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Exploration of *Leuconostoc mesenteroides* Sub sp *mesenteroides* from Indian Fermented Food for Curd Preparation

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A B S T R A C T

Many bacteria are able to produce lactic acid as their end metabolic product during fermentation is known as lactic acid bacteria (LAB). During their metabolism they also produce certain nutraceutical molecules which have many health benefits. Present study aims to explore lactic acid bacteria from fermented food and their health benefits. Out of total LAB isolated from fermented food like idali, dhokala and handavo, one potential isolate was used for further studies. Isolate was identified morphologically and biochemically as *Leuconostoc mesenteroides* sub sp *mesenteroides*. Cells showed maximum growth after 96 hrs of incubation and thereafter growth was declined. It shows sensitivity of the tested antibiotics and it works as antimicrobial against *Escherichia coli*, *Micrococcus lutues*, *Serratia marcescens* and *Listeria monocytogenes* pathogenic microbes. When *Leuconostoc mesenteroides* sub sp *mesenteroides* was used as probiotic in curd preparation, it lowers carbohydrate and fat content compared to control one. Thus, it can be used for the preparation of curd for low carbohydrate content.

Introduction

India is known for its traditional fermented foods like idali, dhosa, khaman, dhokal etc. These fermented foods contain LAB which is responsible for production of lactic acid in food. Due to fermentation it provides typical taste and has high health benefits.

These fermented foods contain lactic acid producing microbes. Generally, lactic acid bacteria are Gram-positive bacteria that do not form spores and which are able to grow both in the presence and absence of oxygen. Another common trait of lactic acid bacteria is their inability to manufacture the many compounds that they need to survive and grow.

LAB are also widely used at commercial level for production of fermented food products, such as yogurt (*Streptococcus* spp. and *Lactobacillus* spp.), cheeses (*Lactococcus* spp.), sauerkraut (*Leuconostoc* spp.), fermented milk (*L.casei* strain Shirota) and sausage. LAB are amongst the best studied microorganisms for human health advantageous effects and fermentation. Significant novel developments have been made in the research of lactic acid bacteria in the areas of multidrug resistance, bacteriocins, osmoregulation, autolysins and bacteriophages. Advancement has also been made in the production of food grade genetically modified LAB. They are also

widely used as probiotics. Probiotics are live microorganisms which are defined by the World Health Organization/ Food and Agricultural Organization (2001) as: "Live microorganisms whose administration in adequate amount to the body is able to confer a health beneficial effect on the host". The most common types of microbes which are used as probiotics are lactic acid bacteria (LAB) and *Bifidobacteria.* Hence, present study aims to isolate LAB from fermented food and their application as probiotic in curd preparation.

Materials and Methods

Isolation of bacterial strains and culture conditions

For isolation of LAB, different batter samples of idali, khaman and handavo which are traditionally used in Indian home were used. For isolation of LAB, samples were serially diluted up to 10^{-7}in sterile distilled water and plated on De Man, Rogosa and Sharpe (MRS) agar plates (Yang *et al.,* 2012). Plates were incubated at 37 ± 2 °C for 2 - 4 days. Based on colony morphology, different bacteria were isolated in pure culture and stored on MRS slant at 4 °C until the use.

Screening for lactic acid bacteria (LAB)

Plate assay

To examine the lactic acid producing ability, cells were primarily screened on agar plate. For screening, an agar plate method was developed in laboratory in which MRS agar was supplemented with $CaCO_3$. Isolate which produced higher lactic acid showed zone of clearance on agar plate. Thus it was easily identified based on clearance zone. Isolated pure cultures were streaked on these plates and plates were incubated at 37 ± 2 °C. Lactic acid producing bacteria showed zone of clearance on MRS were selected for further study.

Quantification of lactic acid production

Isolates which showed highest zone of clearance were selected for their lactic acid production efficacy. 1 ml of 18 hrs old inoculum was inoculated in MRS broth. After incubation, cells were removed by centrifugation and supernatant was used for lactic acid determination. The amount of lactic acid in fermentation broth was determined by transferring 10 ml of supernatant into 100 ml flask along with 1 ml of phenolphthalein indicator (0.5% in 5% alcohol). Sample was titrated with 0.25 M NaOH for the appearance of pink color. The amount of NaOH used was calculated and corresponding lactic acid % W/V was determined (Fortina *et al.,* 1973).

Identification of isolate

Morphological and biochemical characterization

Isolate which produced higher lactic acid was identified by morphological and biochemical characterization according to Bergey's Manual of Systematic Bacteriology (De Vos *et al.,* 2001). Identification was further confirmed by VITEC analyzer.

Antimicrobial activity

LAB are generally produced bacteriocin and other antimicrobial compounds. Their antimicrobial activity was examined against *Escherichia coli*, *Micrococus lutues* ATCC 10240, *Serratia marcescens* ATCC 14756, *Yersinia enterocolitica* ATCC 23715, *Staphylococcus aureus* ATCC 11632, *Listeria monocytogenes(4b)* ATCC 13932, *Bacillus cereus* ATCC 11778, *Proteus vulgaris* ATCC 33420, *Salmonella poona* ATCC 4840,

Enterococcus faecalis ATCC 14506. Antimicrobial activity was examined by well diffusion assay (Schillinger U, Lucke FK (1987). 18 hrs old culture of test pathogen was streaked on Muller Hinton agar. Wells were prepared in agar plate with sterile cup borer and supernatant of potential lactic acid producing isolate was place in well. Plates were incubated at 37°C for 24 hrs. Zone of inhibition was measured in mm. The antimicrobial activity was determined by measuring the zone of inhibition around the well.

Determination of antibiotic resistance of the isolates

To evaluate the antibiotic susceptibility, disc diffusion method of Kirby-Bauer assay (Bauer *et al.,* 1959) was performed. Octodiscs (Combi 61, G-I-minus, Combi 506) (HiMedia Laboratory Pvt Ltd, India) were used for the assay of antibiotic sensitivity. Octodisc was placed on agar plate spread with tested organism. The plates were incubated at 37 °C for 24 hours. The resistances were determined according to the zone of inhibition.

Application in curd preparation

To evaluate the efficacy of LAB bacteria as probiotics, culture was used for curd preparation. 18 hrs old culture broth was used as inoculum. Cells from the broth were harvested by centrifugation and washed twice with distilled water. 1 % inoculums was used for the study. Four different treatment were used for this experiment viz. T1= Milk + starter culture (control), T2 = *Leuconostoc mesenteroides* + Milk (closed system), T3 = *Leuconostoc mesenteroides* + Milk (open system) and T4 = *Leuconostoc mesenteroides* + Milk+ Starter culture. Milk sample after inoculation of bacterial culture incubated overnight.

Next day samples were analyzed for total carbohydrate, protein, fat and moisture content.

Results and Discussion

LAB are among the most important groups of microorganisms used in fermented food. They provide a typical taste and quality to fermented foods. They are widely use for preservation as they produce lactic acid which lowers the pH and thus beneficial in the preservation of food. The lowered pH inhibits the growth of most other food spoilage microorganisms. Along with lactic acid they are also able to produce growth inhibiting substances. For isolation of LAB, batter (idali, khaman and handavo) was collected from different home and local market.

MRS agar was used for the isolation of LAB and cells grown on agar were selected. Total 25 isolates based on their colony characters were selected and purified. Isolated samples were stored on MRS at 4 °C until the use. Isolates were designated as L1 to L25.

All isolates were morphologically characterized by Gram's staining. Data suggests that all isolates were Gram positive and have irregular coccoid to oval morphology. All produce metachormatic granules during their growth. These isolates were primarily screened by growing them on MRS agar contain calcium carbonate. Organic acid producing isolates lowers the pH and thus solubilizer calcium carbonate making clear zone around colony (Fig. 1). Eleven isolates which showed higher clearance zone on MRS were selected for further studies. All these 11 isolates were screened for their quantitative lactic acid production. Lactic acid production was measured by titration. Highest lactic acid was produced by L11 (443 ppm) and least was by L2 (102 ppm). Thus potential isolate L11 was used for further

studies and identified by biochemical test.
L11 was identified as described in Bergey's Manual of Systematic Bacteriology. Oxidase and catalase tests were negative. L11 is able to ferment glucose, lactose, sucrose, xylose, mannose and maltose sugar. Cells showed positive esculin test. Based on the morphological and biochemical tests, L11 was identified as *Leuconostoc mesenteroides*.

Identification was further confirmed by sending culture for identification by VITEK analyzer (Advance Diagnostic Lab, Surat). L11 was confirmed as *Leuconostoc mesenteroides* subsp. *mesenteroides.*

LAB are generally slow grower and required complex media to grow. To observe growth pattern, cells were grown in MRS broth and optical density was monitored at 600 nm upto 120 hrs. Growth starts from 24 hrs and reached its maximum up to 96 hrs and their after declined (Fig. 2). Antimicrobial activity of the isolate was examined against some pathogenic microbes. It inhibits growth of *Escherichia coli*, *Micrococus lutues* ATCC 10240, *Serratia marcescens* ATCC 14756, *Staphylococcus aureus* ATCC 11632 *and Listeria monocytogenes* ATCC 13932 (Table 1). However, it does not inhibit the growth of *Yersinia enterocolitica* ATCC 23715, *Proteus vulgaris* ATCC 33420, *Salmonella poona* ATCC 4840, *Enterococcus faecalis* ATCC 1450.

Isolate was tested for their antimicrobial resistant against different antibiotics using octadisc. Cells were sensitive to all tested antibiotic viz. Imipenem, Meropenem, Ciprofloxacin, Tobramycin, Moxifloxacin, Ofloxacin, Sparfloxacin, Levofloxacin, Ciprofloxacin, Ofloxacin, Sparfloxacin, Gatifloxacin, Teicoplanin, Azithromycin, Vancomycin, DoxcyclineHcl, Ampicillin, Ciprofloxacin, Colistin, Co-Trimoxazole, Gentamicin, Nitrofurantoin, Streptomycin, Tetracycline (Table 2).

Fig.1 Growth of *Leuconostoc mesenteroides* sub sp. *mesenteroides* on MRS agar

Fig.2 Growth pattern of *Leuconostoc mesenteroides* subsp. *mesenteroides*

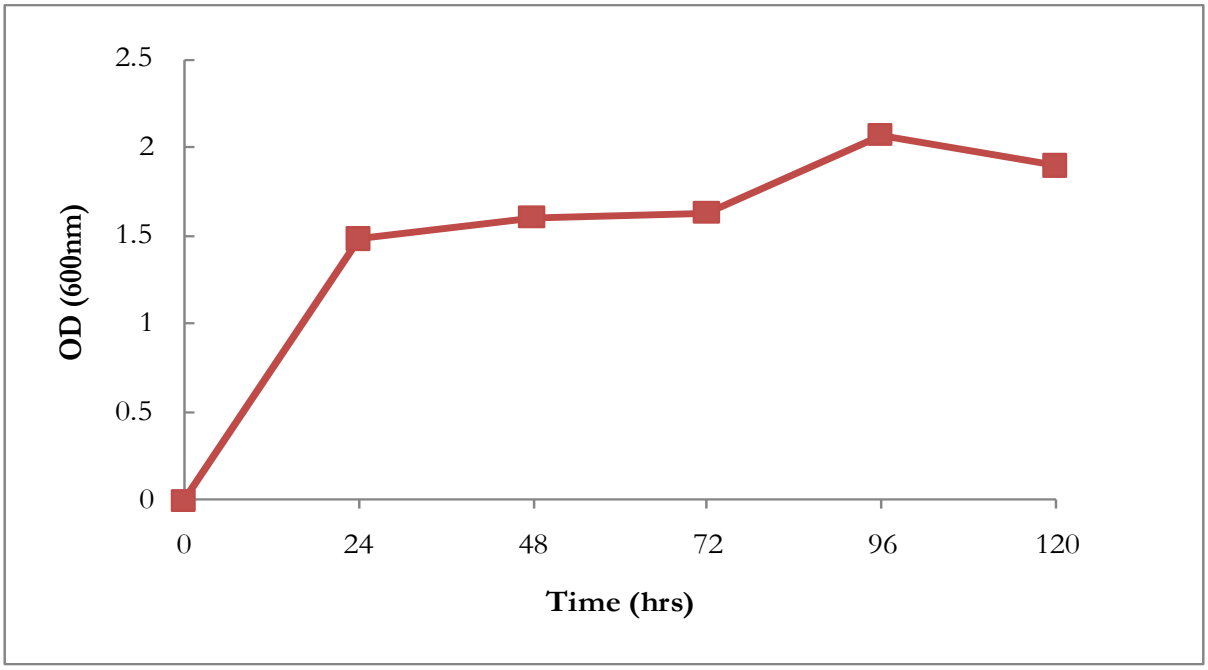

Table.1 Antimicrobial activity of *Leuconostoc mesenteroides* subsp. *Mesenteroides* against test microbes

Pathogen	Inhibition Zone (mm)
Escherichia coli	12
Micrococcus lutues	14
Serratia marcescens	15
Yersinia enterocolitica	Ab
Staphylococcus aureus	19
Listeria monocytogenes	12
Bacillus cereus	Ab
Proteus vulgaris	Ab
Salmonella poona	Ab
Enterococcus faecalis	Ab

Table.2 Antibiotic sensitivity assay of *Leuconostoc mesenteroides* sub sp. *Mesenteroides*

Antibiotics	Concentration (µg)	Senstive / Resistance
Imipenem	10	S
Meropenem	10	S
Ciprofloxacin	5	S
Tobramycin	10	S
Moxifloxacin	5	S
Ofloxacin	5	S
Sparfloxacin	5	S
Levofloxacin	5	S
Ciprofloxacin	5	S
Ofloxacin	5	S
Sparfloxacin	5	S
Gatifloxacin	5	S
Teicoplanin	30	S
Azithromycin	15	S
Vancomycin	30	S
DoxcyclineHcl	30	S
Ampicillin	10	S
Ciprofloxacin	10	S
Colistin	10	S
Co-Trimoxazole	25	S
Gentamicin	10	S
Nitrofurantoin	300	S
Streptomycin	10	S
Tetracycline	30	S

The traditional fermented dairy products can be possibly a good source of potential probiotic organisms. Here curd prepared using *Leuconostoc mesenteroides* showed good organolaptic properties. *Leuconostoc mesenteroides* containing curd has thick consistency and flavor compared to control. Moisture content was almost similar in al treatment ranging from 88.7 to 89 %. Carbohydrate content was higher in T4 followed by T3 and T2. T1 contain lowest carbohydrate content. Protein content was higher in T1 (3.25 %) followed by T2 (3.14 %) and T3 (2.95 %) and least content was in T4 (2.07). Fat content was maximum in T4 (1.7 g %) followed by T3 (1.5 %) and T1 with same content of 1.5 g %. Least 0.9 g % was found in T2 treatment.

The term Lactic Acid Bacteria is conventionally reserved for genera in the order *Lactobacillales*, which includes *Lactobacillus, Leuconostoc, Pediococcus, Lactococcus* and *Streptococcus*, in addition to *Carnobacterium, Enterococcus, Oenococcus, Tetragenococcus, Vagococcus*, and *Weisella*. Lactic acid bacteria (LAB) are generally recognized as safe (GRAS), a cluster of related bacteria that ferment carbohydrates and produce lactic acid (Ashe and Paul,

2010). Lactic acid fermentation of cereal-based foods is a traditional technology used by Indian. It is widely used for preparation of idali, dhosa, dhokla, handvo etc. in India.

Chopra and Mehra (2015) have isolated two strain of *L. plantarum* and two strain *L. fermentum* from dosa and idli batter. These isolates produce bacteriocins which have molecular weights between 16 – 48 KD.

Lactic acid bacteria produce antibacterial compounds that are known as bacteriocins. Bacteriocins act by punching holes through the membrane that surrounds the bacteria. Thus, bacteriocins activity is usually lethal to the bacteria. Examples of bacteriocins are nisin and leucocin. Nisin inhibits the growth of most gram-positive bacteria, particularly spore-formers (e.g., Clostridium botulinum. This bacteriocin has been approved for use as a food preservative in the United States since 1989. Leucocin is inhibitory to the growth of *Listeria monocytogenes*.

Leuconostoc mesenteroides is the most commonly encountered bacterium not only in Indian fermented food (Nout and Sarkar 1999; Kumar *et al.,* 2012; Mukherjee *et al.*, 1965) but also found in various countries of world (Lee, 1994; Lee *et al.*, 1983; Orillo and Pederson, 1968; Souane *et al.*, 1987). LAB contributes lactic acid and acetoin, imparting sour taste and a pleasant flavour (Aidoo *et al.*, 2006). As per FAO report, *Leuconostoc mesenteroides* subsp. *mesenteroides* serves as the main flavouring agent in cultured milk. It produces diacetyl, acetic acid, acetaldehyde and other flavour compounds but less carbon dioxide.

Leuconostoc mesenteroides subsp. *Mesenteroides* is able to inhibit the growth of *Listeria monocytogenes* ATCC 13932. Bellil *et al.*, (2014) has also reported inhibition of *Listeria innocua* ATCC 33090 by *Leuconostoc mesenteroides. Leuconostoc mesenteroides subsp. mesenteroides* used for curd preparation shows that it lowers the carbohydrate and fat content and have high protein content.

Out of all LAB isolated from different fermented food, isolate L11 showed higher lactic acid production. Morphological and biochemical characterization revealed that it is *Leuconostoc mesenteroides subsp. mesenteroides.* It inhibits the growth of potential food borne pathogen *Listeria monocytogenes.* It lowers the carbohydrate and fat content of curd and also provides thick consistency and flavor to curd. Thus it can be used as probiotic for curd preparation.

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