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Research Article

Antimicrobial Profiling of *Lactobacillus* Spp. Isolated from Indian Fermented Food

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ABSTRACT

The present study involved isolation of Lactobacillus spp. from fermented Indian foods and characterization of their antimicrobial activity which might offer significant information about their probiotic potential and future use. From 4 samples of homemade curd and 3 samples of homemade buttermilk, 7 bacterial cultures were isolated based on their colony morphology. The isolates were evaluated for their physiological and biochemical properties and based on these they were assigned to the genus Lactobacillus. These 7 isolates were tested for their antagonistic activity against 7 pathogens including Bacillus subtilis, Bacillus cereus, Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, E. coli and Candida albicans by well diffusion method. Strain 4A showed antagonistic activity against maximum no. of pathogens followed by 21C and 101A. The isolates were further tested for arginine hydrolysis along with their ability to produce extracellular enzymes including amylase, phytase, protease, lipase and gelatinase.

Key words: *Probiotics, Lactobacillus, Antimicrobial activity, Lactic acid bacteria, Pathogens, Enzymes.*

INTRODUCTION

Fermented foods are indigenous part of human diet because of their uncountable benefits. Fermentation is an inexpensive technology used for food preservation, increased digestibility and pharmacological values, enhancement of sensory qualities and nutritional value[15]. Traditionally fermented food products are rich source of microorganisms and are defined as products prepared by native people by means of their hereditary knowledge and skilled technology from locally available plant and animal raw materials. Fermented foods are made either naturally or by addition of starter culture composed of proficient microbes which convert the substrates into generally acceptable edible products[19].

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Amongst fermented foods, dairy products are regarded as the chief source of probiotic bacteria. Food product containing probiotic bacteria are called functional foods, which are defined as foods with positive effect on human health. Such products have gained worldwide popularity and approval[18]. Lactic acid bacteria (LAB) have immense contribution in the production of fermented foods as well as in manufacturing of preparations containing probiotics or health promoting bacteria. Probiotics are living microbes that are able to tolerate and survive the environment of gastrointestinal tract and exert beneficial effects on the host by balancing the gut microbiota[16].

LAB are the chief probiotic bacteria which exert favorable effects in the gut and include genera *Lactobacillus, Lactococcus, Streptococcus, Leuconostoc* and *Pediococcoccus*. They are Gram-positive rods or cocci, non-spore-forming, usually non-motile, catalase, nitrate and oxidase negative, unable to liquefy gelatin and produce indole. They are able to grow in both absence as well as in presence of oxygen and major end-product of their fermentation is lactic acid. They have an extensive history of appliance in fermented foods because of their valuable contribution to organoleptic, nutritional, and shelf-life properties[23]. LAB can produce antimicrobial compounds such as hydrogen peroxide, organic acid, bacteriocins and diacetyls which have antagonistic activity against a large number of food-borne and enteric pathogens[17]. Additionally, they are also known to produce a no. of enzymes like amylase, lipase, protease, phytase, peptidases etc. For this reason along with their 'generally recognized as safe' (GRAS) status, LAB especially *Lactobacillus* have received much attention for their use as probiotics in recent years[5].

The main aim of the current study was the isolation and screening of lactobacilli from local fermented food products and evaluation of their antimicrobial activity and ability to produce various enzymes.

MATERIAL AND METHODS

Isolation and of LAB

Four curd samples and three buttermilk samples were collected from local and nearby area of district Meerut, Uttar Pradesh, India. 1 ml of sample was diluted to 10^{-3} in 0.85% saline and 100 µL aliquot from last dilution was spreaded on freshly prepared Man Rogosa Sharpe (MRS) agar plates and incubated for 24-48 hr at 37°C. Selected colonies with diverse morphological characteristics were sub cultured on fresh MRS plates and the isolates were preserved as glycerol stocks at -20°C[11,10].

Identification of *Lactobacillus* species

The isolates were identified as *Lactobacillus* species by performing various morphological, cultural, biochemical tests described in Bergey's Manual of Bacteriology[8]. The colonies were characterized according to their shape and color. Selected isolates were further characterized by Gram's staining, endospore staining, catalase test, oxidase test[12].

Antimicrobial activity of the isolates

In this test, the antagonistic activity of the isolates was tested against pathogenic bacteria such *as Bacillus subtilis, Bacillus cereus, Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, E. coli* and yeast *Candida albicans* by well diffusion method. The selected isolates were inoculated in MRS broth and incubated at 37°C for 72 h. The cultures were centrifuged at 6000xg for 20 min at 4˚C and supernatant was sterilized using 0.2 µm micro filters. 24 h old cultures of the pathogens were swab on the Muller-Hinton agar (MHA) plates and wells were made using sterile cork borer. Wells were filled with 100 µl of the supernatant of each isolate and plates were incubated at 37°C for 24 h. The diameter of inhibition zone was measured. The test was performed in duplicate and mean diameter for the inhibition zone was recorded[9].

Arginine hydrolysis

Ammonia production from arginine was tested by inoculating the isolates in arginine broth. After 24 h of incubation, an equal volume of inoculated arginine broth and Nessler's reagent were added in a test tube. Instant emergence of dark orange color indicated the

presence of ammonia due to arginine hydrolysis[22].

Amylase test

For detection of amylase activity, 24 h old cultures of the selected isolates were inoculated on MRS agar containing 0.25% starch and the plates were incubated for 48 h t 37°C. The zone of amylase activity was detected by flooding plates with Gram's iodine. Appearance of clear halo around the colony was indicative of amylolytic activity[21].

Phytase test

The phytase activity was determined in phytate agar plates containing calcium phytate (0.5%). Phytate agar plates were inoculated with active cultures and incubated at 37°C for 48 h. Presence of clear zone around the colony was suggestive of phytase activity of the isolate[1].

Protease test

To detect protease activity the isolates were grown in MRS broth and were inoculated on skim milk agar plates and incubated for 48 h. Afterwards, plates were observed for the appearance of clear zone around the colony indicating the positive protease activity[21].

Lipase test

Lipase activity was examined on MRS agar plates containing 1% trybutyrin. 24 h old culture of the isolate was inoculated on the plate and incubated for 48 h. Following which, the plates were observed for clear zone around the colony, which is indicative of positive lipase activity[1].

Gelatinase test

For testing the gelatinase activity of the isolates method described by Thakkar *et al.*[22], was used with slight modification. Nutrient gelatin agar plates were inoculated with 6 h old culture of the isolates. The plates were incubated at 37°C for 48 h, were flooded with saturated solution of ammonium sulphate and then examined for clear zones around the colonies.

RESULTS AND DISCUSSION

Isolation and identification of *Lactobacillus* species

Colonies with distinct morphology were successfully isolated by serial dilution method on MRS agar plates. Colony characteristics of each isolate were studied by obtaining pure culture of each isolate by streak plate method. The colonies were oval or circular, creamy white, smooth, glistening, convex with entire margins. Microscopically, the isolates were found out to be gram positive and endospore negative rods (Fig. 1). Biochemically, isolates showed negative results for catalase and oxidase tests. On the basis of morphological and biochemical characterization, the isolates were identified as *Lactobacillus* species[8]. Similar findings were reported by Kumar *et al.*[13], and Chakraborty *et al.*[2]. 7 strains namely *4A, 20A, 21C, 47A, 100A, 101A* and *102A,* which gave positive results for Gram's staining and negative results for endospore staining, catalase and oxidase tests, were selected for further tests. Table 1 enlists their distinguishing characteristics.

Table 1: Physiological and biochemical characterization of the isolated *Lactobacillus* strains

Strain No.	Gram's staining Characteristics	Endospore Staining Characterisctics	Catalase test	Oxidase test
4A	+ve rods	-ve	-ve	-ve
20A	+ve rods	-ve	-ve	-ve
21C	+ve rods	-ve	-ve	-ve
47A	+ve rods	-ve	-ve	-ve
100A	+ve rods	-ve	-ve	-ve
101A	+ve rods	-ve	-ve	-ve
102A	+ve rods	-ve	-ve	-ve

Antimicrobial activity of the isolates

Antimicrobial activity is an important characteristic feature of probiotic bacteria. *Lactobacillus* isolates were tested for antagonistic effect against common pathogens like *Bacillus subtilis, Bacillus cereus, Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, E. coli* and yeast *Candida albicans* (Table 2) (Fig. 2). Three strains *4A, 21C* and 101A showed antimicrobial activity against maximum no. of pathogens. Damodharan *et al.*[3], and Yadav *et al.*[24], reported that *Lactobacillus* spp. isolated from various fermented dairy products showed varying level of antimicrobial activity against various pathogens. The antimicrobial activity of *Lactobacillus* strains is mainly attributed to the production of antimicrobial substances like bacteriocins, ethanol, hydrogen peroxide, diacetyl, biosurfactants and organic acids and helps in competitive exclusion of pathogen in the intestine[7].

Table 2: Antagonistic activity of *Lactobacillus* strains against various pathogens

Strain No.	Tested pathogenic strains with zone of diameter in mm						
	Bacillus subtilis	***Bacillus cereus***	***Staphylococcus aureus***	***Staphylococcus epidermidis***	***P. aeruginosa***	***E. coli***	***Candida albicans***
4A	++ 12 mm	+ 8 mm	++ 15 mm	-	+++ 17 mm	++ 14mm	++ 10 mm
20A	-	-	++ 11 mm	+++ 16 mm	-	-	++ 5 mm
21C	+ 9 mm	-	++ 12 mm	+ 8 mm	-	++ 11 mm	++ 12 mm
47A	++ 13 mm	-	-	++ 10 mm	-	+ 6mm	15 mm +++
100A	-	+ 8 mm	-	-	+ 9 mm	++13mm	-
101A	+ 9 mm	++ 10 mm	+6 mm	+ 7 mm	-	-	12 mm ++
102A	-	+ 9 mm	+ 7 mm	-	-	-	-

+++ diameter of zone of inhibition between 15-20 mm, ++ diameter of zone of inhibition between 10-15 mm, + diameter of zone of inhibition less than 10 mm, - no effect detected.

Arginine hydrolysis

In current study, three strains namely *20A, 100A* and *102A* out of seven were able to hydrolyze arginine (Table 3). Spano *et al.*[20], have reported that *Lactobacillus* vary in their ability to degrade arginine and those able to derive energy from arginine catabolism may be more competitive in the stressful environment of wine (presence of acid and alcohol) than those strains unable to degrade arginine[14].

Amylase test

Ability to produce β-galactosidase is highly preferred attribute of probiotics. Amylase producing *Lactobacillus* spp. may play important role in the gut of mammals like horse, pig, rabbit, chicken and human including infant by alleviating symptoms of lactose intolerance. Total 5 isolates among 7, namely *4A, 21C, 47A, 100A* and *101A* showed clear zone of amylase activity (Table 3). Tallapragada *et al.*[21], isolated amylase producing *Lactobacillus spp.* from different samples of milk, curd etc.

Phytase test

Phytase activity is an advantageous characteristic for probiotic bacteria especially when they are used as feed for monogastric animals like poultry, fish that are unable to digest dietary phytate. Further, phytase improves the feed or food quality by reducing the antinutritional effects of phytate-rich foods. Isolates *4A, 21C, 47A, 101A* and *102A* were found to be phytase producers as indicated by the halo zones surrounding the colonies (Table 3) (Fig. 3). The results are in agreement with the findings of Andrabi *et al.*[1].

Protease test

Proteolytic activity is an essential requirement in the production of good quality fermented dairy products as the peptides and amino acids produced have straight influence on the organoleptic properties of the products[4]. Only three isolates namely *20A, 101A* and *102A*

were considered protease positive because of clear zone of milk hydrolysis around the colonies (Table 3). Similar results were reported by Thakkar *et al.*[22], where they isolated protease producing *Lactobacillus* spp. from different fermented food samples.

Lipase test

Lipolytic activity leads to the production of free fatty acids which impart characteristic flavor and aroma to some dairy products[4]. Isolates *20A*, *21C*, *47A* and *100A* showed halo zones surrounding the colonies indicating presence of production of extracellular lipase enzyme (Table 3). This is in concurrence with the findings of Andrabi *et al.*[1], who reported lipase activity in some of their strains.

Gelatinase test

Gelatinase activity of bacteria helps them in damaging mucoid lining and thereby causing damage to the underlying tissue. Hence, absence of gelatinase activity is an important criterion for determining the non-pathogenic nature of the isolates[5]. None of the isolates showed zone around the colony except *101A* thereby indicating its positive gelatinase activity and pathogenic nature (Table 3). In a similar study, a species of *Lactobacillus* isolated from curd was found to be gelatinase-negative[2].

Table 3: Extracellular enzyme producing ability of the isolated *Lactobacillus* strains

Strain No.	Arginine hydrolysis	Amylase test	Phytase test	Protease test	Lipase test	Gelatinase test
4A	-ve	+ve	+ve	-ve	-ve	-ve
20A	+ve	-ve	-ve	+ve	+ve	-ve
21C	-ve	+ve	+ve	-ve	+ve	-ve
47A	-ve	+ve	+ve	-ve	+ve	-ve
100A	+ve	+ve	-ve	-ve	+ve	-ve
101A	-ve	+ve	+ve	+ve	-ve	+ve
102A	+ve	-ve	+ve	+ve	-ve	-ve

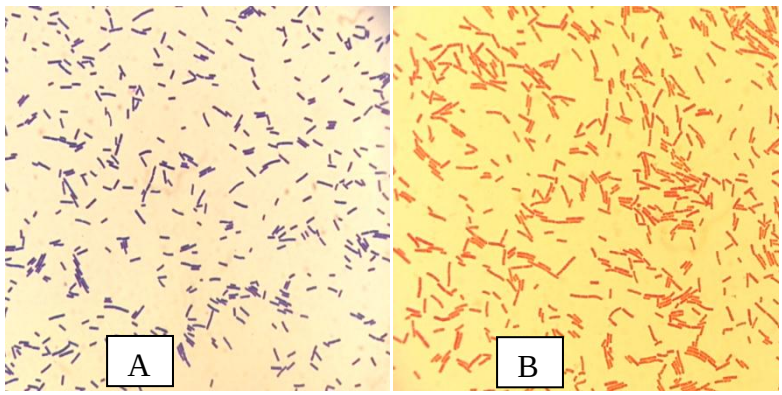

Fig. 1: (a) Microscopic view of the isolate *4A* after Gram's staining (b) endospore staining

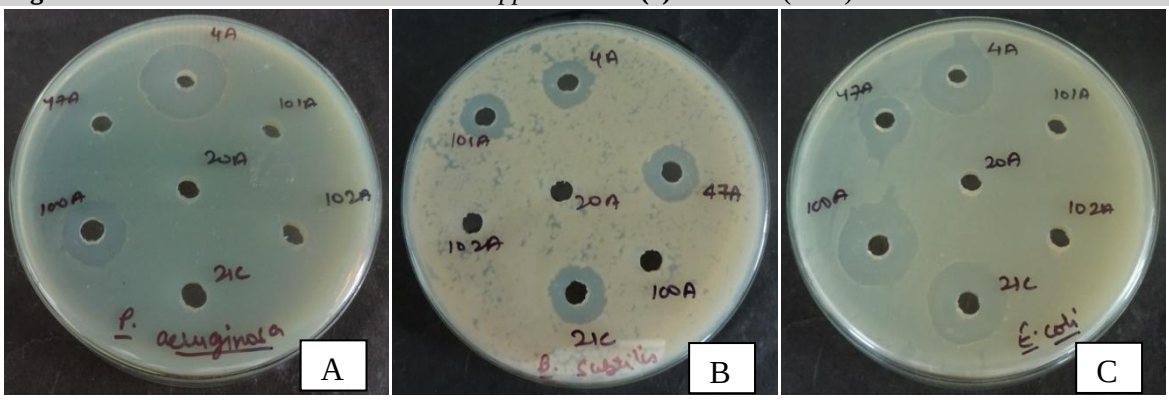

Fig. 2: Antagonistic spectrum of *Lactobacillus* strains against (a) *Pseudomonas aeruginosa* (b) *Bacillus subtilis* (c) *E. coli*

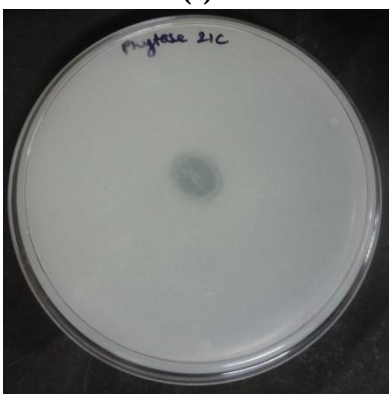

Fig. 3: Phytase activity of *21C*

CONCLUSION

The present study comprehensively documented the isolation and screening of seven *Lactobacillus* spp. from indigenous fermented food products. Probiotics candidates are supposed to have antagonistic activity against pathogenic microorganisms. This study revealed that isolate 4A has highest antimicrobial activity followed by *21C* and *101A*. Ability to produce a variety of hydrolytic enzymes like phytase, lipase, amylase and protease is an advantageous attribute of probiotics because these enzymes play important role in nutrients digestion and hence improving overall health of humans, animals and birds. *4A* was found positive for production of amylase and phytase. *21C* showed positive results for amylase, phytase and lipase activity. *101A* gave positive results for amylase, phytase, protease and gelatinase production. Moreover, *4A* and *21C* were found out to be gelatinase negative, indicating their non-pathogenic nature. Strains *4A* and *21C* may be regarded as good candidate for in *vivo studies* and can be further explored to validate their possible health benefits and applications as probiotics.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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