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MOLECULAR CHARACTERIZATION AND DETERMINATION OF ANTIMICROBIAL POTENTIAL OF PROBIOTIC BACTERIA ISOLATED FROM TRADITIONAL DAIRY AND COMMERCIAL PRODUCTS

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ABSTRACT

The objective of the present study was to isolate, identify, and characterize probiotic Lactic acid bacterial strains from traditional dairy and commercial products and determine their antagonistic activity against pathogenic bacteria. Fifty-five lactic acid bacterial (LAB) strains were isolated, and fifteen strains were selected based on probiotic potential after that antimicrobial activity was measured against bacterial pathogens. Among fifteen isolates, five isolates showed good (above 15 mm zone) antimicrobial activities against the tested pathogenic strains of humans. After the preliminary antimicrobial activity, the best five isolates were selected for the production of different antibacterial compounds such as bacteriocin, hydrogen peroxide, and organic acid. Two isolates showed bacteriocin production and one isolate gave maximum bacteriocin activity against most pathogens and was further identified as *Enterococcus faecalis* JY32 using 16 S rRNA sequencing. The study reveals that in comparison to commercial products, traditional products of dairy could be an alternative and readily available resource for probiotic bacteria with interesting functional characteristics.

Keywords: Antimicrobial, Lactic acid bacteria, Pathogens, Probiotic

INTRODUCTION

The term probiotic originated from the Greek word "biotikos," which means "for life," and is defined as "living microorganisms that when administered in a specified amount provide health advantage to the host" **(Gaspar *et al.*, 2018)**. Probiotic food is believed to improve the quality of life. Probiotic bacteria are non-pathogenic microorganisms that can tolerate gastric environments such as low pH and the presence of bile salts. They produce lactic acid which inhibits the growth of pathogenic microorganisms **(Murry *et al.*, 2004)**. The main sources of probiotics are traditional dairy products such as butter, cheese, yogurt, and milk **(Prabhurajeshwar and Chandrakanth, 2019)**. Other sources include commercially manufactured products supplemented with specific probiotic microflora. Such probiotic bacteria produce a variety of antimicrobial agents i.e. diacetyl, acetoin, organic acids, hydrogen peroxide, and bacteriocin which are responsible for the inhibition of pathogenic microorganisms. Some of the common infections of the human body such as bowel syndrome, UTI, and antibiotic-associated diarrhea could repetitively occur due to a lack of good intestinal microflora. Therefore, the consumption of probiotics daily is a natural way to treat and control the harmful infections (**Guarner *et al.*, 2005**). *Lactobacillus* (**Halder *et al.*, 2017**), *Streptococcus* (**Ferrer *et al.*, 2020**), *Bifidobacterium* (**Lukjancenko *et al.*, 2012**), and Enterococcus (**Guarner *et al.*, 2012**) are some examples of commonly exploited and used probiotic bacteria. Nowadays the genera Enterococcus has gained significant attention, particularly in the field of environmental and food research (Sharma et al. 2012). There are numerous species in the Enterococcus genus, but only a small number of these are known to be probiotics, including *E. faecalis*, *E. faecium*, and *E. lactis*. In the food industry, Enterococci have been utilized as starter cultures or as probiotics (**Franz *et al.*, 2003**). These microorganisms are capable of synthesizing useful antimicrobial peptides like bacteriocin (**Franz *et al.*, 2007**). Bacteriocin are a type of ribosomal synthesized low molecular weight antimicrobial peptides that inhibit the emergence of a variety of bacterial infections (**Ahmadova *et al.*, 2013**). Further, it has been observed that most of the pathogenic bacteria have become resistant to antibiotics due to their indiscriminate consumption. The probiotic bacteria are capable of producing numerous antimicrobial peptides and hence, these can be utilized as an effective alternative to the synthetic drugs that may ease the burden of drug resistance. The present investigation was conducted to identify and characterize potential probiotic lactic acid bacteria from traditional dairy and commercial products which was further tested for their antagonistic activity against pathogenic bacterial strains.

MATERIALS AND METHODS

Collection of sample and Preservation

12 Different traditional dairy samples of paneer, curd, milk, and butter were collected based on their popularity among consumers in Dehradun, India. All samples were collected in clean and sterilized sample containers for serial dilution. Five commercial probiotic products such as probiotic health drinks, probiotic curd, two different probiotic tablets, and Greek yogurt were purchased from the market and stored in the refrigerator for serial dilution. Approx. 1 gm sample was suspended in 9 ml of sterile 0.86% normal saline, and serially diluted. Each sample was incubated on MRS agar media at 37 °C for 18–24 hours.

Primary screening and isolation of Lactic acid bacteria (LAB)

The spread plate technique was used to isolate the organisms.1 ml of the sample was serially diluted to 10^{-1} to 10^{-9} using distilled water and plated on MRS (Man, Rogosa, and Sharpe) agar. All the samples were incubated at 37°C for 24-48 hours under anaerobic conditions. Each batch of samples was run along with a control plate using MRS media. The individual colonies were selected and purified by the streak plate method on MRS agar. For additional experimental work, the selected bacterial colonies were kept on MRS agar slants at 4ºC.

Morphological and Biochemical characterization of Lactic acid bacteria

Isolates were identified by morphological and cultural techniques. Catalase test, simple staining and Gram staining were used for morphological identification. Gram positive and catalase negative rods were chosen for biochemical testing, which includes oxidase test, Methyl Red (MR), Voges Proskauer (VP), Indole, Citrate utilization, Carbohydrate fermentation, and 0.4% agar motility test. All the strains were further preserved in MRS broth with glycerol (30%) at −20 °C for assessment of probiotic potential.

Carbohydrate fermentation

Overnight grown culture of bacteria was inoculated separately into sterilized sugars (dextrose, lactose, sorbitol, and xylose) fermentation broth (peptone-10 g, sodium chloride-15 g, phenol red dye-0.018 g, sugar-5 g and distilled water-1L, pH 7.0) and incubated at 37ºC for 24-48 hours. The acid production by bacterial cultures was observed based on the change in color from red to yellow and gas production (formation of a gas bubble in the Durham tube) during fermentation **(Ahmed and Kanwal, 2004)**.

Hemolytic activity

Overnight grown culture of each isolate was transferred as a spot onto Blood Agar plates containing 10% of fresh human blood and incubated at 37ºC for 24 h. The zone of hemolysis around colonies was measured. Staphylococcus aureus was taken as a positive control **(Harrigan *et al*., 1998)**.

Tolerance of pH

Cells from each isolate were sub-cultured in MRS broth at 1% (v/v) with pH ranging from 2, 4, 6, and 8, and were then incubated at 37°C for 24 hours to determine the best growth of each LAB isolate at each pH level. The ability of LAB isolates to thrive at various pH levels was then observed by measuring the absorbance at 600 nm of bacteria versus un-inoculated broth using a spectrophotometer **(Turgay and Erbilir, 2006)**.

Tolerance of Bile salt

The strains' tolerance to bile salts was assessed using a modified version of Gilliland et al (1984) methodology. This test displays the best growth after individually inoculating several isolates into MRS broth tubes with 0.3% bile salts. Bacterial growth was evaluated using a 600 nm absorbance measurement following a 24-hour incubation period at 37 °C. MRS broth devoid of bile salt was utilized as the experiment's control **(Gilliland *et al*., 1985)**.

Tolerance of different salt concentration

All the isolates were cultured in MRS broth at various NaCl concentrations, such as 1%, 3%, 5%, and 7%, in order to test salt tolerance. 10 µl of an overnight culture of each isolate was added to the broth, which was then incubated anaerobically at 37 °C for 18 to 24 hours. Each isolate's optical density was calculated using absorbance at 600 nm. The control was MRS broth devoid of NaCl **(Gardini *et al*., 2001)**.

Antibiotic susceptibility test

The disc diffusion assay was used to perform antibiotic susceptibility tests. The procedure was first standardized in accordance with ISO 10932/IDF 233 standards with a few modifications. The agar plates were swabbed with test cultures and then discs of the antibiotics were placed on it. The antibiotic discs procured from Hi Media, India were used and which includes Norfloxacin (10 µg), Chloramphenicol (30 µg), Imipenem (30 µg), Gentamicin (10 µg), Erythromycin (15 µg), Nalidixic acid (30 µg), Amikacin (30 µg), Ceftriaxone (30 µg), Trimethoprim (5 µg), Nitrofurantoin (200 µg). After 24 hours of incubation at 37°C, the inhibition zone was measured **(Prabhurajeshwar and Chandrakanth, 2019)**.

Antimicrobial activity of LAB isolates against pathogenic bacteria

Agar well diffusion method was used for assessment of antimicrobial method of the selected bacterial isolates against some pathogenic MTCC culture. Five bacteria (MTCC8911) *Klebsiella pneumonia,* (MTCC736) *Bacillus subtilus,* (MTCC8076) *Pseudomonas aeruginosa*, (MTCC7443) Staphylococcus aureus, and (MTCC11451) *Acinetobacter baumannii* were procured from Institute of Microbial Technology (IMTECH), Chandigarh (India) and used as test pathogens. 100 micro liter of supernatant (without cells) of each LAB isolate was poured in 6 mm diameter well in nutrient agar containing test pathogens. Zone of inhibition was measured against test pathogens after 24 hours of incubation. The experiment was performed in triplicate **(Samedi and Charles, 2019)**.

Determination of antimicrobial substances

The generation of antimicrobial compounds like organic acids, bacteriocin, and hydrogen peroxide was further investigated in probiotic isolates with possible antibacterial action **(Toure *et al*., 2003)**. For separate investigations, the MRS broth of each isolate was split into three equal fractions. Trypsin (1 mg/ml) was added to one fraction of the supernatant to measure the activity of the bacteriocin, 1 N NaOH was used to adjust another fraction to pH 6.5 0.1 to measure the production of organic acids, and 5 ml of supernatant was treated with catalase reagent (0.5 mg/ml) to measure the activity of the hydrogen peroxide. After treatment, 0.22 mm pore-size filters were used to filter the treated supernatants. In 6 mm diameter wells, 50–100 micro liter of each supernatant was added, and 1% (v/v) overnight-grown cultures of each test pathogen were added to the plates as inoculants. After 24 hours of incubation at 37 °C, inhibitory effect was seen, and the zone of inhibition was recorded.

Molecular identification by 16s r RNA

In this work, the best bacterial strain exhibiting antimicrobial activity with bacteriocin activity was identified and confirmed using 16S r RNA sequencing as a technique. For identifying unknown sequences at the molecular level, this approach is quick and accurate. An isolated LAB sequence was commercially sequenced at Biokart laboratory, Bangalore, India.

RESULTS

Isolation and identification of isolated Lactic acid bacteria

12 Different traditional dairy samples were collected from the local dairy and 5 commercial probiotic products were purchased from a local market. Small white shiny colonies of Lactic acid bacteria on MRS agar plate (Figure 1) were selected. Total 55 strains of lactic acid bacteria were isolated (Table 1) and subjected to morphological identification. 15 (27.27%) gram positive and catalase negative bacilli were selected for biochemical characterization (Table 2). The isolates were named ISO 2, ISO 4, ISO 6, ISO 13, ISO 14, ISO 27, ISO 29, ISO 30, ISO 31, ISO 41, ISO 42, ISO 45, ISO 47, ISO 51, and ISO 52. These isolates were grown on MRS in broth containing 30% glycerol and kept at 20 °C.

Table 1 Origin and Number of isolates from traditional and commercial dairy products

Source of traditional dairy products	**No isolates**	**No of catalase negative Rod shaped Lactic acid bacteria**
Milk	6	**2**
Curd	11	**1**
Paneer	16	**5**
Butter	6	**2**
Commercial Probiotic Products	**No isolates**	**No of catalase negative Rod shaped Lactic acid bacteria**
Probiotic drink	1	**1**
Probiotic curd	2	**1**
Probiotic Tablet 1D	4	**2**
Greek yogurt	2	**1**
Probiotic Tablet 2V	7	**0**

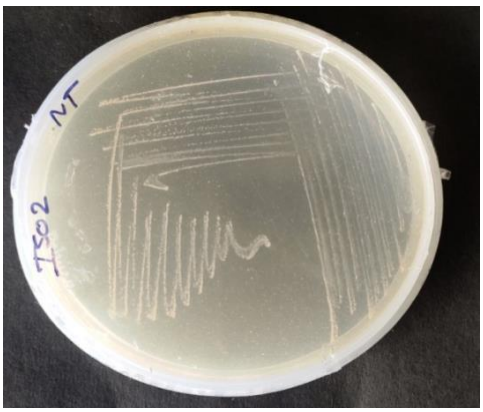

Figure 1 Colonies of Lactic acid bacteria on MRS Agar

Table 2 Morphological, Biochemical, and Cultural characteristics of isolated LAB (Lactic acid Bacteria) from traditional and commercial probiotic samples.

Sr.No.	Selected isolates	Gram's staining	Morphological & culture characteristics	Motility Test	Catalase	MR	VP	Citrate	Oxidase	indole
1	ISO 2	Gram (+ve) Bacilli	Small ,0.5mm Circular and Creamy	Non -Motile	(-ve)	(+ve)	(-ve)	(-ve)	(-ve)	(-ve)
2	ISO 4	Gram (+ve) Bacilli	1mm,Shiny,White Powdery Round	Non -Motile	(-ve)	(+ve)	(-ve)	(-ve)	(-ve)	(-ve)
3	ISO 6	Gram (+ve) Bacilli	Small ,0.5mm, Powdery Round	Non -Motile	(-ve)	(+ve)	(-ve)	(-ve)	(-ve)	(-ve)
4	ISO 13	Gram (+ve) Bacilli	0.5mm,Creamy Viscous White	Non -Motile	(-ve)	(+ve)	(-ve)	(-ve)	(-ve)	(-ve)
5	ISO 14	Gram (+ve) Bacilli	0.5mm,Creamy Viscous White	Non -Motile	(-ve)	(+ve)	(-ve)	(-ve)	(-ve)	(-ve)
6	ISO 27	Gram (+ve) Bacilli	1mm, Creamy, Round	Non -Motile	(-ve)	(+ve)	(-ve)	(-ve)	(-ve)	(-ve)
7	ISO 29	Gram (+ve) Bacilli	Small,0.5mm Creamy White &Circular	Non -Motile	(-ve)	(+ve)	(-ve)	(-ve)	(-ve)	(-ve)
8	ISO 30	Gram (+ve) Bacilli	0.5 to 1 mm, Viscous Creamy White	Non -Motile	(-ve)	(+ve)	(-ve)	(-ve)	(-ve)	(-ve)
9	ISO 31	Gram (+ve) Bacilli	Small, Circular, White	Non -Motile	(-ve)	(+ve)	(-ve)	(-ve)	(-ve)	(-ve)
10	ISO 41	Gram (+ve) Bacilli	1 mm, Irregular White	Non -Motile	(-ve)	(+ve)	(-ve)	(-ve)	(-ve)	(-ve)
11	ISO 42	Gram (+ve) Bacilli	1mm,Round,off -White	Non -Motile	(-ve)	(+ve)	(-ve)	(-ve)	(-ve)	(-ve)
12	ISO 45	Gram (+ve) Bacilli	0.5mm, Round, Powdery-White	Non -Motile	(-ve)	(+ve)	(-ve)	(-ve)	(-ve)	(-ve)
13	ISO 47	Gram (+ve) Bacilli	0.5mm, Small, Irregular White	Non -Motile	(-ve)	(+ve)	(-ve)	(-ve)	(-ve)	(-ve)
14	ISO 51	Gram (+ve) Bacilli	1mm, Round, Powdery White	Non -Motile	(-ve)	(+ve)	(-ve)	(-ve)	(-ve)	(-ve)
15	ISO 52	Gram (+ve) Bacilli	0.5 mm, Irregular, Powdery White	Non -Motile	(-ve)	(+ve)	(-ve)	(-ve)	(-ve)	(-ve)

MR- Methyl Red; VP-Voges Proskauer, Negative (–ve), Positive (+ve)

Carbohydrate Fermentation and gas production

All 15 isolates were inoculated with four different fermenting sugars dextrose, lactose, sorbitol, and xylose. Most of the isolates gave positive result for sugar fermentation, only isolate 42 showed gas production with xylose and sorbitol sugar after 24-48 hours of incubation (Table 3).

Table 3 Results of Carbohydrate fermentation tests of isolated Lactic acid bacteria

Sr.No	Name of isolate	Dextrose	Lactose	Sorbitol	Xylose
1	ISO 2	(+ve)	(+ve)	(+ve)	(+ve)
2	ISO 4	(-ve)	(+ve)	(+ve)	(+ve)
3	ISO 6	(+ve)	(+ve)	(+ve)	(+ve)
4	ISO 13	(+ve)	(+ve)	(+ve)	(+ve)
5	ISO 14	(+ve)	(+ve)	(-ve)	(-ve)
6	ISO 27	(+ve)	(+ve)	(-ve)	(+ve)
7	ISO 29	(+ve)	(+ve)	(-ve)	(-ve)
8	ISO 30	(+ve)	(+ve)	(-ve)	(-ve)
9	ISO 31	(+ve)	(+ve)	(-ve)	(-ve)
10	ISO 41	(+ve)	(+ve)	(-ve)	(-ve)
11	ISO 42	(-ve)	(-ve)	(+ve) with gas	(+ve) with gas
12	ISO 45	(-ve)	(+ve)	(-ve)	(-ve)
13	ISO 47	(+ve)	(+ve)	(+ve)	(-ve)
14	ISO 51	(-ve)	(-ve)	(-ve)	(-ve)
15	ISO 52	(+ve)	(+ve)	(+ve)	(-ve)

Positive (+ve) indicates acid production during carbohydrate fermentation, Negative (-ve) indicates no acid production and no utilization of sugar

Assessment of probiotic properties

Hemolytic activity

The selected Lactic acid bacteria were tested for hemolytic activity. After incubation of 24 hours no zone of hemolysis was observed around the colonies of selected isolates indicating non hemolytic characteristic of the isolated bacteria.

Determination of growth at different pH

Selected LAB isolates from traditional and commercial samples have shown maximum growth at pH 6. Most of the isolates tolerate 2 pH but the ISO 47 (isolated from homemade butter) showed maximum growth at pH 2. ISO 06, ISO31, and ISO47 showed maximum growth at pH 4 and ISO 41 isolated from paneer and ISO 52 isolated from Probiotic drink showed maximum growth at pH 8. The O.D (600nm) taken in triplicate samples was plotted against pH as shown in (Figure 2). All the samples were observed at 600 nm by spectrophotometer.

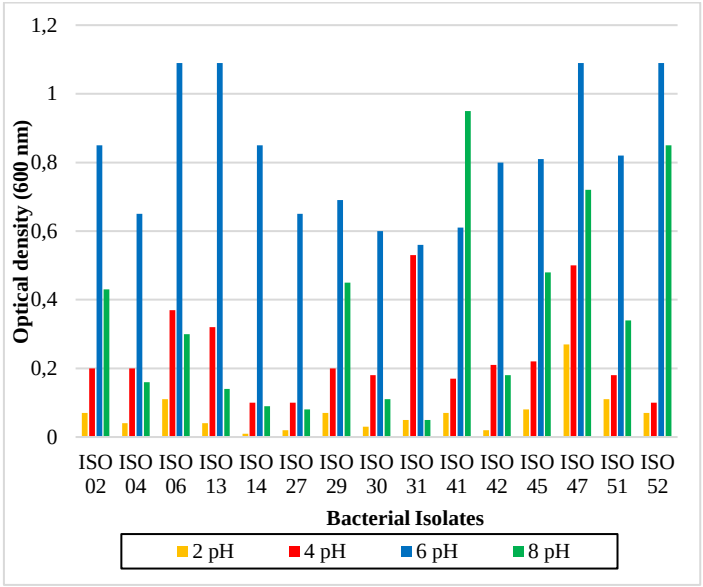

Figure 2 Growth of isolated LAB at different pH

Tolerance to bile salt

The selected LAB isolates were capable of surviving in 0.3% bile salt. At this concentration, growth of all LAB strain was monitored hourly till 8 hrs. ISO 6, ISO13, ISO 30, ISO 47, and ISO 52 showed maximum division rate during 8 hours of incubation (Figure 3). Growth of the samples was observed at 600 nm by spectrophotometer.

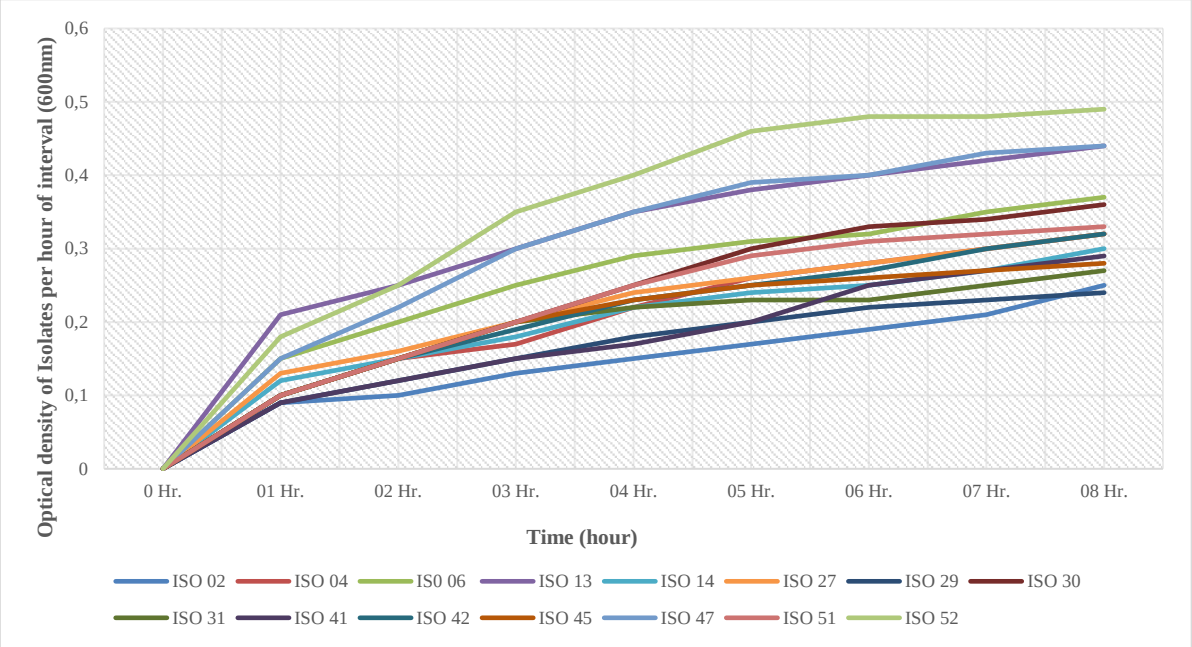

Figure 3 Growth of LAB isolates at 0.3% bile salt concentration.

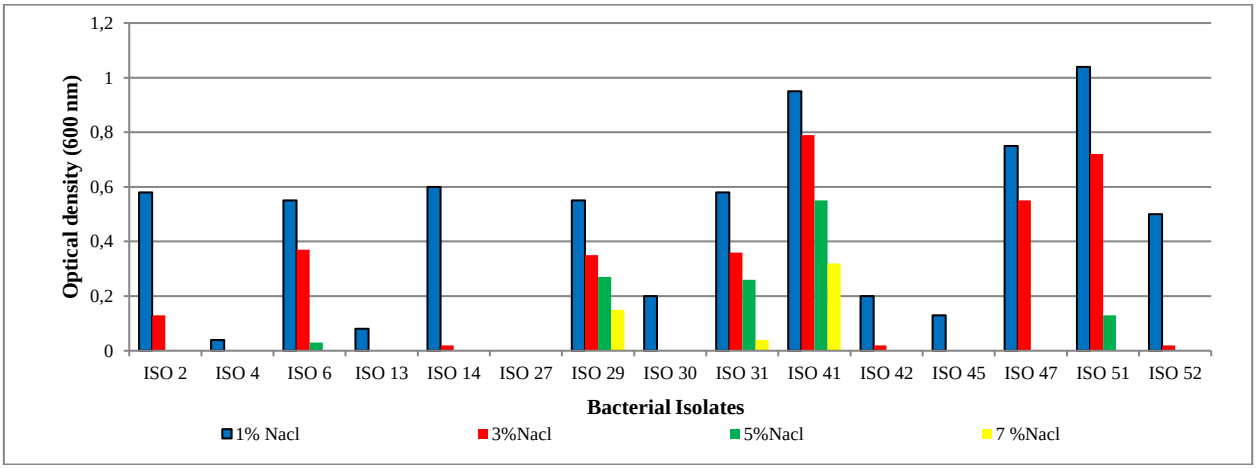

Figure 4 Growth of isolates against different salt concentration

Tolerance to NaCl

The selected LAB was tested for salt tolerance. All the isolates were grown at 1%, 3%, 5%, and 7% NaCl concentration. The growth was measured by taking O.D. at 600 nm as shown in (Figure 4)

Antibiotic sensitivity test

15 putative probiotic LAB strains that were positive were tested for antibiotic sensitivity against 10 different antibiotics from various groups. Isolated strains were found susceptible to Norfloxacin (10 µg), Chloramphenicol (30 µg), Ceftriaxone (30 mg), Imipenem (30 mg), Erythromycin (15 mg), Gentamicin (10 mg), Nalidixic acid (30 mg), Trimethoprim (5 mg), Nitrofurantoin (200 mg), and Amikacin (30 mg) (Table 4, Figure 5).

Table 4 Antibiotic susceptibility test result of 15 Lactic acid bacterial isolates against 10 antibiotics (zone of inhibition in mm ± SD)

Name of Isolates	Norfloxacin 10 (ug)	Amikacin 30 (mg)	Imipeme m 30 (mg)	Erythromy cin 15 (mg)	Chloramp henicol 30 (ug)	Gentamicin 10 (mg)	Nalidixic acid 30 (mg)	Ceftriaxone 30 (mg)	Trimethoprim 5 (mg)	Nitrofuran toin 200 (mg)
ISO 2	18±0.007	5±0.005	20±0.010	-	15±0.012	13±0.012	-	22±0.018	-	15±0.013
ISO4	16±0.012	5±0.015	18±0.014	-	18±0.012	12±0.010	-	25±0.011	20±0.013	-
ISO 6	15±0.014	8±0.008	15±0.012	-	18±0.014	10±0.015	-	20±0.012	19±0.011	13±0.015
ISO 13	16±0.013	14±0.010	15±0.014	25±0.012	-	25±0.012	-	25±0.013	-	-
ISO14	25±0.015	29±0.012	26±0.011	35±0.09	25±0.012	29±0.013	-	34±0.010	5±0.012	-
ISO27	20±0.012	15±0.017	25±0.012	28±0.013	-	15±0.012	-	26±0.011	-	-
ISO29	15±0.011	15±0.014	18±0.012	27±0.011	-	17±0.010	-	28±0.010	-	-
ISO30	4±0.011	6±0.018	7±0.015	5±0.012	5±0.011	7±0.014		5±0.012	8±0.014	7±0.012
ISO31	7±0.009	10±0.012	6±0.011	12±0.017	4±0.011	10±0.019	-	15 ±0.010	8±0.019	5±0.013
ISO41	22±0.011	20±0.013	30±0.011	30±0.011	-	25±0.017	-	34±0.018	-	-
ISO42	20±0.013	20±0.009	25±0.09	20±0.08		20±0.014	-	25±0.012	-	-
ISO45	20±0.012	30±0.012	30±0.011	35±0.017	30±0.013	30±0.012	-	40±0.011	-	15±0.017
ISO47	18±0.012	16±0.015	20±0.013	-	18±0.010	15±0.014	-	25±0.013	20±0.012	-
ISO51	25±0.013	30±0.014	25±0.013	28±0.012	30±0.014	30±0.015	10±0.011	35±0.010	-	-
ISO52	27±0.009	22±0.012	-	35±0.013	10±0.012	27±0.014	-	30±0.013	20±0.011	10±0.016

Figure 5 Antibiotic Susceptibility pattern of ISO 29

Antimicrobial activity

The LAB isolates were exposed to pathogenic microorganisms *S. aureus* (MTCC 7443), *K. pneumonia* (MTCC 8911), *Pseudomonas aeruginosa* (MTCC 8076), *Bacillus subtilus* (MTCC 736), and *Acinetobacter baumannii* (MTCC 11451). All 15 LAB strains showed antibacterial effects against these pathogenic bacteria, but the zone of inhibition varied. All the isolated LAB strains showed an average zone of inhibition (15–20 mm) on the growth of test pathogens, but the ISO 6, ISO 13, ISO 14, ISO 47 and ISO 51 isolates were the most potential ones in inhibiting all the test pathogens (15–22 mm) (Table 5, Fig 6)

Table 5 Antimicrobial activity of 15 Lactic acid bacterial isolates against MTCC test Pathogens (zone of inhibition in mm± SD)

S.No	Name of isolate	MTCC 8911 *K. pneumonia*	MTCC 736 *Bacillus subtilus*	MTCC 7443 *S. aureus*	MTCC 11451 *Acinetobacter baumannii*	MTCC 8076 *Pseudomonas aeruginosa*
1	ISO 2	12±0.016	12±0.017	18±0.010	10±0.014	10±0.016
2	ISO 4	12±0.012	19±0.014	19±0.013	10±0.014	19±0.019
3	ISO 6	16±0.011	15±0.010	18±0.015	15±0.011	15±0.015
4	ISO 13	18±0.014	19±0.013	18±0.011	15±0.019	15±0.021
5	ISO 14	19±0.025	16±0.018	18±0.018	22±0.011	15±0.015
6	ISO 27	18±0.012	15±0.014	15±0.014	19±0,018	12±0.020
7	ISO 29	12±0.010	15±0.016	16±0.015	10±0.019	8±0.011
8	ISO 30	14±0.020	16±0.011	20±0.021	19±0.015	12±0.012
9	ISO 31	10±0.013	16±0.016	20±0.018	0±0.00	7±0.012
10	ISO 41	14±0.024	15±0.021	19±0.017	0±0.00	0±0.00
11	ISO 42	15±0.017	12±0.018	14±0.010	20±0.019	0±0.00
12	ISO 45	17±0.017	10±0.014	16±0.017	21±0.012	0±0.00
13	ISO 47	15±0.012	18±0.013	19±0.017	15±0.025	18±0.027
14	ISO 51	15±0.016	18±0.017	20±0.023	22±0.024	15±0.017
15	ISO 52	14±0.019	19±0.021	19±0.023	10±0.024	10±0.025

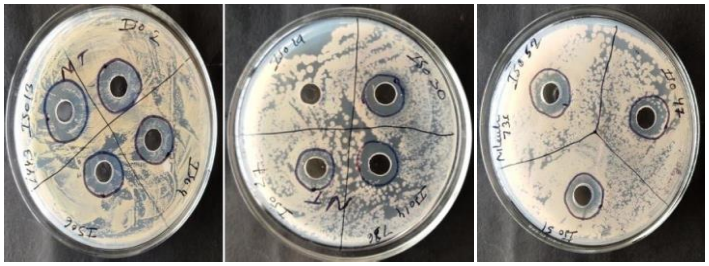

Figure 6 Antagonistic activity of LAB isolates against test pathogens

Characterization of antimicrobial compound

Further research was conducted on potential LAB isolates from conventional and commercial probiotic products to characterize the presence of antimicrobial agents like hydrogen peroxide, organic acid, and bacteriocin. By using the agar well-diffusion method, the antagonistic activity produced by all 15 LAB was evaluated against various indicator strains; and the best 5 strains of LAB were chosen for the identification of antimicrobial metabolites (Table 5, Figure 7). The findings showed that trypsin (1 mg/ml) treatment blocks the inhibitory activities of all five LAB isolates, ISO 6, ISO 13, ISO 14, ISO 47, and ISO 51, in culture supernatants. This demonstrates that the synthesis of bacteriocin was the cause of the inhibitory activity of LAB isolates for the indicator strain. Catalase-treated culture supernatants also inhibit the LAB isolates' inhibitory activity against indicator bacteria. This demonstrated that hydrogen peroxide generation was the cause of the LAB isolates' suppression of the indicator strain. The neutralized supernatant (pH 6.5) of all five LAB, however, had no inhibitory effect on the indicator strain because it prevented the formation of organic acids (Table 6, Figure 7). According to this study's findings, ISO 6 isolated from cow milk was responsible for the production of bacteriocin against *K. pneumonia* as well as of organic acid and bacteriocin against *Pseudomonas aeruginosa*, and ISO 13 isolated from home-made paneer was responsible for the production of both bacteriocin and organic acid against *K. pneumonia*, *Pseudomonas aeruginosa*, and *Bacillus subtilus*. The commercial probiotic curd isolate ISO 15 was in charge of producing peroxide to combat all five infections. ISO 14 isolated from homemade paneer was responsible for the production of organic acid against *Pseudomonas aeruginosa*. ISO 47 isolated from probiotic tablets was responsible for organic acid production against *K. pneumoniae* and *Bacillus subtilus*.

Figure 7 Anti-metabolite activity of ISO 13 against *Pseudomonas aeruginosa*

Table 6 Characterization of antimicrobial substances of selected isolates against MTCC pathogens (zone in mm± SD)

Zone of inhibition (in mm)						
		K. pneumonia	*Bacillus subtilus*	*S. aureus*	*Acinetobacter baumannii*	*Pseudmonas aeruginosa*
ISO 6	**Bacteriocin**	-	5±0.019	12±0.013	6±0.013	-
	Organic acid Assay	5±0.023	5±0.007	10±0.017	5±0.012	-
	Peroxidase Assay	22±0.042	20±0.006	18±0.018	22±0,013	22±0.007
ISO 13	**Bacteriocin Assay**	-	-	10±0.011	4±0.011	-
	Organic acid Assay	-	-	10±0.010	5±0.010	-
	Peroxidase Assay	22±0.025	21±0.012	15±0.008	23±0.011	22±0.015
ISO 14	**Bacteriocin Assay**	5±0.013	6±0.013	10±0.012	5±0.009	5±0.012
	Organic acid Assay	5±0.016	6±0.010	10±0.011	5±0.007	-
	Peroxidase Assay	15±0.018	10±0.015	12±0.012	5±0.012	20±0.011
ISO 47	**Bacteriocin Assay**	5±0.012	6±0.011	6±0.016	5±0.013	10±0.011
	Organic acid Assay	-	-	6±0.007	6±0.011	7±0.018
	Peroxidase Assay	5±0.09	5±0.012	5±0.019	5±0.012	10±0.009
ISO 51	**Bacteriocin Assay**	5±0.015	12±0.009	12±0.018	10±0.008	10±0.008
	Organic acid Assay	5±0.016	12±0.010	12±0.017	6±0.015	9±0.010
	Peroxidase Assay	-	-	-	-	-

Molecular identification of LAB isolates

After preliminary analysis at NCBI and RDP II (http//:rdp.cme.msu.edu), the sequences were downloaded, and a phylogenetic tree was built using phylogenetic tree builder software, showing the phylogenetic relationship of the isolates (Figure 8). This allowed for the molecular characterization of ISO 13, which was the most potential lactic acid bacterium in terms of antimicrobial activity and bacteriocin production. Because of the 99.38% match between the sequence found in the analysis and the sequences of *Enterococcus spp*., ISO 13 was identified as *Enterococcus faecalis* JY32.

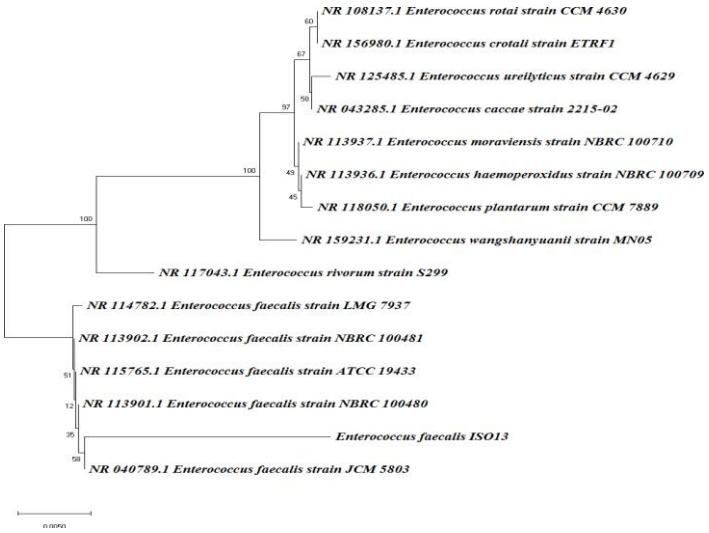

Figure 8 Phylogenetic tree based on neighbor-joining method of 16S r-RNA gene sequencing of isolated Lactic acid bacteria

DISCUSSION

The motive of this study was to test the putative probiotic Lactic acid bacteria's antagonistic power against common pathogenic bacteria by isolating and characterizing them from commercially available probiotic products and traditional dairy products from Dehradun, India. After isolation and Gram-staining, bacteria showed different morphological features such as bacillus-shaped, convex, smooth, rough, shiny, powdery, circular, white and off-white colonies, Gram-positive, non-spore-forming, facultative anaerobic, which indicate them to be the member of Lactic acid bacteria spp. Based on the catalase test, 15 catalase negative bacilli shaped LAB isolates were chosen for further biochemical testing. The productive growth of isolates on MRS-agar plates at pH 6.8 with anaerobic conditions and catalase negative profile indicate their identification as *Lactobacillus* or *Enterococcus* spp. **(Dhanasekaran *et al*., 2010)**. Selective isolates when tested for motility, oxidase, and IMViC yielded negative results. The isolates were citrate, VP, indole, catalase, and oxidase negative but MR positive **(Qian *et al*., 2018)** Sugar fermentation test was used for all 15 isolates. Most of the isolates were able to ferment dextrose except ISO 4, ISO 42, ISO 45, and ISO 51 and all isolates ferment lactose except ISO 42 and ISO 51. Sorbitol and xylose fermentation was accomplished by some isolates named as ISO 2, ISO4, ISO6, ISO13, ISO 42 with gas production, ISO 47 and ISO 52 showed growth in a variety of carbohydrates and produce acid **(Wassie and Wassie, 2016)**. One of the key elements influencing bacterial growth is pH. Probiotic organisms have the unique ability to withstand the low pH of the human stomach. Human stomach pH ranges from 2 to 4 depending on nutrition, feeding schedule, food quality, and individual ability to digest. All of the isolated LAB species had great pH tolerance, with the majority of isolates tolerating a range of pH between 2 and 8, and growing well in neutral pH, as shown in (Fig 2). Bile salt tolerance is an important factor for the growth of a good probiotic bacteria **(Begley *et al*., 2005)** because 0.3% is the maximum bile concentration seen in human guts, it was added to the bacterial growth medium with CaCl2 **(Graciela & Maria, 2001)**. All the isolates develop at 0.3% bile salt concentration; however, ISO 6, ISO 13, ISO 30, ISO 47, and ISO 52 show the best growth. When introduced at varied concentrations to a growing medium, NaCl can prevent the growth of some bacteria, while probiotic species can tolerate high salt concentrations in the human gut. The current investigation showed that LAB isolates were able to withstand concentrations of 1 to 7% NaCl, with good tolerance being seen at 1 and 3% NaCl concentrations. All 15 isolates showed no hemolysis on Blood agar plates after 24 hrs. of incubation no alpha beta and gamma hemolysis were present. One of the primary selection factors for potent and cutting-edge probiotics is the antagonistic ability of strains. The antagonistic activity of isolates is due to antibacterial metabolites such as hydrogen peroxide, organic acids (lactic, acetic, propionic, succinic, etc.), and low-molecular weight antimicrobial compounds like bacteriocin **(Prabhurajeshwar and Chandrakanth, 2019)**. ISO 6 isolated from cow milk was responsible for bacteriocin production against *K. pneumonia* and for both organic acid and bacteriocin production against *Pseudomonas aeruginosa*. ISO 13 isolated from homemade paneer was responsible for both bacteriocin and organic acid production against *K. pneumonia*, *Pseudomonas aeruginosa* and *Bacillus subtilus*. ISO 14 isolated from homemade paneer was responsible for the production of organic acid against *Pseudomonas aeruginosa*. ISO 47 isolated from commercial probiotic was responsible for organic acid production against *K. pneumoniae* and *Bacillus subtilus* (Table 1) ISO 51 isolated from commercial probiotic curd was responsible for the production of hydrogen peroxide against all five pathogens. Based on maximum antimicrobial activity and bacteriocin production ISO 13 was selected for molecular identification by 16srRNA technique. ISO13 showed 99.38% resemblance with the sequence reported on *Enterococcus* genus with species faecalis JY32. *Lactobacillus*, *Enterococcus*, and *Streptococcus* spp. are well-known probiotics used against the growth of a wide range of intestinal pathogens in humans **(Guarner *et al*., 2012)**. According to 2017 Uraporn Phumisantiphong study, *Enterococcus spp*. isolated from human feces illustrate remarkable antimicrobial activity against some clinically important MDRE and VRE strains **(Phumisantiphong *et al*., 2017)**. According to Kunduhoglu (2018) study *Enterococcus faecalis* KT11 isolated from traditional Karg-Tulum cheese showed antagonistic activity because of bacteriocin production against human pathogens. The result demonstrated that Bacteriocin KT11 has an antagonistic ability against various the Abanoz and types of common pathogens, including vancomycin- and/or methicillin-resistant bacteria, such as *E. coli*, *Salmonella typhi*, *P. aeruginosa*, *Shigella species*, *E. faecalis*, *S. aureus* and *K. pneumoniae* **(Abanoz *et al*., 2018)**.

CONCLUSION

Lactic acid bacterial strains isolated in this research from traditional dairy samples and commercial probiotic samples available in Dehradun India with in vitro

assessment make them potential nominees for probiotic application. The strains of the isolated lactic acid bacteria showed excellent probiotic properties, such as salt tolerance, pH tolerance, and bile tolerance, anti-hemolytic activity, and suppression of pathogen growth due to the production of antimicrobial compounds under in vitro conditions. Some tested strains of lactic acid bacteria were susceptible to several clinically effective antibiotics, and some were resisting antibiotics. The outcome of the study suggests that isolated LAB have potential properties of novel probiotics and in comparison, of commercial and traditional products, traditional products show maximum possibilities to making good probiotic supplements. It has been observed that most of the pathogenic bacteria have become resistant to antibiotics due to their indiscriminate consumption, so this research provides support for the formation of novel probiotics as antimicrobial agents or supplements for the prevention of bacterial pathogens and their related disease.

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Author contributions: NT designed experiment and collected the data, AM and IR contributed in first draft of manuscript and SB and SS designed objectives and contributed in finally proof read the manuscript.

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Consent to Publish: Yes

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