

Examination of Haloversatile Bacteria on Salted Goatskin and Inactivation of Haloversatile Bacteria via Direct Electric Current

by

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Abstract

Haloversatile bacteria are among the commonly found microorganisms that have the potential to damage hides and skins in the leather industry. Therefore, the objective of this study was to investigate the presence of haloversatile bacteria on salted goatskins, to characterize these microorganisms through the use of molecular and conventional test methods, to detect their impact on the skins, and finally find an effective solution to inactivate these microorganisms. Haloversatile bacteria were common inhabitants at salted goatskin samples obtained from the Tuzla Organized Leather Industry Zone in Türkiye. Total numbers of haloversatile bacteria, proteolytic haloversatile bacteria, and lipolytic haloversatile bacteria on ten salted goatskin samples ranged from 7×10^4 to 2.7×10^5 CFU/g, 1×10^4 to 8×10^4 CFU/g, and 1×10^4 to 1.3×10^5 CFU/g, respectively. In the present study, 88% of the isolates were protease-positive, 69% were lipase-positive, 8% were xylanase-positive, 27% were caseinase-positive, 23% were amylase-positive, 8% were DNase-positive, 31% were cellulase-positive, 54% were urease-positive, 100% were catalase-positive, and 54% were oxidase-positive. The bacterial isolates showed positive reactions for the utilization of different amino acids such as glycine, L-cysteine, L-proline, and L-threonine, having the highest rates of 88%, 80%, 80%, and 80%, respectively. However, L-histidine had a lower positive reaction rate of 31%. The halophilic bacterial isolates exhibited positive reactions for the utilization and acid production from different types of sugar, with glucose having the highest positive reaction rate of 81%, followed by maltose at 73%, xylose at 58%, galactose at 46%, and lactose at 42%. Haloversatile enzyme-producing bacteria were identified using biochemical and molecular methods, resulting in the identification of 17 different species. Micrographs obtained from the scanning electron microscope revealed the damage inflicted on the fresh goatskin structure by haloversatile bacteria. A direct electric current of 2.2 A was applied to the mixed culture of haloversatile bacteria for 25 minutes to find an effective inactivation method. The total count of the mixed culture of haloversatile bacteria decreased from 7.3×10^6 CFU/mL to 4 CFU/mL within 16 minutes. All seventeen haloversatile bacteria in the mixed culture were killed within 19 minutes.

Introduction

The leather industry plays a significant role in the global economy by utilizing waste products from the meat industry to produce durable and valuable commercial products. Especially the longevity of leather products makes them a sustainable choice compared to some synthetic alternatives. Different leather goods such as footwear, jacket, bag, shoe, wallet, and garment are produced by animal skins and hides.^{1,2} Due to the presence of proteins (collagen, glycoproteins, keratin, elastin, globulins and albumins), carbohydrates, lipids, mineral salts and water in animal hides and skin microorganisms can grow on the raw hides and skins.^{3,4} The contamination of brine cured hides, salted hides, salted sheepskins, salted goatskins, and preservation salt with extremely halophilic archaea,⁵ moderately halophilic bacteria,^{5,6,10-14} halotolerant bacteria,¹⁵⁻¹⁹ and fungal species²⁰ were previously reported. The metabolic activities of these microorganisms cause grain peeling, hair slip, bad odor, loose grain break, grain sueding, disintegration of the structure of raw skins and hides.^{5,15,17,21} In this regard, the destructive effects of microorganisms may cause major economic loss in the leather industry.

Although there are studies reporting the presence of halotolerant bacteria,¹⁵⁻¹⁹ moderately halophilic bacteria,^{5,6,10-14} extremely halophilic archaea⁵⁻⁹ and fungi²⁰ in brine cured hides, salted hides, salted sheepskins, salted goatskins, and preservation salt, haloversatile bacteria found in salted goatskin samples in the leather industry have not been searched.

Previous study showed that haloversatile bacteria were also the other microorganisms found in the leather industry. It was reported that haloversatile *Terribacillus halophilus*, *Brevibacterium luteolum*, *Bacillus australimaris*, *Bacillus siamensis*, and *Bacillus mojavensis* species were isolated from soak liquor samples of leather industry.²²

While haloversatile bacteria can grow the medium with NaCl and the medium containing up to 3M NaCl, optimum NaCl concentrations for haloversatile bacteria are 0.2-0.5 M NaCl.^{23,24} The ability of accumulation compatible solutes allows haloversatile bacterial cells to survive under high osmotic stress.^{25,26} Cold-

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loving haloversatile bacterial species (*Carnobacterium funditum*, *Methanococcoides burtonii*, *Halomonas meridiana*, *Halobacterium lacusprofundi*, *Flavobacterium salegens*, *Carnobacterium alterfunditum*, *Halomonas subglaciescola*, *Flectobacillus glomeratus*, *Flavobacterium gondwanense*, *Vesiculatum antarcticum*) obtained from seawater of Antarctica were firstly reported by Ellis-Evans in 1985.²⁷ The production of compatible solutes by a haloversatile, psychropiezophile deep-sea bacterium *Photobacterium profundum* (isolate code SS9) was also stated.²⁸ Kaye et al. (2011)²⁹ studied the biodiversity of haloversatile *Marinobacter* species in seawater to investigate their biotechnological importance. Two haloversatile isolates which were obtained from mangroves in the Red Sea were reported by the researchers.³⁰

The haloversatile bacterial strains belonging to the genus *Bacillus*, *Kocuria*, *Paracoccus*, *Micrococcus*, *Microbacterium*, *Brevibacterium*, *Staphylococcus*, *Acinetobacter*, *Exiguobacterium*, *Gordonia*, *Microbacterium*, *Pseudomonas*, *Agrococcus*, *Sanguibacter*, *Virgibacillus*, *Rhodococcus*, *Marinobacter*, *Vibrio*, *Marinomonas Salinivibrio* were isolated from salt and brine samples of Camalti Saltern.^{31,32} The salt obtained from Camalti Saltern is also used in the leather industry to preserve hides and skins. The presence of proteolytic and lipolytic haloversatile bacteria in curing salt may cause contamination of salted hides and skins. To prevent the damage of salted hides and skins due to these microorganisms is very important. In 2006, Birbir and Birbir detected that low-level direct electric current was highly effective in inactivating various species of extremely halophilic archaea that were isolated from salt samples. The researchers reported that this technique could be used in the leather industry.³³ Direct electric current^{9,33-35} and combined application of alternating and direct electric currents³⁶⁻⁴⁰ were used for inactivating of different microorganisms found on the samples collected from the leather industry. Also, fresh orange juice,⁴¹ drinking water⁴² and seawater⁴³ were treated with direct electric current to control microorganisms. The inactivation of bacteria and archaea by direct electric current has garnered significant interest due to its high efficiency, cost-effectiveness, and applicability.

Although the presence of haloversatile bacteria in sea water,^{27,29} mangroves in the Red Sea,³⁰ salt and saltern^{31,32} and soak liquor²² was previously reported by few researchers, the presence of the haloversatile bacteria in the salted goatskin samples were not reported yet. Hence, the aims of the present study were to collect salted goatskin samples from the Tuzla Leather Organized Industrial Zone (Istanbul, Türkiye); to detect the total number of haloversatile bacteria, the total numbers of proteolytic and lipolytic haloversatile bacteria on the salted goatskin samples; to identify haloversatile bacterial isolates with conventional and molecular methods; to examine the damage caused by haloversatile bacteria on the fresh goatskin using Scanning Electron Microscope and to inactivate these isolates via direct electric current treatment.

Experimental

Collection of Salted Goatskin Samples from Tanneries and Measurement of pH Values

In this study, ten salted goatskin samples were obtained from the tanneries in the Tuzla Leather Organized Industrial Zone (Istanbul, Türkiye) and placed in sterile bags. Subsequently, the goatskin samples were placed in an insulated box with ice and they were immediately transported to the laboratory. To determine pH of salted goatskin samples, 20 grams of each sample were put into Erlenmeyer flasks containing 180 mL of sterilized sodium chloride solution (1.17% w/v). The Erlenmeyer flasks were placed into a shaking incubator for one hour at 90 rpm at 20°C. After this period, pH values were measured with pH-meter (PT 10, Sartorius Professional Meter PP-50 AG, Göttingen, Germany).⁴⁴

Determination of the Total Numbers of Haloversatile Bacteria, Proteolytic, and Lipolytic Haloversatile Bacteria on the Salted Goatskin Samples

To determine the total number of haloversatile bacteria found on salted goatskin samples, 20 grams of each salted goatskin sample were placed into Erlenmeyer flasks containing 180 mL of sodium chloride solution (1.17%, w/v). Then the Erlenmeyer flasks were put into a shaking incubator for one hour at 20°C, 90 rpm. The total numbers of haloversatile bacteria on the salted goatskin samples were determined by using spread plate technique on Halophilic Agar Medium (HAM) containing 11.7 g NaCl, 1 g MgSO₄·7H₂O, 0.36 g CaCl₂·2H₂O, 5 g protease peptone, 2 g KCl, 0.23 g NaBr, 1 g glucose, 10 g yeast extract, 0.06 g NaHCO₃, 20 g agar, 100 µL FeCl trace element, and 1000 mL distilled water.³¹ Direct and serial dilutions of bacterial suspensions (10⁻¹-10⁻⁶) were spread on the surface of HAM. After inoculation, the Petri dishes were incubated at 35°C for 24 hours. After the incubation period, the haloversatile bacterial colonies grown on HAM were counted. The bacterial suspensions were also spread on the surface of Gelatin Agar Medium (GAM) containing 2% gelatin (w/v) and 1.17% NaCl to determine the total number of proteolytic haloversatile bacteria of the salted goatskin samples. After 24 hours of incubation at 35°C, Frazier's reagent was poured over the bacterial colonies grown on GAM. The presence of transparent zones around the bacterial colonies indicated positive protease activity. Protease-positive bacterial colonies were counted.^{11,31,45} To determine the total number of lipolytic haloversatile bacteria of the salted goatskin samples, the bacterial suspensions were spread on Tween 80 Agar Medium (TAM) containing 5 g yeast extract, 11.7 g NaCl, 10 mL Tween 80, 20 g agar, and 1000 mL of sterile distilled water. The plates were incubated at 35°C for 24 hours. Lipase activity was indicated by the presence of opaque zones around the bacterial colonies grown on TAM. Lipase-positive colonies were counted.⁴⁵ Colony-forming units (CFU) were used to calculate the quantity of viable bacterial cells in the samples. The pH of all test media was adjusted to 7.0.

Isolation of Haloversatile Bacteria

Twenty grams of each salted goatskin sample were added into 180 mL of sterile physiological saline solution containing 1.17% NaCl. The suspensions were shaken in a shaking incubator at 35°C, 90 rpm for one hour. The direct and serial dilutions (10^{-1} - 10^{-6}) were prepared using sterile physiological saline solution (1.17% NaCl). To isolate haloversatile bacteria, 100 μ L of bacterial dilution spread over Petri dishes containing HAM. The inoculated Petri dishes were incubated in an incubator at 35°C for 24 hours. After the incubation period, each bacterial colony which was grown on HAM was picked with sterile loops using streak plate technique until pure haloversatile bacterial colonies were obtained.^{46,47}

Haloversatile Bacterial Identification

The DNA isolation process of haloversatile bacteria obtained from salted goatskin samples was carried out using the QIAamp DNA Mini Kit (Qiagen) DNA isolation kit.⁴⁸ The forward primer 16F27 (5'-AGAGTTTGATCMTGGCTCAG-3') and the reverse primer 16R1488 (5'-CGGTTACCTTGTTAGGACTTCACC-3') were used for the bacterial identification.⁴⁹ Subsequently, amplification was carried out using the Polymerase Chain Reaction (PCR) Technique. The 16S rRNA products were purified using the QIAquick PCR Purification Kit (Qiagen). After the 16S rRNA sequence analyses of the isolates, our isolates were identified using the 16S rRNA sequence database on the NCBI website.

Nucleotide Accession Numbers

The 16S rRNA gene sequence data of the isolates reported in this article, namely S1a, S1b, S2a, S2b, S2c, S3a, S3b, S3c, S4a, S4b, S4c, S4d, S5a, S5b, S6a, S6b, S6c, S7a, S7b, S8a, S8b, S8c, S9a, S10a, S10b, and S10c have been submitted to the NCBI and GenBank nucleotide sequence databases under the respective accession numbers: OR835202, OR835208, OR835209, OR835214, OR835215, OR835216, OR835217, OR835218, OR835220, OR835221, OR835222, OR835223, OR835224, OR835227, OR835225, OR835229, OR835230, OR835231, OR835234, OR835233, OR835238, OR835237, OR835239, OR835240, OR835241, OR835242.⁵⁰

Determination of Optimal Growth Conditions for Haloversatile Bacteria

To determine the optimal growth conditions for haloversatile bacteria were determined at different salt concentrations (0%, 1.17%, 2%, 3%, 5%, 8%, 10%, 12%, 15%, 17.5%, 20%, 22.5%, 25% NaCl), at different temperature values (4°C, 10°C, 15°C, 20°C, 25°C, 35°C, 37°C, 40°C, 45°C, 50°C, 55°C, 60°C), and at pH different values (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0).³¹ The pH of Halophilic Media were adjusted using 1N HCl and 1N NaOH. After inoculation, the Petri plates prepared with different salt concentrations and different pH values were incubated at 35°C for 24 hours.³¹ At the end of the incubation period, the optimal conditions for each isolate were recorded.³¹

Investigation of Enzymatic Properties of Haloversatile Bacteria

Cellulase activity was determined using agar-based cellulose media containing 10% NaCl and carboxymethyl cellulose. Bacteria were streaked onto the cellulose media. After the incubation period, bacterial colonies on the cellulose media were stained with a 0.1% congo red solution for 30 minutes and then washed with a 1 M NaCl solution. Positive cellulase activity was indicated by the presence of a clear zone around the bacterial colonies.^{51,52} Xylanase activity was assessed by streaking isolates onto sterile xylan media. After the incubation process, the presence of a clear zone around the colonies was considered as a positive indication of xylanase activity.⁴⁵ Urease activity was determined using a Urea Agar medium containing phenol red as an indicator. Isolates were streaked onto the media. The development of a pink color in the agar medium after incubation was considered as a positive indication of urease activity.⁴⁵ Protease activity was assessed by streaking isolates onto sterile Gelatin Agar media. After the incubation process, the media was overlaid with enough Frazier reagent to cover the Petri dish's surface. Clear zones observed around the colonies were accepted as a positive indication of protease activity.⁴⁵ Caseinase activity was assessed by streaking isolates onto sterile Casein Agar media. After the incubation process, the presence of a clear zone around the colonies was evaluated as a positive indication of caseinase activity.⁴⁵ DNase activity was determined by streaking isolates onto sterile DNase media. Then, a few drops of 1 N HCl were added to the media. The formation of a clear zone around the colonies showed positive DNase activity.⁴⁵ Oxidase test was performed by streaking pure bacterial colonies from the media onto filter paper soaked with para-aminodimethylaniline monohydrochloride. The development of a blue-purple color within 5-10 seconds was considered a positive result.^{46,53} Catalase activity was determined by adding a few drops of 3% hydrogen peroxide (H_2O_2) onto pure colonies grown on the media. The formation of gas bubbles on the surface of the colony was accepted as a positive indication of catalase activity.⁴⁵ Pullulanase activity was assessed by streaking isolates onto sterile pullulan media. After incubation, the presence of clear zones around the colonies showed positive pullulanase activity.⁴⁵ Lipase activity was determined by streaking isolates onto sterile Tween 80 Agar media. After incubation period, the presence of opaque zones around the colonies indicated positive lipase activity.⁴⁵ Amylase activity was assessed by streaking haloversatile isolates onto sterile Starch media. After incubation, the entire media was covered with Gram's iodine solution. The presence of clear zones around the colonies was accepted as a positive indication of starch hydrolysis by amylase enzyme.⁴⁵ All test media were incubated at 35°C for 24 hours.

Utilization of Different Amino Acids and Carbon Sources by Haloversatile Bacteria

In this study, metabolic activities of haloversatile bacterial isolates were evaluated using five amino acids and five sugars. The utilization of amino acid sources, including 1% glycine, L-histidine, L-cysteine, L-proline, and L-threonine was examined. The medium for amino

acids contained 0.05% dextrose, 0.5% beef extract, 0.0005% pyridoxal, 0.001% bromocresol purple, 0.5% peptone, and 0.0005% cresol red in 1.17% NaCl solution. Test tubes were incubated at 35°C for 24 hours after bacterial inoculation. A positive result was indicated by a change in color to purple in the test tubes.³¹ The utilization and acid production from different sugar sources by the haloversatile isolates were separately tested in the test medium containing 1% lactose, galactose, maltose, xylose, and glucose. The sugar medium contained 0.001% phenol red and 0.5% yeast extract. A change in color from red to yellow was considered a positive result.¹¹

Examination of the Damage Caused by Enzyme-Producing Haloversatile Bacteria on the Goatskin Sample Using Scanning Electron Microscope

Seventeen different haloversatile bacterial species (*Solibacillus silvestris*, *Bacillus licheniformis*, *Staphylococcus warneri*, *Bacillus sonorensis*, *Kocuria rosea*, *Oceanobacillus polygوني*, *Bacillus pumilus*, *Virgibacillus proomii*, *Staphylococcus hominis* subsp. *hominis*, *Bhargavaea ginsengi*, *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus paralicheniformis*, *Alkalihalobacillus clausii*, *Staphylococcus nepalensis*, *Micrococcus luteus*, *Ornithinibacillus scapharcae*) were used as test isolates in this experiment. Firstly, fresh goatskin samples were cut into two pieces and washed with sterile distilled water three times. As a control group (first piece of fresh goat skin), 10 g of fresh goatskin sample was placed into 30 mL of sterile saline solution (15% NaCl) in an Erlenmeyer flask. To prepare the mixed bacterial culture, the turbidity of each test isolate was adjusted to McFarland 0.5 turbidity standard tube (10^8 CFU/mL). Each bacterial solution was diluted one-tenth (10^7 CFU/mL) using a sterile 1.17% NaCl solution. Then, 1.7 mL of each test isolate (17 different isolate) was mixed to prepare the the mixed haloversatile bacterial culture. The second piece of fresh goatskin (10 g) was treated with the mixed culture of test isolates (10^7 CFU/mL) in an Erlenmeyer flask. The goatskin samples were shaken for 15 hours at 45 rpm, 24°C. After that, the samples were stored for 40 days to examine the adverse effects of bacterial enzymes on the skin structure. Furthermore, organoleptic characteristics such as unacceptable odor, sticky appearance, and hair loss were investigated on the goatskin samples during the storage period. After the storage period, the goatskin samples were prepared for examination under the scanning electron microscope. Raw goatskin samples, measuring 5 mm × 5 mm, were excised using a scalpel. The skins were thoroughly cleared of any impurities and hairs. First, the skin samples were fixed in glutaraldehyde solution (4%) prepared with phosphate buffer (pH 7.2, 0.1M) for 30 min, and then the samples were washed three times in phosphate buffer (0.1M) for ten minutes. The osmium tetroxide solution (1%) prepared with phosphate buffer (0.1M) was poured on the samples at 24°C for one hour. Afterward, the skin samples were washed twice with sterile distilled water, 35%, 50%, 75%, 95%, and absolute ethanol. The skin samples were respectively treated with the mix solution of ethanol-hexamethyldisilazane (HMDS) [1:1 (v/v)] (1x30 min), ethanol-HMDS [1:2 (v/v)] (1x30 min), and HMDS (2x30 min) for the air-drying process. After air-drying process,

the goatskin samples were placed in a desiccator for 14 hours for removing HMDS. The dried samples were placed on the stubs of the electron microscope using double-sided adhesive tape and examined under an electron microscope (Thermo Fisher Scientific Quattro S, Bogazici University, Istanbul, Türkiye).⁵⁴

Destruction of Haloversatile Bacteria Using Electric Current

The mixed culture of 17 haloversatile bacterial species (*Solibacillus silvestris*, *Bacillus licheniformis*, *Staphylococcus warneri*, *Bacillus sonorensis*, *Kocuria rosea*, *Oceanobacillus polygوني*, *Bacillus pumilus*, *Virgibacillus proomii*, *Staphylococcus hominis* subsp. *hominis*, *Bhargavaea ginsengi*, *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus paralicheniformis*, *Alkalihalobacillus clausii*, *Staphylococcus nepalensis*, *Micrococcus luteus*, *Ornithinibacillus scapharcae*) were used for the direct electric current treatment. First, the isolates were separately inoculated into test tubes containing a Halophilic Growth Medium. The test tubes were incubated at 35°C overnight. The density of each bacterial solution was adjusted to 10^7 CFU/mL using sterile saline solution (1.17% NaCl). Then, 20 mL of mixed culture was prepared by combining equal volumes from each of the 17 bacterial solutions. Subsequently, 20 mL of the mixed bacterial culture was added into a glass beaker containing 180 mL of 15% NaCl solution. Platinum wire electrodes were placed inside the glass beaker and 2.2 A direct electric current was applied to the test medium for 25 minutes. Throughout the experiment, 100 µL samples were taken from the test medium at 1, 4, 7, 10, 13, 16, 19, 22, and 25-minute intervals. These samples were then diluted and uniformly spreaded over the Halophilic Agar Medium (HAM) surface to determine the count of viable cells in the mixed culture. Colonies grown on HAM were counted after incubation at 35°C for 24 hours. At the beginning of the experiment, the pH of the test medium was 7.2, and the temperature was 22°C. The pH and temperature were measured at 1, 4, 7, 10, 13, 16, 19, 22, and 25-minute intervals during the experiment.^{11,33,36,43}

Examination of Haloversatile Bacterial Cells Before and After Treatment with Direct Electric Current Using Scanning Electron Microscopy

The mixed culture of haloversatile bacterial cells was centrifuged at 10,000 rpm for 5 minutes. Subsequently, after discarding the supernatant, the bacterial cells in the pellets were washed with 0.2 M Na-phosphate-buffered saline (PBS) prepared with 20.44 g Na_2HPO_4 , 6.72 g NaH_2PO_4 , and 500 mL dH_2O . The bacterial cells were fixed with 0.25% glutaraldehyde and incubated at 25°C for 30 minutes, followed by 37°C for 24 hours. The bacterial cells were washed three times with Na-PBS (pH 7.2) and centrifuged to collect the pellet. After that, the pellet was subjected to a series of ethanol washes and incubated in absolute ethanol at room temperature. Then, 10 µL of the pellet was spread on a sterile slide coated with gold using a gold-coating machine at the Department of Biomedical Engineering, Marmara University.^{55,56} Finally, the gold-coated samples were examined by Thermo Fisher Scientific-Quattro SEM at Bogazici University, Istanbul, Türkiye.

Results and Discussion

In the present study, we examined ten salted goatskin samples obtained from the Tuzla Leather Organized Industrial Zone. The pH values of the salted goatskin samples were found as 6.50-6.88. We determined that ten salted goatskin samples contained haloversatile bacteria on Halophilic Agar Medium between 7×10^4 and 2.7×10^5 CFU/g (Table I). The total proteolytic haloversatile bacterial counts on salted goatskin samples were found as between 1×10^4 and 8×10^4 CFU/g on Gelatin Agar Medium and the total lipolytic haloversatile bacterial counts on salted goatskin samples were found as between 1×10^4 and 1.3×10^5 CFU/g on Tween 80 Agar Medium (Table I). In a previous study conducted by Ozbay and Caglayan (2022)²² on haloversatile bacteria, the total counts of haloversatile bacteria in the soaking liquids collected from tanneries were found to be in the range of 3.8×10^4 to 1.1×10^6 CFU/mL on Oligotrophic Agar Medium and 6.7×10^4 to 1.6×10^6 CFU/mL on Halophilic Agar Medium. In that study, the researchers also reported that the total proteolytic haloversatile bacterial counts in the samples were found to be in the range of 1.2×10^4 to 5.8×10^5 CFU/mL on Gelatin Agar Medium.²² Additionally, the total lipolytic haloversatile bacterial counts were determined to be in the range of 2.3×10^4 to 4.6×10^5 CFU/mL on Tween 80 Agar Medium and 6.0×10^3 to 1.5×10^5 CFU/mL on Rhodamine B-Olive Oil Agar Medium.²² In the present study, haloversatile, proteolytic haloversatile and lipolytic haloversatile bacteria were found on all salted goatskin samples. The pH values of the salted goatskin samples were found as 6.50-6.88 (Table I).

A total of 26 isolates were obtained from salted goat skin samples. These isolates were identified as *Staphylococcus warneri*, *Alkalihalobacillus clausii*, *Solibacillus silvestris*, *Bacillus*

licheniformis, *Bacillus pumilus*, *Ornithinibacillus scapharcae*, *Kocuria rosea*, *Oceanobacillus polygona*, *Virgibacillus proomii*, *Staphylococcus hominis* subsp. *hominis*, *Bhargavaea ginsengi*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus cereus*, *Bacillus paralicheniformis*, *Staphylococcus nepalensis*, and *Micrococcus luteus*. While four, three, and two strains of *Staphylococcus warneri*, *Alkalihalobacillus clausii*, and *Solibacillus silvestris* were respectively isolated from the salted goat skin samples, only one strain of the other bacterial species was recovered from the salted goat skin samples (Table II).

In previous study, Caglayan (2019)³¹ isolated different haloversatile species such as *Exiguobacterium sibiricum*, *Bacillus subtilis* subsp. *stercoris*, *Bacillus haynesii*, *Paracoccus marcusii*, *Kocuria polaris*, *Acinetobacter radioresistens*, *Micrococcus aloeverae*, *Bacillus pumilus*, *Microbacterium maritypicum*, *Microbacterium aurantiacum*, *Brevibacterium frigoritolerans*, *Staphylococcus lentus*, *Paracoccus hibiscisoli*, *Staphylococcus hominis* subsp. *novobiosepticus*, *Bacillus velezensis*, *Staphylococcus equorum* subsp. *equorum*, *Bacillus safensis*, *Kocuria sediminis*, *Staphylococcus petrasii* subsp. *jettensis*, *Staphylococcus pasteurii*, *Bacillus thioparans*, *Staphylococcus warneri*, *Virgibacillus salarius*, *Bacillus paraflexus*, *Pseudomonas songnenensis*, *Microbacterium saccharophilum*, *Bacillus altitudinis*, *Staphylococcus saprophyticus* subsp. *saprophyticus*, *Gordonia alkanivorans*, *Micrococcus yunnanensis*, *Agrococcus lahaulensis*, *Kocuria rosea*, *Sanguibacter inulinus*, *Bacillus nealsonii*, *Staphylococcus cohnii* subsp. *urealyticus*, *Bacillus oryzaecorticis*, *Exiguobacterium artemiae*, *Exiguobacterium indicum*, *Bacillus siamensis*, and *Staphylococcus epidermidis* from 20 salt samples collected from Camalti Saltern. Caglayan (2022)³¹ also isolated and identified 16 haloversatile bacteria

Table I

The Salted Skin Sample Codes, pH of Salted Skin Samples, Total Haloversatile, Total Proteolytic Haloversatile, Total Lipolytic Haloversatile Bacterial Counts on Salted Goatskin Samples (CFU/g)

Sample no	Skin sample code	pH of skin	Total counts of haloversatile bacteria (CFU/g)	Total counts of proteolytic haloversatile bacteria (CFU/g)	Total counts of lipolytic haloversatile bacteria (CFU/g)
1	SSS1	6.79	1.3×10^5	1×10^4	1×10^4
2	SSS2	6.64	8×10^4	3×10^4	2×10^4
3	SSS3	6.76	1.5×10^5	5×10^4	1.3×10^5
4	SSS4	6.68	1.6×10^5	4×10^4	6×10^4
5	SSS5	6.68	8×10^4	2×10^4	1×10^4
6	SSS6	6.86	1×10^5	8×10^4	8×10^4
7	SSS7	6.88	2.7×10^5	5×10^4	2×10^4
8	SSS8	6.75	9×10^4	1×10^4	6×10^4
9	SSS9	6.61	1.5×10^5	3×10^4	4×10^4
10	SSS10	6.50	7×10^4	1×10^4	3×10^4

SGS: Salted Goatskin Sample

Table II
The Isolate Codes, Phylogenetically Similar Species, Length (bp), Similarity (%),
Accession Number of Haloversatile Isolates obtained from Salted Goat Skin Samples

	Isolate code	Phylogenetically similar species	Length (bp)	Similarity (%)	Accession Number
1	S1a	<i>Solibacillus silvestris</i>	1371	99.40	MT013412
2	S1b	<i>Bacillus licheniformis</i>	1451	99.24	MN368292
3	S2a	<i>Staphylococcus warneri</i>	1495	100	MT642942
4	S2b	<i>Bacillus sonorensis</i>	1451	99.50	MN710423
5	S2c	<i>Kocuria rosea</i>	1457	99.37	MT225716
6	S3a	<i>Oceanobacillus polygona</i>	1518	99.65	NR114348
7	S3b	<i>Bacillus pumilus</i>	1263	99.74	KR006242
8	S3c	<i>Virgibacillus proomii</i>	1405	99.80	KT893316
9	S4a	<i>Staphylococcus hominis</i> subsp. <i>hominis</i>	1463	99.55	MT585539
10	S4b	<i>Bhargavaea ginsengi</i>	1506	99.56	MN121192
11	S4c	<i>Bacillus amyloliquefaciens</i>	1498	99.45	MT133342
12	S4d	<i>Bacillus licheniformis</i>	1145	99.81	MT492090
13	S5a	<i>Bacillus cereus</i>	1297	99.90	HE660038
14	S5b	<i>Bacillus paralicheniformis</i>	1178	99.73	OP700359
15	S6a	<i>Alkalihalobacillus clausii</i>	1407	99.70	MH938303
16	S6b	<i>Solibacillus silvestris</i>	1371	99.77	MT013412
17	S6c	<i>Staphylococcus nepalensis</i>	1340	99.91	LC511694
18	S7a	<i>Staphylococcus warneri</i>	1276	99.62	MK491025
19	S7b	<i>Staphylococcus warneri</i>	1495	99.70	MT642942
20	S8a	<i>Micrococcus luteus</i>	1455	99.47	MH669309
21	S8b	<i>Ornithinibacillus scapharcae</i>	1434	99.63	MK860001
22	S8c	<i>Alkalihalobacillus clausii</i>	1370	99.81	MT604989
23	S9a	<i>Staphylococcus warneri</i>	1495	99.61	MT642942
24	S10a	<i>Ornithinibacillus scapharcae</i>	1434	99.03	MK860001
25	S10b	<i>Alkalihalobacillus clausii</i>	1407	99.70	MH938303
26	S10c	<i>Bacillus pumilus</i>	1094	99.32	MK377090

which were obtained from brine samples of Camalti Saltern. Those isolates belong to the species *Rhodococcus enclensis*, *Bacillus haynesii*, *Kocuria sediminis*, *Bacillus simplex*, *Salinivibrio costicola* subsp. *vallismortis*, *Bacillus subtilis* subsp. *stercoris*, *Vibrio olivae*, *Bacillus pumilus*, *Staphylococcus saprophyticus* subsp. *saprophyticus*, *Staphylococcus petrasii* subsp. *jettensis*, *Marinomonas communis*, *Marinobacter hydrocarbonoclasticus*, *Vibrio neocaledonicus*, and *Pseudomonas psychrotolerans*.³² Ozbay and Caglayan (2022)²² reported that six haloversatile bacterial isolates were obtained from soak liquor samples collected from leather industry and identified as *Bacillus mojavenensis*, *Bacillus australimaris*, *Brevibacterium luteolum*, *Bacillus siamensis*, and *Terribacillus halophilus*. Haloversatile

Staphylococcus, *Bacillus*, and *Kocuria* species were also isolated by other researchers^{22,31,32} but the isolation of haloversatile *Oceanobacillus polygona*, *Virgibacillus proomii*, *Bhargavaea ginsengi*, *Ornithinibacillus scapharcae*, and *Alkalihalobacillus clausii* from the salted goat skin samples have first been reported in the present study.

All isolates were Gram-positive and grew optimally at pH 7, 1.17% NaCl, 35°C (Table III). Also, *Bacillus paralicheniformis* isolate S5b grew optimally at pH 8 (Table III). Isolates S1a, S2a, S4b, S4c, S4d, S5a, S5b S6b, S6c, S7a, S7b, S9a, S10c; isolates S1b, S2b, S3a, S3b, S6a, S8c, S10b; isolates S2c, S3c, S8a, S8b, S10a were respectively showed growth at pH 5-11, pH 5-12, pH 6-11 (Table

Table III
The Effects of pH, NaCl Contents and Temperature Values on the Growth of Haloversatile Bacterial Isolates

	Isolate code	Isolate name	Optimum pH	Range of pH	Optimum NaCl (%)	Range of NaCl (%)	Optimum temperature(°C)	Range of temperature (°C)	Gram Stain
1	S1a	<i>Solibacillus silvestris</i>	7	5-11	1.17	0-17.5	35	20-50	+
2	S1b	<i>Bacillus licheniformis</i>	7	5-12	1.17	0-17.5	35	15-55	+
3	S2a	<i>Staphylococcus warneri</i>	7	5-11	1.17	0-17.5	35	20-50	+
4	S2b	<i>Bacillus sonorensis</i>	7	5-12	1.17	0-17.5	35	15-55	+
5	S2c	<i>Kocuria rosea</i>	7	6-11	1.17	0-17.5	35	20-45	+
6	S3a	<i>Oceanobacillus polygona</i>	7	5-12	1.17	0-17.5	35	15-55	+
7	S3b	<i>Bacillus pumilus</i>	7	5-12	1.17	0-17.5	35	15-55	+
8	S3c	<i>Virgibacillus proomii</i>	7	6-11	1.17	0-17.5	35	20-50	+
9	S4a	<i>Staphylococcus hominis</i> subsp. <i>hominis</i>	7	6-12	1.17	0-17.5	35	20-45	+
10	S4b	<i>Bhargavaea ginsengi</i>	7	5-11	1.17	0-17.5	35	20-45	+
11	S4c	<i>Bacillus amyloliquefaciens</i>	7	5-11	1.17	0-17.5	35	15-55	+
12	S4d	<i>Bacillus licheniformis</i>	7	5-11	1.17	0-17.5	35	15-55	+
13	S5a	<i>Bacillus cereus</i>	7	5-11	1.17	0-17.5	35	15-55	+
14	S5b	<i>Bacillus paralicheniformis</i>	7-8	5-11	1.17	0-17.5	35-37	15-55	+
15	S6a	<i>Alkalihalobacillus clausii</i>	7	5-12	1.17	0-17.5	35	20-45	+
16	S6b	<i>Solibacillus silvestris</i>	7	5-11	1.17	0-17.5	35	20-45	+
17	S6c	<i>Staphylococcus nepalensis</i>	7	5-11	1.17	0-17.5	35	20-45	+
18	S7a	<i>Staphylococcus warneri</i>	7	5-11	1.17	0-17.5	35	20-50	+
19	S7b	<i>Staphylococcus warneri</i>	7	5-11	1.17	0-17.5	35	20-50	+
20	S8a	<i>Micrococcus luteus</i>	7	6-11	1.17	0-17.5	35	20-45	+
21	S8b	<i>Ornithinibacillus scapharcae</i>	7	6-11	1.17	0-17.5	35	20-45	+
22	S8c	<i>Alkalihalobacillus clausii</i>	7	5-12	1.17	0-17.5	35	20-45	+
23	S9a	<i>Staphylococcus warneri</i>	7	5-11	1.17	0-17.5	35	20-50	+
24	S10a	<i>Ornithinibacillus scapharcae</i>	7	6-11	1.17	0-17.5	35	20-50	+
25	S10b	<i>Alkalihalobacillus clausii</i>	7	5-12	1.17	0-17.5	35	20-45	+
26	S10c	<i>Bacillus pumilus</i>	7	5-11	1.17	0-17.5	35	15-55	+

III). Although all isolates were able to grow at 0-17.5% NaCl, the optimum growth value was 1.17% NaCl. Hence, all isolates were accepted as haloversatile.^{25,26} The growth temperature values of the isolates were slightly different from each other. Isolates S1b, S2b, S3a, S3b, S4c, S4d, S5a, S5b, S10c; isolates S1a, S2a, S3c, S7a, S7b, S9a, S10a; isolates S2c, S4a, S4b, S6a, S6b, S6c, S8a, S8b, S8c, S10b were respectively exhibited growth at 15-55°C, 20-50°C, 20-45°C (Table III).

Staphylococcus saprophyticus subsp. *saprophyticus*, *Bacillus simplex*, *Bacillus pumilus*, *Marinobacter hydrocarbonoclasticus*, *Staphylococcus*

petrasii subsp. *jettensis*, *Vibrio neocaledonicus*, *Salinivibrio costicola* subsp. *vallismortis*, *Bacillus haynesii*, *Kocuria sediminis*, *Pseudomonas psychrotolerans*, *Rhodococcus enclensis*, *Marinomonas communis*, *Vibrio olivae*, *Bacillus subtilis* subsp. *stercoris* which were previously isolated from the brine samples of Camalti Saltern were able to grow at 0-3 M NaCl (optimum growth at 0.2 M NaCl), pH 5-12 and 10-55°C.³²

When we examined the enzymatic activities of 26 haloversatile bacteria isolated from salted goatskin samples, they exhibited enzymatic activities as follows: 88% protease-positive, 69% lipase-

Table IV
Enzymatic Characteristics of Haloversatile Isolates obtained from Salted Goatskin Samples

	Isolate code	Haloversatile species	Protease	Lipase	Xylanase	Pullulanase	Caseinase	Amylase	DNase	Cellulase	Urease	Catalase	Oxidase
1	S1a	<i>Solibacillus silvestris</i>	+	-	-	-	-	-	-	-	+	+	-
2	S1b	<i>Bacillus licheniformis</i>	+	+	-	-	+	+	-	+	-	+	+
3	S2a	<i>Staphylococcus warneri</i>	+	+	-	-	-	-	-	-	+	+	-
4	S2b	<i>Bacillus sonorensis</i>	+	+	-	-	-	+	-	+	-	+	+
5	S2c	<i>Kocuria rosea</i>	+	-	-	-	-	-	-	-	-	+	+
6	S3a	<i>Oceanobacillus polygoni</i>	-	-	-	-	-	-	-	-	+	+	+
7	S3b	<i>Bacillus pumilus</i>	+	+	-	-	+	-	-	+	+	+	+
8	S3c	<i>Virgibacillus proomii</i>	+	+	-	-	-	-	+	-	+	+	-
9	S4a	<i>Staphylococcus hominis</i> subsp. <i>hominis</i>	-	-	-	-	-	-	-	-	-	+	-
10	S4b	<i>Bhargavaea ginsengi</i>	+	-	-	-	-	-	+	-	-	+	-
11	S4c	<i>Bacillus amyloliquefaciens</i>	+	-	-	-	-	+	-	+	-	+	+
12	S4d	<i>Bacillus licheniformis</i>	+	+	-	-	+	+	-	+	-	+	+
13	S5a	<i>Bacillus cereus</i>	+	+	-	-	+	+	-	-	-	+	-
14	S5b	<i>Bacillus paralicheniformis</i>	+	+	-	-	-	+	-	-	-	+	-
15	S6a	<i>Alkalihalobacillus clausii</i>	+	+	-	-	-	-	-	-	+	+	+
16	S6b	<i>Solibacillus silvestris</i>	+	-	-	-	-	-	-	-	+	+	-
17	S6c	<i>Staphylococcus nepalensis</i>	-	+	-	-	-	-	-	-	+	+	-
18	S7a	<i>Staphylococcus warneri</i>	+	+	-	-	-	-	-	-	+	+	-
19	S7b	<i>Staphylococcus warneri</i>	+	+	-	-	-	-	-	-	+	+	-
20	S8a	<i>Micrococcus luteus</i>	+	-	-	-	-	-	-	-	-	+	+
21	S8b	<i>Ornithinibacillus scapharcae</i>	+	+	+	-	+	-	-	+	-	+	+
22	S8c	<i>Alkalihalobacillus clausii</i>	+	+	-	-	-	-	-	-	+	+	+
23	S9a	<i>Staphylococcus warneri</i>	+	+	-	-	-	-	-	-	+	+	-
24	S10a	<i>Ornithinibacillus scapharcae</i>	+	+	+	-	+	-	-	+	-	+	+
25	S10b	<i>Alkalihalobacillus clausii</i>	+	+	-	-	-	-	-	-	+	+	+
26	S10c	<i>Bacillus pumilus</i>	+	+	-	-	+	-	-	+	+	+	+

positive, 8% xylanase-positive, 27% caseinase-positive, 23% amylase-positive, 8% DNase-positive, 31% cellulase-positive, 54% urease-positive, 100% catalase-positive, 54% oxidase-positive, and 100% pullulanase-negative (Table IV).

Haloversatile isolates reported by Ozbay and Caglayan (2022)²² were able to produce protease (83%), lipase (83%), caseinase (67%), amylase (50%), cellulase (17%). However, those isolates did not produce xylanase, DNase, pullulanase and urease.²²

Table V shows the utilization of different sugars (lactose, galactose, xylose, glucose, maltose) and different amino acids (L-cysteine, L-proline, L-threonine, L-histidine, glycine) by haloversatile bacterial isolates. The bacterial isolates showed positive results for lactose at 42%. For galactose, the isolates showed a positive result of 46%. For xylose, the positivity rate was 58%. As for glucose, 81% of the isolates gave a positive result, while maltose resulted in 73% positivity (Table V). These results provide insights into how microorganisms metabolize different sugars. It was concluded that

Table V
Utilization of Different Sugars and Amino Acids by Haloversatile Isolates

	Isolate code	Haloversatile species	Lactose	Galactose	Xylose	Glucose	Maltose	L-cysteine	L-proline	L-threonine	L-histidine	Glycine
1	S1a	<i>Solibacillus silvestris</i>	-	-	-	-	-	-	+	+	-	+
2	S1b	<i>Bacillus licheniformis</i>	+	-	+	+	+	-	-	+	-	+
3	S2a	<i>Staphylococcus warneri</i>	-	-	-	+	+	+	+	+	+	+
4	S2b	<i>Bacillus sonorensis</i>	-	+	+	+	+	+	-	-	-	-
5	S2c	<i>Kocuria rosea</i>	-	-	+	+	-	+	+	+	+	+
6	S3a	<i>Oceanobacillus polygona</i>	-	-	-	-	+	+	+	-	-	+
7	S3b	<i>Bacillus pumilus</i>	+	+	+	+	-	+	+	+	-	+
8	S3c	<i>Virgibacillus proomii</i>	-	+	-	+	+	+	+	+	+	+
9	S4a	<i>Staphylococcus hominis</i> subsp. <i>hominis</i>	+	+	-	+	+	+	+	+	-	+
10	S4b	<i>Bhargavaea ginsengi</i>	-	-	+	+	+	+	+	+	+	+
11	S4c	<i>Bacillus amyloliquefaciens</i>	+	+	+	+	+	+	+	+	-	-
12	S4d	<i>Bacillus licheniformis</i>	+	-	+	+	+	-	-	+	-	+
13	S5a	<i>Bacillus cereus</i>	-	-	-	+	+	+	+	-	+	+
14	S5b	<i>Bacillus paralicheniformis</i>	+	-	+	+	+	+	-	+	-	-
15	S6a	<i>Alkalihalobacillus clausii</i>	+	+	+	+	+	+	+	-	-	+
16	S6b	<i>Solibacillus silvestris</i>	-	-	-	-	-	-	+	+	-	+
17	S6c	<i>Staphylococcus nepalensis</i>	+	+	+	+	+	-	-	+	-	+
18	S7a	<i>Staphylococcus warneri</i>	-	-	-	+	+	+	+	+	+	+
19	S7b	<i>Staphylococcus warneri</i>	-	-	-	+	+	+	+	+	+	+
20	S8a	<i>Micrococcus luteus</i>	-	-	-	+	+	+	+	+	-	+
21	S8b	<i>Ornithinibacillus scapharcae</i>	-	+	+	-	-	+	+	+	-	+
22	S8c	<i>Alkalihalobacillus clausii</i>	+	+	+	+	+	+	+	-	-	+
23	S9a	<i>Staphylococcus warneri</i>	-	-	-	+	+	+	+	+	+	+
24	S10a	<i>Ornithinibacillus scapharcae</i>	-	+	+	-	-	+	+	+	-	+
25	S10b	<i>Alkalihalobacillus clausii</i>	+	+	+	+	+	+	+	-	-	+
26	S10c	<i>Bacillus pumilus</i>	+	+	+	+	-	+	+	+	-	+

the haloversatile bacteria in the skin primarily utilize glucose and have the least utilization of lactose.

It has been known that collagen types I and III contain alanine, arginine, asparagine, aspartic acid, glutamic acid, glutamine, glycine, histidine, hydroxylysine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine.⁵⁷ While alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, hydroxyproline, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine, and valine⁵⁷ are found in

elastin, keratin contains alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, tryptophan, and valine.⁵⁷

The bacterial isolates showed positive results for L-cysteine at 80%. For L-proline, the isolates yielded an 80% positive result. L-threonine resulted in 80% positivity as well. However, only 31% of the isolates showed a positive result for L-histidine. As for glycine, 88% of the isolates demonstrated a positive result (Table V). These results provide insights into how microorganisms metabolize

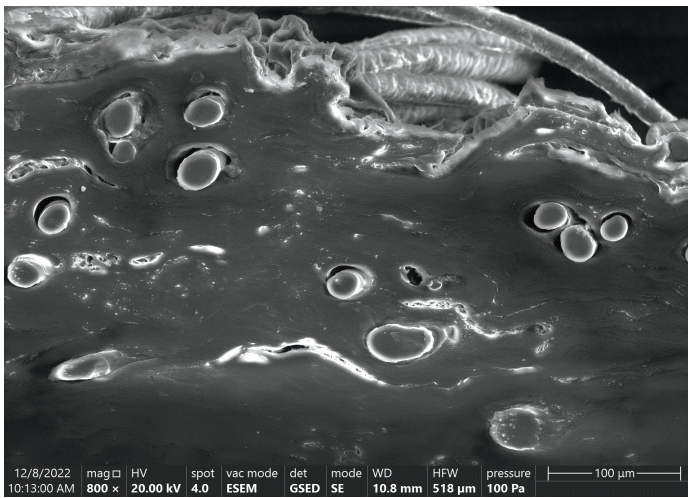


Figure 1. SEM micrograph of the longitudinal section of undamaged goatskin treated with sterile brine solution stored for 40 days at 24°C (Control).
The bar: 100 µm.

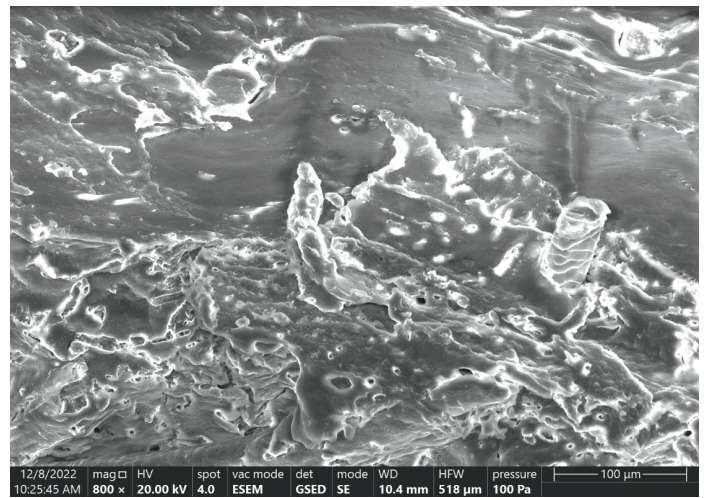


Figure 2. SEM micrograph of the longitudinal section of damaged goatskin treated with mixed culture of haloversatile isolates stored for 40 days at 24°C.
The bar: 100 µm.

different amino acids. It was determined that the haloversatile bacteria in the skin primarily utilize glycine and have the least utilization of L-histidine. These results showed that proteolytic haloversatile strains have the potential utilization of amino acids as nitrogen sources to grow on the skin after the breakdown of protein into amino acids.

Different sugar sources such as lactose (100%), dextrose (100%), myo-inositol (100%), cellobiose (100%), adonitol (100%), salicin (100%), dulcitol (100%), mannose (100%), xylitol (83%), arabinose (83%), mannitol (67%), fructose (67%), trehalose (67%), ribose (50%), melezitose (33%), sucrose (17%) and different amino acid sources such as L-serine (100%), L-glutamic acid (67%), L-phenylalanine (67%), trans-4-hydroxy-L-proline (67%), L-proline

(67%), glycine (50%), L-ornithine (50%), L-aspartic acid (33%), L-phenylalanine (33%), L-arginine (17%), L-histidine (17%), L-lysine (17%), L-threonine (17%) were used by haloversatile bacteria isolated from the soak liquors.²² However, sorbitol, galactose, maltose, xylose and melibiose, L-isoleucine, L-cystine, L-alanine, leucine, L-methionine, L-tyrosine, L-valine were not utilized by the isolates.²²

At the end of the 40-days storage period, the changes on the structure of the goatskin control sample (Figure 1) and the goatskin sample treated with the mixed culture of 17 different haloversatile bacterial species (Figure 2) caused by the haloversatile bacteria were investigated under scanning electron microscope. On the goatskin control sample, it was clearly observed that there was no hair loss, and the skin integrity was preserved (Figure 1).

TABLE VI

The pH values, temperature, voltage and the total counts (CFU/mL) of enzyme producing haloversatile bacteria in the direct electric current treatment

Time	pH value	Temperature (°C)	Voltage (V)	Total counts of mixed culture (CFU/mL)
Before experiment	7.2	22	6.5	7.3×10^6
1 min	7.2	22	6.5	1.4×10^5
4 min	7.3	22	6.4	2.5×10^4
7 min	7.3	23	6.2	5.4×10^3
10 min	7.4	24	5.9	1.6×10^2
13 min	7.4	25	5.7	8×10^1
16 min	7.5	26	5.5	4
19 min	7.5	27	5.3	-
22 min	7.6	28	5.1	-
25 min	7.6	29	4.9	-

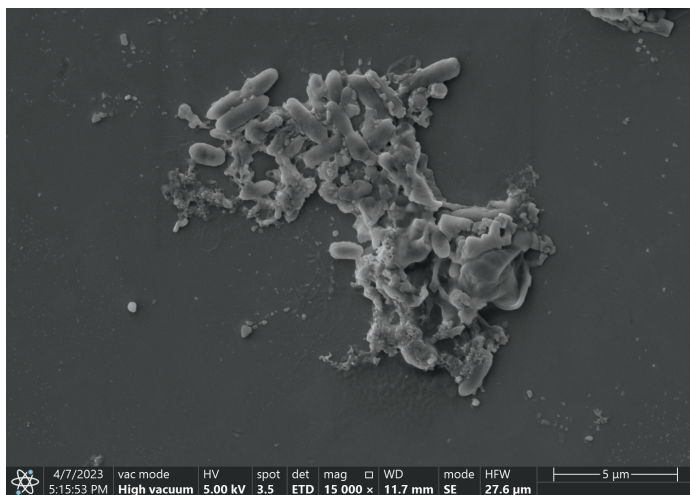


Figure 3. SEM micrograph of the mixed culture of enzyme producing haloversatile bacterial cells before direct electric current treatment. The bar: 5 μm .

Softening and decomposition were observed on the goatskin sample treated with the mixed culture of test bacteria (Figure 2). In addition, environmental scanning electron microscope image clearly showed that the hair follicles in the goatskin were damaged, hairs were broken, and the fibrous structure of the skin was disrupted (Figure 2).

Figure 2 shows the damage caused by the mixed culture of enzyme-producing haloversatile bacteria to the structure of goatskin. Hair loss can be observed in the goatskin (Figure 2). Scanning electron micrograph demonstrates that the haloversatile isolates in the mixed culture destroy the collagen fibers and hairs of the skin.

Before the electric current treatment, the total viable cell count of the mixed culture was 7.3×10^6 CFU/mL (Table VI). During the electric current treatment, the mixed haloversatile bacterial cells were gradually killed. After 16 minutes, it decreased to 4 CFU/mL. After 19 minutes, all test bacteria in the mixed culture were completely killed. While the pH was 7.2 before the experiment, the pH was reached to 7.6 at the end of the experiment. The temperature increased from 22°C to 29°C (Table VI). The voltage values were slightly decreased from 6.5 to 4.9 (Table VI).

Figure 3 clearly shows the morphological structures of enzyme-producing haloversatile bacterial cells in the mixed culture before the direct electric current treatment in the SEM micrograph.

Figure 4 shows the damaged cell structure of enzyme-producing haloversatile bacteria in the mixed culture after direct electric current treatment in the SEM micrograph.

In a study conducted by Birbir and colleagues in 2008, inactivation of extremely halophilic bacteria in salt samples was achieved

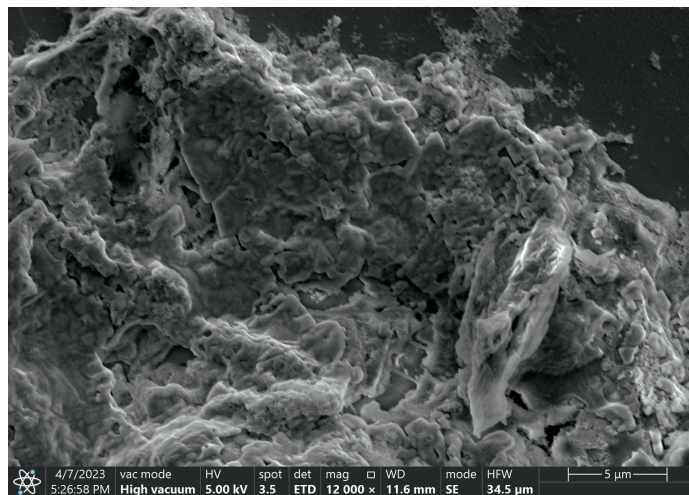


Figure 4. SEM micrograph of damaged bacterial cells in the mixed culture after direct electric current treatment. The bar: 5 μm .

with a direct electric current of 0.5 A.^{34,35} In another study, 1.5 A alternating electric current was used to eliminate a mixed culture of *Enterobacter cloacae*, *Pseudomonas luteola*, and *Vibrio fluvialis*, which were Gram-negative bacteria isolated from hides. The mixed culture of bacteria in the liquid medium containing 25% NaCl was eradicated within 1 minute using a 1.5 A alternating electric current.⁵⁸

The negative effects of protease and lipase positive extremely halophilic archaea against sheepskin structure were previously reported by Birbir and her colleagues in 2020.⁹ In a different research, the detrimental impacts of Gram-positive haloversatile bacteria isolated from soak liquor samples on the skin samples were proven.²² The results obtained from other studies were also similar to the results of this study. The haloversatile bacteria are able to survive under harsh conditions and damage goatskin structure. However, the application of electric current was found to be very effective in killing different species of haloversatile bacteria. In just 19 minutes, all 17 haloversatile bacteria in the mixed culture were successfully eliminated. This impressive outcome demonstrates the effectiveness of the electric current inactivation method and highlights its potential for use in the leather industry.

Conclusion

This study is the first to contribute to the characterization of haloversatile bacteria found in salted goatskin samples, the investigation of the harmful effects of enzyme-producing haloversatile bacteria on the skin under environmental scanning electron microscopy, and the application of direct electric current to kill bacteria in a mixed culture of enzyme-producing haloversatile isolates. We detected that haloversatile bacteria

produced different enzymes and utilized different amino acid and sugar sources. Based on the results of 16S rRNA sequencing, the 26 isolated haloversatile bacterial isolates were found to belong to 17 different species, including *Solibacillus silvestris*, *Bacillus licheniformis*, *Staphylococcus warneri*, *Bacillus sonorensis*, *Kocuria rosea*, *Oceanobacillus polygona*, *Bacillus pumilus*, *Virgibacillus proomii*, *Staphylococcus hominis* subsp. *hominis*, *Bhargavaea ginsengi*, *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus paralicheniformis*, *Alkalihalobacillus clausii*, *Staphylococcus nepalensis*, *Micrococcus luteus*, *Ornithinibacillus scapharcae*. According to our experimental results, we have found that the application of direct electric current can easily kill haloversatile bacterial species belonging to Gram-positive genera, including *Solibacillus*, *Staphylococcus*, *Kocuria*, *Oceanobacillus*, *Virgibacillus*, *Bhargavaea*, *Alkalihalobacillus*, *Micrococcus*, *Ornithinibacillus*, and *Bacillus*. This result suggests that this method can be a potential method to eliminate bacteria. This technique can be used in the leather industry, providing a simple, safe, broad-spectrum antibacterial activity and cost-effective solution. Therefore, we plan to conduct more detailed research in our further experiments to explore its effective use in brine curing of hides and skins.

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