LB-III (1.0%).

3.4 Antibiotic resistance test of *Lactobacillus* isolates

The antibiotic resistances of the *Lactobacillus* isolates were tested against certain antibiotics such as Ampicillin, Gentamicin, Nalidixic acid and Tetracycline at a concentration of 10 ppm and diameter of the inhibition zone was

measured and given in Table 3. The results revealed that the resistant of LB-IV and LB-II was showed resistant to all types of the antibiotics tested. Whereas LB-I showed resistant to ampicillin and gentamicin, while sensitive to Nalidixic acid and Tetracycline. LB-III showed sensitive to all antibiotics tested except Nalidixic acid. The LB- V showed resistant to ampicillin and Nalidixic acid while sensitive to gentamicin and tetracycline.

Table2. Detection of antibacterial activities of *Lactobacillus* isolates

S. No	*Lactobacillus* isolates	Zone of inhibition of growth in mm²				
		E. coli	*Staph. aureus*	*B. subtilis*	*B. cereus*	*S. typhi*
1	LB-I	16	7	8	10	12
2	LB-II	17	8	11	12	10
3	LB-III	14	-	10	8	-
4	LB-IV	18	13	14	12	16
5	LB-V	15	10	12	8	13

Table3. Antibiotic resistance of *Lactobacillus* isolates

S. No	Name of the Antibiotics	Name of the Isolates				
		LB-I	LB –II	LB –III	LB –IV	LB-V
1	Ampicillin	R	R	S	R	R
2	Gentamicin	R	R	S	R	S
3	Nalidixic acid	S	R	R	R	R
4	Tetracycline	S	R	S	R	S

Table 4. Antibacterial activity of crude bacteriocin

S. No	*Lactobacillus* Isolates	Zone of inhibition of growth in mm²				
		E. coli	*S. aureus*	*B. subtilis*	*B. cereus*	*S. typhi*
1	**LB-I**	21	24	20	22	26
2	**LB-II**	20	21	19	20	23
3	**LB-III**	19	22	17	21	20
4	**LB-IV**	23	25	24	23	27
5	**LB-V**	21	23	22	20	24

3.4.1 Antibacterial activity of crude bacteriocin against food borne pathogens

The antibacterial activity of crude bacteriocin from all the five probiotic *Lactobacillus* isolates were tested against some food borne pathogens *Viz*., *Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Bacillus cereus* and *Salmonel-la typhi*. Bacteriocin from all the five isolates had wide anti-bacterial activity. Bacteriocin from LB-IV showed better ac-tivity against *Salmonella typhi* other isolates Table 4 and shown in Fig 4.

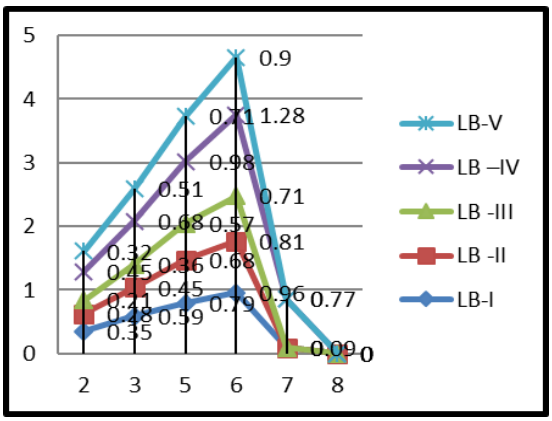

Fig 2. Acid tolerances of LB isolates

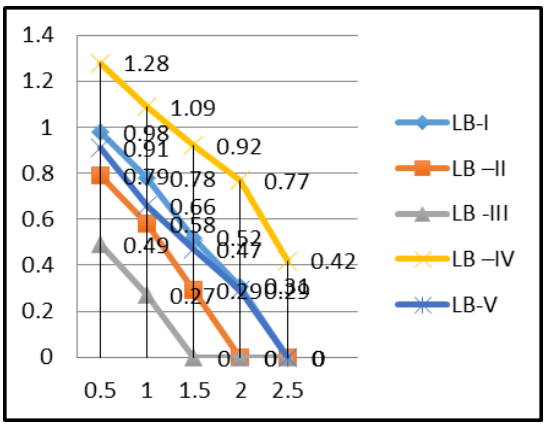

Fig3. Bile salt tolerances of LB isolates

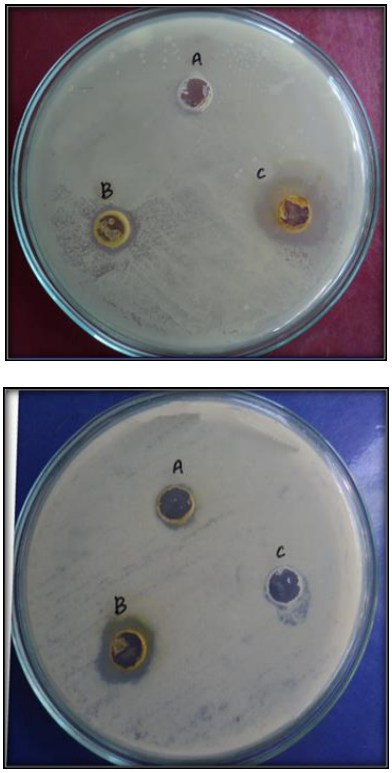

Fig4. Antibacterial activities of crude bacteriocin from *lactobacillus* isolates against food pathogens

3.5 Results and Discussion.

Probiotics can be used in the treatment and prevention of enteric infections and chronic inflammatory disorders of the gastrointestinal tract [13,15]. Bacteriocins are small peptides contains 30-60 amino acid with antimicrobial properties against bacteria usually of the same or closely related species (narrow spectrum), and sometimes against a wide spectrum of species.

Raw milk represents a source of new strains of *Lactobacilli* with probiotic potential to inhibit undesirable micro-flora in the gut. *Lactobacilli* constitute a major part of the natural microflora of human intestine. These probiotic or-ganisms when present in sufficient number can create a healthy equilibrium between beneficial and potentially harmful microflora in the gut by creating unfavorable con-ditions for the growth of commonly occurring intestinal pathogen [1,16].

In present study, cow milk samples were used to isolate probiotic *Lactobacillus* species. Antimicrobial effects of all *Lactobacillus* isolates are sustained by producing some substances such as organic acids (lactic, acetic, propionic acids, succinic acid etc), hydrogen peroxide, low molecular weight antimicrobial substances and bacteriocins [16,17]. Probiotics including *Lactobacillus*, *Bifidobacterium* and *Streptococcus* sp. are known to be inhibitory to the growth of a wide range of intestinal pathogens in human [11].

However, some reports have been supported to our findings that certain lactic acid bacteriocins, especially the Class 2 bacteriocin pediocin, can inhibit a limited number of Gram negative bacteria including *Shigella* sp, *Salmonella* sp, *Pseudomonas* sp and *Shigella flexneri* [18]. In the present study also, isolated strains (LB-I to LB-V) were screened for the antagonistic activity against some food borne patho-gens *Viz*., *E. coli, Salmonella, S. aureus, Bacillus subtilis* and *B. cereus* as indicator strain. Although all isolates seemed to capable of producing zones of inhibition, the isolate LB-IV produced maximum zone of inhibition against all the path-ogens testet reported that, the isolated five probiotics showed acid (pH-2) tolerance and bile salt (2%) tolerance which is considered a prerequisite for colonization and metabolic activity of bacteria in the small intestine of the host. *Lactobacillus* sp isolated in this study was resistant to 0.5% bile salt. All of the isolates are able to survive and grow in 0.5% bile salt concentration [11]. In our present study revealed that, the *Lactobacillus* isolate (LB-IV) shows similar level of acid tolerance and bile tolerance. Interest-ingly, the isolate LB-IV had both acid and bile tolerance. Wasfi *et al* reported that the antagonistic activity of L. sali-varius on Strep. mutans was linked to peroxide. The results of acid tolerance showed that all four selected *Lactobacillus* isolates exhibited good acid resistance at pH 3 for 4 h, with L. plantarum MW-18CGZ showing a better acid tolerance than the other [19].

In present study, screened antibiotic resistance test against different antibiotic, finally all the five isolates (LB-I to LB-V) found to have different sensitivity pattern for all antibiotics tested. This result was agreed with El-Naggar, (4), he reported that, resistance of the probiotic strains to some antibiotics could be used for both preventive and therapeutic purposes in controlling intestinal infections.

4 Conclusion

The present study revealed that, the probiotics Lactic Acid bacteria (LAB) isolates was presented in cow milk that isolates showed strong antibacterial activity against food borne pathogens. The isolates showed better results against pH, acid, bile and antibiotic resistance test. Therefore LAB probiotics was used to food preservation techniques.

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