

PROBIOTIC CHARACTERIZATION OF LACTIC ACID BACTERIA ISOLATED FROM FERMENTED FOODS AND PARTIAL PURIFICATION OF ITS BACTERIOCIN

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ABSTRACT

The present research was focused on probiotic characterization of lactic acid bacteria from fermented foods and beverage of Kinnaur and partial purification of bacteriocin. Lactic acid bacterial isolates were examined *in-vitro* for potential probiotic properties based on their low pH tolerance, bile-salt resistance, production of antimicrobial substances, exopolysaccharide production, proteolytic activity and haemolytic activity. LAB isolates L5, L1 and L10 showed high acid tolerance at pH 3.0 and 2.0 and withstand high bile concentration as compared to the control (pH 7.0). All LAB isolates were able to show antimicrobial activity against some pathogens. None of them showed haemolytic activity. Putative probiotic isolate L5 was partially purified for the bacteriocins production and it was found to have molecular weight of approx. 10 kD. The selected isolate L5 has all the potential probiotic properties. Therefore, isolated strain is thought to survive through the intestinal ecosystem and is considered to be suitable as a biopreservatives and can be used as development of various probiotic food products.

Keywords: Probiotic, Lactic Acid Bacteria, Bile Tolerance, Acid Tolerance, Bacteriocins

INTRODUCTION

Probiotics are “live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host” (WHO/FAO, 2001). Lactic acid bacteria (LAB) are most commonly studied probiotic for the past few decades and they produce desirable microflora of the gastro intestinal tract (GIT) thus called ‘Generally Regarded As Safe’ (Tannock, 1997). LAB present in fermented foods have been associated with various probiotic properties such as improvements in lactose intolerance, increase in natural resistance to infectious disease in the gastrointestinal tract, suppression of cancer, improved digestion, prevention of urogenital infections and reduction in cholesterol level in the serum (Liong and Shah, 2005). Significant contribution in the production of traditional fermented foods and beverages and its importance as functional and probiotic foods have been reported in various studies (Kumari *et al.*, 2016b; Angmo *et al.*, 2016).

One of the concerns in food industry is the contamination by pathogens causing various food borne diseases like diarrhea (Parada *et al.*, 2007). Among biopreservatives, bacteriocins have received increasing attention due to unique properties of inhibiting food-borne pathogens and spoilage causing microorganisms (Klaenhammer *et al.*, 1993).

Bacteriocins produced by LAB have been evaluated for the preservation of milk, meat and vegetables due to their capacity to inhibit the growth of narrow range of pathogenic, spoilage causing bacteria (Gautam and Sharma, 2009).

In the Indian subcontinent, making and use of traditional fermented food and beverages using local food crops and other biological resources is very common among natives of Himalayas (Roy, 2004). Traditional fermented foods and beverages are very popular in the tribal and rural areas of Himachal Pradesh. The fermented products that are unique to the tribal and rural belts of Himachal are *Bhaturu*, *Siddu*, *Chilra*, *Churpe*, *Manna*, *Marchu*, *Bagpinni*, *Seera*, *Dosha*, *Sepubari*, *Sura*, *Chhang*, *Lugri*, *Daru*, *Angoori* and *Behmi* (Navdeep *et al.*, 2004). Production of these lesser-known ethnic fermented products is mainly restricted to the unorganized sectors and individual households, so the indigenous knowledge for production of variety of ethnic fermented milk products needs to be well documented (Dewan and Tamang, 2007).

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However, little effort has been made for isolation and characterization of lactic acid bacteria (LAB) with probiotic properties from the traditional fermented foods and beverages of Kinnaur. Therefore, present work is designed to study the lactic acid bacteria of indigenous fermented foods and beverages (*Chhang* and *Churpe*) of Kinnaur and to exploit its probiotic potential.

MATERIALS AND METHODS

Isolation and Culture Conditions

A total number of 10 LAB isolates (Table 1) were isolated from two traditional fermented food and beverage (*Chhang* and *Churpe*) which were collected from different regions of Kinnaur district of Himachal Pradesh. 10 g/ml of each sample was diluted with 90 ml of a sterile saline solution (0.9% NaCl) and homogenized for 60 s. The homogenates were serially diluted in saline solution and plated onto MRS (de Man Rogosa Sharpe, Hi-Media) medium and incubated at 30 °C for 24-48 h. Isolates were presumptively identified as LAB by culturing on MRS agar and examined for colony and cell appearance, catalase activity, motility, Gram stain and endospore formation. All LAB isolates were grown routinely in Ellikerbroth, and stored in MRS agar plates at 4 °C.

Biochemical and Physiological Characterization

Biochemical characteristics were observed included gas production, sugar fermentation, resistance to pH and NaCl. Lactobacilli were inoculated into carbohydrate fermentation medium containing different sugars (sucrose, raffinose, trihalose, xylose, maltose, fructose, galactose, ribose, dextrose, mannitol, starch and lactose) for determination of sugar fermentation pattern. Growth of lactobacilli at different pH values (2.5, 3.5, 8.5 and 9.5), NaCl concentration (2, 4 and 6.5 %) and temperature (15, 37 and 45 °C) were also checked.

Probiotic Characterisation

Tolerance to Acidic pH, Bile Salt

Acid (pH 2, 3 and 7) and bile (0.5, 1 and 2 %) tolerance at 3 h and 12 h, respectively was assayed by the method of Maragkoudakis *et al.*, (2006). Survival of LAB isolates in acid and bile was calculated in terms of Log cfu/mL.

Antibiotic Susceptibility

Antibiotic resistance of LAB were determined on MRS agar by disk diffusion method (Turchi *et al.*, 2013). The following antimicrobial agents viz inhibitors of cell wall synthesis- penicillin G (P; 10 Units), ampicillin (A; 10 µg), vancomycin (VA; 30 µg); inhibitor of nucleic acid synthesis- norfloxacin (NX; 10 µg); inhibitor of protein synthesis-chloramphenicol (C; 30 µg), erythromycin (E; 15µg); inhibitor of folate synthesis- Co-trimoxazole (COT; 25 µg) have been tested.

Antimicrobial Activity

Antimicrobial effects of LAB isolates on Gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram negative bacteria (*Shigelladysenteriae* and *Escherichia coli*) as test organisms were determined by the well diffusion assay (Schillinger and Lucke, 1989). Diameter of the inhibition zone was measured in millimetres (mm).

Haemolytic Activity (Lombardi *et al.*, 2004)

Blood haemolysis was evaluated by incubating the Columbia agar plates (Oxoid) supplemented with 5% sheep blood at 37°C for 24 h.

Exopolysaccharide Production

Exopolysaccharide production was evaluated according to the procedure of Mora *et al.*, (2002).

Proteolytic Activity

The isolates were qualitative tested for their proteolytic activity on skim milk agar according to Essid *et al.*, (2009). The proteolytic activity was determined by the measurement of the diameter of clear zones around the spots (mm).

Production of Crude Bacteriocin and Detection of its Antimicrobial Activity

LAB isolates were inoculated individually in 1000 ml Elliker's broth (pH 6.8) and incubated for 48h at 30°C in static incubator with sealed plugs. For extraction of bacteriocin, a cell-free culture supernatant

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was obtained after centrifugation of culture broth (10,000 'g' for 20 min at 4°C). As the bacteriocin was produced extra cellularly, the pellets were discarded and supernatant was adjusted to pH 7.0 by means of 1N NaOH to exclude the antimicrobial effect of organic acid, followed by filtration of the supernatant through a 0.22 µm pore-size filter. The supernatant was assayed for primary antimicrobial activity against *S. aureus*. The antimicrobial activity of the supernatant was determined by agar well diffusion assay. After 24 h, the diameter (mm) of the growth inhibition zones were measured.

Partial Purification of Bacteriocin

The bacteriocin produced from the selected presumptive *Lactobacillus* isolate was partially purified by using ammonium sulphate precipitation method (Ogunbanwo *et al.*, 2003). Ammonium sulfate was added with continuous stirring until the precipitates were formed and stored at 4°C for 45 min. The precipitated proteins were recovered by centrifugation at 10,000 g for 20 min and the pellet obtained was resuspended in 0.3 M (pH 7.0) potassium phosphate buffer and bacteriocin assay was performed against test organism. Active protein (with bacteriocin activity) was loaded onto a gel filtration column of Sephadex G-25. Fractions were collected and tested for antibacterial activity by spotting aliquots (10 µl) on a lawn of *S. aureus*. Fractions showing inhibitory activity were pooled and SDS-PAGE was run to check the purity of sample and also to determine molecular mass of the sample. The protein estimation at every step was carried out by following the procedure of Bradford (1976).

RESULTS AND DISCUSSION

LAB isolates have been characterized for cell morphology, Gram's character and catalase activity. These colonies on MRS agar plates were of small sizes mostly tiny dots. These types of colonies are typically formed by LAB (Kandler and Weiss, 1986). A number of bacterial isolates isolated from fermented foods and beverages in the present investigation were gram positive cocci and rods, catalase negative, non-spore forming, which were identified as lactic acid bacteria (Figure 1).

Biochemical and Technological Characterization

Two LAB isolates were homo-fermentative as the end product of fermentation was only lactic acid and rest of the eight isolates were hetero-fermentative as they produced gas (carbon dioxide) from glucose fermentation. Growth of LAB isolates at different temperatures (15, 37 and 45 °C) was checked. From the results of 7 days observation, only 6 isolates grew at 45 °C and 15 °C. The bacterial isolates were tested for growth at different NaCl concentrations at 2, 4 and 6.5 % as shown in Table 1. All LAB isolates showed growth at 2 % and 4 % NaCl concentration. Four LAB isolates showed no growth at 6.5 % NaCl concentration, however, rest of six exhibited fair growth at this concentration. All LAB isolates fermented dextrose, lactose, mannitol, galactose and maltose. The growths of five isolates were completely inhibited at pH 2.5 and high alkaline pH 9.5. The selected five LAB isolates were technologically relevant and these were further characterized for *in-vitro* probiotic properties.

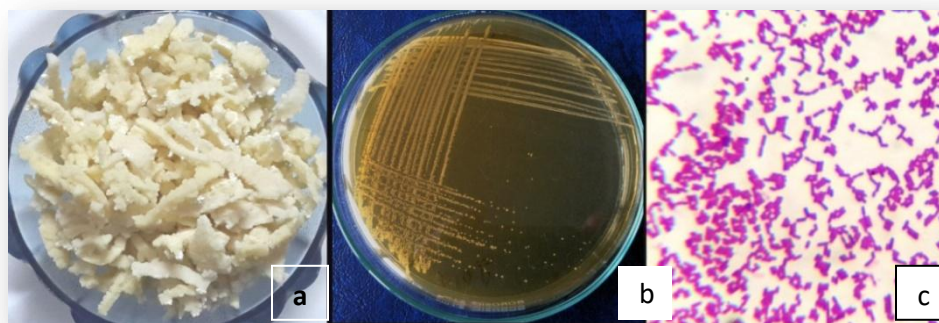


Figure 1: a) Churpe, Traditional Fermented Food; b) LAB Isolate on MRS Plate; c) Microscopic View of LAB

Table 1: Physiological and Technological Characteristics of LAB Isolates

Isolates	Cell Forms	Colony on Plates	pH	Temperature (°C)						NaCl (%)			Homo/Hetero Fermentation	
				2.5	3.5	8.5	9.5	15	37	45	2	4		6.5
L1	Long rods	White, colonies	big	+	++	+++	+++	+++	+++	++	+++	+++	+	Hetero
L2	Short rods	Slimy, colonies	big	-	+++	++	-	-	+++	-	+++	+++	-	Homo
L3	Short rods	White, pointed	pin	-	+	+++	+++	-	+++	-	+++	+++	-	Hetero
L4	Cocci	White, pointed	pin	+	+	+++	-	+++	+++	+++	+++	+++	+	Hetero
L5	Long rods	Big colonies		++	++	++	+	+++	+++	+++	+++	+++	+	Homo
L6	Cocci	Big, white colonies	slimy	-	-	+++	-	+++	+++	+++	+++	+++	+	Hetero
L7	Long rods	White, colonies	small	-	-	++	-	-	+++	-	+++	+++	-	Hetero
L8	Long rods	White, colonies	big	-	-	+	-	-	+++	-	+++	+++	-	Hetero
L9	Short rods	Pin pointed, transparent		-	+	++	++	++	+++	++	+++	+++	+	Hetero
L10	Short rods	Slimy, colonies	big	+	+	+++	-	+++	+++	++	+++	+++	+	Hetero

+ Mild growth; ++ moderate growth; +++ good growth; - no growth

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Probiotic Characterisation

Acid Tolerance

Resistances to acid and bile are generally considered essential assessment criteria for probiotic evaluation since the organisms have to survive the harsh conditions in the stomach and the small intestine. The results of survival of 5 LAB isolates in acidic conditions are illustrated in Table 2. These results showed that most isolates are quite tolerant to pH 3.0. However, the critical limit to survive the exposure to acidic conditions was pH 2.0, which was effective in the survival of most of the microbes present in food. Isolate L5 showed good acid tolerance at pH 2.0 and 3.0 for 3 h as compared to the control whereas decrease in viability of the isolates L4 and L9 was observed at pH 2.0. These results were similar with those of the previous studies, where *Lactobacillus* strains were viable after being exposed to low pH for 3 h (Kumari et al., 2016b and Angmo et al., 2016).

Tolerance to Bile Salt

The gastrointestinal systems have varying concentrations of bile. The rate of secretion and the concentration of bile in different regions of the intestine vary from 0.5 to 2 % during the first hour of digestion depending mainly on the type of food consumed. Most foods pass through the small intestine by 12 hr (Clark and Martin, 1994). Hence, tolerances of LAB isolates were evaluated by exposing the cells to 0.5 to 2 % bile salt solution upto 12 h at 30 °C. All LAB isolates were found to be significantly resistant to bile salt at different concentrations (0.5, 1 and 2 %) after 12 h of exposure retaining their viability with negligible reduction in viable counts (≤ 1 log cycle) as shown in Table 2. The reason behind bile tolerance might be some of the Lactobacillare able to hydrolyze toxic bile salts with bile salt hydrolase enzyme which weakens their detergent effect (Erkkila and Petaja, 2000).

Antibiotic Susceptibility

One of the most desired property by which an organism can be considered as a potential probiotic organism is that it must be safe for human consumption. Such safety includes among other features that it does not harbour acquired and transferable antibiotic resistances (Vizoso-Pinto et al., 2006). All LAB isolates were sensitive to different antibiotics except vancomycin and same sensitivity profile has been reported by Kumari et al., (2016b) as shown in Table 3.

Resistance to vancomycin by *Lactobacillus* strains has been attributed to the presence of D-Ala-D-lactate in their peptidoglycan instead of the normal dipeptide D-Ala-D-Ala, which is the target of the antibiotic (Coppola et al., 2005).

Antimicrobial Activity

Another essential condition for LAB with probiotic activity is the inhibitory effect against the growth of pathogenic bacteria. The 5 LAB isolates selected were examined for their antibacterial activity against different food borne pathogens and spoilage bacteria. All 5 isolates exhibited the antimicrobial activity (Table 3) against *E. coli* (with an inhibition zone of 8.0-14.0 mm in diameter), *S. aureus* (9.2-16.3 mm), *B. subtilis* (11.2-17.3 mm) and *S. dysenteriae* (10.1-13.4 mm). The possible mechanisms of bactericidal action includes diminished pH levels, competition for substrates, the production of substances with abactericidal or bacteriostatic action, including bacteriocins and bacteriocin-like substances (Pan et al., 2009).

Exopolysaccharide Production

LAB isolates often produce polymeric substances such as exopolysaccharide (EPS) which enhance the colonization of probiotic bacteria by cell-cell interactions in gastrointestinal tract (Kanmani et al., 2013). Exopolysaccharide are a major component of the bacterial biofilm with a well-documented impression on adherence of bacteria to host cells (Ciszek-Lenda et al., 2011). All LAB isolates produced EPS on skimmed milk-ruthidium red plates as shown in Figure 2.

Haemolytic Activity

Absence of haemolytic activity is considered as a safety prerequisite for the selection of a probiotic strain (FAO/WHO, 2001). None of the examined isolates exhibited α and β haemolytic activity when grown in Columbia sheep blood agar plates. It has been reported by various workers that haemolysis is rarely present in LAB isolated from fermented foods (Santini et al., 2010).

Table 2: Acid and Bile Tolerance of LAB Isolates

Acid Tolerance (Log cfu/mL)				Bile Tolerance (Log cfu/mL)			
Isolates	pH 7.0	pH 3.0	pH 2.0	Isolates	0.5 %	1 %	2 %
L1	8.47±0.17	7.32±0.12	6.04±0.07	L1	8.35±0.09	8.30±0.05	7.82±0.21
L4	8.73±0.05	7.59±0.10	4.39±0.34	L4	8.10±0.04	8.06±0.05	6.86±0.11
L5	8.21±0.08	7.73±0.11	7.15±0.07	L5	8.14±0.08	8.60±0.09	8.16±0.16
L9	8.67±0.06	7.33±0.05	4.65±0.06	L9	8.26±0.06	8.23±0.05	6.45±0.11
L10	7.72±0.05	7.53±0.06	6.98±0.05	L10	6.54±0.12	6.14±0.05	5.87±0.12

Presented values are means of triplicate determinations; ± indicates standard deviations from the mean;

- : No zone of inhibition

Table 3: Antibiotic Susceptibility Profile, Antimicrobial Activity and Proteolytic Activity of Lactic Acid Bacteria Isolates

Isolates	P	E	A	V	C	NX	COT	<i>S. Aureus</i> (mm)	<i>B. Subtilis</i> (mm)	<i>S. Dysenteriae</i> (mm)	<i>E. Coli</i> (mm)	Proteolytic Activity (mm)
L1	S	S	S	R	S	S	S	9.2±0.15	15.5±0.15	13.4±0.15	12.2±0.1	16.2±0.1
L4	S	S	S	R	S	S	S	13.1±0.1	-	-	8.5±0.01	-
L5	S	S	S	R	S	S	S	16.3±0.15	17.3±0.2	10.1±0.5	14.0±0.05	21.2±0.2
L9	S	S	S	R	S	S	S	12.5±0.15	11.4±0.2	-	11.1±0.25	10.3±0.15
L10	S	S	S	R	S	S	S	15.1±0.15	11.2±0.1	-	10.2±0.2	8.4±0.1

Presented values are means of triplicate determinations; ± indicates standard deviations from the mean; - No zone of inhibition; R-Resistant; S-Sensitive

Table 4: Purification Table for Bacteriocin of Isolate L5

Purification Steps	Volume (ml)	Bacteriocin Activity (AU/ml)	Total Bacteriocin Activity (AU)	Protein (mg/ml)	Specific Activity (AU/mg)	Yield %	Purification Factor
Crude supernatant	1000	4 x 10 ⁵	4.0 x 10 ⁸	0.5	8 x 10 ⁵	100	1.0
Ammonium Sulphate precipitation	20	40 x 10 ⁵	8.0 x 10 ⁷	2.7	14.8 x 10 ⁵	20	1.85
Gel permeation	1	9 x 10 ⁶	9 x 10 ⁶	1.9	47.3 x 10 ⁵	2.25	5.91

AU-arbitrary units, ml- milliliters, mg-milligrams, %- percentage

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Proteolytic Activity

Highest proteolytic activity was recorded in L5 (21.2 mm in diameter) followed by L1 (16.2 mm) and no hydrolysis was observed in L4 (Figure 2 & Table 3). Same results were reported by Kumari *et al.*, (2016a) on skimmed milk agar plates.



Figure 2: Plates Showing Antibiotic Sensitivity, Antimicrobial Activity against *S. aureus* Exopolysaccharide Production and Proteolytic Activity

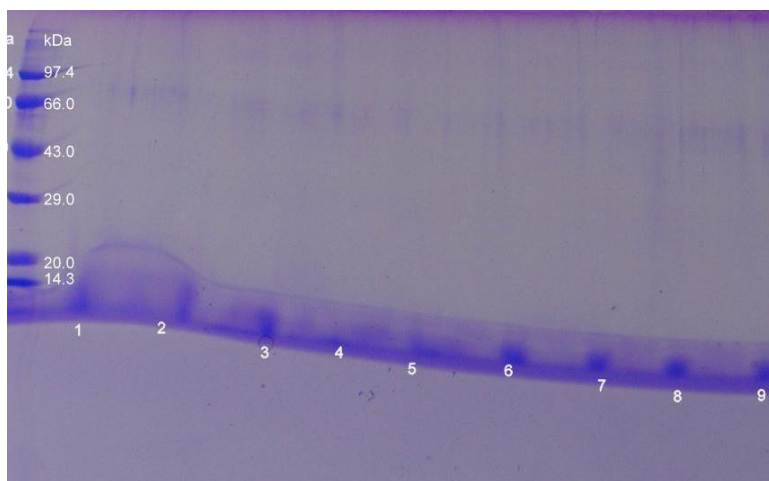


Figure 3: SDS- PAGE of Partially Purified Bacteriocin

Extreme Left Well was loaded with Following Protein Molecular Mass Standards: Phosphorylase b (97.4 kDa), Bovine Serum Albumin (66 kDa), Ovalbumin (43 kDa), Carbonic Anhydrase (29kDa), Soyabean Trypsin Inhibitor (20 kDa), Lysozyme (14.3 kDa)

Crude Enzyme (Lane 1, 2), Pooled Fraction of Gel Permeation (Lane 3), Lanes 4-9 having Fractions of Gel Permeation

Partial Purification of Bacteriocin

The strong antagonism against a number of challenging foodborne and spoilage causing microorganisms recommended the possibility of using bacteriocin as an effective preservative in foods. The selected

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isolate L5 was used to produce extracellular bacteriocin against pathogenic organism in Elliker's broth. The cell free extract of L5 was subjected to sequential ammonium sulphate saturations from 0 to 80 %. The bacteriocin was recovered at 30-40 % ammonium sulphate saturation level and the protein was 0.5 mg/ml (Bradford, 1976). The recovered proteins were then fractionated by gel filtration chromatography using 0.05 M potassium phosphate buffer in Sephadex G-25. The bacteriocin rich fractions (14-16) were pooled and yield of 2.25% with 5.91 purification fold was achieved (Table 4). Sankar *et al.*, (2012) had reported 13.5 fold bacteriocin purification and yield of 21.3% bacteriocin with specific activity of 1023 AU/mg and molecular weight of 9.5 kDa from *Lactobacillus plantarum*. The results of SDS-PAGE of bacteriocin at various stages of purification were shown in Figure 3. The partially purified fractions from gel permeation chromatography showed the partially purified bacteriocin has a molecular mass below 10 kD.

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