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Antibacterial Activity and Optimisation of Bacteriocin Producing Lactic Acid Bacteria Isolated from Beef (Red Meat) Samples

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Abstract. Bacteriocin producing bacteria are commonly found in meat products to enhance their shelf-life. In the present study, bacterial species were isolated from meat samples (beef) from different localities of Lahore, Pakistan. MRS agar medium was used to isolate lactic acid bacteria (LAB) through spread and streak methods (incubated for 72 h at 37 °C). Identification of bacteriocinogenic LAB strains was done by using staining techniques, morphology based characteristics and biochemical tests. These strains were BSH 1b, BSH 3a, BIP 4a, BIP 3a, BIP 1b and BRR 3a. Antibacterial activity of LAB was performed against food borne pathogens *viz*., *Escherichia coli* and *Staphylococcus aureus* through paper disc diffusion method. Three bacterial strains showed maximum inhibition and characterised by ribotyping *viz*., BIP 4a was identified as *Lactobacillus curvatus*, BIP 3a was *Staphylococcus warneri* and BIP 1b was *Lactobacillus graminis*. Optimum pH 5-6.5 and 30-37 °C temperature for isolated bacterial strains was recorded. Protein concentration measured was 0.07 mg/mL for BSH 1b, 0.065 mg/mL for BSH 3a, 0.057 mg/mL for BIP 4a, 0.062 mg/mL for BIP 1b, 0.065 mg/mL for BIP 3a and for BRR 3a 0.078 mg/mL, respectively. Bacteriocin of all isolates except BIP 3a was found to be sensitive towards pepsin and resistant towards Rnase. Bacteriocin production was stable at between pH 5.0 and 6.0 and resistant temperature was 40 °C. It was concluded that lactic acid bacteria (LAB) from meat can be helpful as antibacterial agents against food-borne bacterial pathogens because of thermostable producing bacteriocin.

Keywords: antibacterial activity, bacteriocin, lactic acid bacteria, ribotyping

Introduction

Meat is a major source of protein in many parts of the world, necessary for our everyday activities, growth, repair and maintenance of body cells (Hassan *et al*., 2006). Meat is highly perishable food because of its chemical composition and biological characteristics. On the other hand meat is an excellent source for the growth of many precarious microorganisms which cause spoilage of meat and is responsible for infections in humans and economic losses (Kim and Rajagopal, 2001). Recent outbreak of food poisoning has renovated interest to develop new strategies for reducing pathogens on red meat (Mermelstein, 1993).

Now-a-days, meat industry is forced to produce meat with long shelf-life to fulfill consumer and logistic demands (Nattress and Jeremiah, 2000). In food production, it is crucial to take proper measures to ensure stability and safety of food during shelf-life. Demand of consumer is product with extended shelf-life, free of preservatives, safe, high quality and minimally processed meat (Brul and Coote, 1999). Despite of modern technologies and safety measures, food-poisoning and food-borne illnesses are on the rise (Holzapfel *et al*., 1995).

Biological preservation provides a new concept and novel approach for improvement of microbial safety of food. Biological preservation refers to the use of antagonistic microorganisms to inhibit pathogenic microorganisms in food. For thousands of years, Lactic acid bacteria (LAB) have been used to produce desirable changes in flavour, texture, taste of food, to destroy pathogenic bacteria and to inhibit spoilage microorganisms. LAB extend the shelf-life and ensure the safety of food by producing lactic acid, acetic acid, hydrogen peroxide, carbon dioxide, and bacteriocins. All of these referred as antimicrobials which kill pathogenic and spoilage microorganisms (Mehra *et al*., 2012; Dhewa *et al*., 2011; Dhewa and Goyal, 2009).

The increased public health concern is of microbial food safety worldwide (Zhao *et al*., 2001). Possibility of usage of lactic acid bacteria (LAB) as substrates of other chemical preservatives of food and production of bacteriocin which is of great economic interest had been

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reported earlier (Admas, 1999). From meat and dairy products LAB were isolated which produced bacteriocin. These bacteriocins prolonged the shelf-life and promised for safety of food products (Dhewa, 2012; Berry *et al*., 1990). A variety of other antibacterial agents such as diacetyl, hydrogen peroxide and organic acids are also produced by lactic acid bacteria (Gilliland, 1988). Lactic acid bacteria are also known as Food Grade organisms and widely used as protective cultures in food systems (Holzapfel *et al*., 1995). Bacteriocins as heat stable and amenable to proteolytic inactivation found to have antimicrobial activity against food-pathogens.

Reduction of bacterial pathogens on meat products by acid treatment limits its use in food systems. This method also has drawbacks for meat safety and shelf-life of meat products (Greer and Jones, 1991). Gram negative bacteria such as *Escherichia coli*, *Salmonella serovars* and *Campylobacter* sp. are commonly found on meat products (Mead *et al*., 1999). Olasupo *et al*. (1997, 1995) discovered that *Lactobacillus* sp. (bacteriocin producing strain) is active against bacterial pathogens.

Bacteriocins are bioactive peptide complexes (30-60 amino acids) that on other species have a bacteriocidal effect. They are produced in ribosomes, released extra-cellular and act as active protein molecules. Production of bacteriocin of gram negative bacteria is a lethal event, whereas, the production of bacteriocin of gram positive bacteria is not lethal and they are diverse in nature. Some of gram positive bacteria use sec-dependent export pathway while some have disclosed a bacteriocin specific transport system. Lactic acid bacteria among gram positive bacteria are the main producer of bacteriocin (Margaret and John, 2002).

An objective of present study was the screening and identification of bacteriocin producing LAB from meat samples and to examine antibacterial activity of isolated bacteria (Lewus *et al*., 1991). The study was conducted to isolate bacteriocin producing LAB from red meat samples. Both qualitative and quantitative methods were done for bacteriocin production. The sensitivity of bacteriocin to proteolytic enzyme produced by bacterial pathogens was also studied. The aim was to investigate LAB antibacterial activity and finally to select interesting strains for the role of bio-preservation.

Materials and Methods

Sample collection. Minced beef pieces were purchased from butcher's shops of seven different localities in Lahore, such as Shahdra, Islampura, Jiamusa, Metro Shahdra, Begum Kot, and Ravi Road of Lahore, Pakistan early in the morning and collected in sterilized polythene bags with the help of sterilized spatula then stored at 4 °C for a maximum of 24 h before analysis to isolate LAB.

MRS broth and agar medium preparation. For the preparation of MRS broth, 10.0 g/L of peptone, 10.0 g/L of beef extract, 5.0 g/L of yeast extract, 20.0 g/L of glucose, 1.0 g/L of polysorbate 80 (Tween-80), 2.0 g/L of ammonium citrate, 5.0 g/L of sodium acetate, 0.1 g/L of magnesium sulphate, 0.05 g/L of manganese sulphate and 2.0 g/L of dipotassium hydrogen phosphate were measured and dissolved in 1000 mL of distilled water. For the preparation of MRS agar, 15 g agar was also used. The pH of the medium was 6.0-7.0 and both media were sterilized in autoclave at 121 °C for 15 min.

Bacterial isolates. From seven beef samples, about thirty cultures of LAB were isolated and named as BSH 1a, BSH 3a, BSH 2a, BSH 1b, BSH 4a, BSH 4c, BIP 1a, BIP 1b, BIP 3a, BIP 3b, BIP 4a, BJM 2a, BJM 4c, BJM 4d, BMS 1a, BMS 3b, BMS 4c, BBK 1a, BBK 2a, BBK 3b, BBK 4d, BRR 1b, BRR 2c, BRR 3a, BRR 4b, BGR 1a, BGR 1c, BGR 2b, BGR 3c and BGR 4a.

Screening for pathogenic bacteria as indicator strains. To screen indicator pathogenic bacterial isolates blood agar test was performed (Vanderzant and Splittstoesser, 1992) which used for assaying antibacterial activity. After solidification of medium, streaking of bacterial isolates on plates was done. These blood agar plates were kept inverted and incubated overnight at 37 °C in incubator.

Determination of antibacterial activity of LAB. The inoculation of selected lactic acid bacteria (LAB) isolates from slants to autoclaved 100 mL MRS broth was done and incubated for 48-72 h at 37 °C. The culture broth of each LAB isolate was taken in autoclaved centrifuge tube and centrifuged separately at 10,000 × g for about 20 min. After centrifugation the supernatant was collected and passed through 0.2 m filter paper. This cell free supernatant broth was collected for the antibacterial activity against selected food-borne pathogens *viz*., *Escherichia coli* and *Staphylococcus aureus* through paper disc diffusion method (Baur *et al*., 1966). Sterilized petri plates were taken and poured with nutrient agar plates. A lawn of indicator strains (10^7cfu/mL) was prepared by spreading cultures of the selected strains of pathogens on nutrient agar medium with the help of

spreader. The plates were kept for drying and then discs of 5 mm were punched from sterilized filter paper. The discs were then dipped in 100 L of cell free filtrate of LAB isolates. By using sterilized forceps, disks were placed on agar plates. Agar plates were incubated for 24 h at 37 °C. After 24 h, plates were observed for the zones of inhibition which were formed around the discs on agar plates and these zones were measured. Bacteriocin activity was taken in diameters (mm) of inhibition zones. If the diameter of zone of inhibition (ZOI) was greater than 1.0 mm results were considered positive.

Characterisation of bacterial isolates. The obtained bacterial colonies were characterized by employing different test. Both morphological and biochemical tests were performed for the characterization of obtained bacterial isolates (Cheesbrough, 2002; Collins *et al*., 1989). Bacterial isolates were observed for colony morphology including shape of bacterial colonies, colour of bacterial colonies, texture of colonies and studied microscopically by gram's staining, endospore staining and motility test.

Production of antimicrobial agent by LAB. To prepare inoculums, 10 mL of MRS broth was prepared for each isolate and pH was adjusted to 6.0-7.0. For sterilisation placed in autoclave for 15-20 min at 121 °C and 15 lbs. Then inoculated with 0.1 mL of freshly prepared culture of lactic acid bacteria (LAB) and incubated at 37 °C for 72 h. After incubation of 72 h, this broth was transferred to autoclaved falcon's tube. The isolates propagated in MRS broth media were centrifuged at 8000 rpm for 30 min at 4 °C. With the help of filter paper the supernatant was filtered. This filtered supernatant was crude extract.

Protein estimation. Bradford method was employed for determining protein concentrations (Bradford, 1976). Bradford's reagent (Dissolved 50 mg of Coomassie-Brilliant blue G250 in 50 mL of methanol and added 100 mL of 85% phosphoric acid to the 1st solution. Now added this 2nd solution into 500 mL of water and mixed, then filtered it to remove precipitates. Now additional 350 mL of water was added into it and stored at 4 °C) was made according to recipe. Bovine serum albumin (BSA) 1 mg/mL was employed as standard in this experiment. 100 µL of protein sample in the form of cell free supernatant (CFS) was added to 0.9 mL of Bradford reagent with the help of micropipette and placed at room temperature for 15 min and stand for 15 min at room temperature. Bradford reagent (0.9 mL) was mixed with 100 µL of distilled water which was considered as blank. The O.D (optical density) was taken within 45 min at 595 nm with the help of spectrophotometer.

Characterisation of antimicrobial agent by LAB strains. ***Sensitivity to proteolytic enzymes*.** To check the proteinacious nature of antimicrobial agent by LAB strains, its sensitivity against proteolytic enzyme pepsin was checked. Proteolytic enzyme with final concentration of 2 mg/mL was treated with crude bacteriocin in 0.01 M phosphate buffer at pH 7.0. 0.15 mL (150 µL) phosphate buffer (0.5 M, pH 7.0), 0.15 mL (150 µL) of bacteriocin and 0.15 mL (150 µL) of pepsin (0.25 mg/mL) were taken in a test tube. A control was run by taking only 0.15 mL (150 µL) of bacteriocin and 0.15 mL (150 µL) of distilled water. These samples were kept in an incubator for 2 h at 37 °C and then boiled at 100 °C for 3 to 5 min. No zone of inhibition indicates proteinaceous nature of antimicrobial substances produced by LAB strains.

***Sensitivity to non-proteolytic enzymes*.** Sensitivity of antimicrobial agent against non-proteolytic enzyme was also determined to compare its activity by zones of inhibition. Non-proteolytic enzyme with final concentration of 2 mg/mL was treated with crude bacteriocin in 0.01 M phosphate buffer at pH 7.0. 0.15 mL (150 µL) phosphate buffer (0.5 M, pH 7.0), 0.15 mL (150 µL) of bacteriocin and 0.15 mL (150 µL) of Rnase (0.25 mg/mL) were taken in a test tube. A control was run by taking only 0.15 mL (150 µL) of bacteriocin and 0.15 mL (150 µL) of distilled water. These samples were kept in an incubator for 2 h at 37 °C and then boiled at 100 °C for 3 to 5 min. The presence of inhibition zones indicate the antimicrobial substance is not sensitive to Rnase.

***Sensitivity to pH*.** The pH sensitivity of bacteriocin was checked by adding 4.5 mL of autoclaved sterile MRS broth in each test tube. Then pH was adjusted to different ranges from 5.0 to 10.0 by HCl and NaOH then 0.5 mL of bacteriocin was added to each test tube. These samples were placed at room temperature for one hour. Then these samples were tested for antimicrobial activity.

***Heat resistance*.** The stability of bacteriocin activity against heat was tested at 40-90 °C in a thermostatically controlled water bath. Sterile MRS broth (4.5 mL) was taken in different test tubes and 0.5 mL of bacteriocin was added in each test tube and heated to the temperatures mentioned above. Then samples were cooled

at room temperature in ice slurry water for 15 min immediately. These samples were tested for antimicrobial activity.

Determination of optimal growth conditions. ***Growth curve.*** In order to obtain the growth curve of bacterial isolates, 300 mL of the MRS broth as a starter culture was prepared in 500 mL conical flask. Flasks were properly labeled, cotton plugged and wrapped with aluminum foil and placed in autoclave for 15-20 min at 121 °C and 15 lbs. Triplicates were made to carry out this experiment. Medium was inoculated with bacterial isolates (10^7 colony forming units cfu/mL), placed in shaking incubator for 48 h at 37 °C. About 800 µL of this culture was poured in cuvette by using micropipette under the sterile condition (laminar air flow) after every 1 h. Optical density was taken at 595 nm by using spectrophotometer. To plot the growth curve, the O.D was taken on Y-axis and incubation time on X-axis. MRS medium without bacterial isolate was used as control.

Effect of pH. In order to determine the effect of optimum pH, 250 mL of MRS broth was prepared in a 500 mL conical flask. Then broth was equally distributed in 5 parts to adjust the pH of the broth to 5, 6, 7, 8, and 9 pH and placed in autoclave for 15-20 min at 121 °C and 15 lbs. Media was transferred in 30 test tubes and then inoculated with bacterial isolates and placed in incubator for 72 h at 37 °C. About 800 µL of this culture was poured in cuvette by using micropipette under the sterile conditions (laminar air flow). The optical density was taken after 72 h with the help of spectrophotometer at 595 nm. The graph was plotted by taking optical density (O.D) on Y-axis and pH on X-axis.

Effect of temperature. To ascertain the effect of temperature, 150 mL of MRS broth was prepared in a 200 mL conical flask. Cotton plugged and wrapped its mouth with aluminum foil. For sterilisation, flasks were placed in autoclave for 15-20 min at 121 °C and 15 lbs. Medium was inoculated with bacterial isolates (10^7 colony forming units cfu/mL) and placed in incubator for 72 h at 37 °C. Temperature was adjusted to 25 °C, 30 °C, 37 °C and 45 °C. Experiment was run in triplicates. About 800 µL of this culture was poured in cuvette by using micropipette under the sterile conditions (laminar air flow). The optical density was taken after 72 h with the help of spectrophotometer at 595 nm. The graph was plotted by taking optical density (O.D) on Y-axis and temperature on X-axis.

Effect of different source of carbohydrate. To check the effect on bacterial growth by changing source of carbohydrate, 200 mL of MRS broth was prepared in a 500 mL conical flask. Broth was divided into 4 flask of 200 mL. Source of carbohydrate in 4 flask is as described; Glucose, Lactose, Sucrose and Galactose. Cotton plugged and wrapped with aluminum foil. For sterilisation, flasks were placed in autoclave for 15-20 min at 121 °C and 15 lbs. Medium was inoculated with bacterial isolates and placed in incubator for 72 h at 37 °C. About 800 µL of this culture was poured in cuvette by using micropipette under the sterile conditions (laminar air flow). The optical density was taken after 72 h with the help of spectrophotometer at 595 nm. The graph was plotted by taking optical density (O.D) on Y-axis and source of carbohydrate on X-axis.

Genomic DNA isolation. DNA was isolated by phenol chloroform method. Agarose gel electrophoresis (1%) was done to assure that the samples contain isolated genomic DNA. After running gel, DNA bands in the gel were visualized using short wave ultraviolet light provided by a trans-illuminator and photograph was taken through Stratagene Eagle Eye still video system.

Ribotyping. Ribotyping is aimed at molecular characterization of pathogenic milk isolates, so their 16SrDNA was partially amplified by using primers i.e. 16S-27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 16S-1522R (5'-AAGGAGGTGATCCAGCCGCA-3') (Penicon) through polymerase chain reaction. Polymerase chain reaction (PCR) was performed in a thermocycler for 35 reaction cycles. Total reaction mixture (50 µL) was taken. Initial denaturation was done at 94 °C for 5 min, annealing was done at 52 °C and elongation at 72 °C for 30 sec, 40 sec and 30 sec, respectively. Final extension was given at 72 °C for 10 min.

Amplified DNA extraction and gel electrophoresis. To confirm the amplification, PCR products were loaded in 1% agarose gel. Gel was run following same procedure for half h at 80 volts and the bands of amplified DNA were visualized under the UV light by using the trans-illuminator. After amplification anticipated band were eluted/cut and kept in eppendrofs. Then using GF-1 DNA recovery kit by Vivantis, gene was cleaned.

Sequencing. The amplified product was then sent to 1st Base Laboratory, Malaysia for sequence analysis. The nucleotide sequences were Basic Local Alignment Search Tool (BLAST) searched for blastn and or/ blastx algorithms in NCBI.

Results and Discussion

In the present study, 7 samples of beef were purchased from butcher's shop of different areas of Lahore, Pakistan. Three best samples who gave maximum zone of inhibition against food borne bacterial pathogens were biochemically and molecularly characterized and recognized through Ribotyping. The growth curves, pH effect, temperature effect, effect of different carbohydrate sources, protein estimation, bacteriocin sensitivity towards pepsin and Rnase, different pH and temperatures were also checked. The isolated bacterial strains were symbolically named as BSH 1b, BSH 3a, BIP 4a, BIP 3a, BIP 1b and BRR 3a.

Antibacterial activity test. Antibacterial activity of isolated bacterial strains against food-borne pathogens was determined through paper disc diffusion method. It was found that out of thirty bacterial isolates, six bacterial isolates i.e., BSH 1b, BSH 3a, BIP 4a, BIP 3a, BIP 1b and BRR 3a gave positive results range between 1.8 - 3 mm (Table 1).

Characterization of bacterial isolates. *BSH 1b* (*Lactobacillus* sp.). Its colony appeared off-white, small, smooth/elevated and wet had given positive result for gram staining and negative result for both endospore stains and motility test (Table 1). BSH 1b (*Lactobacillus* sp.) characterized on the basis of biochemical tests whose antibacterial activity zone was of 2 mm found to be negative for catalase test (no bubble formation), negative for indole test, and negative for nitrate reduction test, citrate test, methyl red Voges-Proskeur test, casein hydrolysis test and for starch agar test (Table 1). But it was found to be positive for glucose, lactose and sucrose fermentation. Bacteriocin produced by BSH 1b (*Lactobacillus* sp.) was of 0.07 mg/mL concentration.

***BSH 3a* (*Lactobacillus* sp.).** The colony appeared off-white, medium sized, sharp/elevated, wet and was found to be gram positive, non-spore former and non-motile bacteria (Table 1). BSH 3a (*Lactobacillus* sp.) identified on the basis of biochemical tests whose antibacterial activity measured through inhibition zone was of 2 mm found to be negative for indole test (green layer at the top of test tube) and starch agar test. It also showed negative result for catalase test, nitrate reduction test, citrate test, methyl red Voges-Proskeur test, casein hydrolysis test but positive result for glucose, lactose and sucrose fermentation (Table 1). Bacteriocin produced by BSH 3a (*Lactobacillus* sp.) was of 0.065 mg/mL concentration.

Table 1. Antibacterial activity and characterisation of antimicrobial agent produced by LAB strains

Samples no.	CC	CS	CE	CT	GS	ES	MT	CatT	IndT	NRT	CitT	MRVPT	CHT	SAT	GF	LF	SF	ZIN (mm)	Sensitivity to pepsin (Proteolytic enzyme)	Sesitivity to Rnase (Non-proteolytic)
BSH 1b	Off-white	Small	Smooth/ elevated	Wet	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	2.0	Sensitive	Resistant
BSH 3a	Off-white	Medium	Sharp/ elevated	Wet	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	2.0	Sensitive	Resistant
BIP 4a	Off-white	Medium	Smooth/ elevated	Wet	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	2.5	Sensitive	Resistant
BIP 1b	Off-white	Large	Smooth/ elevated	Wet	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	3.0	Sensitive	Resistant
BIP 3a	Pale yellow	Medium	Smooth/ elevated	Wet	+ve	-ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	2.0	Resistant	Resistant
BRR 3a	Off-white	Small	Smooth/ elevated	Dry	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	1.8	Sensitive	Resistant

CC = colony colour; CS = colony size; CE = colony edge; CT = colon texture; GS = gram's staining; ES = endospore staining; MT = motility test; CatT = catalase test; IndT = indole test; NRT = nitrate reduction test; CitT = citrate test; MRVPT = methyl red Voges-Proskeur test; CHT = casein hydrolysis test; SAT = starch agar test; GF = glucose fermentation; LF = lactose fermentation; SF = sucrose fermentation; ZIN = zone of inhibition.

***BIP 4a* (*Lactobacillus curvatus*).** It was appeared as off-white, medium sized, smooth/elevated and wet had given positive result for gram staining but negative result for endospore staining and motility test. BIP 4a (*L. curvatus*) characterised through molecular characterization was found to be negative for catalase test, indole test (green layer is formed at the top of test tube), nitrate reduction test, citrate test (no blue colour in test tube), methyl red Voges-Proskeur test (no red colour appears in test tube), casein hydrolysis test (not able to produce protease enzyme) and for starch agar test (not able to produce amylase enzyme) (Table 1). But it had been found positive for glucose, lactose and sucrose fermentation and its antimicrobial activity was recorded 2.5 mm. Bacteriocin produced by BIP 4a (*L. curvatus*) was of 0.057 mg/mL concentration.

***BIP 1b* (*Lactobacillus graminis*).** It had given negative result for endospore staining and motility test but was found to be gram positive bacteria through gram staining (Table 1) and its colony was appeared as off-white, large sized, smooth/elevated and wet. BIP 1 b (*L. graminis*) which was molecularly characterised and had 3 mm zone of inhibition. It was found to be positive for glucose, lactose and sucrose fermentation (red colour appeared in test tube) but found to be negative for catalase test (no bubbles were formed), indole test (green layer is formed at the top of test tube), nitrate reduction test, citrate test (green-forest colour in test tube), methyl red Voges-Proskeur test (no red colour appears in test tube), casein hydrolysis test and for starch agar test (no clear zone formation) as shown in Table 1. Bacteriocin produced by BIP 1 b (*L. graminis*) was of 0.062 mg/mL concentration.

BIP 3a (Staphylococcus warneri). Through gram staining, endospore staining and motility test BIP 3a was found to be gram positive, non-spore former and non-motile bacteria, its colony appeared was pale-yellow, medium sized, smooth/elevated and wet (Table 1). BIP 3a (*S. warneri*) was characterised molecularly and had antimicrobial activity of 2.0 mm found to be positive for catalase test (bubbles were formed on glass slide), nitrate reduction test and for fermentation tests of glucose, lactose and sucrose (red colour appeared). It had been found to be negative for indole test (no blue color), citrate test, methyl red Voges-Proskeur test (no red color), casein hydrolysis test (no clear zone) and for starch agar test. Bacteriocin produced by BIP 3a (*Staphylococcus warneri*) was of 0.065 mg/mL concentration.

***BRR 3a* (*Lactobacillus* sp.).** Its colony appeared as off-white, small, smooth/elevated and dry was found to be gram positive bacteria, non-spore former and non-motile through gram staining, endospore staining and motlity test (Table 1). BRR 3a (*Lactobacillus* sp.) identified on the basis of biochemical tests had antibacterial activity of 1.8 mm and found to be negative for catalase test (no bubble formation), indole test (green layer formed in test tube), citrate test (green forest colour in test tube), methyl red Voges-Proskeur test (Fig. 6), for nitrate reduction test (no red color formed in test tube), casein hydrolysis test (clear zone was not formed) and for starch agar test (no clear zone). But it showed positive result for glucose, lactose and sucrose fermentation test as shown in Table 1. Bacteriocin produced by BRR 3a (*Lactobacillus* sp.) was of 0.078 mg/mL concentration.

Determination of optimal growth conditions. *Bacterial growth curve*. In order to determine the incubation time for maximum growth of bacterial isolates, the isolated cultures were grown at 37 °C and their growth was determined after every 1 h. It was found that all selected bacterial strains i.e., BSH 1b, BSH 3a, BIP 4a, BIP 3a, BIP 1b and BRR 3a gave maximum growth between 60-72 h (Fig. 1).

Determination of optimum pH. In order to determine the optimum pH the bacterial isolates were grown in a medium having pH 5 - 9. As a result, 3 bacterial strains BSH 3a, BIP 4a and BRR 3a gave maximum growth at pH 7.0, whereas BSH 1b and BIP 1b gave maximum growth at pH 6.0. But optimum pH for BIP 3a was 5.0. And bacterial isolates grown in pH 8.0 and 9.0 showed very little growth (Fig. 2).

***Determination of optimum temperature*.** In order to determine the optimum temperature, the bacterial strains were grown at temperature ranges from 25 to 45 °C. It was found was that out of 6 bacterial isolates, 3 isolates i.e., BSH 3a, BIP 4a and BIP 3a gave maximum growth at temperature of 37 °C, whereas other 3 bacterial isolates i.e., BSH 1b, BIP 1b and BRR 3a had optimum temperature of 30 °C (Fig. 3).

Determination of carbohydrate source. It has been found that the best source of carbohydrate for 2 strains i.e., BSH 3a and BIP 3a was galactose, whereas for BSH 1b the best carbohydrate source was lactose and for BIP 4a, BIP 1b and BRR 3a glucose was the best carbohydrate source (Fig. 4).

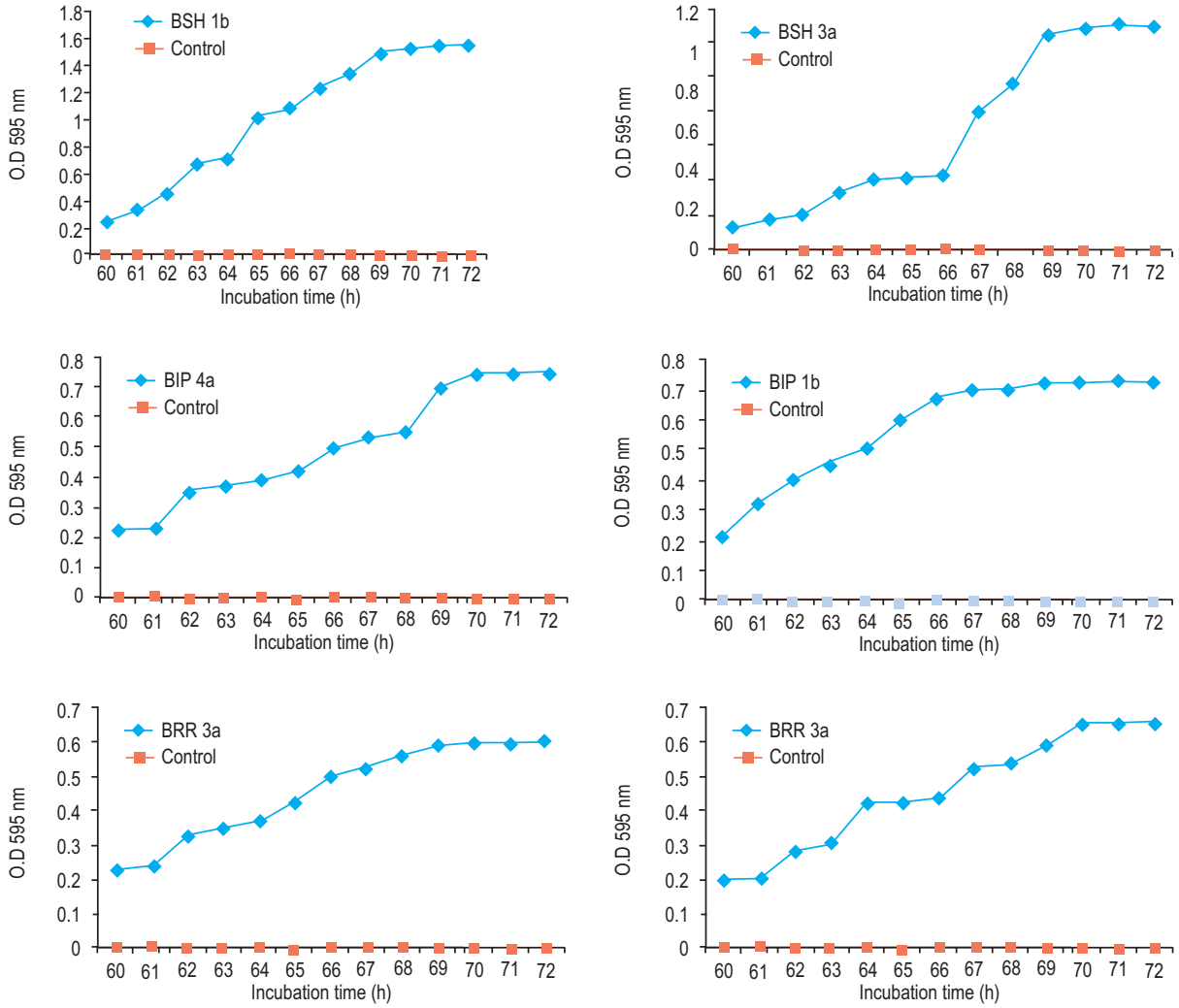


Fig. 1. Growth pattern of bacterial isolates in MRS broth medium at 37 °C for various incubation periods.

Characterisation of the purified bacteriocins. ***Sensitivity to proteolytic enzymes*****.** Bacteriocins of 5 isolates out of 6 i.e., BSH 1b, BSH 3a, BIP 4a, BIP 1b and BRR 3a were found to be fully or partially inactivated by pepsin that was a proteolytic enzyme (Table 1). This is due to their proteinaceous nature. Whereas bacteriocin of BIP 3a had retained its activity when treated with pepsin but a significant decrease in its activity.

Sensitivity to Rnase**.** The sensitivity against Rnase has been checked (Table 1 and it had been found that all the bacterial strains were resistant against Rnase, as zones of inhibition had been found).

Sensitivity to pH**.** The sensitivity of bacteriocin against pH had been determined (Table 2). It was observed that bacteriocin from all bacterial isolates showed sensitivity at pH 9 and 10 while had resistant effect at pH 5 and 6. Mostly variation exist at pH 8 as indicated in Table 2.

Sensitivity to temperatures**.** The sensitivity of bacteriocin was checked at various temperatures. It was observed that bacteriocin from all bacterial isolates showed sensitivity at 60, 70 and 80 °C, whereas resistant at 40 °C (Table 3).

Molecular characterisation. Three bacterial isolates such as BIP 4a, BIP 3a and BIP 1b were selected for molecular identification. The sharp bands of DNA of BIP 4a, BIP 3a and BIP 1b were visualized through 0.8% agarose gel electrophoresis (Fig. 5). By using forward and reverse primers, the conserved region of 16S rDNA gene were amplified from genomic DNA and PCR products were visualized in 1.2% agarose gel

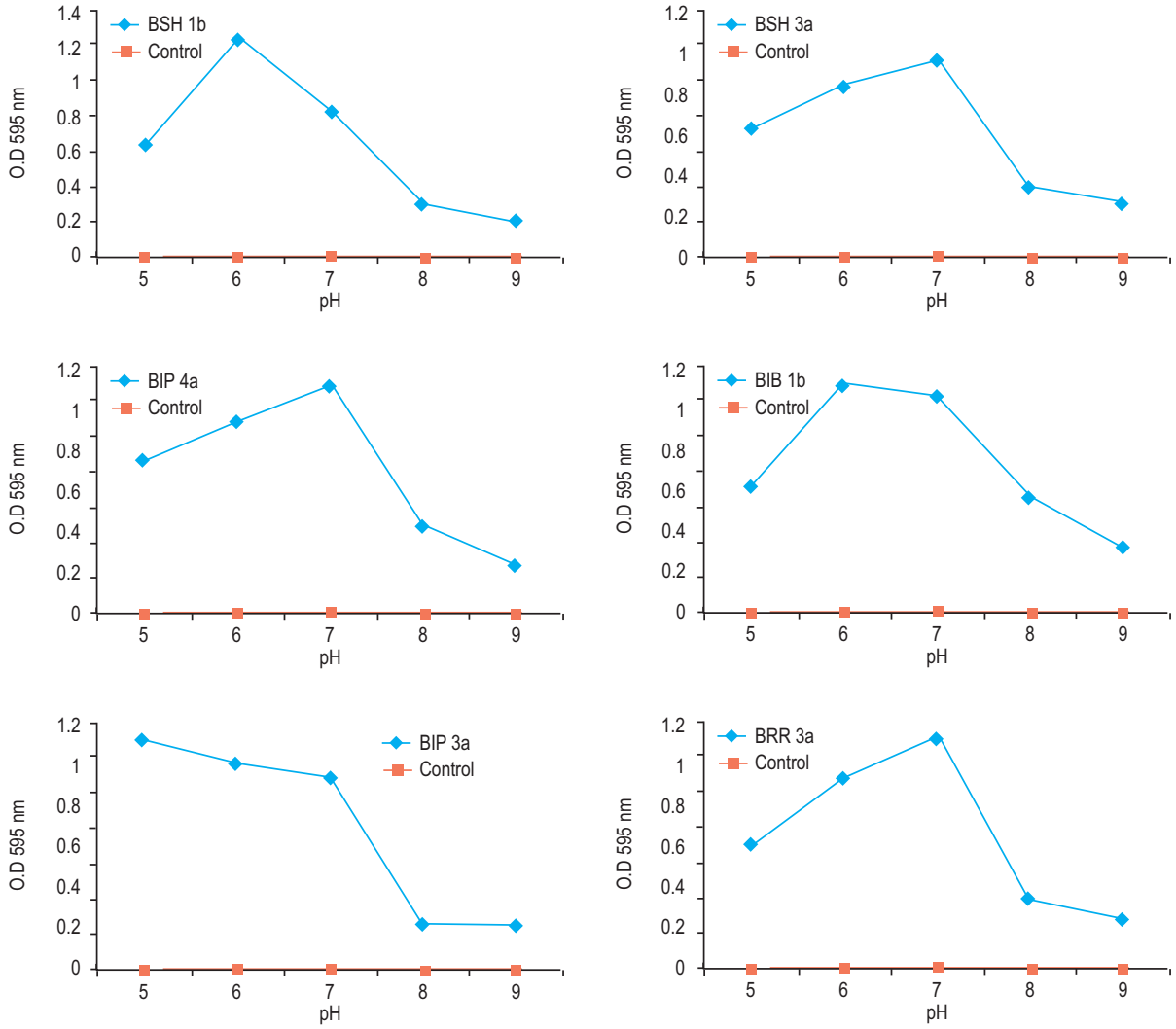


Fig. 2. Effect of different pH values on growth of bacterial isolates.

(Fig. 5). The amplified product was then sent to 1st Base Laboratory, Malaysia for sequencing. After sequencing it was found that BIP 4a contain 1543 base pairs and NCBI BLAST analyzed that it belongs to *Lactobacillus curvatus* (Accession no: KP117256), BIP 3a contain 1525 base pairs and belongs to *S. warneri* (Accession no: KP117257), and BIP 1b has 1567 base pairs and belongs to *L. graminis* (Accession no: KP117258). Dendogram analysis of BIP 4a, BIP 3a, and BIP 1b bacterial isolates is shown in Fig. 6.

Table 2. Sensitivity of bacteriocin of bacterial isolates from beef samples to different pH

Bacterial strains	Sensitivity to different pH					
	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10
BSH 1b	Resistant	Resistant	Resistant	Sensitive	Sensitive	Sensitive
BSH 3a	Resistant	Resistant	Resistant	Resistant	Sensitive	Sensitive
BIP 4a	Resistant	Resistant	Resistant	Sensitive	Sensitive	Sensitive
BIP 1b	Resistant	Resistant	Resistant	Sensitive	Sensitive	Sensitive
BIP 3a	Resistant	Resistant	Resistant	Resistant	Sensitive	Sensitive
BRR 3a	Resistant	Resistant	Sensitive	Sensitive	Sensitive	Sensitive

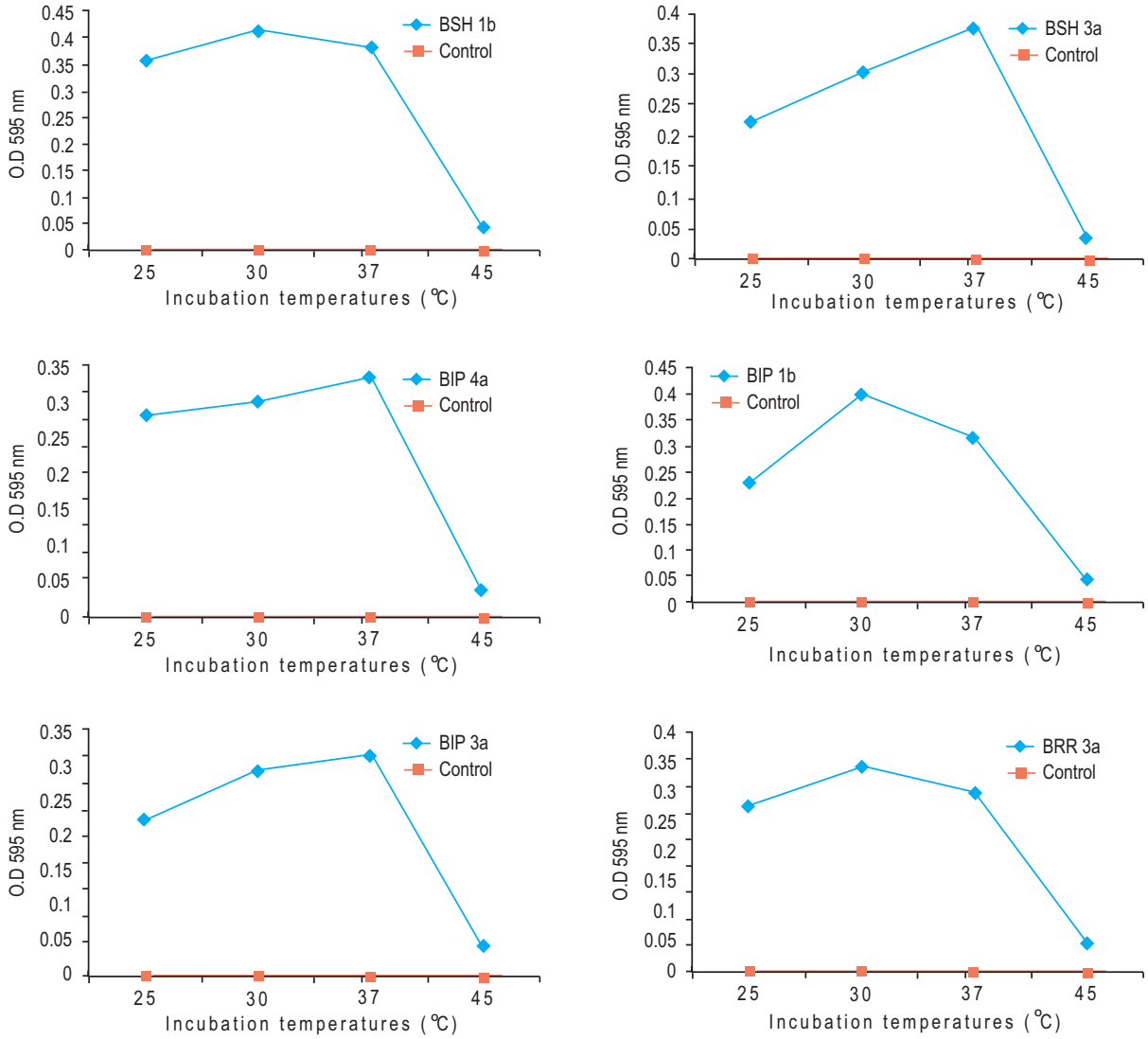


Fig. 3. Effect of different values of temperature on growth of bacterial isolates.

In present study, seven meat samples from different butchers shop were collected to screen out LAB which produce bacteriocins. LAB are also isolated from other than meat samples such as milk, yogurt, vegetables, pickles and found to produce bacteriocins with inhibitory activity against pathogenic bacteria (Nakhdjavani *et al*., 1996).

Table 3. Sensitivity of bacteriocin of bacterial isolates from beef samples to different temperatures

Bacterial strains	Sensitivity to different temperatures °C (in 15 min)				
	40	50	60	70	80
BSH 1b	Resistant	Resistant	Sensitive	Sensitive	Sensitive
BSH 3a	Resistant	Resistant	Sensitive	Sensitive	Sensitive
BIP 4a	Resistant	Sensitive	Sensitive	Sensitive	Sensitive
BIP 1b	Resistant	Sensitive	Sensitive	Sensitive	Sensitive
BIP 3a	Resistant	Resistant	Sensitive	Sensitive	Sensitive
BRR 3a	Resistant	Resistant	Sensitive	Sensitive	Sensitive

The bacterial strains were isolated by using pour plate technique. In this way, 30 LAB strains were isolated on MRS agar plates, the media specific for LAB. From these 30 isolates, 6 bacterial isolates were screened out by performing antibacterial activity test. Dhewa (2012) isolated 20 samples and only 7 of them showed anti-microbial acitivity against bacterial pathogens. De Klerk (1967) studied that 54% of LAB were potential inhibitor against gram positive bacteria and gram negative bacteria were found to be inhibited by only 30% of LAB (Barefoot and Klaenhammer, 1983).

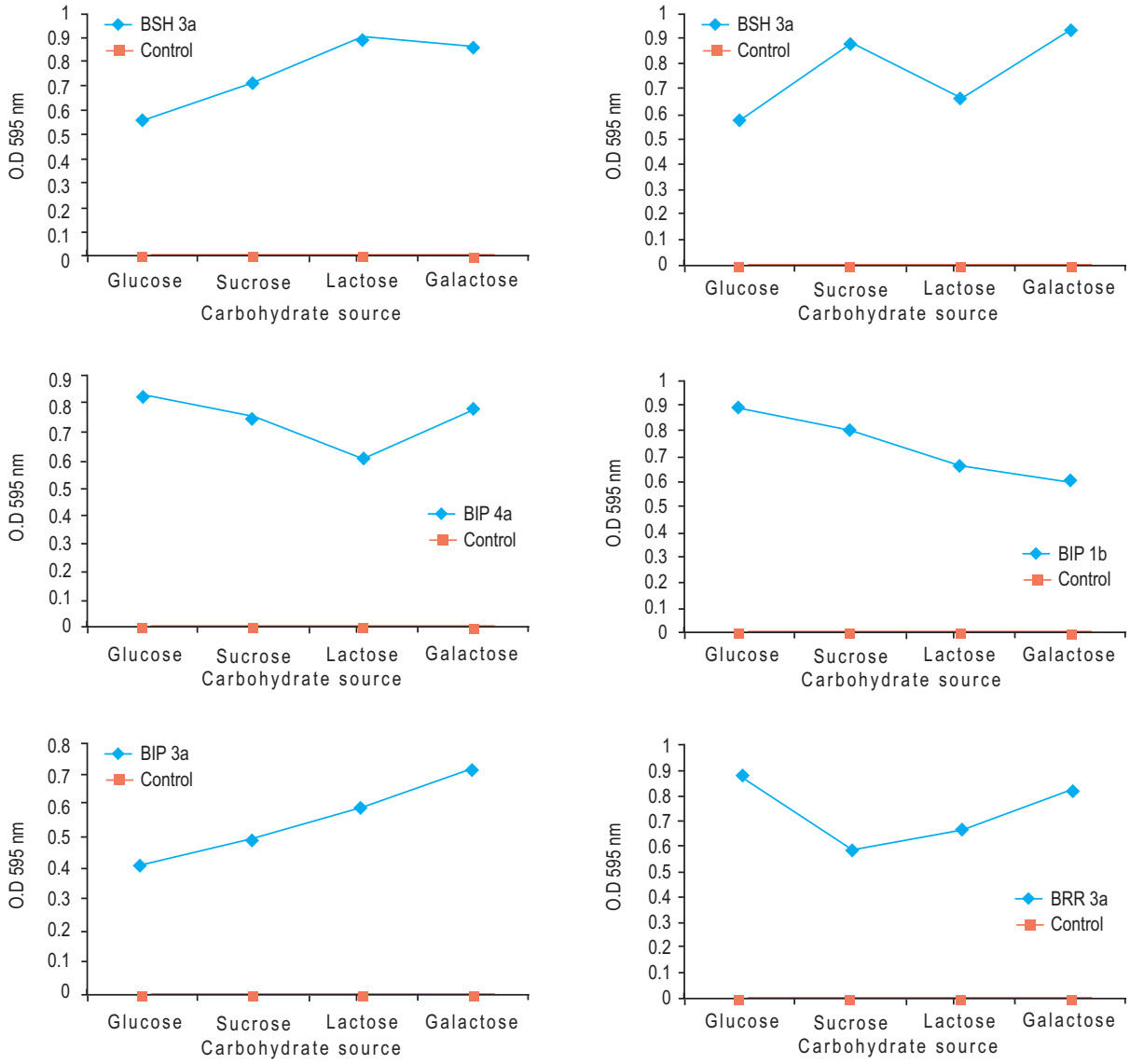


Fig. 4. Effect of different source of carbohydrates on growth of bacterial isolates.

The isolates which had produced inhibition zone greater than 1.0 mm were considered to be best producer of bacteriocin i.e., BSH 1b (*Lactobacillus* sp.), BSH 3a (*Lactobacillus* sp.), BIP 4a (*L. curvatus*), BIP 3a (*S. warneri*), BIP 1b (*L. graminis*) and BRR 3a (*Lactobacillus* sp.). Al-Allaf *et al*. (2009) also isolated 6 lactic acid bacterial strains which had inhibition zone greater than 1 mm against pathogens.

The strains were determined according to "Bergey's Manual Key". All the bacterial strains that had shown antimicrobial activity were characterised morphologically and biochemically (Cullimore, 2008). Then three out of six bacterial strains were proved by Ribotyping (Macrina *et al*., 1978). Lactic acid bacterial strains DNA cannot be easily detected because of slow growth and methods of isolation (Casey and Jimeno, 1989). Ribotyping was performed to characterise bacteria molecularly and on the basis of 16S rRNA gene sequencing, BIP 4a was 99% homologous to *L. curvatus*, BIP 3a was 98% homologous to *S. warneri* and BIP 1b was 99% homologous to *L. graminis*.

To get probiotic bacteria with maximum beneficial effect, make it sure that bacteria must be alive when administrated. Pascual *et al*. (1999) revealed that storage

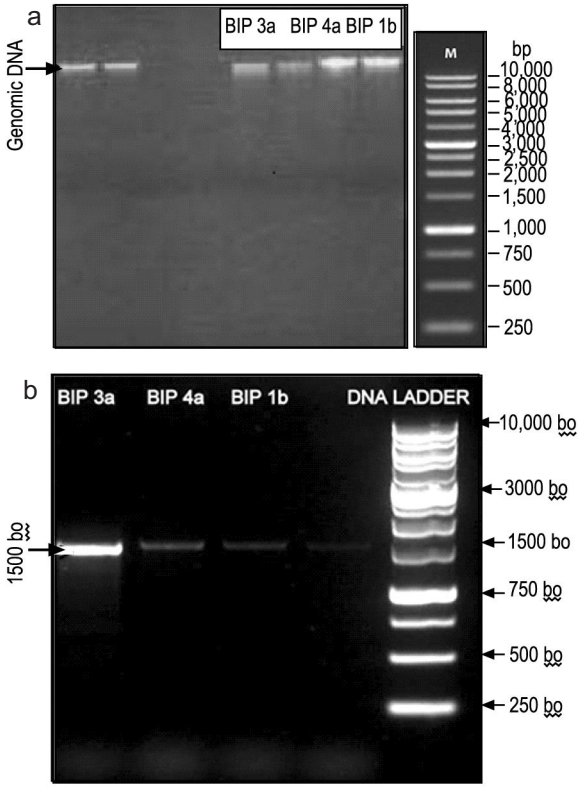


Fig. 5(a-b). Genomic DNA extraction and ribotyping through polymerase chain reaction using 16S rDNA primers. (a) Genomic DNA extraction from bacterial isolates. (b) Ribotyping through PCR.

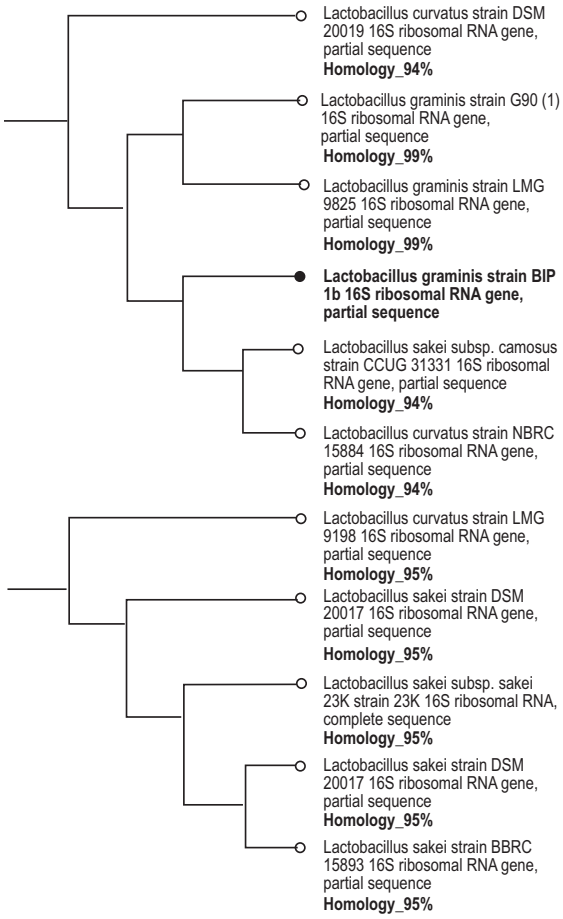
of lactic bacterial culture is important for its maximum beneficiary effect. Glycerol stocks of lactic culture stored at -20 °C had greater viability than stored at room temperature for more than six weeks.

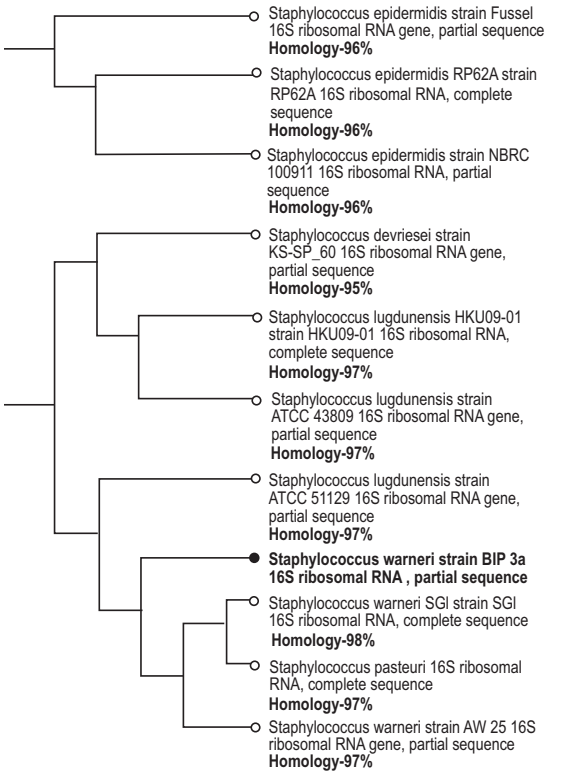
Optimal growth conditions for isolated bacterial strains were found to be between 60 -72 h at 37 °C. In 24 h no bacteriocin produced and no growth was observed because of limited early growth phase (Van den Berghe *et al*., 2006). Optimum temperature for bacterial strains that showed antimicrobial activity was 37 °C for BSH 3a (*Lactobacillus* sp.), BIP 4a (*L. curvatus*) and BIP 3a (*S. warneri*) but three strains BSH 1b (*Lactobacillus* sp.), BIP 1b (*L. graminis*) and BRR 3a (*Lactobacillus* sp.) had optimum temperature of 30 °C while 40 °C do not support any bacteriocin production. pH for optimum growth for isolated bacterial culture was 6.0-7.0 but BIP 3a (*S. warneri*) had optimum pH of 5.0 and pH 8.0 and 9.0 also do not support any bacteriocin production. Dhewa (2012) tested that lactic acid bacteria had optimum pH of 5.5 for *Lactobacillus* and *Lactococcus* but for *Pediocccus* pH of 6.5. Bacteria cultures that had shown antibacterial activity were grown in different media by varying source of carbohydrates. Best media for the growth of BIP 4a (*L. curvatus*), BIP 1b (*L. graminis*) and BRR 3a (*Lactobacillus* sp.) was of media having glucose as source of carbohydrate. BSH 3a (*Lactobacillus* sp.) and BIP 3a (*S. warneri*) grown best in MRS media having galactose as carbohydrate source and lactose for BSH 1b (*Lactobacillus* sp.).

Bacterial cultures were then tested for their potential to produce bacteriocin. Bacteriocin was extracted from bacterial culture according to Al-Zahrani and Al-Zahrani, (2006) method and protein concentration was measured in mg/mL for BSH 1b (*Lactobacillus* sp.) 0.07 mg/mL, BSH 3a (*Lactobacillus* sp.) 0.065 mg/mL, BIP 4a (*L. curvatus*) 0.057 mg/mL, BIP 1b (*L. graminins*) 0.062 mg/mL, BIP 3a (*S. warneri*) 0.065 mg/mL and BRR 3a (*Lactobacillus* sp.) 0.078 mg/mL, respectively.

Sensitivity towards proteolytic (Pepsin) and non-proteolytic (Rnase) was also found when bacteriocin treated with these two enzymes. Except BIP 3a (*S. warneri*) all bacteriocin production was found to be resistant towards pepsin indicating that it had proteinacious nature and found to be resistant against Rnase. Gautam and Sharma (2009) found that *Lactococcus* and *Pediococcus* when treated with proteolytic enzymes became completely inactivated. This was the proof that they had proteinaceous nature or protein in nature. This is of great economic importance for the use of Lactic Acid Bacteria in food industry because it will not cause any damage to gastrointestinal tract of living organisms because of its proteinaceous nature (Gillor *et al*., 2009).

The pH has a very sensitive and crucial role on bacteriocin production (Cakir, 2003). Bacteriocin production was evaluated on different pH ranging from 5.0 to 10.0. Bacteriocin production was stable at pH 5.0 and showed maximum activity and stability. Due to its resistance at low pH, it is used as probiotic because it has to pass through stomach acidic conditions. pH is not only the property of bacteriocin production but temperature also has a very sensitive role on bacteriocin production (Franz *et al*., 1996). The effect of temperature was carried out on the production of bacteriocin. It was found that maximum activity of bacteriocin was observed at 40 °C and no activity was observed at 60 °C and above 60 °C while BIP 4a and BIP 1b were also found to be sensitive at temperature of 50 °C.





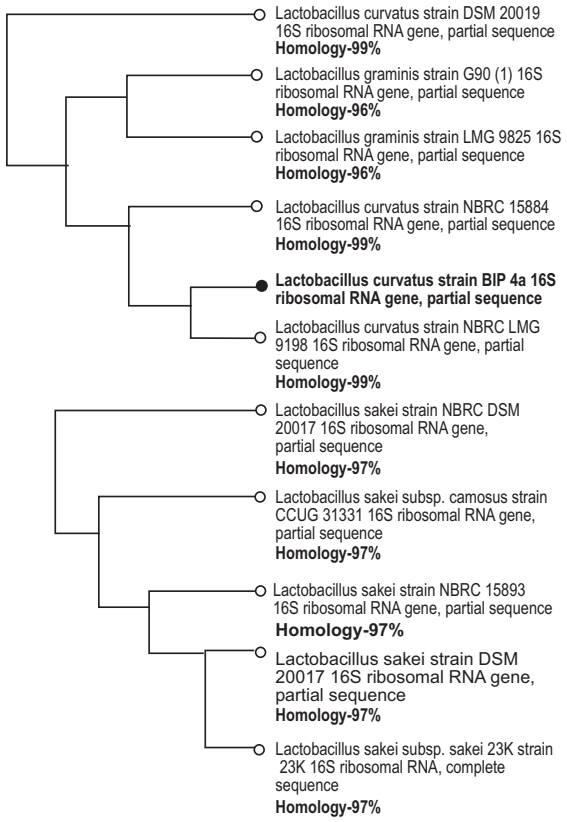


Fig. 6. Dendrogram of BIP 4a, BIP 3a and BIP 1b bacterial isolates.

Conclusion

It can be concluded that bacteriocinogenic LAB from red meat can be helpful as antibacterial agent against food-borne bacterial pathogens. These microbes could be used as a bio-preservative and increased the food shelf life.

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