Characterization and Antibacterial Activity of Bacteriocin Producing *Lactobacillus* Isolated from Raw Cattle Milk Sample

A. Mohankumar (Correponding author)
Department of Zoology, Chikkanna Government Arts College
Tirupur-641 602, Tamilnadu, India
E-mail: moniver@satyam.net.in

N. Murugalatha
Department of Microbiology, Hindusthan College of Arts & Science
Coimbatore- 614 028, Tamilnadu, India

Received: April 15, 2011 Accepted: April 30, 2011 doi:10.5539/ijb.v3n3p128

Abstract

The intent of the study is to determine the antimicrobial activity of *Lactobacillus* producing bacteriocin isolated from raw milk of cattle's like cow, buffalo and goat and to characterize the bacteriocin. Hundred *Lactobacillus* isolates (50 isolates from cow, 22 isolates from buffalo and 28 isolates from goat) based upon the distinct morphology were isolated from the samples and identified as Lactobacilli according to phenotypic characteristics. Bacteriocin producing organisms were screened by Agar spot assay test. Ten strains were able to produce bacteriocin whose antibacterial activity was analyzed by agar well diffusion assay test against indicator organisms and pathogenic organisms. *Bacillus mycoides, Staphylococcus aureus, Streptococcus faecalis, Klebsiella pnuemoniae* and *Proteus vulgaris* were inhibited by the isolates. *Bacillus amyloliquifaciens, Bacillus cereus, Salmonella typhi* and *Pseudomonas aeruginosa,* were resistant to the isolates producing antibacterial substances. The antibacterial protein bacteriocin was characterized based on the sensitivity to heat, different pH values, acid neutralization test, sensitivity to chloroform, NaCl and incubation period. Lactobacilli from raw milk samples that inhibited certain pathogenic organisms by producing bacteriocin may be beneficial for a probiotic culture to be triumphant in colonizing and to contend with pathogens.

Keywords: Raw milk, Lactic acid bacteria, Antibacterial activity and bacteriocin characterization

1. Introduction

Lactic acid bacteria are a group of Gram-positive bacteria united by a constellation of morphological, metabolic, physiological characteristics (Coeuret *et al*., 2003). They produce lactic acid either through homofermentative or heterofermentative pathway and are wide spread in nature and also found in human digestive system. Lactobacilli are considered especially as beneficial bacteria because they have their ability to break down proteins, carbohydrates and fats in food and help in absorption of necessary elements and nutrients such as minerals, aminoacids and vitamins required for the survival of humans and other animals. Lactic acid bacteria exert a strong antagonistic activity against many food-contaminating microorganisms as a result of the production of organic acids, hydrogen peroxide, diacetyl, inhibitory enzymes and bacteriocins (Piard and Desmazeaud, 1991). *Lactobacillus* exerts a positive documentation in the prevention and treatment of gastrointestinal disorders. Although anti-microbial agents are generally effective at eradicating these infections, there is a high incidence of recurrence. Certain Lactobacilli synthesize antimicrobial compounds that are related to the bacteriocin family (Jack *et al.,* 1995; Klaenhammer, 1993). Antimicrobial substances produced by Lactic acid bacteria (LAB) are used in association with selective insensitive starter to inhibit competitive microflora (Scannell *et al*., 2000). The term bacteriocin refers to protein of the colicin type, characterized by lethal biosynthesis, intraspecific activity and absorption to specific receptors and the bacteriocins produced by *Lactobacillus* fit closely to the classical colicin model (Tagg *et al.,* 1976). Lactobacilli produce many different bacteriocins of similar activity and are usually predominant species.

This study was focused on isolation and characterization of bacteriocin-producing *Lactobacillus* from cattle milk like cow, buffalo, goat and its inhibitory nature against common human pathogens.

2. Materials and Methods

2.1 Isolation of Lactic acid bacteria

Raw unpasteurized hundred milk samples of Cow, Buffalo and Goat were collected from the local area of Coimbatore during lactation period under aseptic conditions in a sterile screw cap tubes, processed within three hours and used for further studies.

Milk samples were serially diluted in peptone medium and incubated at 23°C for 30 min before plating by which 50% of recovery of LAB was increased. Diluted samples were plated onto De Man Rogosa Sharpe (MRS) medium for *Lactobacillus* isolation and incubated at 37°C for 48-72 hrs. 100 numbers of well-isolated colonies with typical characteristics namely pure white, small (2-3mm diameter) with entire margins were picked from each plate and transferred to MRS broth. The isolates were designated as CWL (Cow), BFL (Buffalo) and GAL (Goat).

2.2 Identification of the Bacterial strains

Further identification of the *Lactobacilli* was performed according to their morphological, cultural, physiological and biochemical characteristics (Sharpe, 1979; Kandler and Weiss, 1986): Gram reaction, production of catalase, carbohydrate fermentation patterns, growth at 15°C and 45°C in the lactobacilli De Mann Rogosa and Sharpe (MRS) broth as described by Bergey's Manual of Systematic Bacteriology (Kandler and Weiss, 1986), methyl red and Voges-Proskauer test in MR-VP medium, nitrate reduction in nitrate broth, indole production in Tryptone broth. Purified cultures were maintained at -20°C in MRS broth with 10% glycerol and enriched in MRS broth incubated at 37°C for 24 hrs. The identified genus *Lactobacillus* was further classified to the species level based on their ability to ferment sugars (Singh. And Rakesh Roshan Sharma, 2009).

2.3 Detection of Inhibitory activity

2.3.1 Agar Spot Assay Test

100 Lactic acid bacterial isolates were cultured in 5ml of MRS broth at 30° C for 16 hrs. Aliquots (2µl) of the culture were spotted onto agar plates containing 10ml of MRS medium. After 18 hrs at 30°C, the plates were overlaid with 5ml of the appropriate soft agar (1% agar) inoculated with the cell suspension of the indicator strain *Lactobacillus acidophilus* at a final concentration of 10^5 CFU/ml (Kilic *et al*., 1996). The plates were incubated at 37°C for 24-72 hrs, depending on the growth of the indicator strain and the appearance of inhibitory zones were observed. Inhibition was scored positive if the zone was wider than 2mm in diameter.

2.3.2 Agar-Well Diffusion Assay

The strains that were selected as potential bacteriocin producers were grown in MRS broth at 37ºC for 48 hrs. Cells were separated by centrifugation at 5000 rpm for 10 min at room temperature. Around 6mm diameter wells were made on preinoculated agar media and each well was inoculated with 100 µl of culture supernatant of bacteriocin producing *Lactobacillus* strains after neutralization with NaOH (Toba *et al*., 1991). Inhibitory activity was performed against certain Gram positive and Gram negative organisms: *Bacillus amyloliquifaciens* (MTCC 1270), *Bacillus cereus* (MTCC 1272), *Bacillus mycoides* (MTCC 645), *Lactobacillus acidophilus* (MTCC 447), *Lactobacillus delbrueckii* sub sp. *lactis* (MTCC 911), *Lactococcus lactis* sub sp. *lactis* (MTCC 440), *Streptococcus faecalis* (MTCC 459), *Staphylococcus aureus* (MTCC 740), *Klebsiella pneumoniae* (MTCC 3384), *Proteus vulgaris* (MTCC 744), *Pseudomonas aeruginosa* (MTCC 647) and *Salmonella typhi* (MTCC 531). Inhibition zones around the wells were measured and recorded.

2.4 Characterization of Bacteriocin

2.4.1 Sensitivity to heat

100 µl of culture supernatant was heated for 10 min at 60º C, 70º C, 80º C and 90º C. The agar spot assay test was performed to detect residual activity. The resistant culture supernatants were further heated for 10, 30 and 60 min at 100º C and the residual activity was assayed (Larsen *et al.,* 1993).

2.4.2 Sensitivity to different pH values

The pH of culture supernatants was adjusted to 3.0, 4.5, 7.0 and 9.0 and then kept at room temperature for 4 hrs. Residual activity was determined by the agar-spot method as described (Larsen *et al*., 1993).

2.4.3 Acid neutralization test

This test was performed by agar well diffusion assay (Alpay *et al*., 1991). In addition to 100 µl of supernatants buffered with NaOH to pH 7.0, 75 µl of *Lactobacillus* suspension and 25 µl of 10% $CaCO_3$ solution were mixed and placed into the well. The original culture supernatants were used as control samples. When the inhibition

zone was determined around the wells of the control and buffered samples, the inhibitory effect was assumed to be due to bacteriocin or H_2O_2 (Diaz *et al*., 1993).

2.4.4 Sensitivity to chloroform

The culture supernatant was mixed with an equal volume of chloroform and kept at room temperature for 4 hrs before antimicrobial activity testing (Diaz *et al*., 1993).

2.4.5 Effect of NaCl on bacteriocin production

MRS broth with 1%, 3%, 4% NaCl and without NaCl was sterilized by autoclaving and were inoculated with 10% of the overnight bacteriocin producing culture and incubated at 37˚C for 24 hrs. Bacteriocin activity was assayed by inoculating the culture supernatant against indicator organism (Alpay *et al*., 2003).

2.4.6 Effect of incubation period on bacteriocin production

Active cultures of producer organisms (1% v/v) were inoculated in 100 ml aliquots of sterile composed media. Inoculated flasks were incubated at 37^{o} C for 15, 24, 48 and 72 hrs and at the end of each incubation period, bacteriocin activity was observed by inoculating culture supernatant against indicator organism (Lade *et al*., 2006).

2.4.7 Effect of different concentrations of carbon and nitrogen source on bacteriocin production

The effect of different concentrations of medium ingredients on bacteriocin production was evaluated using composed MRS medium. The carbon sources studied were glucose (1% – 3%) and lactose (1% – 3%) while nitrogen sources were tryptone (1 %– 3%), peptone (1% – 3%) and yeast extract (0.5% – 2%) (Lade *et al*., 2006).

3. Results

100 *Lactobacillus* colonies from milk samples (50 from cow milk, 22 from buffalo milk and 28 from goat milk) with typical characteristics namely pure white, small (2-3 mm diameter) with entire margins were picked from each plate and transferred to MRS broth which was then subjected to classification onto the genera *Lactobacillus* based on morphological and biochemical characters.

All strains reacted positively to gram staining under a light microscope. Lactobacilli are generally long rods some times they are short rods, coccoid. It happens that cells of coccoid form strains were not able to show growth at 45°C. Few isolates from buffalo and goat were able to utilize citrate indicating the passage of citrate into a cell with aid of citrate permease**.** *Lactobacillus* donot possess flagella and donot create endospores, nitrates are not reduced, gelatin is not liquefied, indole is not produced, acidic and non acidic end products are not produced and are catalase negative. The isolates were found to be homofermentative that produce lactic acid from glucose, lactose and mannitol (Tables 1, 2 &3).

The identified genus *Lactobacillus* was further classified to the species level. Strains were able to ferment sugars at different percentages which were much significant for identification of the species. Among ten different sugars sucrose was fermented by all the isolated strains. Trehalose was utilized by 94% of the isolates and 60-80% of the isolates fermented all other sugars. The differentiating characters of *Lactobacillus* are given in Tables 4, 5 and 6 and based on the above characters it was concluded that *Lactobacillus casei*, *Lactobacillus fermentum, Lactobacillus lactis* were found commonly in all cattle's milk. *Lactobacillus plantarum* was specifically found in cow milk. *Lactobacillus brevis* was found in buffalo milk. *Lactobacillus delbrueckii* was found in goat mik. *Lactobacillus lactis* was dominating the *Lactobacillus casei* and *Lactobacillus fermentum* in the cow milk sample. Similarly the same species was dominating the *Lactobacillus casei*, *Lactobacillus delbrueckii* and *Lactobacillus fermentum* in case of a buffalo and goat milk sample.

3.1 Bacteriocin Assay

3.1.1 Agar Spot Assay Test

The culture supernatants obtained from a total of 100 Lactobacilli isolates of all the milk samples were tested for antibacterial activity against the same group of lactobacilli. Ten isolates have shown clear zone of inhibition against the indicator organism and they were selected as potential bacteriocin producers. 5 isolates from cow milk sample, 2 isolates from buffalo and 3 isolates from goat milk sample were able to show inhibitory action against the indicator strain.

3.1.2 Agar Well Diffusion Assay

The culture supernatant obtained from ten bacteriocin producer strains were tested for antibacterial activity against certain Gram positive and Gram negative bacteria. Bacteriocins obtained from the isolates showed inhibitory activity against *Bacillus mycoides, Streptococcus faecalis, Staphylococcus aureus* and *Proteus*

vulgaris among the sensitive bacteria tested. *Bacillus amyloliquifaciens, Bacillus cereus, Pseudomonas aeruginosa*, and *Salmonella typhi* were resistant to bacteriocin producers. The resistant activity varied with each strain. The degree of inhibition was designated as very strong inhibition (15-18mm), strong inhibition (10-14mm), moderate inhibition (6-9mm) and no inhibition. BFLI and GAL2 showed very strong activity against *Bacillus mycoides* with the zone of inhibition of 15-18mm in diameter. *Staphylococcus aureus* was moderately inhibited by BFL2, GAL2 and GAL3 with a zone of inhibition 6-9mm in diameter and very strongly inhibited by CWL1, CWL17, CWL25, CWL29, BFL1 and GAL1. *Streptococcus faecalis* and *Proteus vulgaris* were inhibited at a higher range by the bacteriocin isolates when compared to other strains. The two strains BFL1 and GAL1 showed very strong activity against *Klebsiella pneumoniae* (Table 7).

3.2 Characterization of Bacteriocin

Bacteriocin sensitivity to physical conditions and chemical substances was also evaluated. Among ten bacteriocins CWL25 and GAL2 strains were identified as lipid containing bacteriocins because of their sensitivity to chloroform and boiling. These two bacteriocins were also inactivated by heating at 60°C for ten min. On the other hand the remaining strains were found to be resistant to chloroform and resistant to boiling for at least 10 minutes. These bacteriocins belong to low – molecular weight non-lipid containing bacteriocins. All the ten bacteriocins were stable between pH 4.5 and 7.0 but sensitive to pH 9.0. Except for CWL25, BFL2, GAL2 and GAL3, strains the remaining strains were found to be active at pH 3.0. In acid neutralization test the inhibitory zones was determined around the wells of both the control (original culture supernatants) and buffered samples (buffered with NaOH) (Table 8).

3.2.1 Effect of NaCl on bacteriocin production

The effect of NaCl on the production of the ten bacteriocin were studied. 1% NaCl increased the production of bacteriocins in almost all strains. GAL1, BFL1 showed superior activity in presence of 3% NaCl when compared to other isolates, but this activity was lost at 4% NaCl. CWL25 was inhibited by more than 1% NaCl in MRS media and did not show any increase in their activity (Fig 1).

3.2.2 Effect of incubation period on bacteriocin production

The effect of incubation period at 37° C on bacteriocin production was also studied and it was observed that there was no growth after 15 hrs of incubation and at the end of 24 hrs the activity was found the maximum, while at 48 and 72 hrs the inhibitory action was found to be comparatively less (Fig 2).

3.2.3 Effect of different concentrations of carbon and nitrogen source on bacteriocin production

The influence of culture medium components on the production of bacteriocin was investigated using *Lactobacillus delbrueckii* as an indicator organism. The result of this study revealed an increase in bacteriocin production in MRS medium containing 1% glucose and 1% peptone to normal MRS medium which was found to be optimum. Glucose was found to be better carbon source than lactose. It can be stated that variation in the concentration of constituents might have an influence on the amount of bacteriocin produced (Fig 3).

4. Discussion

Milk samples from cow, goat and buffalo were collected from different local areas of coimbatore and processed for isolation of LAB. The colonies from raw milk sample are expected to be little higher than real microflora. This is due to contamination from the animal, especially the exterior of the udder and the adjacent areas; bacteria found in manure, soil and water may enter (Garbutt, 1997).

From the tested samples hundred bacterial cultures were isolated to draw conclusion about the resident lactobacilli of the milk of particular cattle's. *Lactobacillus* was found higher in cow milk when compared to goat and buffalo milk. Singh and Rakesh Roshan Sharma (2009) have stated in their research that *Leuconostoc* and *Lactobacillus* both were found higher in number in camel milk as compared to cow, buffalo and goat milk. But the total number of bacteria was found higher in cow milk.

The LAB isolates were classified into the genera *Lactobacillus* based on their morphological and biochemical characters (Sharpe, 1979). Bacillary and cocci forms were positive to Gram reactions under a light microscope. In the present study, isolates were able to grow at 15ºC and coccoid forms were not able to withstand 45ºC. *L. alimentarius* and *L. animalis* which are cocci in morphology were able to grow at 15ºC but were not able to with stand at 45ºC (Parvathy Seema Nair and Puthuvallil Kumaran Surendaran, 2005). Some *Lactobacillus* were found to be irregular, short, even coccoid rods with roun tappered ends, sometimes longer also (Kandler *et al.,* 1983a). Few strains were able to utilize citrate and were found to be non motile; catalase, indole, MR-VP and citrate negative; nitrates are not reduced and gelatin was not liquefied. Isolated strains were homofermentative, fermenting glucose, lactose, sorbitol and mannitol. 41.6% of isolated strains were able to ferment sucrose and 21.6% were able to ferment mannitol. Kandler and Weiss (1986) have classified *Lactobacillus* isolates from

temperate regions according to their morphology, physiology and molecular characteristics. Coppolla *et al.* (2000) studied the morphological characters of raw milk, natural whey starter and cheese. De Man *et al.* (1960) stated that Lactobacilli are generally isolated on rich media such as MRS which is routinely used for the isolation and counting of Lactobacilli for most fermented food products. The addition of the reducing agent such as cysteine (0.05%) to MRS improve the specificity of the medium for *Lactobacillus* isolation (Hetremink *et al.,* 1997; Lankaputhra *et al*., 1995; Shah, 2000).

Lactobacillus plantarum, Lactobacillus lactis, Lactobacills fermentum and *Lactobacillus casei* were found in cow milk and *Lactobacillus brevis* and *Lactobacillus delbrueckii* were found in buffalo milk. *Lactobacillus lactis, Lactobacills fermentum* and *Lactobacillus casei* were found in goat milk. *Lactobacillus lactis* was dominating the *Lactobacillus brevis* in the buffalo milk sample. Similarly the same species was dominating the *Lactobacillus fermentum, Lactobacillus casei and Lactobacillus delbrueckii* in case of cow and goat milk sample also. Singh and Rakesh Roshan Sharma (2009) have stated that *Lactobacillus fermentum* and *Lactobacillus casei* were found in cow milk, *Lactobacillus lactis, Lactobacillus acidophilus* were found in buffalo milk sample.

In vitro assay was carried to characterize the antimicrobial potential of the culture supernatant to inhibit some pathogenic bacteria. Hundred LAB isolates were screened for bacteriocin producers by Agar spot assay test (Kilic *et al.,* 1996). Isolates inhibiting the indicator organism by clear zone of inhibition were selected as bacteriocin producers. Total inhibition diameter was calculated for each LAB strain as the sum of the inhibition diameter against the indicator strain. The results of the Agar spot assay showed that a total of 10 % of the isolates were able to inhibit the indicator organism by producing bacteriocin. Bacteriocin producers were found to be in a higher percentage of 12% from goat's milk when compared to the other cattle's milk. Nowroozi1 *et al*. (2004) has stated that antibacterial activities were done by an agar spot in which only 14.3% of strains made known to produce bacteriocin.

Active supernatants of *Lactobacillus* sp. were examined for acid and bacteriocin production; ten had inhibitory effects on sensitive bacteria including *Lactobacillus* strains and some common pathogenic bacteria. Among the bacteriocins tested, bacteriocins from BFL1 and GAL1 strains had a broader host range. It was observed that all the ten bacteriocins had an inhibitory effect on *Bacillus mycoides, Klebsiella pnuemoniae, Staphylococcus aureus*, *Streptococcus faecalis* and *Proteus vulgaris* were inhibited moderately. However, none of them affected *Bacillus cereus, Bacillus amyloliquifaciens, Pseudomonas aeruginosa* and *Salmonella typhi.* An expanded host range has been noted recently for a number of *Lactobacillus* bacteriocins, which kill *E. faecalis, L. monocytogenes*, *C. botulinum, C. tyrobutyricum, S. aureus* and *A. hydrophila* (Klaenhammer, 1993). Toba *et al.* (1991) determined bacteriocins in *L. gasseri, L. acidophilus* JCM 1132 and *L. acidophilus* LAPT 1060 strains from infant feces were active against other *Lactobacillus* strains.

The inhibitory effect was assumed to be due to bacteriocin not H_2O_2 since there was no oxidizing effect on bacterial cells which will destroy the basic molecular structure of cell proteins (Zsolt Zalan *et al.,* 2005) and bacteriocin form the pores in the membrane of sensitive cells and deplete the transmembrane potential and/or the pH gradient, resulting in the leakage of cellular materials (Chikindas *et al*., 1993; McAuliffe *et al*., 2001).

Kanatani *et al.* (1995) has stated that a bacteriocin (acidocin A) from *L. acidophilus* TK9201 had inhibitory effect on closely related lactic acid bacteria and food borne pathogens including *L. monocytogenes*. Itoh *et al.* (1995) indicated that gassericin A produced by *L. gasseri* LA39 was one of the most active bacteriocins for use against enteric pathogens. Silva *et al*. (1987) isolated a low molecular weight substance from the *Lactobacillus* GC strain from the feaces of a healthy person with a potent inhibitory activity against a wide range of Gram positive and Gram negative bacteria.

Bacteriocin were characterized and tested for their *in vitro* antimicrobial activity against a group of organisms. The antimicrobial activities of bacteriocin producing isolates were differentiated from pH 3.0 to 9.0 and H_2O_2 by standard methods. Bacteriocin isolated from CWL29, CWL1, CWL17, CWL6, BFL2, BFL1, GAL1 and GAL3, is considered to be resistant to chloroform and boiling. However the other two bacteriocin deserve for further study and all the bacteriocins characterized in this study were found to show antibacterial activity at a pH range of 4.0 to 7.0. Tagg *et al.* (1976) reported that most bacteriocins are resistant to acidic pH more than basic pH. The inhibitory activity of the bacteriocin isolated from *L. acidophilus* LB strain occurred between pH 3.0 and 5.0 and the inhibitory activity was lost when the pH was raised to 5.0-3.0 (Barefoot and Klaenhammer, 1984). Plantaricin S produced by *L. plantarum* LPCO10 showed inhibitory activity at 4% NaCl (pH 3.0 to 7.0) (Diaz *et al.,* 1993). Further more, it was observed from our present study that 1% NaCl enhanced the bacteriocin production of all the ten bacteriocin isolates. Larsen *et al.* (1993) detected bavaricin A from *L. bavaricus* that showed no changes at 1% NaCl, but production was inhibited with increasing amount of NaCl. It was stated that

strains P1-001 and P8-002 from swine showed better tolerance at 4% NaCl, but not at 8 % NaCl when compared to the control (MRS), with characteristics of *L. fermentum*.

The effect of incubation period on bacteriocin production was also studied and it was observed that at the end of 48 hrs pH sensitivity test, the activity was found to be maximum. Further studies were carried out at different temperatures and pH sensitivity test of bacteriocin. The bacteriocins of all isolates were stable at 100ºC for 10 minutes. The work carried out by Lade *et al.* (2006) has stated that bacteriocin isolates were stable at 100ºC for 10 min and bacteriocin of *Lactobacillus lactis* was stable in acidic to neutral range i.e. from pH 4.0 to 7.0, but, inactive in the alkaline range. The result of our study revealed an increase in bacteriocin production in MRS medium containing 1% glucose and 1% peptone concentration. Glucose was found to be better carbon source than lactose. Thus, variation in the concentration of constituents might have an influence on the amount of bacteriocin produced. Ogunbanwo *et al.* (2003) obtained maximum bacteriocin production by supplementing 1 % glucose and 1% peptone to normal MRS media. However it was observed from the present study that glucose and peptone gave better bacteriocin production. Lade *et al*. (2006) obtained maximum production by supplementing 3% lactose and 2% peptone. Lactose was found to be better carbon source than glucose.

Viable Lactobacilli can inhibit food borne and enteric pathogenic microorganisms by producing lactic acid and other antimicrobial substances. Yogurt and acidophilus milk have been considered to be healthy probiotic diets (Eschenbach *et al*., 1989).

These studies have shown the isolates are defensive and they are been labeled as exceptional bacteria as they have shown their constructive role on human pathogens by inhibiting their growth for which they are said to be the second immune system of the body.

In conclusion the microbiota from milk is efficient in inhibiting the pathogenic organism and will act as a barrier by developing its antimicrobial activities in the host system of defense. The inhibitory spectrum of the antimicrobial substance has a potential application as a biopreservative in food industry. The occurrence of minority atypical resistance to certain antibiotics demonstrates that not all strains are suitable for use as probiotics or biotherapeutic agents. Spontaneous resistance nature of lactobacilli to a wide range of clinically important microorganism may enable the development of probiotic therapies for several infections and for the development of infant probiotic products.

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Table 1. Biochemical characterization of *Lactobacillus* isolated from cow milk

S.No	Milk Sample	Gram Reaction	Motility	Growth		Indole	MR	VP	Citrate	Catalase	Nitrate Reduction	Gelatin
				15^{0}C	45^{0}C							
1	CWL1	Rod	-	+	-	-	-	-	-	-	-	-
2	CWL2	Rod	-	+	-	-	-	-	-	-	-	-
3	CWL3	Rod	-	+	-	-	-	-	-	-	-	-
4	CWL4	CB	-	+	-	-	-	-	-	-	-	-
5	CWL5	Rod	-	+	-	-	-	-	-	-	-	-
6	CWL6	Rod	-	+	-	-	-	-	-	-	-	-
7	CWL7	Rod	-	+	-	-	-	-	-	-	-	
8	CWL8	Rod	-	+	-	-	-	-	-	-	-	-
9	CWL9	CB	-	+	-	-	-	-	-	-	-	-
10	CWL10	CB	-	+	-	-	-	-	-	-	-	-
11	CWL11	Rod	-	+	-	-	-	-	-	-	-	-
12	CWL12	Rod	-	+	-	-	-	-	-	-	-	-
13	CWL13	Rod	-	+	-	-	-	-	-	-	-	-
14	CWL14	Rod	-	+	-	-	-	-	-	-	-	-
15	CWL15	Rod	-	+	-	-	-	-	-	-	-	-
16	CWL16	CB	-	+	-	-	-	-	-	-	-	-
17	CWL17	Rod	-	+	-	-	-	-	-	-	-	-
18	CWL18	Rod	-	+	-	-	-	-	-	-	-	-
19	CWL19	Rod	-	+	-	-	-	-	-	-	-	-
20	CWL20	Rod	-	+	-	-	-	-	-	-	-	-
21	CWL21	Rod	-	+	-	-	-	-	-	-	-	-
22	CWL22	Rod	-	+	-	-	-	-	-	-	-	-
23	CWL23	Rod	-	+	-	-	-	-	-	-	-	-
24	CWL24	Rod	-	+	-	-	-	-	-	-	-	-
25	CWL25	CB	-	+	-	-	-	-	-	-	-	-
26	CWL26	Rod	-	+	-	-	-	-	-	-	-	-
27	CWL27	Rod	-	+	-	-	-	-	-	-	-	-
28	CWL28	Rod	-	+	-	-	-	-	-	-	-	-
29	CWL29	Rod	-	+	-	-	-	-	-	-	-	-
30	CWL30	Rod	-	+	-	-	-	-	-	-	-	-
31	CWL31	Rod	-	+	-	-	-	-	-	-	-	-
32	CWL32	Rod	-	+	-	-	-	-	-	-	-	-
33	CWL33	Rod	-	+	-	-	-	-	-	-	-	-
34	CWL34	Rod	-	+	-	-	-	-	-	-	-	-
35	CWL35	Rod	-	+	-	-	-	-	-	-	-	-
36	CWL36	Rod	-	+	-	-	-	-	-	-	-	-
37	CWL37	Rod	-	+	-	-	-	-	-	-	-	-
38	CWL38	Rod	-	+	-	-	-	-	-	-	-	-
39	CWL39	Rod	-	+	-	-	-	-	-	-	-	-
40	CWL40	Rod	-	+	-	-	-	-	-	-	-	-
41	CWL41	Rod	-	+	-	-	-	-	-	-	-	-
42	CWL42	Rod	-	+	-	-	-	-	-	-	-	-
43	CWL43	Rod	-	+	-	-	-	-	-	-	-	-
44	CWL44	Rod	-	+	-	-	-	-	-	-	-	-
45	CWL45	Rod	-	+	-	-	-	-	-	-	-	-
46	CWL46	Rod	-	+	-	-	-	-	-	-	-	-
47	CWL47	Rod	-	+	-	-	-	-	-	-	-	-
48	CWL48	Rod	-	+	-	-	-	-	-	-	-	-
49	CWL49	Rod	-	+	-	-	-	-	-	-	-	-
50	CWL50	Rod	-	+	-	-	-	-	-	-	-	-

Table 2. Biochemical characterization of *Lactobacillus* isolated from Buffalo Milk sample

S.No	Milk Sample	Strain	Morphology	Gram Reaction	Motility	Growth		Indole	MR	VP	Citrate	Catalase	Nitrate Reduction	Gelatin
						15°C	45°C							
	Buffalo	BFL1	CB	+	-	+	-	-	-	-	-	-	-	-
		BFL2	Rod	+	-	+	-	-	-	-	-	-	-	-
		BFL3	Rod	+	-	+	-	-	-	-	-	-	-	-
		BFL4	Rod	+	-	+	+	-	-	-	-	-	-	-
		BFL5	Rod	+	-	+	+	-	-	-	-	-	-	-
		BFL6	CB	+	-	+	-	-	-	-	-	-	-	-
		BFL7	Rod	+	-	+	-	-	-	-	-	-	-	-
		BFL8	Rod	+	-	+	-	-	-	-	-	-	-	-
		BFL9	Rod	+	-	+	+	-	-	-	-	-	-	-
		BFL10	Rod	+	-	+		-	-	-	-	-	-	-
		BFL11	Rod	+	-	+	+	-	-	-	+	-	-	-
		BFL12	Rod	+	-	+	-	-	-	-	-	-	-	-
		BFL13	CB	+	-	+	-	-	-	-	-	-	-	-
		BFL14	Rod	+	-	+	-	-	-	-	-	-	-	-
		BFL15	Rod	+	-	+	-	-	-	-	-	-	-	-
		BFL16	Rod	+	-	+	-	-	-	-	-	-	-	-
		BFL17	Rod	+	-	+	+	-	-	-	-	-	-	-
		BFL18	CB	+	-	+	+	-	-	-	-	-	-	-
		BFL19	Rod	+	-	+	+	-	-	-	-	-	-	-
		BFL20	Rod	+	-	+	-	-	-	-	-	-	-	-
		BFL 21	Rod	+	-	+	-	-	-	-	-	-	-	-
		BFL 22	Rod	+	-	+	+	-	-	-	-	-	-	-

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Table 3. Biochemical characterization of *Lactobacillus* isolated from Goat Milk sample

S.No	Milk Sample	Strain	Morphology	Gram Reaction	Motility	Growth		Indole	MR	VP	Citrate	Catalase	Nitrate Reduction	Gelatin
						15°C	45°C							
1.	Goat	GAL1	CB	+	-	+	+	-	-	-	-	-	-	-
2.		GAL2	Rod	+	-	+	+	-	-	-	-	-	-	-
3.		GAL3	Rod	+	-	+	+	-	-	-	-	-	-	-
4.		GAL4	Rod	+	-	+	+	-	-	-	-	-	-	-
5.		GAL5	Rod	+	-	+	-	-	-	-	-	-	-	-
6.		GAL6	Rod	+	-	+	-	-	-	-	-	-	-	-
7.		GAL7	Rod	+	-	+	-	-	-	-	-	-	-	-
8.		GAL8	Rod	+	-	+	+	-	-	-	-	-	-	-
9.		GAL9	Rod	+	-	+	+	-	-	-	-	-	-	-
10		GAL10	Rod	+	-	+	-	-	-	-	-	-	-	-
11		GAL11	Rod	+	-	+	-	-	-	-	-	-	-	-
12		GAL12	Rod	+	-	+	-	-	-	-	-	-	-	-
13		GAL13	Rod	+	-	+	+	-	-	-	-	-	-	-
14		GAL14	Rod	+	-	+	+	-	-	-	-	-	-	-
15		GAL15	Rod	+	-	+	+	-	-	-	-	-	-	-
16		GAL16	Rod	+	-	+	-	-	-	-	+	-	-	-
17		GAL17	Rod	+	-	+	-	-	-	-	-	-	-	-
18		GAL18	Rod	+	-	+	-	-	-	-	-	-	-	-
19		GAL19	CB	+	-	+	-	-	-	-	-	-	-	-
20		GAL20	Rod	+	-	+	-	-	-	-	-	-	-	-
21		GAL21	Rod	+	-	+	+	-	-	-	-	-	-	-
22		GAL22	Rod	+	-	+	+	-	-	-	-	-	-	-
23		GAL23	Rod	+	-	+	+	-	-	-	-	-	-	-
24		GAL24	Rod	+	-	+	-	-	-	-	-	-	-	-
25		GAL25	Rod	+	-	+	-	-	-	-	-	-	-	-
26		GAL26	Rod	+	-	+	+	-	-	-	-	-	-	-
27		GAL27	Rod	+	-	+	+	-	-	-	-	-	-	-
28		GAL28	Rod	+	-	+	+	-	-	-	+	-	-	-

Table 4. Phenotypic profile of *Lactobacillus* from Cow milk sample

S. No	Isolates	Arabinose	Cellobiose	Lactose	Mannitol	Melibiose	Salicin	Sorbitol	Sucrose	Raffinose	Trehalose	Probable Identity
1	CWL1	-	+	+	-	+	-	-	+	+	+	*Lb.fermentum*
2	CWL2	-	+	-	+	-	+	+	+	-	+	*Lb.casei*
3	CWL3	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
4	CWL4	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
5	CWL5	-	+	+	-	+	-	-	+	+	+	*Lb.fermentum*
6	CWL6	-	+	+	+	+	+	+	+	+	+	*Lb.plantarum*
7	CWL7	-	+	+	-	+	-	-	+	+	+	*Lb.fermentum*
8	CWL8	-	+	-	+	-	+	+	+	-	+	*Lb.casei*
9	CWL9	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
10	CWL10	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
11	CWL11	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
12	CWL12	-	+	+	-	+	-	-	+	+	+	*Lb.fermentum*
13	CWL13	-	+	+	-	+	-	-	+	+	+	*Lb.fermentum*
14	CWL14	-	+	-	+	-	+	+	+	-	+	*Lb.casei*
15	CWL15	-	+	+	-	+	-	-	+	+	+	*Lb.fermentum*
16	CWL16	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
17	CWL17	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
18	CWL18	-	+	+	-	+	-	-	+	+	+	*Lb.fermentum*
19	CWL19	-	+	+	-	+	-	-	+	+	+	*Lb.fermentum*
20	CWL20	-	+	-	+	-	+	+	+	-	+	*Lb.casei*
21	CWL21	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
22	CWL22	-	+	+	-	+	-	-	+	+	+	*Lb.fermentum*
23	CWL23	-	+	-	+	-	+	+	+	-	+	*Lb.casei*
24	CWL24	-	+	+	-	+	-	-	+	+	+	*Lb.fermentum*
25	CWL25	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
26	CWL26	-	+	-	+	-	+	+	+	-	+	*Lb.casei*
27	CWL27	-	+	-	+	-	+	+	+	-	+	*Lb.casei*
28	CWL28	-	+	-	+	-	+	+	+	-	+	*Lb.casei*
30	CWL29	-	+	-	+	-	+	+	+	-	+	*Lb.casei*
31	CWL30	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
32	CWL31	-	+	+	-	+	-	-	+	+	+	*Lb.fermentum*
33	CWL32	-	+	+	-	+	-	-	+	+	+	*Lb.fermentum*
34	CWL33	-	+	+	-	+	-	-	+	+	+	*Lb.fermentum*
35	CWL34	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
36	CWL35	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
37	CWL36	-	+	-	+	-	+	+	+	-	+	*Lb.casei*
38	CWL37	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
39	CWL39	-	+	-	+	-	+	+	+	-	+	*Lb.casei*
40	CWL40	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
41	CWL41	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
42	CWL42	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
43	CWL43	-	+	-	+	-	+	+	+	-	+	*Lb.casei*
44	CWL44	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
45	CWL45	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
46	CWL46	-	+	-	+	-	+	+	+	-	+	*Lb.casei*
47	CWL47	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
48	CWL48	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
49	CWL49	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
50	CWL50	-	+	-	+	-	+	+	+	-	+	*Lb.casei*

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Table 5. Phenotypic profile of *Lactobacillus* from Buffalo milk sample

S No.	Isolates	Ara-binose	Cellobiose	Lactose	Mannitol	Melibiose	Salicin	Sorbitol	Sucrose	Raffinose	Trehalose	Probable Identity
1	BFL1	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
2	BFL2	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
3	BFL3	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
4	BFL4	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
5	BFL5	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
6	BFL6	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
7	BFL7	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
8	BFL8	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
9	BFL9	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
10	BFL10	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
11	BFL11	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
12	BFL12	+	-	-	+	-	+	-	+	+	-	*Lb.brevis*
13	BFL13	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
14	BFL14	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
15	BFL15	+	-	-	+	-	+	-	+	+	-	*Lb.brevis*
16	BFL16	+	-	-	+	-	+	-	+	+	-	*Lb.brevis*
17	BFL17	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
18	BFL18	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
19	BFL19	+	-	-	+	-	+	-	+	+	-	*Lb.brevis*
20	BFL20	+	-	-	+	-	+	-	+	+	-	*Lb.brevis*
21	BFL 21	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
22	BFL 22	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*

Table 6. Phenotypic profile of *Lactobacillus* from Goat milk sample

S No.	Isolates	Ara-binose	Cellobiose	Lactose	Mannitol	Melibiose	Salicin	Sorbitol	Sucrose	Raffinose	Trehalose	Probable Identity
23	GAL1	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
24	GAL2	-	+	+	-	+	-	-	+	+	+	*Lb.fermentum*
25	GAL3	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
26	GAL4	-	+	-	+	-	+	+	+	-	+	*Lb.casei*
27	GAL5	-	+	-	+	-	+	+	+	-	+	*Lb.casei*
28	GAL6	-	+	-	-	-	-	-	+	-	+	*Lb.delbrueckii*
30	GAL7	-	+	-	-	-	-	-	+	-	+	*Lb.delbrueckii*
31	GAL8	-	+	-	-	-	-	-	+	-	+	*Lb.delbrueckii*
32	GAL9	-	+	+	-	+	-	-	+	+	+	*Lb.fermentum*
33	GAL10	-	+	+	-	+	-	-	+	+	+	*Lb.fermentum*
34	GAL11	-	+	+	-	+	-	-	+	+	+	*Lb.fermentum*
35	GAL12	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
36	GAL13	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
37	GAL14	-	+	-	+	-	+	+	+	-	+	*Lb.casei*
38	GAL15	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
39	GAL16	-	+	-	+	-	+	+	+	-	+	*Lb.casei*
40	GAL17	-	+	-	-	-	-	-	+	-	+	*Lb.delbrueckii*
41	GAL18	-	+	-	-	-	-	-	+	-	+	*Lb.delbrueckii*
42	GAL19	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
43	GAL20	-	+	-	+	-	+	+	+	-	+	*Lb.casei*
44	GAL21	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
45	GAL22	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
46	GAL23	-	+	-	-	-	-	-	+	-	+	*Lb.delbrueckii*
47	GAL24	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
48	GAL25	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
49	GAL26	-	+	+	-	-	+	+	+	-	+	*Lb.lactis*
50	GAL27	-	+	-	-	-	-	-	+	-	+	*Lb.delbrueckii*

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Table 7. Effect of Ten Bacteriocins on the growth of bacteria on Agar plates

Indicator Strain		**Bacteriocin Producing *Lactobacillus* strains**								
	CWL1	**CWL6**	**CWL17**	**CWL25**	**CWL29**	**BFL1**	**BFL2**	**GAL1**	**GAL2**	**GAL3**
Lactobacillus acidophilus (MTCC 447)	VSI	VSI	VSI	VSI	VSI	VSI	VSI	VSI	VSI	VSI
Lactobacillus delbrueckii subsp. *lactis* (MTCC 911)	VSI	SI	VSI	VSI	SI	VSI	SI	SI	VSI	SI
Lactococcus lactis subsp.lactis (MTCC 440)	SI	VSI	SI	VSI	SI	VSI	VSI	SI	VSI	SI
Bacillus amyloliquifaciens (MTCC 1270)	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
Bacillus cereus (MTCC 1272)	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
Bacillus mycoides (MTCC 645*)*	SI	SI	SI	SI	SI	VSI	SI	SI	VSI	SI
Klebsiella pnuemoniae (MTCC 3384)	NI	SI	SI	SI	SI	VSI	NI	VSI	NI	NI
Staphylococcus aureus (MTCC 740)	VSI	SI	VSI	VSI	VSI	VSI	MI	VSI	MI	MI
Streptococcus faecalis (MTCC 459)	SI	VSI	VSI	VSI	VSI	VSI	SI	VSI	VSI	MI
Proteus vulgaris (MTCC 744)	VSI	SI	SI	SI	SI	VSI	VSI	VSI	VSI	VSI
Psuedomonas aeruginosa (MTCCC 647)	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
Salmonella typhi (MTCC 531)	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI

*CWL-Cow milk,Lactobacilli, BFL-Buffalo milk Lactobacilli, GAL- Goat milk Lactobacilli

Degree of inhibition: MI = Moderate inhibition Zone (6-9mm), SI = Strong inhibition Zone (10-14mm), VSI =very strong inhibition Zone (15-18mm), NI =No inhibition zone.

Table 8. Effects of chloroform, pH and heat treatment on bacteriocin of lactobacilli isolated from raw milk.

Bacteriocin producing *Lactobacillus* strains	**Sensitivity to chloroform**	**Resistance to heating (10 min) Temperature (°C)**				**Resistance to boiling 100°C (min)**			**Sensitivity to different pH values**			
		60	**70**	**80**	**90**	**10**	**30**	**60**	**3.0**	**4.5**	**7.0**	**9.0**
CWL1	R	R	R	R	R	R	S	S	R	R	R	S
CWL6	R	R	R	R	R	R	S	S	R	R	R	S
CWL17	R	R	R	R	R	R	S	S	R	R	R	S
CWL25	S	S	S	S	S	S	S	S	S	R	R	S
CWL29	R	R	S	S	S	R	S	S	R	R	R	S
BFLI	R	R	R	R	R	R	S	S	R	R	R	S
BFL2	R	R	R	R	R	R	S	S	S	R	R	S
GAL1	R	R	R	R	R	R	S	S	R	R	R	S
GAL2	S	S	S	S	S	S	S	S	S	R	R	S
GAL3	R	R	R	R	R	R	S	S	S	R	R	S

*CWL-Cow milk, Lactobacilli, BFL-Buffalo milk Lactobacilli, GAL- Goat milk Lactobacilli

R= Resistance = Un inhibited bacteriocin activity, S= Sensitive = Inhibited bacteriocin activity

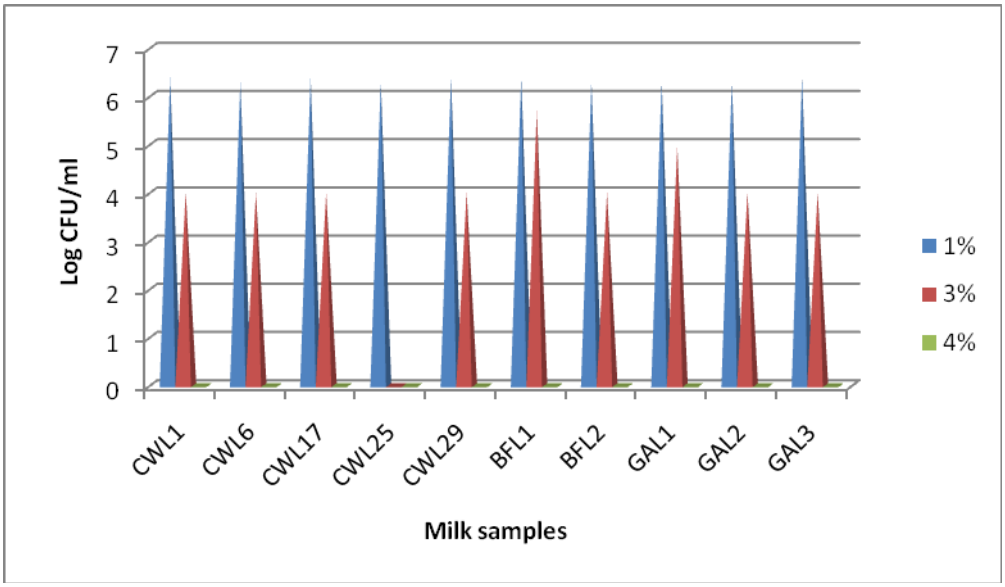

Figure 1. Effect of NaCl concentration on Bacteriocin production

ISSN 1916-9671 E-ISSN 1916-968X

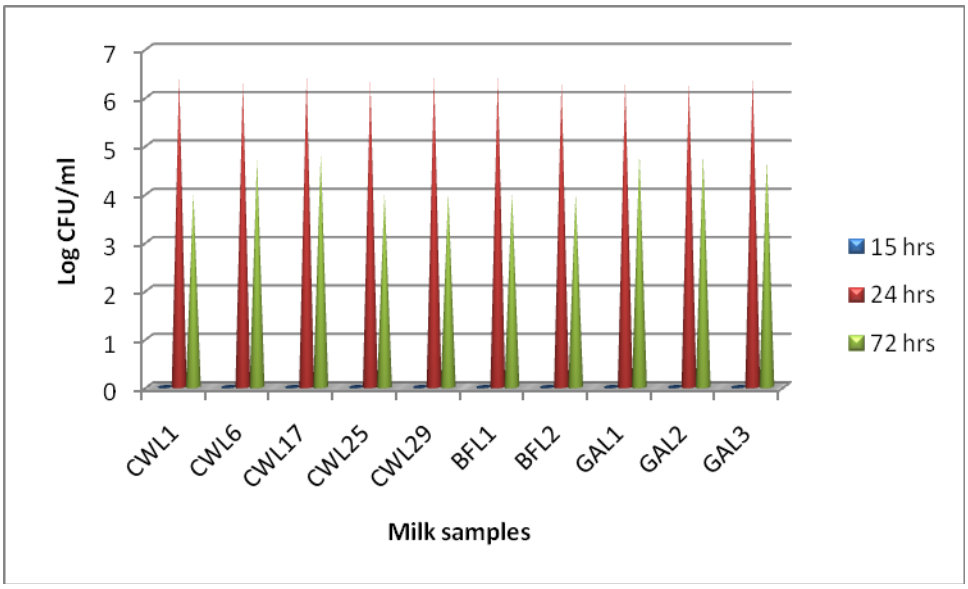

Figure 2. Effect of incubation period (37^{o}C) on Bacteriocin production

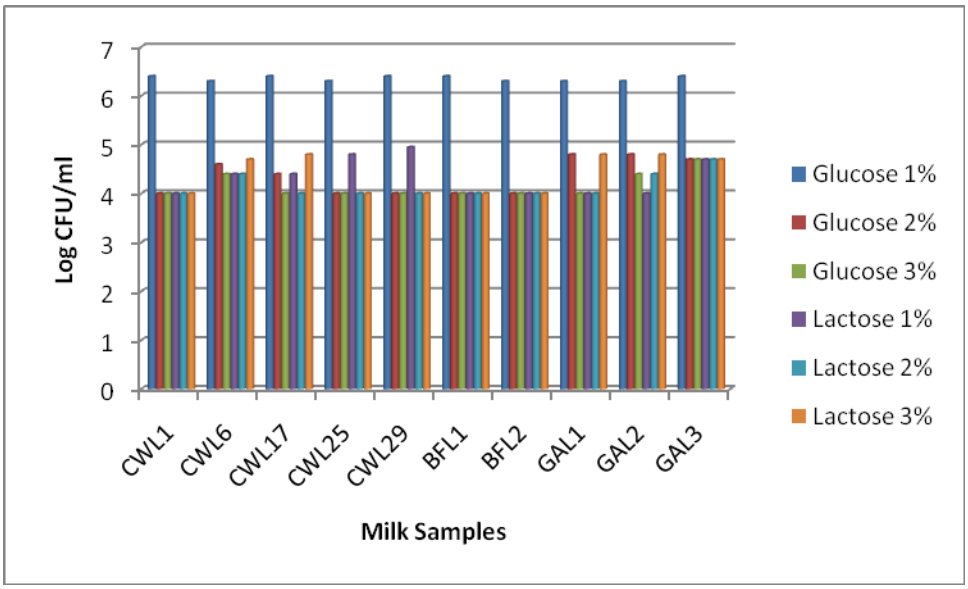

Figure 3. Effect of carbon sources on Bacteriocin production