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In Vitro Cholesterol Assimilation and Functional Enzymatic Activities of Putative Probiotic *Lactobacillus* sp. İsolated from Fermented Foods/ Beverages of North West India

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Abstract

In the present investigation 5 putative probiotic lactobacilli strains isolated from ethnic fermented foods from tribal regions in North West India were explored for their capabilities to assimilate cholesterol in the presence of various bile salts. In addition, β-Galactosidase, β-Glucosidase, protease, amylase, phytase activity of lactobacilli strains were also evaluated. All the lactobacilli strains were able assimilate cholesterol and it was found to be significantly higher in the presence of bile salts. The highest cholesterol removal (47.70 µg/ml) was observed by *L. casei* PLA12 and *L. casei* PLA5 in the presence of cholic acid. Cholesterol removal by resting and dead cells was also investigated and it was found to be 2.08 and 4.74 µg/ml respectively. *L. casei* PLA5 produced highest β-glucosidase (14.97 ± 0.2 U/mg dcw); proteolytic (26 ± 0.5 U/mg) and phytase (23 ± 0.5 mm) activity and *L. casei* PLA12 was reported to produce highest β-galactosidase (0.80 ± 0.01 U/mg dcw) and amylase (13.06 ± 0.25 U/mg) activity. Moreover, none of the strains showed haemolytic, urease and bile salt hydrolase activity. Screening for enzymatic activities among lactic acid bacteria is important for the preparation and improvement of biological activity of food product and in the selection of microorganisms used in the growing industry of functional foods and beverages. This paper is the first report on functional characterization on lactic acid bacteria from some lesser-known ethnic fermented products of the North West Himalayas.

Keywords: *Lactobacillus*; Cholesterol assimilation; Functional enzymes; Fermented foods; Ethnic foods

Introduction

Cholesterol is an essential structural component of animal cell membrane. However, elevated serum cholesterol is associated with major risk for coronary heart diseases. Consumption of probiotics products has been proposed to lower serum cholesterol. It has also been proposed by many studies that probiotic microbes directly assimilate cholesterol. Adhesion of cholesterol to cell surface and incorporation of cholesterol into cellular membrane are the most frequently suggested mechanisms of bacteria activity on cholesterol level *in vitro* [1]. The use of probiotic bacteria to reduce serum cholesterol levels has attracted wide attention of the consumers, researchers and the physicians. Therefore, interest in the use of probiotics for lowering blood cholesterol levels has been increasing from past few decades.

It was postulated that cholesterol assimilation was associated with the presence of bile salts, and it increased with increasing concentration of bile salts in medium. Significant higher cholesterol assimilation in the presence of bile was reported in various *in vitro* studies [2,3]. Cholesterol adhered to the bacterial cells would be less available for absorption from the intestine into the blood. It has been also demonstrated that the ability of some strains to take up cholesterol was growth associated because resting cells did not exhibit interactions with cholesterol.

Utilization of indigestible fibers and oligosaccharides, not digestible by human enzymes, has been recognized as an important attribute of probiotics [4]. Lactobacilli have been reported to produce several functional enzymes exhibiting beneficial impact on human health by digesting indigestible fibers and complex carbohydrates. A health benefit can also arise from the ability of an ingested microorganism to contribute an enzyme to the small intestine e.g. β-galactosidase (lactase) that many adults lack [5]. β-glucosidases hydrolyses cellulose which is important for both industry and human health as humans are unable to digest cellulose due to the low levels of cellulases in the gut. It is also used in the production of ethanol from cellulose and in food fermentation to release the aromatic compounds [6]. Phytate acts as an antinutrient which binds with proteins, lipids, carbohydrates, and metal ions. Phytate degrading activity in humans is relatively low (mainly in the small intestine), so phytate degrading enzymes (phytase) from microbial source are required.

Species of *Lactobacillus* that produce functional enzymes such as β-galactosidase, β-glucosidase, amylase, protease and phytase could have an important impact on human health. However, the production capacity of such hydrolysing enzymes by *Lactobacillus* is strain specific. Therefore, the aim of this study was to evaluate the *in-vitro* cholesterol assimilation and enzymatic activities (β-galactosidase and β-glucosidase activities, proteolyitic, amylase and phytase capacities) of selected five putative probiotic strains of LAB in order to select strains of technological interest that might be used as starters in the manufacture of functional fermented foods.

Materials and Method

Lactobacillus strains were isolated from indigenous fermented foods/beverages (*chhura, chhang, femur, angoori*, and *daru*) of tribal regions of Himachal Pradesh, India. Lactobacilli were isolated by spread plating on MRS (de Mann Rogosa Sharpe) agar (Himedia), and the plates were incubated at 30°C for 24-48 hr. Colonies with different morphologies on the MRS agar plate were selected, identified and further sub-cultured in order to obtain a pure colony. Glycerol stock of isolates were prepared and stored at -80°C. Provisional or tentative identification of genera was made by Gram staining, cell morphology and catalase reaction. Selected five *Lactobacillus* strains were further identified by sequence analysis of the amplified chromosomal 16S rDNA and these sequences were deposited in the National Center for Biotechnology Information (NCBI) nucleotide sequence database. GenBank accession number for the 16S rRNA gene sequence of five strains are KJ726650, KM410930, KJ726656, KJ726658 and KM410931 for *L. casei* PLA5, *L. brevis* PLA7, *L. casei* PLA12, *L. brevis* PLA14 and *L. kefiri* PLA15 respectively. These Lactobacilli strains were found to have potential probiotic properties (data not shown).

In-vitro cholesterol assimilation

Cholesterol assimilation of LAB isolates was done by o-phthalaldehyde method [7]. Freshly prepared MRS broth was supplemented with 0.30% oxgall, cholic acid and taurocholic acid. Cholesterol (polyoxyethanyl cholesteryl sebacate) was filter-sterilized by using a 0.45 mm filter (Millipore Corporation, Bedford, MA) and added to the broth at a final concentration of 100 µg/ml, inoculated with each isolates (at 1%), and incubated at 30°C for 20 hr. After the incubation period, cells were centrifuged and the remaining cholesterol concentration in the broth was determined using a colorimetric method. All experiments were replicated twice.

Cholesterol removal by dead and resting cells

Cholesterol removal by dead and resting cells was done according to Liong and Shah [8]. Cholesterol assimilation by growing, resting and dead cells was expressed in dry weight to obtain uniformity in all treatments. The following equation was used:

Cholesterol assimilation = (C1-C2) / (W2-W1),

Where C1 and C2 were the amount of cholesterol present in the fermentation broths at time=0 and 20 hr, respectively, and W1 and W2 were the dry weight of the individual culture at time=0 and 20 hr.

Statistical analysis was performed on the data by SPSS 19.0 Bivariate Correlation Analysis (SPSS Inc., Chicago, Ill., USA). All experiments were repeated three times. The Tukey's test was used for paired comparisons.

Enzymatic activity of *Lactobacillus* species

β-Galactosidase activity: Primary (qualitative) screening of β-galactosidase activity was done on MRS agar plates containing 60 µl of 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D galactopyranoside) and 40 µl of 1 mg/ml IPTG (iso-propyl-thio-β-D-galactopyranoside) solution as an inducer [9]. The appearance of the characteristic blue colonies on MRS agar supplemented with X- gal and IPTG indicated the presence of β-galactosidase activity.

For secondary (quantitative) screening, β-galactosidase activity was determined according to the method of Bhowmik and Marth [10]. Protein concentrations in cell extracts were measured using the Bradford method [11]. One unit of the enzyme activity was defined as the amount of β-galactosidase that released 1 µmol of o-nitrophenol from the substrate ONPG per millilitre per minute under assay conditions.

Proteolytic activity: Primary (qualitative) screening of LAB for their proteolytic activity on skim milk agar was done according to Essid et al. [12]. The proteolytic activity was determined by the measurement of the diameter of clear zones around the spots (mm). Secondary (quantitative) screening was done according to Manachini et al. [13]. Protein concentrations in cell extracts were measured using the Bradford method [11]. One unit of the enzyme activity is defined as the amount of enzyme required to release one µg of tyrosine/ml under assay conditions.

β-Glucosidase activity: β-glucosidase was determined by monitoring the rate of hydrolysis of ρ-nitrophenyl-β-D-glucopyranoside (β-PNPG) according to Mahajan et al. [14]. Protein concentrations in cell extracts were measured using the Bradford method [11]. One unit of the enzyme activity was defined as the amount of β-glucosidase that released 1 µmol of p-nitrophenol from the substrate p-NPG per millilitre per minute under assay conditions.

Amylase activity: Amylase activity was assayed by employing the 3, 5-dinitrosalicylic acid (DNS) method given by Bernfeld [15]. Protein concentrations in cell extracts were measured using the Bradford method [11].One unit of enzyme activity was defined as the amount of enzyme required to release one µmole of enzyme/mg/min under the assay conditions.

Phytase activity: The phytase activity of LAB isolates was detected using a specific method described by Bae et al. [16]. To eliminate false positive results caused by microbial acid production, the petri plates were flooded with 2% (w/v) aqueous cobalt chloride solution. After 5 min of incubation at room temperature, the cobalt chloride solution was removed and then freshly prepared solution containing equal volumes of a 6.25% (w/v) aqueous ammonium molybdate solution and a 0.42% (w/v) ammonium vanadate solution were added. Following 5 min of incubation, the ammonium molybdate/ammonium vanadate solution was removed and the plate was examined for zones of phytate hydrolysis.

Urease activity: Urease assay was done according to the method given by Fawcett and Scott [17]. Protein concentrations in cell extracts were measured using the Bradford method [11]. One unit of urease activity is defined as that amount of enzyme, which released 1 µmol of ammonia per min under standard assay conditions.

Haemolytic activity: *Lactobacillus* were sub-cultured in MRS and then streaked on Columbia agar plates, containing 5% of sheep blood and incubated for 24 h at 30°C. The isolates that produced green-hued zones around the colonies or did not produce any effect on the blood plates were considered non hemolytic. The isolates showing blood lysis zones (β-hemolysis) around the colonies were classified as hemolytic [18].

Bile salt hydrolase activity: Salt deconjugation ability of lactobacilli was determined by the qualitative direct plate assay [19]. Overnight cultures were spotted on MRS agar plates containing 0.37 g/L $CaCl_2$ and 0.5% sodium salt of taurodeoxycholic acid (TDCA). Plates were incubated at 30°C for 72 hr. Presence of halos around colonies or a white opaque colony indicated positive bile salt hydrolase activity.

Results and Discussion

In-vitro cholesterol assimilation

The *in-vitro* studies on cholesterol removal by lactobacilli have been considered as an important parameter for the selection of probiotic strains with diverse health promoting benefits. Levels of cholesterol assimilation in the presence of different bile salts during 20 hr of growth of the LAB isolates varied among strains ($p<0.05$) and ranged from 18.18-47.70 µg/ml (Table 1). In broth containing bile salts, overall cholesterol removal was observed to be significantly higher than control. *Lactobacillus casei* PLA12 exhibited greater cholesterol removal in the media containing cholic acid (which is deconjugated bile) which is 47.70 µg/ml, in contrary to the taurocholic acid (conjugated bile) and oxbile (contain both conjugated and deconjugated bile) which assimilated 27.52 and 30.68 µg/ml cholesterol respectively and this result was in agreement with the reports of Liong and Shah [8]. One of the possible reasons of higher assimilation in deconjugated bile might be due to greater solubility, detergent activity and more inhibitory effect of conjugated bile (taurocholic acid) towards lactobacilli compared with deconjugated bile (cholic acid) and oxgall [8].

Isolates	Control	Cholic Acid	Taurocholate (TC)	Oxbile	Dead cells	Resting cells
L. casei PLA5	37.34 ± 0.11^{qC}	46.84 ± 0.11^{qD}	28.65 ± 0.07^{vA}	33.26 ± 0.05^{jklB}	2.08 ± 0.02^{mno}	4.74 ± 0.05^{qr}
L. brevis PLA7	29.75 ± 0.10^{nB}	39.59 ± 0.05^{oD}	20.11 ± 0.03^{tA}	$21.93 \pm 0.05^{bcdefghijklB}$	1.25 ± 0.05^{efghijk}	4.23 ± 0.07^{opq}
L. casei PLA12	33.52 ± 0.06^{pC}	47.70 ± 0.03^{qD}	27.51 ± 0.07^{vA}	30.68 ± 0.07^{hijklB}	1.42 ± 0.08^{fghijkl}	4.60 ± 0.13^{pqr}
L. brevis PLA14	30.70 ± 0.04^{noC}	33.80 ± 0.04^{nD}	28.25 ± 0.06^{vB}	$26.58 \pm 0.16^{fghijklA}$	0.92 ± 0.06^{bcdefg}	2.21 ± 0.10^{cdefgh}
L. kefiri PLA15	26.58 ± 0.06^{mB}	29.93 ± 0.11^{mC}	18.18 ± 0.09^{sA}	36.02 ± 0.53^{lD}	1.13 ± 0.07^{defghi}	2.28 ± 0.03^{defgh}

Table 1: Cholesterol removal (µg/ml) by growing, resting and heat-killed cells of some LAB isolates. Values represented as mean ± SD; for each column, different subscripts lowercase letters, significantly different at $p<0.000001$ for Cholic Acid; for Taurocholate at $p<0.001$; for Control at $p<0.0001$ and for Oxbile at $p<0.0001$; and upper case letters for each row indicate significantly different at $p<0.05$ as measured by 2-sided Tukey's HSD between different isolates and cholesterol respectively. Values represented as mean ± SD; for each column, different subscripts lowercase letters indicate significantly different at p ± 0.00001 for dead and resting cells, as measured by 2-sided Tukey's HSD between isolates.

Significantly higher cholesterol assimilation by growing cells was reported in comparison to their resting and dead counterparts; however, there was no significant difference ($p<0.05$) in the level of cholesterol removal by resting and dead cells. Heat-killed (dead) and resting cells showed a small degree of cholesterol removal, ranging from 0.92 to 2.08 µg/ml for dead cells and 2.21 to 4.74 µg/ml for resting cells as compared with growing cells. The capability of dead and resting stage *Lactobacillus* to assimilate some cholesterol indicated that cholesterol might also be removed via binding on the cell surface [8].

Enzymatic activities of *Lactobacillus*

β-Galactosidase and β-glucosidase: All the 5 *Lactobacillus* produced blue colour colonies on X-Gal plates indicating the presence of β-galactosidase enzyme (Figure 1).

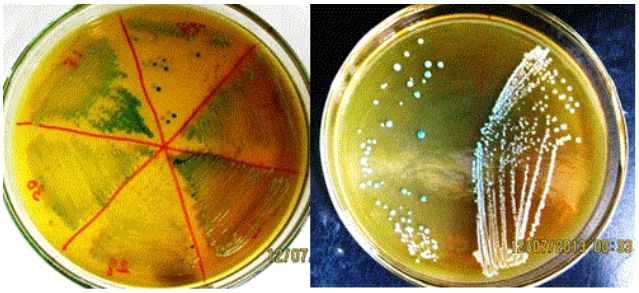

Figure 1: Blue colonies of *Lactobacillus* sp. on X-gal MRS agar plates showing β-Galactosidase activity.

Secondary (quantitative) screening was done by ONPG method, specific activity of β-galactosidase ranged from of 0.1 to 0.8 (U/mg dcw) in 5 *Lactobacillus* strains shown in Table 2. The activity of β-galactosidase was highest in *L. casei* PLA12 (0.8 U/mg dcw). Results obtained by X-gal and ONPG methods confirmed each other which were similar to the previous reports [20,21]. In conclusion, LAB possesses β-galactosidase, and utilizes lactose slowly because of the possible slow transport of lactose into the microorganism.

β-D-glucosidase activity plays a substantial role in the interaction with the human host and releases a wide range of plant secondary metabolites (having dietary and sensory properties) from their β-D-glucosylated precursors [22]. *L. casei* PLA5 exhibited the highest β-glucosidase activity (14.97 U/mg dcw) and lowest was observed in *L. casei* PLA12 ranging 1.20 U/mg dcw.

Proteolytic activity: LAB possesses a complex system of proteinases and peptidases which enable them to use casein as a source of amino acids and nitrogen. The first step in casein degradation is mediated by cell wall located proteases, which cleave casein to oligopeptides [23]. Evaluation of proteolytic activity of LAB isolates were evaluated and best activity was observed in *L. casei* PLA5. The proteolytic activity of LAB isolates was reported by Scolari et al. [24]. Primary screening on the skimmed agar plates showed zone of hydrolysis by all tested isolates (Figure 2). In Quantitative screening, protease activity of all *Lactobacillus* ranged from 3.92-26 U/mg, significantly highest specific activity was measured in *L. casei* PLA5 (26 U/mg) and lowest was observed in *L. brevis* PLA7 (3.92 U/mg) as given in Table 2.

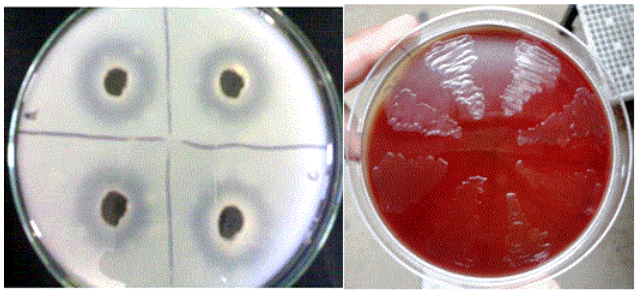

Figure 2: Plates showing proteolytic (left) and ɤ-haemolysis (right) activity of *Lactobacillus* sp.

Amylase activity: Amylolytic LAB utilizes starchy biomass and converts it into lactic acid in single step fermentation. Although most LAB are unable to degrade starch because of the lack of the amylolytic activity, a few isolated from cereal based fermented foods/beverages exhibit this activity and are qualified as amylolytic lactic acid bacteria (ALAB) which are able to decompose starchy material through the amylase production during the fermentation processes [25]. *Lactobacillus* were screened for amylase production and specific activity of all isolates varied from 0.01-0.57 U/mg. Some of the LAB isolates tested exhibited amylase activity and same was reported by Fossi and Tavea [26]. Highest specific activity was observed in *L. casei* PLA12 (0.5 U/mg) and lowest was observed in *L. brevis* PLA14 which was measured to the 0.1 U/mg. *L. brevis* PLA7 does not showed amylase activity (Table 2).

Isolates	β-Galactosidase (U/mg dcw)	β-Glucosidase (U/mg dcw)	Proteolytic (U/mg)	Amylase (U/mg)	Phytase (mm)
L. casei PLA5	0.10 ± 0.01	14.97 ± 0.2	26 ± 0.5	0.16 ± 0.02	23 ± 0.5
L. brevis PLA7	0.40 ± 0.02	3.08 ± 0.08	3.92 ± 0.17	ND	ND
L. casei PLA12	0.80 ± 0.01	1.20 ± 0.07	13.06 ± 0.25	0.57 ± 0.03	24 ± 0.45
L. brevis PLA14	0.30 ± 0.01	3.98 ± 0.1	4.44 ± 0.2	0.01 ± 0.01	ND
L. kefiri PLA15	0.20 ± 0.02	1.95 ± 0.06	7.84 ± 0.25	0.10 ± 0.01	ND

Table 2: Enzymatic activities of LAB isolates. The results are expressed as the mean of triplicate samples from three independent experiments ± SD. ND-Not determined.

Phytase activity: Phytate is a principal storage form of phosphorus found in cereals, legumes, and nuts, and acts as an antinutrient binding with proteins, lipids, carbohydrates, and metal ions like zinc, iron, calcium, and magnesium. Outsourcing of phytate degrading enzymes are required because of relatively low amount of phytase in humans mainly in the small intestine [27]. Microbial sources of such functional enzymes could be the most promising sources for human health. Although microbial phytases are considered of a great value in upgrading the nutritional quality of plant foods, very few studies have dealt with lactic acid bacteria.

Lactobacillus were screened for their phytase activity on the sodium phytate agar plates and all isolates showed clear, opaque zones as shown in Figure 3. To eliminate false positive results caused by microbial acid production, the zone of clearing remained around all the positive isolates confirming the existence of phytase activity in the solid medium. Highest activity was observed in *Lactobacillus casei* PLA12 (24 mm), followed by *Lactobacillus casei* PLA5 (23 mm) zones on sodium phytate plates was similar to the studies reported by Raghavendra and Halami [28], De Angelis et al. [29].

None of the isolates from fermented foods and beverages in the present investigation displayed bile salt hydrolase activity which was similar to the findings reported Quezada et al. [30]. Tanaka et al. [31] studied more than 300 lactic acid bacterial strains and reported that BSH activity was found primarily in organisms isolated from the gastrointestinal tracts of mammals, while organisms isolated from fermented milk preparations and vegetables did not exhibit BSH activity.

Haemolysis is a known virulence factor among pathogenic microorganisms. The examined isolates in the present investigation were ɤ-haemolytic (i.e., no haemolysis) on blood agar plates (Figure 2). Santini et al. [32] and Cosentino et al. [33] have observed that none of the probiotic strains possess haemolytic activity and reported that haemolysis is rarely present in fermented food LAB.

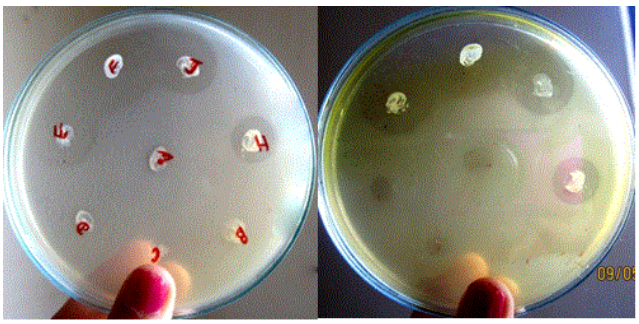

Figure 3: Zone of phytate hydrolysis by *Lactobacillus sp.* before the counterstaining treatment (left petri plate) and after the counterstaining treatment (right petri plates).

Conclusion

Results of present study demonstrate the capability to assimilate cholesterol and useful enzymatic activities of lactobacilli. These

potential strains could be further utilized for preliminary screening in order to identify potentially functional lactobacilli suitable for human use. The two selected strains (*L. casei* PLA5 and *L. casei* PLA12) which possess the good cholesterol-lowering and enzymatic property have a potential to be used as a starter culture for the preparation of functional fermented probiotic foods. However, the production of these lesser-known unexplored ethnic food products could be commercialized for their health benefits.

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