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Probiotic Studies in Colostrum of Buffalo

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Abstract: The genus *Lactobacillus* - a type of Lactic Acid Bacteria is the most widely encountered as probiotics because they display numerous health benefits for man and animals. Inhibitory effect of *Lactobacillus* sp against bacterial pathogens is mainly due to the production of antimicrobial metabolites such as bacteriocin. Bacteriocins are proteinaceous compounds produced by bacteria that exhibit a bactericidal activity against closely related as well as unrelated organisms. They exhibit number of applications. The purpose of this work was to document the nature of distribution of *Lactobacillus* sp in the colostrum of buffalo. Microbiological analysis indicated the presence of three different types of *Lactobacillus* sp such as *L.casei* (27%), *L.delbrukii* (43%) and *L.fermentum* (30%). Bacteriocin producing ability was found with all the three types of probiotic bacteria and they inhibited common food borne pathogens such as *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Bacillus subtilis* and *Staphylococcus aureus*. Production of bacteriocin was done and purification was carried out using ammonium sulphate precipitation method at 40 % level. Thermostability (60°C, 70°C and 80°C) and pH (5, 6 and 9) sensitivity studies were conducted. The results indicated that bacteriocin was able to show wide spectrum of action even at harsh temperature and pH. All the three types of *Lactobacillus* sp showed resistance against Cotrimaxazole and Norfloxacin and sensitivity for Tetracycline. Result of the current study supported the role of *Lactobacillus* sp in bio-preservation and further work at molecular level may throw some more useful information.

Key words: Buffalo milk · *Lactobacillus* sp · Bacteriocin · Indicator · Antibiotics

INTRODUCTION

Milk is a white liquid produced by the mammary glands of mammals [1]. It is the primary source of nutrition in the world with it's high nutritious property. Presence of versatile nutrients and huge amount of water make milk as an excellent media for microbes exhibiting harmful and beneficial roles [2]. Lactic acid bacteria is one of the probiotic bacteria with tremendous health benefits.

Probiotics are beneficial bacteria in that they favourably alter the intestinal micro flora balance, inhibit the growth of harmful bacteria, promote good digestion, boost immune function and increase resistance to infections [3]. Other physiological benefits of probiotics include removal of carcinogens, lowering of cholesterol, immunostimulating and allergy lowering effect, synthesis and enhancing the bioavailability of nutrients, alleviation of lactose intolerance [4]. In order to exert their beneficial effect, probiotics must survive in the gastrointestinal (GI) tract, persist in the host and prove safety for consumer [5]. To survive in the gut, the organisms must be tolerant to low pH and bile toxicity prevalent in the upper digestive tract. Besides, quality assurance programmes associated with research, development, production and validation of the health benefits of these bacteria, require their relevant characterization and identification. Over the world, the research of novel probiotic strains is important in order to satisfy the increasing request of the market and to obtain new functional products. These new functional products must contain probiotic cultures more active and with better probiotic characteristics comparing to those already present on the market. At this back drop, the present study was carried out to screen the profile of *Lactobacillus* sp in the colostrum collected from a buffalo, to assess their probiotic effect over common pathogens, to study the effect of selected physical factors and to screen the effect of antibiotics over the probionts.

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MATERIALS AND METHODS

Location and Nature of Sampling: The milk-colostrum samples were collected from Alwarkurichi, Tirunelveli, Tamailnadu, India. Sampling was done for a period of one week from a buffalo from day one immediately after giving birth of a calf. The samples were subjected for microbiological tests within 2-6 hours.

Enumeration of Total Heterotrophic Bacterial Population (THBP): Milk sample was serially diluted and spread plated on nutrient agar medium and incubated under aerobic conditions at 37°C for 24 hours for determining the THBP.

Isolation and Enumeration of *Lactobacillus* Sp: Milk samples were serially diluted and spread plated on Man Rogosa Sharpe (MRS) - Hi-Media agar medium and incubated at 37°C for 24 hours for determining and isolating *Lactobacillus* sp. Well isolated bacterial strains were picked up and stored in MRS agar slants for further studies.

Screening the Suitability of Media: Different media such as MRS and Milk agar were tested to find their suitability in the cultivation of *Lactobacillus* sp by streaking the *Lactobacillus* sp. The streaked plates were incubated at 37°C for 24 hours. Suitability was understood by observing the luxurious nature of growth of the culture in the plate.

Identification of the *Lactobacillus* Sp: Well isolated colonies exhibiting the specific morphology were selected as per the guidelines of the manufacturer and confirmed by microbiological and biochemical tests [6, 7]. The nature of tests performed and the results are tabulated (Table -1).

Purification of Bacteriocin by Ammonium Sulphate Precipitation: The crude bacteriocin sample was treated with ammonium sulphate at 40% saturation [8] and stirred at 4°C for 24hours. Then the precipitate was re suspended in 25ml of 0.05 M phosphate buffer. The mixture was kept for 18 hours at 4°C. The action of bacteriocin was checked against selected indicator organisms.

Indicator Bacterial Pathogens: In the current study, MTCC cultures such as *E.coli*, *E.aerogenes, K. pneumonia, B.subtilis* and *S.aureus* were used as indicator organism to understand the antibacterial effect of bacteriocin.

Table 1: Identification of Employed Tests

	Lactobacillus sp		
	L.casei,	*L. delbrukii*	*L. fermentum*
Gram`s staining	+ve, rod	+ve, rod	+ve, rod
Motility test	Motile	Non motile	Motile
Catalase activity	-	-	-
Oxidase test	-	-	-
Carbohydrate fermentation test			
Dextrose	+	+	+*
Mannitol	+	-	+
Sucrose	+	-	+*
Maltose	+	+	+
Lactose	+	+	+*

Determination of Thermostability of Bacteriocin: The thermo stability of the bacteriocin produced by *Lactobacillus* sp isolates was checked at different temperature through agar well diffusion method. The supernatant obtained after centrifugation of the *Lactobacillus* sp broth culture was exposed to 60°C, 70°C and 80°C for a period 15 minutes [9]. The exposed supernatants were loaded in the wells of agar plates which were previously seeded with indicator organisms and incubated at 37°C for 24 hours. The effect of different temperature was understood by the lack of zone formation.

Determination of Ph Effect on Bacteriocin: Effect of different pH such as 5, 6 and 9 was determined on basis of the bacteriocin produced by *Lactobacillus* sp isolates was checked at different pH through agar well diffusion method. The supernatant obtained after centrifugation of the *Lactobacillus* sp broth culture was adjusted to 5, 6 and 9. The supernatants set with different pH were loaded in the wells of agar plates which were previously seeded with indicator organisms and incubated at 37°C for 24 hours. The effect of different pH was understood by the lack of zone formation.

Effect of Antibiotics on *Lactobacillus* Sp: Antibiotics such as Co- trimoxazole (25µg), Norfloxacin (10 µg) and Tetracycline (10µg) were tested against *Lactobacillus sp* to find out their effect. The *Lactobacillus* strains were swabbed on previously prepared Muller Hinton agar plates and different antibiotics were incorporated for zone formation. It was incubated at 37° C for 24 hrs for zone formation.

RESULTS AND DISCUSSION

Buffalo milk has become a research subject and received increasing attention in many countries due to its rich nutrient content. Compared to cow milk, buffalo milk has a richer taste due to it's contents of milk fat, protein, lactose, total dry matter, vitamin and minerals. These properties allow a wider variety for buffalo milk as raw material for milk products like cheese, butter and ice-cream [10]. There has been an increasing demand for cheese made of buffalo milk in many countries throughout the world as it is an organic product [11]. It is reported that global buffalo milk was 90 million tons in 2009 and accounted for 13% of total milk production. The same report stated that more than 90% of total buffalo milk in the world is produced by India and Pakistan [12].

Milk quality is directly associated with its composition and hygiene [13, 14].

Qualitative and quantitative analysis of microbes in milk could be used as an indicator for assessing the quality and safety of milk. The most important indicators for microbiological quality include total bacteria number [15].

Count of THBP and *Lactobacillus* sp was made for a period of one week in the milk sample of buffalo immediately after giving birth. THBP and *Lactobacillus* sp load was found to be the maximum in the day one. The trend continued in THBP until four days at the selected dilution. A fall in the level of *Lactobacillus* sp was noted (Figure -1).

The load of both THBP and *Lactobacillus* sp in the current study is found to be higher, it may be due to the collection of colostrum. Similar type of results were obtained on previous studies [6]. The presence of more amount of *Lactobacillus* sp in the milk may help the survival of the calf through easy digestion, keeping them away from intestinal infections and by boosting their immunity.

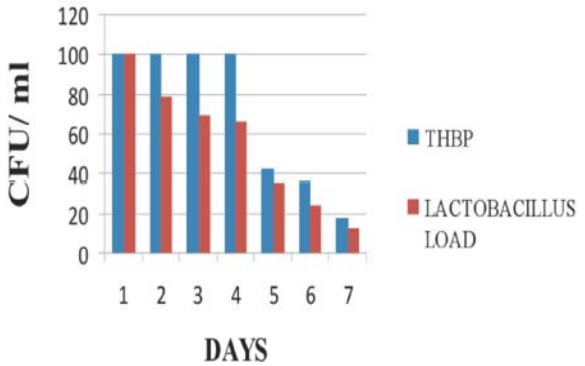

Fig 1: Enumeration Of THBP and *Lactobacillus* sp In Buffalo Colostrum

Table 2: Antibacterial Effect Of Bacteriocin Against Indicator Organisms

		Zone of Inhibition (mm)		
No	Pathogens	*L.casei*	*L.delbrukii*	*L.fermentum*
1	*E. coli*	16	14	12
2	*E. aerogens*	19	16	-
3	*K. pneumonia*	15	18	14
4	*B. subtilis*	17	14	16
5	*S. aureus*	15	10	-

THBP shall involve commensal, saprophytes, probionts, pathogens etc., saprophytic microorganisms in milk could spoil buffalo milk, while the presence of pathogenic bacteria could pose a potential health threat [16].

Among the two media used to cultivate *Lactobacillus* sp, MRS was found to be the best. Frindliness of MRS in *Lactobacillus* sp cultivation has been already documented by Hoque *et al*. [17]. Basic microbiological and biochemical tests have been used by different workers in the detection of bacteria [18, 19].

Tests employed for the Identification of *Lactobacillus* sp are listed in the Table -1. Figure-2 explored the presence of three species of *Lactobacillus* including *L.casei* (27%), *L.delbrukii* (43%) and *L.fermentum* (30%).

Singh and Sharma [6] have reported the distribution of *L.acidophilus* and *L.lactis* in the buffalo milk.

Different reports showed that most lactobacilli strains produce substances that inhibit pathogenic, non-pathogenic and spoilage organisms in fermenting foods and beverages. In general, the antimicrobial activity of lactobacilli may be due to organic acids, hydrogen peroxide, bacteriocins or other inhibitory substances from metabolites [20-22].

Using agar well diffusion method, probiotic effect of the identified three *Lactobacillus* sp was tested over potent selected Gram positive and negative bacteria normally found as a contaminant in foods.

The effect of the three *Lactobacillus* sp could be understood from the Table-2. *L.casei* and *L.delbrukii* were able to inhibit all the selected pathogenic bacteria such as *E.coli*, *E.aerogenes*, *K.pneumonieae*, *B.subtilis* and *S.aureus*. Whereas *L.fermentum* failed to show effect over *Enterobacter* sp and *Staphylococcus* sp.

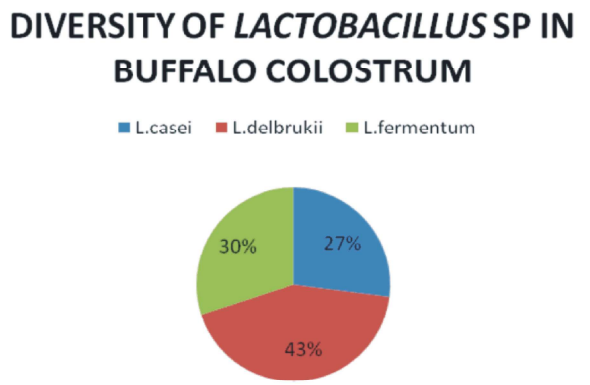

Fig 2: Diversity Of *Lactobacillus* sp In Buffalo Colostrum

The inhibitory effect in *Lactobacillus* sp against *E.coli, E. faecalis*, *Streptococcus mutants*, *S.aureus, K.pneumonieae*. and *Pseduomonas aeruginosa* were documented [23]. Role of bacteriocin in donating probiotic effect has been documented by many workers [24-27].

The findings of the present study indicated the presence of wide spectrum activity in the antimicrobial compound produced by the three *Lactobacillus* sp. Further studies should be done in order to determine the nature of the antimicrobial compound exactly. The effectiveness of antibacterial substance produced by the beneficial bacteria could be impaired by several factors like temperature, pH, bile salt etc., [23]. Thermostability of bacteriocin produced by the *Lactobacillus* sp was screened at varied temperature (Table-3). Loss of activity of bacteriocin was noted only at 80°C against only Gram positive organisms.

Bacteriocin of *Lactobacillus* sp origin may be exposed to different temperature before it reaches the action point in the host. So, it should be able to survive at varied temperature and retain it's activity against pathogens to add value for it.

The impact of different pH on the activity of bacteriocin produced by *Lactobacillus* sp could be understood from Table-4. Loss of activity was noted only at pH 9 only against *E.aerogenes* and *B.subtilis*. Where a notable inhibitory effect was observed against all the tested pathogens in the other two points of pH.

Presence of activity at varied higher temperatures and pH checked in the present study proved that their worthy nature.

Antibiotic resistance of microorganisms used as probiotic agents is an area of growing concern. Out of three antibiotics tested over the *Lactobacillus* sp, resistance was noted against Cotrimaxazole and Norfloxacin and sensitivity was found against tetracycline (Table-5). Our present findings are similar to previous work [21] who also reported sensitivity and resistance in

Table 3: Thermostability Of Bacteriocin Of Buffalo Colostrum

		L.casein (mm)			*L.casein* (mm)			*L.casein* (mm)		
No	PATHOGENS	60°C	70°C	80°C	60°C	70°C	80°C	60°C	70°C	80°C
1	*E. coli*	16	19	10	14	10	12	13	10	11
2	*E. aerogens*	13	10	12	13	11	8	10	9	12
3	*K. pneumoniae*	15	11	13	14	12	9	14	12	10
4	*B. subtilis*	10	14	16	-	15	10	14	9	-
5	*S. aureus*	15	10	-	10	12	-	11	13	10

Table 4: Effect Of pH On Bacteriocin Activity Of Buffalo Colostrum

		L.casein (mm)			*L.delbrukii* (mm)			*L.fermentum* (mm)		
No	PATHOGENS	pH 5	pH 6	pH 9	pH5	pH6	pH9	pH5	pH6	pH9
1	*E. coli*	11	14	16	9	10	12	-	10	11
2	*E. aerogens*	16	12	11	14	11	15	10	-	-
3	*K. pneumoniae*	12	10	14	15	10	12	11	9	7
4	*B. subtilis*	15	12	10	11	9	-	10	10	-
5	*S. aureus*	10	14	11	13	15	10	11	9	10

Table 5: Antibiotic Activity Against *Lactobacillus* sp

		L.casein			*L.delbrukii*			*L.fermentum*		
No	ANTIBIOTICS									
1	CO- CO TRIMOXAZOLE (25µg)	R	R	R	R	R	R	R	R	R
2	NX- NORFLOXACIN (10 µg)	R	R	R	R	R	R	R	R	R
3	T- TETRACYCLINE (10 µg)	S	S	S	S	S	S	S	S	S

R- RESISTANCE S- SENSITIVE

Lactobacillus sp against tetracycline and Cotrimaxazole respectively. Similar type of results were reported resistance against tetracycline in *Lactobacillus* sp isolated from food products in Europe [28].

It is believed that antibiotic used for food-producing animals can promote the emergence of antibiotic resistance in bacteria present in the intestinal microflora. Then, the antibiotic-resistant bacteria can transfer the resistance factor to other pathogenic bacteria through the exchange of genetic material [29]. One of the safety considerations in probiotic studies is the verification that a potential probiotic strain does not contain transferable resistance genes.

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