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Assessment of potential probiotic bacteria isolated from milk of domestic animals

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ABSTRACT

Objectives: Pathogens and over use of antibiotics disturb the normal intestinal microbial flora and probiotics help to normalize it. This study attempted to isolate *Lactobacillus* strains with probiotic potential from milk of domestic animals.

Methodology and results: 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, 43 were recognized as probiotics which included prominently *L. acidophilus*, *L. bulgaricus, L. plantarum*, *L. lactis* and *L. rhamnosus*.

Conclusion: Use of the bacteria with probiotics potential could help to protect and improve intestinal microbial flora. Resistance of the probiotic strains to some antibiotics could be used for both preventive and therapeutic purposes in controlling intestinal infections and thus has potential applications in the production of fermented foods.

Key words: Probiotics, lactic acid bacteria, acid tolerance, bile salt tolerance

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INTRODUCTION

Human beings as well as animals are normally born sterile but shortly after birth, colonization begins with different body parts being occupied by the fittest microbes from the environment, thus creating a balanced ecological system. The normal microflora changes dramatically during the lifetime of the host. The Lactic Acid Bacteria (LAB), if present, constitutes the dominant flora population due to their ability to colonize the human and animal intestinal tract. However, exogenous bacteria, either of probiotic or pathogenic origin, influence the intestinal bacterial flora. The acidity of the stomach maintains a low concentration of bacteria in the upper part of the digestive tract and destroys pathogens. Interactions that occur between various bacterial species are also important in maintaining the equilibrium of the intestinal microflora (Olanrewaju, 2007).

Milk and milk products are usually associated with LAB, which provide supplements in maintaining beneficial intestinal balance (Isolauri, 2001). Generally, the LAB are the most implicated of the probiotic organisms with respect to intestinal bacterial colonization particularly those of *Lactobacilli* and *Bifidobacterium,* which attributes antagonistic property by secreting acids to lower the pH, thereby creating an environment unfavorable to disease-causing bacteria. LAB, a probiotic, must tolerate low pH and high bile concentration, which enables selected strains to survive, grow and perform their therapeutic roles in the intestinal tract (Gilliland & Walker, 1989; Usman & Hosono, 1999), and produce substances that inhibit pathogenic, non-pathogenic and spoilage organisms in fermenting foods and beverages.

LABs have been used successfully to prevent various types of diarrhoea, recurrent *Clostridium difficile* disease and reduce chance of infection from common pathogens (Biller *et al.,* 1995). Extensive use of antibiotics in human health, animal husbandry and agriculture has resulted in emergence of antibiotic resistance in pathogenic as well as commensal bacteria and use of LAB isolated from humans and animal could be used for both preventive and therapeutic purposes in controlling intestinal infections (Moustafa, 2004). Hence, in this study an attempt was made to isolate *Lactobacillus* strains with probiotic potential from milk of domestic animals.

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 from farmers directly. 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 (Whatman No.1, 10mm) were dipped into the culture broth of isolated *Lactobacillus* sp inoculated for 48h 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) and *Salmonella typhi* (MTCC 734). 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 inoculum was transferred to MRS agar by pour plate method and incubated at 37°C for 48h. The growth of LABs on MRS agar plate was used to designate isolates as acid or bile salt tolerant.

Antibiotic resistance: The antibiotic resistance of isolated LABs 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 overlayed on bottom layers of MRS agar. Resistance was assessed against Ampicillin (1ug), Cephalothin (30ug), Co-Trimoxazole (25ug), Gentamicin (10ug), Nalidixic acid (30ug), Nitrofurantoin (300ug), Norfloxacin (10ug), and tetracycline (25ug) as per Halami *et al.,* (1999).

RESULTS AND DISCUSSION

From the 120 milk samples analysed, 110 *Lactobacillus* species isolates were identified that included *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 isolates were reported by Guessas *et al.*, (2004) from raw goat's milk.

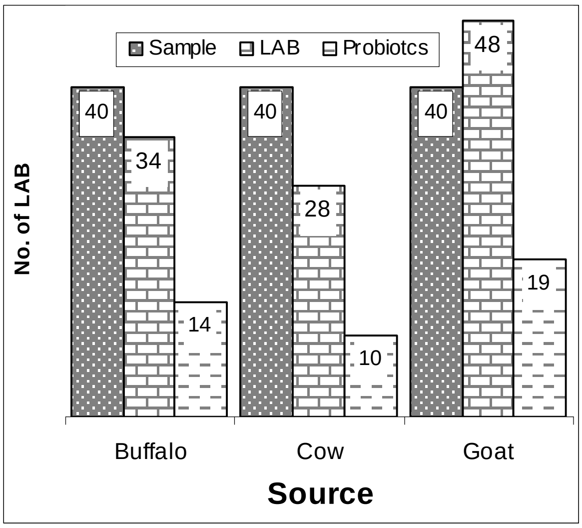
Antagonistic activity: The antagonistic activity of these isolates was determined against selected enteric pathogens. Out of the 110 LAB isolated, 43 isolates exhibited strong inhibition in the disc diffusion test, which suggests that they produce acetic and/or lactic acids that lowered the pH of the medium. Inhibition may also be through competition for nutrients, production of bacteriocin or other antibacterial compounds (Bezkorvainy, 2001). Obadina *et al.* (2006) observed that the fermentation process which involved *L. plantarum* caused a reduction in the level of pathogens such as *S. typhi, E. coli* and *S. aureus.* In the present study all the isolates of *L. rhamnosus* and *L. plantarum* showed high antibacterial potential against *S. typhi*, *Pr. vulgaris* and *Kl. pneumoniae* (Table 1). Olarte (2000) noted that the presence of *L. plantarum* during production of cheese Cameros from goat's milk decreased the number of enterobacteria and fecal coliforms in the final product.

Acid and bile salt tolerance: Results showed that 43 LABs survived high bile salt concentration and low pH. Gilliland and Walker (1989) and Gotcheva *et al.* (2002) reported that probiotic microorganisms such as *L. acidophilus, L. casei* and *L. plantarum* are able to reach the gastrointestinal tract (GIT) and remain viable for 4h or more at pH 3.0, which helps the strains to reach the small intestine and colon and thus contribute to balancing of the intestinal microflora.

Antibiotic resistance: Most of the 43 LAB that showed antagonistic activity, acid and bile salt tolerance were resistant to Ampicillin, Nalidixic acid, Norfloxacin, Cephalothin and Co-Trimoxazole (table 1). *L. plantarum* isolates C1, C4, C7, G7, G8 and *L. rhamnosus* G18 were resistant to all the 8 antibiotics tested. *L. plantarum* isolate B14 and *L. rhamnosus* isolates B13, C5, G4, and G10 were resistant to 7 antibiotics but sensitive to Tetracycline (Table 1). The data shows that all the isolates are different with respect to antibiotic sensitivity.

Since LAB has probiotic applications, occurrence of antibiotic resistance in probiotic LAB strains may pose a threat to human health because plasmid mediated transfer of antibiotic resistance among gut flora has been demonstrated (Charlotte *et al.,* 1985). On the other hand, the resistance to antibiotics indicates the isolates can potentially minimize the negative effects of antibiotic therapy on the host bacterial ecosystem (El-Naggar, 2004).

A total of 43 LAB were recognized as probiotics based on their ability to inhibit the growth of selected enteric pathogenic bacteria, tolerate acid at pH 2 and 2% bile salt, and resist antibiotics. Of the 43 isolates, 14 were from buffalo milk, 10 from cow milk and 19 from goat milk (Fig.1). The 43 isolates comprised of *L. acidophilus* (19%), *L. brevis* (5%), *L. bulgaricus* (12%), *L. plantarum* (23%), *L. lactis* (9%), *L. rhamnosus* (21%), *L. helveticus* (2%), *L. casei* (2%), and *L. fermentum* (2%) (Fig. 2). Mathara *et al.* (2004) isolated *L. plantarum, L. acidophilus*, *L. rhamnosus* and *L. fermentum* from fermented products and showed that these species represent more than 60% of the isolated *Lactobacilli*.



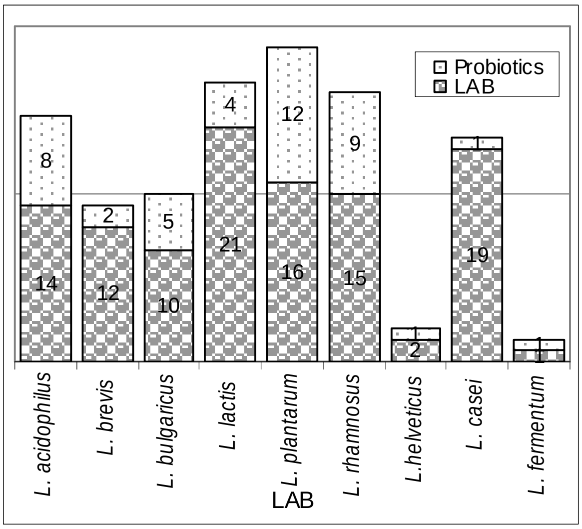


Figure 1 (left): Proportion of milk samples from domestic animals with probiotics lactobacillus species isolates (LAB); Figure 2 (right): Proportion of probiotic isolates amongst LAB isolated from milk of domestic animals.

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)					Antibiotics Susceptibility								
					Escherichia coli	*Enterobacter aerogenes*	*Klebsiella pneumoniae*	*Proteus vulgaris*	*Salmonella typhi*	A	Ch	Co	G	Na	Nf	Nx	T	Total Resistant
Buffalo	*L. bulgaricus*	B1	+	+	15	18	21	17	23	R	R	S	S	R	S	R	S	4
	L. plantarum	B2	+	+	16	16	23	16	21	R	S	R	R	R	S	S	S	4
	L. bulgaricus	B3	+	+	15	20	23	18	21	R	R	S	S	R	S	R	S	4
	L. plantarum	B4	+	+	16	20	22	19	22	R	R	S	S	R	S	R	S	4
	L. lactis	B5	+	+	16	20	23	21	20	S	R	R	R	R	R	R	S	6
	L. acidophilus	B6	+	+	15	18	23	20	21	R	S	R	R	S	R	S	R	5
	L. plantarum	B7	+	+	16	20	20	22	21	R	S	R	R	R	S	R	S	5
	L. plantarum	B8	+	+	16	16	21	17	20	R	S	R	R	R	S	S	S	4
	L. lactis	B9	+	+	16	21	22	24	22	R	R	S	R	R	S	R	S	5
	L. brevis	B10	+	+	16	18	18	19	18	R	R	S	S	R	R	R	S	5
	L. plantarum	B11	+	+	16	17	16	18	17	R	R	S	S	R	S	R	S	4
	L. acidophilus	B12	+	+	16	16	18	17	18	R	S	R	R	R	S	R	S	5
	L. rhamnosus	B13	+	+	18	20	21	23	23	R	R	R	R	R	R	R	S	7
	L. plantarum	B14	+	+	19	21	21	22	24	R	R	R	R	R	R	R	S	7
Cow	*L. plantarum*	C1	+	+	18	22	24	21	23	R	R	R	R	R	R	R	R	8
	L. rhamnosus	C2	+	+	18	22	24	20	21	R	R	R	R	R	S	S	S	5
	L. acidophilus	C3	+	+	18	24	25	22	23	R	S	R	R	R	S	R	S	5
	L. plantarum	C4	+	+	18	22	25	23	23	R	R	R	R	R	R	R	R	8
	L. rhamnosus	C5	+	+	19	24	24	22	24	R	R	R	R	R	R	R	S	7
	L. acidophilus	C6	+	+	18	23	24	24	23	R	S	R	S	R	R	R	S	5
	L. plantarum	C7	+	+	17	23	23	21	24	R	R	R	R	R	R	R	R	8
	L. casei	C8	+	+	15	19	22	22	22	S	R	R	S	R	R	R	S	5
	L. fermentum	C9	+	+	15	18	23	20	24	R	R	S	R	R	S	R	S	5
	L. acidophilus	C10	+	+	17	18	17	17	19	R	S	R	R	R	S	R	S	5
Goat	*L. bulagaricus*	G1	+	+	18	20	18	19	19	R	R	S	S	R	S	R	S	4
	L. bulagaricus	G2	+	+	17	19	16	18	18	R	R	S	S	R	S	R	S	4
	L. rhamnosus	G3	+	+	19	18	20	19	18	R	R	R	R	R	S	S	S	5
	L. rhamnosus	G4	+	+	17	22	23	22	24	R	R	R	R	R	R	R	S	7
	L. rhamnosus	G5	+	+	16	18	18	18	19	R	R	R	R	R	S	S	S	5
	L. lactis	G6	+	+	17	17	16	18	17	R	R	S	S	R	S	R	S	4
	L. plantarum	G7	+	+	19	21	20	22	25	R	R	R	R	R	R	R	R	8
	L. plantarum	G8	+	+	18	22	21	21	24	R	R	R	R	R	R	R	R	8
	L. plantarum	G9	+	+	19	18	18	17	19	R	S	R	R	R	S	R	S	5
	L. rhamnosus	G10	+	+	18	18	23	22	25	R	R	R	R	R	R	R	S	7
	L. acidophilus	G11	+	+	16	15	15	15	16	R	S	R	S	R	R	R	S	5
	L. rhamnosus	G12	+	+	18	18	17	16	18	R	R	R	R	R	R	S	S	6
	L. acidophilus	G13	+	+	17	16	16	16	16	R	S	R	R	R	R	R	S	6
	L. bulagaricus	G14	+	+	15	15	16	15	17	R	R	S	R	R	S	R	S	5
	L. helveticus	G15	+	+	18	17	18	18	19	R	R	R	S	R	R	R	S	6
	L. acidophilus	G16	+	+	18	17	17	16	17	R	S	R	R	S	R	S	R	5
	L. brevis	G17	+	+	16	18	18	17	19	R	R	R	S	R	R	R	S	6
	L. rhamnosus	G18	+	+	19	21	22	20	23	R	R	R	R	R	R	R	R	8
	L. lactis	G19	+	+	15	15	16	15	16	R	R	S	S	R	S	R	S	4
	Total									41	31	31	29	41	22	35	8	238

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

CONCLUSION

Isolated *Lactobacillus* species exhibited good probiotic characteristics and can therefore be used for food or dairy fermentations and contribute health benefits to consumers. These LABs can help to stabilize the gut microbial environment and the intestine permeability barrier, thereby promoting the immunological barrier to gut mucosa. They also hold great promise for the prevention and treatment of clinical conditions associated with impaired gut mucosal barrier functions and sustained inflammatory responses. Probiotics approach is suitable for reconstituting natural condition by repairing a deficiency caused by addition of foreign chemicals to the body, e.g. antibiotics and compromise subsequent therapy. Resistance of the probiotic strains to antibiotics could be used for both preventive and therapeutic purposes in controlling intestinal infections.

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