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Screening and characterization of bacteriocin producing lactic acid bacteria as probiotic from cow and buffalo milk

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Abstract
Bacteriocins are natural peptides secreted by many varieties of bacteria for the purpose of killing other bacteria. This provides them with a competitive advantage in their environment, eliminating competitors to gain resources. In this study, a total of 51 bacterial strains were isolated from the 65 milk samples different regions, Dharmapuri district. 28 isolates were isolated from buffalo milk samples and 23 isolates were isolated from cow milk samples.C-1, B-4, B-13 and B-14, which had the highest activity to both indicators, were inoculated over it and incubated for 24 hours at 30 ºC. The production of lactobacilli C-1, B-4, used in optimized in different concentration of lactose, different pH, different temperature. The weight of the protein bands was approximately 97kDa to below 14.3 kDa by using SDS Page electrophoresis.

Keywords: Bacteriocins, *Lactobacillus*, buffalo milk, cow milk, antibacterial activity, probiotic *Lactobacillus*

1. Introduction
Bacteriocins are ribosomally-synthesized peptides or proteins produced by bacteria that kill or inhibit the growth of other bacteria. Many lactic acid bacteria (LAB) produce bacteriocins with rather broad spectra of inhibition. These Bacteriocins- are produced by LAB found in numerous fermented and non-fermented foods in the food industry. To reduce the addition of chemical preservatives, the intensity of heat treatments, resulting in foods which are more naturally preserved and richer organoleptic and nutritional properties and also to develop "NOVAL" food products (E.g. less acidic or with a lower salt content) (Antonio Galvesz *et al.*, 2007). Bacteriocins act on target cell membranes resulting pore formation, leads to a rapid efflux of small cytoplasmic molecules, ions from the target cells and the collapse of the proton motive Force (PMF), leading to the cell death. Due to the different modes of antibacterial action, these antibacterial peptides (ABP) circumvent the problems related to the development of antibiotic resistance (Kruszewska *et al.*, 2004).
Bacteriocins are natural peptides secreted by many varieties of bacteria for the purpose of killing other bacteria. This provides them with a competitive advantage in their environment, eliminating competitors to gain resources. These peptides are ribosomally synthesized, although some are extensively post-translationally modified (Sivaramasamy Elayaraja *et al.*, 2014).
Environmental pathogens are found in the immediate surroundings of the cow, such as the sawdust and bedding of housed cows, the manure of cattle and the soil. Bacteria include *streptococcal* strains other than *S. agalactiae*, such as *Streptococcus dysgalactiae*, *Streptococcus uberis* and *Streptococcus bovis*, *Enterococcus faecium* and *Enterococcus faecalis* and coli forms such as *Escherichia coli*, *Klebsiella pneumonia* and *Enterobacter aerogenes.* Mastitis caused by environmental organisms is essentially opportunistic in nature and becomes established if the immune system of the host is compromised or if sanitation and hygiene is not adequately practiced (Renee Pieterse *et al*., 2010).
These proteins are also considered to be a possible treatment for cancer. It has shown promise as an agent for diagnosis of cancer. However, whether this protein can be used for treatment is dependent on experiments. The doubt about therapeutic capabilities of this protein remains due to lack of clarity about the Bacteriocin mechanism of action and the supposition that they are incapable of destroying tumor cells in mammals (Duche *et al.*, 2007).
Since lactic acid bacteria are naturally found in food, they have drawn particular attention for their potential in the production of antibacterial substances for food preservation. Nisin, the first lactic acid bacteria bacteriocin, is now approved for use in over 40 countries and has been

used as a food preservative for over 50 years. In recent years, several other antimicrobial substances produced by lactic acid bacteria have also been reported. Nevertheless, more research efforts are needed to develop novel natural antimicrobial substances with broad antimicrobial spectrum and improved yield (Jianyin Miao *et al*, 2015) [16].

2. Materials and Methods

2.1 Collection of Milk Samples

A total of 65 milk samples were collected in sterilized screw capped bottles from Cow and Buffalo indifferent regions of Dharmapuri surroundings. Cow milk looking like yellow color water form and buffalo milk looking like pure white color thick in nature.

2.2 Isolation of Lactic Acid Bacteria

Isolation of lactic acid bacteria from collected milk samples by Serial dilution. 1ml of milk sample was serially diluted separately in 9ml of sterile distilled water up to10^{-1} to 10^{-7}. The serially diluted samples were placed on MRS medium by using spread plate method. The Plates were incubated for 24hrs at 37 ºC. Based colony morphology and microscopic observation the isolates were purified and maintained on MRS and nutrient agar slant for further use (Khedida *et al*., 2009) [18].

2.3 Identification of *Lactobacillus*

The isolated bacterial strains were identified based on the morphological and biochemical characterization. The biochemical characterizations were performed by various tests like Indole, Methyl Red, Voges Proskauer, Triple Sugar Iron, Citrate Utilization, Catalase and Oxidase test.

2.4 Screening of Bacteriocin Dot Plate Methods

MHA agar plates were prepared, *E. coli* ATCC 25922 cultures (48hrs) was swab in agar medium, the isolated *Lactobacilli* ssp. was inoculated over it and incubated for 24hrs at 30 ºC, The Lactobacilli producing zone of inhibition indicates the production of bacteriocin, Measure the zone of inhibition result.

2.5 Antimicrobial Activity assay

The lactobacillus showing antimicrobial activity are selected and grown in nutrient broth medium incubate 48 hrs. After growth the culture medium was centrifuged and cell free supernatant was taken, Maintain the PH 1N NAOH were prepared added with supernatant and mixed well inoculated in the agar well method, MHA was prepared and the test *Staphylococcus aureus* and *E. coli* ATCC 25922 was swab over it, A hole was made with 6 to 8 mm diameter using sterile cork-borer and 20-100µl Lactobacilli added, Incubated at 37 ºC, and 24-48hrs, The size of zone of inhibition was measured and compared with standard chart.

2.6 Probiotic Characterization of bacteriocin

2.6.1 Tolerance to NaCl

The isolates were tested for tolerance to NaCl concentrations between 3%, 4% and 5% according to the method. To this 10 ml MRS broth, 1ml of culture aliquot was inoculated. Samples were retrieved every 1 h time interval for 3h. Total viable counts were determined by standard plate count in MRS agar and incubated aerobically at 37 ºC for 24hrs. The result was expressed as log CFU/ml.

2.6.2 Acid Tolerance

Acid tolerance of selected strain was evaluated under acidic conditions. The MRS broth was adjusted to pH 2, 3, 4 and 7 with 3 M hydrochloric acid and sterilized (121 ºC, 20 min). To this 10ml MRS broth, 1 ml of culture aliquot was inoculated. Samples were retrieved every 1 h time interval for 3h. Total viable counts were determined by standard plate count in MRS agar and incubated aerobically at 37 ºC for 24 hrs. The result was expressed as log CFU/ml.

2.6.3 Gas Production

The gas production from glucose, lactose, fructose, maltose, MRS broth with 1% was used and added to Durham tubes according to the method of inoculated stain culture were incubated 37 ºC for 24 hrs.

2.7 Screening of Virulence

2.7.1 Coagulates test

The coagulates test, 0.3 mL of each C-1, B-4,B-13,AND B-14 culture of isolates was transferred to sterile tubes containing 0.3 mL of rabbit plasma (plasma-Coagula Labor Clin) and incubated at 36 °C ± 1 °C for 6 h. The formation of a large and organized clot or total coagulation was considered a positive result for the test.

2.7.2 Hemolytic Test

The LAB isolates were cultured in MRS broth at 37 ºC for 15 hrs and then transferred onto blood agar (Himedia) plates supplemented with 5% defibrinated whole horse blood (Oxide). After 48/72 h, the hemolytic reaction was evaluated by observing both the partial hydrolysis of red blood cells and the production of a green zone (α-hemolytic), as well as the total hydrolysis of red blood cells producing a clear zone around bacterial colony (β-hemolytic) or no reaction (γ-hemolytic).

2.7.3 Antibiotic Susceptibility Test

The antibiotic susceptibility against clinically important antibiotics, such as, Ampicillin (10 µg), Ceftazidime (30 µg), Chlorampheni-col (30 µg), Amikacin (30 µg), Tetracycline (30 µg), and Ciprofloxacin (5 µg).The LAB strains were grown in MRS broth and incubated at 37 ºC for 24 h turbidity standard. A swab was used to spread the inoculums across the surface of Muller Hinton agar, and then antibiotic disks were applied to the plate. Antimicrobial susceptibility was assessed by measuring the zone of inhibition of bacterial growth after incubation for 24h at 37 ºC. *Lactobacillus* sp. were used for quality control testing.

2.8 Optimization of Carbon source for bacteriocin Production

The MRS broth contains peptone, beef extract, yeast extract, glucose, polysorbate 80, ammonium citrate, sodium acetate, magnesium sulfate, manganese sulfate, and Dipotassium phosphate. For selection of the best carbon source, some carbon sources were tested individually: lactose. Carbon source was added at 0.5%, 1%, 1.5%, 2%, 2.5% (w/v) of the basal MRS medium, replacing the 2% glucose. After inoculation of *Lactobacillus* sp. (1% of inoculums), incubation was performed at 20 ºC, 30 ºC, 37 ºC, 45 ºC, 50 ºC. During the 24 to 48 h of growth cycle, samples were taken at every 24h intervals and measured pH, and optical density (OD) under 600 nm wavelength using spectrophotometer. The antimicrobial activity was also measured by using agar well

diffusion test. Ten mL of cultures were aseptically taken and centrifuged (10,000 g, 15 min at 4 ºC,). The pH of supernatant was adjusted to 6.5 and filtered through 0.22 µl-pore size acetate filter. Cell-free supernatant was assayed using serial 2-fold dilution. MHA was prepared and the test *Staph aureus* and *E. coli ATCC* 25922 was swab over it, a hole was made with 6 to 8 mm diameter using sterile cork-borer and 20-100µl supernatant added; Incubated at 37 ºC, and 24-48hrs, The size of zone of inhibition was measured and compared with standard chart (Mi-Hee Kim *et al.,* 2006).

2.9 Molecular Characterization of Bacteriocin SDS-PAGE analysis

The test sample was prepared by diluting (1:1) in the sample solubilizing buffer and the samples were spun for 1min. (This step does not apply to the protein marker) and medium range protein marker (Genei, Bangalore) was used. The separating gel solution was gently mixed and poured into the glass plate using a pipette up to a level of about 3cm from the top. The gel was allowed to stand for 15 to 20 minutes to polymerize. The stacking gel solution was gently mixed and poured the solution into the glass plate using a pipette, and then the comb was gently inserted between gel plates without air bubbles. The gel was allowed to stand for 10 min to polymerize. The glass plate was fixed with the gel tank tightly with clips on both sides. The upper and lower chamber was filled with running buffer until marked buffer level and the comb were gently removed from the gel. The 40µl of prepared whole cell protein sample were loaded into each well using micropipette. The gel was run at 100V until the tracking dye reached approximately 0.5cm from the bottom of the gel. After electrophoresis, the gel was stained with 0.25% coo massive, brilliant blue for 30 minutes. The stained gel was treated with a destaining solution for two times with 15 minutes interval. The stained gels were placed on white Transilluminator observe the bands and make photocopies.

3. Results

3.1 Isolation of bacteria from cow milk and buffalo milk

In this study, a total of 51 bacterial strains were isolated from the 65 milk samples different regions, Dharmapuri district. 28 isolates were isolated from buffalo milk samples and 23 isolates were isolated from cow milk samples (Table 1, Table 2).

3.2 Identification of *Lactobacillus*

In gram staining, identify 15 bacterial stain cocci positive, 10 bacterial stain short rods gram positive. 14 bacterial strains were isolated from cow milk and 12 bacterial strains were isolated from buffalo milk samples based on the colony morphology the selected strain totally 24 was identified as *Lactobacillus* taken by further study (Table 3).

Each isolate was activated in 5 ml MRS broth for 24 h at 30 ºC before use. Therefore, overnight cultures were used during all the identification procedures. Physiological and biochemical identifications were performed according to the standard methods.

3.3 Antibacterial activity of LAB

From 26 isolates of milk samples were tested for the antimicrobial activity against the clinical pathogens (*E. coli ATCC 25922* and *staphylococcus aureus*) using the dot-plate method, C-1, B-4, B-13 and B-14, which had the highest activity to both indicators, were inoculated over it and incubated for 24hurs at 30 ºC (Table 4).

3.4 Agar well diffusion method

From a total of 26 isolates, 4 bacterial isolates showed antimicrobial activity against the clinical pathogens *E. coli* ATCC 25922 and *Staphylococcus aureus*. Among the 4 isolates bacterial stain B-4, B-13, B-14 and C-1. The zone of inhibition produced against the selected pathogenic bacteria by the culture filtrate of the isolates after incubating them at 37 °C was considered for antimicrobial activity (Table 5).

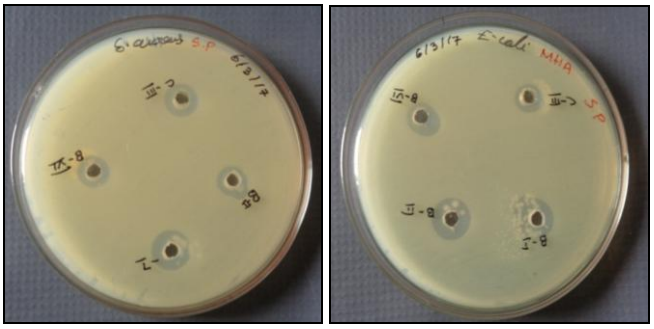


Table 5: Agar well diffusion method

S. No	Strain No	*E. coli*	*Staphylococcus aureus*
1	Buffalo-4	14mm	14mm
2	Buffalo-13	14mm	10mm
3	Buffalo-14	13mm	1mm
4	Cow -1	9mm	12mm

3.5 Probiotic Characterization of bacteriocin producing bacteria

3.5.1 Acid Tolerance

Two main important potential characteristics of probiotics are ability to with stand at acidic condition of the stomach and acids secreted in the upper intestine. At pH2 and 4which leads to the destruction of the microbes C-1, B-4, B-13 and B-14 showed better tolerance and survived well at pH 2 and pH 4 of 3hrs exposure. Moderate survival was observed at pH 2.5 with the log reduction of 4 logs CFU/ml after 3 h of exposure. *Lactobacillus* food isolate showed maximum survival at pH 2rather than at 7 shows in results.

3.5.2 NaCl Tolerance

The 4 isolated probiotic bacteria were capable to grow optimally at 37 °C. C-1, B-4, B-13 and B-14 were capable to grow at 3% NaCl concentration at 3-5% NaCl concentration. *Lactobacillus* strains from river buffalo milk, cow milk, which as well found to tolerate 3-5% NaCl. All LAB isolates were tolerant to the NaCl concentration used in this study. For industrial application of LAB as starter cultures in raw milk, these microorganisms must be capable of tolerating stressful conditions such as acidity, temperature, salt stress and freeze-drying. To tolerate NaCl, various mechanisms developed by LAB are described, for example the uptake or synthesis of a limited number of solutes.

3.5.3 Gas Production

The 4 isolated bacterial strains were used to sugar fermentation test. Phenol red broth base medium was used as an indicator to differentiate the bacteria according to their patterns of carbohydrate utilization. Among the bacterial isolates (B-4, B-13, B-14 and C-1) were ferment the different sugars like sucrose, lactose and glucose. The carbohydrate fermentation was confirmed to the color change like red to yellow and also observed gas production.

3.6 Screening of virulence

1. Coagulates test

In the characterization of virulence factors of microorganisms, all tested strains were negative for coagulates.

2. Hemolytic activity

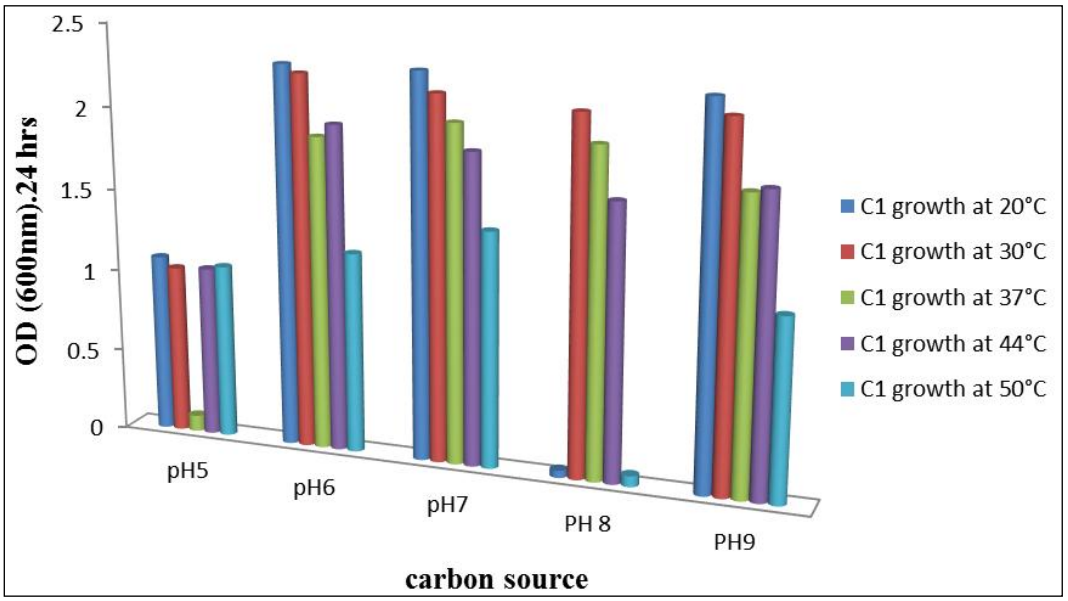
The isolate LAB did not exhibited any effect (γ-hemolysis) and (β-hemolysis). In the blood agar, the LAB isolates were produced green color colony and (α-hemolysis) after 48hrs incubation in blood agar plates.

3. Antibiotic Susceptibility Test

In the antimicrobial susceptibility of strains was high. The 4 antimicrobials tested, 4 isolates were resistant to six antibiotics (54%), 2 were five antibiotics (18%), 6 were susceptible to four antibiotics (12%) and 6 were susceptible to three antibiotics (12%). Lower sensitivity was presented by isolates for antimicrobial, where 4 isolates were resistant. 4 isolates were susceptible to chlorampheni-col. According to, LAB strains are generally susceptible to this antimicrobial. The investigation of the resistance pattern of LAB isolates candidates for probiotic use is essential. Bacteria may serve as hosts of antibiotic resistance genes, which can be transferred to pathogenic bacteria (Table 6).

3.7 Optimization of Carbon source for bacteriocin Production

The effect of the carbon source on cell growth and bacteriocin production was determined using MRS medium supplemented with different carbon sources in replace lactose. For selection of the best carbon source, some carbon sources were tested individually: lactose. Carbon source was added at 0.5%, 1%, 1.5%, 2%, 2.5% (w/v) of the basal MRS medium, replacing the 2% lactose incubation was performed at 20 ºC, 30 ºC, 37 ºC, 45 ºC, and 50 ºC. During the 24 to 48 and 72hrs of growth cycle, samples were taken at every 24 h intervals and measured pH, and optical density (OD) under 600 nm wavelength using spectrophotometer show in the (graph 9, 10). The antimicrobial activity was also measured (Table 7, 8). Among the isolates B4, strain from buffalo milk sample was high amount of bacteriocin production in pH 7, temperature 45 ºC at 2% of lactose concentration.



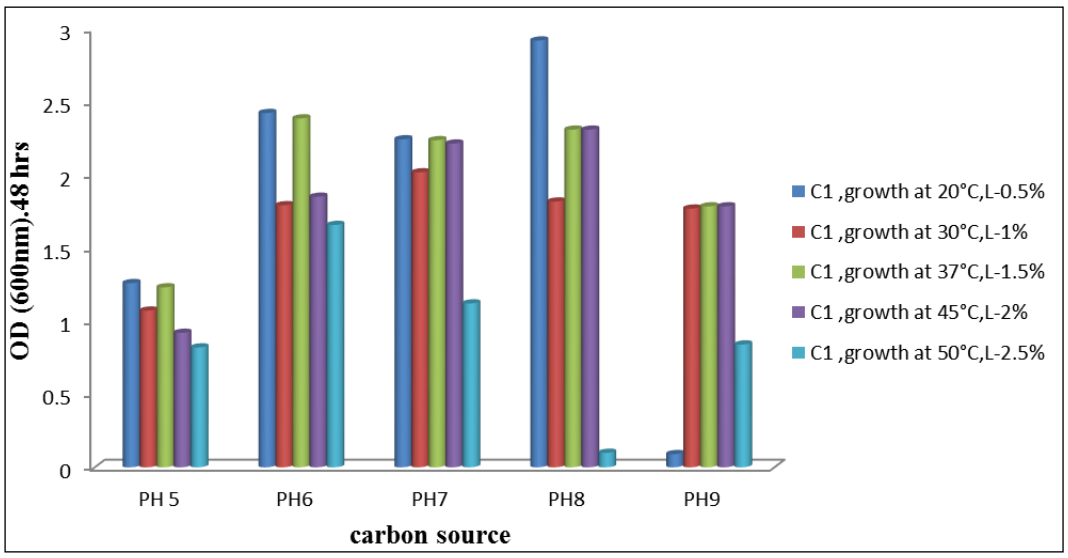
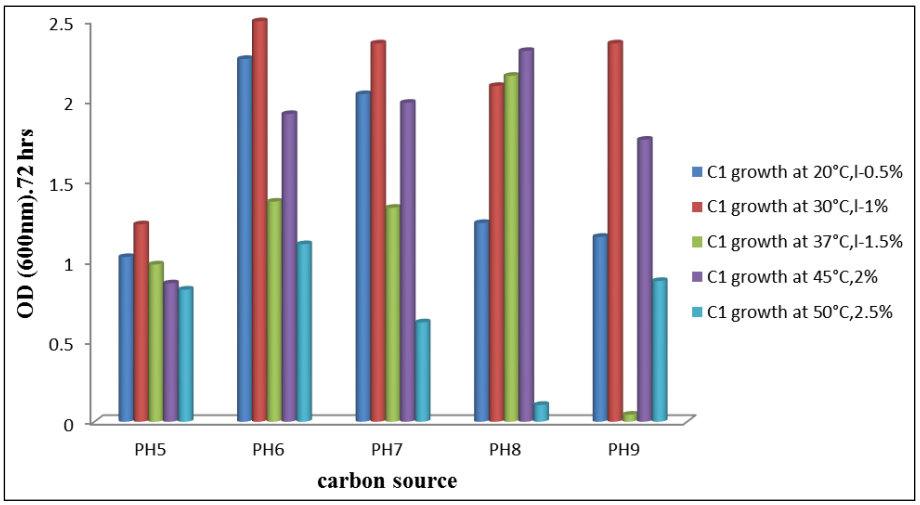
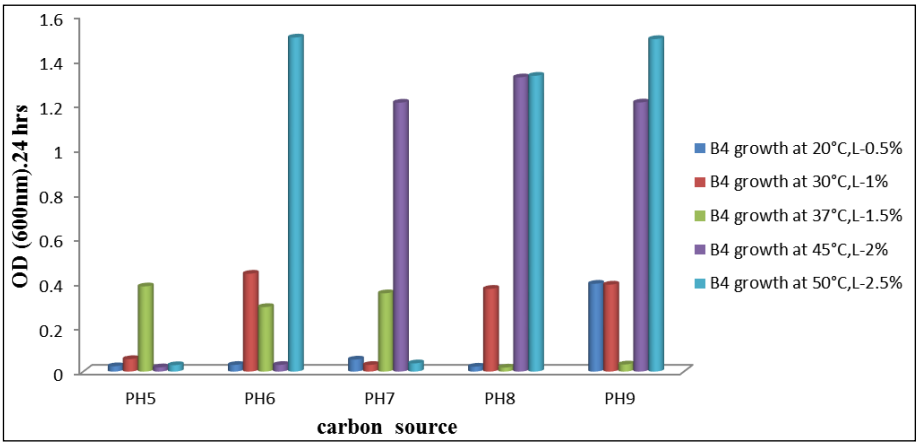
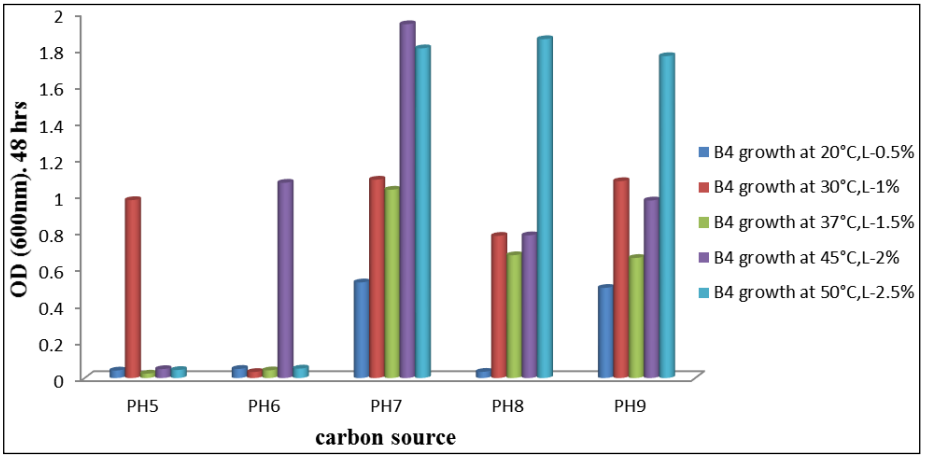


Table 9: Effects of Carbon Sources on c-1







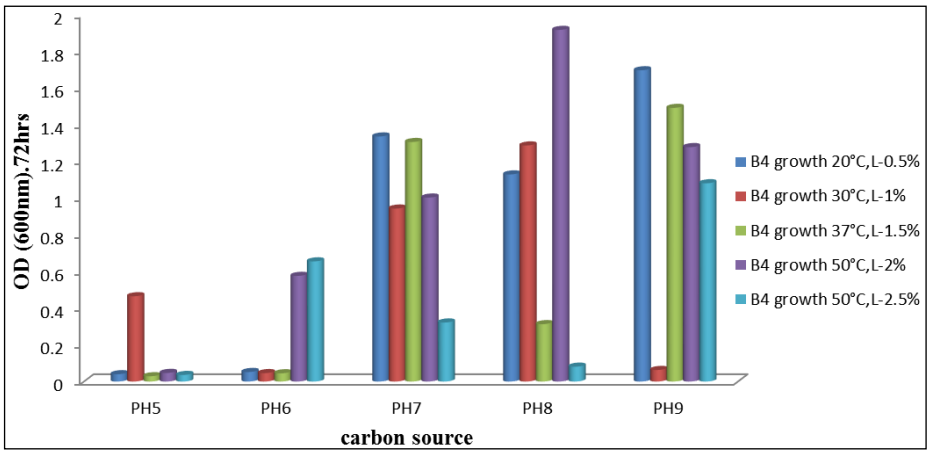


Table 10: Effect of carbon sources on B-4

3.8 Purification of Bacteriocin

The molecular characterization of the bacteriocin was analysis by SDS PAGE method. After de stained the gel, number of protein bands were observed, protein band was approximately 97kd to below 14.3 kDa. The weight of the protein bands was determinate by using standard protein marker. In this study, four isolates producing bacteriocin were subjected to PAGE analysis.

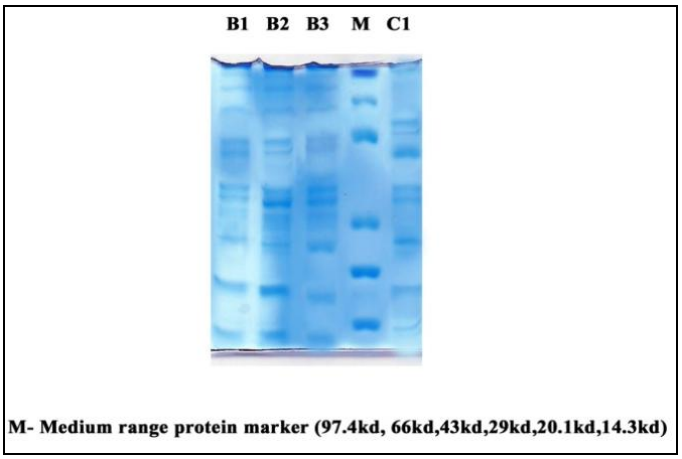


Fig 8: Molecular characterization of Bacteriocin

4. Discussion

In our study 51 isolates were isolated from 65 milk samples both cow and buffalo. 14 as *Lactobacilli* from cow milk and 12 from buffalo milk samples. Axel's son, 1993 also cultivated LAB. LAB isolates were obtained from the 127 collected samples of cow (n = 80), buffalo (n = 35) and goat (n = 12) milk, and from the 29 samples of cow (n = 17), buffalo (n = 5) and goat (n = 7) cheeses. Also, Gram-positive cocci and rods were isolated from 28 out of 29 cheese samples (96.5%). Together, a total of 815 colonies were obtained from MRS and LAMVAB agar plates, and most of them were Gram positive and catalase negative isolates, a presumptive identification for LAB. In my study all LAB isolates are also Gram positive and catalase negative isolates.

24 isolates of milk samples were tested for the antimicrobial activity against the clinical pathogens (*E. coli ATCC 25922* and *staphylococcus aureus*). B-4, B-13, B-14 and C-1 strains have showed the antibacterial activity against *E. coli* ATCC 25922 and *Staphylococcus aureus* in Dot plate assay and Agar well diffusion methods. Kunzes *et al.*, 2016 delivered same results of our study. Isolate 40 showed an evident zone of inhibition (>5 mm) against B. cereus, S. aureus and S. dysenteriae whereas, isolate 11 exhibited inhibitory activity towards S. aureus and S. dysenteriae (5 mm zone of inhibition). Isolates 12, 20, 29, 38 and 55 were found to have antimicrobial activity against B. cereus and L. monocytogenes, although their inhibitory extents were variable. In addition, a few LAB isolates were observed to have weak to medium antimicrobial against E. coli (isolates 30, 45, 52 and 75) and S. aureus (isolates 11, 40 and 68).

In Kunzes *et al.*, 2016 Most of the LAB isolates were found to be susceptible to all However, none of the isolates showed any antimicrobial activity against Pseudomonas sp. tested antibiotics such as Penicillin G, Clindamycin, Co-trimoxazole, Erythromycin and Ampicillin except Vancomycin. Isolates 11, 20 and40 were moderately resistant to penicillin G and isolates 20, 52, 55and 63 toward Ampicillin. Our results were not similar to previous results; B-4, B-13, B-14 and C-1 were showing resistant to all antibiotics except Chlorampheni-col.

Lactobacillus casei against different pH values was checked in Deshpande *et al.*, 2014. They were exposed to gastric juice of pH 2, 3 and 4for 0min,30min, 60min and 90min.pH4 for 90min showed significant results in terms of no. of colonies. This indicates that *Lactobacillus* can survive in upper intestine at lowest pH for at least 90min. The same results were obtained in our study, B-4, B-13, B-14 and C-1 can survive in low pH 2 and 4. These isolates also showed higher activity in 1% Lactose and 40 ^{o}C.

Optimization of C-1, B-4 was done for the production of bacteriocin; B-4 was producing good quality of compound in 37 ^{o}C, pH 9 and 1% of carbon source against *E. coli* (ATCC25922) and *Staphylococcus aureus.* C-1 was producing compound in 30 ^{o}C, pH 7 and 1% of carbon source. In Mona *et al*., 2017. Optimization of bacteriocin production by Lb. plantarum PM4 was studied in MRS broth using temperature, pH and length of incubation period (time) as variables. Accordingly, the best combination of incubation conditions for production of the inhibitory activity by *L. plantarum* M4 appears to be at pH 5.5 for 48 h whether incubated at 25 ˚C, 30 ˚C or37 ˚C.

The results of Ravi Sankar *et al.*, 2012 the purification procedure were summarized in table 1. After final purification step, the bacteriocin was purified 13.5-fold with a recovery of 21.3%. The purified bacteriocin appeared as a single band in SDS-PAGE with molecular weight approximately 9.5 kDa. In our study B-4, B-13, B-14 and C-1 are producing clear bands in the gel in same molecular weight between 20.1 kDa to 14.3 kDa.

5. Conclusion

Results of this study indicated that the LAB strains isolated from Cow and Buffalo milk exhibited promising antimicrobial activity against both Gram positive and Gram negative bacteria, and could be used as a starter culture in the processing of milk as well as bio-preservative, in addition to good manufacturing practices, to the inhibition of food pathogens such as *E. coli* and *S. aureus* and can ensure safe and improved product quality. Bacteriocin-producing lactic acid bacteria isolated and characterized in our study extend the number of available cultures, and probably the number of available bacteriocins, offering a useful protection against eventual contamination of milk or curd with pathogenic or spoilage microorganisms.

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