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EFFICIENCY OF NON-RIBOSOMAL LIPOPEPTIDES (NRPS) PRODUCED BY SALT TOLERANT BACTERIA AGAINST SOME PATHOGENIC BACTERIA.

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ABSTRACT: Halophilic bacteria are micro-organisms living in hypersaline environments. They have many potential in several fields in life such as industry and agriculture. In this study two isolates were obtained from saline ponds belonging to local company for salts and minerals, Qarun Lake, Fayoum, Egypt, on nutrient agar (NA) medium prepared using pond water. The 16S rRNA sequence analysis showed that the isolate QSLA16 had 80.15% similarity to Uncultured bacterium clone QAMU23 as well as Sphingomonas sp. LE-239 with 79.9% similarity, while the other isolate QSLA17 was similar to Bacillus sp. strain 6 with 74.05%. QSLA16 cell was rod shaped, not spore-forming and gram negative, while QSLA17 cell was sporulated long rod and gram positive. The obtained results of halotolerance assay showed that QSLA16 isolate is a halophilic bacteria as it wasn't able to grow in the absence of salt (0% NaCl). While isolate QSLA17 could grow in the absence of salt (0% NaCl) and therefore considered as halotolerant. The presence of the genes encoding for non-ribosomal lipopeptides (NRPs) was detected in both isolates using PCR technique and these NRPs were determined using high performance liquid chromatography (HPLC). The lipopeptides surfactin and fengycin were produced by both tested isolates, while mycosubtilin was only produced by QSLA17. The antibacterial activities of these salt tolerant isolates against halotolerant clinical pathogens (Staphylococcus aureus, Salmonella typhi and Acinetobacter baumannii) were determined and their potential use in controlling these pathogens as contaminants of sterilized salty whey was examined.

Key words: Halophilic bacteria; non-ribosomal lipopeptides; anti-pathogens; solar salterns; salty whey.

INTRODUCTION

Hypersaline ecosystems are widely distributed habitats including a variety of terrestrial lakes and deep-sea basins with salt concentrations exceeding three times seawater up to saturation. Hypersaline habitats can be divided into two main types, thalassohaline and athalassohaline waters (Naghoni *et al* 2017). Thalassohaline waters or brines are of marine origin and have ionic composition similar to that of seawater. Athalassohaline waters or brines such as the Dead Sea and soda lakes are often found inland and therefore not directly connected to marine waters. Solar salterns are widely distributed and can be found at sea level in arid and semi-arid regions, both as naturally occurring or human-made salterns (Ventosa and Arahal, 2011).

Halophiles are salt loving microorganisms present in saline habitats as normal inhabitants (Ventosa and Nieto, 1995) and can be found in all three domains of life Archaea, Bacteria, and Eukarya (Edbeib *et al* 2016). They considered a highly promising source for discovering novel biosurfactant molecules that used currently for pharmacological, food, and cosmetics applications (Kennedy *et al* 2011). Also biosurfactant molecules such as lipopeptide biosurfactants (surfactin, fengycin and iturin) had antimicrobial activities in a way of preventing biofilm formation by bacterial clinical pathogens due to its anti-adhesion activity (Donio *et al* 2013).

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Whey is a liquid producing during making cheese by acid or proteolytic enzyme method (rennet enzymes) (Hammam *et al* 2017). Whey and whey components are value-added ingredients in many foods including dairy, infant formulas, sports nutrition foods, meats, bakery, confections, snack foods, beverages and other food products to develop functionality of foods in the food industry. Some studies observed that it's considered as a natural medium for spoilage and pathogenic bacteria (Leroy, 2004). Benkerroum and Tamime, (2004) revealed that *Lactococcus, Leuconoctoc,* and Enteroccoci species were isolated from Moroccan whey, with pathogenic microorganisms such as coliforms, *Escherchia coli, streptococci, Staphylococcus aureus* and *Listeria monocytogenes*. Bouymajane *et al* 2018 studied the microbiological quality of whey marketed by street traders in Meknes city of Morocco. The percentages of pathogenic bacteria found in the total of lben samples analyzed are 75% for *Escherchia coli*, 75% for *Staphylococcus aureus*, 55.55% for *Clostridium perfringens* and 19.88 % for *Listeria monocytogenes*.

Donio *et al* (2013) isolated halophilic *Bacillus* sp. BS3 from solar salt works in Thamaraikulam, Kanyakumari district, Tamilnadu, India. Screening for biosurfactants using simple methods such as oil spreading test and emulsification activity supported for positive production of lipopeptide biosurfactants. The anti-adhesion activity of lipopeptide bisurfactants were effectively inhibited the growth of the pathogenic bacteria (*Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella typhi*). Romanenko *et al* (2007) isolated an aerobic, Gram-negative, yellow-pigmented, halophilic strain *Sphingomonas molluscorum* sp. nov. from a marine bivalve (*Anadara broughtoni*) collected from Peter the Great Bay, Sea of Japan. It was found to exert a remarkable inhibitory activity against a number of Gram-positive micro-organisms.

Qarun Lake is located in north of Fayoum Governorate and currently saline, turbid without surface outflow (Edbeib *et al* 2016). Solar salterns of Qarun Lake typically consist of several ponds interconnected to form the so-called multipond system. These ponds are used by local company for salt and minerals production. These different ponds has a different salt concentrations, therefore has a different microbial community.

In this study two halophilic bacterial strains were isolated from solar saltern ponds belonging to local company for salts and minerals, Qarun Lake, Fayoum. The characteristics of the metabolities of the halophilic isolates, such as non-ribosomal lipopeptides (NRPs) biosurfactants production as well as biotechnological application such as biocontroling clinical pathogens are also considered during this study.

MATERIALS AND METHODS

Bacterial isolates

During September 2019, water samples were collected from saline ponds belonging to local company for salts and minerals, Qarun Lake, Fayoum. There were four solar salterns ponds with different salt concentrations (4, 8.2, 17.2, 24.2 %) as measured by rafractometer. Ten liters of water were collected from each pond in a sterile plastic jars and placed in ice packs before their transfer to the laboratory of Biotechnology, Botany Department, Faulty of Agriculture, Menoufia University. The collected water samples were filtered to remove impurities, analyzed for salt concentration by rafractometer, and pH by pH meter, before use.

A sterilized Nutrient agar medium (NA) was used for bacterial isolation by streaking 100 µl of water sample on NA plates. The plates were incubated at 30°C for 7-30 days (Benito et al 2004). A subsequent streaking and sub culturing were performed on NA medium prepared by pond water for purification and the purified isolates were kept in 30% glycerol in deep freezer till further use.

Halotolerant clinical pathogens

A clinical pathogens *Staphylococcus aureus, Klebsiella pneumonia, Escherichia coli,*

Salmonella typhi, Acinetobacter baumannii and *Proteus mirabilis* were obtained from Medical laboratories of Zagazig University, Egypt.

Morphological and physiological characterization of the isolates

Colonies developed on the plates were morphologically examined for their shape, pigmentation, elevation and optical properties. In addition shape, gram staining and endospore formation (Hucker & Conn 1923), motility by hanging drop method (Goszczynska *et al* 2000) and catalase activity (whittenbury 1964) were also tested.

Bacterial isolates were screened for salt tolerance using NA medium supplemented with various levels of NaCl (0, 7, 12.5, 20 and 22%). The plates were incubated for 7 days at 30°C and the growth were recorded (Ramadoss *et al* 2013).

Molecular Identification of the isolates by 16S rDNA

DNA Extraction

Genomic DNA was extracted from bacterial cells grown aerobically in nutrient broth according to the protocol of Broderick *et al.* (2004). The extracted DNA was purified and visualized under UV by staining with ethidium bromide (Sambrook *et al* 1989) and kept at -20°C until use (Mwirichia *et al* 2010).

Polymerase Chain Reaction (PCR)

Amplification of the 16S rRNA gene sequence, using bacterial primers 27F and 1492R Table (1), was performed using a model PTC-100 thermal cycler (MJ research inc, USA). The PCR mix consisted of 40 µl mixture containing 0.25 µl of genescript Taq, 1.0 µl (5-pmol) of 27F forward primer, 1.0 µl (5-pmol) of 1492R reverse primer, 1 µl of template DNA, 2.5 µl of dNTPs mix (2.5mM), 4.0 µl PCR 10x buffer (genescript) and 30.25 µl of PCR water. The PCR cycling consisted of an initial activation of the enzyme at 94°C for 5 minutes followed by 35 cycles of a denaturation step at 94°C for 45 seconds, primer annealing step at 53°C for 60 seconds, chain elongation step at 72°C for 2 minutes and a final extension at 72°C for 5 minutes (Roux, 1995). The presence of amplified products was confirmed by applying 7 µl of PCR product on 1% agarose gel in 1X TAE buffer containing ethidium bromide stain then visualized by Gel documentation system (Bio-Rad Laboratories) (Sambrook *et al* 1989). The PCR products obtained were purified using the QIA quick PCR purification Kit protocol (Qiagen) and sent for sequencing.

16S rRNA sequencing

Sequence was carried out by Colors Laboratories (El-Etihad Square, Maadi-Cairo-Egypt) according to the laboratory instructions. The sequences were edited by eliminating all gaps (Complete deletion option), using the CHROMAS PRO software 1.5 version.

Evolutionary relationships of taxa

The 16S rRNA gene sequences were then compared with those published in GenBank databases using Basic Local Alignment Search Tool (BLAST) in the National Centre for Biotechnology Information (NCBI) website (htt//www.ncbi.nih.gov). Alignment was done using CLUSTAL W 1.6 software (Altschul *et al* 1997). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). Evolutionary analyses were conducted in MEGA X (Kumar *et al* 2018). The sequences were aligned using the embedded muscle algorithm and the output was used to build a phylogenetic tree by calculating distance matrices for Neighbor-Joining (NJ) analysis.

Table (1): Primers used for PCR 16S rRNA sequencing analysis (van der Lelie *et al* 2011).

Primer name	Orientation	Priming site	Sequence (5´- 3´)
27F	Forward	8-27	AGAGTTTGATCCTGGCTCAG
1492R	Reverse	1492-1513	GGTTACCTTGTTACGACTT

Detection of non-ribosomal lipopeptides (NRPs) genes in both isolates using polymerase chain reaction (PCR)

The isolates QSLA16 and QSLA17 were checked for presence of NRPs synthetase genes using PCR. In this respect, four genes were amplified *Srf* (surfactin-surfactin), *Pps* (plipastatin-fengycin), *Myc* (mycosubtilin-iturin) and *Kur* (kurstakin- kurstakin) by four degenerated primers, As1-F/ Ts1-R; Ap1-F/ Tp1-R; Am1-F/ Tm1-R and AKs-F/ TKs- R, respectively (Tapi *et al* 2010), Table (2). The PCR conditions were performed as described by (Abderrahmani *et al* 2011 and Hussein & Fahim 2017). The initial denaturation step was performed at 95°C for 2 min, followed by 35 cycles of three steps; denaturation at 95°C for 30 s; annealing step for 30 sec, at 43°C with (As1-F/ Ts1-R), 44.4 °C with (AKs-F/ TKs-R) and at 58°C with (Ap1-F/ Tp1-R), an extension step of 45 sec at 72°C except with Ap1-F/Tp1-R and Aks-F/Tks-R primers, extension step performed for 75 sec at 72°C, at the end 5 min of extension step was lanced at 72°C.

Determination of Lipopeptides (NRPs) using high performance liquid chromatography (HPLC)

Bacteria were cultured in Landy modified medium for 48 h (stationary phase); the bacterial cells were discarded from culture medium by centrifugation with 14.000 r.p.m for 20 min at 5°C. For lipopeptides extraction, 0.5 ml supernatants samples were passing through clean C_{18} cartridges (protocol of Alltech, Fr). The concentrations of lipopeptides families were determined by the spectrum of (HPLC) reverse phase according to the method used by Fahim, (2017).

Antagonistic effect of used isolates against salty whey contaminating pathogens on nutrient agar plates

The antagonistic effect of the isolates QSLA16 and QSLA17 was examined against used pathogens to define their potential use as whey preservative agents.

Salty whey was obtained from Faculty of Agriculture, Menoufia University, Egypt. The salinity of whey was estimated with rafractometer and found to be 9.5%. NA medium (3.5% NaCl) was prepared and sterilized, then cooled at 55°C and inoculated with studied pathogens in rate of 10 µl/ 30 ml medium, then poured into petri dishes. After solidification, 3 sterilized filter paper discs (6 mm. diam.) were layed on the surface of the medium. Ten µl of a pasteurized salty whey (70°C for 20 min.) mixed (11) with serial dilutions (10^{-4} or 10^{-5} or 10^{-6}) of bacterial culture (OD_{630} .6) were applied on sterilized discs and checked for clear zones after incubation for 3 days at 30°C. Whey only and undiluted culture of the isolates were examined separately against used clinical pathogens and used as a control. The inhibition zone of culture dilutions and zones detected around salty whey only (if any) were calculated as follows:

Culture dilution inhibition zone = diameter of total inhibition zone- inhibition zone of whey

Table (2) Degenerated primers used for detection of non-ribosomal lipopeptides genes.

Primers Names	Sequences of primers	Expected Fragment size (bp)	(NRLPs) Identified	References
As1-F	CGCGGMTACCGVATYGAGC	893/929	Surfactins	Tapi *et al.,* (2010)
Ts1-R	ATBCCTTTBTWDGAATGTCCGCC			
Ap1- F	AGMCAGCKSGCMASATCMCC	416/419	Plipastatin	
Tp1- R	GCKATWWTGAARRCCGGCGG			
Am1-F	CAKCARGTSAAAATYCGMGG	422/425/431	Mycosubtilins	
Tm1-R	CCDASATCAAARAADTTATC			
Aks-F	TCHACWGGRAATCCAAAGGG	Not detected	Kurstakins	Abderrahmani *et al.,* (2011)
Tks-R	CCACCDKTCAAAKAARKWATC			

Statistical analysis

The obtained data were further subjected to statistical analysis using analysis of variance (ANOVA), and the differences between means was compared by a high-range statistical domain using Tukey's test and Tukey's post hoc analysis in order to distinguish homogeneous–heterogeneous groups among different variables. The multiple comparisons of means were considered at probability level $p = 0.05$. The obtained results are presented as the average means ± standard deviations (SD) of triplicate.

RESULTS AND DISCUSSION

Isolation and purification of halophilic/halotolerant bacteria from solar salterns water

Collection sites

Two bacterial isolates, QSLA16 and QSLA17 were obtained from two diferent saline ponds. The first was isolated from the fourth pond with 24.2% salt concentration, while the second isolated from the second pond with 8.2% salt concentration on nutrient agar (NA) medium that prepared by dissolving their component in saline water taken from the pond water samples instead of distilled water.

Morphological and Biochemical characterization of used isolates

Morphological, colonial and biochemical characterizations, which aids in partial identification of microorganism (Suthar et al 2017), were checked. The results showed that isolates QSLA16 and QSLA17 had round, ragged, translucent, flat, small and shinny colonies. The colony color of isolate QSLA16 was yellow, while QSLA17 had a creamy color. The isolate QSLA16 was rod shaped and gram negative, while isolate QSLA17 was long rod and gram positive when stained by Gram reaction and examined by microscope according to (Hopwood, 1960). The two isolates were motile when examined by hanging drop method. Pasteurization of pure culture showed that QSLA16 isolate not spore-former, while QSLA17 capable of spore formation. They were catalase producer when examined by hydrogen peroxide (H_2O_2).

Results showed that QSLA16 wasn't able to grow in the absence of salt (0% NaCl) and in high salt concentrations (20 & 22%), while grew well on NA medium with 7 & 12.5% NaCl. On the other hand QSLA17 could grow in the absence (0% NaCl) and in presence (7 and 12.5% NaCl) of salt. Mohamedin *et al* (2018) showed that moderate halophiles growing optimally in medium with 0.5 to 2.5 M NaCl (3% to 15% NaCl) and can't grow in the absence of salt. Halotolerant capable of growth in the absence and presence of salt, but tolerant of varying concentrations of salt (0-5% NaCl) (Ventosa and Nieto, 1995). Accordingly QSLA16 was considered as moderately halophilic while QSLA17 considered as halotolerant bacteria.

Although QSLA16 strain was isolated from saline water pond with 24.2% salt concentration, it couldn't grow on NA medium prepared using distilled water and 20% NaCL. The NaCl requirement of these organisms has been known for many years and NaCl could not be replaced by other salts. Also potassium was essential for optimum growth and required for normal pigmentation. Magnesium was another essential ion for optimal growth, more of the rods appeared "normal" on medium containing the original concentration of magnesium. At lower concentrations the organisms grew as spheres and seemed to adapt to the low concentrations of Mg^{2+}. The spherical form was retained when these cells were returned to medium containing the original concentration of magnesium (Gibbons, 1969). Using shake cultures, Sehgal and Gibbons (1960) found that 10 ppm of Fe^{2+} increased the yield of cells. Small amounts of manganese (0.05 ppm) provided some stimulation of growth and a marked increase in pigmentation. So isolate QSLA16 couldn't grow on NA medium prepared using distilled water and high salt concentration of NaCL (20%)

because of the absence of other essential elements such as potassium, magnesium, iron and manganese which all required for optimum growth.

Naghoni *et al* (2017) isolated halobacterial strains from the three basins of Lake Meyghan which have a different salinities (a green ~50 g/l salinity brine, a red ~180 g/l salinity brine and a white ~300 g/l salinity brine).

Molecular identification of the isolates by 16S rDNA genes

Beside morphological and biochemical characterization of the isolates, the 16S rDNA gene were sequenced and analyzed. The resultant sequences were compared with those identified and deposited in NCBI BLAST database. The sequence analyses of the 16S rRNA gene of the isolates showed that isolate QSLA16 was most closely related to strain Uncultured bacterium clone QAMU 23 and *sphingomonas sp*. LE-239 with 80.15% and 79.9% similarity respectively, while QSLA17 was similar to *Bacillus* sp. strain 6 with similarity of 74.05%.

Phylogenetic affiliation showed that isolate QSLA16 belonging to class Alphaproteobacteria, while QSLA17 was affiliated to phyla firmicutes as shown in Figure (1). Naghoni *et al* (2017) isolated 361 halobacterial strains from the three basins of Lake Meyghan which have a different salinities. These strains belonging to several classes such as Gammaproteobacteria, Alphaproteobacteria, Bacteroidetes and Firmicutes with different genera.

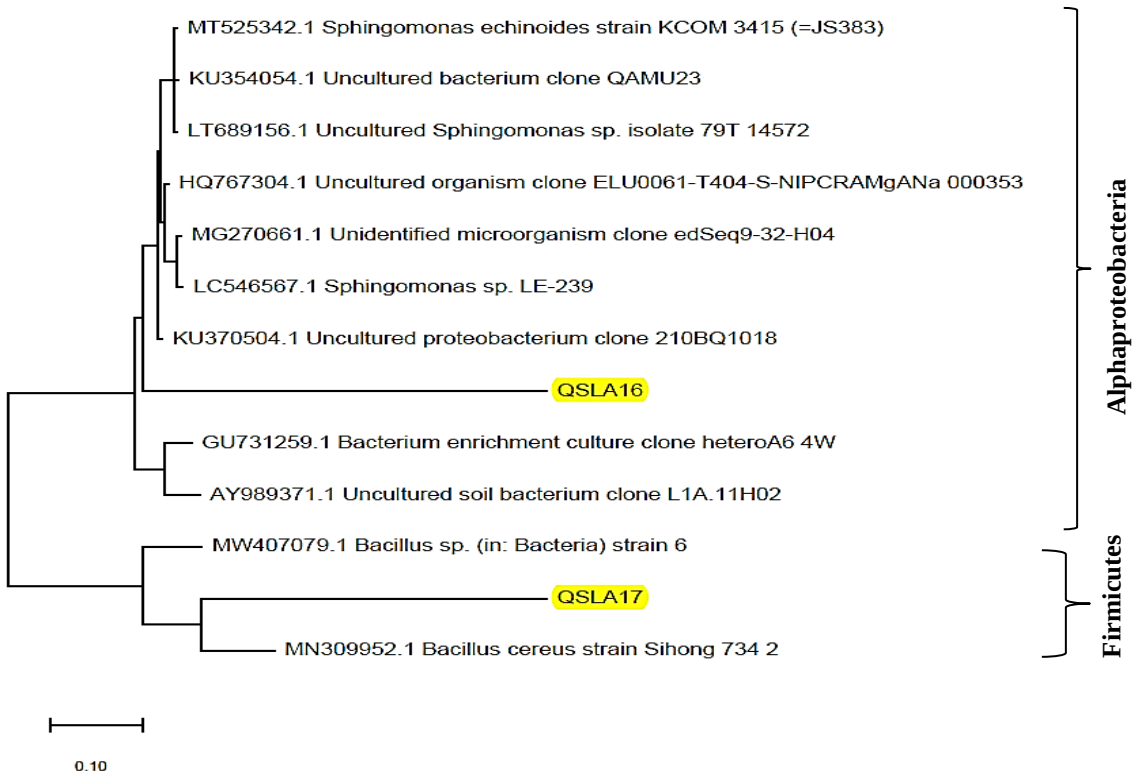

Figure (1): Phylogenetic tree constructed using neighbor-joining analysis, based on 16S rDNA sequences, of QSLA16 and QSLA17 isolates and most closely related sequences deposited in the GenBank.

An aerobic, Gram-negative, yellow-pigmented, designated strain KMM 3882 was isolated from a marine bivalve (*Anadara broughtoni*) collected from Peter the Great Bay, Sea of Japan. Strain KMM 3882 was found to exert a remarkable inhibitory activity against a number of Gram-positive microorganisms. According to the phylogenetic analysis based on 16S rRNA sequences strain KMM 3882 showed the highest 16S rRNA sequence similarity to *Sphingomonas dokdonensis* DS-4 (97.3 %); similarities of 96.5–96.7% were obtained with *Sphingomonas pituitosa* DSM 13101, *Sphingomonas azotifigens* NBRC 15497, *Sphingomonas asaccharolytica* NBRC 15499, *Sphingomonas trueperi* DSM 7225 and *Sphingomonas panni* DSM 15761. It was classified as a novel specie of the genus *Sphingomonas*, for which the name *Sphingomonas molluscorum* sp. nov. is proposed (Romanenko *et al* 2007).

Sphingomonas japonica sp. nov. is a novel halobacterial strain isolated from a marine crustacean specimen obtained from the Sea of Japan by Romanenko *et al* (2009) and subjected to a polyphasic study. Comparative 16S rRNA sequence analysis positioned the novel strain in the genus *Sphingomonas* with 96.1 % similarity to *S. trueperi LMG 2142*, *Sphingomonas dokdonensis DS-4* and *S. azotifigens NBRC 15497*.

Rathakrishnan *et al* (2022) isolated twenty-eight moderately halophilic bacteria from three salterns in Tamil Nadu, Kerala and Goa, India. Among them nine isolates showed 97–99% similarity to genus *Bacillus* and one isolate was most closely related to *Oceanobacillus* with 98% similarity, while twelve isolates showing 96-99% similarity to genus *Staphylococcus*, two isolates belonged to *Pseudomonas* with 98% similarity, one isolate showed 98% similarity to genus *Enterobacter* and three isolates were most closely related to *Enterobacter*, *Ochrabactrum* and *Stenotrophomonas* with 98%, 98% and 96% similarity respectively.

74 halophilic bacteria isolated by Menasria *et al* (2019) from Algerian saline lakes ecosystems (Sebkha and Chott) located in arid and semi-arid ecoclimate zones. Out of them 16 isolates were most closely related to *Halomonas*, 18 isolates belonged to *Bacillus*, 7 strains were similar to *Oceanobacillus*, 5 isolates were identified as *Virgibacillus*, 10 isolates were related to *Halobacillus*, 2 isolates belonged to *Thalassobacillus*, one isolate was most closely related to *Lentibacillus* with 99-100% similarity.

Detection of non-ribosomal lipopeptides (NRPs) genes in used isolates using polymerase chain reaction (PCR)

The presence of the genes encoding for NRPs in the two isolates QSLA16 and QSLA17 was examined using PCR technique. Four genes were amplified *Srf* (surfactin-surfactin), *Pps* (plipastatin-fengycin), *Myc* (mycosubtilin-iturin) and *Kur* (kurstakin- kurstakin) using degenerated primers Table (2). The primers set 1 used for surfactin detection amplified one main fragment of 450 bp, while primers set 2 used for fengycin detection amplified one main fragment of 950 bp. Degenerate primers set 3 used for mycosubtilin detection amplified one main fragment of 419 bp (Figure, 2). Degenerate primers set 4 used for kurstakin detection, hadn't amplified any fragments, thus the gene encoding for kurstakin wan't present in any isolate.

QSLA16 and QSLA17 were found to contain surfactin and fengycin genes while mycosubtilin gene was detected only in QSLA17 isolate.

The non-ribosomal lipopeptides (NRPs) are cyclic lipopeptides (CLPs) and considered as common secondary metabolites. The CLPs are a class of metabolites with structural diversity produced by multifarious bacterial genera (Xiao *et al* 2022). They are built up by huge multi-modular enzymes called non-ribosomal peptide synthetases. These synthetases are organized in modules constituted of adenylation, thiolation, and condensation core domains. As such, each module governs, according to the collinearity rule, the incorporation of a monomer within the growing peptide. The release of the peptide from the assembly chain is finally performed by a terminal core thioesterase domain (Duban *et al* 2022).

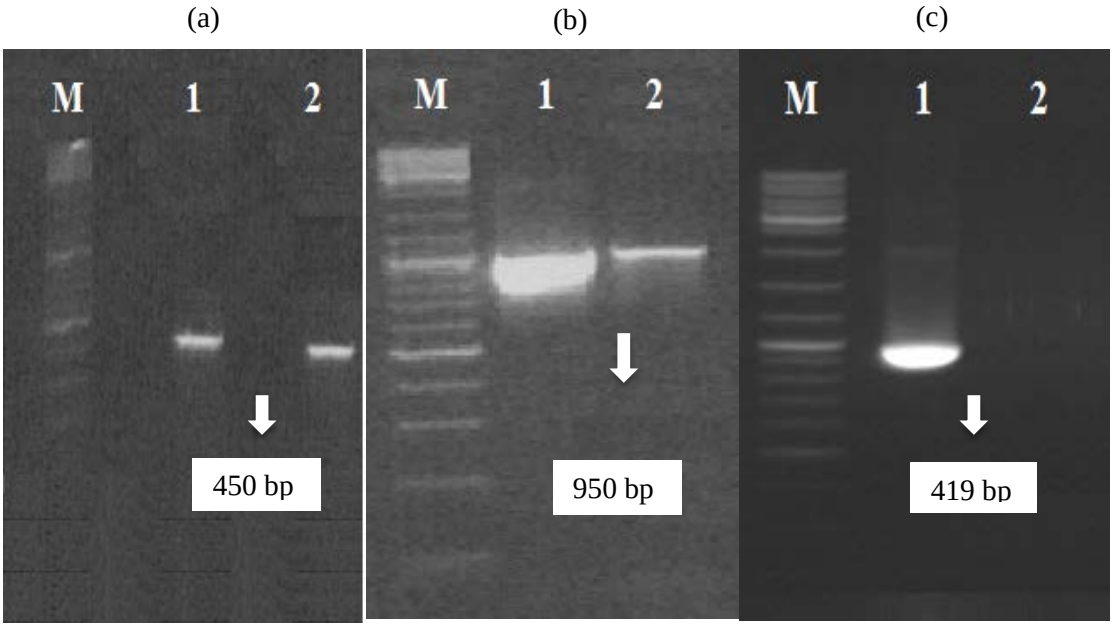

Figure (2): PCR showing amplificatin of 450 bp (a), 950 bp (b) and 419 bp (c) gene encoding for surfactin, fengycin and iturin production, respectively by QSLA17 (lane1) and QSLA16 (lane 2).

There are three families of NRPs/CLPs being of particular importance, namely surfactins, iturins and plipastatins (fengysin), all consisting of a short cyclic oligopeptide (hydrophilic head) linked to the tail of a fatty acid (hydrophobic tail). Surfactin sequences comprise of seven amino acids and a *ß*-hydroxy fatty acid chain containing 12–16 carbons (Figure, 3). The iturin family sequences are composed of heptapeptides and a *ß*-amino fatty acid chain of 14–17 carbon atoms, which consists of bacillomycin D, F, L, Lc, iturin A, AL, C and mycosubtilin (Figure, 4). The plipastatin family comprise of ten amino acids and a *ß*-hydroxy fatty acid containing 14–18 carbon atoms (Figure 5) (Xiao *et al* 2022).

Tapi *et al.* (2010) reported that the use of degenerated primers is helpful in screening for various non-ribosomal genes which supported to detect a new non-ribosomal molecules and it facilitate the study and genetic potential knowledge of lipopeptide molecules biosynthesis.

These lipopeptides as biosurfactants have been used in biomedical and pharmaceutical applications as antitumor, anti-mycoplasma, anti-adhesive, and anti-microbial (biocontrol agents) because of their property of inhibition of growth of a variety of microorganisms including phytopathogens such as *Pythium ultimum* , *Botrytis cinerea*, *Pseudomonas syringae*, *Fusarium Graminearum*, *Rhizoctonia solani*, *Xanthomonas axonopodis* pv. *glycines*, *Sclerotinia sclerotiorum* and *Magnaporthe grisea.* Also they can be used as preservative agents in fermented food products, but the uses of lipopeptides as antimicrobial agents or food preservative are limited because of their sensitivity to proteases. This sensitivity can be prevented using cyclic peptides such as non-ribosomal lipopeptides/ CLPs (Raj Meena and Kanwar, 2014).

Determination of lipopeptides (NRPs) using high performance liquid chromatography (HPLC)

HPLC results showed that isolate QSLA16 produced surfactin and fengycins, Figure, (6), with concentrations of 98 $mg.l^{-1}$ and 45 $mg.l^{-1}$, respectively, while isolate QSLA17 produced surfactin, fengycins and iturin, Figure, (7), with concentrations of 112 $mg.l^{-1}$, 38 $mg.l^{-1}$ and 67 $mg.l^{-1}$, respectively, (Table, 3).

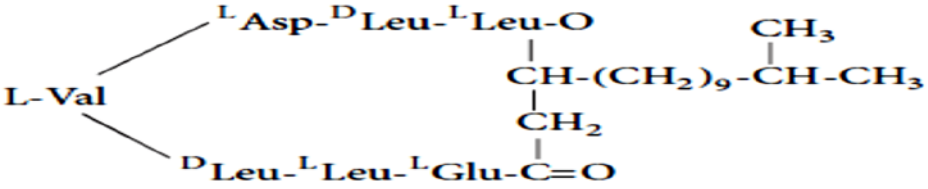

Figure (3): Heptapeptide cyclic structure of surfactin, containing both hydrophobic and hydrophilic amino acids. The structure containing amino acids two D-amino acids (Leu, Leu) and five L-amino acids (Val, Asp, Leu, Glu, and Leu), indicates its amphipathic nature (Raj Meena and Kanwar 2014).

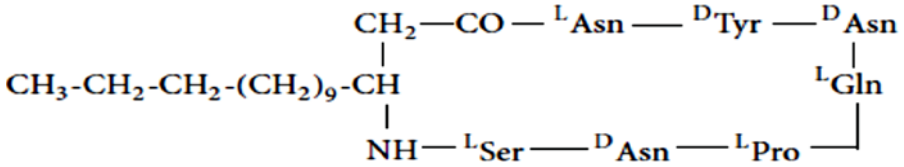

Figure (4): Cyclic structure of lipopeptide iturin, containing seven amino acid residues attached to a 14-carbon chain indicates its amphiphilic nature. The amino acids involved in this structure are three D-amino acids (Tyr, Asn, and Asn) and the four L-amino acids (Pro, Ser, Asn, and Gln) (Raj Meena and Kanwar 2014).

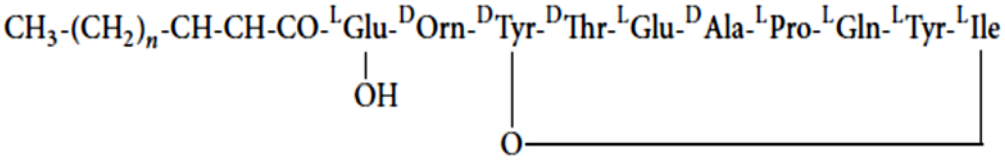

Figure (5): Primary cyclic structure of fengycin A. Structure containing peptide chain of ten amino acids and a β-hydroxy fatty acid chain that can vary according to Fengycin isomer fromC-14 to C-17 carbons. In the structure, the amino acids are six L-amino acids (Glu, Glu, Pro, Gln, Tyr, and Ile) and four D-amino acids (Tyr, Orn, and Thr, Ala). (Raj Meena and Kanwar 2014).

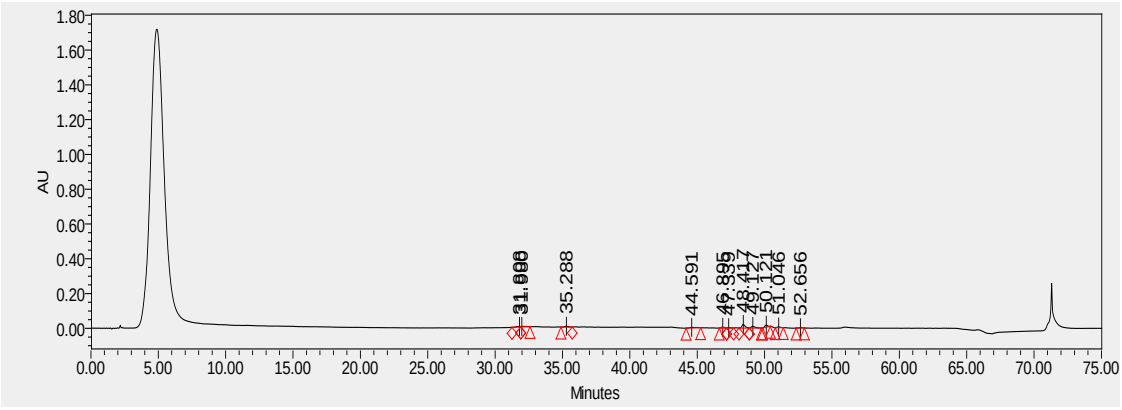

Figure (6): HPLC chromatogram of non-ribosomal lipopeptides in the supernatant of QSLA16. Surfactin determined between 5-35 minutes while fengycin appeared between 35-55 minutes.

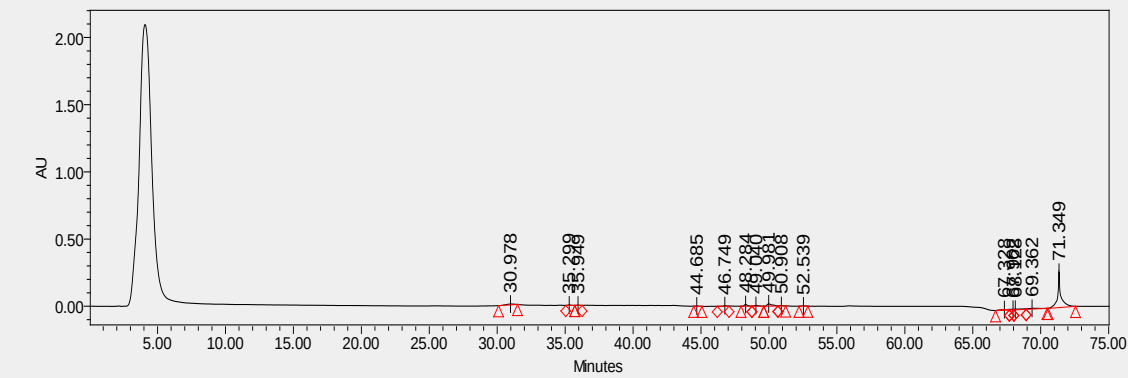

Figure (7): The HPLC chromatogram of non-ribosomal lipopeptides in the supernatant of QSLA17. Surfactin determined between 5-35 minutes, fengycin appeared between 35-55 minutes and iturin determined after 55-75 minutes.

Table (3): The NRPs concentrations as determined by HPLC in QSLA16 and QSLA17.

Lipopeptides types / Isolates	Concentrations detected by HPLC		
	Surfactin Type	**Fengycin Type**	**Mycosubtilin Types**
QSLA17	112 mg.l^{-1}	38 mg.l^{-1}	67 mg.l^{-1}
QSLA16	98 mg.l^{-1}	45 mg.l^{-1}	Not detected

These results proved that the isolates QSLA16 and QSLA17 are producers of two families of lipopeptides at least; surfactin, and fengycin. These compounds known as biosurfactants and a good alternative antimicrobial (Fahim and Hussein, 2016).

Antagonistic effect of used isolates against salty whey contaminating pathogens on nutrient agar plates

QSLA16 showed antibacterial activity against *salmonella typhi*, *Acinetobacter baumanni* (Figure, 8 and Table, 4) and *Staphylococcus aureus* but it had no effect on *Escherichia coli*, *Klebsiella pneumonia* and *Proteus mirabilis*, while QSLA17 didn't show any antagonistic activity against tested clinical pathogens (Figure, 8). The discs filled only with 10 µl volume pasteurized salty whey showed a very small clear zones when applied on NA agar medium inoculated with *salmonella typhi* and the others inoculated with *Acinetobacter baumanni*, but hadn't affected on *staphylococcus aureus* (Figure, 9), *Escherichia coli*, *Klebsiella pneumonia* and *Proteus mirabilis* growth (Table, 4).

On the other hand discs filled with 10 µl volume of isolate QSLA16 culture dilutions mixed separately with same volume of pasteurized salty whey showed clear zone when applied on agar medium inoculated with *salmonella*, *Acinetobacter*, Figure (9) and *staphylococcus*. The results showed that there were significant differences between undiluted culture and culture dilutions in all treatments. It was observed that culture diluted up to 10^{-4} showed the highest antagonistic effect against *salmonella*, *Acinetobacter* and *staphylococcus* with .63 cm, .66cm and 1.17cm area of clear zone around the disc, respectively and this dilution (10^{-4}) was significantly different from other dilutions. An antagonistic effect of QSLA16 culture dilution 10^{-5} was also detected against used pathogens as it showed aclear zone of 0.42 cm, 0.38 cm, and 1 cm around *Acinetobacter*, *Salmonella* and *Staphylococcus* respectively. While culture dilution 10^{-6} showed clear zones of 0.33 cm, 0.29 cm and 0.88 cm around *Acinetobacter*, *Salmonella* and

Staphylococcus respectively. In this regard the effect of treatment by 10^{-5} dilution was significantly different from 10^{-6} dilution when applied against *Salmonella* and *Staphylococcus*, however this difference wasn't significant against *Acinetobacter*. The results were shown in Table (5) and Figures (10, 11 and 12).

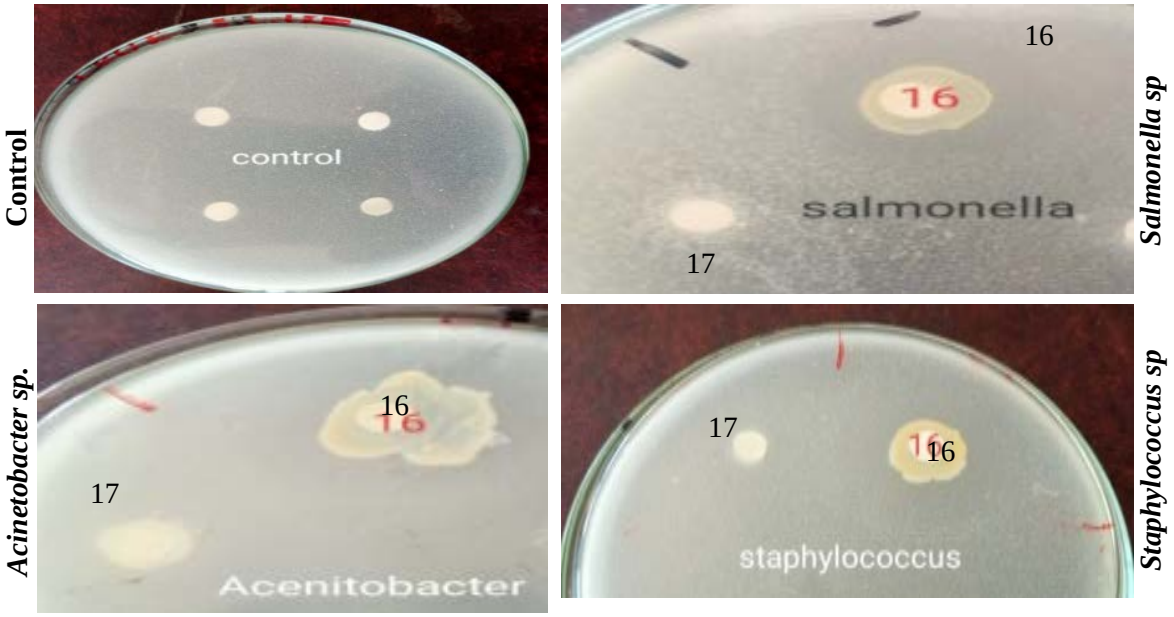

Figure (8) Antagonism of tested isolates QSLA16 and QSLA17 against *Staphylococcus aureus, Acinetobacter baumanni* and *salmonella typhi* (3.5%Nacl).

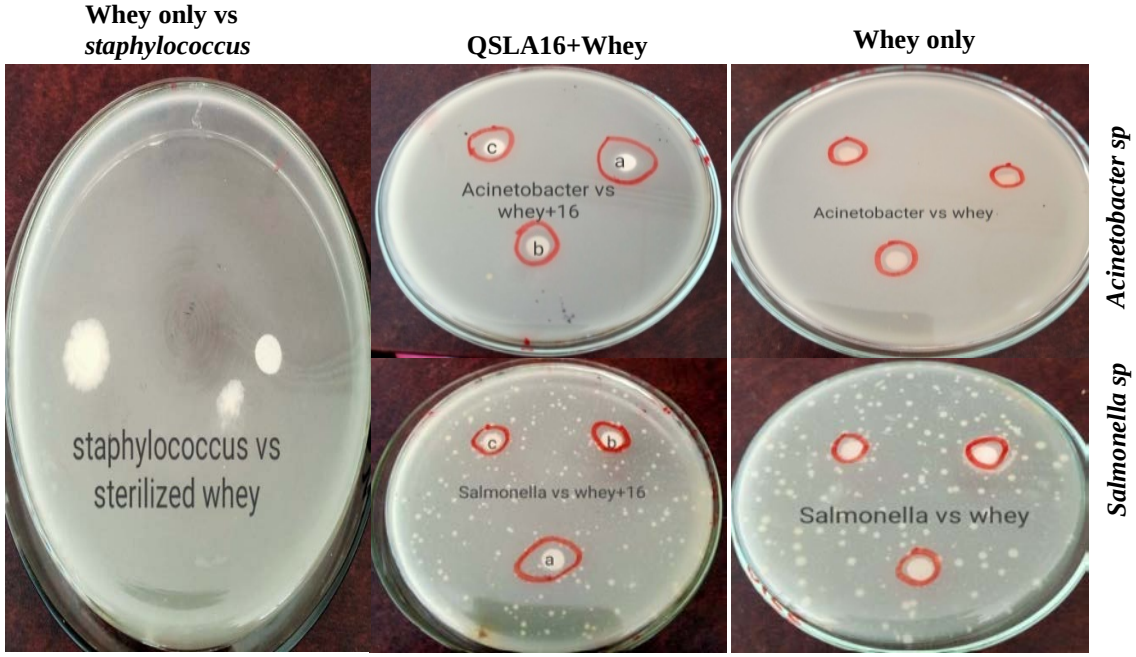

Figure (9): Effect of QSLA16 culture mixed with salty whey and whey only on tested pathogens.
- Letters (a) = 10^{-4}, (b) =10^{-5} and (c) = 10^{-6} were serial dilutions of culture medium.
- Whey had no antagonistic effect against QSLA16 growth.

Table (4) Effect of the isolates QSLA16 & QSLA17 and whey on tested pathogens-

Tested pathogens / Treatments	*Salmonella typhi*	*Acinetobacter baumannii*	*Staphylococcus aureus*	*Escherichia coli*	*Klebsiella pneumonia*	*Proteus mirabilis*
QSLA16	+	+	+	-	-	-
QSLA17	-	-	-	-	-	-
Whey	+	+	-	-	-	-

Table (5): Inhibition zone diameter of selected isolate (QSLA16) against human pathogens *Acinetobacter, Salmonella* and *Staphylococcus* after subtracting whey effect-

Treatments		Inhibition zone diameter (cm)		
		Acinetobacter baumannii	*Salmonella typhi*	*Staphylococcus aureus*
Undiluted QSLA16		1.24±.07^{c}	0.98±.04^{d}	1.67±.07^{d}
QSLA16	a (10^{-4})	0.66±.06^{b}	0.63±.04^{c}	1.17±.06^{c}
	b (10^{-5})	0.42±.04^{a}	0.38±.02^{b}	1.00^{b}
	c (10^{-6})	0.33±.03^{a}	0.29±.02^{a}	0.88±.01^{a}

- Culture dilution inhibition zone = diameter of total inhibition zone- inhibition zone of whey
- Values are means±standard deviation of three replicates
- d= higher value a= lower value

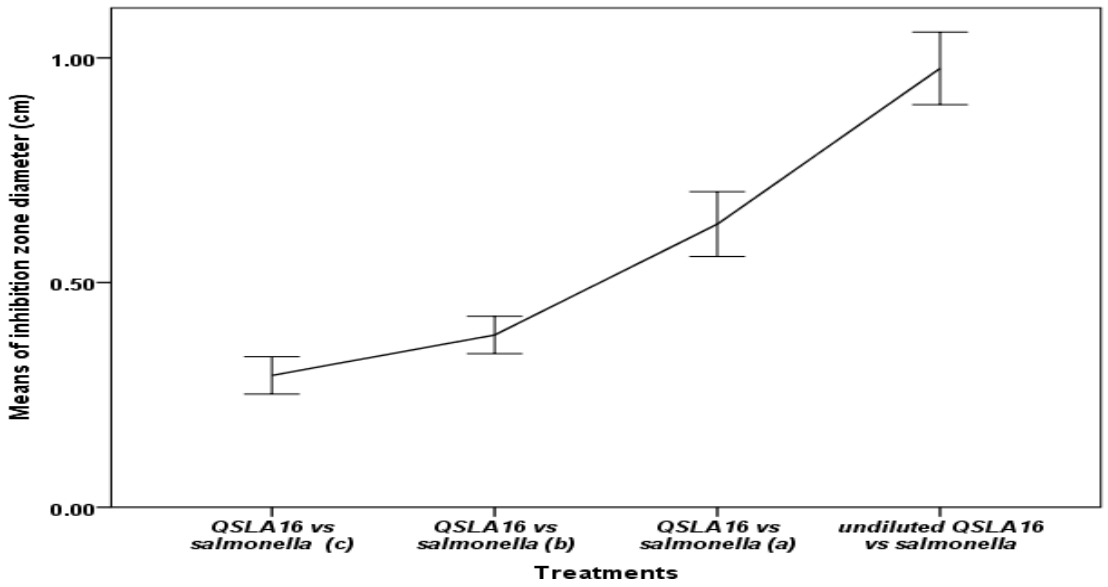

Figure (10): In vitro antagonistic effects of different dilutions of QSLA16 isolate on *Salmonella* sp.
- Culture dilution inhibition zone = diameter of total inhibition zone- inhibition zone of whey.
- Letters (a) = 10^{-4}, (b) =10^{-5} and (c) = 10^{-6} were serial dilutions of culture medium.
- Whey had no antagonistic effect against QSLA16 growth.

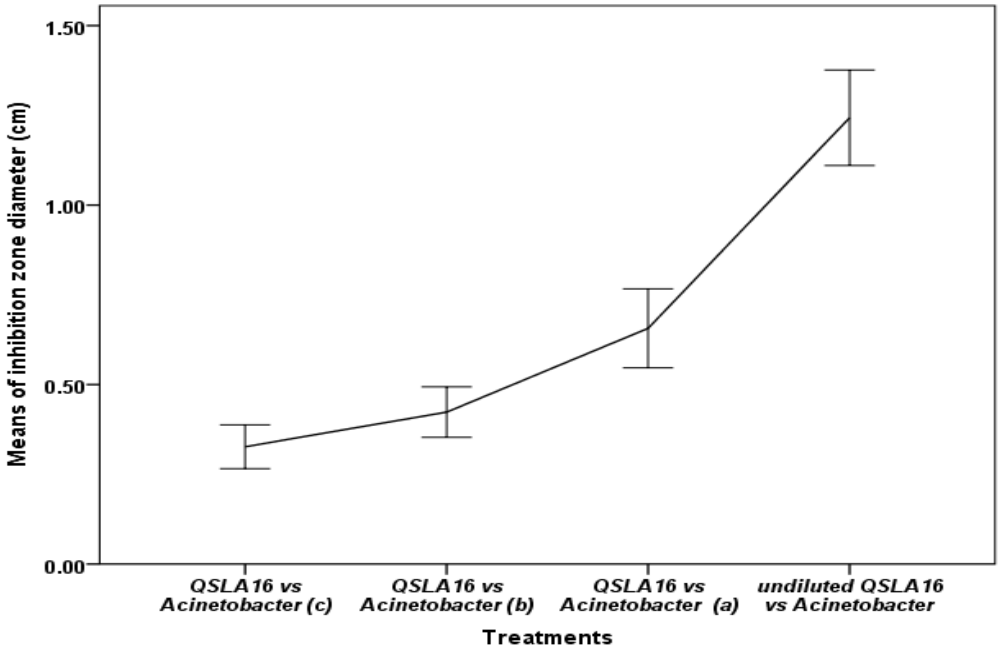

Figure (11): In vitro antagonistic effects of different dilutions of QSLA16 isolate on *Acinetobacter* sp.

- Culture dilution inhibition zone = diameter of total inhibition zone- inhibition zone of whey.

- Letters (a) = 10^{-4}, (b) =10^{-5} and (c) = 10^{-6} were serial dilutions of culture medium.

- Whey had no antagonistic effect against QSLA16 growth.

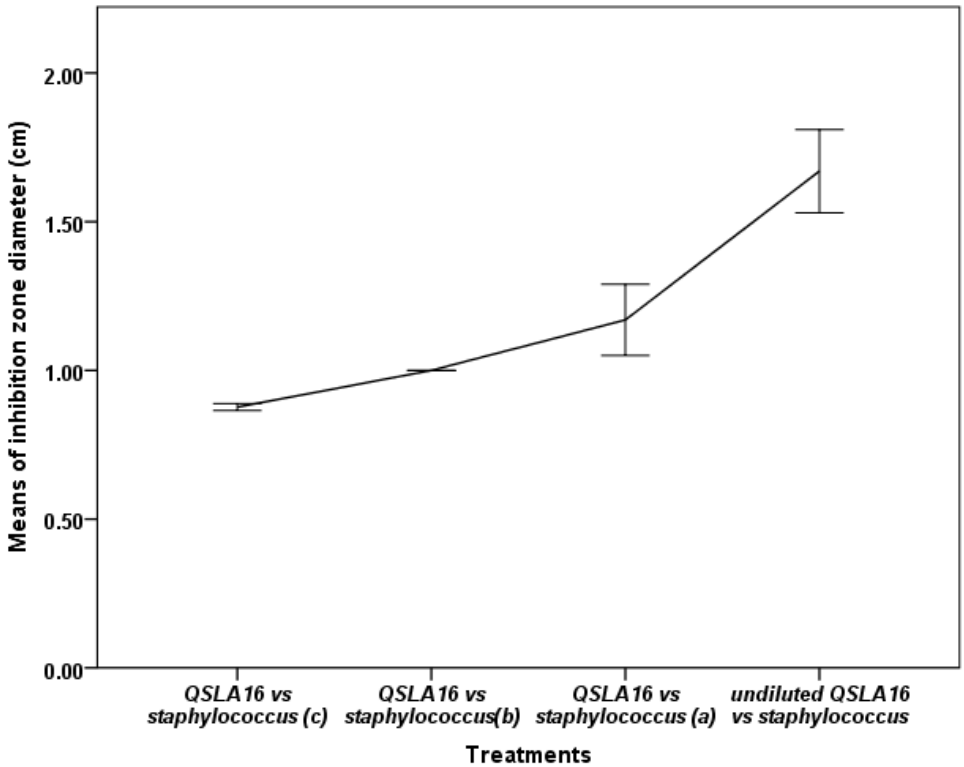

Figure (12): In vitro antagonistic effects of different dilutions of QSLA16 isolate on *staphylococcus* sp.

- Culture dilution inhibition zone = diameter of total inhibition zone- inhibition zone of whey.

- Letters (a) = 10^{-4}, (b) =10^{-5} and (c) = 10^{-6} were serial dilutions of culture medium.

- Whey had no antagonistic effect against QSLA16 growth.

A halophilic *Bacillus* sp. BS3 isolated by Donio *et al* (2013) from solar salt works in Thamaraikulam, Kanyakumari district, Tamilnadu, India. Screening for biosurfactants using oil spreading test and emulsification activity proved its ability for production of lipopeptide biosurfactants. The anti-adhesion activity of lipopeptide bisurfactants were effectively inhibited the growth of the pathogenic bacteria as well as fungi. The antibacterial activity observed was 16.00, 14.06, 13.10 and 10.10 mm of zone of inhibition against *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella typhi*, respectively.

HPLC results showed that isolate QSLA16 produced surfactin and fengycin (Figure, 6), while QSLA17 produced surfactin, fengycins and iturin (Figure, 7). Fahim and Hussein, (2016) observed that these compounds considered as antimicrobial metabolites. However, QSLA16 showed antagonistic effect against used pathogens, but QSLA17 not. These different antagonistic capabilities of both isolates might be due to the type of biosurfactant which may vary according to the order of amino acids and the size of lipid portion. Where, each family of NRPs contains variants with the same peptide length but with different residues at specific positions. Moreover, each variant can have several homologues of different length and isomery of the fatty acid chain, leading to a remarkable structural heterogeneity (Ongena & Jacques, 2008). Thus biosuurfactant produced from different isolates can had different effects. This might be the reason that, QSLA16 isolate had antagonistic effect against *salmonella typhi, Acinetobacter baumanni* and *Staphylococcus aureus*, while QSLA17 hadn't effect on the growth of these pathogens. Furthermore, the nature of QSLA16 as halophilic bacteria isolated from a pond with high salt concentration (24.2%) is different from QSLA17 isolated from a pond with 8.2% salt concentration and considered as halotolerant. These differences may cause effect on the texture of produced surfactant from the two isolates and its stability.

Surfactin responsible for hemolysis and formation of ion channels in lipid membranes of microorganisms (Raj Meena and Kanwar, 2014) leads to disruption of membrane due to possible pore formation resulting in scattering of cellular contents in their surroundings. This membrane lysis mechanism of action of lipopeptides makes the pathogens highly susceptible by decreasing the ability to develop resistance (Sharma *et al* 2020). Iturin and fengycin are responsiple for membrane damage, especially in the iturin A treatments. Therefore, iturin A and fengycin A caused enormous structural and compositional changes to cell surfaces, cellular contents and cell membrane integrity (Gong *et al* 2015). Lipopeptides secreted by QSLA16 isolate might have one or more of these mechanisms which responsible for antagonistic activity against pathogens.

On the other hand, whey is well known as antibacterial and antiviral product because of its protein components and their peptide portions which exhibit different bioactivity (Gobetti *et al*, 2002). But some studies observed that it's considered as a natural medium for spoilage and pathogens (Leroy, 2004). *Lactococcus Leuconoctoc*, and *Enteroccoci* species were isolated from Moroccan whey, with pathogenic microorganisms such as coliforms, *Escherchia coli, streptococci, Staphylococcus aureus* and *Listeria monocytogenes* (Benkerroum and Tamime, 2004). There were few studies on controling pathogens that habitat salty whey which used in several fields of food industries. In this study, the effect of QSLA16 isolate when added to salty whey displayed antagonistic action against used pathogenic bacteria and therefore can be used as preservative agents for salty whey or other salty foods.

Conclusion

Halophiles are considered a highly promising source for discovering novel molecules that used currently for pharmacological and food applications such as non-ribosomal lipopeptides (NRPs) that possess antimicrobial activities. In the current study two salt tolerant strains

QSLA16 and QSLA17 were isolated from saline ponds of local company for salts and minerals production. Detection of the genes encoding for NRPs by PCR technique observed that both isolates contain lipopeptides, surfactin and fengycin while mycosubtilin was detected only in QSLA17 and these molecules were determined by HPLC. As salty whey is a natural medium for spoilage by many pathogenic bacteria. The isolate QSLA16 showed efficiency in controlling the growth of halo-tolerant pathogens contaminating salty whey (*Staphylococcus aureus*, *Salmonella typhi* and *Acinetobacter baumannii*). Therefore QSLA16 have the potential to be used as whey preservative against halo-tolerant clinical pathogens.

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فعالية الليبوببتيدات غير الريبوسومية المنتجة بواسطة البكتريا المقاومة للملوحة ضد بعض البكتريا المرضية.

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الملخص العربى

البكتريا المحبة والمتحملة للملوحة كائنات حية دقيقة تعيش فى البيئات ذات التركيزات الملحية العالية ولديها العديد من الاستخدامات فى كثير من المجالات مثل مجالى الصناعة والزراعة. فى هذه الدراسة تم عزل سلالتان QSLA16 وQSLA17 من تلك البكتريا على بيئة الأجار المغذى وذلك من المياه الملحية الموجودة فى أحواض تستخدم فى إنتاج الملح وهى من مياه بحيرة قارون الموجودة بالفيوم. وبتعريف العزلات من خلال جين 16S rRNA وجد أن العزلة QSLA16 تنتمى لمجموعة Alphaproteobacteria حيث أنها تشبه سلالتى Uncultured bacterium clone QAMU2 و *Sphingomonas* sp. LE-239 بنسبة ٨٠,١٥٪ و ٧٩,٩٪ على التوالى، بينما العزلة QSLA17 تنتمى لمجموعة Firmicutes حيث لديها نسبة تشابه ٧٤,٠٥٪ مع السلالة *Bacillus* sp. strain 6. بالفحص المورفولوجى تبين أن العزلة QSLA16 عصوية غير متجرثمة سالبة لجرام, بينما العزلة QSLA17 عصوية طويلة متجرثمة موجبة لصبغة جرام. وأظهرت النتائج أن QSLA16 بكتريا محبة للملوحة حيث لم تنموإلا فى وجود الملح, بينما استطاعت العزلة QSLA17 النمو فى غياب الملح لذلك تعتبر متحملة للملوحة. وأظهراستخدام تفاعل البلمرة المتسلسل (PCR) قدرة العزلتين على إنتاج السرفكتين والفينجيسين, بينما الميكوسبتلين تم إنتاجه بواسطة العزلة QSLA17 فقط وتم تقدير الكميات المنتجة من هذه المواد باستخدام جهاز HPLC. وقد تم تقدير نشاط العزلتان ضد الميكروبات المرضية (*Staphylococcus aureus* و *Klebsiella pneumonia* و *Acinetobacter baumannii* و *Escherichia coli* و *Salmonella typhi* و *Proteus mirabilis*) لدراسة امكانية استخدامهما فى مقاومة هذه الممرضات الملوثة لشرش اللبن المالح. وأظهرت النتائج قدرة العزلة QSLA16 على تثبيط نمو *Staphylococcus aureus* و *Salmonella typhi* و *Acinetobacter baumanni* , بينما لم تظهر العزلة QSLA17 أى تأثيرعلى البكتريا الممرضة المختبرة.