Characterization and Control of Antibiotic-Resistant Camalti Saltern's Isolates with Bacteriocins

by
P. Caglayan
Division of Plant Diseases and Microbiology, Department of Biology, Faculty of Arts and Sciences, Marmara University, Istanbul, Turkey

Abstract

Camalti Saltern is the largest solar saltern in Izmir, Turkey. The salt obtained from Camalti Saltern is used in food and leather industries to prevent bacterial growth. In this saltern, seawater is pumped into shallow ponds. Then, the sun and wind cause evaporation and crystallization, finally sea salt is produced. Due to the fact that Camalti Saltern contains various halophilic bacteria, the goals of the present study were to isolate and identify haloversatile bacteria from Camalti Saltern's brine samples, to examine their antibiotic resistance profiles, to determine antimicrobial activities under optimum environmental conditions, to determine bacteriocin concentration by Bradford Method, to detect Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) of bacteriocins against multidrug-resistant isolates, and to observe the cell structure of bacteriocin-treated bacteria under SEM. Sixteen bacterial isolates were recovered from Camalti Saltern's brine samples and were identified as 14 different species (*Bacillus haynesii*, *Bacillus simplex*, *Bacillus subtilis* subsp. *stercoris*, *Bacillus pumilus*, *Staphylococcus petrasii* subsp. *jettensis*, *Staphylococcus saprophyticus* subsp. *saprophyticus*, *Kocuria sediminis*, *Rhodococcus enclensis*, *Marinobacter hydrocarbonoclasticus*, *Vibrio olivae*, *Marinomonas communis*, *Pseudomonas psychrotolerans*, *Salinivibrio costicola* subsp. *vallismortis*, *Vibrio neocaledonicus*). Percentages of antibiotic resistance of isolates were 63% to aztreonam, 50% to amoxicillin/clavulanic acid, 44% to ampicillin, 44% to cefadroxil, 31% to imipenem, 19% to ampicillin/sulbactam, 6% to chloramphenicol, 6% to tetracycline, 6% to mupirocin, 6% to meropenem. The bacteriocin concentrations of *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis* were measured as 1.02 mg/mL and 1.25 mg/mL, respectively. Bacteriocins of *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis*, which were not resistant to any antibiotics tested, exhibited the inhibitory effect against *Kocuria sediminis* resistant to ten antibiotics and *Bacillus pumilus* resistant to four antibiotics. Bacteriocin of *Salinivibrio costicola* subsp. *vallismortis* also demonstrated the inhibitory effect against *Pseudomonas psychrotolerans* resistant to five antibiotics. Scanning electron micrographs showed that cell morphologies of bacteriocin-treated isolates (*Kocuria sediminis*, *Bacillus pumilus*, *Pseudomonas psychrotolerans*) were damaged. In conclusion, bacteriocins produced from the haloversatile Camalti saltern isolates may be used in the leather industry to prevent the growth of antibiotic-resistant haloversatile bacteria.

Introduction

Camalti Saltern is the biggest seawater-based saltern in Izmir (Turkey). In this saltern, 500,000 ton of NaCl per year is produced since 1863. There is a multipond system with 182 ponds covering 58 km^2 area.[1] Solar evaporation is the oldest salt production method. In this system, seawater from the Aegean Sea is pumped at certain times into these shallow ponds in the Camalti Saltern.[2,3] The sun and dry wind cause evaporation of seawater. While the seawater salinity is about 0.35%, the salinity is reached to 26.5% after holding in the pools. Then, the salt begins to crystallize once supersaturation is reached. The salt obtained from Camalti Saltern is used in pickling, brine curing, olive salting, curing fish, as well as hide and skin preservation. The food, hide and skin materials are ideal environments for bacteria found in salt. When the salt containing harmful bacteria is used for food and hide preservation, undesirable changes in smell, taste, appearance, hair, color, grain surface may result from bacterial growth.[4,5] These problems will negatively affect economy, people and environment.

Microbial adaptation has a major role in multipond solar salterns that have a wide range of salt concentrations. Halophiles can thrive in saline environments such as salt lakes, sea, seawater-based salterns, salterns, saline soils, salted hides, and salt mines.[6-10] Among the halophilic microorganisms, haloversatile bacteria are capable of growth in the absence of salt and in saturated salt concentrations (>3M NaCl). These bacteria are able to grow optimally in media with 0.2-0.5 M NaCl.[8,11]

Although the presence of halophilic microorganisms in the Camalti Saltern was reported in previous studies,[1,12-14] the presence of bacteria showing haloversatile properties in Camalti Saltern's brine samples and their antibiotic susceptibilities were not reported yet. Only in two research papers, the haloversatile bacteria were reported in salt samples collected from Camalti Saltern.[15,16]

Antibiotics, natural microbial products, are commonly used in animals and humans for disease treatment, growth promotion

*Corresponding author: pinar.caglayan@marmara.edu.tr
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and meat production.[17-20] According to the joint results of World Organisation for Animal Health,[21] World Health Organization,[22] the Food and Agriculture Organization of the United Nations,[23] antibiotic resistance was reported as a global health problem due to the misuse and overuse of antimicrobial agents.[21-23] It was recommended that antibiotics should not be routinely used in the food industry, animal husbandry, veterinary and human medicine.[21-23] Although less information is available about antibiotic resistance of bacteria in natural environments, it is known that the antibiotic-resistant bacteria present in manure and wastewater may pass through the soil, and from there to water sources.[24] These bacteria may transfer to humans and animals during washing and cleaning, air-conditioning, transportation, storage, packaging of food products, and leather processing.[25,26]

Due to the overuse of antibiotics in human and animal health, bacteriocins which are ribosomally synthesized by different microorganisms are used to kill or inhibit microorganisms as an alternative to antibiotics in recent years.[27,29,30] Bacteriocins may be potential candidates instead of antibiotics and chemicals in food preservation, hide and skin preservation, cancer therapy, treating multidrug-resistant bacteria in the food industry, leather industry and medicine.[27-30]

Since antibiotic-resistant bacteria may be found in the Camalti Saltern, the goals of the present study were to investigate: 1) haloversatile bacteria at species level using phenotypic and genotypic characteristics found in Camalti Saltern's brine samples; 2) their antibiotic resistance profiles against different antibiotics; 3) antimicrobial activities of test isolates against each other; 4) optimum environmental conditions for maximum bacteriocin production by *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis*; 5) effect of temperature, pH, NaCl concentration, effect of enzymes (Proteinase K and Lipase) on antimicrobial activities of bacteriocins obtained from *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis*; 6) Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) of bacteriocins against multidrug-resistant isolates; 7) the cell structure observation of bacteriocin-treated bacteria under SEM.

Experimental

Sample collection

Five brine samples were collected from the surfaces of shallow seawater pools at the Camalti Saltern, Izmir/Turkey into sterile bottles in June 2018. Then, the brine samples were placed in a lightproof insulated box containing ice-packs and immediately transported to the laboratory.

Isolation of bacteria

All brine samples were diluted in a physiological saline solution containing 1.17% (w/v) NaCl (0.2 M NaCl). An aliquot of 0.1 mL of each direct and serial dilutions (10^{-1}-10^{-6}) was spread onto surface of Oligotrophic Agar Medium (OAM) plates (18.2 g R2A, 5 g agar, 11.7 g NaCl, 1000 mL distilled water).[15] The plates were incubated at 32°C for 24 hours. Then, different bacterial colonies were selected and restreaked several times to obtain pure cultures.[31,32]

Characterization of the isolates

Exponentially grown isolates were examined for cell morphology and pigmentation. Catalase, oxidase activities, Gram staining were performed using earlier described procedures.[33] Salt requirement and salt tolerance of the test isolates were investigated on both OAM plates without salt and OAM plates containing different NaCl concentrations such as 0.17 M, 0.2 M, 0.34 M, 0.51 M, 0.85 M, 1.36 M, 1.7 M, 2 M, 2.5 M, 3 M, 3.4 M, 4.2 M and 5.1 M. The pH tolerance of isolates were tested on OAM plates (0.2 M NaCl) and adjusted to pH values 4, 5, 6, 7, 8, 9, 10, 11, 12, 13. The pH values of the media were measured using a pH meter (Sartorius Professional Meter PT-10P, Goettingen, Germany). To determine optimum growth temperature of isolates, the plates inoculated with each isolate were incubated at different temperatures (4, 10, 20, 25, 28, 32, 37, 40, 45, 50, 55, 60 °C).

Amplification and sequencing of 16S rRNA genes

Chromosomal DNA was isolated and purified by QIAamp DNA Mini Kit (Qiagen) and QIAquick PCR Purification Kit (Qiagen). The 16S rRNA genes of the isolates were amplified by PCR using universal primers: 1492R (5'TACGGYTACCTTGTTACGACTT3') and 27F (5'AGAGTTTGATCMTGGCTCAG3').[34] The 16S rRNA gene sequences (1000-1380 bp) were determined by IONTEK Laboratory (Turkey). It was further determined 16S rRNA gene similarities (98.7%-100%) between isolates and closely related species using ChromasPro and EzTaxon-e tool.[35]

Nucleotide accession number

16S rRNA sequence data of the brine isolates SW1a, SW1b, SW2a, SW2b, SW3a, SW3b, SW3c, SW3d, SW3e, SW4a, SW4b, SW4c, SW4d, SW5a, SW5b, SW5c, reported in this article have been deposited in NCBI and GenBank nucleotide sequence database under the respective accession numbers: MH748797, MH748778, MH748707, MH748694, MH753648, MH748717, MH753655, MH748716, MH748723, MH748691, MH748690, MH748687, MH748684, MH748683, MH748680, MH748674.

Antibiotic susceptibility tests

Resistance to antibiotics was determined by the Kirby-Bauer disc diffusion method.[36] The isolates were grown in Mueller Hinton Broth (MHB) containing 1.17% NaCl. After incubation at 32°C, the optical density of overnight culture was adjusted to McFarland Standard No 0.5 (1×10^8 CFU/mL) with sterile saline solution (1.17% NaCl). Then, suspensions of isolates were evenly spread on Mueller Hinton Agar (MHA). Chloramphenicol, cefadroxil, ampicillin, tetracycline, mupirocin, imipenem, meropenem, aztreonam, ampicillin/sulbactam and amoxycillin/clavulanic acid discs (Oxoid,

UK) were placed on the surface of inoculated MHA plates. The plates were incubated at 32°C for 24h. At the end of the incubation period, the diameters of the inhibition zones were measured.

Preparation of cell-free supernatant

The isolates were grown in 20 mL Nutrient Broth (NB) (pH 7.0, 1.17% NaCl) at 32°C for 24 hours. Bacterial cells were removed by centrifugation (8000 g for 15 min at 4°C) to obtain a cell-free supernatant (CFS). The pH of CFS was adjusted to 7.0 using 1N NaOH to prevent the inhibitory effect of organic acids on bacterial growth. Each supernatant was filtered through a 0.22 µm membrane filter (Millipore) to produce sterile crude bacteriocin.[37]

Screening of antimicrobial activity among the test isolates

Antimicrobial activity of bacteriocin against each test isolate was examined. Each bacterial concentration was separately adjusted to 10^8 CFU/mL. Aliquots of 100 µL of the each of the adjusted bacterial suspensions were spread on the petri plates containing Nutrient Agar (NA) medium. Aliquots of 3 µL of each bacteriocin were placed on these inoculated petri plates. After 24 hours incubation at 32°C, the inhibition zone diameters (mm) of the bacteriocin substance against each test isolate were measured.[38] According to the obtained experimental results, bacteriocin-producing isolates, bacteriocin-sensitive and bacteriocin-resistant isolates were determined. Later, bacteriocins produced by *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis* were investigated against multidrug-resistant isolates *Kocuria sediminis*, *Bacillus pumilus*, *Pseudomonas psychrotolerans*.

Determination of protein concentration using Bradford Method

The Bradford method was used to determine the amounts of protein in the supernatants of *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis* at 32°C, pH 7, 1.17% NaCl concentration.[39] Bradford reagent and Quick Start Bovine Serum Albumin (BSA), which are commercial product of BIO-RAD (BIO-RAD Laboratories, Hercules CA, USA), were used in this experiment. The optical density of bacteriocins were measured at 595 nm with spectrophotometer (UV Mini 1240, Shimadzu, Kyoto, Japan).

Optimum conditions for bacteriocin production from *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis*

To investigate the impact of environmental factors on bacteriocin production by *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis*, bacterial concentration of each isolate was adjusted to 10^8 CFU/mL. Then, 3 mL of each bacterial solution was inoculated into flasks containing 50 mL of NB and the flasks were incubated at different temperatures (20, 28, 30, 32, 37, 40°C), different pH values (5.0, 6.0, 7.0, 8.0, 9.0, 10.0), different NaCl concentrations (1.17, 3, 5, 10, 15, 20, 25%) for 24 hours. Bacterial concentration of the multidrug-resistant isolates was separately adjusted to 10^8 CFU/mL. Subsequently, 100 µL aliquots from each multidrug-resistant isolate were spread on the petri plates containing NA. Aliquots of 3 µL of each bacteriocin were placed on the inoculated petri plates. The inhibition zone diameters of bacteriocin against each test isolate were measured after 24 hours incubation at 32°C.[38] The experimental results were evaluated to detect the optimum temperature, pH and salt concentration required for the highest bacteriocin production by isolates.

Effect of heat on bacteriocins produced by *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis*

The bacteriocin of each isolate was incubated at different temperatures (4, 10, 20, 25, 28, 32, 37, 40, 50, 60, 70, 80, 90, 100°C) for 15 min. Later, the heat-treated bacteriocin samples were tested for antimicrobial activity against multidrug-resistant isolates by agar spot diffusion assay. The plates were incubated at 32°C for 24 hours. After the incubation period, the inhibition zone diameters of the bacteriocin against the multidrug-resistant test isolates were measured.

Effect of pH on bacteriocins produced by *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis*

The pH of bacteriocin belonging to each isolate was adjusted to 4-12. Bacteriocin samples were kept at 25°C for 4 hours. Then, the bacteriocin samples were tested for antimicrobial activity against multidrug-resistant isolates by agar spot diffusion assay. The inoculated petri plates were incubated at 32°C for 24 hours. After incubation period, the inhibition zone diameters of the bacteriocin against the test isolates were measured.

Effect of NaCl concentration on bacteriocins produced by *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis*

NaCl concentration of the bacteriocin of each isolate was adjusted to 1.17, 3, 5, 10, 15, 20, 25% NaCl. The bacteriocin samples were tested for antibacterial activity against multidrug-resistant isolates by agar spot diffusion assay. The plates were incubated at 32°C for 24 hours. After incubation, the inhibition zone diameters were measured. The results were evaluated to determine the effect of heat, pH and NaCl concentration on the bacteriocins.

Effect of enzymes (Proteinase K and Lipase) on bacteriocins produced by *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis*

The effects of protease and lipase enzymes on bacteriocins produced by *R.enclensis* and *S.costicola* subsp. *vallismortis*, proteinase K of *Tritirachium album* and lipase of *Candida rugosa* were purchased from Sigma-Aldrich (St Louis, MO).[37] Proteinase K and lipase were respectively prepared in 10 mM phosphate buffer (pH 7) and 0.05 M Tris HCl, 0.01 M $CaCl_2$, pH 8, and filtered through 0.22 µm pore size filter paper (HiMedia, Mumbai, India). One mL of sterile enzyme solution was added to the supernatant and this mixture was incubated for two hours at 37°C. The activities of bacteriocins treated with enzyme were examined by the agar spot diffusion method using

the multidrug-resistant isolates. The plates were incubated at 32°C for 24 hours. After incubation period, the inhibition zone diameters of the bacteriocin against the test isolates were measured. Phosphate buffer (10 mM) and Tris HCl buffer (0.05 M) were used as negative controls of Proteinase K and lipase, respectively.[40]

Minimum inhibitory concentrations and minimum bactericidal concentrations of bacteriocins against multidrug-resistant isolates

In order to detect the Minimum Inhibitory Concentrations of bacteriocin produced from *Rhodococcus enclensis* against *Kocuria sediminis* and *Bacillus pumilus*, and bacteriocin produced from *Salinivibrio costicola* subsp. *vallismortis* against *Kocuria sediminis*, *Bacillus pumilus* and *Pseudomonas psychrotolerans*, broth microdilution in 96-well plate method was performed. Two-fold serial dilutions of each bacteriocin (Column 1: 1/2, Column 2: 1/4, Column 3: 1/8, Column 4: 1/16, Column 5: 1/32, Column 6: 1/64, Column 7: 1/128, Column 8: 1/256, Column 9: 1/512, Column 10: 1/1024) were prepared in 96-well microdilution plate containing Mueller Hinton Broth. A multichannel pipette was used to transfer and mix bacteriocin from Columns 1-10. While Column 11 contained 200 µL Mueller Hinton Broth only as a sterility control, Column 12 contained 100 µL Mueller Hinton Broth and 100 µL of each test isolate (*Kocuria sediminis*, *Bacillus pumilus*, *Pseudomonas psychrotolerans*) for the normal bacterial growth. The multidrug-resistant test isolates (*Kocuria sediminis*, *Bacillus pumilus*, *Pseudomonas psychrotolerans*) were streaked onto Mueller Hinton Agar plate and incubated at 32°C for 24 hours. Then, each suspension of test isolate was prepared in sterile Mueller Hinton Broth and adjusted to equal turbidity of 10^8 CFU/mL. An aliquot of 100 µL bacterial suspension of multidrug-resistant test isolates was added into each well on the Columns 1-12 (except Column 11 which is accepted as sterilization control) (totally 200 µL per well). After 24 hours incubation at 32°C, 30 µL of resazurin solution (0.015%) was added into each well on the Columns 1-12. Subsequently, 96-well plates were left for 4 hours at 25°C and the wells were checked for visible growth (turbity and pink colour).[41] Resazurin, which has a blue color, is irreversibly reduced to the pink-color by the respiration of metabolically active bacterial cells, and it is used as an indicator of cell viability.[41] Also, in which well the color of resazurin did not turn from blue to pink, it means that in that well the bacterial cells are killed or controlled by the bacteriocin, and that bacteriocin concentration is accepted as Minimum Inhibitory Concentration (MIC). The lowest concentration that inhibited the growth of the test isolates (blue colour) in the wells were accepted as MIC of bacteriocins. In order to determine Minimum Bactericidal Concentration (MBC), the visible growth of *Kocuria sediminis* and *Bacillus pumilus* effected by bacteriocin produced from *Rhodococcus enclensis*, and the visible growth of *Kocuria sediminis*, *Bacillus pumilus* and *Pseudomonas psychrotolerans* effected by bacteriocin produced from *Salinivibrio costicola* subsp. *vallismortis* were investigated from the wells belonging to MIC endpoint. Hence, 100 µL of each suspension from the each test well belonging to MIC (containing blue color resazurin) was spread on the Mueller Hinton Agar plates with glass spreader. The plates were incubated at 32°C for 24 hours. After incubation period, when the bacterial colonies did not observed on the plates, it was accepted as the bacteriocins have bactericidal effect on the test isolates. All experiments were performed in triplicate.

Preparation of bacteriocin-treated and bacteriocin-untreated cells for Scanning Electron Microscopy

Bacteriocin-treated (from wells belonging to MIC endpoint) and untreated (control) cultures of the multidrug-resistant bacterial isolates were separately dropped on filter membranes (0.45 µm) and air dried. The filter membranes were fixed in 2.5% glutaraldehyde solution prepared in 0.1 M phosphate buffer (pH 7.2) for 1 hour. The filters were rinsed three times with 0.1 M PBS buffer for 15 min, and then treated with 1% OsO_4 in 0.1 M PBS buffer at room temperature for 1 hour. The filters were rinsed with distilled water for 15 min and then, were dehydrated with an ethanol series (30%, 50%, 70%, 80%, 100%), 10 min for each rinse. Then, the samples were examined using a Scanning Electron Microscope (Fei Quanta 450 FEG ESEM SEM) sample stub with double-sided sticky tape.[42]

Results and Discussion

In the present study, bacterial isolates were found in all the brine samples of shallow seawater ponds obtained from the Camalti Saltern, which is the largest seawater-based saltern in Izmir (Turkey). The temperature, pH and salt concentration of brine samples were respectively recorded between 24°C-26°C, 8.43-8.67, and 4.2%-5.0%. 14 different bacterial species such as *Bacillus haynesii* (isolates SW1a, SW3a, SW3c), *Staphylococcus petrasii* subsp. *jettensis* (isolate SW1b), *Kocuria sediminis* (isolate SW3b), *Bacillus simplex* (isolate SW3d), *Bacillus subtilis* subsp. *stercoris* (isolate SW3e), *Marinobacter hydrocarbonoclasticus* (isolate SW2a), *Vibrio olivae* (isolate SW2b), *Bacillus pumilus* (isolate SW4c), *Rhodococcus enclensis* (isolate SW4e), *Marinomonas communis* (isolate SW4f), *Staphylococcus saprophyticus* subsp. *saprophyticus* (isolate SW4g), *Pseudomonas psychrotolerans* (isolate SW5a), *Salinivibrio costicola* subsp. *vallismortis* (isolate SW5b), *Vibrio neocaledonicus* (isolate SW5c) were isolated and identified from five brine samples. The isolated species in this study were first isolated and identified from brine samples obtained from the Camalti Saltern. While the most abundant species in the water samples was *Bacillus haynesii* (n=3), the other species were not abundant (n=1). All isolates were able to grow at 0-3 M NaCl concentrations, but optimally grew at 0.2 M NaCl (Table I). Therefore, these isolates were accepted as haloversatile.[43-45] All isolates were able to grow at pH 5-12 and between 10-55°C (Table I). Gram reactions, cell morphologies, effects of NaCl, temperature and pH on bacterial growth, oxidase and catalase activities were shown in Table I.

Some of the isolated strains in the present study were previously reported by other researchers. For instance, *Bacillus haynesii*,

Table I

Characteristics of haloversatile bacteria isolated from brine samples of the Camalti Saltern

Characteristics	*Bacillus haynesii*	*Staphylococcus petrasii subsp. jettensis*	*Marinobacter hydrocarbonoclasticus*	*Vibrio olivae*	*Kocuria sediminis*	*Bacillus simplex*	*Bacillus subtilis subsp. stercoris*
Isolate number	3	1	1	1	1	1	1
Gram staining	+*	+	–**	–	+	+	+
Cell morphology	rod-shaped	coccus	rod-shaped	curved-rod shape	coccus	rod-shaped	rod-shaped
NaCl range (M)	0-3	0-3	0-3	0-3	0-3	0-3	0-3
Optimum NaCl (M)	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Temperature range (°C)	20-55	20-55	10-55	20-45	10-50	10-55	10-55
Optimum temperature (°C)	32	32	28-32	32	32	32	32
pH range	5-12	5-12	5-12	5-12	6-12	5-12	5-12
Optimum pH	6-8	7-8	7-8	7-8	7	7-8	6-8
Oxidase	–	+	+	+	+	–	–
Catalase	+	+	+	+	+	+	+

Characteristics	*Bacillus pumilus*	*Rhodococcus enclensis*	*Marinomonas communis*	*Staphylococcus saprophyticus subsp. saprophyticus*	*Pseudomonas psychrotolerans*	*Salinivibrio costicola subsp. vallismortis*	*Vibrio neocaledonicus*
Isolate number	1	1	1	1	1	1	1
Gram staining	+	+	–	+	–	–	–
Cell morphology	rod-shaped	rod-coccoid	rod-shaped	coccus	rod-shaped	curved-rod shape	curved-rod shape
NaCl range (M)	0-3	0-3	0-3	0-3	0-3	0-3	0-3.0
Optimum NaCl (M)	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Temperature range (°C)	20-45	10-45	20-45	20-45	10-50	10-45	20-55
Optimum temperature (°C)	32	32	28-32	32	32	32	32
pH range	5-11	5-12	6-12	5-11	6-12	5-11	5-12
Optimum pH	6-8	6-8	7-8	6-8	7-9	7-8	6-8
Oxidase	–	–	+	+	–	+	+
Catalase	+	+	+	+	+	+	+

+*: Positive, –**: Negative

Staphylococcus petrasii subsp. *jettensis*, *Kocuria sediminis*, *Bacillus subtilis* subsp. *stercoris*, *Bacillus pumilus* and *Staphylococcus saprophyticus* subsp. *saprophyticus* were isolated from salt samples of Camalti Saltern and they were reported as haloversatile.[15] In another study, a raw salt sample collected from Camalti Saltern was examined, and six haloversatile bacteria (*Arthrobacter ginsengisoli*, *Arthrobacter psychrochitiniphilus*, *Pseudarthrobacter polychromogenes*, *Glutamicibacter arilaitensis*, *Arthrobacter agilis*) were isolated from that raw salt sample.[16] According to the experimental results, it is clear that the haloversatile bacteria found in the brine of Camalti Saltern, may contaminate the salt crystals during salt production process in evaporation ponds and crystallization ponds.

Among the test isolates, *Bacillus haynesii*, *Bacillus simplex*, *Bacillus subtilis* subsp. *stercoris*, *Bacillus pumilus* have endospores which are resistant to UV radiation, antimicrobial agents, cold, heat, desiccation.[46-49] In another study, *Staphylococcus petrasii* subsp. *jettensis* was found to be resistant to penicillin, cefoxitin, gentamicin, erythromycin and clindamycin.[32] *Salinivibrio costicola* subsp. *vallismortis*, *Pseudomonas psychrotolerans*, *Marinomonas communis*, *Vibrio olivae*, *Vibrio neocaledonicus*, *Rhodococcus enclensis*, *Staphylococcus saprophyticus* subsp. *saprophyticus* and *Kocuria sediminis* were able to use different carbon and energy sources with different enzymes.[48,50-57] *Marinobacter hydrocarbonoclasticus* uses several hydrocarbons as the sole source of carbon and energy.[58] Other studies also suggest that these isolates are metabolically active. While the salt containing these isolates is used in the preservation of hides and skins in the leather industry, they may damage the structure of hides and skin. Bad odor, red heat, hair slip, grain damage are seen due to the catabolic activity of bacteria on the skin and hide structures.[59-61] Due to the huge economic importance of the leather industry in the world, destructive and antibiotic resistant hide and skin bacteria found in the preservation salt should be killed to prevent their damage to the structures of hides and skin, and to prevent financial losses to the leather industry.

Susceptibility of bacteria to antibiotics is also determined by standardized antimicrobial disc diffusion results, in which the inhibition zones around the antibiotic discs are measured and evaluated according to the standards reported by the Clinical and Laboratory Standards Institute and European Committee on Antimicrobial Susceptibility Testing.[62,63] However, there is no standard regarding the antibiotic susceptibilities of the test isolates. Hence, the antibiotic test results were expressed as inhibition zone diameter measurements (mm) in the present study. The experimental results of the resistance of test bacteria to ten different antibiotics are presented in Table II. Multidrug-resistant bacteria, which are able to survive and grow in the presence of more than two antibiotics were detected in the present study. Among the test isolates, *Kocuria sediminis* was resistant to all antibiotics tested (Table II). However, *Vibrio olivae*, *Bacillus subtilis* subsp. *stercoris*, *Marinomonas communis* were resistant to only one antibiotic (Table II). *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis* were susceptible to all antibiotics tested (Table II).

A low percentage (35.7%) of the isolates produced bacteriocin against to each other. Although *Bacillus haynesii*, *Staphylococcus petrasii* subsp. *jettensis*, *Marinobacter hydrocarbonoclasticus*, *Vibrio*

Table II

Inhibition zone diameter measurements (mm) of haloversatile bacteria isolated from brine samples of the Camalti Saltern

	Bacillus haynesii	*Staphylococcus petrasii subsp. jettensis*	*Marinobacter hydrocarbonoclasticus*	*Vibrio olivae*	*Kocuria sediminis*	*Bacillus simplex*	*Bacillus subtilis subsp. stercoris*	*Bacillus pumilus*	*Rhodococcus enclensis*	*Marinomonas communis*	*Staphylococcus saprophyticus subsp. saprophyticus*	*Pseudomonas psychrotolerans*	*Salinivibrio costicola subsp. vallismortis*	*Vibrio neocaledonicus*	**Total number of isolates**
Number of isolates	3	1	1	1	1	1	1	1	1	1	1	1	1	1	**16**
Antibiotics															R%
Chloramphenicol (30 µg)	17	28	23	32	0	20	31	17	22	32	28	21	32	24	**6**
Cefadroxil (30 µg)	29	11	0	0	0	0	36	28	11	0	11	0	12	0	**44**
Ampicillin (10 µg)	14	0	0	20	0	0	24	11	10	28	0	0	20	0	**44**
Tetracycline (30 µg)	15	26	20	30	0	22	26	16	23	31	26	24	30	24	**6**
Mupirocin (20 µg)	39	21	18	20	0	20	30	28	20	31	21	16	20	14	**6**
Imipenem (10 µg)	0	25	24	34	0	22	42	0	23	37	25	18	34	18	**31**
Meropenem (10 µg)	34	25	24	29	0	23	36	34	23	35	25	19	29	11	**6**
Aztreonam (30 µg)	0	0	20	34	0	0	0	0	17	28	0	0	34	15	**63**
Ampicillin/sulbactam (10/10 µg)	15	19	16	29	0	13	27	0	13	27	19	0	29	12	**19**
Amoxycillin/clavulanic acid (20/10 µg)	0	0	12	23	0	0	22	0	10	21	0	0	23	0	**50**
Number of antibiotics that the species are resistant	**3**	**3**	**2**	**1**	**10**	**4**	**1**	**4**	**0**	**1**	**3**	**5**	**0**	**3**	

R (0 mm): Resistance

olivae, *Bacillus simplex*, *Bacillus subtilis* subsp. *stercoris*, *Bacillus pumilus*, *Staphylococcus saprophyticus* subsp. *saprophyticus*, *Vibrio neocaledonicus* did not produce bacteriocin, *Kocuria sediminis*, *Rhodococcus enclensis*, *Marinomonas communis*, *Pseudomonas psychrotolerans*, *Salinivibrio costicola* subsp. *vallismortis* produced bacteriocin (Table III). In this study, *Kocuria sediminis*, *Rhodococcus enclensis*, *Marinomonas communis*, *Pseudomonas psychrotolerans*, *Salinivibrio costicola* subsp. *vallismortis* respectively produced bacteriocin against *Bacillus subtilis* subsp. *stercoris*; *Kocuria sediminis*, *Bacillus pumilus*, *Marinomonas communis*; *Bacillus pumilus*; *Kocuria sediminis*, *Bacillus pumilus*, *Salinivibrio costicola* subsp. *vallismortis*; *Kocuria sediminis*, *Bacillus pumilus*, *Pseudomonas psychrotolerans*, *Vibrio neocaledonicus*. While bacteriocins of *Kocuria sediminis*, *Marinococcus tarijensis* and *Marinomonas communis* showed narrow antibacterial spectrum (inhibiting 1 test isolate), whereas bacteriocins of *Rhodococcus enclensis*, *Pseudomonas psychrotolerans*, *Salinivibrio costicola* subsp. *vallismortis* presented a wide antibacterial spectrum (inhibiting 3-4 test isolates) (Table III). These results are consistent with what has been reported in previous studies.[27-29] The researchers reported that 99% of bacteria produced at least one bacteriocin.[27]

To determine the effects of environmental conditions (°C, pH, NaCl concentration) on bacteriocin production, the bacteriocins produced by *Rhodococcus enclensis*, and *Salinivibrio costicola* subsp. *vallismortis* against antibiotic-resistant *Kocuria sediminis*, *Bacillus pumilus* and *Pseudomonas psychrotolerans* were further studied (Table IV). The inhibition zone diameters of the isolates were measured as 5-10 mm, 8-12 mm, 14-17 mm, 9-13 mm, 5-7 mm respectively for 28°C, 30°C, 32°C, 37°C, 40°C; 6-8 mm, 14-17 mm, 8-11 mm respectively for pH 6, pH 7, pH 8; 14-17 mm, 10-14 mm, 8-11 mm, 5-9 mm respectively for 1.17%, 3%, 5%, 10% NaCl concentration. However, bacteriocin was not produced at 20°C, pH 5.0, 9.0, 10.0 and 15%, 20%, 25% NaCl concentration (Table IV). The

Table III

Inhibitory effect of bacteriocin produced by bacterial isolates against each other

	Bacteriocin producers	***Bacillus haynesii***	***Staphylococcus petrasii* subsp. *jettensis***	***Marinobacter hydrocarbonoclasticus***	***Vibrio olivae***	***Kocuria sediminis***	***Bacillus simplex***	***Bacillus subtilis* subsp. *stercoris***	***Bacillus pumilus***	***Rhodococcus enclensis***	***Marinomonas communis***	***Staphylococcus saprophyticus* subsp. *saprophyticus***	***Pseudomonas psychrotolerans***	***Salinivibrio costicola* subsp. *vallismortis***	***Vibrio neocaledonicus***	**Number of bacteriocin affected by isolate**
Species affected from bacteriocins	Bacillus haynesii	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0
	Staphylococcus petrasii subsp. *jettensis*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0
	Marinobacter hydrocarbonoclasticus	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0
	Vibrio olivae	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0
	Kocuria sediminis	–	–	–	–	–	–	–	–	14	–	–	10	15	–	3
	Bacillus simplex	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0
	Bacillus subtilis subsp. *stercoris*	–	–	–	–	16	–	–	–	–	–	–	–	–	–	1
	Bacillus pumilus	–	–	–	–	–	–	–	–	15	13	–	10	17	–	4
	Rhodococcus enclensis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0
	Marinomonas communis	–	–	–	–	–	–	–	–	14	–	–	–	–	–	1
	Staphylococcus saprophyticus subsp. *saprophyticus*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0
	Pseudomonas psychrotolerans	–	–	–	–	–	–	–	–	–	–	–	–	12	–	1
	Salinivibrio costicola subsp. *vallismortis*	–	–	–	–	–	–	–	–	–	–	–	12	–	–	1
	Vibrio neocaledonicus	–	–	–	–	–	–	–	–	–	–	–	–	10	–	1

largest inhibition zone (17 mm) was measured for *Bacillus pumilus* against bacteriocin produced from *Salinivibrio costicola* subsp. *vallismortis* at 32°C, pH 7.0 and 1.17% NaCl. According to these results, optimum temperature, pH and NaCl concentration values of highest bacteriocin production were detected as 32°C, pH 7.0, 1.17% NaCl concentration (Table IV). The concentrations of crude bacteriocins produced by *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis* were respectively found as 1.02 mg/mL and 1.25 mg/mL. The amount of bacteriocins were found to be directly proportional to the inhibition zones of *Rhodococcus enclensis* (15 mm) and *Salinivibrio costicola* subsp. *vallismortis* (17 mm).

The antibacterial activities of bacteriocins produced from *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis* did not change after the treatments of heat temperatures (10-50°C for 15 min), pH ranges (6-8), NaCl concentrations (1.17-15%) (Table V). Although the antimicrobial activities of bacteriocins against all test microorganisms were not detected after treatment with Proteinase K at 37°C for 2 hours, these bacteriocins were resistant to treatment with lipase enzyme at 37°C for 2 hours. These results suggest that the antimicrobial compounds were proteinaceous substances (Table V).

Minimum Inhibitory Concentrations (MIC) of bacteriocins against multidrug-resistant isolates (*Kocuria sediminis*, *Bacillus pumilus*,

Table IV

Inhibition zone diameters (mm) of multidrug-resistant haloversatile bacteria against bacteriocins obtained from *Rhodococcus enclensis*, *Salinivibrio costicola* subsp. *vallismortis* at different incubation temperatures, pH and NaCl concentrations

Antibiotic resistant bacteria		*Kocuria sediminis*		*Bacillus pumilus*		*Pseudomonas psychrotolerans*
Bacteriocin producing isolates		*Rhodococcus enclensis*	*Salinivibrio costicola subsp. vallismortis*	*Rhodococcus enclensis*	*Salinivibrio costicola subsp. vallismortis*	*Salinivibrio costicola subsp. vallismortis*
Incubation temperature (°C)	20	–*	–	–	–	–
	28	8	6	5	10	5
	30	12	10	8	12	8
	32	**14**	**15**	**15**	**17**	**12**
	37	11	9	12	13	9
	40	6	5	7	6	6
pH	5	–	–	–	–	–
	6	8	8	6	7	5
	7	**14**	**15**	**15**	**17**	**12**
	8	9	11	8	9	6
	9	–	–	–	–	–
	10	–	–	–	–	–
NaCl concentration (%)	1.17	**14**	**15**	**15**	**17**	**12**
	3	10	12	13	14	10
	5	8	9	8	11	8
	10	5	5	6	9	5
	15	–	–	–	–	–
	20	–	–	–	–	–
	25	–	–	–	–	–

mm: milimeter, –*: absence of antibacterial effect

Table V

The effects of different temperatures, pH, NaCl concentrations, proteinase K and lipase enzymes on antibacterial effect of bacteriocins

		Kocuria sediminis		*Bacillus pumilus*		*Pseudomonas psychrotolerans*
		Rhodococcus enclensis	*Salinivibrio costicola* subsp. *vallismortis*	*Rhodococcus enclensis*	*Salinivibrio costicola* subsp. *vallismortis*	*Salinivibrio costicola* subsp. *vallismortis*
Temperature (°C) (15 min)						
Incubation temperature (°C)	4	+*	+	–**	+	–
	10	+	+	+	+	+
	20	+	+	+	+	+
	25	+	+	+	+	+
	28	+	+	+	+	+
	30	+	+	+	+	+
	32	+	+	+	+	+
	37	+	+	+	+	+
	40	+	+	+	+	+
	50	+	+	+	+	+
	60	–	+	–	+	–
	70	–	+	–	–	–
	80	–	–	–	–	–
	90	–	–	–	–	–
	100	–	–	–	–	–
pH (25°C, 4 h)						
pH values	4	–	–	–	–	–
	5	–	–	–	–	–
	6	+	+	+	+	+
	7	+	+	+	+	+
	8	+	+	+	+	+
	9	+	+	–	–	+
	10	–	–	–	–	–
	11	–	–	–	–	–
	12	–	–	–	–	–
	13	–	–	–	–	–
	14	–	–	–	–	–
% NaCl concentration (pH 7.0)						
Salt concentration (%)	1.17	+	+	+	+	+
	3	+	+	+	+	+
	5	+	+	+	+	+
	10	+	+	+	+	+
	15	+	+	+	+	+
	20	–	+	–	–	+
	25	–	–	–	–	–
Enzyme (2 h, 37°C)						
Proteinase K	–	–	–	–	–	–
Lipase	+	+	+	+	+	+

+*: presence of antibacterial effect; –**: absence of antibacterial effect

Table VI

The Minimum Inhibitory Concentrations of bacteriocin produced from *Rhodococcus enclensis* against *Kocuria sediminis* and *Bacillus pumilus*

Column number of 96-well plate	1	2	3	4	5	6	7	8	9	10	11	12
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	medium sterilization control	bacterial growth control
Kocuria sediminis	b[a]	p[b]	p	p	p	p	p	p	p	p	b	p
Bacillus pumilus	b	p	p	p	p	p	p	p	p	p	b	p

b[a]: Blue; p[b]: Pink

Pseudomonas psychrotolerans) were also detected in the present study. The MIC of bacteriocin produced from *Rhodococcus enclensis* against *Kocuria sediminis* and *Bacillus pumilus*, and the MIC of bacteriocin produced from *Salinivibrio costicola* subsp. *vallismortis* against *Kocuria sediminis*, *Bacillus pumilus* and *Pseudomonas psychrotolerans* were found as 1/2 (Tables VI-VII). The blue (**b**) colors in the Column 1 (Tables VI-VII) showed that the color of resazurin did not change to pink color due to the concentration of bacteriocins (1/2) were effective on the test bacteria and the cells of test bacteria were not alive. This bacteriocin concentration (1/2), which is the lowest concentration that inhibited the growth of the test isolates in the wells were accepted as MIC of bacteriocins (Tables VI-VII).

The growth of *Kocuria sediminis* and *Bacillus pumilus* which were exposed to the bacteriocin of *Rhodococcus enclensis*, and the growth of *Kocuria sediminis*, *Bacillus pumilus* and *Pseudomonas psychrotolerans* which were exposed to the bacteriocin of *Salinivibrio costicola* subsp. *vallismortis* were examined from the wells of Column 1 belonging to MIC endpoint to detect Minimum Bactericidal Concentration (MBC) (Tables VI-VII). Although, the tested bacteriocin concentrations of *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis* from 1/4 to 1/1024 did not effected the multidrug-resistant bacteria, the 1/2 bacteriocin concentration of *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis* was found to be effective on the test isolates. The bacterial growth was not observed on the plates after plating from the MIC (1/2) endpoint wells. Therefore, it was accepted that the bacteriocins of *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis* have bactericidal effect on the test isolates (Tables VI-VII).

To examine morphological changes in bacteriocin-treated isolates, the multidrug-resistant test bacteria were examined for morphological changes using a scanning electron microscope after the treatment of bacteriocin (Figures 1-3). The length of untreated *Kocuria sediminis*, *Bacillus pumilus*, *Pseudomonas psychrotolerans* cells were respectively recorded as 1.38 µm, 6.77 µm, 2.61 µm. After bacteriocin treatment, the round cell structure of *Kocuria sediminis* was lost (Fig.1a-c).

Table VII

The Minimum Inhibitory Concentrations of bacteriocin produced from *Salinivibrio costicola* subsp. *vallismortis* against *Kocuria sediminis*, *Bacillus pumilus* and *Pseudomonas psychrotolerans*

Column number of 96-well plate	1	2	3	4	5	6	7	8	9	10	11	12
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	medium sterilization control	bacterial growth control
Kocuria sediminis	b	p	p	p	p	p	p	p	p	p	b	p
Bacillus pumilus	b	p	p	p	p	p	p	p	p	p	b	p
Pseudomonas psychrotolerans	b	p	p	p	p	p	p	p	p	p	b	p

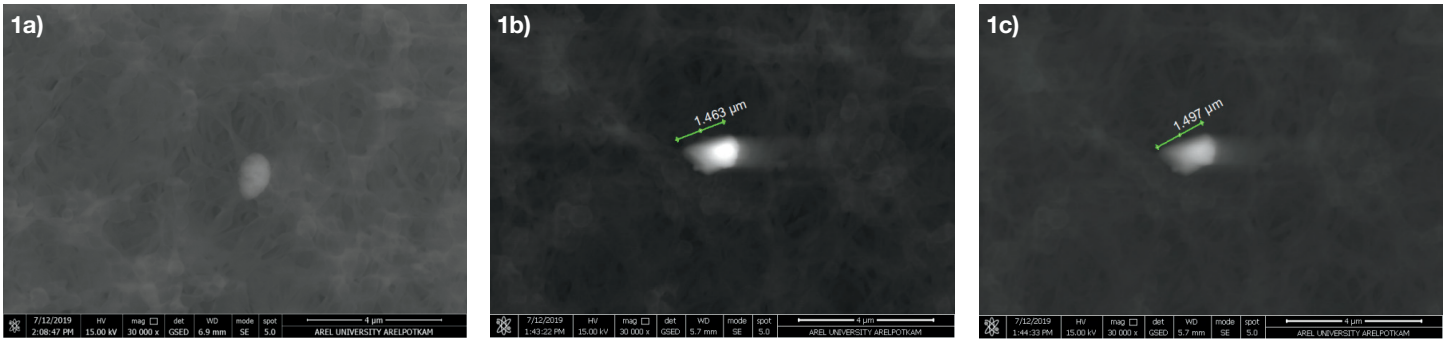

Figure 1. SEM micrographs of *Kocuria sediminis* on membrane filters without bacteriocin treatment **(a)**, after treatment with bacteriocin of *Rhodococcus enclensis* **(b)**, after treatment with bacteriocin of *Salinivibrio costicola* subsp. *vallismortis* **(c)**. The bar = 4 µm (a, b, c).

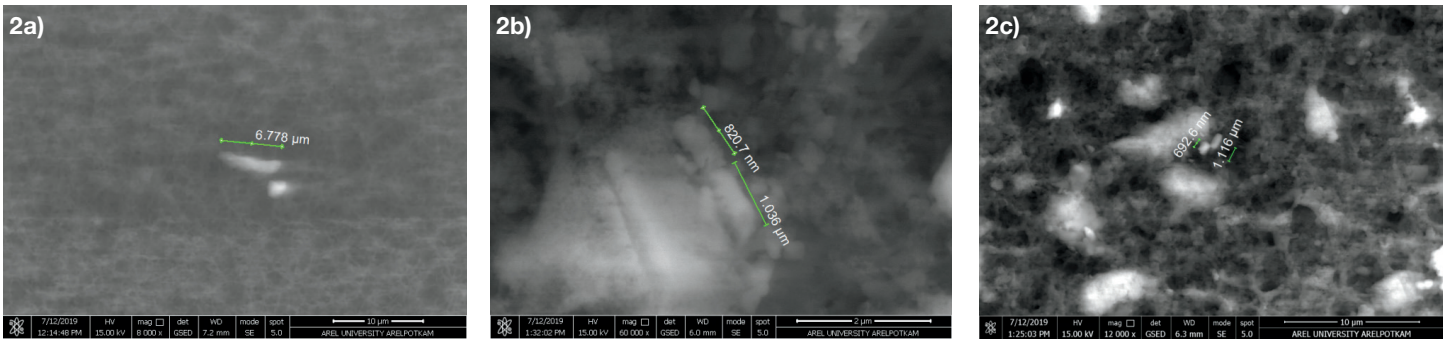

Figure 2. SEM micrographs of *Bacillus pumilus* on membrane filters without bacteriocin treatment **(a)**, after treatment with bacteriocin of *Rhodococcus enclensis* **(b)**, after treatment with *Salinivibrio costicola* subsp. *vallismortis* **(c)**. The bar = 10 µm (a), 2 µm (b), 10 µm (c).

The length of *Bacillus pumilus* cells after treatment with bacteriocin of *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis* were respectively measured as 1.03 µm-820 nm and 1.11 µm-692 nm (Fig.2a-c).

The length of *Pseudomonas psychrotolerans* cells after treatment with bacteriocin of *Salinivibrio costicola* subsp. *vallismortis* were recorded as 1.24 µm-692 nm (Fig.3a-b).

In leather industry, although salt is used for curing process, the experimental data of the researchers showed that salt contaminates hides and skins with various microorganisms. Mesophilic bacteria[64,65], moderately halophilic bacteria[32,33,66,67], enteric bacteria[68], halotolerant bacteria[49], extremely halophilic archaea[66] were previously reported on the salted hides, salted skins and preservation salt samples. In addition, antibiotic-resistant faecal indicator bacteria[69], antibiotic-resistant *Enterobacteriaceae*[70], antibiotic-resistant moderately halophilic bacteria[71] isolated from salted and soaked hides and skins were reported.

It has been known that antimicrobial agents are widely used in different industrial processes to prevent the bacterial growth and damage. Owing to randomly and misuse of these antimicrobial agents, some of the microorganisms may be resistant to these agents. Bacteria resistant to commonly used antimicrobial agents in different industries have been isolated in previous

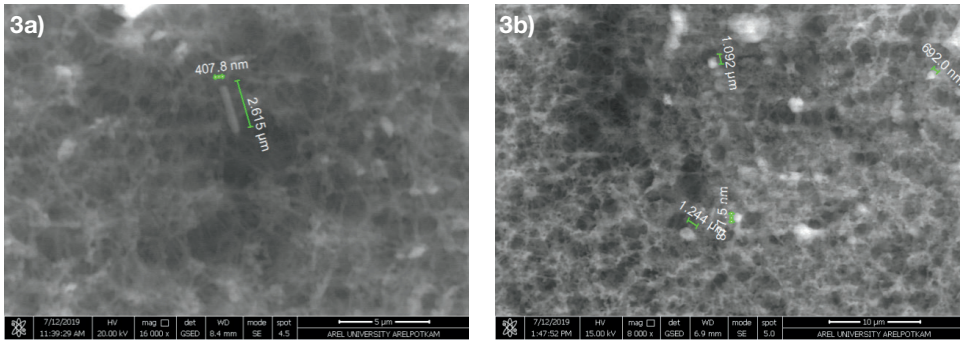

Figure 3. SEM micrographs of *Pseudomonas psychrotolerans* on membrane filters without bacteriocin treatment **(a)**, after treatment with bacteriocin of *Salinivibrio costicola* subsp. *vallismortis* **(b)**. The bar = 5 µm (a), 10 µm (b).

studies. These bacteria may have become resistant to antibiotics that entering their natural environment in different ways. To prevent spread of antibiotic resistance among bacteria in natural ecosystems, effective wastewater treatment, water disinfection and environmental regulations are essential. The researchers emphasized that bacteriocins may be an alternative to other antibacterial compounds due to their potency, low toxicity, broad or narrow spectrum of antibacterial activity, inhibition of pathogens and amenability to molecular manipulations.[28] The application of some bacteriocins were reported in previous studies. For instance, subtilosin A produced by *Bacillus subtilis* was used as anti-viral agent.[72] In another studies, it was reported that nisin inhibited the growths of *Listeria*, *Bacillus*, *Micrococcus*, *Alicyclobacillus*, *Pediococcus*, *Clostridium*, *Sporolactobacilllus*, *Desulfotomaculum*, *Enterococcus*, *Lactobacillus* and *Leuconostoc*[73] and it was used as biopreservative agent[74]. Sakacin A produced by *Lactobacillus curvatus* was used for controlling of *Listeria monocytogenes* and pediocin PA produced by *Pediococcus acidilactici* was used as a meat starter.[75,76] The applications of bacteriocins as cleaner-preservation methods to reduce salt pollution in leather industry were also reported by the researchers.[77-79] Bacteriocin produced from *Lactobacillus plantarum* was found to be effective against goat skin contaminated with *Pseudomonas aeruginosa* and *Bacillus putrefaciens*.[77] Bacteriocins produced by moderately halophilic non-enzyme producing *Salimicrobium salexigens*, *Halomonas halodenitrificans* and *Halomonas venusta* were inhibited the growth of moderately halophilic enzyme-producing *Gracilibacillus dipsosauri*, *Staphylococcus arlettae*, *Planococcus rifietoensis*, *Marinococcus tarijensis*, *Salinivibrio costicola subsp. alkaliphilus*, *Halomonas halmophila*, *Idiomarina loihiensis*, *Halomonas eurihalina*.[79] Rapid developement in genetics will allow to develope bacteriocins into the next generation antibiotics.[76]

Conclusion

This is the first study that contributes in screening and detection of bacteriocin producing haloversatile bacteria and multidrug-resistant haloversatile bacteria in the brine samples of Camalti Saltern. The experimental results showed that multidrug-resistant haloversatile bacterial species were found in brine samples collected from the Camalti Saltern. These species may survive during evaporation and salt production process at the Camalti Saltern due to their ability to live in harsh conditions, high salt concentrations, and wide ranges of pH and temperature values. When this salt is used in the leather industry, it may contaminate hide products with antibiotic-resistant bacteria. In order to combat antibiotic resistance, inappropriate and frequent antibiotic use must be reduced. Before discharging the wastewater to the sea, antibiotics and antibiotic resistant bacteria should be effectively removed from the wastewater. Due to the antimicrobial activities of bacteriocins and the stability of antimicrobial properties under various conditions, the bacteriocins produced by haloversatile bacteria may have potential application in different industries. Bacteriocins produced from *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis* showed broad spectrum inhibition and they may play an important role to decrease detrimental effects of multidrug resistant or enzyme-producing bacteria in leather industry during salting, brine curing or soaking processes of hides and skins. These results highlight their potential use in different industries such as leather and food.

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