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The Destructive Effects of Extremely Halophilic Archaeal Strains on Sheepskins, and Proposals for Remedial Curing Processes

Use of sterile brine or direct electric current to prevent red heat damage on salted sheepskins

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Proteolytic and lipolytic extremely halophilic archaea found in curing salt may contaminate skins during the brine curing process and damage skin structure. In the present study, three proteolytic and lipolytic extremely halophilic archaea were isolated from deteriorated salted sheepskins and characterised using conventional and molecular methods. Each test strain (*Haloarcula salaria* AT1, *Halobacterium salinarum* 22T6, *Haloarcula tradensis* 7T3), a mixed culture of these strains and the mixed culture treated with 1.5 A direct current (DC) were used for brine curing processes of fresh sheepskins and examined during 47 days of storage to evaluate the degree of destruction wreaked by these microorganisms. Both organoleptic properties and scanning electron microscopy (SEM) images of sheepskins proved that each separate test strain and the mixed culture caused serious damage. However, the mixed culture of strains treated with electric current did not damage sheepskin structure. Therefore, we highly recommend sterilisation of brine using DC to prevent archaeal damage on cured hides and skins in the leather industry.

1. Introduction

Extremely halophilic archaea have been found in hypersaline salt lakes, salterns, salt mines, salted foods and salted hides. There have been numerous studies on the presence of extremely halophilic archaea in these hypersaline environments (1–12). Due to the high salt requirements of extreme halophiles (15–30% NaCl), these microorganisms have been denominated as extremely halophilic archaea (13, 14). Cells of *Haloarchaea* staining Gram-negatively are irregular rods, cocci, pleomorphic rods, cups, irregular disks, flattened disks, irregular triangles, rectangles and squares (2, 5, 15). Chemoorganotroph extremely halophilic archaea, which can be motile or non-motile, grow aerobically and use different amino acids. Colonies of these microorganisms are pink, red and orange due to C_{50}-carotenoid pigments called bacterioruberins (15, 16).

Observation of red or violet discolorations on the flesh side of salted hides and skins is the key for detecting extremely halophilic archaea in the leather industry. These discolorations are a sign of bacterial deterioration of hides and skins (17, 18). Previous experiments reported that microorganisms in curing salts and raceway brines contaminated hides and skins and caused red heat (10). The brine cured hides and skins were often stored in hot warehouses, trucks or ships, and these high temperature conditions, combined with moisture, offer an ideal medium for proteolytic extremely halophilic archaea to grow and potentially digest collagen fibres in the hides and skins (10).

Extremely halophilic archaea (10^2–10^5 colony forming units (CFU) g^{-1}), proteolytic (10^2–10^4 CFU g^{-1}) and lipolytic (10^2–10^4 CFU g^{-1}) extremely halophilic archaea were detected in 40 curing salt samples collected from different tanneries in Turkey (19). Almost all salted hides and skins contained extremely halophilic archaea, proteolytic and lipolytic extremely halophilic archaea originating in the curing salt. Extremely halophilic archaea were also detected on 94% of 131 brine-cured cattle hides collected from USA, 91% of 35 salted hides cured in France and Russia and all salted hides cured in Turkey, Greece, the UK, USA, Serbia, Bulgaria, Russia, South Africa and Australia (20–22). Five extremely halophilic archaeal species, *Halorubrum saccharovorum*, *Halorubrum tebenquichense*, *Halorubrum lacusprofundi*, *Natrinema pallidum* and *Natrinema gari* were isolated from five salted hides originating in England and Australia (22). Also, 101 extremely halophilic archaeal strains (*Halorubrum tebenquichense*, *Halorubrum saccharovorum*, *Halorubrum kocurii*, *Halorubrum terrestre*, *Halorubrum lipolyticum, Halococcus dombrowskii*, *Halococcus qingdaonensis*, *Halococcus morrhuae*, *Natrinema pellirubrum*, *Natrinema versiforme, Halostagnicola larsenii* and *Haloterrigena saccharevitans*) were isolated from four salted sheepskin samples (Spain) exhibiting bad odour, a slimy layer, hair slip, red and yellow discolorations (23). Moreover, 28 extremely halophilic archaeal strains (*Natrialba aegyptia*, *Halovivax asiaticus*, *Halococcus morrhuae, Halococcus thailandensis*, *Natrinema pallidum*, *Halococcus dombrowskii*, *Halomicrobium zhouii*, *Natronococcus jeotgali*, *Haloterrigena thermotolerans*, *Natrinema versiforme* and *Halobacterium noricense*) were isolated from eight salted hide and skin samples from Turkey, Iraq, Turkmenistan and Kazakhstan (24).

While there are many reports that detect the presence of extremely halophilic archaea on salted hides and skins (10, 17, 20–25), the destructive effects of these microorganisms on salted hides have been studied much less (25, 26). In our previous investigation, we found that extremely halophilic archaeal strains, isolated from hides brine cured in the USA, damaged grain the surface of hides at 41°C after 49 days (25). An experiment with extremely halophilic *Haloferax gibbonsii* (ATCC® 33959™) and *Haloarcula hispanica* (ATCC® 33960™) obtained from American Type Culture Collection (ATCC), USA, demonstrated that *Haloferax gibbonsii* caused hair slip, loss of hide substance and deterioration of brine cured hide after 45 days at 40°C (26).

The adverse effects of extremely halophilic archaeal hide isolates and ATCC strains of extremely halophilic archaea on brine cured hides have been reported in these studies, respectively (25, 26). However, the destructive effects of salted sheepskin strains of extremely halophilic *Haloarcula salaria*, *Halobacterium salinarum* and *Haloarcula tradensis* on brine cured sheepskins have not been examined yet. Therefore, the aim of this study was to examine adverse effects of proteolytic and lipolytic archaeal sheepskin strains (*Haloarcula salaria* AT1, *Halobacterium salinarum* 22T6, *Haloarcula tradensis* 7T3) and the mixed culture of these strains on sheepskins during a 47-day storage period at 33°C. We also investigated effective curing methods to prevent the destructive effects of these microorganisms on sheepskins. Additionally, we evaluated pH values, ash contents, moisture contents, salt saturations, total counts of extremely halophilic archaea and organoleptic properties of the brine cured sheepskin samples during different storage periods to determine the brine curing procedure's efficiency and the test microorganisms' adverse effects of on sheepskins.

2. Materials and Methods

2.1 Isolation of Extremely Halophilic Archaeal Strains from Deteriorated Salted Sheepskins

Two deteriorated salted sheepskins containing red discolorations were collected from two tanneries in the Istanbul Leather Organized Industrial Zone (40°52'39.7"N,29°20'25.3"E) in Tuzla, Turkey. The samples were immediately placed into sterile sample bags and transported on ice to the laboratory. Then, 20 g of the salt-pack cured sheepskin samples were weighed and separately soaked in flasks containing 180 ml 30% NaCl (Merck KGaA, Germany) solution. The flasks were placed into a shaking incubator at 90 rpm, 24°C for 3 h. The suspension of the skin was diluted with sterile physiological saline water (30% NaCl). An aliquot of 100 µl each of direct and serial skin suspension dilutions was spread onto the surface of modified Brown agar media containing (per litre): 1 g $CaCl_2{\cdot}H_2O$, 2 g KCl, 20 g $MgSO_4{\cdot}7H_2O$, 3 g trisodium citrate, 250 g NaCl, 5 g yeast extract, 20 g agar, pH 7 (5, 27). The plates were incubated at 39°C for 10 days. Following incubation, red pigmented colonies on the agar media were selected and restreaked several times to obtain pure cultures. A total of 22 isolates were obtained from the sheepskins and then, these strains were

examined the proteolytic and lipolytic activities. Proteolytic activity of each strain was detected on gelatin agar medium containing 2% gelatin. After incubation, clear zones around the colonies on the gelatin agar medium indicated protease production (5, 10). Lipolytic activity of each strain was screened on Tween® 80 agar medium containing 1% Tween® 80. After growth was obtained, opaque zones around the colonies were interpreted as positive lipase activity (5). In the present study three red pigmented proteolytic and lipolytic strains (AT1, 22T6 and 7T3) were obtained from two salted sheepskins and these strains were used in the present study.

2.2 Phenotypic Characteristics of Test Strains

Exponentially growing pure cultures of three strains designated as AT1, 22T6 and 7T3 were used in all experiments. First, the strains' salt requirement and salt tolerance were examined on Brown agar plates containing different salt concentrations (0%, 0.5%, 3%, 5%, 7.5%, 10%, 12.5%, 15%, 20%, 25% and 30%) (27). After detection of optimum salt concentration for each strain, pH and temperature ranges for growth of each strain (AT1, 22T6, 7T3) were respectively examined at Brown agar plates with different pH values (pH 4, pH 5, pH 6, pH 7, pH 7.5, pH 8, pH 9, pH 10, pH 11 and pH 12) and different temperatures (4°C, 10°C, 15°C, 24°C, 28°C, 35°C, 37°C, 39°C, 45°C, 50°C, 55°C, 60°C) according to the methods described in Proposed Minimal Standards for Description of New Taxa in the Order *Halobacteriales* (28). Based on the pH, and temperature range of each test strain, the optimal pH and growth temperature of each test strain were determined.

Pigmentation, size, margin, elevation and opacity of colonies of the strains grown on Brown agar media were examined under optimal growth conditions (28). Cell morphology, cell length, cell width and motility of each strain were examined using both light microscopy and electron microscopy. Microscopic observation of each strain was made by using freshly prepared wet mount (28). For SEM observations, 20 ml of each test strain were separately passed through 0.2 µm pore size cellulose nitrate membrane filter placed in the stainless steel funnel *via* vacuum pump (Sartorius AG, Germany). The archaeal cells of each strain trapped on the membrane filters were observed under SEM (Quanta™ 450 FEG (FEI, USA)). Gram staining was performed with acetic acid-fixed slides (28–30). Catalase and oxidase activities, indole production, methyl red test, H_2S and NH_3 productions of each strain were investigated according to the procedures described previously (4, 28, 31). Furthermore, each strain's caseinase activity was determined on the agar medium containing 2% skim milk. After incubation, clear zones around the colonies were evidence of positive caseinase activity (4). Urease production was investigated on Christensen urea agar medium. The tubes were examined for pink or red colour change in the medium after seven days of incubation (28, 31). β-galactosidase activity was screened in test tubes containing *ortho*-nitrophenyl-β-galactoside (ONPG) discs and 1 ml of sterile saline water (30% NaCl). The yellow colour formation in the test tube was accepted as positive β-galactosidase activity (5, 31). Amino acid utilisation of each strain was examined in the test medium containing 1% amino acid, 0.5% beef extract, 0.5% peptone, 0.05% dextrose, 0.0005% cresol red, 0.001% bromocresol purple, 0.0005% pyridoxal and saline water (30% NaCl). Purple colour formation in the test tube containing archaeal culture was accepted as a positive test after 10 days incubation period at 39°C (31).

2.3 Amplification and Sequencing of 16S rRNA Genes of Test Strains

Chromosomal DNA was isolated by QIAamp DNA Mini Kit (Qiagen, Germany) and purified by QIAquick PCR Purification Kit (Qiagen, Germany) according to the manufacturer's directions. The 16S rRNA genes of the strains were amplified by polymerase chain reaction (PCR) using forward primer 21F and reverse primer 1492R (32). The 16S rRNA gene sequences of three strains (AT1, 22T6 and 7T3) were determined by IONTEK Laboratory (Turkey). The sequences of these strains were analysed using ChromasPro v.2.1.8 software (Technelysium, Australia) and then compared with the sequence on the EZBioCloud Database (ChunLab, South Korea) (33).

2.4 Preparation of Test Strains and Sheepskin Samples for Brine Curing Treatments

2.4.1 Preparation of Strains and Cultures Used in Brine Curing Processes

Pure cultures of each test strain (AT1, 22T6, 7T3) were separately grown in liquid Brown test medium containing 30% NaCl for 10 days at 39°C.

Each archaeal cell suspension's turbidity was adjusted to 0.5 McFarland standard (10^8 CFU ml^{-1}) using densitometer (DEN-1, BIOSAN, Latvia). Each cell suspension was diluted in sterile saline solution (30% NaCl) to adjust the cell suspension to 10^7 CFU ml^{-1}. In addition, mixed cultures of these strains (10^7 CFU ml^{-1}) were prepared. Then, 20 ml of each test strain, 20 ml of the mixed culture were used in the brine curing solutions of T1–T4 (**Table I**).

To prepare brine curing solution containing electrically inactivated mixed culture (T5), 20 ml of the mixed culture containing AT1, 22T6, 7T3 strains (10^7 CFU ml^{-1}) were placed into the electrolysis cell consisting of a glass beaker having two internally attached platinum wire electrodes and 180 ml of sterile brine solution (30% NaCl) (34, 35). To detect the archaeal numbers of the mixed culture in the electrolysis cell before the electric current application, 100 µl of the test medium was removed from the electrolysis cell and diluted to 10^{-2}–10^{-4} using sterile 30% NaCl solution. The diluted archaeal suspensions were spread over the Brown agar media. Then, 1.5 A DC was applied to the electrolysis cell for 22 min (**Figure 1**). A 100 µl quantity of test medium was removed from the cell at intervals of 1 min, 4 min, 7 min, 10 min, 13 min, 16 min, 19 min and 22 min of electric current application. Direct and diluted suspensions of electrically inactivated the mixed culture were spread over Brown agar media. All inoculated Brown media were incubated for 10 days at 39°C, and colonies on the agar plates were counted. This test medium was used for curing process of the sheepskin (T5) after 22 min of electric current application on the mixed culture (**Table I**).

Table I Protocol for Brine Curing Treatments of Sheepskins Brine Curing Compositions

Control Treatments	59.5 g sheepskin sample + 200 ml sterile brine solution
T1	59.5 g sheepskin sample + 180 ml sterile brine solution + 20 ml strain AT1 (10^7 CFU ml^{-1})
T2	59.5 g sheepskin sample + 180 ml sterile brine solution + 20 ml strain 22T6 (10^7 CFU ml^{-1})
T3	59.5 g sheepskin sample + 180 ml sterile brine solution + 20 ml strain 7T3 (10^7 CFU ml^{-1})
T4	59.5 g sheepskin sample + 180 ml sterile brine solution + 20 ml mixed culture (10^7 CFU ml^{-1})
T5	59.5 g sheepskin sample + 180 ml sterile brine solution containing 20 ml electrically inactivated mixed culture

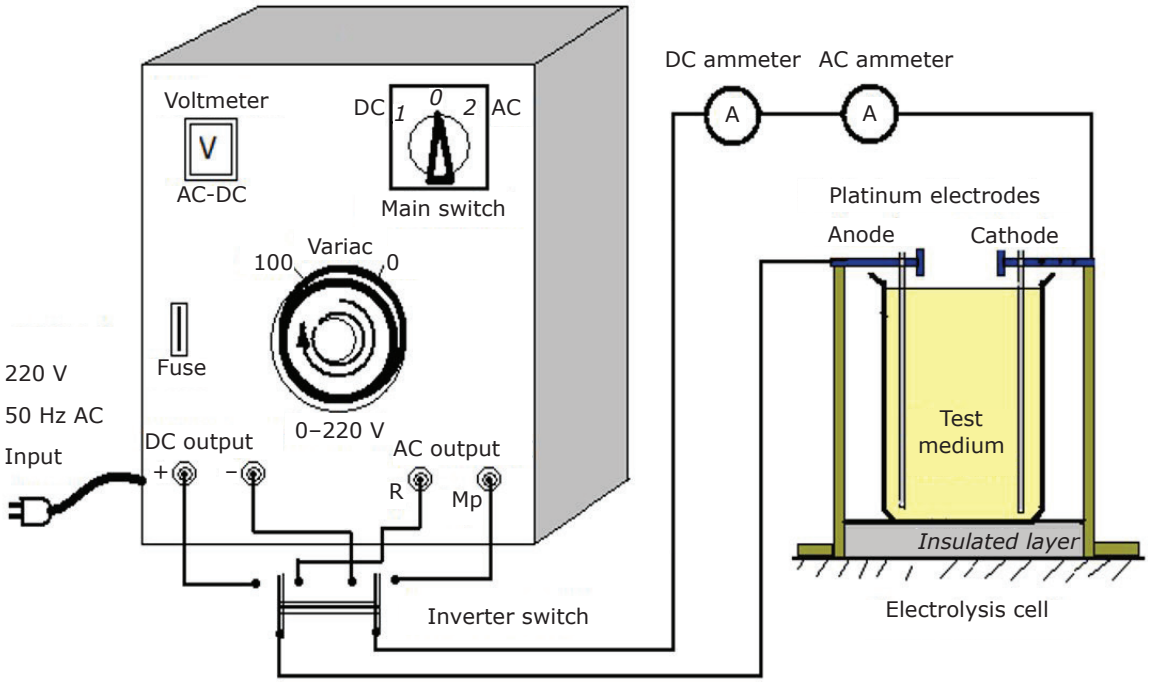

Fig. 1. Electrolysis cell system used 1.5 A DC treatment in this study (R: phase, Mp: ground)

2.4.2 Preparation of Sheepskin Samples for Brine Curing Treatments

One freshly slaughtered, de-fleshed whole sheepskin sample was obtained from a slaughterhouse in Istanbul, Turkey. Then, the sheepskin sample was immediately placed into sterile sample bag and transported on ice to the laboratory. The sheepskin was cut into six pieces perpendicular to backbone, from backbone to belly. Next, we carried out the following six treatments for brine curing of the sheepskin samples. In each treatment, sterile 30% NaCl (Merck KGaA) solution was used. In all treatments, a 400% float of the brine solution (238 g of the brines without test strain, with each test strain or mixed culture/59.5 g of sheepskin) was used (25). Sterile 30% NaCl solution containing the sheepskin sample was used as Control. The sheepskin samples (T1–T4) were separately placed in a glass beaker containing the brine solution, each test strain or mixed culture (T1–T4, **Table I**). In the Treatment 5, the sheepskin sample was placed in a glass beaker containing the brine solution with electrically inactivated mixed culture (T5, **Table I**).

The curing processes of all sheepskins were carried out the protocol described in **Table I**. The sheepskin samples were separately cured in the brine solutions at 90 rpm for 18 h at 24°C. After the curing processes, all sheepskins were taken from the brine solutions and stored for 47 days at 33°C.

2.5 Determination of Extremely Halophilic Archaeal Counts in Curing Solutions and Cured Sheepskin Samples

To determine total counts of extremely halophilic archaea in the curing solutions before the curing processes, 100 µl of the test medium was removed from the each curing solution and diluted to 10^{-2}–10^{-4} using sterile 30% NaCl solution. The diluted archaeal suspensions were spread over the Brown agar media. In addition, subsequent to each brine curing process detailed above (T1–T5), the suspensions of cured sheepskin samples were prepared at intervals of 5 days, 16 days, 28 days and 47 days of storage. 2 g of each skin sample were put into a flask containing 18 ml sterile 30% NaCl solution and incubated for 1 h at 24°C and 100 rpm. Direct and serial dilutions of the suspensions were spread onto the surface of Brown agar media. All inoculated Brown media were incubated at 39°C for 10 days and the colonies grown on the test media were counted.

2.6 Determination of pH, Moisture Content, Ash Content and Salt Saturation of Cured Sheepskin Samples

After curing processes, 5 g of the sheepskins were cut and placed into flasks containing 100 ml of sterile distilled water. The flasks were placed in a shaking incubator for 1 h at 100 rpm and then pH was measured with a pH meter. Hairs and dirt on the samples were removed to properly determine the samples' moisture content. 3 g of the samples were placed into an oven at 102°C for 6 h. The dried samples were weighed, returned to the oven for 1 h, and then were weighed again. The drying procedure was repeated until the first dry weight was equal to the second dry weight. The samples were put into a desiccator for 30 min to cool. Next, we calculated the skins' moisture contents (20, 21). The dry sheepskins samples were placed in ceramic crucibles and ashed in a muffle furnace at 600°C for 8 h. After cooling, the samples were weighed to determine ash content. Moisture content, ash content and salt saturations of skin samples were calculated according to the aforementioned methods (30, 36). The pH value, ash content, moisture content and salt saturation of all cured sheepskin samples were examined at different storage periods.

2.7 Organoleptic Examination of Brine Cured Sheepskin Samples During Storage Periods

All cured sheepskin samples were examined organoleptically (hair slip, deterioration of skins, bad odour, sticky appearance, red heat, hole formation) during different storage periods.

2.8 Preparation of Sheepskin Samples for Scanning Electron Microscopy Observation

After a 47-day storage period, the sheepskin samples were prepared for SEM observation. The samples were fixed in 4% glutaraldehyde solution prepared in 0.1 M phosphate buffer (pH 7.2) for 30 min. The samples were washed three times with 0.1 M phosphate buffer for 10 min and were treated with 1% OsO_4 prepared in 0.1 M phosphate buffer at room temperature for 1 h. The samples were washed two times in sterile distilled water for 10 min. Then, the water in the sheepskins was gradually removed by 35%, 50%, 75%, 95% and absolute ethanol. The mixtures

of ethanol-hexamethyldisilazane (ethanol-HMDS) [1:1 (v/v)] (1 × 30 min), ethanol-HMDS [1:2 (v/v)] (1 × 30 min) and HMDS (2 × 30 min) were used for air drying process. After drying, HMDS was poured from petri dishes and the samples were placed in a desiccator for 12 h. Later, the sheepskin samples were examined under SEM (Quanta™ 450 FEG) using sample stub with double-sided sticky tape (37).

3. Results and Discussion

3.1 Isolation and Selection of Test Strains from Sheepskins

A total of 22 red coloured strains were isolated from two deteriorated salted sheepskin samples obtained from two tanneries in the Istanbul Leather Organized Industrial Zone in Tuzla, Turkey. While nine, seven and three strains respectively produced protease, lipase, both protease and lipase, three strains did not produce either lipase or protease enzymes. The red coloured three extremely halophilic strains producing both protease and lipase enzymes were selected and used as test strains (AT1, 22T6 and 7T3) in the present study.

3.2 Phenotypic Characteristics of Test Strains

Strains AT1, 22T6 and 7T3 grew at 15–30% NaCl, 15–30% NaCl, 20–30% NaCl concentrations, respectively. Optimum salt concentrations of strains AT1, 22T6 and 7T3 were determined as 25% NaCl. Hence, these strains were accepted as extremely halophilic archaea. The pH and temperature ranges for growth of strains AT1, 22T6 and 7T3 were respectively found as pH 6–11 and 20–50°C, pH 6–11 and 15–55°C, pH 5–11 and 15–55°C. All extremely halophilic archaeal strains optimally grew at 39°C and pH 7. The colony pigmentation, size, margin, elevation and opacity of strains AT1, 22T6, 7T3 were respectively observed as: red, 0.6–2 mm, entire, convex, translucent; red, 1–2 mm, entire, convex, translucent; red, 0.8–1.9 mm, entire, convex, translucent. The cells of strains AT1 (**Figure 2(a)**) and 7T3 (**Figure 2(c)**) were non-motile, extremely pleomorphic (triangle, square,

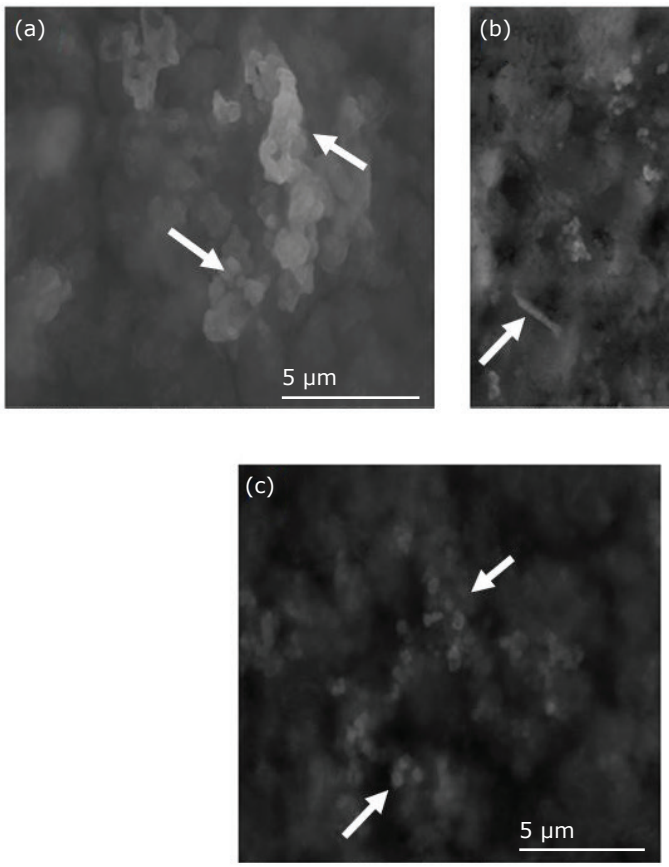

Fig. 2. SEM micrographs of pleomorphic test strains of (a) *Haloarcula salaria* (AT1) cells; (b) *Halobacterium salinarum* (22T6) cells; (c) *Haloarcula tradensis* (7T3) cells trapped on the membrane filter

irregular disk, short rod). The cells of strains AT1 and 7T3 were approximately 0.4–1.3 µm × 0.4–2.0 µm and 0.3–0.7 µm × 0.3–4 µm, respectively. The cells of strain 22T6 (**Figure 2(b)**) were motile, pleomorphic rods, approximately 0.5–1.2 µm × 3.2–6.6 µm. All strains were Gram-negative (**Table II**). While all strains showed positive catalase, oxidase, protease, lipase activities, indole production, the methyl red, caseinase, urease and β-galactosidase reactions of all strains were negative. The strains did not produce H_2S and NH_3 (**Table II**).

Our experimental results showed that *Haloarcula salaria* (AT1), *Halobacterium salinarum* (22T6), *Haloarcula tradensis* (7T3) strains have protease activities which can breakdown proteins in corium of sheepskin causing loss of skin substance. When the protein structure of salted skins is broken down by proteolytic extremely halophilic archaea, these microorganisms can utilise some amino acids as a source of carbon, nitrogen and energy. *Haloarcula salaria* AT1 and *Halobacterium salinarum* 22T6 utilised most of the amino acids examined. While *Haloarcula salaria* AT1, *Halobacterium salinarum* 22T6 utilised 17 amino acids, *Haloarcula tradensis* 7T3 used only three amino acids (**Table III**). In another study, the liquid test media containing calfskin samples, 30% NaCl and proteolytic red and pink strains of the extremely halophilic archaea were separately prepared to show disintegration of the skin proteins. After an incubation period,

Table II Phenotypic Characteristics of *Haloarcula salaria*, *Halobacterium salinarum*, *Haloarcula tradensis*

Characteristics	*Haloarcula salaria*	*Halobacterium salinarum*	*Haloarcula tradensis*
Strain code	AT1	22T6	7T3
Motility	Non-motile	Motile	Non-motile
Cell morphology	Extremely pleomorphic	Pleomorphic rods	Extremely pleomorphic
Cell width, µm	0.4–1.3	0.5–1.2	0.3–0.7
Cell length, µm	0.4–2	3.2–6.6	0.3–4
Gram staining	Negative	Negative	Negative
Pigmentation	Red	Red	Red
Colony size, mm	0.6–2	1–2	0.8–1.9
Colony margin	Entire	Entire	Entire
Colony elevation	Convex	Convex	Convex
Colony opacity	Translucent	Translucent	Translucent
NaCl concentration, %	15–30	15–30	20–30
pH range	6–11	6–11	5–11
Temperature range, °C	20–50	15–55	15–55
Optimum NaCl	25	25	25
Optimum Temperature, °C	39	39	39
Optimum pH range	7	7	7
Catalase activity	+	+	+
Oxidase activity	+	+	+
Methyl red reaction	–	–	–
Caseinase activity	–	–	–
Urease activity	–	–	–
β-galactosidase activity	–	–	–
Indole production	–	–	–
H_2S production	–	–	–
NH_3 production	–	–	–
Protease activity	+	+	+[a]
Lipase activity	+	+	+

[a] *Haloarcula tradensis* (7T3) showed weak protease activity

Table III Utilisation of Amino Acids by Strains

Amino acids	*Haloarcula salaria* (AT1)	*Halobacterium salinarum* (22T6)	*Haloarcula tradensis* (7T3)
L-arginine	+	+	+
L-cysteine	–	–	–
L-glycine	+	+	–
L-alanine	+	+	–
L-tyrosine	+	+	–
L-proline	+	+	–
L-hydroxyproline	+	+	–
L-glutamic acid	–	–	–
L-methionine	+	+	–
L-serine	+	+	–
L-isoleucine	+	+	–
***myo*-inositol**	+	+	–
L-lysine	+	+	+
L-phenylalanine	+	+	–
L-leucine	+	–	–
L-valine	+	+	–
L-threonine	+	+	–
L-ornithine	+	+	–
L-histidine	+	+	+
L-aspartic acid	–	–	–
L-cystine	–	+	–

decomposition of the skin samples in the media was detected by visual observation. While contents of asparagine, threonine, serine, glutamine, proline, glycine, alanine, valine, isoleucine, leucine, phenylalanine, lysine and arginine in the test tubes were detected at high levels, contents of methionine, tyrosine and histidine were low (10).

Phenotypic features of extremely halophilic AT1, 7T3 and 22T6 strains detected in this study were fairly similar to phenotypic features of *Haloarcula salaria*, *Haloarcula tradensis* and *Halobacterium salinarum* isolated by other researchers (15, 38, 39).

3.3 16S rRNA Gene Sequences of Test Strains

The phylogenetic analysis revealed that three strains shared highly similar identities with their closest phylogenetic relatives. Strains AT1, 22T6, 7T3 were respectively assigned to *Haloarcula salaria* (98.36%-1344 base pairs), *Halobacterium salinarum* (99.78%-1345 base pairs), *Haloarcula tradensis* (98.37%-1355 base pairs). The gene sequence data of the strains AT1, 22T6, 7T3 were respectively deposited in GenBank® (National Center for Biotechnology Information, USA) under accession numbers as MN585896, MN585803, MN585804.

In our previous study, extremely halophilic archaeal strains were isolated from Tuz Lake and its salterns (5). In Turkish leather industry, curing salt is mostly obtained from Tuz Lake and its salterns. Hence, we suspect that contaminations of our sheepskin samples with *Haloarcula salaria* AT1, *Halobacterium salinarum* 22T6 and *Haloarcula tradensis* 7T3 were due to the curing salt obtained from Tuz Lake and its salterns.

3.4 Extremely Halophilic Archaeal Counts in Curing Solutions Before Curing

In the study carried out with 25 salted sheepskin samples (Australia, Bulgaria, Dubai, Greece, Israel, Kuwait, South Africa, Turkey, USA) and 25 salted goat skin samples (Australia, Turkey, Bulgaria, Israel, South Africa, Russia, China, France), proteolytic extremely halophilic archaea and lipolytic extremely halophilic archaea were detected as 10^2–10^5 CFU g^{-1}; 10^2–10^6 CFU g^{-1}

and 10^2–10^6 CFU g^{-1}; 10^2–10^6 CFU g^{-1} on salted sheepskins and goat skins, respectively (40). The highest number of proteolytic and lipolytic extremely halophilic archaea on the salted skins was found as 10^6 CFU g^{-1} (40). Therefore, the archaeal cell numbers of test strains in the brine curing solutions were adjusted to 10^6 CFU ml^{-1}. Before the curing processes of sheepskins, while the archaeal cell numbers in the brine solutions of Treatments 1, 3 and 4 were detected as 2.1×10^6 CFU ml^{-1}, the archaeal cell numbers in the brine solution of Treatment 2 was detected as 2.2×10^6 CFU ml^{-1}.

The archaeal cell numbers in the mixed culture was detected as 2.1×10^6 CFU ml^{-1} in the electrolysis cell before 1.5 A DC application. While the archaeal cell numbers in the mixed culture were reduced from 2.1×10^6 CFU ml^{-1} to 3.2×10^5 CFU ml^{-1} after 1 min of DC treatment, the cell numbers of 1.24×10^2 CFU ml^{-1} was detected after 4 min of DC treatment. All archaeal cells in the mixed culture were completely killed in 7 min of DC treatment. In the present study, $\log_{10}$ value of the mixed culture of extremely halophilic archaea in the brine solution before the DC treatment was 6.32. After 1 min, 4 min and 7 min of 1.5 A DC treatment; 0.82, 4.23 and 6.32 $\log_{10}$ reduction values (CFU ml^{-1}) of the mixed culture in the brine were detected, respectively.

Temperature and pH of the electrolysis cell were respectively measured as 31°C and pH 6 prior to the electric current treatment. After treating the brine solution with the electric current, the temperature of the brine was adjusted to 24°C for using in

Table IV pH, Ash Content, Moisture Content and Salt Saturation Values, Total Extremely Halophilic Archaeal Counts of the Sheepskin Samples After Different Storage Periods

Experiment	pH	Ash content, %	Moisture content, %	Salt saturation, %	Total count of extremely halophilic archaea
After 5 days					
Control	7.55	20	55	>100	0
T1	6.72	24	50	>100	2.0×10^7
T2	6.59	23	50	>100	3.4×10^7
T3	6.65	21	57	>100	2.2×10^7
T4	6.53	26	52	>100	3.8×10^7
T5	7.80	21	57	>100	0
After 16 days					
Control	7.43	25	50	>100	0
T1	6.52	30	47	>100	3.0×10^7
T2	6.70	27	51	>100	6.0×10^7
T3	6.65	22	50	>100	3.4×10^7
T4	6.85	32	46	>100	8.4×10^7
T5	7.32	23	55	>100	0
After 28 days					
Control	7.40	28	45	>100	0
T1	7.70	29	40	>100	1.2×10^7
T2	7.52	29	43	>100	2.0×10^7
T3	7.36	33	44	>100	2.0×10^7
T4	7.51	32	39	>100	3.4×10^7
T5	7.81	29	46	>100	0
After 47 days					
Control	7.26	41	30	>100	0
T1	7.58	34	26	>100	1.0×10^7
T2	7.47	34	35	>100	1.8×10^7
T3	7.31	44	24	>100	1.7×10^7
T4	7.60	37	38	>100	2.0×10^7
T5	7.64	33	33	>100	0

curing process of sheepskin in the Treatment 5. While the temperature and pH of the test medium respectively increased from 31°C to 41°C and from pH 6 to pH 8.5 during the electric current treatment, voltage values slightly decreased from 4.7 V to 4.3 V.

We also demonstrated the inactivation of extremely halophilic strains *via* DC and alternating electric current (AC) in our previous studies (35, 41, 42). A 0.5 A DC was applied for 30 min to several strains of extremely halophilic archaea (10^7 CFU ml^{-1}) isolated from Tuz Lake, Kaldırım and Kayacık salterns (35). While the mixed culture of extremely halophilic archaea was exterminated in 10 min, protease producing extremely halophilic archaea were killed in 5 min. However, lipase or lipase and protease producing extremely halophilic archaea were exterminated in 20 min (35). In another experiment, lipase and protease producing extremely halophilic strains (10^5–10^6 CFU ml^{-1}), separately grown in liquid Brown media, were inactivated by a 10 min treatment with 0.5 A DC (41). It was also detected that 1 min of 2 A AC treatment was enough to kill extremely halophilic archaea found in brine solution (10^2–10^4 CFU ml^{-1}). When 2 A AC was applied to lipolytic extremely halophilic archaea, proteolytic extremely halophilic archaea, both proteolytic and lipolytic extremely halophilic archaea, and a mixed culture of these strains (10^6 CFU ml^{-1}), all test microorganisms found in 25% NaCl solution were exterminated in 5 min (42).

3.5 Extremely Halophilic Archaeal Counts on Cured Sheepskin Samples During Storage

After the curing processes of sheepskins, we did not detect any extremely halophilic archaea on the sheepskin sample cured with the sterile brine solution (Control) and the sheepskin sample cured with the brine solution containing electrically inactivated mixed culture (T5) during the all storage periods.

While extremely halophilic archaeal numbers on both skin samples cured with each strain and the skin sample cured with mixed cultures of the strains slowly increased from 10^6 CFU ml^{-1} to 10^7 CFU during five days and 16 days storage periods, the numbers of these strains slowly decreased 28 days and 47 days storage periods due to attachment of these cells to sheepskins (**Table IV**).

3.6 pH, Moisture Content, Ash Content and Salt Saturation Values of Cured Sheepskin Samples

After the curing processes of skins, pH values of the sheepskin samples were measured as pH 7.35 for Control; pH 6.89 for T1; pH 7.09 for T2; pH 7.05 for T3; pH 7.16 for T4; pH 8.05 for T5. While salt saturation values of all cured sheepskins were higher than 100% during all storage periods, pH, ash content and moisture content values changed during different storage periods. pH, ash content and moisture content values of the cured skins were detected between pH 6.52–7.81, 20–44%, 24–57%, respectively (**Table IV**).

Moisture, minimum and maximum ash contents, salt saturation values of adequately cured salted hides were suggested as 40–48%, 14–48%, higher than 85%, respectively (36). Due to detection of high moisture content in all samples (between 50–57%) after five days storage, sterile salt was added to all sheepskins to reduce their moisture contents according to curing procedure described in the previous study (43). While all skin samples reached the suggested moisture content values (39–46%) after 28 days, the suggested saturation values were detected after five days. The samples' lowest moisture content values were detected after 47 days. Ash contents of all skins (20–44%) were close to suggested values (36). While the skins' pH values changed during storage periods, all values were found sufficient to support the growth of extremely halophilic strains (**Table IV**). The pH, moisture content, ash content and salt saturation values detected in this study were also consistent with pH range (pH 6.53–8.01), moisture content (32–68%), ash content (12–30%) and salt saturation (58–100%) values of 25 salted sheepskin samples determined in the previous experiment (40).

3.7 Organoleptic Characteristics of Brine Cured Sheepskin Samples During Storage

While hair slip and bad odour were detected on the sheepskin samples cured with each strain and the mixed culture after five days at 33°C, sticky appearance and red heat were observed on the cured sheepskin samples after 16 days (T1–T4, **Figure 3**). In addition to the aforementioned organoleptic properties, hole formations were observed on these sheepskin samples after 28 days. However,

we did not detect any organoleptic properties on sheepskin samples cured with sterile brine and the brine treated with 1.5 A DC (Control and T5, **Figure 3**).

In another study, the commercially cured hides stored one year in the USA were also examined for proteolytic activity of extremely halophilic archaea. Experimental results of that study showed that the flesh side of hides containing extremely halophilic archaea had pink discolorations called red heat. When these hides were incubated at 35°C–40°C, bad odour, hair slip and severe grain damage were detected. Damaged grain surfaces were observed on leather made from these hides (10). In another experiment researchers emphasised that temperatures of the brines and hides should be maintained below 20°C to prevent growth of extremely halophilic archaea (44).

3.8 Scanning Electron Microscopy Observation of Mixed Culture and Treated Sheepskin Samples

Figure 4 shows extremely halophilic archaeal cells of the mixed culture on 0.2 µm pore-size cellulose nitrate membrane filter in pleomorphic shapes such as triangle, square, irregular disk and rod. As seen in the SEM micrograph, 1.5 A DC treatment significantly debilitated structural integrity of the cells in the mixed culture trapped on the filter (**Figure 5**). The SEM images clearly showed that electric current application damaged cell structures of each strain in the mixed culture (**Figure 5**). As seen in **Figure 6**, the sterile brine curing process protected the sheepskin against microbial damage during 47 days of storage.

Attachment of *Haloarcula salaria* AT1, *Halobacterium salinarum* 22T6 and *Haloarcula tradensis* 7T3 to corium fibres and the consequent destructive effects on sheepskins are seen in **Figures 7–10**. *Haloarcula salaria* AT1, *Halobacterium salinarum* 22T6 and the mixed culture of the strains caused fibres in the corium to split and weaken (**Figures 7, 8 and 10**). In contrast with the skin samples treated with *Haloarcula salaria* AT1, *Halobacterium salinarum* 22T6, skin sample treated with *Haloarcula tradensis* 7T3 had compact appearance, although the shredding of the fibres was still present in corium (**Figure 9**). That damage was due to the proteolytic activities of these microorganisms.

Figure 11 clearly shows that the curing process of sheepskin with the brine containing mixed culture treated with DC prevented extremely halophilic archaea from contaminating the sheepskin and furthermore protected the skin very well against microbial damage during a long storage period.

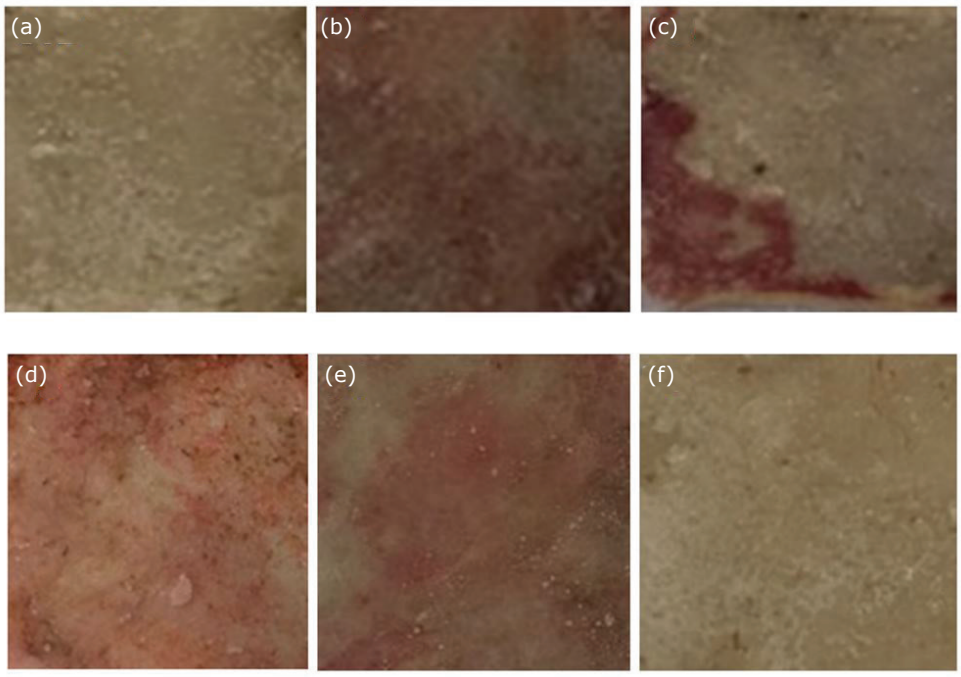

Fig. 3. Organoleptic characteristics of brine cured sheepskin samples after 16 days storage period: (a) Control, sheepskin sample cured with sterile brine (30% NaCl); (b) T1, sheepskin sample cured with brine containing *Haloarcula salaria* AT1; (c) T2, sheepskin sample cured with brine containing *Halobacterium salinarum* 22T6; (d) T3, sheepskin sample cured with brine containing *Haloarcula tradensis* 7T3; (e) T4, sheepskin sample cured with brine containing mixed culture; (f) T5, sheepskin sample cured with brine containing electrically inactivated mixed culture

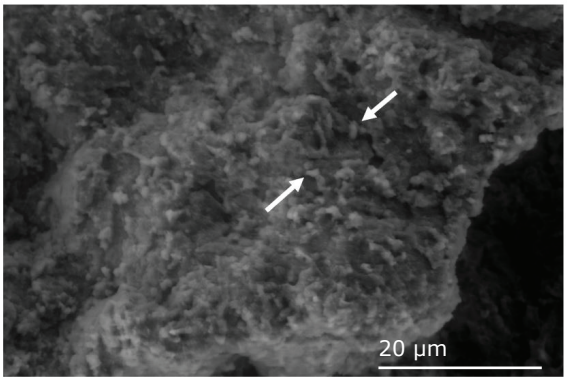

Fig. 4. SEM micrograph of mixed culture of undamaged pleomorphic cells of *Haloarcula salaria* (AT1), *Halobacterium salinarum* (22T6) and *Haloarcula tradensis* (7T3) trapped on the membrane filter

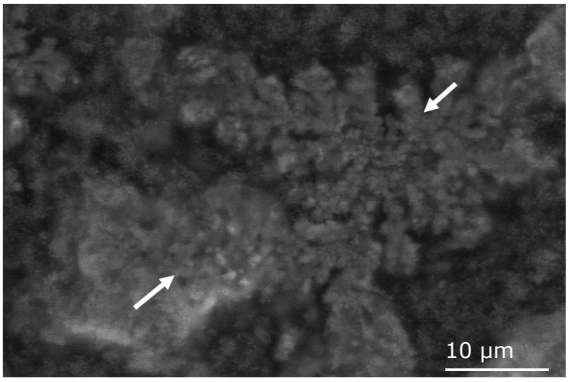

Fig. 5. SEM micrograph of mixed culture of damaged *Haloarcula salaria* (AT1), *Halobacterium salinarum* (22T6) and *Haloarcula tradensis* (7T3) cells treated with 1.5 A DC trapped on the membrane filter

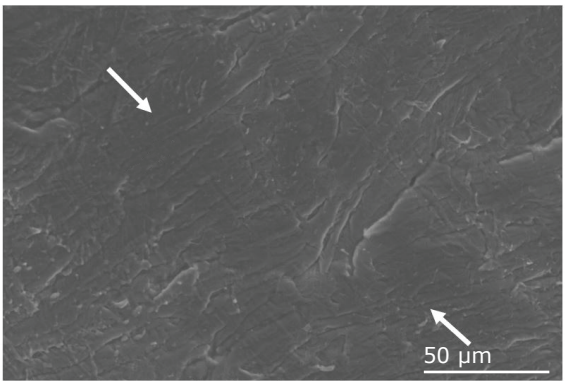

Fig. 6. SEM micrograph of the longitudinal section of undamaged sheepskin structure treated with sterile brine (Control) stored for 47 days at 33°C

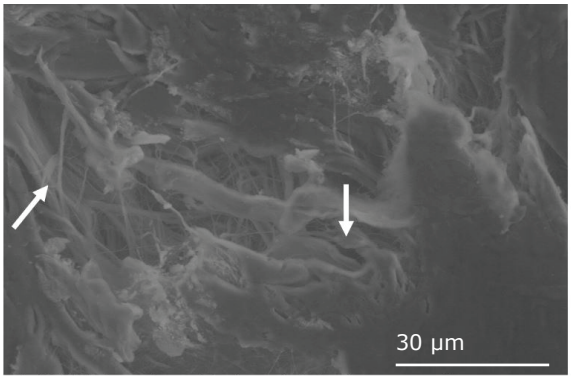

Fig. 7. SEM micrograph of the longitudinal section of damaged corium layer of sheepskin treated with *Haloarcula salaria* (AT1) stored for 47 days at 33°C

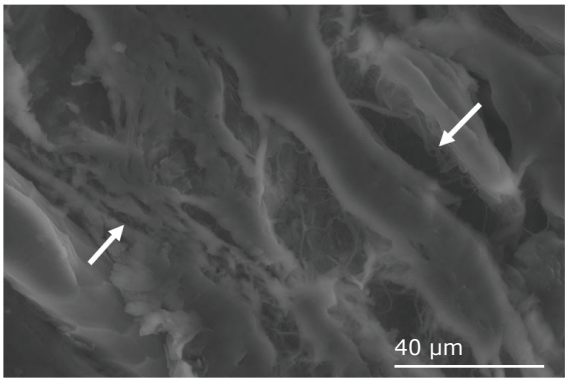

Fig. 8. SEM micrograph of the longitudinal section of damaged corium layer of sheepskin treated with *Halobacterium salinarum* (22T6) stored for 47 days at 33°C

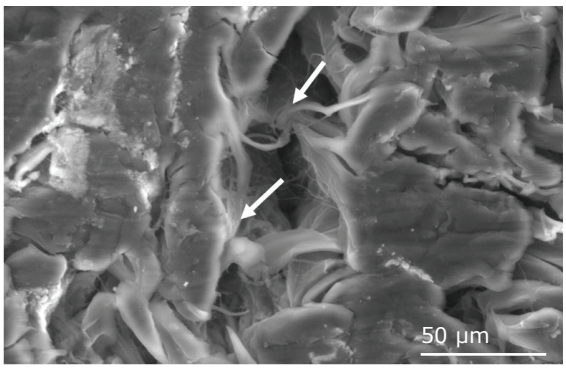

Fig. 9. SEM micrograph of the longitudinal section of damaged corium layer of sheepskin treated with *Haloarcula tradensis* (7T3) stored for 47 days at 33°C

The present study proved that organoleptic changes detected in the sheepskins were closely related to proteolytic and lipolytic activities of extremely halophilic archaeal strains on the skin. Electron micrographs also showed that each test isolate and a mixed culture of extremely halophilic strains destroyed the skins' collagen fibres. We did not detect any difference when assessing the

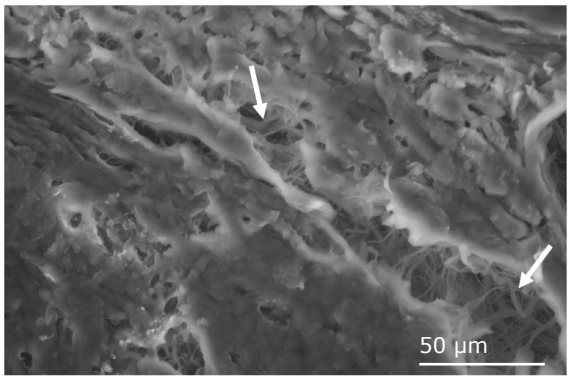

Fig. 10. SEM micrograph of the longitudinal section of damaged corium layer of sheepskin treated with mixed culture of *Haloarcula salaria* (AT1), *Halobacterium salinarum* (22T6), *Haloarcula tradensis* (7T3) stored for 47 days at 33°C

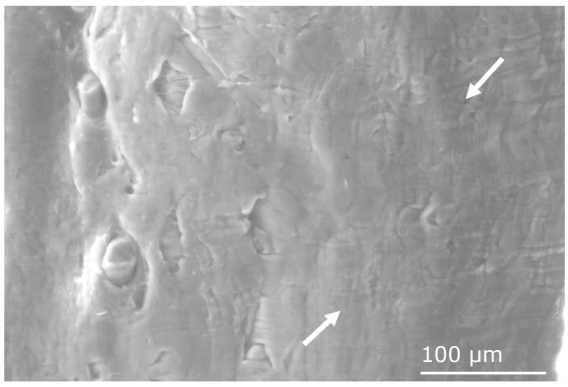

Fig. 11. SEM micrograph of the longitudinal section of undamaged sheepskin structure treated with electrically inactivated mixed culture stored for 47 days at 33°C

efficacy of sterile brine and electrically treated brine curing processes of sheepskin samples throughout 47 days. We did not observe any damage to the compactness of sheepskin structure cured with both the sterile brine and electrically treated brine containing the mixed culture. Both methods were found very effective for preventing archaeal growth and damage on the brine cured sheepskins.

Our results were consistent with those of other experimental studies on the extremely halophilic strains and culture collection strains of extremely halophilic archaea (25, 26). In our previous experiment, SEM images showed that hides cured with proteolytic extremely halophilic archaeal strains had red heat and severe grain damage after 49 days of storage at 41°C (25). In another study, the cured hides with extremely halophilic *Haloferax gibbonsii* (ATCC® 33959™) exhibited hair loss, thinner and flaccid structure; these consequences of deterioration and loss of hide substance. The open fibre structure was also detected in the corium of the hide inoculated with *Haloferax gibbonsii* (27). The SEM images showed that the fibre structures of hide were broken down into the smaller fibres after 43 days (27).

4. Conclusion

This is the first study that detects adverse effects of characterised extremely halophilic archaeal strains on brine cured sheepskins with SEM. SEM images proved that proteolytic and lipolytic *Haloarcula salaria* AT1, *Halobacterium salinarum* 22T6, *Haloarcula tradensis* 7T3 caused corium fibres to split apart in cured sheepskins after 47 days in storage. The mixed culture of proteolytic and lipolytic extremely halophilic archaea originating in curing salt can be exterminated with application of 1.5 A DC treatment in 7 min. Our experimental results proved that a curing process using sterile brine or brine treated with electric current prevented red heat and deterioration of sheepskins during long storage periods. Therefore, an environmentally friendly, easy, cheap, very simple electrolysis system is a very attractive alternative for brine disinfection: (a) it kills different species of microorganisms including proteolytic and lipolytic extremely halophilic archaea; (b) it prevents development of resistant strains in leather factories; (c) it kills very effectively the aggregated microorganisms found in the brine containing high organic substances; (d) it can achieve a reduction factor of more than 6 $\log_{10}$ for proteolytic and lipolytic extremely halophilic archaea; and (e) it has irreversibly lethal action. Hence, we suggest using this effective brine disinfection system in the leather industry after sufficient insulation and grounding are provided by an electrical engineer.

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