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ACID AND BILE TOLERANCE, ANTIBACTERIAL ACTIVITY, ANTIBIOTIC RESISTANCE AND BACTERIOCINS ACTIVITY OF PROBIOTIC *LACTOBACILLUS* SPECIES

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Abstract

Lactobacilli are an extremely important group of probiotic bacteria inhibit undesirable microflora in the gut and create a healthy equilibrium between beneficial and potentially intestinal pathogens. The study was attempted to isolate *Lactobacilli* strains from goat milk to search for a new effective antibacterial probiotic strains. From 40 raw goat milk samples, 48 *Lactobacilli* isolates were isolated, identified and analyzed for their probiotic properties including acid and bile salt tolerance, antibacterial activity against enteric pathogens, antibiotic resistance patterns and production of bacteriocin. Out of these *Lactobacillus* species, five isolates were potential probiotics, and bacteriocins produced by them showed demonstrable antibacterial activity against the test pathogens such as *S. typhi*, *Pr. vulgaris*, *K. pneumoniae*, *Sh. flexneri*, *E. aerogenes* and *E. coli*. The bacteriocin produced by *L. plantarum* (G95a) and *L. rhamnosus* (G119b) was prominent antibacterial, resistant to heat at 1210C and tolerated acidic pH 3 but sensitive to pH 9. The present study suggested that isolated Lactobacillus strains have an excellent probiotic potential and control of enteric infections and restoration of microbial gut flora.

Keywords: Lactobacillus, Acid tolerance, Bile tolerance, Probiotic, Bacteriocin, Antibacterial activity

Introduction

Goat raw milk represents a source of new strains of Lactobacilli with probiotic potential to inhibit undesirable microflora in the gut. Lactobacilli constitute a major part of the natural microflora of human intestine. These probiotic organisms when present in sufficient number can create a healthy equilibrium between beneficial and potentially harmful microflora in the gut by creating unfavorable conditions for the growth of commonly occurring intestinal pathogen (Tagg and McGiven, 1971; Salminen et al., 1998). Probiotics can be used in the treatment and prevention of enteric infections and chronic inflammatory disorders of the gastrointestinal tract (Gill and Guarner, 2004). They are non-pathogenic, acid and bile tolerant, adhere to the gut epithelial tissue and produce antimicrobial substances, including organic acids, hydrogen peroxide and bacteriocins (Dunne et al., 2001). Bacteriocins are proteinaceous antibacterial compounds that are bactericidal to many pathogens associated with food spoilage and food borne illnesses including *E. coli*, *Salmonella* sp., *Shigella* sp., *B. cereus*, *Cl. botulinum*, *Cl. perfringes*, *S. aureus* etc (Van der Kaaij et al, 2004). They are degraded by the proteolytic enzymes of the gastrointestinal tract and seem to be non-toxic and non-antigenic to animals. Thus, they can be used to enhance the safety and shelf life of many foods (Ogunshe et al., 2007). Antibiotic resistant Lactobacilli strains have been isolated from animals or human and can be used as the antimicrobial agents for therapy and prophylaxis of bacterial infections (Salminen et al, 1998). Antibiotics tolerance probiotic is of great interest due to their possible use to reconstitute the intestinal microflora of patients suffering from antibiotic-associated colitis (Danielsen and Wind, 2003).

The objective of the study was to investigate the probiotic potential of the *Lactobacilli* sp from goat milk against selected enteric bacterial pathogens and to reduce their pathological consequences for the host. On the other hand, the tolerance of each probiotic strain to bile salts, acidity was investigated to demonstrate its survivability in the small intestine and colon to contribute in the balance of the intestinal microbiota. Moreover, their tolerance to antibiotics was investigated to clarify their potential in minimizing the negative effects of antibiotic therapy on the host bacterial ecosystem. Bacteriocin produced by Lactobacilli are characterized and investigated for their antibacterial potential against test pathogens.

Materials and Methods

Isolation and identification of *Lactobacillus* species

40 goat milk samples were randomly collected in sterilized glass bottles. Milk was serially diluted and plated on to sterile de-Mann, Rogosa and Sharpe (MRS) agar. The MRS plates were maintained in

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microaerophilic condition and incubated at 37°C for 48h. The isolates were identified using standard morphological, cultural and biochemical reactions (Howells, 1992).

Detection of antibacterial activities

The antibacterial activities of isolated *Lactobacillus* species were determined by modifying the disc diffusion method. Sterile blotting paper discs were dipped into isolated 48h *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 kept at 4°C for 1h for diffusion and then incubated at 37°C for 24h and zones of growth inhibition measured in mm (Bauer et al., 1966).

Acid and bile salt tolerance

Isolated *Lactobacillus* sp. were inoculated into MRS medium of varying pH, i.e. pH 2-5; as well as broth with varying concentrations of bile salt (0.5-2.0%), and incubated at 37°C for 48h. Then 0.1mL inoculums transferred to MRS agar by pour plate method and incubated at 37^{0}C for 48h. The growth of *Lactobacillus* sp. on MRS agar plate was used to designate isolates as acid or bile salt tolerant.

Antibiotic resistance

The antibiotic resistance of isolated *Lactobacillus* sp. was assessed using antibiotic discs (Hi media Laboratories Pvt. Ltd. Mumbai, India) on MRS agar plates. The antibiotic discs were placed on the surface of agar and the plates were kept at 4^{0}C for 1h for diffusion, and then incubated at 37^{0}C 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 culture supernatants for bacteriocins assay

The prominent probiotic *Lactobacillus* strains were selected as potential bacteriocins producers were grown in MRS broth at 37^{0}C 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 pH. The antagonistic activity of bacteriocins was determined by disc diffusion method (Tagg and McGiven, 1971).

Heat and pH sensitivity

To test the heat sensitivity, culture supernatant was heated for 10 min. at 60^{0}C, 70^{0}C, 80^{0}C, 90^{0}C, 100^{0}C and 121^{0}C and bacteriocins activity was detected against *E. coli.* Similarly, sensitivity of bacteriocins to different pH values was tested by adjusting the pH of culture supernatant in the range of pH 4.0 to 9.0 and bacteriocins antibacterial activity was detected by disc diffusion method against *E. coli* (Ogunbanwo et al., 2003).

Results

In present study, a total of 40 goat milk samples were analyzed, from which 48 *Lactobacillus* species were identified as *L. acidophilus* (15%), *L. brevis* (13%), *L. bulgaricus* (13%), *L. lactis* (17%), *L. plantarum* (13%), *L. rhamnosus* (21%), *L. helveticus* (4%) and *L. casei* (6%). Out of these 48 *Lactobacillus* species, five isolates including *L. plantarum* (G95a and G96a) and *L. rhamnosus* (G92, G99c and G119b) were recognized as prominent probiotics that showed strong antibacterial (Table 1). These 5 probiotic isolates showed acid tolerance at pH-2 and bile salt tolerance at 2% (Table 1).

Table 1: Antibacterial activity and antibiotic susceptibility of *Lactobacillus* isolates

	Antibacterial activity as zone of inhibition of growth in mm						Antibiotics resistance								
Lactobacillus isolates	*E. coli*	*E. aerogenes*	*K. pneumoniae*	*Pr. vulgaris*	*S. typhi*	*Sh. flexneri*	Ampicillin	Cephalothin	Co-Trimoxazole	Gentamicin	Nalidixic acid	Nitrofurantoin	Norfloxacin	Tetracycline	Total Resistant
L. rhamnosus G92	17	22	23	22	24	21	R	R	R	R	R	R	R	S	7
L. plantarum G95a	19	24	23	24	25	24	R	R	R	R	R	R	R	R	8
L. plantarum G96a	18	22	21	21	24	20	R	R	R	R	R	R	R	R	8
L. rhamnosus G99c	18	18	23	22	25	21	R	R	R	R	R	R	R	S	7
L. rhamnosus G119b	19	24	24	24	25	24	R	R	R	R	R	R	R	R	8

Note: All these *Lactobacillus* isolates were acid tolerance at pH 2 and bile salt tolerance at 2% (bile salt concentration),
R= Resistance and S= Sensitive

Probiotic species of *L. plantarum* (G95a and G96a) and *L. rhamnosus* (G119b) were showed resistant to eight test antibiotics while *L. rhamnosus* (G92, and G99c) were sensitive to tetracycline only but resistant to remaining seven antibiotics. These five prominent probiotics were characterized and performed antimicrobial activity of bacteriocins. These isolates include G92, G95a, G96a, G99c and G119b of *L. rhamnosus* and *L. plantarum.* Bacteriocins of all the five organisms have wide antibacterial activity towards selected enteric pathogens (Table 2). Bacteriocin from of all the 5 isolates showed heat stability at up to 90^0C for 10 min. Out of these, 4 isolates showed heat stability at 100^0C while 2 isolates showed heat stability at 121^0C which includes G95a of *L. plantarum* and G119b of *L. rhamnosus*.

Table 2: Characterization of bacteriocins produced by *Lactobacillus* isolates

	Antibacterial activity as zone of inhibition of growth in mm						Resistance to heat						Sensitivity to Different pH			
Lactobacillus isolates	*E. coli*	*E. aerogenes*	*K. pneumoniae*	*Pr. vulgaris*	*S. typhi*	*Sh. flexneri*	60°C	70°C	80°C	90°C	100°C	121 °C	3	4.5	7	9
L. rhamnosus G92	19	24	24	23	24	23	R	R	R	R	S	S	R	R	R	S
L. plantarum G95a	22	25	25	26	27	27	R	R	R	R	R	R	R	R	R	S
L. plantarum G96a	20	24	23	25	25	23	R	R	R	R	R	S	R	R	R	S
L. rhamnosus G99c	20	23	24	23	25	24	R	R	R	R	R	S	R	R	R	S
L. rhamnosus G119b	23	25	25	26	28	27	R	R	R	R	R	R	R	R	R	S

Note: R= Resistance and S= Sensitive

Discussion

In present study, from 40 goat milk samples, 48 *Lactobacillus* species were identified as *L. acidophilus* (15%), *L. brevis* (13%), *L. bulgaricus* (13%), *L. lactis* (17%), *L. plantarum* (13%), *L. rhamnosus* (21%), *L. helveticus* (4%) and *L. casei* (6%). Medina et al., (2001) similarly isolated some *Lactobacillus* sp. from raw milk and dairy products. Out of these 48 *Lactobacillus* species, five isolates including *L. plantarum* (G95a and G96a) and *L. rhamnosus* (G92, G99c, and G119b) were strong antibacterial (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) reported that fermentation process, which involved L. plantarum, had a broad antimicrobial inhibitory spectrum, with activity against *S. typhi, E. coli,* and *S. aureus.* The isolates, *L. rhamnosus* and *L. plantarum* showed the maximum antibacterial potential against *S. typhi* followed by *Pr. Vulgaris* and *K. pneumoniae*, which indicated that these probiotics could be used in the treatment and prevention of enteric infections. Olarte et al., (2000) noted that the presence of *L. plantarum* in the cheese of Goat's milk decreased the number of the enterobacteria and fecal coliforms in the final product.

The isolated five probiotics showed acid (pH-2) tolerance and bile salt (2%) tolerance which is considered a prerequisite for colonization and metabolic activity of bacteria in the small intestine of the host (Havenaar et al, 1992). Before reaching the intestinal tract, probiotic bacteria must pass through the stomach where the pH can be as low as 1.5 to 2 (Dunne et al., 2001). This acid tolerance will help *Lactobacilli* to reach the small intestine and colon and thus contribute in balancing the intestinal microflora.

Probiotic species of *L. plantarum* (G95a and G96a) and *L. rhamnosus* (G119b) showed resistant to eight test antibiotics while *L. rhamnosus* (G92, and G99c) were sensitive to tetracycline only but resistant to remaining seven antibiotics, which are in accordance with Voravuthikunchai et al., (2006). 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 (El-Naggar, 2004). The antibiotic resistance data supports that all the isolates are different with respect to antibiotic sensitivity pattern.

The bacteriocins from G92, G95a, G96a, G99c and G119b of *L. rhamnosus* and *L. plantarum* were antimicrobial. These bacteriocin showed prominent inhibitory activity against *S. typhi, Pr. vulgaris, K. pneumoniae, Sh. flexneri* and followed by *E. aerogenes* and *E. coli*. Itoh et al., (1995) also reported the inhibition of food borne bacteria by bacteriocins from *L. gasseri.* It was observed that bacteriocin stable at the highest temperature but at that temperature the

antibacterial activity became low but it did not destroyed after heat treatment. These results suggested that the inhibitory compounds produced by these isolates were heat stable proteinaceous compounds. Bacteriocin 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 in accordance with Lade et al., (2007).

The study concluded that isolated *Lactobacillus* species from goat milk meet several of the probiotic criteria, which includes acid and bile tolerances, as well as the production of antimicrobial substances. These characteristics may be advantageous for a probiotic culture to be successful in colonizing and compete with pathogens in gastrointestinal environment. The ability of these isolates to survive in acidic conditions, bile resistance, and the production of bacteriocin that is active against enteric pathogens and useful in prevention of enteric infections. 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. Thus, these probiotic strains could be used for both preventive and therapeutic purpose in controlling intestinal infection.

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