JOURNAL OF PURE AND APPLIED MICROBIOLOGY, April 2011. Vol. **5**(1), p. 179-184

Lactobacillus and *Leuconostoc* of Cattle Milk and their Bacteriocinogenic Potential

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(Received: 15 October 2010; accepted: 24 December 2010)

Lactic Acid Bacteria (LAB) are widely distributed in the nature and occurring naturally as indigenous micro flora in raw milk that play an important role in many foods and feed fermentations. LAB are also known to produce bacteriocins and have great potential in food bio preservatives. The purpose of this research was to study the potential of cattle (cow, buffalo, camel and goat) milk's LAB to produce bacteriocins. Total 80 strains of lactobacilli and leuconostocs were achieved to isolate and identify by using MRS and sodium azide agar medium. Among the 40 strains of each LAB genera i.e. Lactobacillus and Leuconostoc, 6 spp. of lactobacilli viz.- Lactobacillus brevis, Lactobacillus casei, Lactobacillus fermentum, Lactobacillus lactis, Lactobacillus acidophilus and Lactobacillus delbrueckii and 5 spp. of leuconostocs viz. – Leuconostoc dextranicum, Leuconostoc lactis, Leuconostoc paramesenteroids, Leuconostoc mesenteroids and Leuconostoc cremoris were observed. For all 80 strains the influence of antibacterial activities was obtained by using the agar well diffusion method against 3 Gram positive and 3 Gram negative food spoilage causing bacteria. Inhibition was shown by 36% strains but only 5% revealed the possible bacteriocinogeny. Further studies will be undertaken for the bacteriocinogenic strains.

Key words: Agar well diffusion, Bacteriocin, Dominant species, Inhibition zone, Lactic acid bacteria.

Food safety is an important issue of international concern (Park and Fujisaiua, 2003). Prepacked food items available in market contain variety of chemical preservatives (Silva *et al*., 2002) which may alter chemical constituents, nutritional and organoleptic qualities of foods thus may have serious adverse effects on health (Messi *et al*., 2003). Thus bio preservation of foods has emerged as an attractive and safe approach.

Lactic acid bacteria are a group of Gram positive, non-sporing, non-respiring cocci or rods which produce lactic acid as the major end product during the fermentation of carbohydrates and are used as starter culture.

The genus *Lactobacillus, Lactococcus, Pediococcus* and *Leuconostoc* are included in the group. The lactic acid fermentation, which these bacteria carry out, has long been known and applied by humans for making different foodstuffs.

Listeria monocytogenes is one of the pathogens of concern to food safety due its widespread present in the environment, its high virulence and its resistance in stressful conditions (Thevenot *et al*., 2005). *L. monocytogenes* has been shown to be sensitive to many bacteriocins produced by LAB. In particular, all bacteriocins of

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the lantibiotics and the subclass II a are, by definition, inhibitory to *L. monocytogenes* (Klaenhammer, 1993; Cotter *et al*., 2005).*Staphylococcus aureus* is Gram positive, food borne pathogen capable of growing in foods at refrigeration temperature.

These conditions have initiated a search for naturally produced bio preservatives.Many studies were carried out in Nigeria, Adeskan (2008) using poultry meat isolated LAB and studied its antimicrobial activity against several microorganisms. The results showed that LAB inhibited *Staphylococcus aureus*, *E.coli*, *Pseudomonas aerouginosa* with the exception of *Candida albicans* and *Proteus vulgaris* (Adeskan *et al.*, 2008).

Inhibition quality of LAB is due to a combination of many factors produced by LAB e.g. production of lactic acid which reduces pH of the surroundings and also other inhibitory substances such as bacteriocins which are responsible for the most antimicrobial activity (Ogunbanwo, 2005).

Some of the metabolites of these bacteria have an antimicrobial effect against many food spoilage and pathogenic bacteria; include lactic acid, diacetyls, hydrogen peroxide, and proteinaceous substances bacteriocins (Barefoot and Klaenhammer, 1983; Daeschel, 1989).

Different reports show that most lactic acid bacteria (LAB) produce substances that inhibit pathogenic, non-pathogenic and spoilage organisms in fermenting foods and beverages (Gilliland and Speck, 1975; and Schillinger and Lucke, 1989).

Bacteriocin producing *Lactobacillus casei* was previously identified and isolated from infant stool sample (Joshi and Chaudhary 2003).

The aim of this study is, therefore, to evaluate the *in-vitro* bacteriocinogenic activities of *Lactobacillus* and *Leuconostoc* isolated from cattle's milk, on the growth and survival ofsome food borne pathogenic microorganisms. The studies produced some results of interest that will be a matter of further research.

MATERIAL AND METHODS

The present study was conducted following the established methodologies. Most of the media and chemical reagents used were obtained from HiMedia Laboratories Pvt. Limited (India). The inhibitory activity was measured in duplicates throughout the studies.

Collection of samples

Four different cattle milk samples namely camel; cow, buffalo, and goat milk were collected during the lactation process in sterile screw cap tubes and processed within 3 hours. The samples were aseptically collected and brought to the laboratory, via ice box for the isolation of *Lactobacillus* and *Leuconostoc* and further screening of them.

Strains and culture conditions

The test organisms (*Listeria monocytogens* MTCC657, *Bacillus subtilis MTCC441*, *Staphylococcus aureus MTCC96*, *E.coliMTCC119*, *Salmonella typhi MTCC734*and *Proteus vulgaris* MTCC1771) were selected on the basis of the food spoilage caused by them. Both Grampositive and Gram negative bacterial species were selected as test organisms. The organisms were obtained from MTCC Chandigarh. All the above test organism strains were maintained in nutrient agar at 4°C and sub cultured every three weeks.

Isolation of Lactobacillus* and *Leuconostoc

The *Lactobacillus* and *Leuconostoc* strains were isolated from the above collected milk samples. A ten folds dilution of each sample was done with sterile water and was plated. The MRS Agar (De-Man *et al*., 1960) and Sodium Azide Agar media were used for the isolation of *Lactobacillus* and *Leuconostoc* respectively. Thoroughly mixed the samples with media and the media were allowed to solidify. After solidification the plates were incubated. Few of the selected single colonies were transferred into MRS and MMRS broths and were tested and examined morphologically and microscopically for purity and then sub cultured on MRS and MMRS agar respectively.

Cultivation was carried out with appropriate incubation temperature and time required for the growth. It was 37°C for 24 hours for *Lactobacilli* and 25°C for 72 hours for *Leuconostocs*. Isolation of pure cultures was completed by the streak plate method. Bacterial isolates were identified on the basis of morphological, cultural and biochemical characteristics according to Bergey's Manual of

Determinative Bacteriology (Holt *et al*., 1994). *Lactobacillus* and *Leuconostoc* isolates were sub cultured regularly.

Detection of inhibitory activity of *Lactobacillus* and *Leuconostoc*

Cell free culture supernatant was obtained from the *Lactobacillus* and *Leuconostoc by* centrifugation of cultures at 8,000 g at 4°C for 10 minutes. The supernatant was filtered through 0.45 µm pore size filters and stored at -20°C until use.

The inhibitory activity of the supernatant was determined by the agar well diffusion method(Tagg *et al*., 1976). Petri dishes containing 20 ml of Nutrient Agar were prepared previously. Once solidified the dishes were stored for 2 hours in a refrigerator. Four wells of a diameter of 4mm each were made. Lawn culture of the each test organism was prepared by inoculating 0.1 ml of 24 hours activated broth culture of test organisms. The wells were filled using 100 µl of cellfree filtrate of *Lactobacillus* and *Leuconostoc* strains according to (Kalalou *et al*., 2004). Plain broths were used as the control.

The procedure was adopted twice once with un-neutralized and once with neutralized supernatant. The supernatant was adjusted to pH 7.0 with 1N NaOH. Objective of this exercise was to differentiate the inhibition and the bacteriocinogeny.

Petri dishes were incubated at 37°C for 24 hours. Diameter of the inhibition zone was measured with calipers in mm. The inhibition was determined by measuring the clear zone around the wells. Inhibition was recorded as positive if the width of the clear zone around the wells was 0.5 mm or larger (Schillinger and Lucke 1989).

RESULTS

Enumeration, isolation and identification of the dominating species of *Lactobacillus* and *Leuconostoc* in the milk samples of cow, buffalo, camel and goat was accomplished. From the tested samples total 80 strains of *Lactobacillus* and *Leuconostoc* were isolated to draw conclusion about the dominant species of *Lactobacillus* and *Leuconostoc*. In concern of *Lactobacillus* and *Leuconostoc* counts camel milk (Lactobacillus-1.6 x 10^5, *Leuconostoc*- 1.3 x 10^5) was observed rich as compared to other milk samples. The goat milk (Lactobacillus-1.0 x 10^5,*Leuconostoc*- 0.7 x 10^5) was found of poor *Lactobacillus* and *Leuconostoc* counts, while the cow (*Lactobacillus*-1.2 x 10^5, *Leuconostoc*- 0.8 x 10^5) and buffalo milks (*Lactobacillus*-1.4 x 10^5, *Leuconostoc*- 0.9 x 10^5) were average.

The dominating *Lactobacillusspp.* found in cow milk was *Lactobacillus brevis*(60%), in buffalo milk *Lactobacillus lactis* (50%), in camel milk *Lactobacillus acidophilus*(50%) and in goat milk it was *Lactobacillus delbrueckii*(50%). *Leuconostoc dextranicum* was commonly dominant in cow and goat milk (50%) while in buffalo milk (40%) *Leuconostoc lactis* (50%) and in camel milk *Leuconostoc cremoris* (50%) was dominant species of *Leuconostoc*. Other species found at a little difference were *L. casei*, *L. fermentum, L. paramesenteroides* and *Leuconostoc mesenteroides*.

All the 80 strains were tested for their inhibitory ability against the test organisms (*Listeria monocytogens* MTCC657, *Bacillus subtilis MTCC441, Staphylococcus aureus*

Table 1. Frequency of LAB strains showing inhibition against test organisms

Source Animal	Number of inhibitory isolates and type of supernatant		
	Total isolates	un-neutralized supernatant	neutralized supernatant
Cow	20	8	1
Buffalo	20	11	2
Camel	20	4	0
Goat	20	5	1
Total	80	28	4

LAB: Lactic Acid Bacteria i.e. strains of *Lactobacillus* and *Leuconostoc*

MTCC96, *E.coli MTCC119*, *Salmonella typhi MTCC734* and *Proteus vulgaris* MTCC1771). The inhibitory activity of all the isolates was determined by the agar well diffusion method.As results indicate, the diameters of the inhibition zones were varied; it ranged between 0.5 to 1.8 mm. Among the four possible bacteriocinogenic LAB strains there was no any *Leuconostoc* strain, only *Lactobacillus* strains gave positive results. Three homo fermentative strains (URLB18-*Lactobacillus acidophilus*, URLB16 and URLB38-both *Lactobacillus lactis)* and one facultative homo fermentative strain (URLB1- *Lactobacilluscasei*) shown varied inhibition against the test organisms (Table 2).

Table 2. Spectrum of inhibitory activity (in mm) of NaOH treated supernatant of LAB strains

Test organism	Inhibition zone diameter (mm) given by Bacteriocinogenic *Lactobacillus* strains			
	URLB1	URLB16	URLB18	URLB38
Gram positive				
Listeria monocytogens	1.8	1.5	-	1.2
Bacillus subtilis	0.5	0.6	-	-
Staphylococcus aureus	0.7	1.2	0.9	1.0
Gram negative				
E.coli	-	0.6	0.7	-
Salmonella typhi	-	-	-	-
Proteus vulgaris	-	-	-	-

URLB1= Cow milk *Lactobacillus casei,* URLB16= Buffalo milk *Lactobacillus lactis,* URLB18= Buffalo milk *Lactobacillus acidophilus,* URLB38= *Goat milk Lactobacillus lactis*

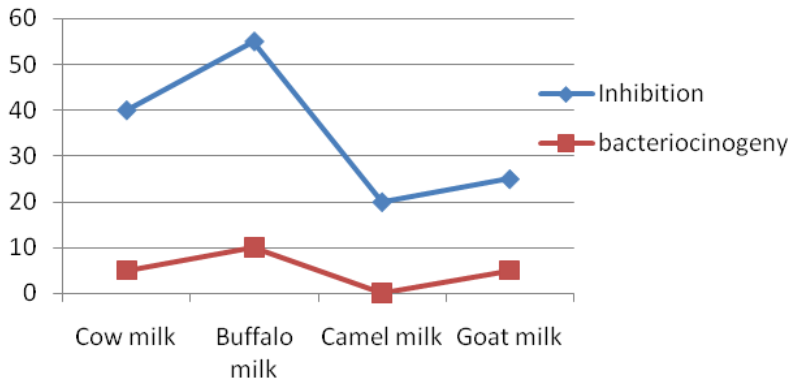

Fig. 1. Inhibition and bacteriocinogency by LAB isolates in %

DISCUSSION

Lactic Acid Bacteria (LAB) have been used to provide an effective form of natural preservation for many centuries. The antimicrobial activity of lactic acid bacteria (LAB) on various food borne pathogens is well documented. This prompted the study to evaluate the *in-vitro* antimicrobial activity of *Lactobacillus* and *Leuconostoc* isolates of cattle's milk on *Listeria monocytogens,Bacillus subtilis, Staphylococcus aureus, E.coli, Salmonella typhi* and *Proteus vulgaris*.

Among the antimicrobial substances produced by microorganisms, bacteriocins have gained an increasing interest in the recent years.

They are small ribosomally synthesized proteins, which are able to kill bacteria, including a number of potential foodborne pathogens and food spoilage microorganisms. The bacteriocins from the Generally Recognized As Safe (GRAS) lactic acid bacteria (LAB) have arisen a great deal of attention as a novel approach to control pathogens in foodstuffs. Data for bacteriocins, produced by LAB, and their application as preservatives in the food industry can be found in several reviews (Sholeva*et al*., 1998). Therefore, use of these antimicrobial substances or their producer strains as a biological means to enhance the control of specific food borne pathogens is worthwhile considering and hence, search for new bacteriocin producing LAB potentially useful to food preservation should continue.

Bacteriocins are proteinaceous compounds that mainly inhibit closely related species (Klaenhammer, 1993). Some bacteriocins have been shown to possess the ability to inhibit the unrelated genera such as *Clostridia, Listeria*, enteropathogenic bacteria and Gramnegative bacteria. For these reasons bacteriocins are promising candidates for bio preservation of foods (Cleveland *et al*., 2001).

The results shown in figure- 1prove the studies of (Sumathi and Reetha, 2009) as LAB strains of buffalo milk had more potential (55%) to inhibit the test organisms. Camel and goat milks were known as of less use for this purpose while the cow milk LAB strains also gave considerable results.

As the LAB strains were grown in broth media containing glucose, the observed inhibition might arise from the acid produced. Varadaraj *et al*.,(1993)observed moderate inhibition of some food borne pathogens and other bacterial species by neutralized culture filtrates of LAB using a well diffusion assay. McLean andMcGroarty (1996) also showed that about 60% of the antimicrobial activity of culture filtrates of LAB was removed when the filtrates were neutralized to pH 6.5 with NaOH.

The inhibitory activity of all the isolates was examined again with neutralized supernatant (pH- 7.0). Frequency of the positive strains decreases with neutralized supernatant means this time the inhibition was not due to the acids but due to some other metabolites which can be bacteriocin (Table 1).

Against Gram negative bacteria inhibition was almost not observed. URLB16 and URLB18 inhibited only *E. coli*. According to Sholeva*et al*.,(1998) bacteriocins, produced by LAB, usually do not exhibit activities against Gram negative strains, although there is information by some authors (Piard and Desmazeaud, 1992) about the production of substances active against *E. coli*. It is not clear, however, whether these compounds are bacteriocins or other agents of inhibition.

Most of the Gram positive bacteria were inhibited by all the four strains. Maximum diameter of inhibition zone was shown by *Lactobacillus casei* against *Listeria monocytogens (*1.8 mm). *Staphylococcus aureus* was inhibited by all four strains. Similar study was carried out in Morocco by Kalalou*et al*.,(2004) who's studied the activity of LAB on some Gram positive and Gram negative pathogenic bacteria such as *E.coli*, *Pseudomonas aueriginosa, Klebsiella pneumoniae*, *Staphylococcus aureus* and *Bacillus cereus* and the inhibition zones were in the range of 1.4 to 2.8 mm.

According to Klaenhammer (1993) 99% of all bacteria may make at least one bacteriocin. It has been extensively reported that the environmental factors including stressful conditions influence the magnitude of bacteriocin production to overcome the competitive strains living in the same environment (Pattnaik *et al*., 2005). On the basis of this study suitable LAB strains isolated from the cattle milk to be optimized for the bacteriocins production in further studies.

CONCLUSIONS

· The data obtained from the presented results show that most of the lactic acid bacteria can show the inhibition against pathogens due to their metabolites but only few of them show the bacteriocinogeny.

· Buffalo milk isolates may be of higher interest to exploit their inhibiyory properties. Genera *Lactobacillus* is more promising to produce bacteriocins rather than *Leuconostoc*.

· Bacteriocins are efficient inhibitors of the growth of the Gram positive bacteria but these rarely affect the Gram negative bacterial growth.

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