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SCREENING AND CHARACTERIZATION OF NOVEL BACTERIOCIN PRODUCING LACTOBACILLI OBTAINED FROM RAW MILK SAMPLES.

Sarangdhar Mithun*, Vora Dipak and Sarang Sheela.

Department of Microbiology, Ramnarain Ruia College, Mumbai, Maharashtra, India.

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***Correspondence for Author**
Sarangdhar Mithun
Department of Microbiology, Ramnarain Ruia College, Mumbai, Maharashtra, India.

ABSTRACT

Lactic acid bacteria are known to produce a variety of compounds including bacteriocin, hydrogen peroxide and organic acids which inhibit the growth of spoilage and pathogenic bacteria. Hence they may have potential applications as natural and safe food preservatives. The present study was aimed at isolation and screening of bacteriocin producing lactobacilli from raw milk samples. A total of 73 isolates were obtained from the 10 milk samples procured from a local dairy. Out of these only four of them, two belonging to each *L.fermentum* and *L. viridescens* showed promising bacteriocin activity against the test cultures. Maximum activity was obtained against *S.aureus*, followed by *E.coli* whereas relatively lower activity was observed against *B.subtilis*. Upon subsequent heat treatment, the activity of the crude bacteriocin preparations diminished gradually and it was completely destroyed at 100°C. Further, antibiograms were generated for these isolates and MAR index was analyzed. All the isolates were found to be resistant to Penicillin, Norfloxacin and Vancomycin whereas they were found to be sensitive to Bacitracin, Amoxycillin and Ceftazidime. Three of the isolates exhibited a high MAR index greater than 0.2. The bacteriocins obtained from these isolates pose an avenue and may be examined for their potential to replace the conventional synthetic chemicals used in preservation of food.

KEYWORDS: Isolation, Identification, Bacteriocin, Agar Well diffusion, Antibiotic Sensitivity test, MAR Index.

INTRODUCTION

Lactic acid bacteria (LAB) is an important group of industrial microorganisms involved in the processing of various fermented foods, dietary adjuncts, probiotics and even cosmetics.[1] The genera that comprise the LAB are at its core *Lactobacillus, Leuconostoc, Pediococcus, Lactococcus,* and *Streptococcus*, as well as the more peripheral *Aerococcus, Carnobacterium, Enterococcus, Oenococcus, Sporolactobacillus, Tetragenococcus, Vagococcus,* and *Weisella*.[2] The LAB are almost ubiquitous and are found in a variety of natural sources like milk and milk products, sewage, meat, fermented food products, fishes, prawns and mucosal surfaces of animals. Isolation and screening of microorganisms from these naturally occurring sources have always been the most powerful means for obtaining useful cultures for scientific and commercial purposes.[3] This certainly holds true for lactic acid bacteria and especially lactobacilli, which are used throughout the world for manufacture of a wide variety of traditional fermented foods. Since they are involved in numerous food fermentations, known to man for millennia, it is assumed that most representatives of this group do not pose any health risk to man and are designated as GRAS (Generally Recognized as Safe) organisms.[3]

The genus Lactobacillus consists of a genetically and physiologically diverse group of rod-shaped, Gram-positive, non-spore forming, non-pigmented, catalase negative and microaerophilic to strictly anaerobic fastidious organisms. Lactobacilli possess two major advantages in that some of them are known to be probiotics [4] and secondly, they also possess the GRAS status. The LAB are widely used in manufacturing fermented food products and can also be safely used for medical and veterinary applications.[5]The most important contribution of lactobacilli to fermented products is to preserve the nutritive qualities of the raw material and inhibit the growth of spoilage and pathogenic bacteria. This inhibitory activity may be due to the production of many metabolites such as organic acids (lactic and acetic acid), hydrogen peroxide, diacetyl and bacteriocins.[6]

In recent years, extensive work has been channelized on bacteriocins and bacteriocin producing strains of LAB for their potential use as biopreservatives.[7] Bacteriocins of LAB are considered as safe natural preservatives or biopreservatives, as it is assumed that they are degraded by the proteases in gastrointestinal tract.[8]

Bacteriocins are extracellularly released peptides or protein molecules, with a bactericidal or bacteriostatic mode of action against closely related species. The inhibitory spectrum of some bacteriocins also includes food spoilage and/or food-borne pathogenic microorganisms.[3]

Classification of Bacteriocins

Four general classes of bacteriocins from LAB have been characterized till date[9]

1. Lantibiotics
2. Small (Less than 13 kDa) hydrophobic heat stable peptides
3. Large (appx 30 kDa) heat labile proteins
4. Complex proteins that require additional carbohydrates or lipid moieties to attain antimicrobial activity.

The majority of those produced by bacteria associated with food belong to classes I and II. Most of the bacteriocins isolated from lactobacilli belong to the class II bacteriocins.[2]

The discovery of nisin, the first bacteriocin used on a commercial scale as a food preservative dates back to the first half of last century but research on bacteriocin of LAB has expanded in the last two decades, searching for novel bacteriocin producing strains from dairy, meat and plant products, as well as traditional fermented products.[10]

Due to current awareness of consumers towards the use of food preservatives, the use of bacteriocin or bacteriocin producing culture as potential 'biopreservatives', and the possibility for replacing chemical preservatives has received much attention. This has led to the discovery of increasing potential sources of these biopreservatives.[1]

The present study deals with the isolation and identification of bacteriocin producing lactobacilli obtained from various raw milk samples. After confirming the purity of the isolates and classifying them as lactobacilli, they were further tested for their ability to produce bacteriocins. Four of the isolates which exhibited promising activity were further studied in detail. The bacteriocin preparations from these isolates were also subjected to heat treatment to study the effect of heat on its inhibitory activity. Also, these isolates were further subjected to a series of antibiotics to study their antibiotic resistance in order to generate antibiograms for individual isolates. MAR index was also calculated for these isolates.

MATERIALS AND METHODS

Collection of Milk Samples

Milk samples were collected in sterile containers at the sheds and were transported to the lab in temperature controlled boxes. The milkmen were advised to sterilize the surface of the udders and their hands before sample collection. Further analyses of the samples were carried out at the lab.

Isolation and Identification of Lactobacillus

The milk samples were processed immediately after reaching the lab. They were diluted appropriately in normal saline and pour plated on MRS agar and incubated at 37°C anaerobically for 24 to 48 hours.[11] At the end of 48 hours, as the colonies became predominant and morphologically distinct, the well isolated colonies were picked and transferred to new MRS agar plates by streaking. Colonies showing typical characteristics of lactobacilli on agar surface were picked up randomly and transferred into MRS broth for further enrichment. Consequently their purity was further checked on MRS agar.

The pure isolates were subjected to biochemical characterization and identification as per the Bergey's Manual of Determinative bacteriology and open source softwares like PIBWin[12] and IDENTAX.[13] Macroscopic appearance, size, shape, colour and texture of the colonies were recorded along with microscopic characteristics to aid in identification.

The isolates were stained by Gram's method and examined under microscope for purity. Those isolates readily identified as Gram positive rods and catalase negative were included for further characterization [14] which included cytochrome oxidase; growth at 15°C and 45°C; acid production from carbohydrates (1 % w/v) - L-arabinose, D-fructose, cellobiose, esculin, lactose, maltose, sucrose, D-galactose, D-mannose, raffinose, salicin, melebiose, melezitose, mannitol, rhamnose, D-ribose, trehalose, sorbitol and D-xylose in MRS broth devoid of glucose and beef extract with phenol red as indicator; production of acid and gas from 1 % glucose (MRS broth without beef extract); methyl red and Voges-Proskauer test in MRVP medium; nitrate reduction in nitrate broth, indole production in tryptone broth, production of ammonia from arginine and growth on acetate agar.[15–17]

Determining the sources of inhibitory activity

Lactic acid bacteria are able to produce several antimicrobial compounds which include hydrogen peroxide, bacteriocins and organic acids.[6] In order to eliminate the possibility of organic acids causing inhibition, the cell free supernatant were neutralized to a final pH of 7.0 with 1N NaOH and then tested for their inhibitory activity. In order to eliminate inhibition by hydrogen peroxide, neutralized cell free supernatants were tested against catalase enzyme before testing for their inhibitory activity. [1]

Test microorganisms

The indicator organisms namely *E.coli NCIM 2068, P.aeruginosa NCIM 5029, B.subtilis NCIM 2063, S.pyogenes NCIM 5280* were procured from National Chemical Laboratory, Pune, India whereas *P.vulgaris,MTCC 426* and *S.aureus, MTCC 9886* were procured from Microbial Type Culture Collection and Gene Bank, Chandigarh, India.

Screening for bacteriocin-producing *lactobacillus* species by Agar Well Diffusion (AWD) assay

A volume of 1% of culture (0.1 O.D) was inoculated into 20 ml MRS broth and incubated at 30°C under anaerobic conditions overnight. Culture supernatants were derived from these overnight broths by centrifugation at 10,000 rpm for 15 mins at 4°C. The cell free supernatants were further neutralized with 1 N NaOH to pH 7 in order to eliminate the possibility of inhibition by acids and also tested for the absence of hydrogen peroxide. The neutralized supernatants were further sterilized by passing through a Millipore filter (0.22 µm) and their antimicrobial activity was carried out by using the agar well diffusion method. A volume of 1% of 0.1 OD culture of the indicator bacteria was inoculated to 20 mL of liquid molten Muller Hinton agar, poured into sterile petri plates and allowed to solidify. Wells of 5 mm diameter were cut into the agar and filled with 50 µL of neutralized cell free supernatant prepared from the lactobacillus strains. Plates were pre-incubated at 4°C for few minutes to allow diffusion of any inhibitory metabolites into the surrounding agar and then incubated at 37°C for 24 h. The plates were later examined for clear zones of inhibition in the agar surrounding the wells.[18,19] All experiments were performed in triplicates.

Sensitivity of bacteriocin to heat treatment

Out of the 73 isolates, four of them showed promising bacteriocin activity and were studied in more detail. The neutralized crude bacteriocin preparations of these strains were subjected

to heat treatment at 45°C, 75°C and 100°C for 10 minutes and then their antimicrobial activity were assayed against the indicator microorganisms using the AWD assay.[18]

Antibiotic Sensitivity Test

Drug sensitivity of the four selected isolates to various antibiotics (HiMedia, Mumbai) were assayed by Kirby Bauer disc diffusion method. A volume of 1% of 0.1 OD culture of the isolate was inoculated to 20 mL of liquid molten MRS agar, poured into sterile petri plates and allowed to solidify. Antibiotic discs were placed on the surface of the agar and pressed to ensure contact with the surface. The plates were then incubated anaerobically at 30°C for 24 hours. Sensitivity was measured as a zone of inhibition around the discs.

RESULTS

Identification

The preliminary investigations included macroscopic analysis, microscopic analysis, Gram-positive bacilli, lactic acid biosynthesis, endospore test, milk coagulation activities and the negative catalase reaction. Microscopically they were Gram-positive rod shaped, non- motile, catalase negative and exhibited absence of endospore. The isolates were tolerant to a range of salt concentrations (1-9%) and also demonstrated the ability to coagulate milk. The results of these tests permitted the classification of the working bacterium into the genus *Lactobacillus*.

Biochemical Characterization

Identification was carried out upto the species level with the help of biochemical tests given in Bergey's Manual of Determinative bacteriology whereas open source softwares like IDENTAX and PIBWin were also used to aid the identification and help develop an identification scheme.[17,20,21] (Table 1) A total of 73 isolates were obtained from the 10 milk samples procured from the local dairy. After acreditting the isolates to genus *Lactobacillus*, they were further subjected to biochemical characterization and identified upto the species level. 69 isolates could be assigned to the species level whereas 4 could not be identified upto the species level by the designed scheme.

Table 1: Scheme used for identification of Lactobacillus

No	*Lactobacillus* spp	Morphology	Growth at			Acid and gas from glucose	NH_3 from arginine	Sugar fermentation														
			15 °C only	45 °C only	15 and 45 °C			Arabinose	Cellobiose	Mannitol	Mannose	Melebiose	Raffinose	Ribose	Salicin	Lactose	Melezitose	Rhamnose	Sorbitol	Xylose	Trehalose
1.	*L. plantarum*	SR	+	-	+	-	-		+	+	+	+	+	+	+						
2.	*L. brevis*	SR	+	-	+	+	+	+						+		+	-	-	-	+	
3.	*L. divergens*	SR	+	-		+	+		+		+	-	-		+	-				+	+
4.	*L. gasseri*	SR	-	+		-	-	-	+		+			-	+			-	+		+
5.	*L. rhamnosus*	SR	-	-	+	-	-		+	+	+	-			+	+	+		+	+	+
6.	*L. fermentum*	SR			+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+
7.	*L. viridescens*	SR			+			-	+	+	+	-	-	-	-	+	+	-	-	-	+
8.	*L. farciminis*	SR			+		+	-	+	+	+	+	-	+	+	-	+	-	+	+	+
9.	*L. buchneri*	SR	+			+	+	-	+	-	-	+	-	+	-	+	-	-	-	+	-
10.	*L. acidophilus*	SR	-	-				+	+	+	+	-	-	-	+	+	+	-	-	+	+
11.	*L. alimentarius*	C	+					+	+	+	+	+	-	+	+	-	+	-	+	+	+
12.	*L. animalis*	C	-	-				+	+	+	+	+	+	-	+	+	-	+	+	-	+
13.	*L. reuteri*	R			+	+	+	+	-	-	-	+	-	+	-	+	-	-	-	+	-

Table 2: Total number of isolates identified

Identified species	No. of Isolates	Percentage
L.fermentum	36	49.32
L.plantarum	13	17.81
L.viridescens	6	8.22
L.brevis	14	19.18
Unidentified	4	5.48
Total	73	100

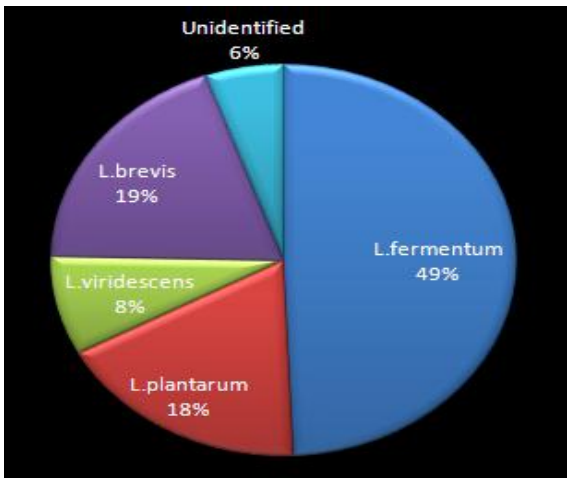

Fig 1: Percentage of lactobacillus species amongst the isolates

Screening for bacteriocin-producing *lactobacillus* species by Agar Well Diffusion (AWD) assay

The isolates obtained were further analyzed to determine their ability to produce bacteriocins. Out of the 73 isolates, only 4 of them showed promising activity against the test organisms. These were Lf1 and Lf2 which belonged to *L.fermentum* and Lv1 and Lv2 belonging to *L.viridescens*. The elimination of acid from the cell free supernatants had no effect on their inhibitory activity as well as testing the supernatants with catalase enzyme eliminated the possibility of inhibition by hydrogen peroxide. Therefore, it can be concluded that the inhibitory action is caused only by the bacteriocin produced by these strains. Maximum activity was recorded against *S.aureus*, followed by *E.coli* whereas relatively lower activity was seen against *B.subtilis.*

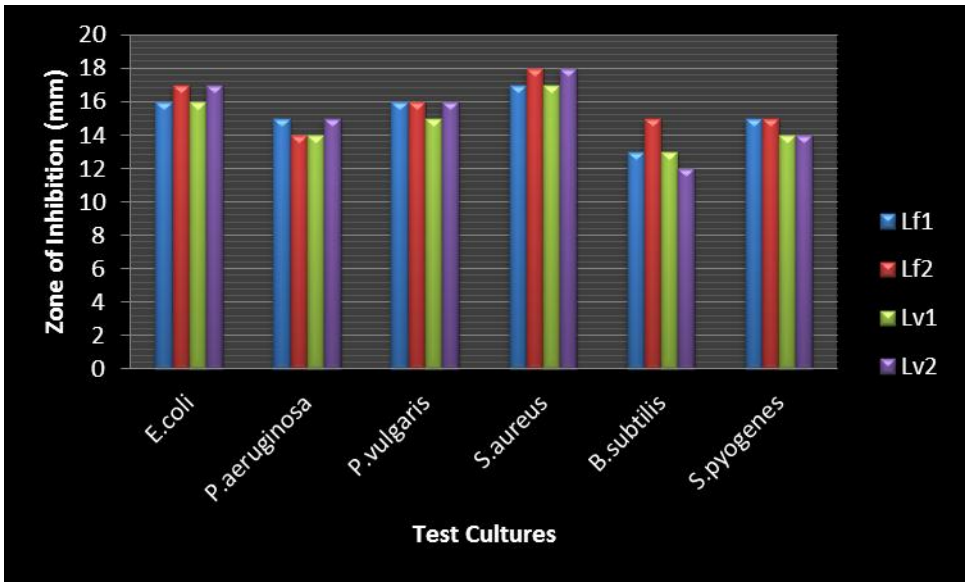

Fig 2: Effect of Crude Bacteriocin preparations on test cultures

Sensitivity of bacteriocin to heat treatment

The crude bacteriocin preparations from the isolates were also subjected to heat treatment before analysing their inhibitory activity. It was quite evident from the results that the bacteriocin activities of all the isolates decreased gradually upon heating and the activity was completely destroyed at 100°C. Hence it can be concluded that the bacteriocins obtained from these isolates are not heat stable and may not be used in processes which involve high holding times at high temperatures.

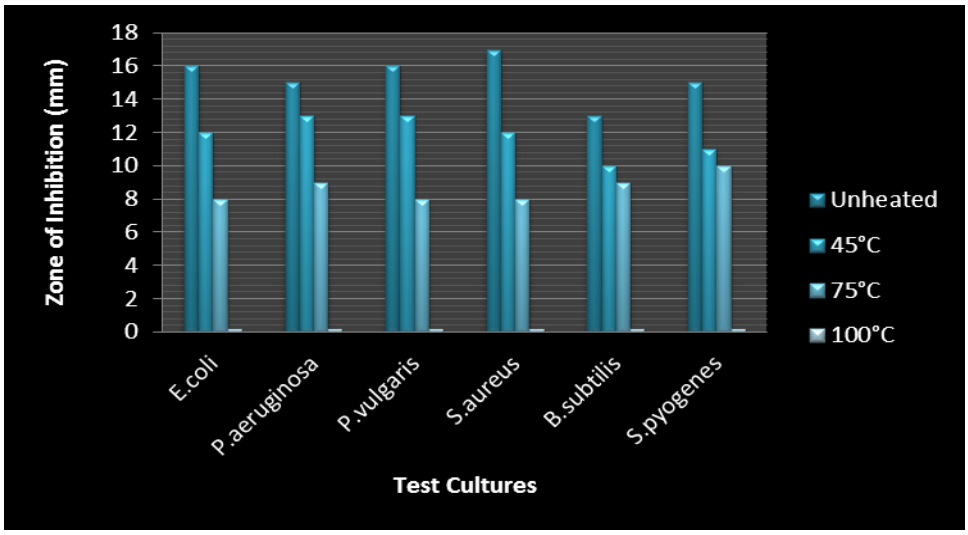

Fig 3: Effect of heat on Crude bacteriocin preparation of Lf1

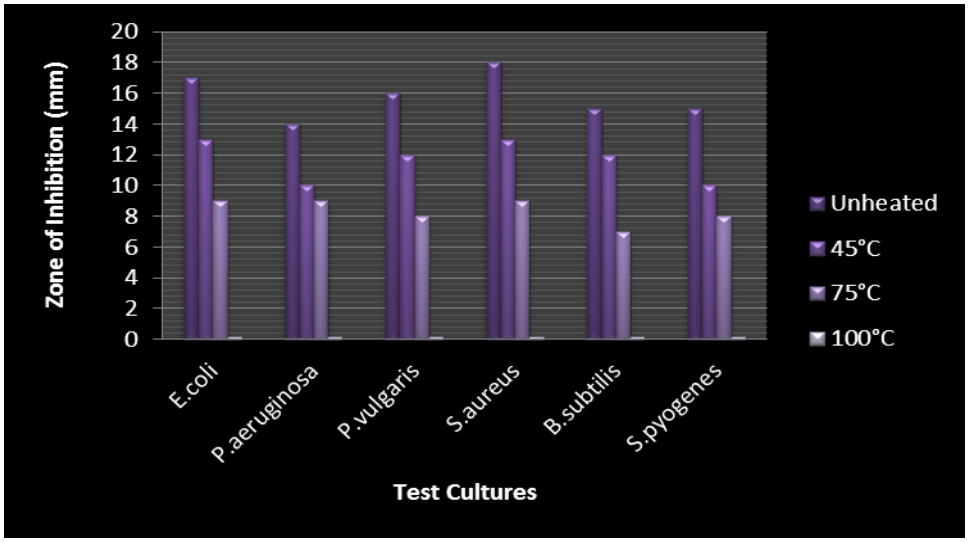

Fig 4: Effect of heat on Crude bacteriocin preparation of Lf2

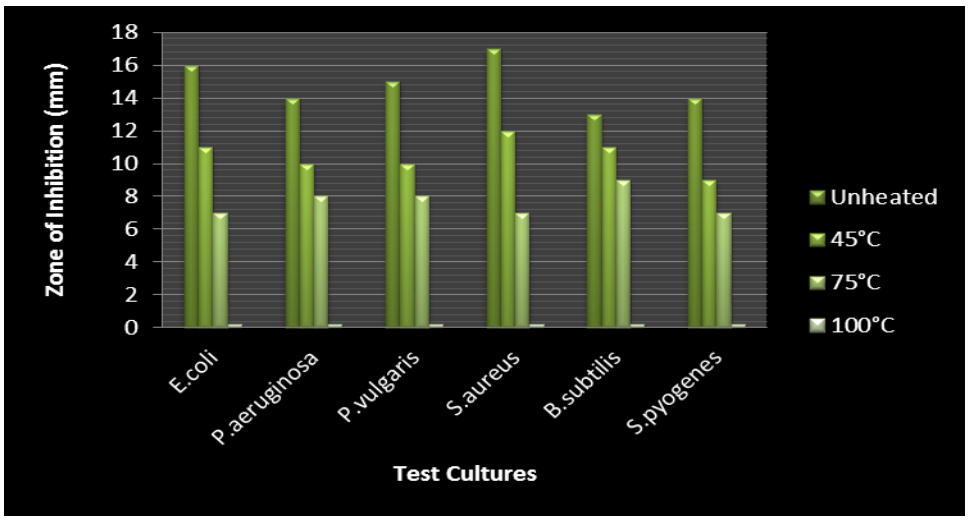

Fig 5: Effect of heat on Crude bacteriocin preparation of Lv1

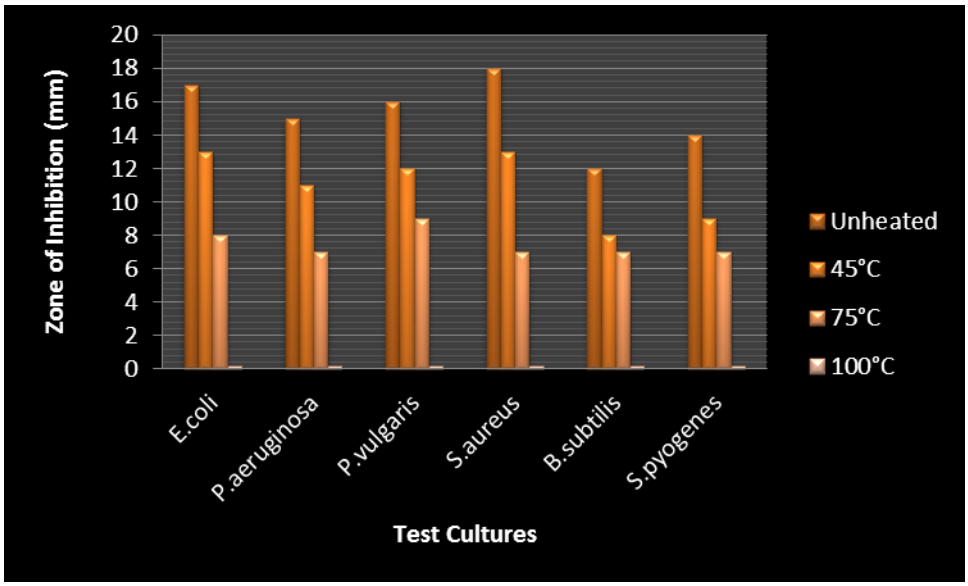

Fig 6: Effect of heat on Crude bacteriocin preparation of Lv2

Antibiotic Sensitivity Test

The antibiotic sensitivity test was performed on the four isolates and antibiograms were generated for them by using sixteen antibiotics belonging to all the four generations. It was found that the isolates were resistant to Penicillin, Norfloxacin and Vancomycin whereas they were found to be intermediate for Methicillin and Erythromycin. The isolates were found to be sensitive to Bacitracin, Amoxycillin and Ceftazidime. Based on the resistance pattern of the isolates, the Multiple Antibiotic Resistance (MAR) Index was calculated. The MAR Index analysis reveals that three of the isolates viz; Lf1, Lf2 and Lv1 have a very high MAR index value (>0.2).Bacteria having MAR Index > 0.2 are assumed to have originated from an environment where several antibiotics may have disseminated.[22]

Table 3: Effect of various antibiotics on the isolates

Antibiotics	**Concentration**	**Isolates**			
	µg	Lf1	Lf2	Lv1	Lv2
Tetracycline	30	**I**	**S**	**S**	**I**
Amikacin	30	**R**	**R**	**S**	**S**
Ampicillin	10	**S**	**S**	**I**	**S**
Penicillin G	10	**R**	**R**	**R**	**R**
Gentamycin	10	**S**	**S**	**I**	**S**
Bacitracin	10	**S**	**S**	**S**	**S**
Amoxycillin	20	**S**	**S**	**S**	**S**
Streptomycin	10	**R**	**S**	**R**	**S**
Ceftazidime	30	**S**	**S**	**S**	**S**
Carbenicillin	100	**S**	**S**	**I**	**I**

Cefotaxime	30	**S**	**S**	**I**	**I**
Norfloxacin	10	**R**	**R**	**R**	**R**
Vancomycin	30	**R**	**R**	**R**	**R**
Methicillin	5	**I**	**I**	**I**	**I**
Clindamycin	2	**S**	**S**	**I**	**I**
Erythromycin	15	**I**	**I**	**I**	**I**

(R= Resistant , S = Sensitive, I = intermediate)

Table 4: MAR index analysis of the isolates

Isolate	No. of Antibiotics to which the isolate is resistant	MAR index
Lf1	5	0.31
Lf2	4	0.25
Lv1	4	0.25
Lv2	3	0.19

DISCUSSION

Milk and milk products have always been an important source exhibiting a wide diversity of lactobacilli and hence screening and isolation of lactobacilli from these sources has been an important mode of obtaining cultures. The present study was aimed at isolating lactobacilli from raw milk samples obtained from a local dairy. A total of 73 isolates were isolated and identified from the 10 milk samples obtained from the dairy. After confirming the purity of the isolates, they were subjected to microscopic and biochemical analysis according to the Bergey's Manual of Determinative bacteriology and also with open source softwares likes IDENTAX and PIBWin. Out of the 73 isolates obtained, 36 were found to belong to *L.fermentum*,13 were found to belong to *L.plantarum*, 6 were found to belong to *L.viridescens*,14 were found to belong to *L.brevis* whereas 4 isolates could not be identified upto the species level. The results exhibit a diversity of lactobacilli in milk and indicate that *L.fermentum* is predominant in the milk samples which may play an important role in the quality of the milk. The obtained isolates were further tested for their ability to produce bacteriocin.

Out of the 73 isolates tested for bacteriocin activity, only 4 viz. Lf1, Lf2, Lv1 and Lv2 showed promising results and hence were further studied. The crude bacteriocin obtained from these isolates showed maximum activity against *S.aureus*, followed by *E.coli* whereas relatively lower activity was seen against *B.subtilis*. Upon subsequent heat treatment, the activity of the crude bacteriocin preparations diminished gradually and was completely destroyed at 100°C.This indicates that the bacteriocins produced by these isolates are heat

labile and may not sustain high temperatures for long time periods. Subsequently, antibiograms were developed and MAR index values were calculated for these isolates. The isolates were found to be resistant to Penicillin, Norfloxacin and Vancomycin whereas they were sensitive to Bacitracin, Amoxycillin and Ceftazidime. The isolates Lf1, Lf2 and Lv1 exhibit high MAR values (>0.2) indicating that the isolates may have spread from a niche of high antibiotic use.

CONCLUSION

The results of this study corroborate the wide diversity of lactobacilli occurring naturally in milk and explores its potential as a natural source for isolating lactobacilli. The study is extremely promising as it ascertains the isolation and identification of novel bacteriocin producing lactobacilli. Since these isolates show good inhibition against the test cultures, they may also possess probiotic properties. Hence these cultures can be further screened and exploited for their probiotic properties which may play an important role in food industry. The bacteriocins obtained from these isolates may replace the conventional food preservatives as well as these isolates may find applications as starter-culture, co-culture and bio protective cultures to improve quality and safety of preserved food and beverages. They may also render a beneficial effect to fermented food products by imparting stability and improving flavor, aroma and texture of the product. Hence these isolates need to be further screened for their probiotic and related properties and exploited for human and economic benefits.

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