Studies on Antimicrobial Activity and Characteristics of Bacteriocins Produced by Lactobacillus strains Isolated from Milk of Domestic Animals

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Citation

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Abstract

Lactobacilli strains were isolated from the milk of domestic animals for potential probiotic. A total of 120 milk samples (40 each from buffalo, cow and goat) were analyzed and 110 Lactic Acid Bacteria (LAB) were identified, out of these 43 were identified as probiotics, (L. acidophilus, L. bulgaricus, L. plantarum, L. lactis and L. rhamnosus). Out of these, 11 isolates were potential probiotics, which includes L. plantarum (C1, C4, C7, G7, G8 and B14) and L. rhamnosus (B13, C5, G4, G10 and G18). Their bacteriocins showed a broad inhibitory spectrum against the indicator organisms tested. The bacteriocins produced by L. plantarum (C4, G7) and L. rhamnosus (G18) showed prominent antimicrobial activity, resistance to heat at 121C and tolerate acidic pH 3 but sensitive to pH 9 indication strong probiotic potential. These isolated LABS, exhibiting excellent probiotic characteristics, can be use in the protection and improvement of intestinal microbial flora and contribute health benefits to consumers.

INTRODUCTION

Lactic acid bacteria (LAB) widely distributed in the nature and occurring indigenous microflora in raw milk that play an important role in many food and feed fermentations with increased shelf life. Within the LAB group, the genus Lactobacillus is the most widely encountered for probiotics because they display numerous antimicrobial activities. This is mainly due to the production of antimicrobial metabolites including organic acids, hydrogen peroxide and bacteriocins. Among these, bacteriocins have gained increasing interest. Bacteriocins, as defined by Ogunbanwo et al, (2003) are proteinaceous compounds produced by bacteria that exhibit a bactericidal mode of action against related as well as unrelated organisms. Bacteriocin generally exert their antimicrobial action by interfering with the cell wall or the membrane of target organisms, either by inhibiting cell wall biosynthesis or causing pore formation, subsequently resulting in death (Sullivan et al, 2002).

The bacteriocin from food grade LAB appear to qualify as ideal food biopreservative primarily because they have proven non-toxic to humans, do not alter the nutritional properties, effective at low concentration, active under refrigerated storage and can be used even in the preservation of foods by the use of bacteriocin produced by LAB. Bacteriocins produced by LAB have been the focus of many investigations because of their particular importance in the dairy industry. Several bacteriocins with industrial potential have been purified and characterized. The highly promising results of these studies underline the important role that functional, bacteriocinogenic LAB strains may play in the food industry as starter cultures, co-cultures, or bioprotective cultures, to improve food quality and safety (De vuyst and Leroy, 2007). Many LAB produce proteinaceous antimicrobial bacteriocins, some of which could provide valuable alternatives to traditional therapeutic antibiotics for the treatment of infectious diseases (Ryan et al, 1989). Bacteriocins are inhibitory towards sensitive strains and are produced by both Gram positive and Gram negative bacteria (Kacem et al, 2005). Bacteriocins have attracted a great interest in food industry due to their application potentiality in food preservation. It is relatively hydrophobic and heat stable (Klaenhammer, 1993). The purpose of this work was to isolate potential probiotic Lactobacillus strains and determined the characterization and antimicrobial activity of bacteriocins.

MATERIALS AND METHODS

Isolation and identification of Lactobacillus species: A total of 120 milk samples (40 each from cow, goat and buffalo) were randomly collected in sterilized glass bottles. Milk was serially diluted to 10^{-5}–10^{-6} using sterile distilled water and 0.1mL plated on to sterile de-Mann, Rogosa and Sharpe (MRS) agar. The MRS plates were maintained in microaerophilic condition and incubated at 37°C for 48h. After incubation well-isolated typical colonies were picked up and transferred to MRS broth and incubated at 37°C for 48h. The isolates were identified using standard morphological, cultural and biochemical reactions (Howells, 1992).

Detection of antagonistic activities: The antagonistic properties of isolated LAB species were determined by modifying the disc diffusion method. Sterile blotting paper discs (10mm) were dipped into 48h incubated Lactobacillus sp. culture broth and then placed on solidified Nutrient Agar seeded with 3h old culture of test pathogens, which included Escherichia coli (MTCC 443), Enterobacter aerogenes (MTCC 111), Klebsiella pneumoniae (MTCC 2653), Proteus vulgaris (MTCC 426), Salmonella typhi (MTCC 734) and Shigella flexneri (MTCC 1457). The plates were kept at 4°C for 1h diffusion and then incubated at 37°C for 24h. Zones of inhibition were measured (Kirby-Bauer, 1966).

Acid and bile salt tolerance: Isolated Lactobacillus sp. were inoculated into MRS medium of varying pH, i.e. pH 2, 3, 4 and 5; as well as broth with varying concentrations of bile salt (0.5, 1.0, 1.5 and 2.0%), and incubated at 37°C for 48h. Then 0.1mL inoculums was transferred to MRS agar by pour plate method and incubated at 37°C for 48h. The growth of LAB on MRS agar plate was used to designate isolates as acid or bile salt tolerant.

Antibiotic resistance: The antibiotic resistance of isolated LAB was assessed using antibiotic discs (Hi media Laboratories Pvt. Ltd. Mumbai, India) on MRS agar plates. A 10^6 cfu/mL suspension of freshly grown test organisms was mixed with 5mL of MRS soft agar (0.5% agar) and over layered on bottom layers of MRS agar. The antibiotic discs were placed on the surface of agar and the plates were kept at 4C for 1h for diffusion, and then incubated at 37C for 24h Halami et al, (1999). Resistance was assessed against Ampicillin (1µg), Cephalothin (30µg), Co-Trimoxazole (25µg), Gentamicin (10µg), Nalidixic acid (30µg), Nitrofurantoin (300µg), Norfloxacin (10µg) and Tetracycline (25µg).

Preparation of bacteriocin assay: The prominent probiotic Lactobacillus strains were selected as potential bacteriocin producers grown in MRS broth at 37C for 48h. Cell suspensions were centrifuged at 5000 rpm for 15 min. The pH of the cell free supernatant was adjusted to pH 6.5-7.0 with 1N NaOH to neutralize the acids in broth culture of probiotics. The antagonistic activity of bacteriocin was determined by disc diffusion method (Tagg and McGiven, 1971).

Heat and pH sensitivity: To test the heat sensitivity, culture supernatant containing bacteriocin was heated for 10 min. at 60C, 70C, 80C, 90C, 100C and 121C and bacteriocin activity was tested against E.coli. Similarly sensitivity of bacteriocins to different pH was tested by adjusting the pH of culture supernatant (containing bacteriocins) in the range of pH 3.0, 4.5, 7.0 and 9.0 then bacteriocin antibacterial activity was detected by disc diffusion method against E. coli (Ogunbanwo et al, 2003).

RESULTS AND DISCUSSION

In present study, a total 120 milk samples (40 each from Cow, Goat and Buffalo) were analyzed, from which 110 Lactobacillus species were identified as L. acidophilus (13%), L. brevis (10%), L. bulgaricus (9%), L. lactis (19%), L. plantarum (15%), L. rhamnosus (14%), L. helveticus (2%), L. casei (17%) and L. fermentum (1%). Some similar Lactobacillus sp. isolated from raw milk and dairy products by Medina et al, (2001). Out of these 11 isolates were recognized as prominent probiotics (Table 1).

Figure 1

Table1

Table 1: Antagonistic activities against bacterial pathogens, Acid tolerance, Bile salt tolerance and antibiotic susceptibility of potential probiotic bacteria isolated from milk of domestic animals

Source	Probiotic	Code	Acid Tolerance (pH-2)	Bile salt Tolerance 2 %	Zone in inhibition against pathogens (Diameter in mm, Average of 3 reading)						Antibiotics Susceptibility (Average of 3 reading)								
					E. coli	*Enterobacter aerogenes*	*Klebsiella pneumoniae*	*Proteus vulgaris*	*Salmonella typhi*	*Shigella flexneri*	A	Ch	Co	G	Na	Nf	Nx	T	Total Resistant
Buffalo	*L. rhamnosus*	B13	+	+	18	20	21	23	23	19	R	R	R	R	R	R	R	S	7
	L. plantarum	B14	+	+	19	21	21	22	23	21	R	R	R	R	R	R	R	S	7
Cow	*L. plantarum*	C1	+	+	18	22	24	21	23	20	R	R	R	R	R	R	R	R	8
	L. plantarum	C4	+	+	18	24	25	23	25	22	R	R	R	R	R	R	R	R	8
	L. rhamnosus	C5	+	+	19	23	23	22	24	20	R	R	R	R	R	R	R	S	7
	L. plantarum	C7	+	+	17	23	23	21	24	20	R	R	R	R	R	R	R	R	8
Goat	*L. rhamnosus*	G4	+	+	17	22	23	22	24	21	R	R	R	R	R	R	R	S	7
	L. plantarum	G7	+	+	19	24	23	24	25	24	R	R	R	R	R	R	R	R	8
	L. plantarum	G8	+	+	18	22	21	21	24	20	R	R	R	R	R	R	R	R	8
	L. rhamnosus	G10	+	+	18	18	23	22	25	21	R	R	R	R	R	R	R	S	7
	L. rhamnosus	G18	+	+	19	24	24	24	25	24	R	R	R	R	R	R	R	R	8

Where:- A-Ampicillin, Ch-Cephalothin, Co-Co-Trimoxazole, G-Gentamicin, Na-Nalidixic acid, Nf-Nitrofurantoin, Nx-Norfloxacin, T-tetracycline

Figure 2

Table2

Table 2: Antibacterial activity of bacteriocin against pathogens

Probable isolate	Code no.	Zone in inhibition (diameter in mm) (Average of 3 reading)					
		E.coli	*Enterobacter aerogenes*	*Klebsiella pneumoniae*	*Proteus vulgaris*	*Salmonella .typhi*	*Shigella flexneri*
L.rhamnosus	B13	18	20	22	23	23	20
L. plantarum	B14	18	22	21	23	23	21
L. plantarum	C1	20	22	23	22	24	20
L. plantarum	C4	21	24	25	24	27	25
L.rhamnosus	C5	19	24	24	23	23	22
L. plantarum	C7	18	23	24	23	24	22
L.rhamnosus	G4	19	24	24	23	24	23
L. plantarum	G7	22	25	25	26	27	27
L. plantarum	G8	20	24	23	23	25	23
L.rhamnosus	G10	20	23	24	23	25	24
L.rhamnosus	G18	23	25	25	26	28	27

Antagonistic activity: The antagonistic activity of isolates was determined against selected enteric bacterial pathogens. Out of these, 11 isolates showed strong inhibition in the disc diffusion test (Table 1). This may be due to the production of acetic and lactic acids that lowered the pH of the medium or competition for nutrients, or due to production of bacteriocin or antibacterial compound (Bezkorvainy, 2001). Obadina et al, (2006) also reported that fermentation process which involved L. plantarum had a broad antimicrobial inhibitory spectrum, against Salmonella typhi, E. coli and S. aureus. Our study showed that L. rhamnosus and L. plantarum had strongest antibacterial potential against Salmonella typhi followed by Proteus vulgaris and Klebsiella pneumoniae. Olarte, (2000) noted that the presence of L. plantarum in the cheese from Goat's milk decreased the number of the enterobacteria and fecal coliforms in the final product.

Acid and Bile salt tolerance: For evaluating the potential of LAB as effective probiotics it is generally considered necessary to evaluate their ability to resist the effects of bile acids (Lee and Salminen, 1995). In this study 11 isolates showed acid tolerance at pH-2 and bile salt tolerance at 2% (Table 1). Before reaching the intestinal tract, probiotic bacteria must first survive transit through the stomach where the pH can be as low as 1.5 to 2 (Dunne et al, 2001). Tolerance to bile salts is considered to be a prerequisite for colonization and metabolic activity of bacteria in the small intestine of the host (Havenaar et al, 1992). This will help Lactobacilli to reach the small intestine and colon and thus contribute in balancing the intestinal microflora.

Antibiotic resistance: Resistance of the probiotic strains to some antibiotics could be used for both preventive and therapeutic purposes in controlling intestinal infections. Moreover, their resistance to antibiotics was clarifying their potential in minimizing the negative effects of antibiotic therapy on the host bacterial ecosystem (EI-Naggar, 2004). In this study, all identified probiotic species of L. plantarum (C1, C4, C7, G7 and G8) and L. rhamnosus (G18) were showed resistance to all these tested 8 antibiotics while L. plantarum (B14) and L. rhamnosus (B13, C5, G4, and G10) were sensitive to tetracycline but resistance to remaining 7 antibiotics in the octa disc which is in accordance with Voravuthikunchai et al, (2006).

Antibacterial activity of bacteriocin: The isolated prominent probiotics 11 LABs strains were screened for antimicrobial potential of produced bacteriocins. These isolates include L. plantarum (B14, C1, C4, C7, G7 and G8) and L. rhamnosus (B13, C5, G4, G10 and G18). Bacteriocins of all the eleven producer organisms have wide antibacterial activity towards selected enteric pathogens (Table 2). These bacteriocin producer isolates showed inhibitory activity against Salmonella typhi, Proteus vulgaris, Klebsiella pneumoniae, Enterobacter aerogenes, and followed by Shigella flexneri and E. coli. Itoh et al, (1995) also reported the inhibition of food borne bacteria by bacteriocins from L. gasseri.

Figure 3

Table 3

Table 3: Effect of heat treatment and pH on bacteriocin

Probable isolate	Code no.	Heat Resistance at						Sensitivity to pH			
		60°C	70°C	80°C	90°C	100°C	121°C	3	4.5	7	9
L. rhamnosus	B13	R	R	R	S	S	S	R	R	R	S
L. plantarum	B14	R	R	R	R	S	S	R	R	R	S
L. plantarum	C1	R	R	R	R	S	S	R	R	R	S
L. plantarum	C4	R	R	R	R	R	R	R	R	R	S
L. rhamnosus	C5	R	R	R	R	S	S	R	R	R	S
L. plantarum	C7	R	R	R	R	R	S	R	R	R	S
L. rhamnosus	G4	R	R	R	R	S	S	R	R	R	S
L. plantarum	G7	R	R	R	R	R	R	R	R	R	S
L. plantarum	G8	R	R	R	R	R	S	R	R	R	S
L. rhamnosus	G10	R	R	R	R	R	S	R	R	R	S
L. rhamnosus	G18	R	R	R	R	R	R	R	R	R	S
Where R=Resistant, S=Sensitive											

Characterization of bacteriocin: Bacteriocin of all the 11 probiotics showed heat stability at 60C, 70C 80C and 90C

for 10 min except B13 which was sensitive at 90C. Out of these, 6 isolates showed heat stability at 100C while 3 isolates showed heat stability at 121C which includes C4, G7 of L. plantarum and G18 of L. rhamnosus. Bacteriocins of all these isolates were stable in acidic to neutral range i.e. from pH 3.0 to 7.0 but it became inactive in alkaline range at pH 9.0. Similar finding was also recorded by Lade et al, (2007) and Toba et al, (1991).

CONCLUSION

The study indicated that the isolated Lactobacillus species meet several of the criteria for use as a probiotic. These characteristics may be advantageous for a probiotic culture to be successful in colonizing and compete with pathogens in gastrointestinal environment. The ability to survive acidic conditions, bile resistance, and the production of bacteriocin that is active against enteric pathogens. These bacteriocins were also stable over a wide range of pH and heat. This heat and pH stability may be useful if the bacteriocin is to be used as an antimicrobial agent in fermented foods or thermally processed foods. Probiotic approach is to reconstitute natural condition by means of repairing a deficiency either by producing organic acids, antimicrobial substance and vitamins etc. and remove foreign chemicals from the body, which may have toxic consequences or, as in the case of antibiotics induce resistance and compromise subsequent therapy. Resistance of the probiotic strains to antibiotics could be used for both preventive and therapeutic purpose in controlling intestinal infection. The bacteriocins produced by L. plantarum (C4, G7) and L. rhamnosus (G18) showed prominent antimicrobial properties, heat resistance and acid tolerant indicating strong probiotic potential hence these isolates can be use in the protection and improvement of intestinal microbial flora and contribute health benefits to consumers.

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