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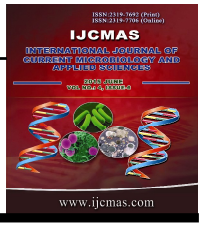

Original Research Article

Antibiotic Resistance pattern and Effect of Some Growth Condition on *Lactococcus lactis* subsp. *lactis* Isolated from Cow Milk

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A B S T R A C T

In this study a total of one hundred forty five-milk sample were collected during the period (July to November 2013) from different fields in Erbil and Duhok province. Screening revealed that 45 out of 145 samples of crude cow milk (31%) were *Lactococcus lactis* subsp. *lactis*. Characterization of these selected isolates was carried out using morphological, biochemical, API 20 system and further confirmation was achieved by PCR assay. Antibiotic sensitivity test performed for *L. lactis* subsp. *lactis* against thirteen antibiotics. The isolates appeared to be varied in their resistance, the number and resistance percent for antibiotics used were as follow: Cephalothin 95.55%, Ampicillin 93.33%, Amoxicillin/ Clavulanic acid (AMC) 93.33%, Sulfamethoxazole/ Trimethoprim 91.11%, Cefotaxime sodium 84.44%, Clindamycin 77.77%, Ceftriaxone 71.11%, Nitrofurantoin 62.22%, Gentamycin 53.33%, Vancomycin 40%, Ciprofloxacin 35.55, Chloramphenicol 24.44%, and Imipenem 2.22%. All isolates extracts exhibited marked activity against five pathogenic bacteria includes *B. subtilis*, *P. aeroginosa*, *E. coli*, *S. aureus* and *C. perfringes* using agar well diffusion method. Isolate 15 and 41 exert maximum activity (20mm) and (16mm) against *B. subtilis* and *S. aureus* whereas isolate 20 showed highest activity (13mm) against *P. aeroginosa*. Generally, all aforementioned isolates were active against gram positive and Gram – negative bacteria. In addition, the effect of pH and, temperature were studied on isolates 10, 26, and 42 extract (bacteriocin) activities against some pathogenic bacteria. The results showed that isolate 42 displayed highest activity at pH 2.5 and temperature at –4°C against pathogenic bacteria under study. Minimum inhibitory concentrations were tested for nisin and the results revealed the MIC of nicin for *S. aureus* was 400µg/ml while for *E. coli* was 500µg /ml.

Keywords

Lactococcus lactis subsp. *lactis*, bacteriocin activity , and MIC

Introduction

Lactic acid bacteria (LAB) are industrially important organisms recognized for their fermentative ability as well as their health and nutritional benefits. The ability of lactic acid bacteria to produce antimicrobial substances has historically long been used to preserve foods. The inhibitory compounds produced by LAB contain organic acid, hydrogen peroxide, carbon dioxide, diacetyl, low molecular weight, antimicrobial substances and bacteriocin (Awla *et al.*, 2014). Microorganisms often develop forms that are resistant to antibiotics. Bacteria always seem to be able to find ways of avoiding being killed by antibiotics (Neu, 1988). Plasmids allow the movement of genetic material, including antimicrobial resistance genes between bacterial species and genera (Murtaza *et al.*, 2012). Bacteriocins especially nisin can use as new antibiotic and bacterial toxin. As the microbes grow, nisin protects these products from germs causing spoilage or food poisoning, including those responsible for botulism and listeriosis (Mitra *et al.*, 2013). Bacteriocin production is influenced by several environmental factors such as pH, temperature, concentration of nitrogen carbohydrate sources and the presence of essential elements such as vitamin and oligo-elements (Mitra *et al.*, 2013). Nisin, a unique "bacteriocidin" has a wide spectrum anti – viral, anti – fungal and anti – bacterial preservative, extracted from *L. lactis.* Nisin is better polypeptides used to fight bacteria at 1000 times than compounds currently in use. Nisin can stop the spread of viruses, bacteria and fungus (Suganthi *et al.*, 2012). Therefore, this study aimed to isolate and identify of bacterial isolates producing nisin (*L. lactis*) from cow milk, extraction of bacteriocin from fermentation broth medium and defines its antimicrobial activity, study the effect of growth conditions on the production of bacteriocin (pH and temperature), and determination the minimum inhibitory concentration of nisin against some pathogenic bacteria.

Materials and Methods

Samples collection

Samples used in this study were raw cow milk that collected from different location within Erbil and Duhok province during the period between August to 2013 November 2013 in sterile 20 ml falcon tubes and transported to laboratory of agriculture college, Dept of food technology, the central laboratory on the same day.

Identification of the isolates

Identification of these isolates was carried out using microscopical, morphological, biochemica test and API 20 system (Barrow and Felthan, 2003).

Molecular technique

For further identification of the different isolates, PCR was applied using specific primers for the conserved gene 16S rDNA. The method was used as stated by (Sambrook *et al.*, 1989).

DNA extraction

Genomic DNA purification protocol

DNA was extracted from *L. lactis* isolates and used for detection of 16S rDNA gene by PCR technique. Three to five ml overnight culture in a 1.5 or 2 ml micro centrifuge tube harvested by centrifugation for 10 min at 10000 rpm, the supernatant discarded. The pellet resuspended in 180 µl of digestion solution, 20 µl of proteinase k solution was added and mixed thoroughly by vortexing or

pipetting to obtain a uniform suspension. The sample incubated at 56 o C for 30 min in shaking water bath, until the cells are completely lysed. Twenty µl of RNase solution was added then mixed by vortexing and the mixture incubated for 10 min at room temperature. Two-hundred µl of lyses solution was added to the sample, mixed thoroughly by vortexing for about 15 sec until homogeneous mixture was obtained. Four-hundred µl of 50% ethanol was added and mixed by pipetting or vortexing. The prepared lysate transferred to Gene JET™ genomic DNA purification column and inserted in a collection tube. The column centrifuged for 1min at 6,000 rpm. The collection tube then discharged containing the flow – through solution. The Gene JET™ genomic DNA purification column placed into a new 2 ml collection tube. Five hundred µl of wash buffer I (with ethanol added) was added then centrifuged for 1 min at 8,000 rpm. The flow – through discarded and the purification column placed back into the collection tube. Five hundred µl of wash buffer II (with ethanol added) was added to the Gene JET™ genomic DNA purification column, Centrifuged for 3 min at maximum speed (≥ 12,000 rpm).Two-hundred µl of elution buffer was added to the center of the Gene JET™ genomic DNA purification column membrane to elute genomic DNA. Incubated for 2 min at room temperature and centrifuged for 1min at 8000 rpm. The purified DNA immediately discarded in downstream applications or stored at –20 ºC.

PCR procedure

Oligonucleotides (Primers)

To detect 16S rDNA homology a pair of bacteria-specific universal primers were used forward 5'AGA GTT TGA TCC TGG CTC AG-E' and reverse 5'-CGG TCA ATT CCT TTG AGT TT-3' (Beasley and Saris, 2004). Primers for *L. lactis* purchased freeze dried from metabion international AG (Germany).

PCR reaction condition

Amplification reaction to confirm the *L.lactis* isolates were performed in 25 µl containing 2 µl of DNA, 2.5 µl of PCR buffer (Vivantics Company, Malaysia). One µl of Mgcl2 (50mM) (Vivantics), 1 unit of taq polymerase, and the volume was completed to 25 µl per reaction and mixed with ddH_2O. The cycle conditions were followed by 35 cycles, the thermo cycle program was as follows: 95ºC for 5 minutes. 95ºC for 1 minute (Denaturation); 45ºC for 1 minute (Annealing); 72ºC for 1 minute (Extension); Final extension step at 72ºC for 10 minutes. This PCR resulted in a DNA fragment of 900 base pairs (Beasley and Saris, 2004).

Agarose gel electrophoresis

Agarose gel with 1.5% was prepared by dissolving 0.375g of agarose (Sigma) in 25 ml 1x Tris EDTA. The mixture was dissolved by heating in microwave oven at 100ºC, and then was left to cool. After that, it was stained with Ethidium bromide 0.5mg/ml (10) of stock solution was added to the gel. Then, the mixture was poured into horizontal electrophoresis apparatus, after sample time to solidify the gel, it was covered with 0.1X TBE buffer. Subsequently, 5 µl of each PCR products were mixed with 2.5 µl of 6x ladder buffer. Two µl of DNA ladder size marker of 100 pb was loaded with its dye for the determination of the amplified DNA product size. Finally, gel electrophoresis run was done at 120 voltage and 25 – 35 mA for 30 minute in 0.1x TBE buffer (Sambrook *et al.*, 1989).

Gel visualization

Following the run completely, the gel was examined using gel documentation system (Gel 2000, Bio Red, and USA). The PCR fragment sizes were estimated from their distance of migration relative to the DNA maker size.

Antimicrobial susceptibility testing

Disc diffusion method, also known as the Kirby- Bauer-method was carried out according to the Clinical and Laboratory Standard institute guidelines [CLSI, formerly the National Committee for Clinical Laboratory Standards (NCCLS)] (Wayne, 2005).

Screening of crude bacterial extracts for antimicrobial activity

Preparation of bacterial extracts

Cells were cultured on MRS broth and incubated at 34ºC for 24hr. then cell free supernatants (CFS) were obtained by centrifugation at 1200 g for 10 minutes at 4ºC (Salih *et al.*, 2011).

Screening of crude bacteria extracts for antimicrobial activity involving two components: (a) determination of sensitivity of a pure bacterial culture to bacterial extract, and (b) explain the effect of pH, temperature and UV on bacterial extract.

Tested bacteria

Five bacterial isolates which include two Gram negative bacteria (G –ve): *E. coli and P. aeruginosa* and three Gram positive bacteria (G +ve): *S. aureus*, *B. subtilis*, and *C. perfringens*, were used as target pathogens to test the antibacterial activity of bacteriocin against these isolates.

Preparation of the tested bacterial suspension

Four morphological similar colonies of the tested organism that had been grown on to solid media were selected and picked up with a wire loop, transferred to a test tube containing 4 ml nutrient broth, and after visible turbidity appeared.

The culture was adjusted with nutrient broth to 0.5 McFarland Standard (Murray *et al.*, 2013). A sterile swab was dipped into each suspension and the excess fluid was removed by rotation of the swab against the side of the tube above the fluid level. The swab was streaked in three directions over the entire surface of the agar.

Inhibitory effect of the *L. lactis* subsp. *Lactis* extract (bacteriocin) against some pathogenic bacteria

The determination of the inhibitory effect of the selection isolates on tested bacteria was carried out according to the agar diffusion method (Héchard and Sahl, 2002). The radius of the inhibition zone was measured in mm.

The pathogens were spread over petri dishes contain Muller-Hinton agar (HiMedia). Wells were made by Cork boirel and 100 µl of the supernatants were poured on each well. The plates were incubated in inverted position after dried at 37ºC for 48 hours, the diameter of growth inhibition zones were measured with a ruler held on the back of the plates.

Characterization of the *L. lactis* subsp. *lactis* extract

According to the result of the inhibition zone sample 10, 26, and 42 had highest activity, and were choose for further study.

Effect of different pH

To determine the effect of pH on the bacteriocin activity, cell free culture supernatants (CFCS) of the isolates were adjusted to pH values of 2.5, 4.5, 6, 5 and 8.5 using NaOH or HCL by pH meter. The samples were assayed for the activity with the bacteriocin sensitive isolates (Awla *et al.,* 2014).

Effect of different temperature

To evaluate the effect of temperature on the bacteriocin activity, (CFCS) were exposed to temperate at –4 ºC, 30 and 100ºCand the results record (Awla *et al.,* 2014).

Isolation of plasmid DNA content from L42 isolate

DNA spin™ Plasmid DNA Purification Kits (Intron/ Korea) procedure is based on alkaline lysis method of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt. The following step describes the DNA-Spi™ Plasmid Purification Kit, which used silka adsorption to quickly and simply purify amount of nucleic acid.

A single colony picked up from a freshly streaked bacterial plate and inoculated into LB plus appropriate antibiotic. The culture was incubated overnight with shaking. Three to five (3 – 5) ml of bacterial culture harvested by centrifugation at 13,000 rpm for 30 sec. at room temperature then the supernatant were discarded. The pellet were resuspended in 250 µl of resuspension Buffer, vortexed or pipetted until no clumps of the cell pellet remain. Two hundred fifty µl of Lysis Buffer were added to resuspended cells. The tube was closed and gently mixed by inverting the tube several times (not exceeded 5min for lysis time). Three hundred fifty µl of Neutralization Buffer were added and gently mixed by inverting the tube several times. Centrifuged at 13,000 rpm for 10 min. at 4ºC during the centrifugation period, the column was inserted into the collection tube. After centrifugation, the supernatant transferred promptly into the column. Centrifuged at 13,000 rpm for 60 seconds the column removed from the collection tube, the filtrate discarded in collection tube. Then the spin column placed back in the same collection tube. Five hundred µl of Washing Buffer A were added and centrifuged at 13,000 rpm for 60 sec. the column removed from the collection tube, the filtrate discarded, and the spin column placed back in the same collection tube. Seven hundred µl of Washing Buffer B were added, centrifuged at 13,000 rpm for 60 sec. the filtrate discarded and the spine column placed back in the same collection tube. Centrifuged at 13,000 rpm for 60 seconds to dry the filter membrane. The columns were put into a clean and sterile centrifuge tube. 50 µl of Elution Buffer were added to the upper reservoir of the column, and left it stands for 1 min. then, the tube centrifuged assembly at 13,000 rpm for 60 sec.

Agarose gel electrophoresis technique

The method described by Sambrook and Russell (2001) with few modifications was followed and include the following steps:

The edges of a clean, dry glass plate sealed with tape to form a mold. The mold was set on a horizontal section of the bench. Sufficient electrophoresis buffer prepared to fill the electrophoresis tank and to cast the gel. A solution of agarose prepared in electrophoresis buffer at a concentration appropriate for separating the particular size fragments expected in the DNA samples (for our experiment 0.7g agarose in 100 ml TBE

buffer). The neck of Erlen Meyer flask plugged with Kim wipes. The slurry heated in microwave oven (at 98 °C for about 2 minutes). The flask transferred into a water bath at 55 °C. When the gel cooled, Ethidiumbromide was added to a final concentration of 0.5µg/ml (8 µl Ethidium bromide to 100 ml agarose). The gel mixed thoroughly. An appropriate comb was chosen for forming the sample slots in the gel. The warm agarose solution poured into the mold. The gel allowed to set completely (left to be solidified at room temperature 30 –45 min) then carefully the comb removed. Just enough electrophoresis buffers added to cover the gel to a depth of about 1mm). Samples of DNA were mixed with appropriate volume of loading dye (bromophenol blue); the standard is 2 µl of loading dye with 5 µl DNA. The sample mixture slowly loaded into the slots of submerged gels using a disposable micropipette. Size standards was loaded into slots on both the right and left sides on the gels. The lid of the gel tank was closed and then the electrical leads attached so that the DNA will migrate toward the positive anode, the gel run until the samples have migrated an appropriate distance through the gel. The electric current turned – off and the lid was removed. Finally we examined the gel by UV-transilluminator, and then photographed.

Sample loading

For our test, ten µl of plasmid DNA sample were mixed with 5µl of loading dye and the mixture was slowly loaded in to the wells on the gel; also a molecular weight marker was loaded too as control.

Running the electrophoresis

The electrophoresis apparatus was jointed to power supply, turned on and the samples electrophoresed at 10 volt/cm for 1 hour. The gel was visualized by UV – transilluminator, and then photographed.

Determination of Minimum Inhibitory Concentration (MIC) of nisin

The MIC of nisin was determined by turbidity method (spectrophotometer method) at 600 nm and the following dilutions were prepared (100, 200, 300, 400, 500, and 600) µg/ml and the MIC were used as a curing agent. The MIC was determined for nisin which inhibited bacterial growth of *E. coli* and *S. aureus*, contrasted with control sample that consisted of 10 ml of nutrient broth and 0.1 ml of activated culture of bacterial suspension, and then it was incubated at 37 ºC for 24 hours (Khoshnaw, 2010).

Results and Discussion

Isolation and identification of *L. Lactis* subsp.*lactis*

Forty – five isolate were identified. The identification of the isolates were carried out using conventional method based on cultural characteristics, cell morphology, Gram stain reaction biochemical properties and API 20 strep in addition to PCR technique as described by Barrow and Felthan (2003). All of the isolates tested had dry opaque colonies and mostly with powder texture. They were all Gram – positive, cocci and immotile. Biochemical characterization of the selected isolates (45) involved 10 diagnostic characters All isolates were found to be catalase and oxidase negative and they showed a small deal of variation in other biochemical characteristics, 93.33% of the isolates were able to hydrolyzed arginine, 88% carbohydrate fermentation, 28% grew in NaCl and 11% to express Nitrate reductase. Blood was haemolysed by 51% of

the isolates, showing a typical haemolysin, 68% of the isolates were able to utilize the carbohydrates such ribose, 37% were able to utilize arabinose, 37% were able to utilize mannitol, 77% were able to utilize sucrose, 82% were able to utilize lactose, lactose, 80% were able to utilize adanitol, 8% were able to utilize sorbitol, 11% were able to utilize trehalose, and 2% were able to utilize raffinose and inulin. The modern method API20 strep confirms that the isolates were *L. lactis* subsp. *lactis* according to the read of the interpretation chart (Table 1).

Molecular identification of *L. lactis* subsp. *lactis*

After extraction of genomic DNA from *L. lactis* subsp. *lactis* according to manufactures protocol, polymerase chain reaction (PCR) and gel electrophoresis were carried out in order to identify *L. lactis* subsp. *lactis* using 16S rDNA. Results indicated that the isolates were *L. lactis* subsp. *lactis*. The specific primers used produced a 900 bp band, which was shown in electrophoresis gel and measured using a 100 bp DNA ladder (Fig.1a and 1b). Similar results obtained by Awla (2012) who used the same technique to confirm that the isolated bacteria belong to *L. lactis* subsp. *lactis.*

To analyses and rapidly identify bacteria from microbial communities, classical physiological and biochemical tests are not adequate because the bacterial populations involved often have similar nutritional requirements and grow under similar environmental conditions. Currently, there is a wide variety of molecular strategies, such as PCR with specific primers, DGGE, RAPD, PFGE, FISH, RFLP, and PCR – ARDRA, which are available to determine the species diversity of *Lactobacillus* (Soto *et al.,* 2010).

The comparison of sequences of the 16S rDNA gene is a very reliable method for sorting and identifying bacterial species indicated to those of *L. lactis* subsp. *lactis*, similar results were reported by Siboukeur and Siboukeur (2012) who used the sequence to identify *L. lactis*. Because these genes are highly conserved and are present in large numbers of copies within each bacterial cell, their use as a molecular target has increased in the recent years (Ventura *et al.*, 2001).

Antibiotic resistance pattern in the bacterial isolates understudy

The antibiotic resistance pattern of seventy (45) *L. lactis* subsp. *lactis* isolates from cow milk were screened for their resistance to thirteen used antibiotics (Ampicillin, Amoxicillin/ Clavulanic acid, Imipenem, Gentamycin, Sulfamethoxazole/ Trimethoprim, Cephalothin, Clindamycin, Nitrofurantoin, Ciprofloxacin, Ceftriaxone, Chloramphenicol, Cefotaxime sodium, and Vancomycin using disc diffusion method also known as Kirby – Bauer method. The results of antibiotic resistance patterns for the bacterial isolates understudy are shown in table 2. From the table, the percentages of resistance for each isolates were obtained, and it is clear that the bacterial isolates revealed remarkable variation in their resistance to antibiotics used. The most resistant isolates were L42 with 100% and L25 with 92.5%. It's obvious from the table, the percentages of resistant to antibiotics were as follow: Cephalothin 95.55%, Ampicillin 93.33%, Amoxicillin/ Clavulanic acid 93.33%, Sulfamethoxazole/ Trimethoprim 91.11%, Cefotaxime sodium 84.44%, Clindamycin 77.77%,Ceftriaxone 71.11%, Nitrofurantoin 62.22%, Gentamycin 53.33%, Vancomycin 40%, Ciprofloxacin 35.55, Chloramphenicol 24.44%, and Imipenem 2.22%.

Klare *et al*. (2007) study the antimicrobial activity for 13 antibiotics against lactic acid bacteria (LAB). Generally, LAB was susceptible to Penicillin, Ampicillin, Ampicillin/ Sulbactam, Quinupristin/ Dalfopristin, Chloramphenicol and Linezolid. LAB exhibited broad or partly species – dependent MIC profiles of trimethoprim, Trimethoprim/ Sulfamethoxazole, Vancomycin, Teicoplanin and Fusidic acid. Three probiotic *Lactobacillus* strains were highly resistant to Streptomycin. Although Erythromycin, Clindamycin and Oxytetracycline possessed high antimicrobial activities, 17 *Lactobacillus* isolates were resistant to one or more of these antibiotics. Eight isolates of *L. lactis* subsp. *lactis* were isolated and identified by phenotypic and molecular characterization out of 23 isolates of lactic acid bacteria (LAB) from different dairy and non – dairy sources. Out of eight strains, four were obtained from dairy and four from non – dairy sources. Antibiotic susceptibility test to 21 different types of antibiotics was evaluated. All the isolates were resistant to Fosfomycin, Cefepime, Amikacin, Kanamycin, Neomycin, Nalidixic acid, Pipemidic acid, Norfloxacin, Sulphadiazine, Colistin, Polymixin, Teicoplanin, Nystatin, and Amphotericin B but susceptible to Ampicillin, Erythromycin, Spiramycin, Spectinomycin, Ciprofloxacin, Rifampicin, and Trimethoprim (Khemariya *et al.,* 2013).

Alrabadi (2012) investigated the antibiotic resistance profile of the *L. garvieae* isolated strains from Jordanian dairy products. Disk diffusion method was used. In particular, different antibiotics were tested against *L. garvieae*; the antibiotic disks were Trimethoprim (5μg), Clindamycin (2μg), Nitrofurantoin (300μg), Erythromycin (15μg), Ampicillin (15μg), Trimethoprim /Sulfamethoxazole (1.25/23.75μg), Polymyxin B(300U) and Tetracycline (30μg). The results indicated high and statistically significant effects of Trimethoprim, Nitrofurantoin and Trimethoprim/ Sulfamethoxazole on *L. garvieae*. Trimethoprim had the greatest antimicrobial effect on *L. garvieae* strains. All of the strains were sensitive to this antibiotic. Trimethoprim showed 20 mm inhibition zone in some strains. On the other hand, there were no antibiotic effects of Clindamycin and Polymyxin B on *L. garvieae.* Slight and statistically insignificant effects were found of other tested antibiotics. Antimicrobials have been used increasingly as a primary intervention for inhibition or inactivation of pathogenic microorganisms in foods (Davidson and Zivanovic, 2003). Generally, food antimicrobial agents are not used alone to control foodborne pathogens, but are included as components of the multiple approaches to microbial control. Lactic acid bacteria (LAB) from fermented products may act as a reservoir of antimicrobial-resistance genes that could be transferred to pathogens, either in the food web or, more importantly, in the gastrointestinal tract of human and animal (Belen Florez *et al.*, 2005). However, there are different views pertaining to the resistance susceptibility breakpoints of most antimicrobials in LAB [24]. Because of their long – time use in various food and feed preparations, LAB has been given the so – called GRAS status (generally recognized as safe).

In practice, this means that such LAB strains are food – grade organisms without imposing a health risk for the consumers or the environment. However, there are several studies that have documented the presence and expression of virulence genes and/or antibiotic resistance genes in food – associated LAB. When located on mobile genetic elements such as plasmids or (conjugative) transposons, antibiotic resistance traits can potentially be

transferred to the human or animal commensal flora and to pathogenic bacteria temporarily residing in the hosts. Therefore, it is very important to verify that probiotic and nutritional LAB strains consumed on a daily basis worldwide lack acquired antimicrobial resistance properties prior to considering them safe for human and animal consumption (Danielsen and Wind, 2003).

Antimicrobial activity of *L. lactis* subsp. *lactis* extract (Bacteriocin) against some pathogenic bacteria

The *L. lactis* subsp. *lactis* isolates were screened for their activities to inhibit the growth of the tested pathogenic bacteria by using the agar well diffusion assay. *E. coli, S. aureus, P. aeruginosa, B. subtilis, C. perfringens*, were used as target pathogens to test the antibacterial sensitivity of the isolate. The diameters of growth inhibition zones for each *L. lactis* isolates against each pathogenic bacteria were measured in millimeters (mm) and recorded (Table 3).

Most of the isolates extract have shown abroad inhibitory spectra since they inhibited four or five of the tested organism. It should be emphasized that *L. lactis* isolates have shown more strong activity against both Gram positive (*B. subtilis, S. aureus* and *C. perfringens*,) and Gram – negative bacteria (*P. aeruginosa* and *E. coli*). All the 45 selected isolated of *L. lactis* exhibited activity against at least two of the tested bacteria. Forty one (91%) of them were active against *B. subtilis*, and *P. aeruginosa*, 39(86%) of them were active against *E. coli*, 33(73%) of them were active *S. aureus*, and 40(88%) of them were active against *C. perfringens*.

It is clear from the table that isolate 15 and 41(20mm) showed a higher inhibition activity against *B. subtilis* followed by isolate 19 (15mm) against *B. subtilis*, whereas isolate 44(15mm) had a higher activity against *S. aureus* while isolate 20(13mm) had a higher activity against *P aeruginosa*, Isolate 4, 5, 8, and 17(9mm) had a higher inhibition activity against *C. perfringens* and isolate 6 and 29(10mm) had a higher activity against *E. coli*.

Among biopreservatives, bacteriocin has caught the attention of food researchers and industries. The bacteriocins are peptides of proteins having an antimicrobial activity against closely related microorganisms. These could be used as a natural food biopreservative due to its antimicrobial activity against food spoilage and pathogenic bacteria. Majority of the research on biopreservatives including bacteriocin focused on using lactic acid bacteria. In recent years, the major research interest is shown globally towards production of bacteriocin from Lactic Acid Bacteria (LAB).

Lactic Acid Bacteria (LAB) are comprised of at least ten genera: *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus* and *Vagococcus* (Yildirim, 2001). Most representatives of this group have been consumed for thousands of years, do not pose any health risk to man and are designated as GRAS (generally recognized as safe) organisms. Lactic acid bacteria play important role in majority of foods (dairy, vegetable, meat and fish) fermentation (Gulhamadov *et al.*, 2009), preservation and flavor development and acts as protective culture in various food systems, so, they can be selected and implemented in the food products. LAB exerts strong antagonistic activity against many microorganisms, including food spoilage organisms and pathogens (Sawa *et al.*, 2009). They are also known to have a probiotic effect on human health (Saxelin *et al.*, 2005).

Probiotic organisms alter the composition of the gastrointestinal flora. Bacteria like *E. coli*, *Streptococcus*, *Clostridium*, *Bacteriodes* and *Salmonella* are inhibited by probiotics. Their additional benefits include production of mucosal nutrients, elimination of toxins and reduction of faecal ammonia to decrease mucosal toxicity (Benkerroum *et al.*, 2007).

Effect of different pH on bacteriocin activity

The bacteriocin was active over a wide pH range 2.5–6.5. At the pH between 2.5 and 4.5, the activity was much higher than at neutral and basic pH values, the activity was decreased at pH 7.5 to 8 (Table 4). Authors Jack *et al.* (1995) and Siboukeur and Siboukeur (2012) reported similar result., affirm reports of other researches (Siboukeur and Siboukeur, 2012), who found that the higher activity of nisin between 2 and 4, similar with Delves-Broughton (2005) who report that the nisin shows increased solubility in an acid environment and becomes less soluble as the pH increases, differ with Badr *et al.* (2005) who stated that the pH of nisin was stable at level 5–6 and also different with the results of Noonpakdee *et al.* (2003) who found that the pH of bacteriocin was stable at the pH between 2–10.

Effect of pH on stability of nisin activity has long been known that nisin is unstable and becomes inactivated at high pH (Alwa *et al.*, 2014). The mechanism of inactivation is unknown but could be consequence of denaturation, chemical modification or combination of both. The dehydro residues are potentially susceptible to modification by nucleophiles that are present at high pH, such as hydroxide ions, deprotonated amines and deprotonated hydroxyl groups.

Reactions with these nucleophiles could be intramolecular or intermolecular, the latter causing cross-linking. Since, there are three dehydroresidues per molecule, large multimolecular aggregates could form by intermolecular reactions (Gross and Morell, 1971). The greater inhibitory effect of nisin at acidic than at neutral pH was probably due mainly to the greater solubility of nisin at acidic pH (Chen and Hoover, 2003). On the other hand, activity of nisin was higher and stable at pH 5 and 6 than other pH values when tested against *S. aureus* and *B. Cereus* reported by (Hurst, 1972).

Effect of different temperature values on bacteriocin activity

A number of physical treatment (heating and freezing) were applied for bacteriocin activity produced by *L. lactis*on both Gram negative and Gram positive bacteria (Table 5). The nisin was stable under the heat at 30ºC and freezing temperature at –4ºC for 15 minutes and the activity was decreased when the temperature increased to 100ºC (Table 5). This result agreed with Rattanachaikunsopotr and Phumkhachorn (2010) who found that nisin was exert maximum activity against pathogenic bacteria understudy at –4ºC and 30 ºC but this activity decreased when the temperature is raised to 100 ºC.

The results are not comparable to the results reported by Siboukeur and Siboukeur (2012) who found that nisin was stable at temperature 100ºC for 5,10,15 and 20 minutes and comparable with Delves-Broughton (2005) who found that nisin was stable at temperature 40ºC for 30 minutes. Nevertheless, Thomas and Wimpenny (2012) found that nisin did not inhibit the bacterium at either temperature of 4 ºC and 37ºC.

The production of bacteriocin by lactic acid bacteria is known to be temperature dependent; bacteriocin varies among different strains of lactic acid bacteria (Rattanachaikunsopotr and Phumkhachorn, 2010). Stability of nisin activity was recorded at 4ºC till 90ºC attained. Nisin was stable to low temperature and for long storage periods. The heat stability implies very compact molecular structure. In addition, lactococcin R proved to be stable to high temperatures at acidic pH values. However, biological activity was decreased at alkaline pH (Schillinger and Lücke, 1989; Yildirim and Johnson 1997). Murtaza *et al.* (2012) study the rate of thermal inactivation of the bacteriocins by heating crude samples of bacteriocin at various temperatures (37ºC, 65ºC, 100ºC and 121ºC) for 15 minutes. The bacteriocin was stable at 37ºC and 65ºC but partially stable at 100ºC and retained its activity. However, bacteriocin completely lost its activity when heated at 121ºC.

In colony count, at 65ºC less colonies of target organism (*S. aureus*) appeared ranging from 30–35 indicating that bacteriocin is active at this temperature and inhibit the growth of target organism i.e. *S. aureus*. At 100ºC, 240–270colonies appeared indicating that bacteriocin is partially active at this temperature but it showed complete inactivation at temperature 121ºC because colonies appeared were greater than 300 showing no inhibition against *S. aureus*. Crude sample of bacteriocin without any heat treatment showed maximum inhibition or activation revealed its proteinaceous nature that denatures at certain temperature.

Table.1 API 20 Strep Identification of *L. lactis* subsp. *lactis* isolates

Test	Result
VP	+
HIP	+
ESC	+
PYRA	+
GAL	-
BGUR	-
BGAL	-
PAL	-
LAP	-
ADH	+
RTB	+
ARA	+
MAN	+
SOR	-
LAC	+
TRE	+
INU	-
RAF	-
AMO	+
GLYG	-
BHEN	-

Table.2 Percentage of antibiotic resistance pattern for *L. lactis* subsp. *lactis*

***L.lactis* isolates**	**CEP**	**AMP**	**AMC**	**SXT**	**CTX**	**CLIN**	**CRO**	**F**	**GM**	**VAN**	**CIP**	**C**	**IPM**
%	**95.55**	**93.33**	**93.33**	**91.11**	**84.44**	**77.77**	**71.11**	**62.22**	**53.33**	**40**	**35.55**	**24.44**	**2.22**

Figure.1a Bacterial isolates showed on Agarose Gel Electrophoresis after amplification of 16srDNA

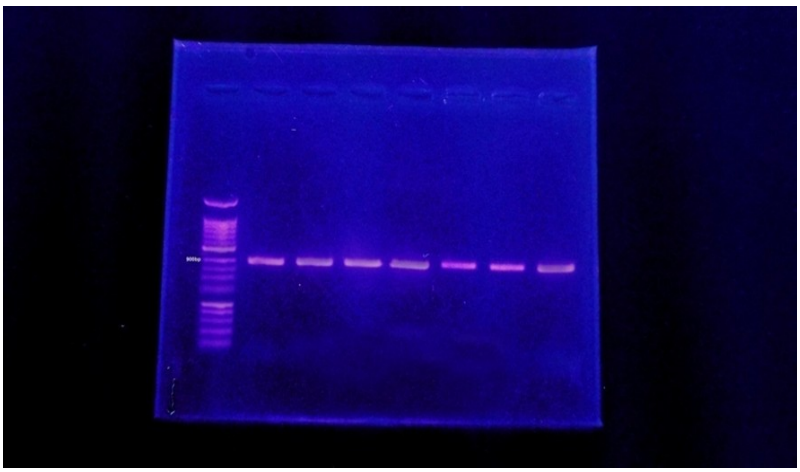

Figure.1b Bacterial isolates showed on Agarose Gel Electrophoresis after amplification of 16s rDNA

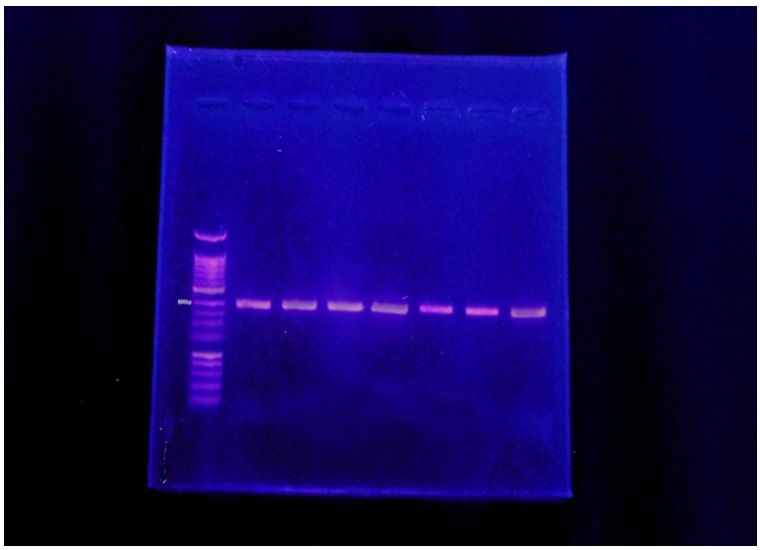

Table.3 Antimicrobial activity of *L.lactis* subsp. *lactis* extract against some pathogenic bacteria

No. of isolate	Diameters of growth inhibition zones in mm				
	B. subtilis	***P. aeruginosa***	***E. coli***	***S. aureus***	***C. perfringens***
Isolate 1	1	10	5	5	8
Isolate 2	3	9	3	6	7
Isolate 3	5	5	9	7	7
Isolate 4	8	7	9	9	9
Isolate 5	4	6	8	6	9
Isolate 6	6	5	10	8	7
Isolate 7	6	9	4	10	7
Isolate 8	9	6	7	7	9
Isolate 9	6	9	2	6	7
Isolate 1o	3	6	5	6	4
Isolate 11	1	10	5	5	1
Isolate12	6	5	7	8	6
Isolate 13	1	7	9	9	8
Isolate14	3	6	7	6	4
Isolate15	20	8	7	16	7
Isolate 16	5	8	0	0	5
Isolate 17	9	9	6	0	9
Isolate18	12	9	8	15	8
Isolate 19	15	4	5	1	7
Isolate20	9	13	6	0	7
Isolate 21	15	10	5	7	8
Isolate22	2	0	3	0	1
Isolate 23	5	3	0	10	4
Isolate 24	0	10	3	10	0
Isolate 26	3	6	1	6	2
Isolate 27	1	2	3	0	0.5
Isolate 28	1	2	5	9	1
Isolate 29	5	5	10	10	0
Isolate30	4	11	7	1	4
Isolate31	1	2	5	0	3
Isolate32	1	0	0	0	5
Isolate33	9	2.5	3	5	4
Isolate34	0	7	2	0	1
Isolate35	0	6	5	5	0
Isolate36	0	0	5	10	5
Isolate37	5	8	0	0	5
Isolate 3 8	5	8	0	0	5
Isolate39	1	2	3	0	0.5
Isolate40	1	0	0	0	5
Isolate41	20	8	7	16	7
` Isolate42	15	10	5	7	8
Isolate43	5	5	6	7	7
Isolate44	12	9	8	15	8
Isolate45	2	0	3	0	1

[25] Found Inhibition was positive if the width of the clear zone around the well was = 0.5mm

Table.4 Effect of pH on Isolate 10, 26, 42 extract activity against some pathogenic bacteria (Inhibition zone, mm)

Extract of isolate	pH	*B. subtilis*	*P. aeruginosa*	*E.coli*	*S. aureus*	*C. perfringens*
	2.5	17	16	13	13	17
	4.5	8	4	9	10	8
Isolate 10	6.5	4	1	6	5	1
	7.5	1	0	1	1	0
	8.5	0	0	0	0	0
	2.5	20	10	11	12	25
	4.5	9	8	8	9	12
Isolate 26	6.5	5	4	6	5	4
	7.5	1	0	1	0	1
	8.5	0	0	0	0	0
	2.5	25	21	15	13	13
	4.5	18	9	8	9	8
Isolate 42	6.5	7	3	2	4	3
	7.5	1	1	0	1	2
	8.5	1	0	0	0	1

Table.5 Effect of different temperature on isolate 10, 26, 42 extract activity against some pathogenic bacteria (inhibition zone, mm)

Extract of isolate	Temp. °C	*B. subtilis*	*P. aeruginosa*	*E. coli*	*S. aureus*	*C. perfringens*
	- 4	11	10	15	0	19
Isolate 10	30	7	6	10	5	8
	100	0	8	0	6	0
	-4	11	8	12	11	19
Isolate 26	30	9	6	10	8	10
	100	0	0	2	6	4
	-4	13	11	10	6	16
Isolate 42	30	17	7	8	5	11
	100	0	0	2	1	1

Table.6 The MIC results of Nisin against S. aureus and *E. coli* isolate at different concentration (µg/ml)

Concentration of nisin (µg/ml)	Optical Density (OD) (600nm)	
	O.D of Bacterial *S. aureus* isolate	O.D of Bacterial *E. coli* isolate
100	0.946	0.937
150	0.936	0.988
200	0.711	0.980
250	0.607	0.875
300	0.575	0.750
350	0.308	0.452
400	0.196	0.208
450	0.000	0.191
500	0.000	0.090
550	0.000	0.000

Determination of Minimum Inhibitory Concentration (MIC) of nisin

The minimum inhibitory concentration of nisin is determined by assaying the ability of bacteria to grow in broth culture containing different concentration of nisin. After examination the turbidity or visible growth, the lowest concentration that reduce the growth of the bacteria is considered as MIC of the nisin. The minimum inhibitory concentration of nisin was determined through the optical density reading at 600nm by spectrophotometer which indicated in table (6). It is clear from the table that MIC for *S. aureus* and *E.coli* was differ depending on the tolerance of these isolates understudy to this agent. The MIC for *S. aureus* is 400 µg/ml in a way that absorbance reading was decreased from 0.196 to 0.000 while for isolate *E.coli* the absorbance reading was reduced from 0.090 to 0.000 that 500µg/ml considered as MIC for mentioned isolate and this may due to that Gram negative bacteria possess outer membrane that make it more tolerant for nisin compared with Gram positive bacteria. MIC of nisin 400 and 500µg/ml.Inhibitory efficacy of nisin (0–20 µg/ml) against *S. aureus* in nutrient broth was investigated. The results revealed that addition of different concentration of nisin (5–20 µg/ml)in bacterial suspension affected *S. aureus* population significantly (P<0.05). *S. aureus* counts were reduced by approximately 4.5 – 4.8 log CFU/ml when nisin at the concentrations of 15–20 µg/ml was applied, whereas there was only a slight decrease of 2.5–3.3 log CFU/ml at nisin concentrations of 5–10 µg/ml at the end of the test period. This finding indicated that the degree of microbial inactivation was directly related to the concentration of nisin (Vongsawasdi *et al.*, 2012).

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