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Screening of lactic acid bacteria from unconventional sources

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Abstract
In the present study, unconventional sources were selected for isolation of LAB. The selected sample was apple, pomegranate and tomato. Isolation was done by following conventional method of isolation, followed by morphological and biochemical characterization. The isolates were Gram-positive, cocco-bacilli in shape and catalase-negative. Further molecular characterization of isolated was done by 16s rRNA sequencing and isolates were confirmed as Lactobacillus acidophilus in molecular characterization.

Keywords: Isolation, lactic acid bacteria, *Lactobacillus acidophilus*, Unconventional, 16s rRNA

Introduction
Microorganisms play a very important role in dairy industry. One of the most important groups of acid producing bacteria in dairy industry is the Lactic Acid Bacteria (LAB). LAB is a group of bacteria that are Gram-positive, non-spore forming, cocci/rod shaped, catalase-negative and fastidious. These are naturally occurring bacteria and categorized as 'Generally Recognized as Safe' (GRAS) as it is non-pathogenic to both humans and animals (Patil *et al.*, 2010 and Raisagar and Shukla, 2022) [18, 21]. LAB are used as starter culture as well as preservatives and probiotics in both food and dairy industries. (Amin *et al.*, 2009 and Kumar and Kumar 2014) [1, 14]. It is aprroved by FAO and WHO that the consumption of probiotics in adequate amounts confers a health benefit on the host (FAO/WHO, 2002) [7]. LAB causes fermentation of foodstuffs and food is the most widely studied environment and favored niche for growth and multiplication of lactic acid bacteria (De Filippis *et al.*, 2020) [5]. Commercial available LAB which is routinely used for human consumption is obtained from traditional or conventional sources. The conventional sources of LAB are dairy products, human feces and human breast milk (Sornplang and Piyadeatsoontorn, 2016) [24] but the consumption of these products as probiotics can be limited by the growing popularity of vegetarianism, high number of individuals with lactose intolerance, individuals with cholesterol-restricted diets, and economic and social conditions (Granato *et al.*, 2010) [10]. Thus, the isolation of LAB from other sources *i.e.*, unconventional sources such as fruits, vegetables and their by-products may used as an alternative of LAB (Peres *et al.*, 2012) [19]. High carbohydrate content, availability of nutrient and acidic environment of fruits and vegetables favours growth of LAB.
Taking into consideration the importance of screening of LAB, the present study aimed at isolation and identification of LAB from selected unconventional sources. The present work will act as foundation work to identified different unconventional sources of LAB and also proves that conventional sources are not the only one to isolate Lactic acid bacteria, but unconventional sources to isolate Lactic acid bacteria are equally potent.

Methodology
Isolation of Lactic acid bacteria (LAB)
For isolation of LAB, unconventional sources *i.e.*, apple, pomegranate and tomato were collected from 5 different localities of Prayagraj. The samples were placed in sterile bags and transported to the laboratory.1g of each sample was taken aseptically and subjected to 10-fold dilution; 0.1 ml of diluted sample was inoculated on MRS agar plates under anaerobic condition and incubated at 37 °C for 24 to 48 hours. The different morphological colonies were isolated and pure cultures were maintained in MRS agar slant at 4° temperature.

Morphological characterization of isolates

For Morphological characterization, Cultural identification and microscopic observation was performed. Cultural characterization of LAB isolates was done on different agar plates. Cultural characteristics *i.e.*, colony colour, margin, form, surface, elevation and optical density were recorded. Microscopic observation was done by Gram's staining. To perform Gram staining, thin smear was prepared on a clear dry slide by heat fixing and staining was done by flooding with Gram's Crystal Violet followed by Gram's Iodine, Gram's Decolorizer and Safranin. After washing and air drying, slide was examined under oil immersion objective for Gram's reaction, cell shape and arrangements. (Aneja, 2003) [2].

Biochemical Characterization of isolates

The isolates were biochemically characterized using tests namely, oxidase test, catalase test, citrate utilization test, Vogus-Proskaur test, methyl red test, nitrate reduction test, urease test and sugars fermentation (Cappuccino and Sherman, 2005) [3].

a. **Oxidase test:** A loop full lactic acid bacterial culture picked from an 18 to 24-hour old culture plate and rub onto a filter paper. Then add a drop of 1% oxidase reagent on the culture. Observe for colour changes. Development of dark bluish- purple within 5 to 10 seconds showed a positive oxidase test (Aneja, 2003) [2].
b. **Catalase test:** A microscope slide was placed inside a petri dish. Using a sterile inoculating loop isolate was collected from an 18- to 24-hour old colony and placed onto the microscope slide. Then a drop of 3% H_2O_2 was added onto the culture onto the slide and immediately the Petri dish was covered with a lid and observed for immediate bubble formation. The formation of bubble showed positive catalase test (Aneja, 2003) [2].
c. **Methyl Red test:** Glucose phosphate broth was inoculated with LAB isolates and incubated at 30 °C for 48 to 72 hrs. 5 drops of Methyl red reagent was added to the broth. Red colour development indicated the positive result and yellow colour showed negative result (Aneja, 2003) [2].
d. **Vogus-Proskaur test:** Glucose phosphate broth was inoculated with LAB isolates and incubated for 24 hrs. at 30 °C. 10 drops of VP reagent A followed by 10 drops of VP reagent B was added. The tube was shake gently to expose the medium to atmospheric oxygen and allowed the tube to remain undisturbed for 10 to 15 min. Pinkish red colour development at the surface of the medium showed positive and yellow colour showed negative VP test (Aneja, 2003) [2].
e. **Citrate test:** For Citrate test Simmons Citrate agar slants was used. The slants were inoculated with LAB isolates and incubated for 24 - 48 hours at 30 °C. Observed the slants for colour change. Blue colour of the slants showed positive Citrate test. The citrate negative slants were remains green in colour (Aneja, 2003) [2].
f. **Nitrate reduction test:** Nitrate broth was inoculated with LAB isolates and incubated for 24-48 hours at 30 ºC. After the incubation, 5 drops of nitrate reagent A and B was added and shake gently to mix the reagents. A positive nitrate reduction was denoted by the appearance of a deep red colour change after the addition of nitrate reagents A and B. Lack of colour development denotes a presumptive negative nitrite reduction test. Then nitrate reagent C was added to confirm the nitrate reduction. Development of red colour showed negative reduction test and lack of colour showed positive reduction test (Aneja, 2003) [2].
g. **Urease test:** LAB isolates were tested for the urease test in urease broth. Inoculated a loopful culture of isolates on urease broth and incubated for 24 hours at 30 °C. Development of pink colour was a positive test for urease and development of yellow colour showed negative urease test (Aneja, 2003) [2].
h. **Sugar fermentation test:** Sugar fermentation broth with phenol red indicator was prepared using different sugar *i.e.*, arabinose, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, ribose and sucrose. After sterilization, the broth was inoculated with LAB isolates and incubated for 24 hrs. at 30 °C. Positive result was yellow after incubation and no colour change /remains reddish was negative fermentation test (Aneja, 2003) [2].

Molecular identification

Isolates were identified at the molecular level in Scangene Labs Pvt. Ltd., Delhi. Molecular identification was done by the Sanger sequencing method. The steps involved in this method were Isolation of Genomic DNA followed by Agarose Gel Electrophoresis, Genomic DNA Quantitation, Amplification of partial 16srRNA by PCR, Gel Purification of amplified product and Automated sequencing of DNA clones.

Results and Discussions

Isolation of lactic Acid Bacteria

For the isolation of lactic acid bacteria, sample of unconventional sources namely apple, pomegranate and tomato were selected. 50 samples in each category were collected from five different localities of Prayagraj city *i.e.*, Naini, Civil lines, Rambagh, Mahewa and Khan choraha. A total of 132 lactic acid bacteria were isolated from 150 samples in which 55 isolates (42%) were isolated from apple samples, 43 isolates (32%) were isolated from pomegranate samples and 34 isolates (26%) were isolated from tomato samples (Figure 1; Table 1). In previous studies, isolation of LAB from unconventional sources were also reported by Hamet *et al.*, 2013 [11]; Tajabadi *et al.*, 2013 [26] and Chen *et al.*, 2005 [4] from grains, honey-comb and soil, respectively. In a study conducted by Naeem *et al.*, 2012 [17] also showed presence of *Lactobacillus plantarum* in fruit juice and *Leuconostoc mesenteroides* in tomatoes. Another study done by Siddiqee *et al.*, 2013 [23] also screened and found LAB in fruit juice, flesh, long grass and vegetables. Junnarkar *et al.*, 2018 [13] also isolated LAB from 3 different unconventional sources, *viz*., plants, fermented foods and beverages, and human feces. Junnarkar *et al.*, 2019 [12] also isolated LAB from vegetable samples including tomato. Ruiz Rodríguez *et al.*, 2019 [22] also reported isolation of LAB from different flowers and fruit sample. In the present study, de Mann Rogosa Sharpe agar was used as selective media for isolation of LAB. Di Cagno *et al.*, 2009 [6] also used deMana Rogosa and Sharpe broth as a standard medium for isolation of LAB from fruit sample.

Table 1: Isolation of LAB isolates

Total No. of Samples (n=150)	No. of isolated LAB
Apple	55
Pomegranate	43
Tomato	34
Total	132

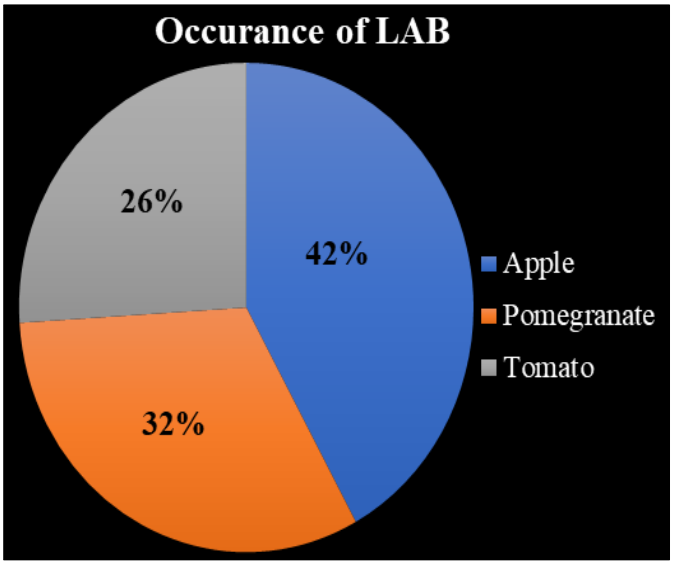

Fig 1: Isolation of LAB isolates

Identification of LAB isolates

Morphological Characterization of LAB isolates: All the isolates were identified by morphological characterization on nutrient agar media, de Mann Rogosa Sharpe agar and blood agar media plate. The parameters used were colony color, elevation, margin, optical density, surface, colony form and microscopic observations (Gram's reaction, shape and arrangement of cells).LAB isolates were found off white, creamy white and gray on Nutrient agar media plate, de Mann Rogosa Sharpe agar and blood agar media plate, respectively. In all the plates, entire margin, circular colony form, convex elevation, opaque optical density and smooth glistening surface was observed. In microscopic observation, all the isolates showed positive Gram's reaction. The cells were rod with rounded ends (Coccobacilli) and arranged in short chains (Table 2).The current findings are in agreement with the report of Goa *et al.*, 2022 [9] who have found LAB in creamy colored colonies in MRS agar plate and Gram-positive cocci and rod-shaped bacteria in microscopic observations. Pyar and Peh, 2014 [20] also identified isolates as *Lactobacillus acidophilus* by observing gram-positive, rod-shaped coccobacilli, in chains. In another study, Linares-Morales *et al.*, 2020 [15] also identified LAB isolated from unconventional sources by white, opaque, small and convex colony characteristic and found Gram-positive in microscopic observation. Occurrence of Gram-positive LAB isolates in vegetables samples was also confirmed by Junnarkar *et al.*, 2019 [12].

Table 2: Morphological Characterization of LAB isolates

Morphological Characters	LAB isolates		
	On Nutrient agar Plate	On de Mann Rogosa sharp Agar Plate	On Blood agar Plate
Colony Colour	Off white	Creamy white	Gray
Colony margins	Entire		
Colony form	Circular		
Elevation	Convex		
Optical density	Opaque		
Surface	Smooth and glistening		
Microscopic observation			
Gram reaction	Gram Positive		
Cell Shape	coccobacilli		
Cell Arrangement	Short chain		

Biochemical Characterization of LAB isolates

After morphological characterization, all the isolates were characterized by using the following biochemical tests: oxidase test, catalase test, Methyl red, Voges-Proskauer test, Citrate test, Nitrate reduction test, Urease test, and carbohydrate fermentation tests (arabinose, fructose, Galactose glucose, lactose, maltose, mannitol, Mannose, raffinose, Ribose and sucrose).The main task of sugar fermentation test is to investigate the ability of bacteria to ferment different types of sugar. Phenol red broth base medium was used as an indicator to differentiate the bacteria according to their patterns of sugar utilization. In the present study, all the isolates were negative for oxidase, catalase, methyl red, Voges-Proskauer, Citrate, Nitrate reduction and urease test. In sugar fermentation test, isolates showed positive for arabinose, fructose, galactose, glucose, lactose, maltose, mannose and sucrose whereas for sugar mannitol and ribose, the isolates showed negative fermentation test (Table 3). The current findings are supported by Goa *et al.*, 2022 [9] who also found catalase and citrate negative and glucose fermentation positive for LAB isolates. Mac Faddin, 2000 [16] and Linares-Morales *et al.*, 2020 [15] also found LAB as catalase-negative in biochemical characterization. Pyar and Peh, 2014 [20] also confirmed negative for catalase and positive for glucose, maltose, lactose and sucrose in *Lactobacillus acidophilus.* Junnarkar *et al.*, 2019 [12] also confirmed catalase and oxidase negative LAB isolates in vegetable samples.

Table 3: Biochemical Characterization of LAB isolates

Biochemical Test	LAB isolates
Oxidase	Negative
Catalase	Negative
Methyl red	Negative
Voges-Proskauer	Negative
Citrate	Negative
Nitrate reduction	Negative
Urease	Negative
Sugar fermentation test	
Arabinose	Positive
Fructose	Positive
Galactose	Positive
Glucose	Positive
Lactose	Positive
Maltose	Positive
Mannitol	Negative
Mannose	Positive
Ribose	Negative
Sucrose	Positive

Molecular identification of isolates

Sequence analysis of the 16S rRNA gene is the most often used method for genotypic identification. This method is based on the presence of specific variable regions of each bacterial species, with subsequent comparison to references from public domain databases, such as Gene Bank. In the present study, after the morphological and biochemical confirmation, isolates were selected for molecular characterization and identified as *Lactobacillus acidophilus* by 16S rRNA sequencing (Figure 2). Earlier, Garcia *et al.* 2016 [8]; Strejcek *et al.*, 2018 [25]; Junnarkar *et al.*, 2019 [12] and Ruiz Rodríguez *et al.*, 2019 [22] also used 16S rRNA sequencing method for identification of *Lactobacillus*.

ATGAGTGCTAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGC
ACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCC
CGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAG
GTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGA
CAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
ACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACT
GCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATG
ACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAG
GTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTG
CGTGAAGCTGGAATCGCTAG TAATCGCGGATCAGCATGCCGCGGTGAATAC GT

Fig 2: Partial 16SrRNA sequencing of *Lactobacillus acidophilus*

Conclusions

In the present study, a total number of 132 Lactic acid bacterial isolates were isolated from selected unconventional sources. The isolates were identified as *Lactobacillus acidophilus* by morphological, biochemical, and molecular characterization. The presence of LAB in unconventional sources is proved as an alternative source of LAB and these LAB are being selected as probiotics for lactose intolerant people. Thus, further study is required to evaluate the probiotic potential and antimicrobial activity of isolated LAB for use as probiotics with different applications.

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