IMPACT: International Journal of Research in Applied, Natural and Social Sciences (IMPACT: IJRANSS)
ISSN (P): 2347-4580; ISSN (E): 2321-8851
Vol. 6, Issue 2, Feb 2018, 69-76

PROBIOTIC PROPERTIES OF LACTOBACILLUS ISOLATED FROM MILK

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Received: 02 Feb 2018 **Accepted:** 17 Feb 2018 **Published:** 24 Feb 2018

ABSTRACT

Nine lactic acid bacterial (LAB) strains were isolated from different milk samples and evaluated for functional and probiotic properties and potentials as starter cultures. All of these isolates were recognized as probiotics on the basis of their acid and bile tolerance, antibacterial activity, antibiotic resistance, antibacterial potential, tolerance to acidic and bile salt conditions. Antimicrobial activity of the probiotic Lactobacillus was determined by well diffusion method. These results suggest that these strains may be used in the future as probiotic starter cultures for manufacturing novel fermented foods.

KEYWORDS: *Lactic Acid Bacterial (LAB) Strains, Probiotic Properties*

INTRODUCTION

Nowadays demand for complimentary and health foods had increased which encourage innovation as well as new and novel product development in the food industries (Abbas and Mahasneh, 2014). The consumption of probiotic bacteria as formulation or fermented food ingredients stimulates growth of beneficial bacteria and reduces pathogen activity has been already established (Chiang and Pan, 2012).

Over the past 10 years, probiotics have become a major focus of research with most attention drawn to the genera *Lactobacillus* and *Bifidobacterium* for improving human health in natural way (Fernandez *et al*., 2003). These organisms exert many beneficial health effects, such as activation of the immune system, prevention of cancer cell growth, maintenance of mucosal integrity and presentation of an antagonistic environment for pathogens (Rashmi and Gayathri, 2014).

Recently, a drive towards non-dairy novel probiotics has been observed to span traditionally fermented foods of vegetable origin (Sanchez *et al*., 2012). An effective probiotic function and survive under a variety of physiological conditions. When consumption of probiotic bacteria is there, it should survive transit in the gastro-intestinal tract where it is open to challenges, such as the low pH environment of stomach and bile salts of the upper intestinal tract (Kechagia *et al*., 2013).

Probiotics must be safe i.e. should not cause lysis of red blood cells. Furthermore, antibiotic resistance is necessary for survival in the presence of co-administered drugs (Mombelli and Gismondo 2000). The genes conferring resistance in probiotics should be innate in nature and non-transferable to other bacteria (Jose *et al.,* 2015). Probiotic bacteria must also be capable of adhering to intestinal epithelial lining in order to provide benefits in the host. Adherence

enables the probiotic bacteria to persist for a longer time in the gut and enhances the host-bacteria interactions (Gueimonde and Salminen 2006). Adherence of probiotic bacteria also helps it to overcome peristalsis activity of stomach (Suvarna and Boby, 2005).

This study aims at isolating and identifying selected Lactobacillus strains from Milk samples and studying their probiotic characteristics such as acid tolerance, tolerance to gastrointestinal juice and bile, and their antagonistic activity against some pathogens.

MATERIALS AND METHODS

Collection of Samples

Cow, Buffalo and Goat Milk samples were collected from Local market of Jaipur. Immediately after the collection, the samples were stored aseptically in low temperature (-4°C) refrigerator to protect from contamination and deterioration.

Media

The bacteria *Lactobacillus* spp. was isolated from milk samples by using modified MRS broth and MRS agar media (Rogosa *et al.*, 1960). Additionally, 0.05% cysteine was added to MRS to improve the specificity of this medium for isolation of *Lactobacillus*. The pH of the media was adjusted to 6.5.

Isolation of *Lactobacilli* Species

Lactobacillus was isolated from milk by using MRS medium using pour plate method. The cultures were subjected to subculture at 37°C onto MRS agar media at pH 4.8.

Preliminary Identification of the Isolates

The isolated bacteria were identified as *Lactobacillus* spp. by Gram staining, motility test, catalase test, endospore test, milk coagulation activities, 0.4% bacteriostactic phenol tolerance test and 1-10% NaCl tolerance test.

Determination of Optimal pH and Acid Tolerance

For the Determination of optimal pH of *Lactobacillus*, 1% (v/v) fresh over night culture of *Lactobacillus* were inoculated into MRS broth with varying pH ranging 5, 4, 3 and 2. The pH was adjusted with 1N HCl and 1 N NaOH. The inoculated broths were incubated in anaerobic condition 24 h at 37°C in the presence of 10% CO_2. After 24 h of incubation growth of the bacteria were measured using a spectrophometer, reading the optical density at 560 nm (OD) against the uninoculated broth.

Preliminary selection of acid tolerant lactobacilli using rapid method was determined according to slightly modified methods as described (Pelinescu *et al*., 2009) to simulate gastric conditions.

Bile Salt Tolerance

The tolerance of lactobacilli to bile salts (BS) was evaluated in MRS supplemented with bile salts with varying concentrations of 0.3%, 0.5% and 0.8% using a modified method described by Dora and Glenn (2002).

Resistance to Antibiotics

The antibiotic susceptibility of selected acidotolerant and bile tolerant isolates was determined towards eight antibiotics, namely, Ampicillin, Cefolaxime, Amikacin, Cephalexin, Augmentin, Oflolaxin, Ciprofloxacin, Cephalexin. Strains selection was based on their performance toward acid and bile salts. Antibiotic susceptibility was determined semi- quantitatively using a modification of the agar overlay diffusion methods of the National Committee for Clinical Laboratory Standards NCCLS (1993).

Gelatinase Activity

Gelatinase activity of the most antibiotics sensitive isolates was investigated as described by Harrigan and McCance (1990).

Haemolysis Activity

Haemolysis activity of gelatinase negative isolates was investigated as described by Gerhardt *et al.* (1981).

In Vitro Cholesterol-Lowering Property

The ability of isolates to assimilate cholesterol was determined by a modified method described by Dora and Glenn (2002). Total cholesterol was analyzed using an enzymatic procedure, which is a modification of the method of Allain *et al*. (1974). The amount of cholesterol removed from the growth medium was expressed as a percentage by the treatment compared with the control (MRS broth supplemented 0.4% bile salt) as follows:

[1- (residual cholesterol in cell-free broth)/ (cholesterol of control broth)] x 100.

Cholesterol assimilation of the isolates was expressed as the amount of cholesterol consumed in milligram per gram of cells.

Antimicrobial Activity

Antimicrobial activity of the selected probiotic isolates was checked by using the agar-spot test (Mami *et al*., 2008). Isolates were screened for production of antimicrobial against Escherichia coli ATCC as the indicator microorganisms.

RESULTS AND DISCUSSIONS

LAB isolation was carried out from different milk samples of Camel, Buffalo and Goat. A total of 9 isolates were found to be gram-positive bacilli and catalase negative that were presumptively identified as *Lactobacillus sp.*

Acid Tolerance (pH)

Lactobacillus growth and viability is reduced and the metabolism is inhibited at low pH environments. Because of hydrochloric acid found in the stomach of human beings which interrupt the biomolecules of cells such as proteins, vitamins, fatty acids and DNA (Hassanzadazar *et al*., 2012). Thus screening and selection of the Lactobacilli isolates under the acidic conditions using rapid selective method was carried out at pH range 2 to 5.

Nine isolates tested showed growth at pH 4 and 5 which were isolated C1, C2, C3, B1, B2, G1 and G2. Among these, nine *Lactobacilli* isolates B1 and C1 showed maximum growth at pH 3, but none of the isolates was able to grow at pH 2. Tolerance to low pH found due to the presence of special enzyme H^+ Translocating ATPase in the

cytoplasmic membrane of *Lactobacillus* sp. which maintains cytoplasmic pH more alkaline then the outside medium.

Table 1: Tolerance of *Lactobacilli* Isolates to Different pH

Lactobacillus Isolates	MRS Broth as Control	O.D at 620 nm			
		pH 2	pH 3	pH 4	pH 5
C1	1.667	0	0.633	1.119	1.442
C2	1.325	0	0.443	0.625	0.882
C3	1.456	0	0.522	0.887	1.085
C4	1.422	0	0	0.741	1.012
C5	1.655	0	0	0.608	0.887
B1	1.426	0	0.779	1.193	0.967
B2	1.753	0	0.454	1.272	1.432
G1	1.579	0	0.428	0.974	1.128
G2	1.475	0	0.321	0.439	0.803

In vitro survival of bacterial strains in low pH is a more accurate indication of the ability of strains to survive passage through the stomach. The organisms taken orally have to face stresses from the host which begin in the stomach, with pH between 1.5 and 3.0 (Corzo and Gilliland, 1999).

Many scientific reviews demonstrated the capacity of *L. plantarum* strains to tolerate gastric acidic conditions. Sirilun *et al.* (2010) reported a viable rate of more than 90% of 43 out of 114 strains at pH 3 after 2h of incubation was found; at the pH 2 a surviving percentage that was higher than 50% could be observed in 27 strains. For strains to survive and colonize the gastrointestinal tract, microorganisms should express tolerance to acid and bile salts (Gibson, 1998).

Bile Salt Tolerance

After exposure to acidic conditions, 9 selected acido tolerant lactobacilli isolates were assayed for bile salt tolerance. All 9 isolates (C1, C2, C3, C4, C5, B1 B2, G1 and G2) showed good capacity to resist bile salts under exposure to 0.3%. Amongst these 9 isolates, C2 and C4 showed maximum turbidity when grown in MRS medium with 0.3% bile salt concentration. Five isolates C1, C3, B2, G1 and G2 showed growth at bile salt 0.5% concentration. None of 9 isolates showed growth at bile salt 1% concentration.

Table 2: Tolerance of *Lactobacilli* Isolates to Different Bile Salt Concentration

Lactobacillus Isolates	MRS Broth as Control		O.D at 620 nm	
		0.30%	0.50%	1%
C1	1.667	0.651	0.302	0
C2	1.325	**1.265**	0	0
C3	1.456	1.08	0.143	0
C4	1.492	**1.046**	0	0
C5	1.524	0.343	0	0
B1	1.796	0.465	0	0
B2	1.887	0.263	0.239	0
G1	1.426	0.896	0.249	0
G2	1.753	0.281	0.204	0

Bile salt tolerance is the second selection criterion for probiotics. Resistance to bile salts is generally considered as an essential property for probiotic strains to survive the conditions in the small intestine. Bile salts are synthesized in the liver from cholesterol and are secreted from the gall bladder into the duodenum in the conjugated form in volumes ranging from 500 to 700ml per day. The relevant physiological concentrations of human bile range from 0.1 to 0.3% (Dunne *et al*., 2001) and 0.5% (Mathara *et al.*, 2008). Thus, it is necessary that efficient probiotic bacteria should be able to grow in bile salt with concentration ranging from 0.15 - 0.30% (w/v) (Šuškovi *et al.*, 2000).

Resistance to Antibiotics

Nine potentially probiotic lactobacilli isolates were subjected to antibiotic susceptibility testing using the agar diffusion method (Table 3). Four isolates (C2, C3, C4 and C5) were resistant to Cefotaxim, ampicillin, Cephalexime, augmentin and Doxycycline. Two isolates (G1 and G2) demonstrated intermediate resistance to Cephalexime and Augmentin.

Table 3: Susceptibility of Potentially Probiotic Lactobacilli Isolates to Antibiotics Using Disc Diffusion Method. CF-Cefotaxime; Am-Ampicillin; AK-Amikicin; NT-Netilmicin; PR—Cephalexime; NZ-Ofloxacin; AG-Augmentin; RC-Ciprofloxacin; DX-Doxycycline; S- Sensitive; I-Intermediate; R-Resistant

Antibiotics / Sample	CF	AM	AK	NT	PR	NZ	AG	RC	DX
	Diameter of Inhibition Zone in mm								
C1	$16_{(S)}$	$27_{(S)}$	$29_{(S)}$	$28_{(S)}$	$10_{(I)}$	$10_{(I)}$	$0_{(R)}$	$10_{(I)}$	$0_{(R)}$
C2	$0_{(R)}$	$0_{(R)}$	$27_{(S)}$	$23_{(S)}$	$0_{(R)}$	$11_{(I)}$	$0_{(R)}$	$12_{(I)}$	$0_{(R)}$
C3	$0_{(R)}$	$0_{(R)}$	$27_{(S)}$	$29_{(S)}$	$10_{(I)}$	$16_{(S)}$	$0_{(R)}$	$13_{(I)}$	$0_{(R)}$
C4	$0_{(R)}$	$0_{(R)}$	$27_{(S)}$	$24_{(S)}$	$0_{(R)}$	$16_{(S)}$	$0_{(R)}$	$12_{(I)}$	$0_{(R)}$
C5	$0_{(R)}$	$0_{(R)}$	$29_{(S)}$	$25_{(S)}$	$0_{(R)}$	$0_{(R)}$	$0_{(R)}$	$0_{(R)}$	$10_{(I)}$
B1	$19_{(S)}$	$29_{(S)}$	$24_{(S)}$	$25_{(S)}$	$17_{(S)}$	$20_{(S)}$	$26_{(S)}$	$26_{(S)}$	$30_{(S)}$
B2	$29_{(S)}$	$23_{(S)}$	$18_{(S)}$	$19_{(S)}$	$17_{(S)}$	$14_{(I)}$	$13_{(I)}$	$0_{(R)}$	$0_{(R)}$
G1	$13_{(I)}$	$22_{(S)}$	$16_{(S)}$	$17_{(S)}$	$12_{(I)}$	$17_{(S)}$	$13_{(I)}$	$16_{(S)}$	$18_{(S)}$
G2	$0_{(R)}$	$10_{(I)}$	$16_{(S)}$	$15_{(I)}$	$15_{(I)}$	$15_{(I)}$	$14_{(I)}$	$21_{(S)}$	$20_{(S)}$

Antibiotic resistance of microorganisms used as probiotic agents is an area of growing concern. It is believed that antibiotic used for food-producing animals can promote the emergence of antibiotic resistance in bacteria present in the intestinal microflora. Then, the antibiotic-resistant bacteria can transfer the resistance factor to other pathogenic bacteria through the exchange of genetic material (Mathur and Singh, 2005).

Haemolysis and Gelatinase Activity

The Nine potentially probiotic lactobacilli isolates were tested for their haemolysis and gelatinase activity. All the isolates showed no positive haemolysis and gelatinase activity. The mucoid lining constitutes the target across which many physiological substances are exchanged. Haemolysis activity would break down the epithelial layer while gelatinase activity would derange the mucoid lining. These impairments interfere with the normal functioning of these very important linings and would cause pathways for infections. Absence of haemolytic and gelatinase activity is a selection criteria for probiotic strains, indicating that these bacteria are non virulent (De Vuyst *et al*., 2003).

Cholesterol Reduction

All the 9 isolates were tested for their ability to reduce cholesterol in-vitro in the presence of bile salts. The amount of cholesterol reduced ranged from 14.11 to 89.6%. Cholesterol reduction of the isolates ranged from 14.11 to 89.6 mg of cholesterol per g of cells. The highest value of cholesterol reduction was recorded in isolate B2 (Table 4).

Table 4: Cholesterol Reduction of Potentially Probiotic *Lactobacilli* Isolates

Isolate No.	Cholesterol Reduction
C1	44.1%
C2	14.1%
C3	31.2%
C4	30.0%
C5	35.4%
G1	36.7%
G2	45.8%
B1	62.5%
B2	89.6%

Hypercholesterolemia is considered as a major risk factor for the development of coronary heart disease. Although therapeutic drugs are available to relieve this problem, they are often expensive and can have side effects. Several studies indicated that Lactobacillus species were able to reduce cholesterol via several mechanisms including bile salt deconjugation (Liong and Shah, 2005). Other hypocholesterolemic mechanism(s) of lactobacilli may be involved in the removal of cholesterol from growth media. The removal of cholesterol by lactobacilli in vitro could be due to an uptake or assimilation of cholesterol by bacterial strains.

Antimicrobial Activity of Isolates

Among all the 9 isolates only 5 *Lactobacilli* showed the antimicrobial activity against *E.coli*. Out of 5 only 2 B2and G1 (21mm) showed highest activity against the *E.coli* (Table 5)

Table 5: Antimicrobial Activity of *Lactobacilli* Isolates Against *E.coli*

Antimicrobial Activity Against *E.coli*	
Isolates	**Zone of inhibition[mm]**
C3	15
C4	14
C5	16
B2	21
G1	21

CONCLUSIONS

The present study indicated that the novel lactic acid strains isolated from fermented dairy products have more effective functions. The examined strains for probiotic traits had a good ability to grow and survive well in the presence of different concentrations of bile salts. All tested strains were survived under acidic conditions (pH 3) till 5 hrs of incubation time at 37 ^{o}C. No haemolytic activities were observed for all tested strains.

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