TECHNOLOGICAL AND FUNCTIONAL PROPERTIES OF *LACTOBACILLUS PLANTARUM* ISOLATED FROM IRANIAN FERMENTED FOOD

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ABSTRACT

In the present study, lactic acid bacteria were isolated from fermented foods (curd, pickle, and tuna fish) used in Iran. Molecular characterization was performed to identify the isolated LABs. Further, the LAB isolates were studied for antimicrobial activity against pathogenic bacteria and spoilage flora, antimicrobial resistance pattern, exoenzyme (lipase and protease) production, and synthesis of biogenic amines. The study revealed that the predominant lactic acid bacteria isolated from fermented foods were *Lactobacillus plantarum.* The isolates showed significant antimicrobial activity against food-borne bacteria. They were capable of producing industrially important enzyme lipases. They do not have resistance to the commonly used antibiotics and were not capable of synthesizing biogenic amines. They can find potential application as starter cultures in the preparation of fermented foods, as preservatives to enhance shelf life in food, and as probiotics for developing functional foods.

Keywords: Lactic acid bacteria,*Lactobacillus plantarum,* Fermented foods, Antimicrobials, Enzymes, Antimicrobial susceptibility, Biogenic amines

INTRODUCTION

Lactic acid bacteria (LAB) have been used in the production of fermented foods as they produce several metabolites that contribute to the flavor, smell, color, and texture of the foods. These bacteria produce antimicrobial substances including bacteriocins which inhibit growth of pathogenic and food spoilage bacteria (**Rattanachaikunsopon and Phumkhachorn, 2010**). LAB was initially used to prevent spoilage and preserve foods through natural fermentations. In recent years, they have found commercial applications as starter cultures in the food and beverages industries due to their ability to produce organic acids, diacetyl, hydrogen peroxide, and bacteriocins or bactericidal proteins, exoenzymes, and exopolysaccharides during fermentation **(Lindgren and Dobrogosz, 1990)**.

LAB produces proteinaceous compounds with efficient antimicrobial effects, which are known as bacteriocins **(Galvez *etal.,* 2007)**. LAB can outcompete and antagonistic activity against other microorganisms. As in recent years, consumers are preferring foods without chemical preservatives, biopreservatives such as LABs, and their metabolites are increasingly used to extend shelf life and enhance the safety of foods **(Ross *et al.,* 2002)**.

However, the antibiotic resistance in lactic acid bacteria (LAB) strains used for food, feed, and probiotic applications has the risk of conferring the resistance genes to the pathogenic bacteria and is a matter of consideration. Hence, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) of the European Food Safety Authority (EFSA) has defined microbiological breakpoints for a representative group of ten antibiotics, to identify genotypes resistant to the most commonly used antimicrobials (EFSA, 2008). Therefore, it is essential to screen the LAB for antibiotic resistance before their application in foods and as probiotics.

The objective of the study was to isolate the predominant lactic acid bacteria from fermented foods used in Iran, evaluate their antimicrobial activity against food-borne bacteria and for antibiotic resistance. Further, they were studied for the production of biogenic amines and enzyme synthesis.

MATERIAL AND METHODS

Isolation of lactic acid bacteria

Lactic acid bacteria (LAB) isolates were obtained from three fermented foods in Iran. The fermented foods such as curd (kashk), pickle, and tuna fish were collected from a commercial market in Iran and analyzed in the Department of Studies in Microbiology, University of Mysore. The samples were stored under refrigeration conditions for subsequent experiments. The samples of curd, pickle, and fish tuna after serial dilutions were inoculated into the MRS broth and incubated at 37°C for 24-48 hours. From MRS broth, the culture was inoculated to the MRS agar for separation of colonies. The selected colonies from MRS agar were isolated and purified for further studies **(Harrigan 1998)**.

Biochemical characterization

To identify the LAB isolates, phenotypic methods including morphological examinations (gram staining), biochemical tests (sugar fermentation), and enzymatic tests (catalase) were performed.

Molecular characterization

Total genomic DNA was extracted by the conventional phenol-chloroform extraction method. Forward primers 27F: GAGTTTGATCCTGGCTCA and the reverse primer 1492R: TACGGCTACCTTGTTACGACTT were used in the study. The amplification of the 16S rDNA was performed using 2 µL DNA template, 0.4 µM of each forward and reverse primers, 0.2mM of each dNTPs, 1.5mM MgCl2, 0.625 units of Taq Pol, 2.5 µl of 10 buffer, 6.5 µl of sterile water. The thermal profile consisted of an initial denaturation step of 5 min at 94˚C, followed by 30 cycles of 15 s at 94˚C, 30 s at 54˚C, 90 s at 72˚C, followed by a final step of 7 min at 72 ˚C. The amplicon was sequenced using the forward primers. The sequence was submitted to the NCBI GenBank nucleotide collection. Sequences with high similarity to the sequence of the isolate were retrieved from the GenBank database using the BLAST program (megablast).

Antimicrobial activity

Bacterial strains of *Salmonella enterica* serovar Typhimurium*, Escherichia coli, Pseudomonas aeruginosa, Pseudomonas fluorescens, Vibrio parahemolyticus,*and *Staphylococcus aureus*(obtained from Microbial Type Culture Collection, India) were sub cultured in nutrient broth. The overnight culture was used to determine the antibacterial activity of LAB isolates by the agar well diffusion method. 100 µl of inoculum was poured and swabbed on sterile nutrient agar plates. Wells were prepared in the plates with a sterile borer (5mm). The 24-hour culture of LAB (50 µl) was introduced into the well and plates were incubated at 37°C for 72 hrs. All samples were tested in triplicates. Antimicrobial activity was determined by measuring the diameter of the zone of inhibition.

Antimicrobial susceptibility

The antibiotics used for susceptibility assay were selected due to their frequent and common usage. A total of 1 ml LAB culture grown in MRS broth was collected by centrifugation at 1000 × g for 5 min. The cell pellet was collected and washed twice using 1 ml of 0.85% (w/v) NaCl, followed by suspending the cell pellet with 0.5 ml of 0.85% (w/v) NaCl. The cell suspension was adjusted to 0.5 Mc Farland by using 2 ml of NaCl 0.85% (w/v) beforethe spread was plated on MRS agar. The antibiotic discs (Himedia, India) were then placed on an MRS agar plate. The diameter of inhibitory zones was measured after 48 h of incubation at 30°C under anaerobic conditions **(Shazali*et al.,* 2014)**.

Exoenzyme activity

Lipolytic and proteolytic activity was detected in the isolated LAB by using de Man-Rogosa-Sharpe (MRS) agar in combination with tributyrin (1%) as well as MRS in combination with skimmed milk (1%), respectively. The LAB strains were streaked on the MRS media supplemented with the substrates (tributyrin and skimmed milk) and incubated for 3 days at 37 ºC. The potent proteolytic and lipolytic activity in the isolates was selected based on the diameter of the zone of clearance or halo production around the colony on the media **(Ramakrishnan *etal.,* 2012)**.

Biogenic amine production

Moeller's decarboxylase medium (Himedia, India) was used as the basal medium (BM). Amino acid decarboxylation was tested by adding 2gr/100 ml of Lysine –Histidine- Tyrosine amino acids to BM. The media were adjusted to pH 6·5 with NaOH and sterilized at 121°C for 15 min. The strains were pre-cultured in BM and then inoculated into media at 5×10^7 cells ml^{-1}.These cultures were incubated statically at 30 °C for 3 days under microaerophilic or anaerobic (by overlaying with paraffin) conditions.

Statistical analysis

All the experiments were carried out in triplicates. The results were expressed as mean ± standard deviation (SD). Statistical analysis was performed using SPSS 20 statistical software (IBM, Armonk, NY, USA).

RESULTS

Isolation of Lactic acid bacteria

Samples (10 g) of each food product were homogenized with 90mL of 0.85% (w/v) sterile physiological saline and serially diluted in the same diluent. The LAB was selectively isolated on MRS agar plates incubated both anaerobically and aerobically at 30 °C for 3 days. The predominant LAB was obtained from MRS plates with the highest sample dilutions, and the studied strains, therefore, represent bacteria present in numbers ≥ 10^6g or ml. Colonies were either selected randomly or all sampled if the plate contained less than 10 colonies. The purity of the isolates was checked by streaking again on fresh MRS agar plates, followed by microscopic examination. Initial characterization of isolates included colony and cell morphology, Gram staining, and catalase tests. Gram-positive, catalase-negative, non-motile cells were presumptively identified as LAB.

Characterization of Lactic acid bacteria

The samples of curd, pickle, and fish tuna after serial dilutions were inoculated into the MRS broth and incubated at 37° C, 42° C and 50° for 24-48 hours. From MRS broth, the culture was inoculated to the MRS agar for separation of colonies. The optimal growth temperature for the growth of the isolated LAB was 37° C. The LAB showed catalase-negative and gram-positive on staining. Molecular characterization revealed that the predominant microorganisms associated with fermented foods used in the study were *Lactobacillus plantarum.* The LAB isolated from fermented foods curd, pickle, and tuna fish were labeled as *Lactobacillus plantarum* LBC1, *Lactobacillus plantarum* LBP1, and *Lactobacillus plantarum* LBT1. The GenBank accession number for the 16S rDNA sequences generated in this study isKP789017 for LBC1, KP789018 for LBP1, andKF660248for LBT1.

LBC1 fermented all the four carbohydrates (glucose, sucrose, lactose, and mannitol) used in the sugar fermentation test producing the only acid with no gas production. LBP1 and LBT1 fermented the three carbohydrates used to produce acid and were unable to ferment mannitol and did not produce gas. *L. plantarum* are homofermentative, so no strain produces gas from glucose. Gas-producing LAB are not suitable for the production of certain fermented foods as the formation of large amounts of carbon dioxide leads to holes of different sizes in the product **(Essid*et al.,* 2009)**.

Antimicrobial activity

The antibacterial activity of the LAB isolates against the pathogenic and spoilage flora is represented in Table 1. The LBP1 isolate can also act as a good substitute for improving the microbiological safety in bio preservation of fermented food as it showed significant inhibitory activity against *E. coli*, *V. parahaemolyticus*, *P. fluorescens,* and *S. aureus*.However, it did not exhibit any activity against the pathogen *S.* Typhimurium*.* The LBC1 isolate showed high inhibitory activity against *V. parahaemolyticus*and*S. aureus* and moderate antimicrobial activity against *E. coli, P. aeruginosa* and *P. fluorescens.* In the present work, the isolate LBT1 revealed strong inhibition of growth against the pathogens *P. aeruginosa, V. parahaemolyticus,S. aureus*. It exhibited moderate inhibitory activity against spoilage flora *P. fluorescens*

and *E. coli* and low activity against *S.* Typhimurium. Thus, in the present study, the *L. plantarum* isolated from fermented food displayed a broad range of antimicrobial activity against the tested pathogens and spoilage flora (Figure 1).

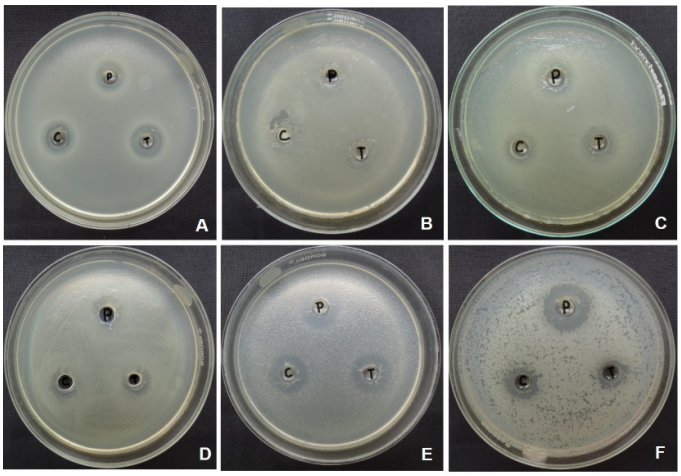

Figure 1 Antimicrobial activity of LAB against food-borne bacteria by agar well diffusion method. *Lactobacillus plantarum* LBC1(C), *L. plantarum* LBP1(P) and *L. plantarum* LBT1(T) isolated from fermented foods showed inhibition of *Vibrio parahaemolyticus* (A), *Salmonella* Typhimurium (B), *Pseudomonas aeruginosa* (C), *Pseudomonas fluorescens* (D), *Staphylococcus aureus* (E) and *Escherichia coli* (F).

Antimicrobial susceptibility

The antibiotics used for susceptibility assay were penicillin-G (10 µg), clindamycin (2 µg), erythromycin (15 µg), cefoxitin (30 µg), chloramphenicol (30 µg), tetracycline (30 µg), rifampicin (5 µg), pristinimycin (5 µg), levofloxacin (5 µg), vancomycin (30 µg), co-trimoxazole (5 µg). Table 1 shows the inhibitory zone of antibiotic susceptibility for the three LAB isolates used in the study. LBC1 showed resistance to vancomycin and rifampicin. The growth of LBP1 was significantly inhibited by clindamycin and pristinomycin. LBT1 showed antimicrobial susceptibility to all the antibiotics used in the experiment.

Table 1 Antibiotic susceptibility profile of *Lactobacillus plantarum* isolates

Antibiotic	Inhibition zone diameter (in mm)			Disk potency (µg/ml)
	*L. plantarum LBC*1	*L. plantarum LBP*1	*L. plantarum LBT*1	
Penicillin-G	28	27	28	10
Vancomycin	10	35	24	30
Cefoxitin	30	24	35	30
Chloramphenicol	40	36	35	30
Rifampin	10	40	35	5
Tetracycline	30	30	40	30
Erythromycin	35	40	35	15
Clindamycin	20	12	35	2
Pristinomycin	40	-	30	5
Levofloxacin	20	35	40	5
Co-Trimoxazole	25	32	30	25

Exoenzyme production

The LAB isolates used in the study were screened for the production of the exoenzymes lipases and proteases. None of the *L. plantarum* isolates were able to produce the protease enzyme. However, the isolates *L. plantarum* LBT1 exhibited significant lipase activity on tributyrin- MRS agar plate (Figure 2). Similarly, the isolate LBP1 also showed lipase activity whereas LBC1 had neither lipase nor protease activity.

Figure 2*Lactobacillusplantarum* LBT1 showinglipase activity on tributyrin- MRS agar plate.

Biogenic amine production

The LAB isolates have no decarboxylase activity with the substrate's lysine, tyrosine, and histidine. It can be implied that they don't produce biogenic amines on utilizing these amino acids or they were not capable of producing any biogenic amines as seen by preliminary screening with decarboxylase activity.

DISCUSSION

LAB was isolated from fermented foods such as curd, pickles, and tuna fish. The predominant LAB isolates belonged to *Lactobacillus species* as identified by biochemical and morphological techniques. Molecular characterization revealed that the predominant microorganisms associated with fermented foods used in the study were *Lactobacillus plantarum*. *L. plantarum* isolates could be competitive against other autochthonous species present in the fermented food samples. It has been reported that the strains harbouring interesting metabolic traits could supersede other biotypes of the same species **(Minervini*et al.,* 2010)**.

Lactic acid bacteria (LAB) play a key role in food fermentations apart from imparting the desired sensory properties they also contribute to the microbiological safety of foods **(Smaoui*et al.,* 2010)**. The antimicrobial effect of LAB is due to the production of organic compounds such as lactic- and aceticacids, as well as propionic-,sorbic-, benzoic-acids, hydrogen peroxide, diacetyl, ethanol, phenolic- and proteinaceous-compounds. The antimicrobial activity of LAB can also be due to the production of bacteriocins **(Cizeikiene*et al.,* 2013)**. Bacteriocins are antimicrobial polypeptides inhibitory to strains closely related to the bacteriocin-producing bacteria. These antimicrobial compounds are thought to provide a selective advantage to the producer strains over other strains **(Hyronimus*et al.,* 2000)**. Lactic acid bacteria (LAB) have been studied extensively for the production of antimicrobial compounds such as bacteriocins for their potential use as biopreservatives in foods **(Hur*et al.,* 2000)**. In this study, LAB isolates were capable of producing metabolites against various pathogenic bacteria and spoilage flora (*S. enterica* svTyphimurium*, E. coli, P. aeruginosa, P. fluorescens, V. parahaemolyticus,* and *S. aureus*). Except for *S. enterica* svTyphimurium, the *L. plantarum* isolates in the study showed broad-spectrum antimicrobial activity. Previous studies have shown that strains of *L. plantarum* had inhibitory activity against *S. aureus* and not against *P. aeruginosa*, *E. coli,*and *Salmonella* spp**(Nieto-Lozano*et al.,* 2002)**. However, *L. plantarum* strains, isolated from fermented sausages, had inhibitory activity against *E. coli* and *S.* Typhimurium**(Klinberg*et al.,* 2005)**. It has also been reported that *L. plantarum* strains have anti-*Listeria monocytogens* activity **(Klinberg*et al.,* 2005)**. Similarly, *L. plantarum,* isolated from a Tunisian traditional salted meat has shown antimicrobial activity against *S. arizonae*, *S. aureus*, *P. aeruginosa*, and *E. coli*. The characterization of the antimicrobial substances showed that none of the strains could produce bacteriocins **(Essid*et al.,* 2009)**. Therefore, the *L. plantarum* strains isolated in the study could be used as biopreservatives in foods to obtain safe products with an extended shelf life.

Lactic acid bacteria widely used as probiotics or in starter cultures have the potential to serve as a host of antibiotic resistance genes with the risk of transferring the genes in many lactic acid bacteria and other pathogenic bacteria. In recent years, increased focus has been given to food as vehicles of antibiotic resistance genes **(Klein *et al.,* 2000)**. The LAB strains in our study showed antibiotic susceptibility to the commonly used antibiotics such as Penicillin-G, Vancomycin, Cefoxitin, Chloramphenicol, Rifampin, tetracycline, Erythromycin, and Levofloxacin. Therefore, these isolates can find safe applications in foods and probiotics.

Biogenic amines in food are mainly produced by microbial decarboxylation of amino acids except for physiological polyamines. Biogenic amines are basic nitrogenous compounds that have undesirable physiological effects. Apart from the hygienic quality of raw materials, the addition of amine-negative starter culture to carry out a controlled fermentation is suggested to prevent excessive amine accumulation **(Nie*et al.,* 2014)**. The LAB isolates in our study did not produce biogenic amines using the precursor amino acids tyrosine, lysine and histamine due to their negative decarboxylase activity and can find safe application in fermented and functional foods.

The LAB used in the study was unable to produce the protease enzyme. However, the isolates were capable of producing enzymes such as lipase showing that they have potential technological properties. Lipases have an essential role during fermentation and in the ripening of fermented foods **(Bautista-Gallego *et al.,* 2013)**. Lipases of microbial origin are used in dairy and meat products to produce free fatty acids from selective hydrolysis of fat triglycerides. These fatty acids serve as either flavors or flavor precursors **(Flores andToldrá, 2011)**. Therefore, *L. plantarum* LBT1 has the potential to be used as a flavor enhancer in fermented foods.

CONCLUSION

In this study, the isolates of lactic acid bacteria (LAB) from fermented foods used in Iran were examined for antimicrobial potential. The antimicrobial activity was assessed against food-borne pathogens *Salmonella enterica* sv Typhimurium, *Escherichia coli*, *Vibrio parahaemolyticus*, *Staphylococcus aureus,* and reference strains *Pseudomonas aeruginosa* and *P. fluorescens*. The metabolites of lactic acid bacteria (LAB) inhibited the growth of pathogenic bacteria. The strains were tested for their susceptibility to a panel of antibiotics. The LAB isolates were also screened for biogenic amine production. Molecular characterization revealed that the predominant microorganisms associated with fermented foods used in the study were *Lactobacillus plantarum*. The isolates have low antibiotic resistance and are not capable of producing biogenic amines. The study shows that the LAB isolates *L. plantarum* has the potential to be used as probiotics and is currently evaluated for its survival during gastric transit, tolerance to bile salt, and adherence to the gut epithelial tissue.

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