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AN EVALUATION OF PROBIOTIC POTENTIAL OF *LACTOBACILLUS* SP. FROM MILK OF DOMESTIC ANIMALS AND COMMERCIAL AVAILABLE PROBIOTIC PREPARATIONS IN PREVENTION OF ENTERIC BACTERIAL INFECTIONS

D.H. Tambekar* and S.A. Bhutada

Department of Microbiology, S.G.B. Amravati University, Amravati, 444602, India

Abstract

Probiotics are the health promoting viable microorganisms that exhibit a beneficial effect on the health of human being by improving the intestinal microbial balance. A total of 120 milk samples (40 each from buffalo, cow, and goat) were analyzed and 110 isolates were identified as Lactic Acid Bacteria (LAB). Out of these 11 isolates were identified as prominent probiotics, among them 3 isolates were excellent probiotics. These excellent probiotics were compared for their probiotic potential with commercial probiotic preparations such as Sporlac powder, LactoBacil plus, P-Biotics kid, Gastroline, Pre-Pro kid and standard probiotic bacterial strains *L. plantarum* (MTCC 2621) and *L. rhamnosus* (MTCC 1048). The isolated LAB exhibited excellent probiotic characteristics than commercial probiotic preparations and standard probiotic bacterial strains. Study suggested that use of these probiotic bacteria from milk of domestic animals can be help to prevent or control the intestinal infections and contributes health benefits to consumers.

Keywords: Probiotics, *Lactobacillus*, Antibacterial activity, Bacteriocin, Commercial probiotic preparations

Introduction

Milk and milk products are usually associated with probiotic bacteria, which provide supplements in maintaining beneficial intestinal balance (Isolauri, 2001). Probiotic is the group of microbes that may help directly for enhancing resistance against intestinal pathogens and in the prevention of diseases. Probiotic bacteria may produce various compounds, which are inhibitory to the pathogen's growth, which include organic acids (lactic and acetic acids), bacteriocins, and reuterin. The organic acids not only lower the pH, thereby affecting the growth of the pathogen, but they can also be toxic to the microbes. *Lactobacilli* are known to produce many types of bacteriocins like acidophilin, acidolin, lactocidin, bulgarican, lactolin, lactobacillin and lactobrevin (Alvarez-Olmos and Oberhelman, 2001).

Infectious diseases are the biggest problem in human being and every year gastrointestinal infections are responsible for significant morbidity and mortality worldwide (Culligan et al, 2009). World Health Organization (WHO, 2004) estimates there to be more than four billion episodes of diarrhoeal disease annually, while there were 2.2 million deaths attributable to enteric infection, making it the fifth leading cause of death at all ages worldwide. Enteric bacteria comprised of *Salmonella* species, *Shigella* species, *Proteus* species, *Klebsiella* species, *E. coli*, *Pseudomonas* species, *Vibrio cholerae* and *S. aureus* which are major etiologic agents of enteric infection (Ballal and Shivananda, 2002). There is increasing evidence that probiotics are beneficial in gastrointestinal disturbances, such as diarrhoea, dysentery, typhoid etc (Fuller, 1991). The rise in antibiotic resistant bacteria has awakened the scientific community to the prophylactic and therapeutic uses of probiotics, and to reconsider them as alternatives to antibiotics (Ahmed, 2003).

Various *Lactobacilli, Bifidobacteria* and *Streptococcus* species have been evaluated for the prevention or treatment of various infectious diseases and these were found to be safe (Chapoy, 1985). Various commercial probiotic preparations are also available in the market in the form of capsules, liquid/gel and powdered that claims for prevention of infectious diseases. Commercially available probiotic preparations include *Lactobacillus* alone (Lactiflora, LactoBacil, Lactocap, Lactovit, Sporlac) or in combination with *Streptococcus* (Lacticin) or *Saccharomyces* (Laviest) and showed benefical effects (Saggioro, 2004).

The functional properties and safety of probiotics of particular strains of *L. casei, L. lactis, L. acidophilus* from various sources have been extensively studied and commercial probiotic preparations also claims its efficiency for prevention of infectious diseases, but probiotics potential from milk of domestic animals viz cow, goat and buffalo, with significance in prevention of enteric infection have not been reported so far. However, the overall efficacy of probiotics from milk of domestic animals and the mechanisms by which

* Corresponding Author, *Email*: diliptambekar@rediffmail.com

probiotics ameliorate enteric infections are mostly unknown. Therefore the an attempt was made to isolate *Lactobacillus* strains as probiotics from milk of domestic animals and compared its probiotics potential with commercial probiotic preparations and standard probiotic bacterial strains in prevention of enteric bacterial infections.

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 37⁰C for 48h. After incubation, well-isolated typical colonies were picked up, transferred to MRS broth, and incubated at 37⁰C for 48h. The isolates were identified using standard morphological, cultural and biochemical reactions (Howells, 1992).

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 37⁰C for 48h. Then 0.1mL inoculums was transferred to MRS agar by pour plate method and incubated at 37⁰C for 48h. The growth of LAB on MRS agar plate was used to designate isolates as acid or bile salt tolerant.

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 (Kirby-Bauer, 1966).

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 4⁰C for 1h for diffusion, and then incubated at 37⁰C 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 37⁰C 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 60⁰C, 70⁰C, 80⁰C, 90⁰C, 100⁰C and 121⁰C 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).

Comparative study of probiotics

Identified excellent probiotic (*L. plantarum* C68a, *L. plantarum* G95a, *L. rhamnosus* G119b) were compared with commercial probiotic preparations available in market (Sporlac powder, LactoBacil plus, P-Biotics Kid, Gastroline, Pre-Pro kid) and standard probiotic bacterial strains (*L. plantarum* MTCC 2621, *L. rhamnosus* MTCC 1048) i.e. acid and bile tolerance, antibacterial activity, antibiotic resistance, antibacterial potential of bacteriocin, acid, alkali, heat tolerance of bacteriocin. Multiple antibiotic resistance (MAR) were determined as following modified formula

$$\text{MAR Index for a antibiotics} = \frac{\text{Number of antibiotics resistance isolates}}{\text{No. of antibiotics tested X No. of isolates}}$$

Calculation of probiotic potential

Each property of isolated probiotics from milk of domestic animals, commercial probiotic preparations and standard probiotic bacterial strains were scored as under

Probiotic characters	Indication	Score
Acid, bile tolerance sensitivity, antibiotic resistant, acid/ alkali/ heat tolerance of bacteriocin	Sensitivity	0
	Resistance	1
Antibacterial potential of probiotics (zone of inhibition of growth in mm)	14-16 mm	1
	17-20 mm	2
	> 21 mm	3

Cumulative probiotic potential is the sum of score of acid, bile tolerance, antibacterial potential, acid and alkali and heat tolerance of bacteriocin. Probiotic potential was calculated as observed score divided by maximum score into hundred.

$$\text{Probiotic potential} = \frac{\text{Observed score}}{\text{Maximum score}} \times 100$$

Results and Discussion

In present study, a total of 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%). Out of these 11 isolates were recognized as probiotics and from these 3 were identified as a excellent probiotics on the basis of their acid and bile tolerance, antibacterial activity, antibiotic resistance, antibacterial potential of bacteriocin, acid, alkali and high temperature tolerance of bacteriocin. These 3 best probiotics were *L. rhamnosus* (G119b), *L. plantarum* (G95a) from goat milk and *L. plantarum* (C68a) from cow milk were compared for their potential with commercial probiotic preparations and standard probiotic bacterial strains (Table 1).

Table 1: Comparative study of isolated probiotics from milk of domestic animals, commercial probiotic preparations and standard probiotic bacterial strains

Probiotics	Acid tolerance	Bile tolerance	Antibacterial activity of probiotics against enteric pathogens (Zone of Inhibition in mm)						Antibiotic susceptibility								Antibacterial activity of bacteriocin against enteric pathogens (Zone of inhibition in mm)						Heat sensitivity of bacteriocin						Sensitivity to different pH			
	pH 2	2%	*E. coli*	*E. aerogenes*	*K. pneumoniae*	*Pr. vulgaris*	*S. typhi*	*Sh. flexneri*	Ampicillin (A)	Cephalothin (Ch)	Co-Trimoxazole (Co)	Gentamicin (G)	Nalidixic acid (Na)	Nitrofurantoin (Nf)	Norfloxacin(Nx)	Tetracycline (T)	*E. coli*	*E. aerogenes*	*K. pneumoniae*	*Pr. vulgaris*	*S. typhi*	*Sh. flexneri*	60^{0}C	70^{0}C	80^{0}C	90^{0}C	100^{0}C	121^{0}C	pH 3	pH 4.5	pH 7	pH 9
L. plantarum (C68a)	R	R	18	24	25	23	25	22	R	R	R	R	R	R	R	R	21	24	25	24	27	25	R	R	R	R	R	R	R	R	R	R
L. plantarum (G95a)	R	R	19	24	23	24	25	24	R	R	R	R	R	R	R	R	22	25	25	26	27	27	R	R	R	R	R	R	R	R	R	R
L. rhamnosus (G119b)	R	R	19	24	24	24	25	24	R	R	R	R	R	R	R	R	23	25	25	26	28	27	R	R	R	R	R	R	R	R	R	R
Sporlac powder	R	R	17	20	21	20	23	21	R	R	S	S	R	S	S	R	21	23	23	22	25	23	R	R	R	R	S	S	R	R	R	S
LactoBacil plus	R	R	20	20	17	19	22	18	R	R	R	R	R	R	R	S	22	23	23	23	25	19	R	R	R	R	S	S	R	R	R	S
P-Biotics Kid	R	R	18	20	23	22	23	20	R	R	R	R	R	R	R	S	20	19	20	23	27	25	R	R	R	R	R	S	R	R	R	S
Gastroline	R	R	16	16	15	17	19	18	R	R	R	R	R	R	S	R	20	22	22	22	25	23	R	R	R	R	S	S	R	R	R	S
Pre-Pro kid	R	R	16	20	23	25	25	22	R	R	R	R	R	S	S	R	19	22	24	23	27	25	R	R	R	R	S	S	R	R	R	S
L. plantarum (MTCC 2621)	R	R	18	20	20	22	20	19	R	R	R	S	R	S	R	R	20	22	24	21	24	23	R	R	R	R	R	S	R	R	R	S
L. rhamnosus (MTCC 1048)	R	R	16	18	18	17	20	18	R	R	R	R	R	S	S	S	20	21	23	21	25	23	R	R	R	R	R	S	R	R	R	S

Where: R=Resistant, S= Sensitive

Acid and Bile salt tolerance

Probiotics potential of LAB is necessarily its ability to resist bile salts and acidic pH (Lee and Salminen, 1995). 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%. 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 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 contribute in balancing the intestinal microflora (Tambekar and Bhutada, 2010).

Antagonistic activity

The isolated probiotics from milk of domestic animals, *L. rhamnosus* (G119b), *L. plantarum* (G95a, C68a) were strong antagonistic (Score 17) followed by commercial probiotic preparations Pre-Pro kid, P-Biotics kid, Sporlac powder, LactoBacil plus, Gastroline containing probiotics (Score 9-15), standard probiotic bacterial strains *L. plantarum* (MTCC 2621), *L. rhamnosus* (MTCC 1048) (Score 11-12) against enteric pathogens (fig.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 (Tambekar *et al,* 2009; Bezkorvainy, 2001). Chuayana *et al,* (2003) reported that different milk products were able to inhibit the growth of *S. aureus*, *E. coli*, *Ps. aeruginosa*, *S. typhi, Serratia marcescens* and *Candida albicans.*

Fig. 1: Antagonistic activity of isolated probiotics, commercial probiotic preparations and standard probiotic bacterial strains against enteric pathogens

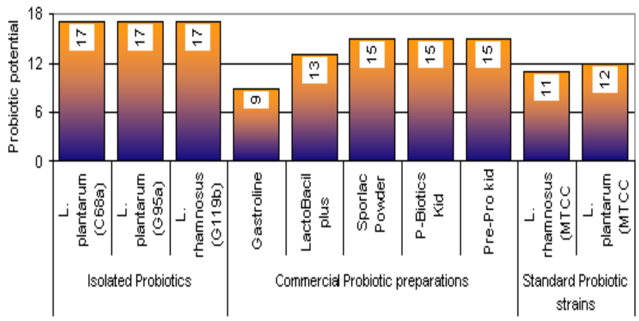


Fig. 2: MAR Index of isolated probiotics, commercial probiotic preparations and standard probiotic bacterial strains

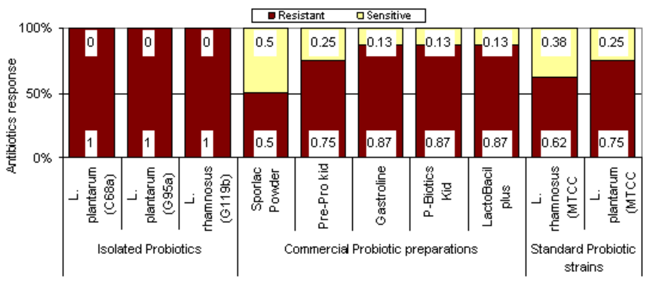
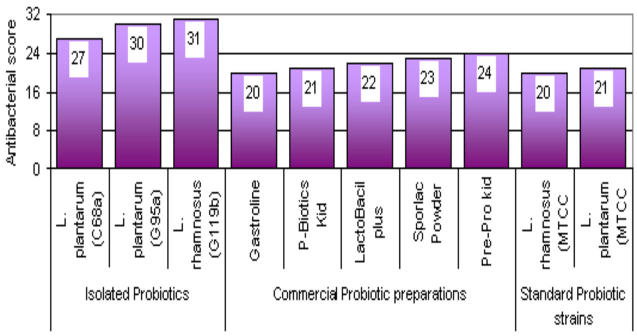


Fig. 3: Antibacterial activity of bacteriocin produced by isolated probiotics, commercial probiotic preparations and standard probiotic



Where: Antibacterial Score between 20- 24 moderate and 27-31 Strong antibacterial

Our study showed that *L. rhamnosus* and *L. plantarum* had strongest antagonistic potential against *Salmonella typhi* followed by *Proteus vulgaris* and *Klebsiella pneumoniae.* 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.* Hence, isolated probiotics can be useful to prevent enteric infections such as diarrhoea, dysentery, typhoid, food poisoning etc.

Antibiotic resistance

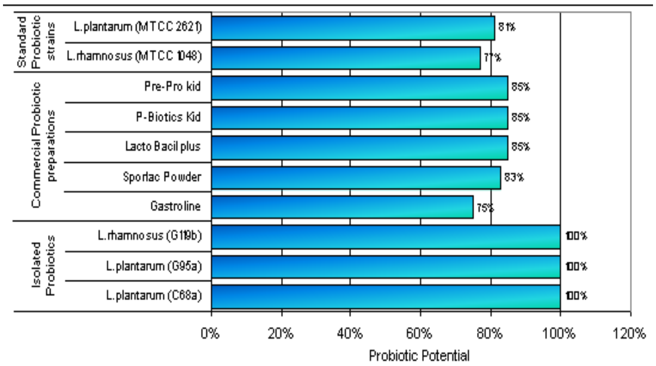
Multiple antibiotic resistance (MAR) index (fig.2) of isolated probiotics from milk of domestic animals was high indicating high resistance to all antibiotics as compared to commercial probiotic preparations (MAR index 0.75-0.87) and standard probiotic bacterial strains (MAR index 0.50-0.62). Such resistance to a wide spectrum of antibiotics indicated that if isolated probiotics induced in patients treated with antibiotic therapy may be helpful in faster recovery of the patients due to rapid establishment of desirable microbial flora. Present studies have lent support the use of selected probiotic agents for the prevention of antimicrobial-associated diarrhoea. Resistance of the probiotic strains to some antibiotics could be used for both preventive and therapeutic purposes in controlling intestinal infections (El-Naggar, 2004).

Antibacterial activity and characterization of bacteriocin

Antibacterial activity of bacteriocin produced by isolated probiotics showed that, *L. rhamnosus* (G119b), *L. plantarum* (G95a, C68a) were strong (Score 27-31) antibacterial than bacteriocin of commercial probiotic preparations Pre-Pro kid, Sporlac powder, LactoBacil plus, P-Biotics kid, Gastroline containing probiotics (Score 20-24) and standard probiotic bacterial culture of *L. plantarum* (MTCC 2621), *L. rhamnosus* (MTCC 1048) (Score 20-21) against enteric bacterial pathogens (fig. 3). Bacteriocins of *L. rhamnosus* (G119b), *L. plantarum* (G95a, C68a) were stable at 121^{0}C and in acidic as well as alkaline pH (3 to 9). Moghaddam *et al*, (2006) reported that bacteriocins of *L. acidophilus*, *L. bulgaricus* were stable between pH 3 and 10 while *L. helveticus* was found to be sensitive to pH 10. Bacteriocins of all the selected commercial probiotic preparations were stable at 90^{0}C and acidic to neutral pH i.e. (3 to 7) except P-Biotics kid was stable up to 100^{0}C. Bacteriocins of standard probiotic strains were stable up to 100^{0}C and pH 3 to 7. Similarly Alpay *et al,* (2003) reported that bacteriocins of *Lactobacilli* were stable at pH 4.5 to 7 but sensitive to pH 9.

The probiotic potential based on cumulative probiotic score of isolated best probiotic, commercial probiotic preparations and standard probiotic bacterial strains were calculated and compared (Fig. 4). Probiotic potential of the isolated probiotic *Lactobacilli* is highest (100%) as compared to commercially available probiotic preparations (75-85%), and standard probiotic bacterial strains (77-81%). Different probiotic species and even different strains within a species exhibit distinctive properties. The standard probiotic bacterial strain, *L. plantarum* (MTCC 2621) was most efficient than *L. rhamnosus* (MTCC 1048). Among the commercial probiotic preparations Pre-Pro kid was found efficient probiotic followed by P-Biotics kid, Sporlac powder, LactoBacil plus, Gastroline, whereas study showed that probiotics *L. plantarum* (G95a) *L. rhamnosus* (G119b) from goat and *L. plantarum* (C68a) from cow milk were more effect probiotic (fig. 4). This may be due to lack of standardization and quality control of probiotic formulation, proper consistency between batches with respect to manufacture, storage of probiotics formulations (Clancy and Pang, 2007; McFarland, 2008). Most commercial products at the time of purchase have low numbers of viable organisms with little clinical benefit. Production and quality control are not regulated as per the guidelines of probiotic products, but few products are likely to achieve the benefits identified in formal clinical trials. Even with better-characterized isolates, trial results can vary reflecting production and stability of the products (Ouwehand et al, 2003). The present study revealed that probiotics from goat and cow milk were acid tolerant at pH 2, bile tolerant at 2% bile salt, antibacterial against enteric pathogens, antibiotics resistant to most of the antibiotics and their bacteriocin stable at temperature 121^0 C and pH 3 to 9 and strong antibacterial against enteric bacterial pathogens. These probiotics follow the criteria of FAO/WHO (2002) and may use to prevent the enteric bacterial infection.

Fig. 4: Comparative study of the excellent probiotics isolated from milk of domestic animals, commercial probiotic preparations and standard probiotic bacterial strains



Conclusion

The present study revealed that probiotics from goat and cow milk, commercial probiotic and standard probiotic bacterial strains were acid tolerant at pH 2, bile salt tolerant at 2% bile salt concentration, antibacterial against enteric pathogens, antibiotics resistant to most of the antibiotics and their bacteriocin stable at temperature 121^{0}C and pH 3 to 9. From comparative study of probiotics it was concluded that goat milk probiotics and their bacteriocins showed strong antibacterial potential as compared to the commercial probiotic preparation, hence probiotic from milk of domestic animals can be used for oral therapy and as prophylactic to prevent the enteric infections such as diarrhoea, gastroenteritis, dysentery, urinary tract infections, food poisoning, typhoid, irritable bowel syndrome etc. Study suggested that these isolated prominent probiotics can be used in milk or milk products supplement to provide restoration and maintenance of normal microbial flora of intestine and prevention of side effect of antibiotics. Study will affirm their use in the development of new pharmaceutical preparations and functional foods that contain milk probiotics for the betterment of the health of the public.

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