

# Isolation and Characterization of *Lactobacillus* spp. of Human Origin for Studying their Prevalence

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## Abstract

A total of 15 samples of healthy human feces 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 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 thirty eight isolates were confirmed to the different species of *Lactobacillus* (i.e., *Lactobacillus plantarum* (4), *Lactobacillus casei* (15), *Lactobacillus rhamnosus* (2), *Lactobacillus fermentum* (4), and *Lactobacillus acidophilus* (10), *Lactobacillus helveticus* (3). Among isolates *Lactobacillus casei* was found to be prevalent in the human gut.

**Keywords:** *Lactobacilli*, biochemical characterization, human gut

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## INTRODUCTION

Healthy human gut is comprised of over five hundred different species of microbes. Most of these microbes are non-pathogenic and protect us against a number of diseases. Therefore, because of quantity and quality of its resident microflora, the colon has become one of the most metabolically active organs of human body. Researchers in past [1-3] had shown that there is a significant role of the intestinal microflora in providing resistance to different diseases. Therefore, the concept of using beneficial microbes for improved health has become the subject of increasing interest among the scientists, industry and consumers [4-9].

*Lactobacillus* species are commonly present in human fecal samples [10] and several beneficial properties for human health are related to the activity of these in the colon

[11-13]. Lactobacilli of human intestinal origin with potential health-associated properties could be used in the production of products containing probiotics.

Probiotics are termed to be a preparation of or a product containing viable, defined microorganisms in sufficient numbers that alter the microflora (by implantation or colonization) in a gastrointestinal compartment of host and thus exerting beneficial health effects [14]. A probiotic for intended use in food and pharmaceuticals must ideally meet the following criteria: preferably be of human origin, non-pathogenic in nature and safe to handle, genetically more stable, highly tolerant to gastric acidity and bile salts. Moreover, it should remain viable and active during technological hurdles, should possess a good shelf-life besides potential health benefits [15-18] to consumers.

Presently, in a health-conscious society, the demands for a health-promoting diet containing probiotics [19–21] are also increasing at an accelerated pace. Probiotics exert benefits beyond inherent nutrition if present at the level of  $10^6$ – $10^7$  live microbes per gram in product at the time of consumption [22–24]. The beneficial effects of probiotics are results of changes in intestinal health and immune system, in addition, anti-carcinogenic, antidiarrheal, antidiabetic and hypocholesterolaemic effects are also reported [25–29]. However, probiotic organisms should reach colon safely and get established there to qualify for these benefits [30]. The objective of this study was to isolate and characterize the *Lactobacillus* sp. of human origin and exploit the prevalent strain as probiotic for human health benefits.

## MATERIALS AND METHODS

All the microbiological and biochemical analysis were carried out in triplicate for ensuring the reproducibility of results.

### Collection of Samples and Isolation of Lactobacilli

Fresh human fecal samples for the isolation of lactobacilli were collected from 15 different healthy young human subjects of 6–20 years of age group that had no recent history (minimum in the last 6 months) of antibiotic therapy and/or known infection(s). Each fecal sample was taken from a single subject in a sterile container and put in a polythene bag aseptically following the standard microbiological practices recommended for sample collection. Before microbiological analysis, the sample from each age group of human subjects (6–10; 11–15 and 16–20 years) was mixed by selecting the samples in the same ratio to get an integrated sample. One gram each of five integrated fecal samples were immediately placed and suspended in 9 mL of sterilized normal saline (0.85%) and vortexed. Each homogeneous sample (1 mL) was enriched in *Lactobacillus* selection broth simulated gastro-intestinal conditions of low pH (2.0) and bile salts (1.5%) concentration for 12 h at 37 °C to screen the suitable probiotics.

The enriched fecal samples were streaked on the Petri plates containing *Lactobacillus*

selection agar with inoculating loop and incubated aerobically at 37 °C for 24–48 h and observed for the growth of lactobacilli. Based on morphology, certain colonies were picked up for further identification and characterization employing an array of biochemical tests.

### Identification and Characterization of Lactobacilli

The identification and biochemical characterization of selected isolates was done with the following tests commonly used in microbiological taxonomy [31].

### Microscopic Examination

The purity and morphological characterization of the isolates as lactobacilli was done microscopically by Gram staining for tentative identification. The colonies confirmed as Gram-positive rods were transferred to MRS broth and incubated overnight at 37 °C for further identification.

### Micrometry

Gram-positive isolates were subjected to microscopic measurements employing ocular and stage micrometers. To determine the exact size (i.e., length and width of a rod) of isolates, the slides were observed under oil-immersion and the number of ocular division occupied by each bacillus was recorded.

### Physiological Characteristics of the Selected Isolates

#### Occurrence of Growth at 15 and 45 °C

The selected isolates were checked for their ability to grow in MRS broth by incubating at 15 °C for 7 days and 45 °C for 24 to 48 h. For this, 10 mL of MRS broth in culture tubes were inoculated at the rate of 1% of lactobacilli cultures. The appearance of turbidity in tubes was considered as the ability of a culture to grow at 15 or 45 °C and results were recorded as positive or negative.

### Requirement for Oxygen

For testing the requirement for oxygen for their growth, the isolates were inoculated in MRS broth and were incubated differently, i.e., under oxygenated environment, in a desiccator with a burning candle (for microaerophilic conditions) and in an anaerobic jar with gas pack (HiMedia, Mumbai) at 37 °C for 24 h. The results were

recorded as per the effect of oxygen on growth of isolates for categorizing these as aerobe, anaerobe and microaerophilic.

### **Growth of Isolates in Presence of NaCl**

The isolates were inoculated in MRS broth containing different concentrations of NaCl (2.0, 4.0 and 6.5%) and incubated at 37 °C for 24 h.

### **Biochemical Characterization**

#### **Catalase Test**

The culture from a typical single colony was placed onto a clean grease-free glass slide. A drop of 3 % hydrogen peroxide solution was added to this culture and observed for the evolution of effervescence. The production of effervescence on the slide indicates a positive catalase reaction [32].

#### **Gas from Glucose and Gluconate**

The sterilized culture tubes having 10 mL of glucose or broth containing Durham's tube (inverted and dipped), were inoculated with isolates at a rate of 1% before incubating at 37 °C for 24 h. The presence of gas in the form of a hollow space in the inoculated Durham's tube was recorded as positive for gas from glucose or gluconate.

#### **Arginine Hydrolysis**

The arginine hydrolysis broth culture tubes were inoculated with the isolated cultures (1%) and incubated at 37 °C for 48 h. After incubation, 3–5 drops of the Nessler's reagent were added to each culture tube and observed for change in color from yellow to orange and this change was interpreted positive for arginine hydrolysis.

#### **Aesculin Hydrolysis**

The bile aesculin agar plates were streaked with the isolated cultures and incubated at 37 °C for 24 h. After incubation, the plates were examined for the presence of a dark-brown to black halo around the bacterial growth and results were recorded.

#### **Nitrate Reduction Test**

The isolates were inoculated with a loop full culture in 5 mL of trypticase nitrate broth and incubated at 37 °C for 24 h. After incubation, 0.5 mL each of sulphanic acid (0.8%, in 5N acetic acid) and  $\alpha$ -naphthylamine (0.5%, in 5N

acetic acid) were added to the culture tubes. The appearance of a red or pink color indicated a positive test for nitrate reduction.

#### **Citrate Utilization Test**

The isolates were inoculated with a loop full culture on Simmons citrate agar and incubated at 37 °C for 24 h. After incubation culture slants were observed for growth and blue coloration. The appearance of blue color from green was recorded positive for citrate utilization.

#### **Carbohydrate Fermentation**

A number of sugars were used to determine the fermentation profile of the selected isolates. For this, cultures were subjected to different sugar fermentation reactions using CHL medium for knowing the pattern of utilization by the production of acid. CHL medium was used as a basal medium and 5 mL of it was taken in each culture tube and sterilized by autoclaving at 121 °C for 15 min. One sugar disc was aseptically added to each culture tube and inoculated with 0.1 mL of inoculum from different isolates followed by incubation at 37 °C for 24–48 h. The results for change in color were recorded as positive or negative. A control using 0.1 mL sterile water as inoculum was used to compare the change in color. The various sugars used in this study to determine the fermentation pattern of isolates were arabinose; cellobiose; fructose; galactose; lactose; maltose; mannitol; mannose, melibiose; raffinose; rhamnose; salicin; sorbitol; sucrose; trehalose and xylose (7, 33).

#### **Maintenance, Propagation and Purity of Cultures**

The standard culture of *Staphylococcus aureus* as an indicator was originally procured by Dhewa *et al.* [7]. The isolated lactobacilli cultures were maintained in chalk litmus milk at refrigeration temperature after overnight growth at 37 °C. All the cultures were routinely sub-cultured at regular intervals in chalk litmus milk and were activated in MRS broth before being used. The *S. aureus* was maintained on mannitol salt agar. Before use, all the cultures were checked for purity by microscopic examinations and catalase test.

## RESULTS AND DISCUSSION

Keeping in view the intended use of isolates as probiotics, the selective pressure in form of low pH and high bile salts were extended to reduce the bulk of colonies during enrichment. Only 38 isolates based on slight variations on colonial morphology (Table 1) were picked having different color such as cream, white and greyish white appeared on *Lactobacillus* selection agar plates. The colonies were also of different shapes (i.e., pin point, circular, smooth, compact, convex), large rough and irregular sizes (i.e., in the range of 0.6 to 2.3 mm) with either entire or undulate margin. This clearly shows diversity among the isolates of same genus *Lactobacillus* leading to the idea that these were of different species for which tests were

conducted to identify and characterize the isolates. The isolates (Table 1) were purple-colored Gram-positive rods of slightly varying shapes and sizes. Each isolate was then subjected to micrometry for confirming the cell dimensions that ranged between 1.8 and 5.1  $\mu\text{m}$  for length and 0.6 and 1.2  $\mu\text{m}$  for width. Some of the lengthier and shorter isolates were observed as singles or also in chains of short length (3–5 cells). These characteristics were matching with genus *Lactobacillus* and thus preliminary results indicate that these isolates belong to the lactobacilli paving the way for further specific tests. These cultures when incubated in MRS broth at 15 °C for 7 days showed turbidity, indicating microbial growth except in few, where no growth was observed (Table 2).

**Table 1:** Colonial Morphology of *Lactobacilli* Isolates from Human Feces on *Lactobacillus* Selection Agar at 37 °C for 24–48 h.

Isolate(s)	Colonial morphology				Cell morphology			
	Colour	Shape	Size (mm)	Margin	Gram reaction	Shape	Size ( $\mu\text{m}$ )	Cell arrangement
<b>HLI01, 07, 18, 36</b>	Cream	Pin point; circular; smooth; compact and convex	0.8	Entire	Gram positive	Straight rods	0.9x3.4	Single
<b>HLI02, 04, 08, 10, 15, 16, 20, 21, 22, 24, 26, 30, 32, 34, 37</b>	White	Circular and smooth	1.2	Entire	Gram positive	Rods	0.9x3.7	Chains
<b>HLI03, 05, 06, 09, 17, 19, 23, 27, 31, 33</b>	White	Circular; large and rough	2.1	Undulate	Gram positive	Rods with rounded ends	0.9x3.8	Single
<b>HLI11, 25, 28</b>	White	Circular	0.8	Entire	Gram positive	Rods	0.8x4.7	Single
<b>HLI12, 14</b>	White	Circular	2.1	Undulate	Gram positive	rods	1.0x3.2	Single
<b>HLI13, 29, 35, 38</b>	Greyish White	Pin point; circular; smooth; compact and convex	0.6	Entire	Gram positive	Rods	0.8x3.7	Single

**Table 2: Physiological Characteristics of Lactobacilli Isolates from Human Feces with Respect to Growth Temperature, Oxygen Requirement and NaCl Tolerance.**

Isolate(s)	Physiological characteristics							
	Growth at temperature (°C)		Oxygen requirement			Effect of NaCl (%)		
	15	45	Aerobic	Microaerophilic	Anaerobic	2.0	4.0	6.5
<b>HLI01, 07, 18, 36</b>	-	+	+	+	+	+	+	-
<b>HLI02, 04, 08, 10, 15, 16, 20, 21, 22, 24, 26, 30, 32, 34, 37</b>	-	+	+	+	+	+	+	-
<b>HLI03, 05, 06, 09, 17, 19, 23, 27, 31, 33</b>	+	-	+	+	+	+	-	-
<b>HLI11, 25, 28</b>	+	+	+	+	-	+	-	-
<b>HLI12, 14</b>	+	+	+	+	+	+	+	-
<b>HLI13, 29, 35, 38</b>	-	+	+	+	+	+	-	-
Isolate(s)	Biochemical characterization							
	Catalase test	Gas from glucose	Gas from gluconate	NH <sub>3</sub> from Arginine hydrolysis	Aesculin hydrolysis	Nitrate reduction	Citrate Utilization	
<b>HLI01, 07, 18, 36</b>	-	-	+	-	+	-	+	
<b>HLI02, 04, 08, 10, 15, 16, 20, 21, 22, 24, 26, 30, 32, 34, 37</b>	-	-	+	-	+	-	+	
<b>HLI03, 05, 06, 09, 17, 19, 23, 27, 31, 33</b>	-	+	-	V	V	-	+	
<b>HLI11, 25, 28</b>	-	-	+	W	+	-	-	
<b>HLI12, 14</b>	-	-	+	-	+	-	-	
<b>HLI13, 29, 35, 38</b>	-	+	+	+	-	-	-	

+ = growth observed (physiological); - = no growth observed (physiological); + = substrate utilization and/ or enzyme produced (biochemical); - = no substrate utilization and/ or enzyme produced (biochemical); v = variable reaction and w = weak reaction.

Similarly, when isolates were incubated at 45 °C for 24–48 h the turbidity was observed in culture tubes containing half of the genera. The results of variations in growth at 15 and 45 °C are in accordance with earlier workers [33, 34]. When isolated cultures were incubated in MRS broth under aerobic condition at 37 °C for 24 h, majority of the isolates showed turbidity, while under microaerophilic condition, the turbidity was observed in all culture tubes. On the other hand, under anaerobic condition five culture tubes showed no turbidity (Table 2). This kind of growth pattern proves the facultative nature

of lactobacilli that have the ability to grow with and without oxygen efficiently making it an ideal probiotic candidate for fermentation processes under varying availability of oxygen.

When isolates were incubated in MRS broth containing different NaCl concentration (2.0, 4.0, 6.5%) at 37 °C for 24 h, at the concentration of 2.0% NaCl, the growth was observed for all the lactobacilli isolates, while in case of 4.0% no growth was observed for 13 isolates (Table 2). The similar variations in results were also reported by Wang *et al.* [34].

In contrast, at 6.5% NaCl concentration, the turbidity was observed only in two isolates and no growth was observed in rest of the culture tubes indicating the inhibitory effect of high concentration of salt, an activity normally occurring in halophiles, and hence, other groups of microbes may not have the ability to counter the high osmotic pressure. Since catalase, an extracellular enzyme secreted by some microorganisms, is a characteristic of pathogenic bacteria hence, its presence or absence can be an important diagnostic tool. In the present study, all the 38 lactobacilli isolates were catalase-negative while the indicator organism (i.e., *S. aureus*) was catalase-positive (Table 2). These results obtained for catalase production further support the identification of lactobacilli and are in accordance with that of earlier workers [7, 9, 33]. Table 3 also represents that the

pattern of gas produced by the isolates in MRS broth containing glucose as the sole source of carbon. The previous reports for glucose fermentation by lactobacilli [7, 33, 34] support the results in this study. It was also observed that all the isolates tested could not produce gas after incubation for 24 h at 37 °C in MRS broth containing gluconate as the sole source of carbon except few isolates. The variable results for gluconate utilization by lactobacilli as an important characteristic were in agreement with Holt *et al.* [35]. Arginine broth was used to check the ability of the isolates to hydrolyze arginine resulting in the production of ammonia. In arginine hydrolysis test, the isolates HLI13, 29, 35 and 38 showed positive reaction and remaining isolates showed either weak hydrolysis or a variable reaction, while few isolates could not hydrolyze arginine (Table 2).

**Table 3: The Biochemical Characteristics of Lactobacilli Isolates from Human Feces.**

Isolate(s)	Ara	Cel	Fru	Gal	Lac	Mal	Mani	Mano	Mel	Raf	Rha	Sal	Sor	Suc	Tre	Xyl	Species Identified
HLI01, 07, 18, 36	V	+	+	+	+	+	+	+	+	+	-	+	+	+	+	v	<i>L. plantarum</i>
HLI02, 04, 08, 10, 15, 16, 20, 21, 22, 24, 26, 30, 32, 34, 37	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	-	<i>L. casei</i>
HLI03, 05, 06, 09, 17, 19, 23, 27, 31, 33	-	+	+	W	+	-	+	+	-	+	+	+	-	+	-	+	<i>L. acidophilus</i>
HLI11, 25, 28	-	-	+	+	+	-	-	+	-	-	-	-	-	+	-	-	<i>L. helveticus</i>
HLI12, 14	-	-	+	+	+	+	+	+	-	+	+	+	-	+	-	+	<i>L. rhamnosus</i>
HLI13, 29, 35, 38	V	v	+	+	+	+	+	-	+	+	-	-	-	+	v	v	<i>L. fermentum</i>

Ara = Arabinose; Cel = Cellobiose; F = Fructose; G = Galactose; Lac = Lactose; Mal = Maltose; Man = Mannitol; Man = Mannose, Mel = Melibiose, Raf = Raffinose; Rha = Rhamnose; Sal = Salicin; Sor = Sorbitol; Suc = Sucrose; Tre = Trehalose; Xyl = Xylose; + = ferment sugar; - = do not ferment sugar; V = variable reaction, W = weak reaction

Arginine hydrolysis is used as a distinguished characteristic of lactobacilli and is indicated by change in color from yellow to orange in presence of ammonia that brings the pH of the broth to alkaline. With respect to arginine hydrolysis, the results obtained in

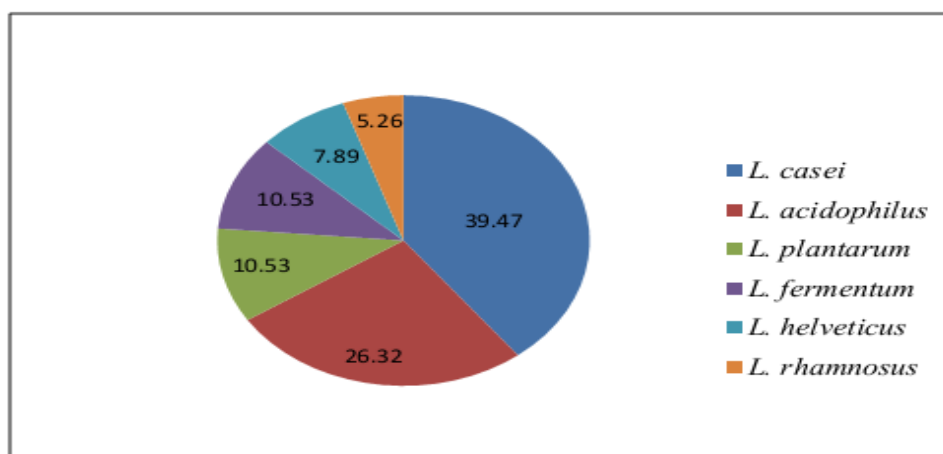
the present study are supported by Dhewa *et al.* [7]. The esculin hydrolysis test was also used to check the ability of the isolated organisms to hydrolyze the glycoside esculin to esculetin and glucose in the presence of 10–40% bile. The esculetin combines with

ferric ions in the medium to form a black complex. In this test, some isolates were negative and some positive that showed dark brown/black color in culture tubes after incubation (Table 2). The results of esculin hydrolysis are nearly in sharp contrast to that of arginine hydrolysis and gas from glucose, though most strains showed variable reaction. For identification of lactobacilli, other researchers also obtained data of similar nature [33, 34]. During nitrate reduction test, all the isolates of present study showed negative reactions as there was no formation of red/pink color after incubation of isolates in nitrate broth at 37 °C for 24 h (Table 2). The negative results for nitrate reduction further confirms the isolates as lactobacilli [7, 33, 35], a characteristic used in Bergey's manual of determinative bacteriology. In dairying, the citrate utilization and development of flavor is an important aspect for product manufacturing. Moreover, it is also used as a characteristic for the bacterial identification as certain bacteria can utilize citrate by secreting enzyme citrate permease and citrase while producing diacetyl as an end-product. Following incubation on Simmon's citrate agar, the citrate-positive isolates were identified by the presence of growth on slants, accompanied by blue coloration. From Table 3, citrate utilization was confirmed in majority of the isolates. For citrate utilization, the results reported by Wang *et al.* [34] are in contrast with the authors' data.

For determining the sugar utilization profile of the isolates deemed to be the lactobacilli after their growth on *Lactobacillus* selective MRS

agar specific for this group of bacteria, the tests for fermentation were performed using CHL basal media with the different sugar discs. A total of 16 sugars used for the complete identification of lactobacilli up to species level were used.

The different isolates showed different types of sugar utilization patterns, as some culture tubes containing the culture and sugar turned yellow whereas the others remained brown, indicating the test as positive and negative, respectively, when these sugar fermentation patterns were compared with those given for *Lactobacillus* species in the *Bergey's Manual of Determinative Bacteriology* [35]. The isolates were identified as *L. plantarum* (HLI01, 07, 18 and 36), *L. fermentum* (HLI13, 29, 35 and 38), *L. helveticus* (HLI11, 25 and 28), *L. rhamnosus* (HLI12 and 14), *L. casei* (HLI02, 04,08, 10, 15, 16, 20–22, 24, 26, 30, 32, 34 and 37) and *L. acidophilus* (HL03, 05, 06, 09, 17, 19, 23, 27, 31 and 33) from human fecal sample. Overall, *L. casei* was predominant in human feces followed by *L. acidophilus* among the total isolates (Figure 1). Dimitrov *et al.* [36] also reported the presence of these species in human feces. The data obtained for genus and species identification comprising a number of morphological, physiological, biochemical and sugar utilization pattern tests were also subjected to analysis through software called PIBWIN [37] to confirm the identification in addition to the matching with *Bergey's Manual of Determinative* were in agreement with Dimitrov *et al.* [36].



**Fig. 1:** Prevalence (%) of *Lactobacillus* Species in Feces of Healthy Human Beings.

## CONCLUSIONS

From the healthy human feces, a total of 38 *Lactobacillus* isolates were distributed among six species and identified as *L. plantarum*, *L. casei*, *L. rhamnosus*, *L. fermentum*, and *L. acidophilus*, *L. helveticus*, where *L. casei* was the most prevalent one that could be exploited as a potential probiotics. After ensuring that out of 38 isolates all were found to be distributed over six different species of lactobacilli, further work was continued with only six isolates randomly selected among the same species. *Lactobacillus* species are commonly present in human fecal samples [10].

Several properties beneficial for human health are related to the activity of *Lactobacillus* strains in the colon [11–13]. Lactobacilli of human intestinal origin with potential health-associated properties could be used in the production of products containing probiotic bacteria. The authors isolated lactic acid bacteria from the feces of healthy donors and species isolated probiotic after investigating its beneficial characteristics.

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