Molecular Identification of Moderately Halophilic Bacteria and Extremely Halophilic Archaea Isolated from Salted Sheep Skins Containing Red and Yellow Discolorations

by

C. Akpolat,[1] A. Ventosa,[2] M. Birbir,[3]* C. Sánchez-Porro[2] And P. Caglayan[3]

[1]*Institute for Graduate Studies in Pure and Applied Science, University of Marmara,*
34722 Istanbul, Turkey

[2]*Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Sevilla,*
41012 Sevilla, Spain

[3]*Division of Plant Diseases and Microbiology, Department of Biology, Faculty of Arts and Sciences, University of Marmara,*
34722 Istanbul, Turkey

Abstract

Salted hides or skins containing red and yellow discolorations have been examined for many years, but much less information is available about the isolation and molecular techniques for identifying moderately halophilic bacteria and extremely halophilic archaea on the salted sheep skins exhibiting these blotches. The deteriorated salted sheep skins were collected from a warehouse in Spain. Moderately halophilic bacteria and extremely halophilic archaea were isolated from these samples and molecular identification of these microorganisms were performed using 16S rRNA gene sequence analysis. Total cell counts of moderately halophilic bacteria and extremely halophilic archaea were found as 10^5-10^8 CFU/g and 10^5-10^7 CFU/g, respectively. According to comparative partial 16S rRNA gene sequence analysis, *Alkalibacillus halophilus*, *Pseudomonas halophila*, *Acinetobacter johnsonii*, *Alkalibacillus salilacus*, *Salimicrobium salexigens*, *Marinococcus luteus* and *Staphylococcus equorum* subsp. *equorum* belonging to moderately halophilic bacteria; and *Halorubrum tebenquichense*, *Halorubrum saccharovorum*, *Halococcus dombrowskii*, *Halococcus qingdaonensis*, *Natrinema pellirubrum*, *Halococcus morrhuae*, *Halorubrum kocurii*, *Halorubrum terrestre*, *Halorubrum lipolyticum*, *Halostagnicola larsenii*, *Haloterrigena saccharevitans* and *Natrinema versiforme* belonging to extremely halophilic archaea were isolated from these sheep skins. *Alkalibacillus halophilus* belonging to moderately halophilic bacteria and *Halorubrum tebenquichense* belonging to extremely halophilic archaea were found as the most common species on the skins. Among the moderately halophilic bacterial isolates, *Acinetobacter johnsonii* showed lipolytic activities. Among the extremely halophilic archaeal isolates, *Halococcus dombrowskii*, *Halococcus morrhuae, Natrinema pellirubrum, Halorubrum lipolyticum* showed proteolytic activity and *Halococcus dombrowskii, Halorubrum lipolyticum, Haloterrigena saccharevitans, Natrinema versiforme* showed lipolytic activity. Hair slip, red and yellow discolorations, slimy layers and bad odor were detected on the skin samples examined. This study confirmed that salted sheep skins were contaminated by preservation salt containing different proteolytic or lipolytic species of mostly extremely halophilic archaea. Therefore, antimicrobial applications during brine curing of skins should be applied to overcome halophilic microbial damage on the salted skins.

Introduction

Red discoloration of salted food products has drawn scientific attention since 1880. Farlow (1880) examined such blotches on salted codfish especially during the summer season.[1] Similar discolorations have also been observed on salted hides. Due to the trade value of fish and hide, scientists were interested in the cause, effect and prevention of red discolorations of salted fish and hide. This aberration, called as red heat in the leather industry, is considered by tanners a sign of deteriorated hides. It may be commonly observed on the flesh side of salted hides or skins as bright red, orange-brown, blue-violet spots, extensive blotches and streaks.[2-5] These areas are usually wet and slimy. *Sarcina lutea*, *Sarcina auriantica*, *Micrococcus roseus*, *Micrococcus tetragenus*, *Bacillus subtilis*, *Proteus* species, and *Actinomyces* species, were isolated and identified from red spots of salted hides by Bergmann in 1929. The researcher stated that these microorganisms produced pink to yellow, red-orange, pink to red pigments. Most of these isolates

*Corresponding author e-mail: mbirbir@marmara.edu.tr
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showed proteolytic activity that would lead to a loss of hide substance.[3] *Micrococcus tetragenus* and *Actinomyces* species produced lipase activity.[3] Hausam (1931) isolated *Bacillus prodigiosus* from salted hides, and reported that this organism caused red stains on the salted hides.[6] The researchers emphasized that the formation of red heat on salted hides was an evidence of bacterial decomposition of the flesh side.[3,7] It has been reported that bacteria causing red spots on the flesh side of salted hides or skins cause hair slip, epidermis and grain damage which diminish the sale value of the leather.[8-10] It was also emphasized that the microorganisms found in the blotches hydrolyzed fats and caused production of unevenly finished leathers.[11,12] In our previous study, we proved that extremely halophilic archaea causing red heat damaged the grain of brine-cured hide within 7 weeks at a temperature of 41°C. This damage was easily observed by the naked eye, and scanning electron microscopy clearly showed that the damage caused by halophilic archaea resembled sueded grain.[9] Moreover, other scientists examined the sequence of events leading to the appearance of red heat on salt-cured hides within 2 hours exposure. In that experiment, red pigment producing extremely halophilic archaea [*Haloarcula hispanica* ATCC 33960, *Haloferax gibbonsii* ATCC 33959] and moderately halophilic bacteria [*Halomonas elongata* ATCC 33173] were used to examine red heat damage on the hides. Investigators observed that most of bacterial and archaeal cells attached to the hides after 30 minutes of exposure. The hide samples infected with *Haloferax gibbonsii* ATCC 33959 deterioted after 45 days of incubation at 40°C. Hair slip was observed on the hide, and the samples had become thinner and quite soft. This condition was related with loss of hide substance. After 14 days of incubation, although cells of *Haloarcula hispanica* and *Haloferax gibbonsii* were present in the hide interior, cells of *Halomonas elongata* were common on hide surface.[10]

Researchers reported that red colorations were produced by halophilic microorganisms found in marine salts. Those microorganisms contaminated the hide during the curing process.[13] It was found that 34 out of 35 samples of crude solar evaporated salts and 25 of 39 open-pan evaporated grainer salts contained red microorganisms.[4] In addition to marine salts, salts from salt lake and salt mines may contain halophilic archaea in red, blood-red, brick red, orange-red, pale-orange red, pink, pale pink colors.[14,15] Researchers stated that the archaeal strains isolated from Tuzkoy salt mine in Turkey mainly belong to the genera *Halobacterium*, *Haloarcula*, *Natrinema* and *Halorubrum*.[14] It was also found that *Halobacterium*, *Haloarcula* and *Halorubrum* were dominant genera in Tuz Lake and its salterns.[15]

In the experiment conducted on imported salted hides, moderately halophilic bacterial species belonging to the genera *Salimicrobium*, *Halomonas*, *Marinococcus*, *Chromohalobacter*, *Oceanobacillus*, *Thalassobacillus* and *Alkalibacillus*, and extremely halophilic archaeal species belonging to the genera *Halorubrum* and *Natrinema* were isolated.[16]

Although there are a few studies about extremely halophilic archaea on salted hides and their deleterious effect,[5,9,10,16-18] the isolation and molecular identification of moderately halophilic bacteria and extremely halophilic archaea on red discolored areas of deteriorated salted hides has not been reported. Using molecular methods to identify halophilic microorganisms on the deteriorated salted sheep skins will provide a valuable information about the microbiota and their destructive potential. Therefore, the goal of this study was to isolate and use molecular methods to identify moderately halophilic bacteria and extremely halophilic archaea in red discolored areas of salt cured hides, and to determine their proteolytic and lipolytic capacities. The information obtained may help to control the growth of moderately halophilic bacteria and extremely halophilic archaea and develop new preservation techniques in the leather industry.

EXPERIMENTAL

Collection of Salted Skins

In the present study, four deteriorated salted sheep skins exhibiting red and yellow discolorations, hair slips, slimy layers and bad odor were collected from a warehouse in Valencia, Spain. Then, these samples were immediately placed into sterile sample bags and containers, and they were transported on ice. Before the experiments, all of the salt-pack cured skin samples were thoroughly cleansed of hair, fat and dirt.

Determination of pH Values of the Salted Skins

Five grams of the salted sheep skins were placed into the flasks containing 100 ml of sterile distilled water at 25°C. The flasks were placed in a shaking incubator (New Brunswick Scientific Co. Inc. U.S.A.) for 1 hour at 200 rpm. The pH was measured by using a pH meter (Crison, Basic 20).[19]

Determination of Moisture Content of the Salted Skins

Three grams of the salted skin samples were thoroughly cleansed of hair, fat and dirt. The skin samples were weighed and placed into an oven at 102°C for 6 hours. The dried skin samples were weighed, put into an oven for 1 hour, and then were weighed again. The drying procedure was repeated until the first dry weight was equal to the second dry weight, and finally mositure contents of the skins were calculated.[9,19]

Determination of Total Bacterial Population of Moderately Halophilic Bacteria and Extremely Halophilic Archaea on the Skin Samples

The spread plate technique was used to determine the total viable cell counts of moderately halophilic bacteria and extremely halophilic archaea on the salted skins.[20] Twenty

grams of salted skin samples were cut and separately placed into flasks containing 180 ml 15% NaCl solution. The flasks were placed in a shaking incubator at 200 rpm for 2 hours at room temperature. Afterwards, the bacterial and archaeal suspensions were diluted with sterilized physiological saline water (15% NaCl). An aliquot of 0.1 ml of direct and serial dilutions (10^{-1} to 10^{-5}) of bacterial and archaeal suspensions were spread onto the surface of the agar plates containing complex media supplemented with 0.5% (w/v) yeast extract and oligotrophic media supplemented with 1.82% R2A agar. The final salt concentrations of the media were adjusted to 15% and 25% in order to determine the total viable cell counts of moderately halophilic bacteria and extremely halophilic archaea, respectively.[21] The agar plates were incubated at 37°C for one week for moderately halophilic bacteria, and three weeks for extremely halophilic archaea. The colonies were counted. Selected colonies differing in shape, size and pigmentation were restreaked several times to obtain pure cultures. In all experiments, the pH of media was adjusted to 7.0.

Morphological, Cultural and Physiological Characteristics of the Isolates

The isolates were examined for cell morphology and pigmentation. Cell morphology of exponentially growing liquid cultures was examined on prepared wet mounts using light microscopy. Gram staining was performed by using acetic acid-fixed slides as described by Dussault (1955).[22]

Determination of Protease Activity

Proteolytic activity of the isolates was screened on Tryptic Soy Agar medium containing 15 g peptone from caseine, 5 g peptone, 40 g gelatin, 15 g agar and 1000 ml salts solution with a final concentration of 15% and 25% (w/v) total salts. The agar plates were incubated at 37°C for one week for moderately halophilic bacteria, and three weeks for extremely halophilic archaea. After incubation, the plates were covered with a saturated solution of ammonium sulfate. Clear zones around the colonies were interpreted as evidence of protease activity.[16,19]

Determination of Lipase Production

Lipolytic activity of the test isolate was screened on Tween 80 agar medium containing 10 g peptone, 0.1 g calcium chloride, 10 g Tween 80, 20 g agar and 1000 ml salts solution with a final concentration of 15% and 25% (w/v) total salts. The agar plates were incubated at 37°C for one week for moderately halophilic bacteria, and three weeks for extremely halophilic archaea. After incubation period, opaque zones around the colonies were interpreted as evidence of lipase activity.[15]

Phylogenetic Analysis

Genomic DNA of the moderately halophilic bacterial and extremely halophilic archeal isolates was prepared using the method described by Marmur (1961).[23,24] The 16S rRNA of moderately halophilic bacteria was amplified by PCR using forward primer 16F27 and reverse primer 16R1488 as described by Mellado and colleagues (1995).[25] The 16S rRNA of extremely halophilic archaea was also amplified by PCR using forward primer 21F and reverse primer 1492R as described by Delong (1992).[26] The 16S rRNA gene sequences were determined by Stabvida Laboratory (Portugal), and the derived 16S rRNA gene sequences were compared with sequences in the GenBank and EMBL databases using the BLAST search program.

Nucleotide Accession Number

16S rRNA sequence data of the moderately halophilic bacterial and extremely halophilic archaeal isolates *Alkalibacillus halophilus*, *Pseudomonas halophila*, *Acinetobacter johnsonii*, *Alkalibacillus salilacus*, *Salimicrobium salexigens*, *Marinococcus luteus*, *Staphylococcus equorum* subsp. *equorum*, *Halorubrum tebenquichense*, *Halorubrum saccharovorum*, *Halococcus dombrowskii*, *Halococcus qingdaonensis*, *Natrinema pellirubrum*, *Halococcus morrhuae*, *Halorubrum kocurii*, *Halorubrum terrestre*, *Halorubrum lipolyticum*, *Halostagnicola larsenii*, *Haloterrigena saccharevitans* and *Natrinema versiforme* reported in this article have been submitted to GenBank nucleotide sequence database under the respective accession numbers: DQ359731, AB021383, X81663, AY671976, FR714935, FJ214659, AB009939, EF468473, U17364, AJ420376, AB009939, AGIN01000009, X00662, DQ072718, AB090169, DQ355814, AM117571, AY820137 and AB023426.

RESULTS AND DISCUSSION

In the present study, four salt cured sheep skins exhibiting intensive red discolorations were collected from a warehouse in Spain. The pH values of the salted hides were between 7.95 and 8.90 (Table I). Researchers reported that alkali pH was a sign of protein degradation by bacteria.[4,5] Moisture contents of the hides ranged from 11 to 17. Although moisture contents were low, red discolorations were observed on the hides (Table I). The presence of halophilic microorganisms on the salted hides

TABLE I
pH values and moisture contents of skins.

Skin samples	pH	Moisture content (%)
1	8.90	11.00
2	8.25	17.00
3	8.44	12.00
4	7.95	14.00

with low moisture contents is closely related with production of compatible solutes and accumulation of K^+ by halophilic microorganisms.[21,27]

Viable cell counts of total moderately halophilic bacteria and total extremely halophilic archaea on the salted skin samples are shown in Table II. The salted skin samples 1, 2, 4 contained moderately halophilic bacteria and extremely halophilic archaea in high numbers. The salted skin sample 3 contained the same number of moderately halophilic bacteria and extremely halophilic archaea (9×10^5 CFU/g). The total viable cell counts of moderately halophilic bacteria were found higher (10^5-10^8 CFU/g) than the total viable cell counts of extremely halophilic archaea (10^5-10^7 CFU/g).

In the studies carried out with hide curing salt, it was observed that 40 salt samples contained 10^2-10^5 CFU of extremely halophilic archaea per gram.[19] These salt samples contained 10^2-10^4 CFU of proteolytic and lipolytic extremely halophilic archaea per gram.[19] Investigators stated that the salted hide samples imported from Greece, England, U.S.A., Serbia, Bulgaria, Russia, Africa and Australia contained 10^2-10^6 CFU/g of proteolytic and lipolytic halophilic archaea. These studies proved that curing salt contained extremely halophilic archaea in red and pink colors, and these microorganisms contaminated hide samples during curing process.[19]

A total of 78 moderately halophilic bacterial isolates, representing 6 genera and 7 species, were isolated and identified from the salted sheep skins. Three, four, three and four different species of moderately halophilic bacteria were isolated from skin sample 1, sample 2, sample 3 and sample 4, respectively (Table III).

According to phenotypic characteristics and comparative partial 16S rRNA sequence analysis, 78 moderately halophilic bacterial isolates obtained from the salted skins were identified as *Alkalibacillus halophilus* (41 isolates), *Pseudomonas halophila* (13 isolates), *Acinetobacter johnsonii* (11 isolates), *Alkalibacillus salilacus* (8 isolates), *Salimicrobium salexigens* (3 isolates), *Marinococcus luteus* (1 isolate), *Staphylococcus equorum* subsp. *equorum* (1 isolate) (Table III). *Alkalibacillus halophilus*, *Alkalibacillus salilacus*, *Salimicrobium salexigens*, *Marinococcus luteus* and *Staphylococcus equorum* subsp. *equorum* were found to be Gram-positive; *Pseudomonas halophila* and *Acinetobacter johnsonii* were found to be Gram-negative (Table IV).

A total of 101 extremely halophilic archaeal isolates, representing 5 genera and 12 species, were isolated and identified from the salted sheep skins. Three, eight, three and three different species of extremely halophilic archaea were isolated from skin sample 1, sample 2, sample 3 and sample 4, respectively (Table V).

TABLE II
Total counts of moderately halophilic bacterial and extremely halophilic archaeal isolates from the four salted sheep skins.

Salted sheep skin samples	Total counts of moderately halophilic bacterial isolates (CFU/g)	Total counts of extremely halophilic archaeal isolates (CFU/g)
1	2.8×10^7	1.1×10^7
2	1.1×10^8	3.4×10^6
3	9.0×10^5	9.0×10^5
4	3.6×10^7	5.8×10^6

TABLE III
Frequency of various species of moderately halophilic bacteria isolated from the four salted sheep skins

Species of moderately halophilic bacteria	Salted sheep skin samples				Total numbers of the isolates
	1	2	3	4	
Alkalibacillus halophilus	10	13	10	8	**41**
Pseudomonas halophila	10	-	3	-	**13**
Acinetobacter johnsonii	-	7	-	4	**11**
Alkalibacillus salilacus	3	1	4	-	**8**
Salimicrobium salexigens	-	-	-	3	**3**
Marinococcus luteus	-	1	-	-	**1**
Staphylococcus equorum subsp. *equorum*	-	-	-	1	**1**
Total	**23**	**22**	**17**	**16**	**78**

TABLE IV
Phenotypic characteristics of the moderately halophilic bacterial isolates.

Species	Number of isolates	Pigmentation	Cell morphology	Gram staining reaction	Proteolytic activity	Lipolytic activity
Alkalibacillus halophilus	41	White	Rods	+	-	-
Pseudomonas halophila	13	Cream	Rods	-	-	-
Acinetobacter johnsonii	11	Cream	Rods	-	-	+
Alkalibacillus salilacus	8	White	Rods	+	-	-
Salimicrobium salexigens	3	Yellow	Cocci	+	-	-
Marinococcus luteus	1	Orange	Cocci	+	-	-
Staphylococcus equorum subsp. *equorum*	1	Cream	Cocci	+	-	-

TABLE V
Extremely halophilic archaeal species isolated from the four salted sheep skins.

Species of extremely halophilic archaeal isolates	Salted sheep skin samples				Total number of isolates
	1	**2**	**3**	**4**	
Halorubrum tebenquichense	28	24	2	-	**54**
Halorubrum saccharovorum	6	16	2	-	**24**
Halococcus dombrowskii	-	-	-	9	**9**
Halococcus qingdaonensis	-	-	-	3	**3**
Natrinema pellirubrum	-	2	1	-	**3**
Halococcus morrhuae	-	-	-	2	**2**
Halorubrum kocurii	1	-	-	-	**1**
Halorubrum terrestre	-	1	-	-	**1**
Halorubrum lipolyticum	-	1	-	-	**1**
Halostagnicola larsenii	-	1	-	-	**1**
Haloterrigena saccharevitans	-	1	-	-	**1**
Natrinema versiforme	-	1	-	-	**1**
Total	**35**	**47**	**5**	**14**	**101**

According to the phenotypic characteristics and comparative partial 16S rRNA sequence analysis, 101 extremely halophilic archaeal isolates were identified as *Halorubrum tebenquichense* (54 isolates), *Halorubrum saccharovorum* (24 isolates), *Halococcus dombrowskii* (9 isolates), *Halococcus qingdaonensis* (3 isolates), *Natrinema pellirubrum* (3 isolates), *Halococcus morrhuae* (2 isolates), *Halorubrum kocurii* (1 isolate), *Halorubrum terrestre* (1 isolate), *Halorubrum lipolyticum* (1 isolate), *Halostagnicola larsenii* (1 isolate), *Haloterrigena saccharevitans* (1 isolate) and *Natrinema versiforme* (1 isolate) (Table V). All extremely halophilic archaeal isolates were found to be Gram-negative (Table VI). Everett and Cordon (1956) isolated halophilic Gram-positive and Gram-negative rods and Gram-positive cocci from the salted hides.[28] Researchers stated that these halophilic microorganisms, grown in the media containing 23% NaCl, were able to digest gelatin.[28] It was also mentioned that halophilic microorganisms that demonstrate proteolytic activity may cause destruction of the skin substance or grain surface.[28]

In our previous study, 13 moderately halophilic bacteria and 5 extremely halophilic archaea were isolated from the salted hides imported from England and Australia. Comparative partial 16S rRNA gene sequence analysis revealed that the isolated strains were identified as *Salimicrobium album* (1 strains), *Salimicrobium halophilum* (1 strain), *Halomonas eurihalina* (1 strain), *Salimicrobium luteum* (1 strain), *Halomonas koreensis* (1 strain), *Halomonas elongata* (1 strain), *Halomonas halmophila* (1 strain), *Halomonas alimentaria* (1 strain), *Marinococcus halophilus* (1 strain), *Halorubrum saccharovorum* (1 strain), *Halorubrum tebenquichense* (1 strain), *Halorubrum lacusprofundi* (1 strain), *Chromohalobacter salexigens* (1 strain), *Oceanobacillus picturae* (1 strain), *Thalassobacillus devorans* (1 strain), *Alkalibacillus salilacus* (1 strain), *Natrinema pallidum* (1 strain) and *Natrinema gari* (1 strain).[16]

Anderson (1954) isolated 25 red pigmented and eight colorless bacteria from hides with red heat.[7] These microorganisms required 15% NaCl for growth. It was found that some red pigmented and colorless microorganisms were able to digest protein. He observed that these microorganisms grew very well in the medium containing sterile hide and 27.5% NaCl; they produced red pigments in 2 weeks at 37 °C. It was reported that after 10 weeks, the hides became soft because of proteolytic bacteria and gave off ammonia odor. Hair slip was not observed in these hides.[7] Anderson (1954) isolated

TABLE VI
Phenotypic characteristics of the extremely halophilic archaeal isolates.

Species	Number of isolates	Pigmentation	Cell morphology	Gram staining reaction	Proteolytic activity	Lipolytic activity
Halorubrum tebenquichense	54	Red	Irregular discs	-	-	-
Halorubrum saccharovorum	24	Red	Rods	-	-	-
Halococcus dombrowskii	9	Red	Cocci	-	+	+
Halococcus qingdaonensis	3	Red	Cocci	-	-	-
Natrinema pellirubrum	3	Red	Rods	-	+	-
Halococcus morrhuae	2	Red	Cocci	-	+	+
Halorubrum kocurii	1	Red	Rods	-	-	-
Halorubrum terrestre	1	Red	Pleomorphic cells	-	-	-
Halorubrum lipolyticum	1	Red	Rods	-	+	+
Halostagnicola larsenii	1	Pink	Pleomorphic rods	-	-	-
Haloterrigena saccharevitans	1	Red	Rods	-	-	+
Natrinema versiforme	1	Red	Pleomorphic rods	-	-	+

Halobacterium salinarum from these hides. Other study showed that hides with red heat were more prone to bacterial attack than normal hides and had extensive damage in hide fibers.[13]

In our study, extensive red discolorations in spots, great blotches, streaks, hair slip and slimy growth of organisms were observed on the sheep skins examined (Table VII). Moderately halophilic bacterial isolates formed white, cream, yellow and orange colonies on the complex and oligotrophic agar media containing 15% (w/v) total salts (Table IV). Extremely halophilic archaeal isolates formed mostly red colored colonies on the complex and oligotrophic agar media containing 25% (w/v) total salts (Table VI).

In the present study, proteolytic and lipolytic activities of the isolates were determined. Among the moderately halophilic bacterial isolates, *Acinetobacter johnsonii* (11 isolates) showed positive lipolytic activities. The other isolates showed neither proteolytic nor lipolytic activity (Table IV). Among the extremely halophilic archaeal isolates *Halcoccus dombrowskii, Halococcus morrhuae* and *Halorubrum lipolyticum* showed both proteolytic and lipolytic activities. *Natrinema pellirubrum* showed positive proteolytic activity, but *Haloterrigena saccharevitans* and *Natrinema versiforme* exhibited only lipolytic activity (Table VI). Protease activity of *Halococcus dombrowskii*,[29] *Halococcus morrhuae*,[30] *Halorubrum lipolyticum*[31] and *Natrinema pellirubrum*[32] was reported on previous studies. It was also stated that *Halococcus* species cause spoilage of salted fish and hides.[27] We consider that the presence of proteolytic and lipolytic halophilic microorganisms on the skin samples may cause hair slip.

Previous study proved that damage began on the hide surface before the red heat became visible. It has been reported that the archaea moved down the hair follicles to attack underlying dermal layer. It was also stated that halophilic archaea destroyed collagen fibers and caused production of sponge like vesicles within the hide. Furthermore, it has been observed that the test bacteria formed thin strands of a web-like material that may link the bacterial cells to the hide surface. It was also proved that halophiles were present on both grain and flesh sides of hides prior to the appearance of red heat. It has been emphasized that halophiles can cause distinct separation between the grain and corium layers of the hides.[10]

In our study, the most frequently isolated genus of moderately halophilic bacteria on the skin samples investigated was *Alkalibacillus* (49 isolates) and the most frequent species was *Alkalibacillus halophilus* (41 isolates). Two different species of the genus *Alkalibacillus* (*Alkalibacillus halophilus* and *Alkalibacillus salilacus*) were isolated from the skins (Table III). The moderately halophilic bacterial isolates isolated from the salted sheep skins in the present study were also isolated from other environments. *Acinetobacter johnsonii* and *Staphylococcus equorum* subsp. *equorum* were isolated from fresh hides,[33,34] *Salimicrobium salexigens* and *Alkalibacillus salilacus* were isolated from salted hides.[16,35] *Alkalibacillus halophilus*, *Pseudomonas halophila*, and *Marinococcus luteus* were isolated from hypersaline soil in China,[36] Great Salt Lake in USA,[37] and saline Barkol Lake in China,[38] respectively.

In our study, the most frequently isolated genus of extremely halophilic archaea on the skin samples was *Halorubrum* (81 isolates); and the most frequent species was *Halorubrum tebenquichense* (54 isolates). A total of five different species belonging to the genus *Halorubrum (Halorubrum tebenquichense, Halorubrum saccharovorum*, *Halorubrum kocurii*, *Halorubrum terrestre* and *Halorubrum lipolyticum*) were isolated from the skins (Table V). We isolated some species of moderately halophilic bacteria and extremely halophilic archaea from the skin samples in fairly low numbers. Only one isolate of *Marinococcus luteus* and *Staphylococcus equorum* subsp. *equorum*, *Halorubrum kocurii*, *Halorubrum terrestre*, *Halorubrum lipolyticum*, *Haloterrigena saccharevitans*, *Halostagnicola larsenii* and *Natrinema versiforme* was isolated from the skin samples.

TABLE VII
Presence of red discoloration, hair slip and slimy growth of microorganism on the skin samples.

Salted sheep skin samples	Red discoloration on flesh side of skin	Presence of hair slip	Slimy growth of microorganisms
1	In spots	+	+
2	In spots, streaks, great blotches	+	+
3	In spots, great blotches	+	+
4	Great blotches	+	+

Most isolates of extremely halophilic archaea produced red colored colonies on complex and oligotrophic media. That is why these archaeal species caused red heat on the skins. The occurrence of these archaeal species in high numbers on the skins was thought to result from their presence in the curing salt. Yellow and red pigment production by halophilic microorganisms that grew on the salted hides has been reported, as well as that when red and yellow pigment producing halophilic microorganisms were found together on the salted hides, they caused more damage.[13]

Our extremely halophilic archaeal isolates were also isolated from different hypersaline environments such as salt lakes, sea salt samples, saline soils, salted hide, salt deposit and saltern. *Halorubrum kocurii*, *Halococcus qingdaonensis*, *Halorubrum terrestre*, *Halorubrum tebenquichense*, *Halococcus dombrowskii*, *Halococcus morrhuae*, *Halorubrum saccharovorum, Natrinema pellirubrum* and *Halostagnicola larsenii* were isolated from saline lake Bagaejinnor in China,[39] sea-salt in China,[40] saline soil,[41] Atacama Saltern in Chile,[42] rock salt in Austria,[29] Red Sea,[30] saltern in USA,[43] salted hide,[32] and saline lake Xilinhot in China,[44] respectively. In addition, *Halorubrum lipolyticum*,[31] *Haloterrigena saccharevitans*,[45] and *Natrinema versiforme*,[46] were isolated from saline lake Aibi in China.

CONCLUSIONS

This is the first detailed study in which moderately halophilic bacteria and extremely halophilic archaea are isolated from red discolorated areas of the salt pack cured sheep skins and characterized these microorganisms using molecular methods. White, cream, yellow, orange, pink and red colored moderately halophilic bacteria and extremely halophilic archaea were isolated from the samples. Although only one species of moderately halophilic bacteria showed lipolytic activity, a few species of extremely halophilic archaea showed proteolytic or lypolytic activities. These species may cause hair slip and red heat on the sheep skins. To the best of our knowledge, although moderately halophilic isolates such as *Acinetobacter johnsonii*,[33] *Alkalibacillus salilacus*,[16] *Staphylococcus equorum* subsp. *equorum*,[34] extremely halophilic archaea such as *Halorubrum saccharovorum, Halorubrum tebenquichense*,[16] and *Natrinema pellirubrum*,[32] isolated from the sheep skins in this study were also isolated from hide samples in previous investigations. Especially *Alkalibacillus halophilus*, *Pseudomonas halophila, Salimicrobium salexigens, Marinococcus luteus, Halorubrum kocurii, Halorubrum terrestre, Halorubrum lipolyticum, Natrinema pellirubrum, Natrinema versiforme, Halostagnicola larsenii, Haloterrigena saccharevitans, Halococcus dombrowskii, Halococcus morrhuae* and *Halococcus qingdaonensis* were species of halophilic microorganisms have been isolated and identified for the first time from salted skins exhibiting red and yellow discolorations in the present study. Therefore, we suggest that inactivation of halophilic microorganisms in curing salt will help to avoid red and yellow discolorations that devalue or even destroy the leather industry's end product.

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