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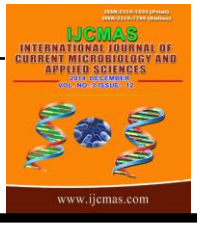

Original Research Article

Isolation and Characterization of Endemic strains of *Lactobacillus sp.* and evaluation of their Probiotic Activity

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A B S T R A C T

Keywords

Lactobacillus, Probiotic activity, antibiotic susceptibility, bacterial antagonism.

In this study 30 different samples of milk ,curd and yoghurt were collected from Noida, for isolation of *Lactobacillus spp*. and evaluating their probiotic potential. A total of eight isolates showed good probiotic activity and were identified as *Lactobacillus acidofarinae, Lactobacillus equi, Lactobacillus aqaticus, Lactobacillus curvatus* and *Lactobacillus floricola and Lactobacillus casei*. They were able to tolerate pH 3, 0.3% bile salts and exhibited good ability to attach to intestinal epithelial cells and were not resistant to the tested antibiotics. They also showed good antimicrobial activities against the tested pathogenic strains of humans. Thus, these eight Lactobacillus strains could be considered as potential antimicrobial and probiotic strains against human pathogens.

Introduction

Lactobacilli are the major type of Lactic acid bacteria which have been shown to act as a preservative as well as a probiotic agent. Probiotics means beneficial effects of microorganisms on Human health. Probiotics are products used as dietary supplements to enhance the growth and health of the humans and animals. They have been shown to be important in disease control, as digestion aids, immune booster and in supplementing or replacing the use of antimicrobial compounds in the field of health and medicine (Chantharasophon et al., 2011, Heller, 2001, O'Mahony et al., 2005.) Due to these factors Lactobacilli sp. have a protective or therapeutic effect Shokryazdan(2014). In India gastrointestinal infections in humans are a major problem. World Health Organization (WHO) estimates that there are more than four million episodes of Diarrhoeal cases every year which in multiple cases lead to death due to enteric infections.

Lactobacilli also produce lactic acid and Bacteriocin. These both products help the organism to act as a biological preservative used in food products against fungal and bacterial infections and spoilage. Thus, lactobacilli are an important class of GRAS microorganisms with multiple end uses. This paper reports the isolation, characterization of endemic strains of Lactobacilli and evaluation of their probiotic activities.

Materials and Methods

Sample

For isolation of Lactobacilli strains cow milk, buffalo milk, goat milk and curd were used. These were collected in sterile bottles.

Isolation of *Lactobacillus*

MRS (de Man Rogosa and Sharpe) medium were used for the isolation of *Lactobacillus sp.*Dilutions of the various types of milk were done in sterile water and then they were spread plated on MRS agar plates. The plates were inoculated at both 37 ^{0}C and 25 ^{0} C for 24 to 48 hours. Distinctive colonies having the characteristic appearance as large, white colonies were picked up and studied further.

Identification of isolates

The isolates were identified initially on the basis of Bergey's Manual of Systematic Bacteriology (Holt *et al*., 1984.) The isolated strains were differentiated on basis of colony characteristics like colour, texture and size of colony and Gram stain. Different Biochemical test were conducted and through Biolog software the lactobacilli strains were identified. The various biochemical test performed were: IMViC, Catalase, Sugar fermentation tests (glucose,fructose, galactose, maltose, mellibiose, lactose, sucrose, raffinose, mannose, arabinose, ribose, amygdalin, adonitol, arabitol, cellobiose, fucose, gluconate, glycogen, glycerol, inositol, trehalose etc) , Gelatin utilization, Casein utilization, Starch Utilization, Urease Test etc.

Probiotic Activity Test

Production of Bacteriocin from *Lactobacillus sp.*: A loopful of each of the different *Lactobacillus* culture were inoculated in freshly prepared MRS broth and were incubated for 48 hrs. at 37^{0}C. After that the cultured broths were centrifuged at 5000 rpm for 15 min. The supernatant were used as Bacteriocin.

Antimicrobial activity: Antimicrbial activity of *lactobacillus* isolates were performed against test organism. Twelve strains that are pathogenic to humans were used as test pathogens to investigate the antagonistic activity of the LAB strains. They were *Escherichia coli* (NCIM 5010), *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (NCIM 2901) and *Helicobacter pylori* (ATCC 43579) *Enterobacter cloacae* (NCIM 2691), *Enterobacter aerogenes* (NCIM 5139) and *Klebsiella pneumoniae* (MTCC 3040) ,*Candida albicans* and *Bacillus Subtilis*

Survival in low pH and Bile: 5ml each of MRS (pH 2.5), MRS containing 0.3% bile salts, and normal MRS, each were inoculated with the different *Lactobacillus* strains. The initial count was around 10^6 cells/ml. As a control, broth without inoculation was used. Changes in optical density at 620 nm (OD_{620}) were measured on different days after incubation at 37°C. Survival under the different conditions was tested after 4 h of incubation at 37°C and plating of 100 µl of the above broth onto MRS agar.

Milk technological properties: *Lactobacillus* have a very strong property of curdling milk. This is due to the formation of organic acids on fermentation of a carbohydrate source. All the 8 isolates were inoculated with into fresh milk and kept in incubator for 24 hrs at 25^{o}C. Change of milk into curd by the *Lactobacilli* strain is also

considered as an indicator for effective probiotic activity.

Effect of different salt concentration on bacterial isolates: 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. The bacterial strains were streaked in the MRS Agar plates containing different levels of NaCl concentration (2%, 3%, and 6.5%) and kept in incubator at 37^{o}C for 24 hrs.

Antibiotic susceptibility: Each different antibiotic were mixed in MRS Agar media (25ml) and was poured on petriplate and after that different lactobacillus culture were swabbed on MRS Agar plates. Then these plates were incubated at 37^{0}C for 24 hrs. The antibiotics resistance activity was also studied by the disc diffusion/ well diffusion methods.

Result and Discussion

Isolation of *Lactobacillus* from different samples-In the present study 30 different samples of curd, cow milk,buffalo milk, goat milk, were used to isolate *Lactobacillus.* Seven *Lactobacilli* isolates were isolated from these samples.(Table : 1)

Identification and characterization of bacterial isolates - The strain were grown on MRS media and gram staining was done for morphological characteristic and further biochemical tests were performed for identification and characterization of bacterial isolates. The results are presented in the Table : 2 show that Isolate 'C' and 'F' were both *Lactobacillus aqaticus, while the other isolates were identified as : (A) Lactobacillus acidofarinae. (B) Lactobacillus equi, (D) Lactobacillus curvatus (E) Lactobacillus floricola (G) Lactobacillus casei (H) Lactobacillus*

The isolates were identified initially on the basis of Bergey's Manual of Systematic Bacteriology **(Holt *et al.*, 1984.)** The isolated strains were differentiated on basis of colony characteristics like colour, texture and size of colony and Gram stain. Different Biochemical test were conducted and through Biolog software the lactobacilli strains were identified. The various biochemical test performed were: IMViC, Catalase, Sugar fermentation tests (glucose,fructose, galactose, maltose, mellibiose, lactose, sucrose, raffinose, mannose, arabinose, ribose, amygdalin, adonitol, arabitol, cellobiose, fucose, gluconate, glycogen, glycerol, inositol, trehalose etc) , Gelatin utilization, Casein utilization, Starch Utilization, Urease Test etc. Finally the isolate were identified as:

(A) Lactobacillus acidofarinae. (B) Lactobacillus equi, (C) Lactobacillus aqaticus, (D) Lactobacillus curvatus (E) Lactobacillus floricola ***(F)*** *Lactobacillus aqaticus (G) Lactobacillus casei (H) Lactobacillus casei*

Probiotic Activity Test

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Survival in low pH and Bile: All the *Lactobacilli* cultures showed growth positive growth at pH- 3, pH- 4 and pH-5 . All eight Lactobacillus strains showed good tolerance to acid (pH 3), but the level of tolerance varied among the strains. Of the eight Lactobacillus strains, *Lactobacillus acidofarinae., Lactobacillus equi, Lactobacillus aqaticus, Lactobacillus floricola, Lactobacillus casei and Lactobacillus* showed good tolerance of acid conditions. The survival was seen by culturing bacterial isolates on MRS plates having pH 3. In addition to this OD was also taken each day for demonstration of tolerance level. *Lactobacillus acidofarinae* , *Lactobacillus curvatus* and *Lactobacillus* casei I strain showed very high tolerance and cell viability as the OD increased to a higher extent on 2nd and 3rd day. In the other isolates the OD of growth was observed in form of a gradual increase. The graph of acid tolerance is given in Figure :1.

When inoculation was done with bile salts, the growth was observed in all except *Lactobacillus aquaticus*. The results of bile tolerance for all nine Lactobacillus strains and the reference strain are shown in Table 4. All eight Lactobacillus strains exhibited tolerance to 0.3% bile (oxgall). However, the degrees of tolerance varied among the strains. *Lactobacillus acidofarinae* and *L. casei* showed the highest tolerance to bile salt, with an increase in cell viability This indicates that almost all the Lactobacilli isolated have a good capacity to survive in the gastrointestinal tract of humans.

Milk technological properties: *Lactobacillus* have a very strong property of curdling milk. This is due to the formation of organic acids on fermentation of a carbohydrate source. All the 8 isolates were inoculated with into fresh milk and kept in incubator for 24 hrs at 25°C. Change of milk into curd by the *Lactobacilli* strain is also considered as an indicator for effective probiotic activity. The rate of curdling was observed to be different in the isolates. Maximum curdling was observed in isolate D, E and G i.e. in *Lactobacillus curvatus* , *Lactobacillus floricola and Lactobacillus casei*. The difference is shown in Figure 2.

Effect of different salt concentration on bacterial isolates: The bacterial strains were streaked in the MRS Agar plates containing different levels of NaCl concentration (2%, 4%, 6.5%) and kept in incubator at 37°C for 24 hrs. The results showed that all the bacterial isolates can prevent osmotic shock, survive and grow at the NaCl concentration of 2% and 4% but even at higher concentration of NaCl i.e., 6.5% except for *Lactobacillus acidofarinae,* all the other *Lactobacilli* isolates were able to survive and grow at a basal level.

Antibiotic susceptibility: The Lactobacilli isolates exhibited varied degrees of susceptibility and resistance towards the antibiotics used for testing. Most of the bacterial isolates showed susceptibility towards the antibiotics : tetracycline, streptomycin, ampicillin, gentamycin, oflaxacillin, angifix, ceferoxime, penicillin and amoxicillin. Apart from these *Lactobacillus curvatus* and *Lactobacillus floricola* showed resistance against cephalexime and *Lactobacillus aqaticus* and *Lactobacillus curvatus* showed resistance against roxythromycin only

In the present study 30 different samples of

locally available curd, cow milk ,buffalo milk and different juices were used to isolate *Lactobacillus* strains. From these samples eight *Lactobacilli* isolates were purified, characterized and identified. The five strains were classified on the basis of biochemical tests and were shown to be Isolate 'A' - *Lactobacillus acidofarinae,* Isolate 'B' - *Lactobacillus equi,* Isolate 'C' and 'F' - *Lactobacillus aqaticus,* Isolate 'D' - *Lactobacillus curvatus* and Isolate 'E' - *Lactobacillus floricola,* Isolate 'G' and 'H' - *Lactobacillus casei.* These isolated strains were evaluated for their potential probiotic characteristics and antimicrobial activity against some human pathogens.

For a bacterial isolate to be used as a potential probiotic strain, it is expected to tolerate the condition of the Gastrointestinal tract of humans, in order to be able to provide its beneficial effect on the host. The ability to tolerate acid, bile, and digestive enzymes and to adhere to the intestinal epithelial cells have been considered as good indicator for the survival of a bacterial strain in the Gastrointestinal tract. These characteristics are are important for the preliminary selection of a probiotic strain.

These in vitro selection studies may not be able to totally mimic the actual in situ conditions in the gut ecosystem but they are still the most powerful tools for rapid screening of potential probiotic strains. They permit an enormous level of simplification of the system under study thus allowing a large number of strains to be screened for probiotic property. Dunne et al (2001) reported that adoption of proper criteria for the in vitro selection of probiotic bacteria can result in the isolation of strains capable of performing effectively in the GIT. In the present study all the *Lactobacilli* strains isolated showed antibacterial activity against both gram negative and gram positive bacteria like *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus* and *Helicobacter pylori Enterobacter cloacae*, *Enterobacter aerogenes* and *Klebsiella pneumoniae* and *Bacillus Subtilis.* The lactobacilli isolates showed very less inhibitory activity against *Candida albicans. Candida* is a pathopgenic yeast which causes diseases in humans. The other bacterial pathogens are mostly gastrointestinal in their presence. In another study by (Saumya et al., 2012) 28 lactobacilli were isolated from different samples and from these only 4 isolates showed antimicrobial effect against different pathogenic bacteria like *S.aureus, Methicillin resistant S. aureus,* and *Pseudomonas aeruginosa.* These strains of *Lactobacilli* showed good activity against both gram negative and gram positive food spoilage and pathogenic bacteria (Saumya et al., 2012).

In the present study, pH 3 was used to investigate the acid tolerance of the Lactobacillus strains as pH 3 has been considered as a standard pH for investigation of acid tolerance of probiotic strains in many studies. The results showed that all eight isolated Lactobacillus strains exhibited good acid tolerance at pH 3. In addition to this OD was also taken each day for demonstration of tolerance level. *Lactobacillus curvatus* strain showed very high tolerance and cell viability as the OD increased to a higher extent on 2nd and 3rd day. Ehrmann et al. (2003) also reported that strains of *L. reuteri, L. salivarius, and L. animalis* were able to tolerate pH 3 for 4 h, but the degrees of tolerance varied among the strains.

The ability to tolerate bile salt at a concentration of 0.3% has a physiological significance because it is a level normally encountered in human intestine.

Table.1 Isolation of *Lactobacillus* in different samples

Name of samples	No. of samples used	Bacterial Isolates
Cow Milk	10	2 (B, E)
Curd	10	2 (C, D)
Goat milk	2	1 (A)
Buffalo milk	5	1 (G)
Pineapple juice	3	1(F)
Total	**30**	**7**

Table.2 Biochemical characterization of Bacterial Isolates

	Characteristics	Bacterial isolates							
		A	B	C	D	E	F	G	H
Biochemical test	Catalase	-	-	+	+	+	+	+	+
	Indole test	-	+	-	-	-	-	-	-
	MR test	-	+	-	-	-	-	+	+
	VP test	-	+	-	-	-	-	-	-
	Citrate utilization	-	+	-	-	-	-	+	+
	Starch hydrolysis test	+	+	+	+	+	+	+	+
	Glycerol test	-	-	-	-	+	-	-	-
	Gelatin test	+	+	+	+	+	+		
	Casein test	+	+	+	+	+	+	+	+
	Urease test	+	+	+	+	+	+		
Carbohydrate test (carbon source)	Lactose	-	A+G	-	-	-	-	A	A
	Mannitol	A+G	A+G	A+G	A+G	-	A+G	A+G	A+G
	Maltose	A	A+G	A+G	A+G	-	A+G	+	+
	Fructose	-	A+G	-	-	A+G	-	+	+
	Glucose	A+G	A+G	-	Acid	A	-	A	A
	Galactose	+	+	-	+	-	-	+	+
	Mellibiose	-	+	-	-	-	-	-	-
	Sucrose	-	+	+	+	-	+	+	+
	Glycogen	-	-	-	-	-	-	-	-
	Trehalose	-	-	+	+/-	-	+	+	+
	Inositol	-	-	-	-	-	-	-	-
	Gluconate	-	-	-	-	-	-	+	+
	Raffinose	-	+	-	-	-	-	-	-
	Mannose	-	+/-	+	+	-	+	+	+
	Arabinose	+/-	+/-	-	-	-	-	-	-
	Ribose	+	+/-	-	+	-	-	+	+
	Amygdalin	-	-	+	+/-	-	+	+	+
	Adonitol	-	-	-	-	-	-	-	-
	Arabitol	+/-	-	-	-	-	-	-	-
	Cellobiose	-	-	+	+/-	-	+	+	+
	Fucose	-	-	-	-	-	-	-	-

Table.3 Zone of Inhibition (in mm) shown by the isolated *Lactobacilli* isolates against different gastrointestinal pathogens

Zone of Inhibition (in mm) against pathogenic bacteria									
Pathogenic strains / *Lactobacilli* strains	*E. coli*	*E. aerogenes*	*S. aureus*	*Bacillus subtilis*	*E. faecalis*	*E. cloacae*	*K. pneumoniae*	*Heliobacter pylori*	*Candida albicans*
(A) *L. acidofarinae*	**29**	16	26	19	22	24	21	17	18
(B) *L.equi*	23	17	19	18	12`	13	09	14	10
(C) *L. aqaticus I*	23	25	20	28	11	18	20	17	06
(D) *L.curvatus*	21	25	19	16	14	13	14	13	17
(E) *L.floricola*	27	18	21	22	13	24	22	10	-
(F) *L. aquaticus II*	**28**	20	22	27	13	24	24	15	-
(G) *L. casei*	27	25	10	06	09	22	23	**23**	-
(H) *L. casei II*	20	23	-	08	18	25	20	16	18

Table.4 Growth OD of the Lactobacilli isolates at pH 3 on different days

S.No.	Bacterial isolates	Day one O.D.	Day two O.D.	Day three O.D.	Day four O.D.	Day five O.D.
1	A	.376	.583	.599	1.695	1.507
2	B	.389	.769	.967	1.786	1.246
3	C	.391	.560	.944	1.807	1.383
4	D	.405	.620	1.534	1.640	1.451
5	E	.405	.640	.699	1.503	1.300
6	F	.350	.560	.96	1.44	1.05
7	G	.380	.70	1.60	1.9	1.70
8	H	.340	.65	1.4	1.75	1.55

Table.5 Growth of *Lactobacilli* isolates at different NaCl concentrations

Bacterial isolates	Different Salt Concentration		
	2%	4%	6.5%
A	+++	+++	-
B	+++	+++	+
C	++	++	+
D	++	++	+
E	++	++	+
F	+++	++	+
G	+++	++	+
H	+++	++	+

Figure.1 Growth curve of all the Lactobacilli isolates at pH 3

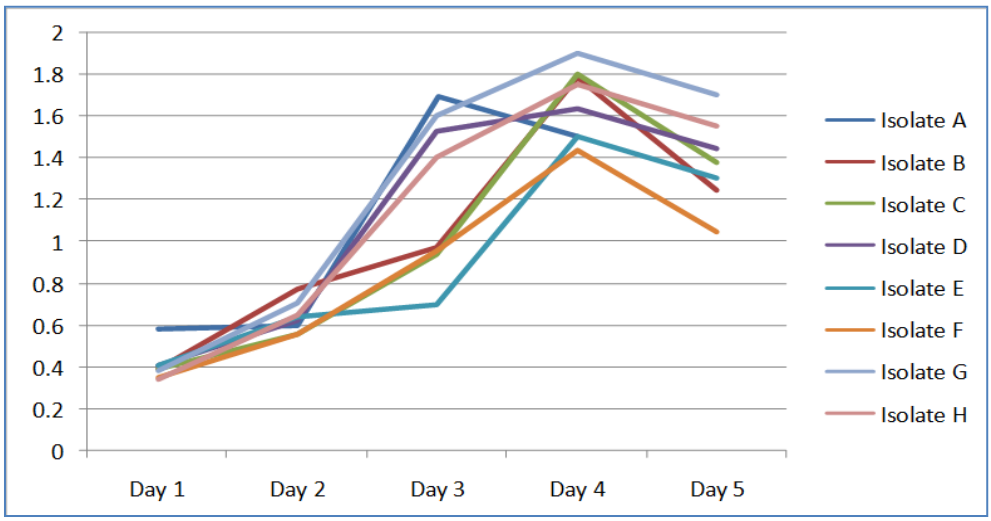
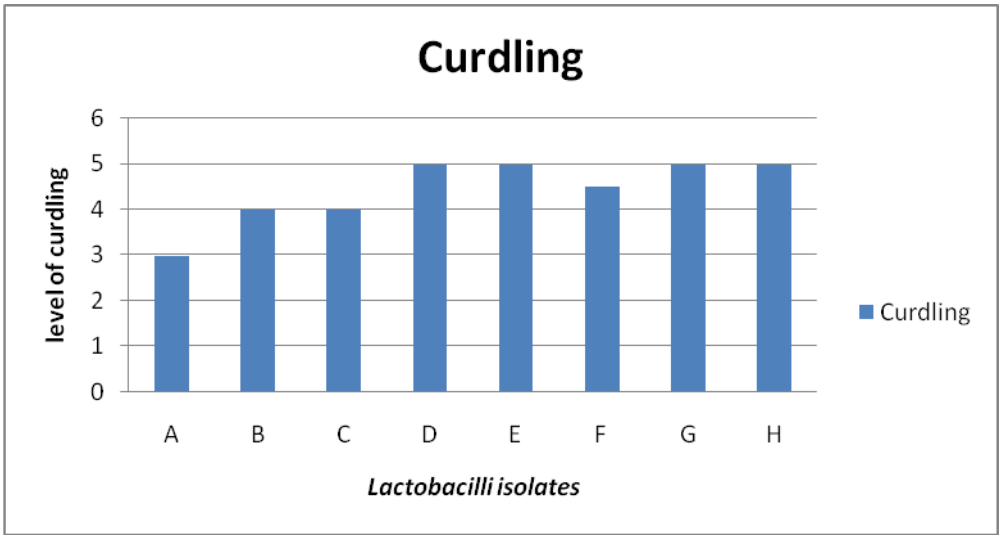


Figure.2 Comparative study of curdling of milk by different isolates



Gilliland et al. (1997) have also reported that the normal concentration of bile salt in human small intestine is 0.3% (w/v), but some studies have suggested that bile concentration is variable and unpredictable, changing according to diet composition and in a close relationship with the secretion of pancreatic enzymes.. Thus, in the present study, 0.3% bile concentration was used for studying the survival of Lactobacilli isolates. All eight isolated Lactobacillus strains showed good bile tolerance at this concentration of bile salt.

The assessment of susceptibility of bacterial strains to antibiotics, showed that most of the bacterial isolates showed susceptibility towards the antibiotics: tetracycline, streptomycin, ampicillin, gentamycin, oflaxacillin, angifix, ceferoxime, penicillin and amoxicillin.

Apart from these *Lactobacillus curvatus* and *Lactobacillus floricola* showed resistance against cephalexime and *Lactobacillus aqaticus* and *Lactobacillus curvatus* showed resistance against roxythromycin only.

Antimicrobial activity against pathogens is another important attribute to be considered in the selection of potential probiotic strains for maintaining a healthy microbial balance in the GIT. In the present study, all eight isolated *Lactobacillus* strains showed antagonistic activity against all the 9 test pathogens, which are pathogenic to humans. The antagonistic activity has mostly been attributed to the production of antimicrobial substances or metabolites such as organic acids, hydrogen peroxide, acetoin, carbon dioxide, reuterin, reutericyclin, and bacteriocins by the probiotic strains. This activity, together with the mechanism of competitive exclusion, in which probiotic strains compete with pathogens for nutrients and attachment sites, would prevent colonization of the intestine by pathogens. Among the antimicrobial substances, organic acids (especially lactic and acetic acids), hydrogen peroxide, and bacteriocins are the most common antimicrobial substances that have been reported to be produced by probiotic strains. Jin (2003) & Shokryazdan (2014) had also found that the inhibitory effects of 12 Lactobacillus strains on pathogenic *Salmonella* and *E.coli* were due to their production of organic acids and bacteriocins.

The results of this in vitro study indicated that all eight Lactobacillus strains were able to survive in the GIT and attached to the epithelial cells, while none of them was antibiotic resistant. Since all eight *Lactobacillus* strains showed strong antagonistic activities against a wide range of pathogens to humans, they could be considered as good potential probiotic candidates for treatment and prevention of infections. They should be studied further as biotherapeutic agents for treatments of specific disease conditions. The strains should also be investigated further for other probiotic bioactivities that have human health benefits.

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