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Isolation and Biochemical Characterization of *Lactobacillus* species Isolated from Dahi

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ABSTRACT

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Dahi (curd) is a fermented milk product, most commonly used by Indian population. Trials are in process to establish dahi as a source of health beneficial organisms (probiotics). Hence, the present study is directed towards the study of prevalence of *Lactobacillus* species in dahi. A total of 40 samples of dahi were collected for the isolation of Lactobacilli using Lactobacillus selection MRS agar. Thirty-eight colonies were randomly picked based on colonial morphology. All the isolates were subjected to cell morphology, physiology and an array of biochemical characterization. The isolates showed different growth patterns at different temperatures (15°C and 45°C), oxygen and at different concentrations of NaCl (2.0, 4.0 and 6.5%). On the basis of physiological tests and sugar utilization pattern, all the three isolates were confirmed to the different species of *Lactobacillus*: *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus lactis*. Among isolates, *L. acidophilus* was found to be prevalent in dahi.

Introduction

Generally, the healthiness of food has been linked to a nutritionally rich diet recommended by specialists and the role of it in totality has been emphasized instead of emphasizing on individual components.

Milk, very first food of mammals including humans, is surrounded with emotional and cultural importance in the society. Men have been habituated to think of milk as nature's most perfect food for them (1). Therefore, milk and dairy products have long been recognized as an important constituent of balanced diet for human beings as these

products provide a wide range of essential nutrients.

The microbial ecology in the gastrointestinal tract influences many functions in our body (i.e., digestion, absorption of nutrients, detoxification and ultimately the functioning of immune system) (2,3). All these aspects make the gut a target organ for development of functional foods that can help in maintaining the relative balance of microorganisms in the gastrointestinal tract. The establishment of microbial balance by shifting it towards a beneficial one with the

help of specific dietary components (i.e., probiotics and prebiotics) has opened the gateway for the development of functional foods ensuring more benefits to the host's health (4, 13, 14).

Probiotics are defined as live microbes which transit the gastro-intestinal tract and in doing so, benefit the health of the consumer. Probiotic microorganisms are found in many food products, especially in the fermented foods. Therefore, the probiotic lactic acid bacteria can be isolated from the fermented milk products like acidophilus milk, yoghurt lactic cultures, is widely consumed throughout South Asia, especially in the Indian subcontinent. The micro-flora of dahi varies from one geographical region to the other and also with seasonal variations of the year. Apart from the starter lactic cultures, dahi can also have some probiotics like *Lactobacillus acidophilus*, *Lactobacillus bulgaricus* etc. Therefore, dahi can be used as a source for isolation of probiotic bacteria and also a product of immense importance for human consumption (5, 6).

Materials and Methods

Isolation of Lactic Acid Bacteria

Dahi samples (40) for the isolation of lactic acid bacteria were collected from different rural and urban locations in order to get a wider diversity of lactic acid bacterial strains. Each sample was taken in a sterile container separately and placed in a polyethylene bag during transportation to the laboratory employing standard conditions for sample collection. One gram of curd sample was immediately processed under aseptic conditions by suspending in 9mL of normal saline (0.85%) and was vortexed for proper mixing. One milliliter of each well-mixed dahi sample was enriched in 9mL of sterile *Lactobacillus* selection

MRS broth for 24h at 37°C. Before inoculation of sample, the pH of MRS broth was adjusted to 6.5±0.2. The enriched samples were streaked on the Petri plates containing *Lactobacillus* selection MRS agar with the help of calibrated inoculating loop and incubated aerobically at 37°C for 48h and observed for the growth of colonies. and dahi. Dahi, naturally fermented milk by characterization of *Lactobacilli* isolates grown on MRS agar was done mainly with the help of the following tests microscopic examination (Gram staining), catalase test, growth at different temperatures 10+1°C and 42+1°C), growth under aerobic and anaerobic conditions, growth at different NaCl concentration, fermentation of different carbohydrates, etc (7, 8, 9, 15, 16).

Microscopic Examination

The purity morphological identification of the isolates as *Lactobacilli* was confirmed microscopically by performing Gram staining, for which single colony of each isolate was picked up and stained as per the standard protocol and viewed under oil immersion for similar type of cells.

Micrometry

Each isolate after Gram staining was subjected to microscopic measurements employing ocular and stage micrometer. To determine the size of *Lactobacilli* isolates, prepared slides were observed under oil immersion objective and number of ocular divisions occupied by each bacillus was recorded and interpreted as per the above formula.

Physiological Characterization of Isolates

After confirming the purity of culture, each isolate was further assessed for growth at two different temperatures.

Growth of Isolates at (10°C and 42°C)

The isolates were tested for their ability to grow in MRS broth at 10°C for 7 days and 42°C by incubating for 24–48h. For this, 10 mL of MRS broth tubes were inoculated at 1% of Lactobacilli cultures. The

Identification of Selected *Lactobacilli*

The identification and further accordingly for the presence or absence of enzyme.

Gas from Glucose

Sterile test tubes of 10mL glucose broth containing Durham's tube (inverted and dipped), were inoculated with Lactobacilli cultures at the at 1% and incubated at 37°C for 24–48h. Gas production that appeared in the form of a hollow space in Durham's tube was recorded as a positive result.

Citrate Utilization Test

The isolates were inoculated in Simmons citrate agar incubated at 37°C for 24h. After incubation, the appearance of blue coloration indicated the positive test for citrate utilization and was recorded accordingly for the isolates tested.

Carbohydrate Fermentation Pattern by the Isolates

Most microorganisms obtain their energy through a series of orderly and integrated enzymatic reactions leading to the bio-oxidation of a substrate, frequently a carbohydrate. Thus, different sugars were used for determining the fermentation profile and further characterization of Lactobacilli isolates. For this, Lactobacilli cultures were subjected to sugar fermentation reactions using CHL medium for knowing their fermentation pattern. CHL medium was used as basal medium. Four

milliliter of the medium was taken in each tube and sterilized by autoclaving. One sugar disc was aseptically added to each tube. Each tube was inoculated with 0.1mL of inoculum, incubated at 37°C for 24–48h and the results of color change were recorded as positive or negative. A control using 0.1mL sterile water development of turbidity in culture tubes was recorded as the ability of isolates to grow at 10°C and 42°C and results were noted as positive or negative.

Effect of NaCl Concentrations on Growth of Isolates

The isolates were inoculated in MRS broth having different NaCl concentration (2.0%, 4.0 % and 6.5%) and incubated at 37°C for 24–48h. The culture tubes were observed for the presence or absence of growth.

Biochemical Characterization of Isolates

Catalase Test

The test was performed in order to determine the ability of the isolated cultures to degrade the hydrogen peroxide by producing the enzyme catalase. The test was carried out as the slide method, using an inoculating needle for this, culture from a typical colony was placed onto a clean grease-free glass slide and drop of 3% hydrogen peroxide solution was added onto the culture and closely observed for the evolution of bubbles. The production of bubbles indicated positive catalase reaction and was recorded

Identification and Characterization of *Lactobacillus* Isolates

The Gram reaction property and cell morphology of all the isolates were examined using standard staining procedure. The isolates were found to be purple colored

Gram-positive rods (i.e., straight rods, irregular rods, rods with rounded ends) of varying sizes and arrangements such as rods in single, or in chains (2–5 cells), under oil immersion microscope. After confirming the Gram positive character each isolate was further characterized for cell dimensions using micrometry. These preliminary results as inoculum was used to compare the color change. Sugars used to determine the fermentation profile of *Lactobacilli* isolates were arabinose, cellobiose, fructose, galactose, lactose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, trehalose, salicin, sorbitol, sucrose and xylose. The cultures were identified based on the pattern of sugar utilization.

Results and Discussion

Isolation of *Lactobacilli* from Dahi from Nearby Vicinity

Out of several colonies developed on agar plates, based on colonial morphology (i.e., color, size, margin and shape of the colony) such as white, greyish white or cream color, size varying from 0.5–2.3mm in diameters, with entire or undulate margins were picked up after plating 1mL of sample that was previously enriched in 9mL of *Lactobacillus* selection MRS broth for 24h at 37°C. The sub-culturing of isolates was done 3–4 times in MRS agar by incubating at 37°C for 24–48h. Finally, the well isolated colonies were streaked on the solidified Petri plates of *Lactobacillus* selection MRS agar with inoculating loop incubated aerobically at 37°C for 48h for further identification and characterization based on different physiological and biochemical tests used routinely in microbial taxonomy.

Biochemical Characterization of *Lactobacilli*

Catalase Production

Catalase, an extracellular enzyme secreted

by several microorganisms, helps in degradation of hydrogen peroxide produced during carbohydrates utilization for energy production, thereby its presence or absence in a microbial cell can be used as a significant indicator that the isolates could belong to the *Lactobacilli* group and paved the way for further characterization through physiological and biochemical means for confirmation.

Physiological Characterization

Growth at 10°C and 42°C

The isolated *Lactobacilli* cultures were assessed for their growth at two different temperatures (i.e., 10°C and 42°C). For this, cultures were incubated in MRS broth at 10°C for 7 days and the turbidity in broth was observed as an indication of microbial growth. The isolates showed good turbidity except that of 30 isolates i.e., *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus lactis* where no growth was observed in terms of turbidity. On the other hand, isolates were also incubated at 42°C for 24 to 48h and turbidity was observed in tubes containing isolates *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus lactis*.

The other *Lactobacilli* could not grow at the elevated temperature of 42°C. Hence, it can be concluded from that 37°C is the optimum temperature for all the isolates and few of these could either survive or grow at 10°C/42°C or at both the temperatures away from the optimum.

Effect of NaCl

The other physiological parameter for growth of a cell is the requirement of sodium chloride as the physiological saline prevents the cell from osmotic shock. For this, isolated *Lactobacilli* cultures were incubated in MRS broth at 37°C for 24h to

assess the effect of different NaCl concentrations (i.e., 2.0%, 4.0%, and 6.5%) in terms of turbidity, as an indication of microbial growth. At the NaCl concentration of 2.0%, the growth was observed in all the *Lactobacilli* isolates. With an increase in NaCl from 2 % to 4 %, turbidity diagnostic tool.

The catalase is involved in catalyzing the breakdown of toxic hydrogen peroxide to produce molecular oxygen that generates vigorously while producing effervescence, when a microbial culture is mixed with an equal volume of 3% solution of hydrogen peroxide. Absence of effervescence is taken as indicative as negative for catalase enzyme production. In the present study, all the isolates were found to be catalase negative. These results obtained for catalase further support the identification of isolates as *Lactobacilli* and pave the way in further characterization of the isolates and are in agreement with.

Gas from Glucose

The microorganisms use carbohydrates in a different pattern depending on their enzyme complement. In fermentation, substrates such as carbohydrates and alcohols undergo anaerobic dissimilation and produce organic acids that may be accompanied by the production of gases such as hydrogen or carbon-dioxide. Therefore, all the *Lactobacilli* were subjected to glucose fermentation test in order to see the production of gas.

It was observed from produced gas in broth containing glucose as the sole source of carbon while other isolates could not produce any gas as shown by a hollow space in the inverted Durham's tubes and, therefore, resulted as negative for gas production from glucose. Thus, the above isolates showed fermentation of glucose in

the medium for their growth. These variations in results were also reported (10, 11, 12).

Citrate Utilization Test

For further characterization and identification, all the isolates were subjected for their potential to utilize citrate as the sole carbon source. Further, it also attributes to the better technological property of the was observed only in three isolates *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus lactis*. The growth pattern at 4% NaCl concentration was also reported and concluded wide variation in growth of *Lactobacilli* cultures. At 6.5% NaCl concentration, not a single isolate could show growth as indicated in basal media with different sugar discs. In total, 16 sugars were used for conformational identification of *Lactobacilli* and these were: arbinose, cellobiose, fructose, galactose, lactose, maltose, manitol, mannose, melibiose, raffinose, rhamnase, salicin, sorbitol, sucrose, trehalose and xylose. Different isolates showed different types of sugar utilization patterns where some tubes containing isolates and sugar turned yellow where as the other remained brown colored, indicating the positive and negative tests, respectively for sugar fermentation. When these sugar utilization patterns were compared with those given for *Lactobacillus* species in the Bergey's Manual of Determinative Bacteriology, the isolates were tentatively identified as *L.acidophilus*, *L.bulgaricus*, *L. lactis*. Hence, nine different species within *Lactobacillus* genera in the dahi sample obtained from rural and urban locations were confirmed in the present results.

Carbohydrate Fermentation Pattern of Isolated Lactobacilli Cultures

For accounting the sugar utilization pattern

of the isolates suspected to be the *Lactobacilli* after their growth on

Lactobacillus selection MRS agar, the sugar utilization tests were performed using CHL.

Table.1 Morphological, Physiological and Biochemical characterization of *Lactobacillus spp*

Characteristics	Strain I	Strain II	<i>Lactobacillus acidophilus</i> Characteristics (Bergy's Manual)
Morphological Characterization			
Colour	Cream-white	Cream-white	Cream-white
Shape	circular	circular	Circular
Size	0.6µm	0.5µm	0.5-0.8µm
Motility	Non-motile	Non-motile	Non-motile
Gram reaction	Positive	Positive	Positive
Shape	rod	Rod	Rod
Physiological characterization			
Growth at different temperature			
15-20 °C	-	-	-
30 °C	+	+	+
35 °C	+	+	+
37 °C	+	+	+
Oxygen requirement			
Aerobic	+	+	+
Anaerobic	-	-	-
Microaerophilic	+	+	+
Biochemical characterization			
Indole	-	-	-
Methyl red	-	-	-
Voges proskauer	-	-	-
Citrate utilization	-	-	-
Urease	-	-	-
Triple sugar iron	-	-	-
Nitrate reduction	-	-	-
Catalase	-	-	-
Oxidase	-	-	-

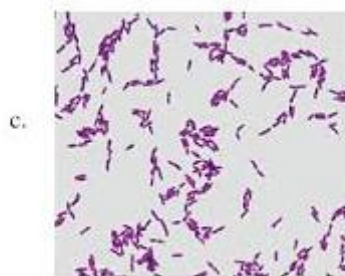
Table.2 Carbohydrate Fermentation of *Lactobacillus spp*

Sugars	Strain I	Strain II	<i>Lactobacillus acidophilus</i> Characteristics (Bergy's manual)
Dextrose	+	+	+
Fructose	+	+	+
Galactose	+	+	+
Glucose	+	+	+
Lactose	+	+	+
Maltose	+	+	+
Mannitol	+	+	+
Sucrose	+	+	+
Xylose	-	-	-

Morphological characterization of *Lactobacillus acidophilus*



Gram Staining



In conclusion, from dahi, a total of 3 *Lactobacillus* isolates were distributed among nine species and identified as, *L. acidophilus*, *L. bulgaricus*, *L. lactis*, where *L. acidophilus*, was the most prevalent. It can be stated that *L. acidophilus* isolated from traditional Indian dairy product (dahi)

could be exploited as a probiotic after investigating its beneficial characteristics. product as citrate utilization leads to a better flavor production in fermented milk production. Certain bacteria utilize citrate with the help of enzyme citrate permease and citrate-producing diacetyl, a flavoring

compound as end product. Following incubation on Simmon's citrate agar, citrate-positive cultures were identified by the presence of growth on the surface of slant, accompanied by blue coloration whereas negative cultures did not show any growth and medium remained green.

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