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PURIFICATION AND CHARACTERIZATION OF BACTERIOCIN PRODUCED BY DIFFERENT *Lactobacillus* SPECIES ISOLATED FROM FERMENTED FOODS

SARANYA S. AND HEMASHENPAGAM N.*
Department of Microbiology, Hindusthan College of Arts and Science, Coimbatore- 641 028, TN, India.
*Corresponding Author: Email- drhemashenpagam@gmail.com

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Abstract- The aim of the study was to isolate and characterize bacteriocin producing Lactic acid bacteria (LAB) from fermented foods products and to exploit their potential as biopreservatives. The two Lactobacillus strains namely *L. plantarum* and *L. fermentum* exhibiting wide spectrum of activity against closely related strain were selected and screened for their bacteriocin producing ability. Cell free supernatant fluid collected from both the isolates and several gram positive and gram negative pathogens such as *S.aureus, E.coli, P.aeruginosa, S.pneumoniae, Klebisella,* and *proteus* were inhibited by the inhibitory action of bacteriocins in study. The bacteriocinogenic potential in these strains appeared non-inducible and increase in their titer was observed after exposure to different concentrations of UV light. The concentrated crude bacteriocin samples subjected to ammonium sulphate precipitation resulted in an increased activity and high protein yield. By non-denaturing gel a band of approximately 8 KDa for *L. fermentum* and band corresponding to 37KDa in *L. plantarum* was seen. Physio-chemical characterization of the partially purified bacteriocin samples indicated heat (121°C for 60 min) and acidic pH stability (pH 2-6) of bacteriocin. Exposure to different carbon and other substations also resulted in increased bacteriocin titer. The high performance liquid chromatography was performed for *L. plantarum.*

Keywords- Lactic acid Bacteria, Antagonistic acivity, Mutation, TLC, HPLC

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Introduction

Lactic acid bacteria are a diverse group of genera which can be characterised as Gram-positive, catalase negative, non-sporulating, non-pigmented mesophils [1]. The tolerated temperature range is generally between 5 and 50°C, with the optimum for most strains being about 30°C [2]. Shape is variable, from cocci through to elongated rods. Although metabolically similar, there is a lack of DNA homology between them. They lack the pathways for nitrate reduction, for the production of catalase and for the production of cytochromes and other pigments. They also have a complex and variable nutritional requirement differing according to species [3].

Lactic acid is an organic acid that is produced as a result of fermentation metabolism by the lactic acid bacteria [4]. Many chemical substances are constantly involved in the biochemical processes going on in living systems. Among these, Lactic acid serves as an important metabolite of an equally important energy yielding process [5]. Lactic acid is a carboxylic acid with a chemical formula $CH_3CHOHCOOH$ and is a colorless liquid organic acid [6]. It is widely used chemical that has found application in many industries and various commercial purposes. It is used in leather tanning and textile dyeing and in making inks, solvents and lacquers [6]. Lactic acid is also used as acidulate, flavoring, pH buffering agent or inhibitor of bacterial spoilage in processed foods. The esters of lactic acid are used in baking foods, as emulsifying agents [6,7]. Lactic acid bacteria produce various compounds such as organic acids, diacetyl, hydrogen peroxide and bacteriocin or bactericidal proteins during lactic acid fermentations [8,9].The production of lactic acid can be done in two ways which are chemical synthesis and carbohydrate fermentation [6].

Lactic acid bacteria exert strong antagonistic activity against many microorganisms including food spoilage organisms and pathogens. In addition, some strains may contribute to the preservation of fermented foods by producing bacteriocins [10]. Research on Bacteriocins from lactic acid bacteria has expanded during the last decades to include the use of bacteriocins or the producer organisms as natural food preservatives [11]. Bacteriocins of LAB have been classified into four structural classes, namely I, II, III and IV [12].

Bacteriocins are ribosomally synthesized, extracellularly released bioactive peptides or peptide complexes (usually 30-60 amino acids) which have a bactericidal or bacteriostatic effect on other (usually closely related) species [13]. Bacteriocins are generally considered to act at the cytoplasmic membrane and dissipate the proton motive force through the formation of pores in the phospholipid bilayer [14].

Antimicrobial substances produced by lactic acid bacteria can be divided into two main groups: low molecular mass substances with molecular mass <1000 Da and high molecular mass substances with molecular mass >1000 Da, such as bacteriocins. All non-bacteriocin antimicrobial substances from LAB are of low molecular mass [15].

The bacteriocins are low molecular mass peptides and it has been reveled that the bacteriocin Bavaricin A consists of 41 amino acid residues and has a calculated molecular mass above 4300 Da; SDS-PAGE reveals a molecular mass of 3500-4000Da [18]. It has been also showed that the molecular mass of reutericyclin is 3100Da, indicating that they form stable multimers in aqueous solutions, even in the presence of denaturing agents or organic solvents [16]. Further purification was performed by thin-layer chromatography [17].

Lactic acid occurs in fermented products as a result of hydrolysis, biochemical metabolism, and microbial activity. Quantitative determination of lactic acid is important in fermented products for technical, nutritional, sensorial, and microbial reasons. Trimetric methods, gas chromatography, colorimetric analysis and enzymatic methods are examples of techniques that are used for analyses of organic acids [19]. However, because simplicity and speed of analysis, the HPLC techniques is an attractive method, which requires a minimum of sample preparation prior to separation and permits quantitative determination of organic acids in short time.

This study was attempted to isolate lactic acid producing strains from fermented food products. These strains were identified and initially tested for their probiotic properties. The inhibitory effect of these strains on both Gram-positive and Gram-negative pathogenic bacteria was further investigated. These aspects are used in the study of antimicrobials for their production and characterization.

Materials and Methods

Isolation and Identification of Lactobacillus Species

A total of 25 fermented milk products were randomly collected in sterilized glass bottles [20]. The strains were stored at -80°C in MRS broth medium [21]. Before experimental use the cultures were propagated twice in MRS at 37°C. The transfer inoculum was 1% (v/v) of 16h culture grown in fresh medium.

The sample was serially diluted to 10^{-5}-10^{-6} using sterile distilled water and 0.1ml were taken for spread plate and 1ml were taken for pour plate and the different diluents was plated on to sterile de-Mann Rogosa and Sharpe (MRS) agar. The MRS plates were maintained in microaerophilic condition and incubated at 37°C for 48 Hrs. The isolates were identified using standard morphological, cultural and biochemical reactions [22].

Morphological Biochemical Characterization

Gram staining was done to identify the morphology structure and biochemical test namely MRVP, oxidase and catalase test was done.

Carbohydrate Fermentation

To determine the products of sugar fermentation, a carbohydrate fermentation broth was prepared at pH 7.4. This broth contains 3 essential ingredients: 0.5%-1.0% of the carbohydrate to be tested (e.g. lactose or glucose), nutrient broth, and the pH indicator phenol red. The nutrient broth, which is a light red color, supports the growth of most organisms whether they are able to ferment the sugar or not.

Growth at 8 and 15°C in tubes containing MRS broth and fermentation of carbohydrates were determined. The carbohydrates tested were D(+) cellobiose (Difco), D(+) galactose (Difco), xylose (Difco), fructose (Difco), maltose (Difco), mannitol (Difco), Trehalose (Difco), D(-) raffinose (Difco), ribose (Difco), sorbitol (Difco) and glucose (Difco).

Determination of Antibiotic Resistance

Antibiotic disc were employed to determine the pattern of antibiotic resistance of *Lactobacillus* strains. The disc includes gentamycin (10µg), ketoconozole (30µg), rifampicin(10µg), novobiocin(10µg), flucozonazole(15µg), chloramphenicol(30µg), and amphotericin (30µg). These discs were placed on the solidified agar surface (Muller Hinton Agar) swabbed with the *lactobacillus* strains (10^{6} cfu/ml suspension of freshly grown strain).

The plates were incubated aerobically for 24 Hrs. at 37°C [23]. Resistance was determined according to the reference zone diameter interpretative standards of National Committee for Clinical Laboratory Standards [24]. Inhibition zones were recorded and compared to standard values.

Assessment of Bacteriocin Production by Antagonistic Activities of the Strains of *Lactobacillus* Spp. Isolates

Antimicrobial effects of presumptive strains of *Lactobacillus spp*. on the test isolates *Escherichia coli, Staphylococcus aureus, Pseudomonas aeroginosa, Streptococcus, Proteus spp, Klebsiella pneumonia* were determined by the agar well diffusion method.

Quantitative Studies on Production of other Antimicrobial Agents by the test Isolates

Quantitative Estimation of Lactic Acid

The production of lactic acid was determined by transferring 25ml of broth cultures of test organisms (*Lactobacillus* strains) into 100ml flasks. This was titrated with 0.1N NaOH and 1ml of phenolphthalein indicator (0.5% in 5% alcohol) till the end point appears pink colour. The titratable acidity was calculated as lactic acid % w/v [35]. Each ml of 1n NaOH is equivalent to 90.08mg of lactic acid. The titratable acidity was then calculated as stated in AOAC [25].

Quantitative Estimation of Diacetyl

Diacetyl production was determined by transferring 25ml of broth cultures of test organisms into 100ml flasks. Hydroxylamine solution (7.5ml) of 1 molar was added to the flask and to a similar flask for residual titration. Both flasks were titrated with 0.1M HCl to a greenish yellow end point using bromophenol blue as indicator [36]. The equivalence factor of HCl to diacetyl is 21.52mg. The concentration of diacetyl produced was calculated using the A.O.A.C. [25].

Quantitative Estimation of Hydrogen Peroxide

Hydrogen peroxide production was determined by measuring 25ml of broth cultures of the test organisms into a 100ml flask. To this was added 25ml of dilute H_2SO_4. This was then titrated with 0.1 M $KMnO_4$ (potassium permanganate). Each milliliter of 0.1N $KMnO_4$ is equivalent to 1.701mg of H_2O_2. A de-colorization of the sample was regarded as the end point. The volume of H_2O_2 produced was then calculated A.O.A.C. [25].

Mutation Studies to Increase the Production of Bacteriocin Production

The effect of mutation was studied under UV light on the bacteriocinogenic potential of the strains. To analyze the activity of mutation,

a 10ml aliquot of cultured broth was placed in a sterile petridish and these plates were exposed to short-wave UV light (254nm) from a electric germicidal bulb at a distance of 20cm. The time of exposure ranges from 0 to 20min [26]. After each time interval, bacteriocin activity was analyzed as described below.

The mutated aliquots from each plate was taken in different concentrations as 25µl, 50µl, 75µl and 100µl respectively and plated on to the solidified agar plates. These plates were incubated at 37°C for 24 Hrs. and the results were compared with the standards and the zone of inhibition was measured.

Optimization Studies on Physio-Chemical Characterization of Partially Purified Bacteriocin Sample

To test the heat sensitivity, culture supernatant (cell-free filtrate mentioned above) containing bacteriocin was heated for 10min at 60°C, 70°C, 80°C, 90°C, 100°C and 121°C and the bacteriocin activity was tested against various bacterial pathogens *Escherichia coli, Staphylococcus aureus, Pseudomonas aeroginosa, Klebsiella pneumonia, Streptococcus* and *Proteus spp* by agar well diffusion assay.

The sensitivity of bacteriocins to different pH was tested by adjusting the pH of culture supernatant (containing bacteriocins) in the range of pH 3.0, 4.5, 7.0 and 9.0 with 4M hydrochloric acid (HCl) and /or 4M sodium hydroxide (NaOH) and then production of bacteriocins was detected by agar well diffusion method against *Escherichia coli, Staphylococcus aureus, Pseudomonas aeroginosa, Klebsiella pneumonia, Salmonella typhimurium* and *Proteus spp.*

Effect of Other Parameters

Overnight grown culture of *L. plantarum* was inoculated into 100ml of MRS broth with varying concentrations of different carbon sources such as xylose 2-4%, glucose 2-4%, sorbitol 2-4%, lactose 2-4%, maltose 2-4%, mannose 2-4%, galactose 2-4%, sucrose 2-4% and fructose 2-4% and incubated at 37°C for 16 Hrs. and *L. plantarum* was inoculated into 100ml of MRS broth with varying concentrations of different substitutes such as yeast extract 0.5-2%, MgSO4 0.5%, Tween80 0-2% and SDS 0.25-0.5% and incubated at 37°C for 16 Hrs.

Estimation of Protein and Molecular Size Determination by SDS-PAGE

The protein content of the *Lactobacillus* strains was estimated using Lowry, et al. method. Partial purification was done using ammonium sulphate. The strains were mixed with ammonium sulphate at different concentration 20%, 40%, 60%, 80% respectively. This mixture was allowed to stand for 1 hr. at 4°C. The sample was centrifuged at 10,000 rpm for 15min. The supernatant was brought to saturation and the pellet was dissolved in 100Mm/L of potassium phosphate buffer and stored at 4°C for further use. The above sample was taken for molecular size determination using SDS-PAGE. The bands were compared with the marker.

To separate the antimicrobial compounds present in the LAB strains TLC was performed as described by sadasivan and Manikam [27]. Further purification of the LAB fractions was done by HPLC with an isocratic gradiant.

Result

In the present study, a total of 25 different fermented food samples like paneer, milk, curd, butter and cheese were collected to isolate *Lactobacillus* species. The mean pH values of these samples were around 6 to 6.5. All isolates were tested for the following as shown below [Table-1], [Fig-1], [Table-2] and [Table-3].

Table 1- LAB species isolated from different food samples

S. No.	Fermented Food Sample	Code	LAB Isolated
1	Cheese	C1	*L.fermentum*
		C2	*L.casei*
		C3	*L.plantarum*
		C4	*L.brevis*
2	Butter	B1	*L.casei*
		B2	*L.fermentum*
		B3	*L.plantarum*
		B4	*L.brevis*
3	Paneer	P1	*L.fermentum*
		P2	*L.casei*
		P3	*L.fermentum*
		P4	*L.plantarum*
4	Milk	M1	*L.casei*
		M2	*L.plantarum*
		M3	*L.fermentum*
		M4	*L.brevis*
5	Curd	CU1	*L.fermentum*
		CU2	*L.plantarum*
		CU3	*L.casei*
		CU4	*L.brevis*

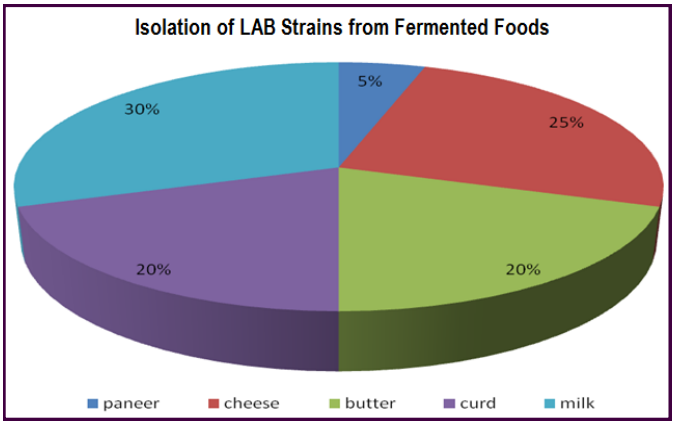

Fig.1- Graphical view of LAB species isolated from different food samples

Presumptive Identification of the Test Isolates

The four presumptive bacteriocin producers were characterized and identified to species level utilizing carbohydrate fermentation profiles, biochemical and physiological characteristics as stated below

Gram Staining

The bacteriocin-producing strains were identified based on the morphologyical studies by Gram staining. All the LAB isolates showed the presence of Gram-positive rods when viewed under the phase contrast microscope. The results interrupted on the basis of staining technique [Table-2].

Table 2- Morphological studies based on Gram staining

S. No	LAB isolates	Morphological features
1	*Lactobacillus casei*	Gram positive rods
2	*L. plantarum*	Gram positive rods
3	*L. brevis*	Gram positive rods
4	*L. fermentum*	Gram positive rods

Biochemical Tests for LAB Isolates

Various biochemical test were performed to isolate and identify the LAB strains. Based on these studies, the test isolates showed the absence of catalase, oxidase, indole production and it showed posi-

tive result for methyl red test Voges proskaur test and nitrate reduction. All the above results showed the presence of LAB isolates indicated in [Table-3].

Table 3- Biochemical test for LAB isolates

No	Lactobacillus spp	Indole	MR	VP	Nitrate reduction	Catalase	Oxidase
1	*L. plantarum*	-ve	+ve	-ve	+ve	-ve	-ve
2	*L. fermentum*	-ve	+ve	-ve	+ve	-ve	-ve
3	*L. casei*	-ve	+ve	-ve	+ve	-ve	-ve
4	*L. brevis*	-ve	+ve	-ve	+ve	-ve	-ve

Carbohydrate and Sugar Fermentation Profiles

Based on the carbohydrate fermentation patterns, the LAB strains showed no gas production from sugars. Out of the 4 isolated LAB strains, *L. plantarum* and *L. fermentum* showed maximum acid production compared to *L. casei* and *L. brevis.* The fermentation profile are listed in [Table-4].

Table 4- Sugar Fermentation test

LAB isolate	Cellu	Glu	Galac	Mann	Malt	Rham	Raffi	Ribo	Sorb	Treh	Fruc	Xylo
S1	+	-	+	+	+	+	+	+	-	-	-	-
S2	+	+	+	+	+	-	+	+	+	+	+	+
S3	+	-	+	+	+	+	+	+	-	+	+	+
S4	-	+	-	-	-	-	-	-	-	-	_	+

S1 - L. plantarum, S2 - L. fermentum, S3 - L. casei, S4 - L. brevis : Cellu-cellulobiose, glu-glucose, galac-galactose, mann-mannitol, malt-maltose, rham-rhamonase, raff-raffinose, ribo-ribose, sorb-sorbitol, treh-trehalose, fruc-fructose, xylo-xylose :(+) - acid production, (-) - no acid production.

On the basis of all the above identification tests the strains isolated from the test samples was identified as *Lactobacillus casei, L. plantarum, L. brevis,* and *L. fermentum*.

Detection of Antibiotic Resistance of *Lactobacillus* Species

The LAB producing strains were then further studied for their antibiotic susceptibility patterns. All the four isolates were susceptible to all of the antibiotic discs used in the study. Results concerning the determination of antibiotic susceptibility of the isolates are given in [Table-5]. In the study, 7 antibiotic discs were used as follows: novobiocin, flucozonazole, gentamycin, rifampicin, ketoconzole, amphotericin and chloramphenicol.

Table 5- Antibiotic sensitivity of different Lactobacillus species

LAB isolates	Novo biocin	Fluco zonazole	Genta mycin	Chloram phenicol	Ampho tericin	Rifam picin	Ketoco nozole	Tetra cycline
L. plantarum	S	R	S	S	R	S	S	R
L. fermentum	S	R	R	R	R	R	R	R
L. casei	R	R	R	R	R	R	R	R
L. brevis	S	R	S	S	R	S	S	R

S - Sensitive; R - Resistant.

On the basis of result shown in [Table-5], it was observed that *L. fermentum* and *L. casei* showed maximum resistance against antibiotics used in the study.

Antagonistic Activity of Test Isolates

The antagonistic effects of the bacteriocins on the growth of other Gram-positive and Gram-negative bacteria were determined using the well-diffusion assay. The inhibitory spectrum of the CFS (cell-free supernantant) obtained from the four isolated bacteriocin-producing LAB in this study were able to inhibit the growth of *S. aureus, E. coli, P. aeruginosa, S. pneumoniae, Klebisella,* and *proteus* [Table-6] [Fig-2]. The spectrum of inhibition by *L. plantarum* was significantly wider than *L. brevis*. Both the producer strains were immune to the inhibitory effect of their own bacteriocin as no inhibition was observed when tested against themselves. It was observed that among the 4 test strains *L. plantarum* possess strongest antagonistic activity of 25mm against *Pseudomonas*. This may be due to the production of lactic acid that lowered the pH of the medium or competition for nutrients, or due to production of bacteriocin or antimicrobial compounds.

Table 6- Antagonistic activity of selected LAB against pathogens

Test pathogens	*L. fermentum*	*L. plantarum*	*L. brevis*	*L. casei*
E. coli	10mm	14mm	6mm	8mm
Klebsiella	10mm	7mm	7mm	7mm
S. aureus	13mm	10mm	6mm	9mm
Proteus	12mm	5mm	15mm	7mm
Pseudomonas	15mm	25mm	14mm	8mm
Streptococcus	5mm	20mm	12mm	7mm

**Zone of inhibition in mm*

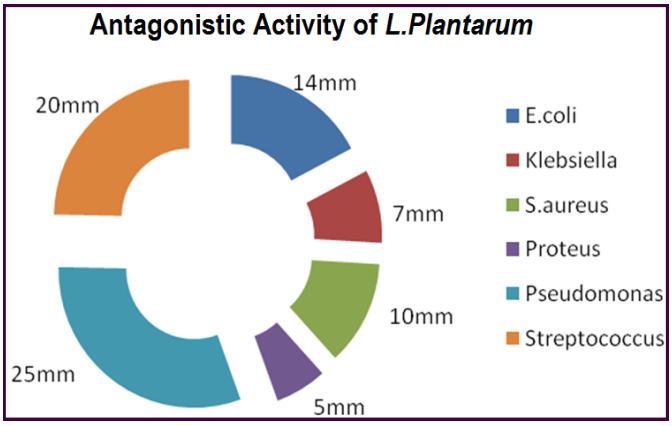

Fig. 2- Graphical view of antagonistic activity of selected LAB against pathogens

Quantitative Studies on Production of Antimicrobial Agents by the Test Isolates

The LAB species were screened for the quantitative production of lactic acid using normal MRS broth. It was observed that *L. plantarum* produced the highest quantity of lactic acid (11.6 ± 0.32 gL-1) at 48 Hrs. compared to all other LAB species used in this work with *L. brevis* having the lowest yield (8.7 ± 0.07 gL-1) after 48 Hrs. of incubation [Fig-3] [Table-7].

The highest quantity of hydrogen peroxide was produced by *L. plantarum* (15.0 gL-1) after 48 Hrs. incubation. After 48 Hrs. incubation, it was estimated that the quantity of diacetyl was 18.5 gL-1 in *L. plantarum* [Table-7].

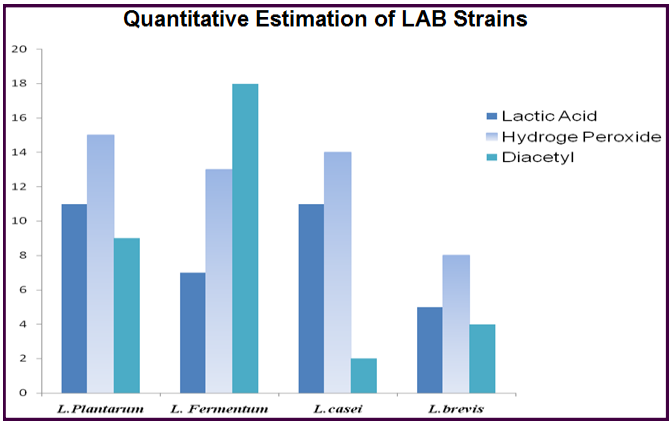

Fig. 3- Antimicrobial production by the LAB isolates (gL-1)

Table 7- Antimicrobial production by the LAB isolates (gL-1)

LAB Isolates	Lactic Acid	Hydrogen Peroxide	Diacetyl
L. plantarum	11.6	15	18.5
L. plantarum	11.6	15	18.5
L. fermentum	11	12	10.4
L. fermentum	11	12	10.4
L. casei	10.5	20	7.5
L. brevis	8.7	10	3

Mutation Studies to Increase the Production of Bacteriocin

[Table-8] shows the effect of mutation on bacteriocin activity. (Exposure to most of UV rays tested resulted in an increase in the bacteriocin titer (by at least one to two fold dilutions).

Table 8- The effect of mutation on bacteriocin activity

LAB isolates	Mutated strain	Wild strain	Control
L.plantarum	17 mm	10 mm	-
L.fermentum	12 mm	9 mm	-
L.casei	10 mm	5 mm	-
L.brevis	8 mm	2 mm	-

**Zone of inhibition in mm, (-) – no growth.*

Temperature and pH Stability of Bacteriocins

The stability of the secreted inhibitory compounds was tested using different temperature treatments [Table-8]. The inhibitory activity was shown to be completely unaffected following heat treatments at 60°C to 120°C. The inhibitory compounds produced by isolates *L. plantarum* and *L. fermentum* were seen to be the most stable to heat treatments up to and beyond 100°C. *L. plantarum* maintains its activity even after treatment at 120°C for 20min, a property which is typical for bacteriocins. The sensitivity and stability at high temperatures therefore conclusively identifies these compounds as bacteriocin.

The stability of the inhibitory activity was tested at different pH values [Table-10]. The bacteriocins produced by isolates, *L. plantarum* and *L. fermentum* showed greater pH tolerance and stability than those secreted by other three isolates.

As shown in [Table-9] [Fig-4] and [Table-10] [Fig-5], it was concluded that the use of constituted medium 30°C incubation temperature, initial pH 5.5 and for 48 to 60 hours favored the best production of antimicrobial by isolates.

Table 9- Optimization of temperature stability

LAB isolates	Temperature in °C (zone of inhibition in mm)				
	60	70	80	90	110
L. plantarum	15	25	17	19	22
L. fermentum	20	17	19	15	17
L. casei	12	19	13	15	19
L. brevis	11	15	15	13	12

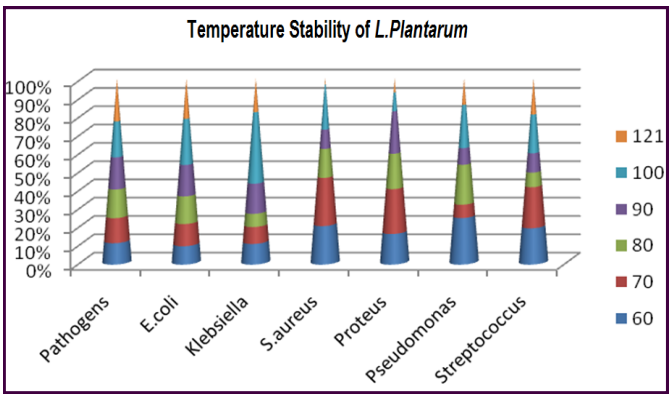

Fig. 4-Graphical view of Optimization of temperature stability

Table 10- Optimization of pH stability

LAB isolates	pH Range (zone of inhibition in mm)			
	3	4.5	7	9
L. plantarum	12	15	25	17
L. fermentum	9	12	17	14
L. casei	13	11	19	12
L. brevis	9	12	18	13

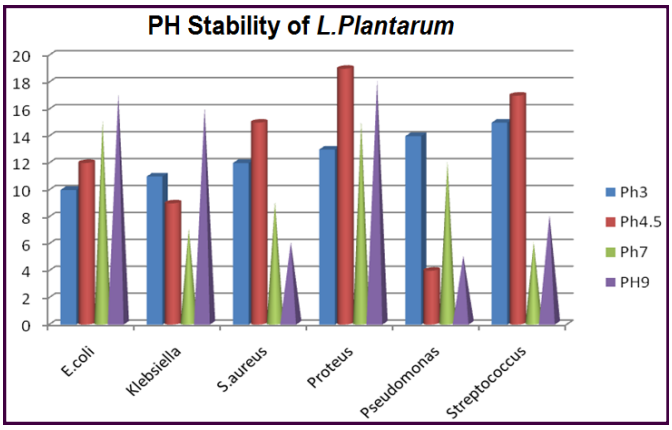

Fig. 5- Graphical view of optimization of pH stability

Effect of Different Carbon Sources and Medium Supplements

Medium composition was also found to play a very important role on bacteriocin production.

Table 11- Bacteriocin activity of Lactobacillus plantarum with different supplements carbon subsitutes in MRS medium.

MRS Substitutes	Concentration (%)	Final pH	Activity (AUMl^{-1})
MRS broth		4.43	50
Xylose	2	4.98	0
	3	4.9	0
	3.5	4.95	0
	4	4.99	0
Lactose	2	4.38	500
	3	4.42	500
	3.5	4.4	2500
	4	4.43	2500
sorbitol	2	4.8	50
	3	4.75	50
	3.5	4.72	50
Sucrose	2	4.49	100
	3	4.5	500
	3.5	4.45	500
	4	4.47	500
Galactose	2	4.7	100
	3	4.65	100
	3.5	4.72	100
	4	4.71	100
fructose	2	4.67	100
	3	4.65	100
	3.5	4.71	100
	4	4.68	100
Mannose	2	4.67	100
	3	4.68	100
	3.5	4.62	100
	4	4.7	100
Maltose	2	4.5	100
	3	4.48	500
	3.5	4.52	500
	4	4.51	500
Glucose	2	4.43	50
	3	4.45	50
	3.5	4.43	100
	4	4.46	100

The components of the production medium peptone, beef extract, yeast extract, glucose, Tween80, Na_2HPo_4, sodium acetate, triammonium citrate, $MgSo_4.7H_2O$ and $MnSO_4$ were used. The pH was adjusted to 6.4. This medium corresponds to a complex medium. Various carbon substitutes were tried out. Maximum activity equivalent to 50 AU ml-1 was recorded in MRS medium using LAB isolates. When MRS medium was substituted with 3.5% of lactose as a carbon source an increase in bacteriocin production to 2500 AU ml-1 was observed. (plate 10). There was a slight increase in bacteriocin activity by addition of 3.0% each of sucrose and maltose in the medium. It was observed that the production organism has an efficient enzyme machinery to utilize disaccharides [Table-11].

As shown in [Table-12] various supplements in MRS medium were also carried out for the production of bacteriocin. It was found that there was no effect on bacteriocin activity by the addition of 0.25-0.50% of SDS and 2% of yeast extract. There was slight increase in the bacteriocin activity by addition of 1% Tween80 and 0.05% of $MgSO_4$.

Table 12- Bacteriocin activity of L. plantarum with different supplements in MRS medium

MRS Substitutes	Concentration (%)	Final pH	Activity (Aumnl^{-1})
MRS+ Yeast extract	2	4.27	50
MRS + Tween80	1	4.19	100
	2	4.09	100
MRS + $MgSo_4$	0.05	4.07	100
	0.25	5.86	50
MRS +SDS	0.5	5.89	50

Quantitative Estimation of Protein using Lowry, et al. Method

The quantitative estimation of protein was studied using Lowry, et al. method. These studies showed that all the 4 LAB isolates showed the presence of protein content and the quantitative estimation was carried out using UV- spectrophotometer with the OD values read at 650nm and the results are shown in [Table-13].

Table 13- Estimation of protein

S. No	LAB isolates	Standard (BSA solution)	OD at 650nm	
			LAB	STD
1	*L.plantarum*	S1(0.2)	1.34	0.12
2	*L.fermenturm*	S2(0.4)	1.19	0.15
3	*L.casei*	S3(0.6)	0.89	0.19
4	*L.brevis*	S4(0.8)	0.92	0.2

Partial Purification and Molecular Size Determination Samples by TRICINE-SDS PAGE

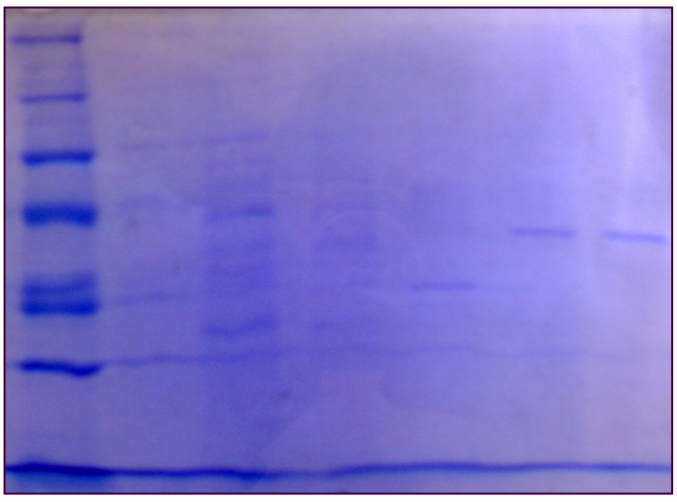

Fig. 6- Molecular weight determination by SDS-PAGE

A significant increase in yield and purification fold of the bacteriocin in study was observed during different purification stages. The crude supernatant fluid of 4 different were concentrated before subjected them to four rounds (0-20, 20-40, 40-60 and 60-80%) of ammonium sulphate precipitations. All the activity was recovered in the pellet at 80% saturation.

Attempts to size the bacteriocin under denaturing conditions were obscured due to diffuse banding. However under non-denaturing conditions the exact location of the protein giving activity was detected. The bacteriocin of *L. plantarum* was resolved as a single band of approximately 37KDa while that of *L. fermentum* appeared to be 8 KDa [Fig-6].

Qualitative Analysis of Antimicrobial Compounds by TLC

After exposure to ninhydrin, pink color spots similar to the standard amino acids were observed. And the Rf value was determined as 0.54, 0.34 and 0.11.

$$\text{Rf value} = \frac{\text{Distance travelled by the solute from the origin}}{\text{Distance travelled by the solvent from the origin}}$$

Table 14- Experimental Rf data for antimicrobial compounds isolated from LAB isolates

S. No	Detector reagent	LAB isolates	Rf values
1	Ninhydrin	*L. plantarum*	0.54
			0.34
			0.11

High Performance Liquid Chromatography

The high performance liquid chromatography was performed for *L. plantarum*. The sample was made to run for 10min and the volume of sample taken for analysis is 10µl and the analysis was made at 200nm-320nm. Methanol was used as the solvent system for the study. After the analysis, peaks was obtained as shown in [Table-15].

Table 15- HPLC for L. plantarum

S. No	RT	Area	% Area	Height
1	3.469	3.5E+07	23.73	1430580
2	3.732	3.1E+07	21.54	1694336
3	4.197	1.9E+07	13.31	1661178
4	4.353	3.9E+07	26.61	1921461
5	4.937	1.5E+07	10.3	471870
6	6.493	3591438	2.47	147201
7	7.029	2979136	2.05	113079

**The results were compared with the standard.*

Discussion

The present investigation highlights the isolation and characterization of bacteriocin producing LAB strains from fermented foods and were screened for bacteriocin production, from which four bacteriocinogenic strains were identified and selected for further study, representing four isolates of *L. plantarum, L. fermentum, L. brevis* and *L. casei*. This would indicate that a wide variety of bacteriocin-producing LAB are present on fermented milk, which therefore represent an abundant resource of such potentially useful bacteria. To date, only few bacteriocin producing LAB has been reported in fermented foods [28].

The bacteriocin produced by *L. plantarum* and *L. fermentum* exhibited a wider spectrum of inhibition compared to the bacteriocin produced by *L. brevis*. The potential of these bacteriocins to inhibit the pathogens such as *E. coli, S. aureus, S. typhi, Pseudomonas* and *Klebsiella* makes it of crucial interest especially in processed foods

where there is risk of food pathogens. Due to the phenomenon of immunity the bacteriocin from the producer organism were resistant to the organism producing it. Their antagonistic property is attributed to the low pH, the undissociated acid and production of other primary and secondary antimicrobial metabolites [29].

The antimicrobial effect exerted by LAB is the production of lactic acid and reduction of pH, and acetic acid, diacetyl, hydrogen peroxide, fatty acids, aldehydes and other compounds [30]. Many LAB are resistant to antibiotics. This resistance's attributes are often intrinsic and nontransmissible [31]. *L. plantarum* was resistant to all of the antibiotics used in this study but *L. brevis* isolate was susceptible to all of the antibiotics.

Exposure of the bacteriocin samples to UV resulted in an increase in the bacteriocin titre. This might be due to the effect of UV on the permeability of the cell membrane. It has also been suggested that the dispersion of the bacteriocin complex into active subunits ultimately results in more lethal hits and consequently enhanced activity is witnessed [32].

According to Tagg, et al., [33] bacteriocins differ greatly with respect to sensitivity to pH. Many of them are considerably more tolerant to acid than alkaline pH values. In the present study, bacteriocin produced by the *L. plantarum* exhibited the same profile and was active at pH values between 3-9. Maximum inhibitory activity was demonstrated at pH values between 5 to 6.5. The optimum temperature and pH for the production of lactic acid by the test isolates was at 60°C and pH 6.5.

The phenomenon of heat stability of LAB bacteriocins have been reported earlier for bacteriocin produced by *L. brevis* 0G1 [2]. The findings are also in agreement with the above mentioned reports as observed heat stability of *L. plantarum* bacteriocin. The retention of activity by this bacteriocin after heating at 121°C for 30min, placed it within heat stable low molecular weight group of bacteriocins. This quality of the bacteriocin makes it superior in processed food stuffs where high heat is applied.

The number of chromatographic steps varies from three or more, for bacteriocin in the present study in which antibacterial activity was recovered after simple precipitation with ammonium sulfate from the cell-free culture supernatant fluid. The final reversed-phase HPLC step of the purification procedure led to isolation of a single active fraction having antibacterial activity. Bioautography on thin-layer chromatograms, a method previously used for detecting antibacterial and antifungal substances for control of pathogens was useful to revel the active fraction [34].

The study indicated that the isolated Lactobacillus species meet several of the criteria for use as a probiotic. The bacteriocins produced by *L. plantarum* and *L. fermentum* showed prominent antimicrobial properties, heat resistance and acid tolerant indicating strong probiotic potential hence these isolates can be use in the protection and improvement of intenstinal microbial flora and contribute health benefits to consumers.

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