



Isolation, Identification and Analysis of Probiotic Characteristics of *Lactobacillus* spp. from Regional Yoghurts from Surendranagar District, Gujarat

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ABSTRACT

Background: Probiotics are good bacterial species. They confer health benefits to the human gastrointestinal tract. *Lactobacillus* spp. and *Bifidobacterium* are the most commonly used probiotic strains.

Methods: In the present study, Lactic acid bacteria were isolated from the different regional yoghurt sample (masti, lite and household fermented dahi). Identification and analysis was done by different morphological characterization, biochemical tests and probiotic characterization like pH tolerance, bile salt tolerance, temperature tolerance and NaCl tolerance etc. of isolated *Lactobacillus* spp.

Result: This study indicated that *Lactobacillus* species from yogurt samples have potential probiotic properties.

Key words: Food supplements, *Lactobacillus* spp., Probiotic, Yoghurt.

INTRODUCTION

Fermentation is one the oldest method of food preservation and the fermented foods are rich in nutritional values. Since decades, fermented food is a part of staple food of human diet. The most common examples of fermented food are beer, yogurt, kimchi, milk, cereals, soybeans, fruits and fish (Wolfe and Dutton, 2015). The presence of microorganisms and their byproducts in fermented food is responsible for the unique texture, color, flavor and aroma. These microorganisms are collectively known as "probiotics". (McFarland, 2015).

Probiotics are living organisms offering multiple health benefits when used as food additives (FAO/WHO, 2006/2008; Plaza-Diaz *et al.*, 2019). The key target of probiotics is gastrointestinal tract which acts as an interface for microbial balance and metabolic pathways (Thakur *et al.*, 2018; Hassanzadazar *et al.*, 2012). They are a part of human micro biota (mouth, intestine and female genital tract). The health benefits of probiotics includes stimulation of the immune system, blood cholesterol reduction, vitamin synthesis, anti-carcinogenesis and anti-bacterial activities (Somnath *et al.*, 2017). They prevent growth and controls of undesirable microorganisms and hence can be considered as natural biopreservatives.

Lactic acid bacteria (LAB) which includes *Lactobacillus* and *Bifidobacterium* are the most common genera of microorganisms used as probiotic foods (Tripathi and Giri, 2014). The genus *Lactobacillus* comprises rod-shaped, Gram-positive, non-spore forming, non-pigmented, catalase negative and microaerophilic bacteria which produce lactic acid as the end product of carbohydrate fermentation (Issazadeh *et al.*, 2013; Azcarate-Peril *et al.*, 2001; Hasan and Frank, 2001; Pelinescu *et al.*, 2009). Food industry is a major consumer of lactic acid produced (approximately 70%)

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due to its role in the production of yogurt, cheese and other dairy products.

The major factor which is considered before selecting probiotic strain is the survival of probiotic bacteria in gastrointestinal tract which includes tolerance under acidic conditions and tolerance against bile salts and its antimicrobial properties against pathogenic bacteria (Petros *et al.*, 2006; Durme *et al.*, 2001; Issazadeh *et al.*, 2013). The potential microorganisms used as probiotics are listed in Table 1.

Yogurt is a fermented milk product that has been prepared traditionally by allowing milk to sour at 40-45°C. Modern yogurt production is a well-controlled process that utilizes ingredients of milk, milk powder, sugar, fruit, flavors, coloring, emulsifiers, stabilizers and specific pure cultures

of lactic acid bacteria (*S. thermophilus* and *L. bulgaricus*) to conduct the fermentation process (Radke-Mitchell and Sandine, 1984).

Despite important role of probiotics on human health, there is a paucity of scientific research regarding emerging uses of *Lactobacillus* spp. as probiotics. Therefore extensive studies are required for finding *Lactobacillus* probiotics for therapeutic benefit from different dairy products. The aim of the present study was isolation, identification and analysis of probiotic characteristics of *Lactobacillus* spp. from regional yoghurts sold in Surendranagar district, Gujarat, India. Morphological identification of lactic acid bacteria was done by gram staining and the characterization was done by performing various biochemical tests and test for probiotic property like temperature sensitivity, pH tolerance, bile salt tolerance and NaCl tolerance.

MATERIALS AND METHODS

Samples collection and enrichment

A total 3 regional brands of yoghurt namely masti, lite and traditional fermented dahi made from pasteurized milk was collected from super market and stored in a fridge at 4°C to 6°C until use.

MRS agar and MRS broth media were used for isolation and growth of LAB and to inhibit the growth of unwanted bacteria. MRS agar and broth were also used for enrichment of LAB culture. All other chemicals and dyes used in this study were of analytical grade, purchased from Merck, India. The bacteriological media were obtained from HiMedia Laboratories Pvt. Ltd., India. The study was carried out

during the period of January 2018 to July 2018, in the Department of Microbiology, C.U. Shah Institute of Life Sciences, CU Shah University, Surendranagar-363030, Gujarat, India.

Isolation of LAB

1 gm of each sample was taken in 9 ml of MRS Broth (Hi-Media, India) and incubated at 37°C for 36-48 h. One loopful broth culture was streaked on MRS agar plates and was incubated for 36-48 hrs. The isolates were screened by morphological and biochemical tests. Pure culture was obtained by multiple sub-culturing and was stored in MRS agar slant for further study.

Screening and identification of *Lactobacillus* spp

The colonies were screened for *Lactobacillus* species according to their morphological, cultural, physiological and biochemical characteristics as described in Bergey's manual of determinative bacteriology, 8th edition. The different biochemical tests carried out were gram reaction, indole test, motility test, production of catalase, endospore test, urease test and sugar fermentation profile.

Bile tolerance test

The isolated strains were grown in MRS broth containing 0.2% of bile salt for 24-36 h under anaerobic conditions at 37°C. Culture broths with turbidity more than 0.5 units at 600 nm were classified as bile tolerant strains. These strains were further grown in MRS broth containing higher concentrations of 0.5 and 1.0% (w/v) of bile salt to assay their survival rate. The survival rate was expressed as the

Table 1: Potential microorganisms used as probiotics (Holzapfel *et al.*, 2001).

<i>Lactobacillus</i> species	<i>Bifidobacterium</i> species	Other lactic acid bacteria	Nonlactic acid bacteria
<i>L. acidophilus</i>	<i>B. adolescentis</i>	<i>E. faecium</i>	<i>Bacillus cereus</i> var. <i>toyoi</i>
<i>L. casei</i>	<i>B. animalis</i>	<i>Leuconostoc mesenteroides</i>	<i>Escherichia coli</i> strain nissle
<i>L. crispatus</i>	<i>B. bifidum</i>	<i>Lactococcus lactis</i> ³	<i>Propionibacterium freudenreichii</i>
<i>L. gasseri</i>	<i>B. breve</i>	<i>Leuconostoc mesenteroides</i>	<i>Saccharomyces cerevisiae</i>
<i>L. johnsonii</i>	<i>B. infantis</i>	<i>Pediococcus acidilactici</i> ³	<i>S. boulardii</i>
<i>L. plantarum</i>	<i>B. longum</i>	<i>Sporolactobacillus inulinus</i> ¹	
<i>L. paracasei</i>	<i>B. lactis</i> ²	<i>Streptococcus thremophilus</i> ³	
<i>L. reuteri</i>			
<i>L. rhamnosus</i>			

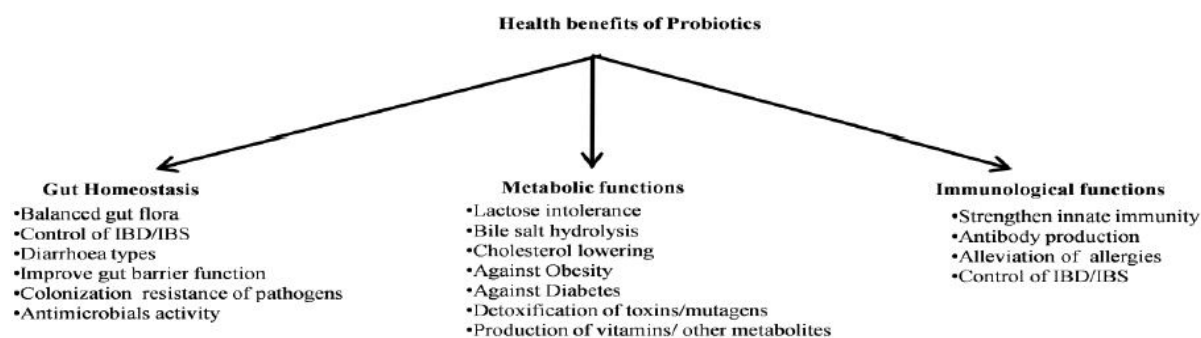


Fig 1: Health benefits of probiotics (Bajaj *et al.*, 2015).

percentage of viable cells in the presence of bile salt compared to that without bile salt.

Acid tolerance test

The isolated strains were grown in MRS broth tubes at different pH ranges, i.e. pH 2, 3, 4, 5, 6 and 7 and incubated at 37°C for 48 - 72 hrs. From each tube 0.1 ml of inoculated culture was poured into MRS agar medium by using pour plate method and incubated at 37°C for 48 hrs. The growth of different culture on MRS agar media was examined to measure the pH tolerance of the isolated culture. Isolates which were growing on the agar were considered to be acid tolerant strains.

NaCl tolerance test

NaCl tolerance of isolated *Lactobacillus* strains was determined by using MRS broth with different NaCl concentrations (1 - 10%) and incubated at 37°C for 24-36 hours. After incubation 0.1 ml of culture from each tube was used to grow in agar medium by pour plate method. The plates were then incubated at 37°C for 24 hours and observed for comparative growth in these plates.

Temperature sensitivity

The selected *Lactobacillus* strains were inoculated into different MRS broth tubes and incubated at different temperatures, i.e. 25, 30, 37, 40 and 45°C for 48 hours. By using pour plate method the isolates then cultured on agar medium. The plates were then incubated at 37°C for 24 hours and observed for comparative growth in these plates.

Sugar fermentation test

The selected *Lactobacillus* strains were evaluated for fermentation of different carbohydrates and sugar alcohols. Different carbohydrates and sugar alcohols used in this fermentation tests were arabinose, glucose, fructose, galactose, sucrose, lactose, starch and mannitol. The test organisms were inoculated into a broth containing the test sugar and incubated at 37°C for 48 to 96 hours. A bright yellow color indicates the production of enough acid products from fermentation of the sugar to drop the pH to 6.9 or less. Gas production was investigated with a Durham tube, a small inverted vial filled with the carbohydrate fermentation broth. Yellow color or yellow color with gas bubble was considered as positive result and red color with no gas bubble was considered as negative result.

RESULTS AND DISCUSSION

Isolation and identification of LAB

Three regional brands of yoghurt samples namely masti, lite and traditional fermented dahi were collected from super market. These strains were identified as *Lactobacillus* spp. (Table 2) after observation of their colony morphology, cultural, physiological and biochemical characterization and further consulting the Bergey's manual of determinative bacteriology, 8th edition.

The samples were cultured on MRS agar plate (Fig 2) for 36-48 h and colonies were selected based on the morphological characteristics and identified as LAB spp. based on their physiological and biochemical characteristics (Table 3, 4 and 5). The result showed that the bacterial isolates were Gram-positive, rod shaped, creamish in color, dull appearance, smooth texture, non motile and catalase negative.

Determination of effect of medium pH, Bile Salt and NaCl on growth of LAB

The LAB isolates showed growth in the pH range of 3-9 with a maximum growth at pH 5 and pH 7 (Table 6). The findings suggest that LAB is well adapted to acidic as well as basic conditions. The isolated LAB spp. survived in 0.5% to 3% bile salt concentration with a maximum growth in 0.5% and 1% (Table 6). The isolates also had tolerance to 1-5% NaCl with maximum NaCl tolerance at 1% and 2% (Table 6). Tolerance to Bile salt and NaCl are one of the major factors in deciding any bacterial isolate as probiotics.

Probiotics are defined as live microorganisms, including *Lactobacillus* species, *Bifidobacterium* species and yeasts that may beneficially affect the host upon ingestion by improving the balance of the intestinal micro-flora. The aim of the present study was to isolate, identify and analyze the probiotic characteristics of *Lactobacillus* spp. from regional yoghurts sold in Surendranagar district, Gujarat, India. A total of six isolates were identified as LAB from different yogurt samples based on their morphological characteristics. The bacterial isolates were rod-shaped, gram positive, creamish appearance, round-circular texture, dull appearance, raised/convex elevation and opaque or translucent. These morphological features indicated that

Table 2: LAB isolates from yoghurt sample.

Yogurt brand	No. of isolates	LAB spp.
Masti	15	2
Lite	13	3
Dahi	21	1
Total	49	06

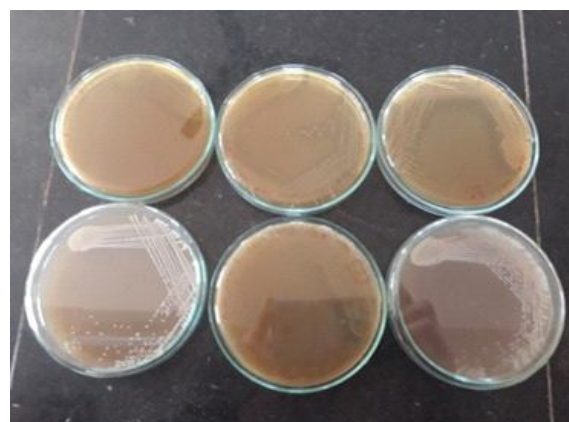


Fig 2: *Lactobacillus* MRS Agar plate with different morphology of LAB.

Table 3: Gram's Staining and morphological characteristics of different LAB on MRS.

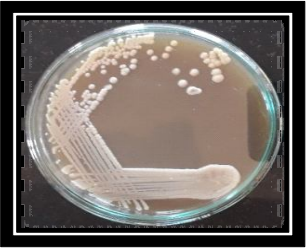
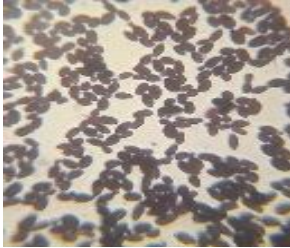
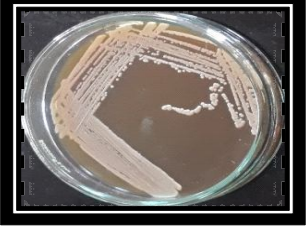
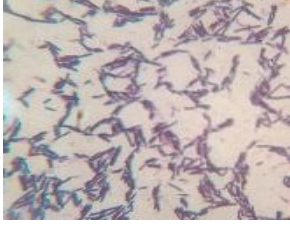
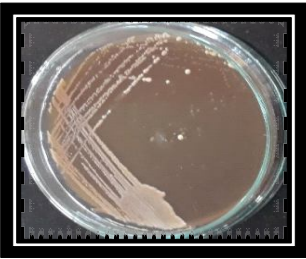
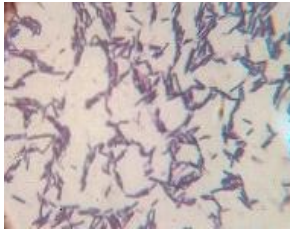
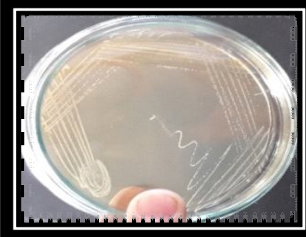
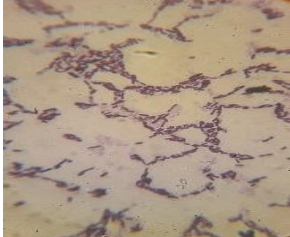
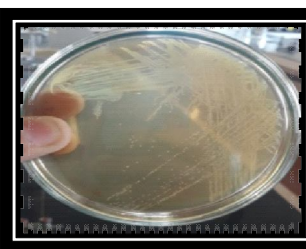
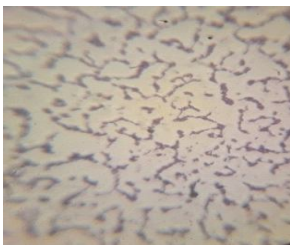
Yoghurt sample	MRS agar plate	Gram's stain	Morphology
Strain A (Dahi)			Gram positive, rod shaped bacteria
Strain B (masti)			Gram positive, rod shaped bacteria
Strain C (masti)			Gram positive, straight rod
Strain D (lite)			Gram positive, rod shaped bacteria
Strain E (lite)			Gram positive, rod shaped bacteria
Strain F (lite)			Gram positive, short rod shaped bacteria

Table 4: Colony characteristics of LAB strains isolated from yoghurts.

Colony characteristics	Strain A	Strain B	Strain C	Strain D	Strain E	Strain F
Gram's staining	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
Color (pigmentation)	Creamish	White	Creamish	White	Creamish	Creamish
Size	Big	Medium	Pinpoint	Medium	Pinpoint	Pinpoint
Shape	Circular	Circular	Round	Circular	Round	Round
Texture	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Appearance	Dull	Dull	Dull	Dull	Dull	Dull
Margin	Entire	Entire	Entire	Entire	Entire	Entire
Elevation	Convex	Raised	Convex	Raised	Flat	Flat
Opacity	Opaque	Opaque	Opaque	Opaque	Translucent	Translucent

+ve: Positive.

Table 5: Biochemical characterization of the isolated LAB strain.

Strain	Indole test	Urease test	Catalase test	Oxidase test	Sugar fermentation	Motility test
Strain A (dahi)	—	+	—	+	Acid and gas	Non motile
Strain B (masti)	—	—	—	+	Acid and gas	Motile
Strain C (masti)	—	—	—	—	Acid	Non motile
Strain D (lite)	—	—	—	+	Acid and gas	Motile
Strain E (lite)	—	—	—	+	Acid	Non motile
Strain F (lite)	—	—	—	—	Acid	Non motile

Table 6: Effect of medium pH, bile salt and NaCl on growth of LAB.

Strain	pH Tolerance			Bile Salt Tolerance				NaCl Tolerance					
	3	5	7	0.5%	1%	2%	3%	1%	2%	3%	4%	5%	10%
Strain A (dahi)	+++	++	+	+++	+++	++	+	+++	+++	++	++	+	Nil
Strain B (masti)	+++	++	+	+++	+++	++	+	+++	+++	++	++	+	Nil
Strain C (masti)	+++	++	+	+++	+++	++	+	+++	+++	++	++	+	Nil
Strain D (lite)	+++	++	+	+++	+++	++	+	+++	+++	++	++	+	Nil
Strain E (lite)	+++	++	+	+++	+++	++	+	+++	+++	++	++	+	Nil
Strain F (lite)	+++	++	+	+++	+++	++	+	+++	+++	++	++	+	Nil

they belong to *Lactobacillus* spp (Holt *et al.*, 1994). The optimum growth of the isolates was at pH 6.5 to 7 on MRS agar plates. The biochemical tests of all the six selected isolates were indole negative, urease negative, catalase negative, oxidase positive, produced gas and acid on sugar fermentation and were non-motile. These findings are similar to the findings of Elizete and Carlos, 2005 and Prabhurajeshwar and Chandrakanth, 2019.

The human gut pH is in acidic range which is one of the critical factors in identification of a LAB spp which can be used as a probiotics. The result of present study shows that the isolates were able to tolerate acidic pH range (Table 5). Another crucial component of human gut is bile salts. In the gut, 0.3% to 0.7% is the maximum tolerable bile salt concentration (Begly *et al.*, 2005; Quwehand and Vesterlund 2004). In the present study, isolates shows resistant in the range of 0.5 to 1% (Table 5). Tolerance to high salt concentration is another parameter used to select LAB spp. as probiotics. Most of the bacterial growth is inhibited by high NaCl concentration. In the present study, the isolates showed growth in the range of 1% to 5% with a maximum in 1% to 2% NaCl. These findings support earlier studies (Prabhurajeshwar and Chandrakanth, 2019).

As the acid production increases, the pH of the media starts decreasing with time. The maximum acid production and decrease in pH was observed after an incubation time of 48 hours which are concurrent with the earlier findings. (Prabhurajeshwar and Chandrakanth, 2019). The isolates were capable of fermenting different sugars used. They showed growth in arabinose, glucose, fructose, galactose, sucrose, lactose, starch and mannitol which indicates their broad range potential to utilize different carbohydrates for their growth.

CONCLUSION

Different microbes, bacteria in particular find multiple applications in food, dairy and pharmaceutical products. In the present study, all the six LAB isolates, showed good potential to be used as a probiotic. and can be recommended in daily diet. Further study on these isolates at molecular level will help to detect the genes responsible for therapeutic activities and this type of research will help to design more probiotic agents to control numerous diseases and at the same time it will ensure the safe and healthy human civilization.

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Conflict of interest

The authors have no conflict of interest.

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