Probiotic Properties of Lactic Acid Bacteria Isolated from Yoghurt: A Traditional Indian Fermented Milk Product

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Abstract: *Lactobacillus* species play a major role in fermented dairy products and also contribute to the therapeutic aspects of human health. The present study was carried out to isolate and identify *Lactobacillus species* in Yoghurt having potential probiotic properties. A total of 40 Yoghurt samples were analyzed and 63 Lactic Acid Bacteria (LAB) were identified, out of these 23 were identified as probiotics, (*L. acidophilus, L. bulgaricus, L. plantarum, L. lactis and L. rhamnosus*). Out of these, 10 isolates were potential probiotics, which includes *L. plantarum* (L11, L13, L24, L29, L33, L34 and L40) and *L. rhamnosus* (L9, L36 and L38). Their bacteriocins showed a broad inhibitory spectrum against the indicator organisms tested. The bacteriocins produced by *L. plantarum* (L24, L33) and *L. rhamnosus* (L36) showed prominent antimicrobial activity, resistance to heat at 121^0C and tolerate acidic pH 3 but sensitive to pH 9 indication strong probiotic potential. The results proved that LABS isolated from Yoghurt exhibited promising probiotic properties can be use in the protection and improvement of intestinal microbial flora and contribute health benefits to consumers.

Key words: Probiotic, Lactobacillus, Antibacterial activity, Bacteriocin, Yoghurt

1. Introduction

Probiotics are, "live microorganisms which when administered in adequate amounts confer a health benefit on the host[1]. Yogurt is the most common source of probiotics. Yogurt consists of milk fermented by bacteria that modify lactose into lactic acid [2]. Lactic acid bacteria (LAB) produce lactic acid either through homofermentive or heterofermentive pathway and are wide spread in nature. Lab bacteria have a probiotic effect on human health[3-4]. Also found in human digestive system. *Lactobacillus* species play a major role in fermented dairy products and food industry and also contribute to the therapeutic aspects of human health[5]. *Lactobacilli* are considered as beneficial bacteria because they have their ability to break down proteins, carbohydrates & fats in food and help in absorption of necessary elements and nutrients such as minerals, amino acids and vitamins required for the survival of humans and other animals Lactobacilli represent a significant part of our intestinal microflora, and their friendship with the general state of human health is under serious investigation[6-7]. The genus *Lactobacillus* is one of the major groups of lactic acid bacteria used in food fermentation and is thus of great economical importance. Lactic acid bacteria (LAB) are a group of Gram-positive, non-spore forming, cocci or rods, catalase-negative. For an organism to be a probiotic, it must essentially be non-pathogenic, be generally regarded as safe (GRAS), tolerate low pH, tolerate high concentrations of bile salts[8-9]. showing a DNA having G+C content less than 50 mol% and organisms of interest in food processing industries because of their typical roles in inhibiting the growth of food spoilage bacteria. The lactic acid bacteria (LAB) are comprised of at least ten genera: *Aerococcus, Carnobacterium ,Enterococcus, Lactobacillus, Leuconostoc, Pediococcus, Streptococcus,tetragenococcus* and *Vagococcus* .Most representatives of these group have been consumed for thousands of years, do not pose any health risk to humans and are designated as GRAS organisms (generally recognized as safe)[10].The search for new bacteriocin producing lactic acid bacteria is of great significance because of their potential use in fermented food and feed industries[11]. LAB exerts a strong antagonistic activity against many food spoilage organisms and food borne pathogens. The aim of the study was to isolate lactic acid bacteria from fermented food Yoghurt and screening it for probiotic properties. Yoghurt consists of milk fermented by bacteria that modify lactose into lactic acid[12]. LAB is the most important group of microorganisms commercially used as starter cultures for the manufacture of dairy based probiotic foods and have been established as a natural consumer.

2. Materials and Methods

Isolation and identification of *Lactobacillus* species:

A total 40 different samples of fresh *Yoghurt* (Indian traditional fermented milk Product) were collected in sterilized screw capped bottles. Yoghurt 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 37^0C for 48h. After incubation, well isolated typical colonies were picked up, transferred to MRS broth, and incubated at 37^0C for 48h. The isolates were identified using standard morphological, cultural and biochemical reactions[13].

Acid and bile salt tolerance

Isolated *Lactobacillus* sp. were inoculated into MRS medium of varying pH, i.e. pH 2, 4, 7 and 11; as well as broth with varying concentrations of bile salt (0.5, 1.0, 1.5 and 2.0%), and incubated at 37^0C for 48h. Then 0.1mL inoculums was transferred to MRS agar by pour plate method and incubated at 37^0C for 48h. The growth of LAB on MRS agar plate was used to designate isolates as acid or bile salt tolerant.

Temperature tolerance

From the yoghurt sample isolate 10 LAB were screened for the temperature tolerance activity of different range. MRS broth were prepared and screened for temperature such as 27^o C, 37^o C, 40^o C, 60^o C, 80^o C.

Preparation of bacteriocin assay

The prominent probiotic *Lactobacillus* strains were selected as potential bacteriocin producers grown in MRS broth at 37^0C for 48h. Cell suspensions were centrifuged at 5000 rpm for 15 min. The pH of the cell free supernatant was adjusted to pH 6.5-7.0with 1N NaOH to neutralize the acids in broth culture of probiotics. The antagonistic activity of bacteriocin was determined by disc diffusion method[14]. Heat and pH sensitivity: To test the heat sensitivity, culture supernatant containing bacteriocin was heated for 10 min. at 60^0C, 70^0C, 80C, 90^0C, 100^0C and 121^0C 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* [15].

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 4^0C for 1h for diffusion, and then incubated at 37^0C for 24h[16]. Resistance was assessed against Ampicillin (1µg), Cephalothin (30µg), Co-Trimoxazole (25µg), Gentamicin (10µg), Nalidixic acid (30µg), Nitrofurantoin (300µg), Norfloxacin (10µg) and Tetracycline (25µg).

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 40C for 1h diffusion and then incubated at 370C for 24h. Zones of inhibition were measured[17].

3. Results and Discussion

In present study, a total 40 yoghurt samples were analyzed and 63 Lactic Acid Bacteria (LAB) were identified, out of these 23 were identified as probiotics, (*L. acidophilus, L. bulgaricus, L. plantarum, L. lactis and L. rhamnosus*). Out of these, 10 isolates were potential probiotics, which includes *L. plantarum* (L11, L13, L24, L29, L33, L34 and L40) and *L. rhamnosus* (L9, L36 and L38). Their bacteriocins showed a broad inhibitory spectrum against the indicator organisms tested. The bacteriocins produced by *L. plantarum* (L24, L33) and *L. rhamnosus* (L36) (Table 1).

4. Acid and Bile salt tolerance

Probiotics potential of LAB is necessarily its ability to resist bile salts and acidic pH[18]. In this study, three isolated excellent probiotic, commercial probiotic and standard probiotic bacterial strains 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[19]. Tolerance to bile salts is a prerequisite for colonization and metabolic activity of bacteria in the small intestine of the host[20]. This will help Lactobacilli to reach the small intestine and colon and contribute in balancing the intestinal microflora[21].

Antibacterial activity of bacteriocin: The isolated prominent probiotics 11 LABs strains were screened for antimicrobial potential of produced bacteriocins. These isolates include (L11, L13, L24, L29, L33, L34 and L40) and *L. rhamnosus* (L9, L36 and L38). Their bacteriocins showed a broad inhibitory spectrum against the indicator organisms tested. The bacteriocins produced by *L. plantarum* (L24, L33) and *L. rhamnosus* (L36). Bacteriocins of all the eleven producer organisms have wide antibacterial activity towards selected enteric pathogens (Fig 1). 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* [22]. (Table 2)

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 (Fig 2). 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[23] 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*[24].

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[25] (Tabel 3).

Characterization of bacteriocin: Bacteriocin of all the 11probiotics showed heat stability at 60^0C, 70^0C 80^0C and 90^0C or 10 min except L13 which was sensitive at 90^0C. Out of these, 6 isolates showed heat stability at 100^0C while 3 isolates showed heat stability at 121^0C which includes L11, L24 of *L. plantarum* and L36 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 apt 9.0. Similar finding was also recorded[26-27] by Lade *et al.*, (2007) and Itoh *et al.*, (1991).

5. 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. The bacteriocins produced by *L. plantarum* and *L. rhamnosus* 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.

Table 1: Probiotics properties of isolated lactic acid bacteria from yoghurt

Probiotics strains	Acid tolerance	Bile tolerance	Antibiotic susceptibility								Heat sensitivity of bacteriocin						Sensitivity to different pH			
	pH 2	2%	Ampicillin (A)	Cephalothin (Ch)	Co-Trimoxazole (Co)	Gentamicin (G)	Nalidixic acid (Na)	Nitrofurantoin (Nf)	Norfloxacin (Nx)	Tetracycline (T)	60^0C	70^0C	80^0C	90^0C	100^0C	121^0C	pH 2	pH 4	pH 7	pH 11
L. plantarum (L11)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
L. plantarum (L13)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
L. plantarum (L24)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
L. plantarum (L29)	R	R	R	R	S	S	R	S	S	R	R	R	R	R	S	S	R	R	R	S
L. plantarum (L33)	R	R	R	R	R	R	R	R	R	S	R	R	R	R	S	S	R	R	R	S
L. plantarum (L34)	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	S	R	R	R	S
L. plantarum (L40)	R	R	R	R	R	R	R	R	S	R	R	R	R	R	S	S	R	R	R	S
L. rhamnosus (L9)	R	R	R	R	R	R	R	S	S	R	R	R	R	R	S	S	R	R	R	S
L. rhamnosus (L36)	R	R	R	R	R	S	R	S	R	R	R	R	R	R	R	S	R	R	R	S
L. rhamnosus (L38)	R	R	R	R	R	R	R	S	S	S	R	R	R	R	R	S	R	R	R	S

Where: R=Resistant, S= Sensitive

Table 2: Antibacterial activity of probiotics against enteric pathogens

Probiotics	Antibacterial activity of probiotics against enteric pathogens (Zone of Inhibition in mm)					
	E. coli	*E. aerogenes*	*K. pneumoniae*	*Pr. vulgaris*	*S. typhi*	*Sh. flexneri*
*L. plantarum (*L11)	18	24	25	23	25	22
L. plantarum (L13)	19	24	23	24	25	24
L. plantarum (L24)	19	24	24	24	25	24
L. plantarum (L29)	17	20	21	20	23	21
L. plantarum (L33)	20	20	17	19	22	18
L. plantarum (L34)	18	20	23	22	23	20
L. plantarum (L40)	16	16	15	17	19	18
L. rhamnosus (L9)	16	20	23	25	25	22
L. rhamnosus (L36)	18	20	20	22	20	19
L. rhamnosus (L38)	16	18	18	17	20	18

Table 3: Antibacterial activity of bacteriocin against enteric pathogens (Zone of inhibition in mm)

Probiotics strains	*E. coli*	*E. aerogenes*	*K. pneumoniae*	*Pr. vulgaris*	*S. typhi*	*Sh. flexneri*
*L. plantarum (*L11)	21	24	25	24	27	25
L. plantarum (L13)	22	25	25	26	27	27
L. plantarum (L24)	23	25	25	26	28	27
L. plantarum (L29)	21	23	23	22	25	23
L. plantarum (L33)	22	23	23	23	25	19
L. plantarum (L34)	20	19	20	23	27	25
L. plantarum (L40)	20	22	22	22	25	23
L. rhamnosus (L9)	19	22	24	23	27	25
L. rhamnosus (L36)	20	22	24	21	24	23
L. rhamnosus (L38)	20	21	23	21	25	23

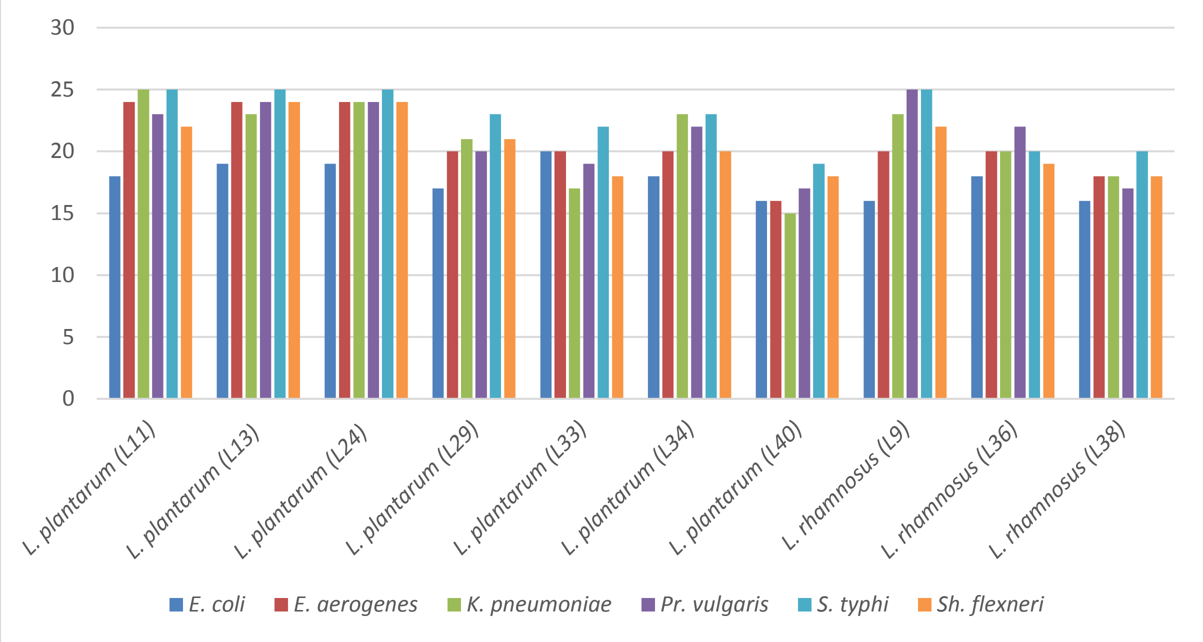

Fig 1: Antibacterial activity of probiotics against enteric pathogens

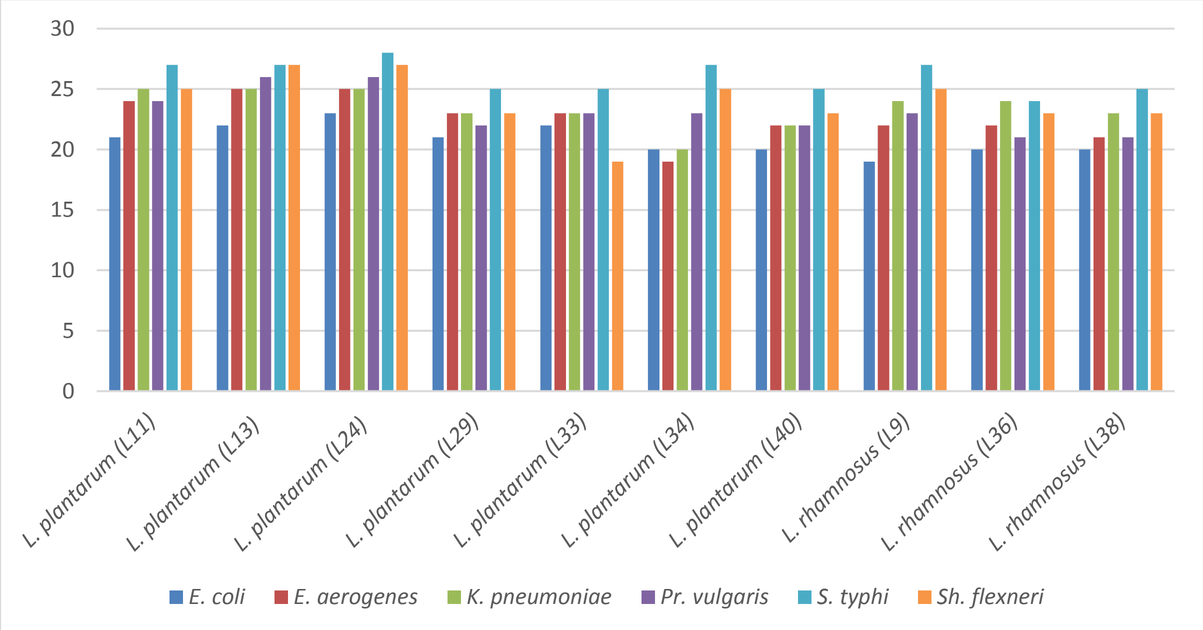

Fig 2: Antibacterial activity of bacteriocin against enteric pathogens

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