Investigation of Moderately Halophilic Bacteria Causing Deterioration of the Salted Sheep and Goat Skins and Their Extermination via Electric Current Applications

by

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

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

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

[3]*Technology Faculty, Electric and Electronic Engineering Department, Marmara University,* Istanbul, Turkey

Abstract

Determination of moderately halophilic bacterial counts on the salted skins and examination of utilization of amino acid and carbon sources by skin isolates offer important information about biodegradation of salted skins. Hence, total counts of moderately halophilic bacteria, total counts of proteolytic and lipolytic moderately halophilic bacteria found on the salted sheep and goat skins belonging to different countries were examined in this study. One hundred-thirty seven moderately halophilic bacterial species closely related to species of genera *Alkalibacillus*, *Bacillus*, *Chromohalobacter*, *Gracilibacillus*, *Halomonas*, *Idiomarina, Marinococcus*, *Oceanobacillus*, *Planococcus*, *Salimicrobium*, *Salinicoccus*, *Staphylococcus* and *Salinivibrio* were used to detect utilization of different amino acids and carbon sources which are related to biodegradation of skins. The values of pH, moisture contents, ash contents and salt saturations of these skins were also investigated to understand the correlation between these parameters and moderately halophilic bacterial activities. All salted skin samples contained moderately halophilic bacteria, proteolytic and lipolytic moderately halophilic bacteria in high numbers. Each of 137 test isolates, obtained from the salted sheep and goat skins, used different amino acids found in the skin structure. While 100% of both sheep and goat skin isolates utilized L-arginine, 86%, 66%, 85%, 64% and 66% of the isolates respectively utilized L-glycine, L-alanine, L-tyrosine, L-proline and L-hydroxyproline amino acids. Ninety-three percent of the isolates used different carbon sources such as dulcitol (13%), D-sorbitol (29%), L-rhamnose (19%), ribose (52%), salicin (40%), *myo*-inositol (27%), xylitol (21%), benzoate (18%), propionate (34%), D-melezitose (31%), butanol (20%), propanol (23%), methanol (23%), formate (23%) and tartrate (12%). While pH values, moisture contents, ash contents and salt saturations of the sheep skins ranged from 6.53 to 8.01, 32 to 68%, 12 to 30% and 58 to 100%, respectively, the pH values, moisture contents, ash contents and salt saturations of the goat skins were between 6.65-8.06, 34-70%, 11-32% and 64-100%, respectively. The values of all skin samples were found to be suitable for the growth and catabolic activities of these bacteria. To prevent the growth and activities of moderately halophilic isolates causing skin deterioration, the annihilation effects of 0.5, 1.0, 1.5 and 2.0 A direct and alternating electric current treatments on the mixed culture of moderately halophilic isolates (*Chromohalobacter israelensis*, *Chromohalobacter canadensis*, *Halomonas halodenitrificans*, *Staphylococcus nepalensis* and *Halomonas halmophila*) were also separately investigated in the present study. Both 0.5, 1.0, 1.5, 2.0 A direct and alternating electric current applications used in this study completely annihilated the mixed culture of moderately halophilic spoilage microorganisms. As a conclusion, we suggest using low-level direct or alternating electric current treatment to eradicate harmful moderately halophilic bacteria in salt which will be used in brine curing of hides and skins in the leather industry.

Introduction

Skin containing water (64%), structural (29% collagen, 2% keratin, 0.3% elastin) and non-structural proteins (1% albumins and globulins, 0.7% mucins and mucoids), fat (2%), mineral salts (0.5%) and other substances (0.5%) provides an excellent environment for the growth of a wide variety of spoilage microorganisms.[1,2] Especially high moisture and protein contents of skins support metabolic activities of microorganisms which cause decomposition of skin substances. Therefore, salt curing method is applied to skins to prevent bacterial growth and their metabolic activities. However, curing salt contaminates skins with moderately halophilic bacteria. These

* Corresponding author e-mail: pinar.caglayan@marmara.edu.tr

Manuscript received November 8, 2017, accepted for publication December 5, 2017.

microorganisms, which are able to grow at 3-15% NaCl, 0-45°C and pH values of 5.0-10.0, are the common inhabitants of salt obtained from saline lakes, salterns, solar salt evaporation ponds.[3-6] The presence of moderately halophilic bacteria on salted hides and skins has drawn scientific attention since 1929.[7-12] Llyod and colleagues (1929) emphasized that halophilic microorganisms found in marine salt contaminate hides during the brine curing process. A more detailed study of halophilic microorganisms on the salted hides was accomplished by Kallenberger in 1985. Kallenberger (1985) reported that moderately halophilic microorganisms were common in the raceways and cured hides. Moderately halophilic bacteria were also isolated from 131 brine cured hides collected from USA, from 35 salt pack cured hides imported from France and Russia, from four salted sheep skins belonging to Spain, from five salted hides imported from England and Australia.[5,9-12]

Researchers pointed out that proteolytic and lipolytic non-halophilic bacteria, moderately halophilic bacteria and extremely halophilic archaea on salted hides or skins may cause red and yellow discolorations of the flesh side of hide or skin, bad odor, hair slip, pin pricks, entire degradation of hair follicle, holes in grain surface, partially removal of grain layer, loose grain, grain peeling, distruption of collagen fibers, and uneven dyeing in leathers.[5,8,10-17] These problems may occur during the brine curing and soaking, the storage of the skins and transportation.

Different skin preservation methods have been suggested by investigators to prevent the growth of these spoilage microorganisms. Major skin preservation methods include curing skin with sodium chloride and boric acid, treating skin with electron beam irridation or antibacterial agents containing dithiocarbamates, isothiazolinones, quaternary ammonium compounds.[18-21] In addition, sophorolipids (palmitic acid, stearic acid, oleic acid) were found very effective to inhibit Gram-positive and Gram-negative bacteria isolated from the salted hides.[22]

In addition to aforementioned applications, electric current treatments were used to kill halophilic archaea in curing salt, Gram-positive and Gram-negative bacteria on salted hides and in soak liquors in the previous studies.[23-27] Firstly, proteolytic and lipolytic extremely halophilic archaea in skin curing salt were separately exterminated via direct electric current and alternating electric current in leather industry.[23-25] Then, Gram-positive and Gram-negative bacteria found on hides and in soak liquors were annihilated using direct and alternating electric currents.[26,27]

Although there are a few studies related to the total counts of moderately halophilic bacteria and their protease and lipase activities on salted hides and sheep skins[11,12], these bacterial counts have not heretofore been examined in detail on sheep and goat skin samples belonging to Australia, Bulgaria, Greece, Israel, South Africa, USA, Dubai, Russia, Kuwait, China, France and Turkey. Moreover, it has not been investigated whether or not the amino acids found in the structure of sheep and goat skins and different carbon sources are used by moderately halophilic bacteria isolated from the salted skins. Hence, the present study investigated total counts of moderately halophilic bacteria, total counts of proteolytic and lipolytic moderately halophilic bacteria on the salted sheep and goat skins belonging to these countries. To understand their catabolic activities on the skins, their abilities to utilize different amino acids and different carbon sources were also examined. In addition, the correlation between pH values, moisture contents, ash contents and salt saturations of the salted sheep and goat skins and moderately halophilic bacterial activities was evaluated. To inhibit the growth and catabolic acitivites of moderately halophilic spoilage microorganisms on the skins, extermination effect of 0.5, 1.0, 1.5 and 2.0 A electric treatments against mixed culture of moderately halophilic isolates were also examined.

Experimental

Skin Samples

Salted skin samples (25 sheep and 25 goat) were obtained from different tanneries in Tuzla and Corlu Leather Organized Tannery Regions, Turkey. While the salted sheep skin samples were belong to 10 different countries and the salted goat skin samples were belong to 8 different countries. The samples used in this study were taken from different parts of sheep and goat such as leg, rump, shoulder, back crotch, back, neck and fore crotch. Then, the skin samples were immediately placed into sterile sample bags and carried on ice during transportation.

Determination of Total Counts of Moderately Halophilic Bacteria on the Skin Samples

The total counts of moderately halophilic bacteria on each skin sample were determined by spread plate technique. Twenty grams of each salted skin sample were put into a flask containing 180 ml sterile 10% NaCl solution, and the flasks were placed into a shaking incubator for 4 h at 25°C and 100 rpm. Direct and serial dilutions of the bacterial suspension were spread onto the surface of Complex Medium-I (CMI) agar plates containing 0.5% (w/v) yeast extract.[12] The final salt concentration of all test media was 10% (w/v) with the following composition (SW10, saline water): 8.1% (w/v) NaCl, 0.7% (w/v) $MgCl_2$, 0.96% (w/v) $MgSO_4$, 0.036% (w/v) $CaCl_2$, 0.2% (w/v) KCl, 0.006% (w/v) $NaHCO_3$, and 0.0026% (w/v) NaBr.[28] The plates were incubated at 37°C for 24 h. After incubation, colonies of moderately halophilic bacteria grown on the plates were counted.

Determination of Total Counts of Proteolytic and Lipolytic Moderately Halophilic Bacteria on the Skins

Total counts of proteolytic and lipolytic moderately halophilic

bacteria on the skins were tested using gelatin agar and Tween 80 agar media, respectively. Direct and serial dilutions of the bacterial suspension, prepared according to the directions above, were spread onto the surface of gelatin agar media containing 2% (w/v) gelatin and Tween 80 agar media containing 1% (w/v) Tween 80 supplemented with 0.5% yeast extract (w/v) and 10% (w/v) total salts. The plates were incubated at 37°C for 48 h. The gelatin agar media were flooded with Frazier solution. Clear zones around the colonies were interpreted as evidence of protease activity.[29] Presence of opaque zones around the colonies was taken as evidence of lipase activity.[29] The pH of all test media used in this study were adjusted to pH 7.0. All experiments were performed in triplicate in the present study.

Utilization of Different Amino Acids and Different Carbon Sources

A total of 137 moderately halophilic bacterial species belonging to genera *Alkalibacillus*, *Bacillus*, *Chromohalobacter*, *Gracilibacillus*, *Halomonas*, *Idiomarina*, *Marinococcus*, *Oceanobacillus*, *Planococcus*, *Salimicrobium*, *Salinicoccus* and *Salinivibrio* were used to examine their catabolic activities. These species were isolated from salted sheep skins (90 isolates) and salted goat skins (47 isolates) and identified using both conventional and molecular techniques in our previous studies.[30,31] These isolates were obtained from the culture collections of Division of Plant Diseases and Microbiology, Department of Biology, Faculty of Arts and Sciences, Marmara University, Turkey.[30,31] Amino acid utilization tests of these isolates were performed using 1% (w/v) L-arginine, L-cysteine, L-glycine, L-alanine, L-tyrosine, L-proline and L-hydroxyproline, 0.5% (w/v) peptone, 0.5% (w/v) beef extract, 0.05% (w/v) dextrose, 0.001% (w/v) bromocresol purple, 0.0005% (w/v) cresol red, 0.0005% (w/v) pyridoxal in SW10. Bromocresol purple was used as pH indicator in these tests. Positive test was indicated by purple color formation in the test tube containing bacterial culture after 24 h incubation at 37°C.[30-32]

Utilization of different carbon sources by these isolates was separately examined using 1% (w/v) dulcitol, D-sorbitol, L-rhamnose, ribose, salicin, *myo*-inositol, xylitol, benzoate, propionate, D-melezitose, butanol, propanol, methanol, formate and tartrate, 0.5% (w/v) yeast extract, and 0.001% (w/v) phenol red. Positive test result was indicated by a color change from red to yellow.[33]

Determination of pH Values of the Salted Skins

Five grams of each skin samples were cut into pieces. Then, the skins were separately transferred into flasks containing 100 ml of sterile distilled water at 20°C. The flasks were placed into a shaking incubator for 1 h at 200 rpm, and then pH values of the skins were measured by using a pH meter.[15]

Determination of Moisture Content, Ash Content and Salt Saturation of the Salted Skins

Three grams of the skins were cut and hair and any adhering flesh of the skins were removed with a razor blade. Then, the samples were cut into small pieces. The moisture content was determined by drying the samples in an oven at 102°C for 6 h. The dried skin samples were put into a dessicator for 30 min to cool. After cooling procedure, the samples were weighed, later placed into an oven for one hour before being weighed again. The drying procedure was repeated until first dry weight was equal to second dry weight, and finally moisture contents of the skins were calculated. Then, the dry 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 the examined skin samples were calculated according to the standard methods.[15,34]

Application of Alternating and Direct Electric Currents on the Mixed Culture

Five characterized moderately halophilic skin isolates (*Chromohalobacter israelensis*, *Chromohalobacter canadensis*, *Halomonas halodenitrificans*, *Staphylococcus nepalensis* and *Halomonas halmophila*) were used in the electric current treatments. These microorganisms were isolated from three salted sheep skins and two salted goat skins in our previous studies.[30,31] The test isolates were separately grown in CMI liquid test medium containing 10% total salts at 37°C for 24 h. Then, each test isolates was diluted in sterile saline solution to adjust cell density to 10^8 CFU/ml. Mixed culture of these isolates was prepared, and 20 ml of the mixed culture was added into the electrolysis cell consisting of a glass beaker having two internally attached platinum wire electrodes and 10% NaCl (180 ml).[23,35] A 100 µl quantity of test medium was removed from the electrolysis cell before the electric current applications and diluted to 10^{-4} with sterile 10% NaCl solution. The diluted bacterial suspension was spread over the test medium and incubated at 37°C for 24 h. After incubation, the colonies grown on the test medium were counted. 0.5A, 1.0A, 1.5A and 2.0A direct current (DC) and 0.5A, 1.0A, 1.5A and 2.0A alternating current (AC) were separately applied to each electrolysis cell for 20 min (Fig.1a-b). A 100 µl quantity of test medium was removed from the cell at intervals of 1, 3, 5, 10, 15 and 20 min. Direct and diluted suspensions of the test medium (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) were spread over CMI agar media, incubated for 24 h at 37°C and colonies on the agar plates were counted. Temperature and pH of each test medium were adjusted to 25°C and 7.0 before the electric current treatments.

Results and Discussion

Moderately Halophilic Bacteria, Proteolytic and Lipolytic Bacteria on Salted Sheep and Goat Skin Samples

All skin samples contained moderately halophilic bacteria, proteolytic and lipolytic moderately halophilic bacteria (Tables I-II). The mean values of moderately halophilic bacterial counts; proteolytic moderately halophilic bacterial counts; lipolytic moderately halophilic bacterial counts were found as $7.9x10^6$, $1.9x10^7$; $1.3x10^6$, $1.5x10^6$; $9.6x10^5$, $1.9x10^6$ CFU/g for sheep and goat skin samples, respectively. Due to inadequate preservation methods, moderately halophilic bacterial counts were found to be high in most of the skin samples in the present study.

In another investigation, viable cell counts of total moderately halophilic bacteria were found between 10^5 and 10^8 CFU/g on four salted skin samples.[11] In our previous study, total moderately halophilic bacterial counts, proteolytic and lipolytic moderately halophilic counts on the salted hide samples imported from England and Australia were respectively detected as 10^2-10^7 CFU/g, 10^2-10^5 CFU/g and 10^5-10^6 CFU/g.[12] Our experimental results were similar to previous studies' results belonging to moderately halophilic bacteria isolated from salted sheep skins and salted hides.[11,12] When moderately halophilic bacterial counts, lipolytic and proteolytic moderately halophilic bacterial counts were high, microbial damage on the salted goat skins will be high and low quality leather will be obtained. Owing to the fact that most skin samples contained proteolytic and lipolytic bacteria, bacterial decomposition of skins may occur during storage and transportation overseas. Presence of moderately halophilic bacteria on the salted sheep and goat skins may be explained by contamination of skin samples with moderately halophilic bacteria found in curing salt.

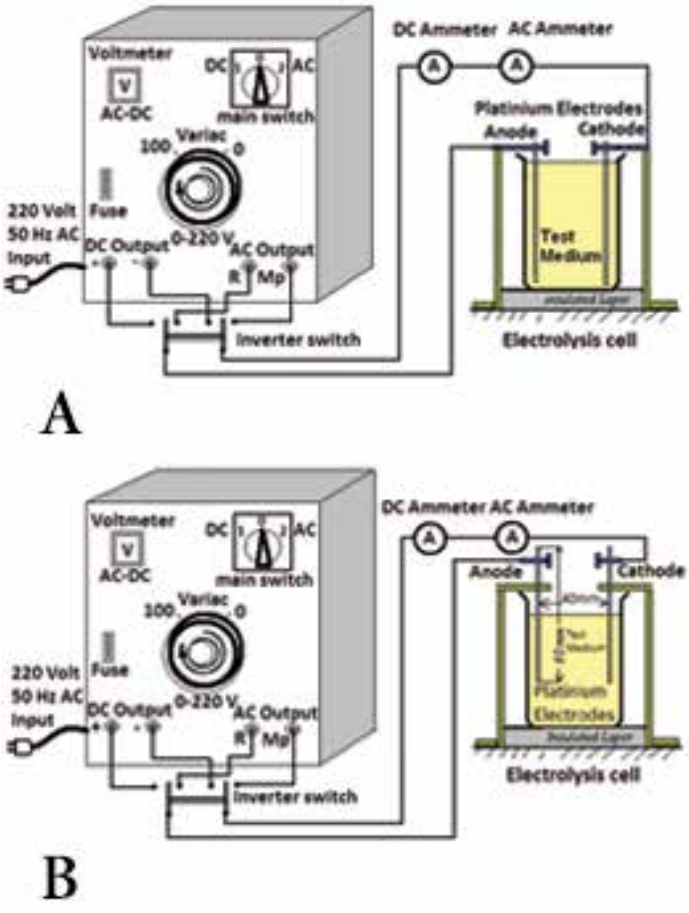

Figure 1. Electrolysis cell system used in this study. (a) DC treatment. (b) AC treatment.[23,35] R: phase, Mp: ground.

Determination of pH Value, Moisture Content, Ash Content and Salt Saturation of the Salted Skins

The temperature of the tanneries, where the sheep and goat skin samples were collected, were between 19°C and 35°C. According to the experimental results obtained from our previous studies[30,31], these temperatures were suitable for the growth of moderately halophilic bacteria (Tables I-II).

In our previous studies we observed that moderately halophilic microorganisms grew in pH values of between 6.0 and 8.0.[30,31] In the present study, pH values of the sheep skin samples (6.53-8.01) and goat skin samples (6.65-8.06) were reasonably appropriate for the growth of moderately halophilic bacteria. Moisture contents of the salted sheep and goat skins in this study ranged from 32 to 68% and 34 to 70%, respectively.

The standard values of moisture content and salt saturation of salted hides and skins were noted as 40-48% and ≥ 85%, respectively.[34,36] According to these standart values, moisture contents of our skin samples were determined low (<40%) for 4 sheep and 6 goat skin samples; normal (40-48%) for 7 sheep and 8 goat skin samples; high (>48%) for 14 sheep and 11 goat skin samples. However, the growth of both moderately halophilic bacteria, proteolytic and lipolytic moderately halophilic bacteria was not affected by low moisture content. It has been known that moderately halophilic bacteria can grow on media containing low moisture content by producing compatible solutes such as glutamate, glutamine, proline, ectoine, taurine, alanine, glycine betaine, mannitol, erythritol, glycerol, sucrose, trehalose and glucose.[6,37] The growth of moderately halophilic bacterial communities on these low moisture contents in our study was related to production of compatible solutes.

Most of the sheep skin samples (18 samples) and all goat skin samples (19 samples) had high salt saturation (>96% and 100%) which allow the growth of our halophilic microorganisms. Minimum and maximum ash content of salted hides and skins were respectively stated as 14% and 48%.[34] While ash contents of the salted sheep skins ranged from 12 to 30%, ash contents of goat skins were between 11-32% (Tables I-II). Our experimental results showed that ash contents of the all skins, which are mostly related to NaCl, were sufficient for the growth of moderately halophilic bacterial populations.

Table I

The origin, parts of skin taken, total counts of moderately halophilic bacterial populations, values of storage temperature, pH, moisture content, ash and salt saturation contents of salted sheep skin samples.

No	Origin	Parts from which sheep skin was taken	MHB[a]	PMHB[b]	LMHB[c]	ST[d] (°C)	pH	MC[e] (%)	AC[f] (%)	SS[g] (%)
1	Australia	fore crotch	$5.3x10^6$	$4.0x10^5$	$1.9x10^5$	19	6.89	48	17	99
2	Australia	rump	$5.0x10^7$	$9.0x10^5$	$5.8x10^4$	31	7.75	56	15	100
3	Australia	shoulder	$6.0x10^5$	$9.0x10^4$	$7.1x10^4$	31	7.43	44	17	100
4	Australia	leg	$1.3x10^6$	$3.2x10^4$	$7.9x10^3$	35	7.61	32	26	100
5	Bulgaria	leg	$4.0x10^6$	$9.9x10^5$	$7.1x10^5$	23	7.62	61	17	78
6	Bulgaria	back	$8.0x10^5$	$5.9x10^4$	$3.6x10^4$	27	7.30	68	19	78
7	Dubai	fore crotch	$4.6x10^6$	$6.0x10^5$	$7.9x10^4$	19	7.24	32	19	100
8	Dubai	neck	$4.0x10^5$	$7.0x10^4$	$4.3x10^4$	19	6.95	52	21	100
9	Dubai	shoulder	$1.1x10^6$	$3.7x10^4$	$1.8x10^4$	31	6.96	67	25	100
10	Greece	leg	$4.0x10^6$	$7.0x10^5$	$7.5x10^5$	23	7.18	51	24	100
11	Greece	back	$3.8x10^7$	$3.9x10^6$	$4.1x10^6$	23	7.29	54	12	62
12	Greece	leg	$1.6x10^6$	$3.0x10^5$	$1.9x10^5$	27	8.01	64	18	78
13	Greece	shoulder	$1.2x10^6$	$1.2x10^5$	$5.2x10^4$	27	7.89	55	12	61
14	Israel	neck	$1.0x10^7$	$8.0x10^6$	$2.6x10^6$	19	6.70	52	19	100
15	Israel	rump	$2.5x10^7$	$4.1x10^6$	$7.5x10^6$	19	6.89	46	30	100
16	Kuwait	leg	$3.9x10^6$	$4.2x10^5$	$2.4x10^5$	19	7.00	48	28	100
17	South Africa	shoulder	$6.2x10^6$	$5.0x10^5$	$7.0x10^5$	23	7.30	47	26	100
18	South Africa	back crotch	$5.0x10^4$	$4.0x10^3$	$3.0x10^2$	27	7.74	58	20	96
19	Turkey	rump	$3.4x10^5$	$4.0x10^4$	$6.2x10^3$	23	6.89	62	13	58
20	Turkey	rump	$4.5x10^6$	$3.3x10^5$	$2.1x10^5$	31	6.53	42	24	100
21	USA	leg	$1.4x10^4$	$3.2x10^3$	$2.6x10^3$	19	7.34	33	25	100
22	USA	leg	$4.5x10^4$	$5.4x10^3$	$1.7x10^3$	19	6.93	58	26	100
23	USA	rump	$7.0x10^3$	$1.5x10^2$	$5.0x10^2$	23	7.20	48	15	87
24	Russia	leg	$1.0x10^7$	$8.0x10^6$	$3.5x10^6$	35	7.40	37	17	100
25	Russia	leg	$2.2x10^7$	$2.2x10^6$	$2.8x10^6$	35	6.91	57	20	96

[a]MHB: Moderately Halophilic Bacteria, [b]PMHB: Proteolytic Moderately Halophilic Bacteria, [c]LMHB: Lipolytic Moderately Halophilic Bacteria, [d]ST: Storage Temperature, [e]MC: Moisture Content, [f]AC: Ash Content, [g]SS: Salt Saturation

Table II

The origin, parts of skin taken, total counts of moderately halophilic bacterial populations, values of storage temperature, pH, moisture content, ash and salt saturation contents of salted goat skin samples.

No	Origin	Parts from which goat skin was taken	MHB	PMHB	LMHB	ST (°C)	pH	MC (%)	AC (%)	SS (%)
1	Australia	neck	$4.7x10^7$	$4.1x10^6$	$2.8x10^6$	32	7.36	53	21	100
2	Australia	back	$3.1x10^7$	$1.2x10^6$	$1.7x10^6$	32	7.82	46	17	100
3	Australia	leg	$3.5x10^7$	$2.5x10^6$	$6.2x10^6$	32	6.78	34	13	100
4	Turkey	leg	$2.5x10^6$	$2.0x10^5$	$1.6x10^5$	29	6.87	40	27	100
5	Turkey	rump	$3.4x10^7$	$1.9x10^6$	$2.3x10^6$	29	7.11	49	20	100
6	Turkey	leg	$8.0x10^7$	$2.0x10^5$	$4.8x10^6$	35	7.57	40	16	100
7	Turkey	back	$6.0x10^3$	$4.5x10^2$	$7.5x10^2$	19	7.50	37	16	73
8	Bulgaria	neck	$3.5x10^7$	$4.7x10^6$	$1.7x10^6$	23	7.24	48	11	64
9	Bulgaria	back	$2.5x10^7$	$1.5x10^6$	$3.8x10^6$	23	8.06	59	22	100
10	Bulgaria	leg	$1.1x10^6$	$3.5x10^5$	$2.1x10^5$	35	8.00	37	23	100
11	Israel	leg	$6.0x10^5$	$8.5x10^4$	$4.4x10^4$	19	6.65	63	32	100
12	Israel	neck	$2.0x10^6$	$2.6x10^5$	$1.8x10^5$	35	7.08	43	25	100
13	South Africa	neck	$2.9x10^7$	$8.0x10^5$	$3.0x10^5$	23	7.01	56	23	100
14	South Africa	back crotch	$3.4x10^6$	$7.4x10^5$	$8.0x10^5$	27	6.82	70	21	84
15	South Africa	back crotch	$4.4x10^7$	$4.5x10^6$	$1.2x10^6$	29	6.98	43	22	100
16	South Africa	shoulder	$2.7x10^7$	$2.3x10^6$	$4.2x10^6$	29	7.45	54	17	88
17	Russia	leg	$7.1x10^6$	$1.0x10^5$	$3.5x10^5$	29	6.80	45	19	100
18	Russia	leg	$2.3x10^7$	$1.3x10^6$	$2.4x10^6$	29	7.00	35	20	100
19	China	rump	$1.5x10^6$	$1.4x10^5$	$2.0x10^5$	35	7.42	68	19	78
20	China	shoulder	$2.1x10^5$	$2.0x10^4$	$1.1x10^4$	35	7.00	58	21	100
21	China	back crotch	$1.6x10^5$	$1.7x10^4$	$1.7x10^4$	35	6.95	66	20	84
22	France	leg	$1.7x10^7$	$3.4x10^6$	$6.8x10^6$	27	7.00	52	23	100
23	France	neck	$1.4x10^7$	$3.2x10^6$	$1.4x10^6$	27	7.51	46	27	100
24	France	back	$1.1x10^7$	$1.6x10^6$	$2.6x10^6$	27	6.93	35	29	100
25	France	fore crotch	$1.6x10^7$	$2.0x10^6$	$3.0x10^6$	27	6.97	37	27	100

Utilization of Different Amino Acids and Different Carbon Sources

Although L-arginine was used by all sheep and goat isolates, utilization of L-cysteine by both sheep and goat isolates was not common. Most of our test isolates utilized L-glycine, L-alanine, L-tyrosine, L-proline and L-hydroxyproline which are found in the skin structure (Tables III-IV). It has been known that skin contains L-arginine (4.9%), L-cysteine (0.1%), L-glycine (32.9%), L-alanine (10.9%), L-tyrosine (0.3%), L-proline (12.6%) and L-hydroxyproline (9.5%).[38] Utilization of these amino acids by the skin isolates was thought to be related to amino acids found in the skins.

While 7% of 90 moderately halophilic sheep skin species were able to use all amino acids examined, 37% of these species were able to use six different amino acid sources. Five, four, three and two different amino acids were utilized by 16%, 8%, 28% and 4% of the sheep species, respectively. Among 47 salted goat skin species, 45%, 21%, 4% and 30% of the isolates used respectively six, five, four and three different amino acids (Tables III-IV).

Nine, eight, seven, six, five, four, three, two and one of these carbon sources were utilized by 6%, 7%, 4%, 13%, 3%, 17%, 16%, 18%, 9% of the sheep skin isolates, respectively. Eight percent of the sheep skin isolates did not use any carbon sources. While six, five, two different carbon sources were used by 15% of the salted goat skin isolates, 4% of them utilized eight and four different carbon sources. Although nine, three and one different carbon sources were respectively used by 6%, 19%, 17% of the salted goat skin isolates, 4% of the goat skin isolates did not use any carbon sources (Tables III-IV).

These results clearly showed that the test microorganisms have ability to use different amino acids and carbon sources. In the utilization of amino acids by bacteria, carboxyl group is removed from amino acids to produce CO_2 and amine. Then, these amines are used as precursors for the synthesis of other needed molecules. In the utilization of carbon sources, some bacteria produce acidic waste products. The enzymes used in utilization of amino acids are activated by low pH. These enzymes remove acid groups from amino acids and produce alkaline amines, which raise the pH of medium making skin more suitable for growth of bacteria.[32] In other words, catabolism of different amino acids and carbon sources are important in order to survive and growth. These results showed that the isolates on both sheep and goat skins were fairly active in skin deterioration.

Application of Alternating and Direct Electric Currents on the Mixed Culture of Moderately Halophilic Spoilage Bacteria

In this study, 0.5, 1.0, 1.5 and 2.0 Amper of direct and alternating electric currents were separately applied to the mixed culture of moderately halophilic *Chromohalobacter israelensis*, *Chromohalobacter canadensis*, *Halomonas halodenitrificans*, *Staphylococcus nepalensis*, *Halomonas halmophila.* Most of these isolates produced protease and lipase enzymes.[30,31] In addition, these isolates utilized different skin amino acids and different carbon sources (Tables II-III). Hence, we especially examined for extermination of these catabolically active moderately halophilic bacteria causing skin deterioration via direct and alternative electric current treatments. Before experiments, the temperatures of all media were adjusted to 25°C. The temperatures of test media increased during the application of direct and alternating electric currents (Tables V-VI). During electrical applications, most voltage values slightly decreased but temperature values increased slowly. However, pH values did not change. The mixed culture of moderately halophilic bacteria was completely killed by 0.5 A DC in 10 min, 1.0 A DC in 5 min, 1.5 A DC in 3 min, and 2.0 A DC in 1 min. (Table V). In addition to DC treatment, AC treatment was also found fairly effective to kill all moderately halophilic bacteria found in the mixed culture. The mixed culture was killed by 0.5 A AC in 20 min, 1.0 A AC in 15 min, 1.5 A AC in 10 min, 2.0 A AC in 5 min (Table VI).

Extermination of hide microorganisms via direct and alternating electric currents applications has received much attention due to its ease of application, low cost and high efficiency. As it is known that metabolically active microorganisms on salted hides reduces the value of the hide as a raw material for leather manufacturing. Every brine curing raceway contaminates hide with halophilic microorganisms. In our previous study, different species of protease, lipase, both protease and lipase producing extremely halophilic archaea, as well as a mixed population of extremely halophilic archaea isolated from the curing salt were killed by 0.5A direct electric current application in 20 min. The temperature and pH of the test medium increased during treatment. The maximum temperature rise was 9 °C, and the pH increased by 4.[23] In another study, we found that proteolytic and lipolytic hide microorganisms found in the both first and main hide-soak liquors were completely inactivated via 2 A direct electrical current treatment in 50 min.[26] These studies and our experimental results showed that direct and alternating electric current treatments were fairly effective for eradication of skin damaging bacteria in the leather industry.

Table III

Utilization of different amino acids and different carbon sources by 90 moderately halophilic sheep skin isolates.

	B. licheniformis	*C. canadensis*	*C. beijerinckii*	*B. pumilus*	*H. halodenitrificans*	*H. halmophila*	*S. nepalensis*	*S. equorum* subsp. *equorum*	*S. salexigens*	*G. dipsosauri*	*S. costicola* subsp. *alkaliphilus*	*H. zhanjiangensis*	*S. cohnii* subsp. *cohnii*	*H. alkaliphila*	*C. japonicus*	*S. xylosus*	*S. lentus*	*B. tequilensis*	*B. safensis*	*S. roseus*	*H. venusta*	*S. saprophyticus* subsp. *saprophyticus*	*B. siamensis*	*P. rifietoensis*	*A. halophilus*	*M. luteus*	*M. tarijensis*	*O. picturae*	*H. eurihalina*	*C. israelensis*	*I. loihiensis*	Positive isolate numbers
Isolate numbers	8	6	6	5	5	5	4	4	4	4	4	4	3	3	3	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	
Amino acids																																
L-arginine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	90
L-cysteine	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	6
L-glycine	+	+	-	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	-	+	73
L-alanine	-	+	+	+	+	+	-	+	+	+	-	+	-	+	+	+	+	+	+	+	+	-	+	+	+	-	+	-	-	-	+	66
L-tyrosine	+	+	+	+	+	-	+	-	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	-	+	-	-	+	+	+	-	71
L-proline	-	+	+	+	+	-	-	+	+	+	-	+	+	-	+	+	+	-	+	+	-	-	+	-	+	-	-	-	+	+	-	56
L-hydroxyproline	-	+	+	+	+	+	-	+	+	+	-	+	-	-	+	+	-	+	+	+	+	-	+	+	+	-	-	+	-	-	-	60
Carbon sources																																
Dulcitol	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	15
D-sorbitol	+	-	-	-	-	-	+	-	-	-	-	+	-	+	-	-	-	+	-	+	+	-	+	-	+	+	-	-	-	-	-	28
L-rhamnose	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+	+	-	-	-	-	19
Ribose	+	-	+	+	+	-	-	+	-	-	+	-	-	-	+	-	-	+	+	+	+	-	-	+	-	+	-	-	-	+	+	47
Salicin	+	-	+	+	-	-	+	-	-	-	+	-	-	+	-	-	+	-	+	-	-	-	+	-	+	+	-	-	+	-	-	38
Myo-inositol	+	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	+	+	+	-	+	+	-	+	-	-	-	-	-	25
Xylitol	-	-	-	-	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	15
Benzoate	-	-	+	-	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	19
Propionate	+	-	+	-	+	+	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	33
D-melezitose	-	+	+	-	-	+	-	-	-	+	+	-	-	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	33
Butanol	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	16
Propanol	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15
Methanol	-	+	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	+	+	-	19
Formate	-	-	+	-	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	20
Tartrate	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8

Table IV

Utilization of different amino acids and different carbon sources by 47 moderately halophilic goat skin isolates.

	S. saprophyticus subsp. *saprophyticus*	*S.arlettae*	*B. pumilus*	*S.nepalensis*	*G. dipsosauri*	*H.halodenitrificans*	*S. roseus*	*B. licheniformis*	*C. beijerinckii*	*S. xylosus*	*H. eurihalina*	*S. equorum* subsp. *equorum*	*H. zhanjiangensis*	*H. venusta*	*C. canadensis*	Positive isolate numbers
Isolate numbers	7	6	6	5	5	3	3	2	2	2	2	1	1	1	1	
Amino acids																
L-arginine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	47
L-cysteine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
L-glycine	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	45
L-alanine	-	-	+	-	+	+	+	-	+	+	-	+	+	+	+	25
L-tyrosine	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	46
L-proline	-	+	+	-	+	+	+	-	+	+	+	+	+	-	+	32
L-hydroxyproline	-	+	+	-	+	+	+	-	+	+	-	+	+	+	+	31
Carbon sources																
Dulcitol	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	3
D-sorbitol	-	-	-	+	-	-	+	+	-	-	-	-	+	+	-	12
L-rhamnose	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	7
Ribose	-	+	+	-	-	+	+	+	+	-	-	+	-	+	-	24
Salicin	-	-	+	+	-	-	-	+	+	-	+	-	-	-	-	17
Myo-inositol	-	-	-	-	+	-	+	+	-	-	-	-	+	+	-	12
Xylitol	+	-	-	+	-	-	-	-	-	-	-	-	+	+	-	14
Benzoate	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	5
Propionate	-	-	-	-	+	+	-	+	+	-	+	-	-	-	-	14
D-melezitose	-	-	-	-	+	-	-	-	+	-	-	-	-	+	+	9
Butanol	-	-	-	-	+	+	-	-	+	-	+	-	-	-	-	12
Propanol	-	+	-	-	+	+	-	-	+	-	-	-	-	-	-	16
Methanol	-	+	-	-	-	+	-	-	-	-	+	-	-	-	+	12
Formate	-	+	-	-	-	+	-	-	+	-	-	-	-	-	-	11
Tartrate	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	9

Table V

Values of temperatures and voltages and bacterial counts of the mixed culture of moderately halophilic bacteria treated with direct electric current.

DC	0.5A DC			1A DC			1.5A DC			2A DC		
ET[a] (min)	°C	V	CFU/ml	°C	V	CFU/ml	°C	V	CFU/ml	°C	V	CFU/ml
BE[b]	25	3.7	$4.0x10^7$	25	5.0	$1.9x10^7$	25	6.1	$2.8x10^7$	25	7.3	$3.0x10^7$
1	25	3.7	$7.0x10^5$	25	5.0	$1.1x10^5$	26	6.1	$4.0x10^4$	27	7.0	-
3	26	3.6	$3.0x10^4$	26	4.9	$3.0x10^2$	28	5.9	-	29	6.8	-
5	27	3.6	$1.0x10^1$	27	4.8	-	30	5.7	-	31	6.6	-
10	28	3.6	-	29	4.7	-	31	5.4	-	32	5.9	-
15	29	3.6	-	32	4.6	-	33	5.1	-	34	5.5	-
20	30	3.6	-	33	4.5	-	34	4.8	-	35	5.4	-

[a]ET: Electric Treatment, [b]BE: Before Experiment

Table VI

Values of temperatures and voltages and bacterial counts of the mixed culture of moderately halophilic bacteria treated with alternating electric current.

AC	0.5A AC			1A AC			1.5A AC			2A AC		
ET (min)	°C	V	CFU/ml	°C	V	CFU/ml	°C	V	CFU/ml	°C	V	CFU/ml
BE	25	2.8	$6.6x10^7$	25	3.9	$3.1x10^7$	25	5.6	$7.5x10^7$	25	6.8	$5.6x10^7$
1	25	2.8	$1.0x10^7$	25	3.9	$9.0x10^6$	25	5.6	$6.2x10^6$	25	6.8	$1.3x10^5$
3	26	2.8	$2.0x10^6$	26	3.9	$1.3x10^6$	26	5.6	$1.4x10^5$	26	6.8	$5.0x10^3$
5	26	2.8	$2.8x10^5$	26	3.9	$8.0x10^4$	26	5.6	$4.0x10^3$	27	6.8	-
10	27	2.8	$1.2x10^3$	27	3.9	$4.0x10^2$	28	5.6	-	29	6.2	-
15	28	2.8	$7.0x10^1$	28	3.9	-	30	4.8	-	30	6.0	-
20	28	2.8	-	28	3.9	-	32	4.8	-	32	5.7	-

Conclusion

In the present study, moderately halophilic bacteria, proteolytic and lipolytic moderately halophilic bacteria were detected in all sheep and goat isolates belonging Australia, Bulgaria, Dubai, Greece, Israel, Kuwait, South Africa, Turkey, USA, China, France and Russia. The counts of moderately halophilic bacteria, proteolytic and lipolytic moderately halophilic bacteria were high enough to cause the biodegradation of the salted sheep and goat skins. While all salted sheep and goat skin isolates used different amino acids found in the skin, fairly high percentage of these isolates utilized different carbon sources. This study results clearly demonstrated how moderately halophilic microorganisms are active player in skin biodeterioration. Biodegradation of

these skins was also supported by the presence of bad odor, cream and yellow discolorations, sticky apperance of the skin samples examined in this study. The pH, moisture content, ash content and salt saturation of both salted sheep and goat skin samples were appropriate for the growth and metabolic activities of these microorganisms. According to our results, the traditional salt curing method applied to the sheep and goat skins in all countries was not adequate to prevent the growth and catabolic activities of moderately halophilic bacteria, proteolytic and lipolytic moderately halophilic bacteria. Hence, electric current treatment system can be easily designed and used in leather industry to eradicate destructive moderately halophilic bacteria found in hide or skin curing salt.

Acknowledgements

This work was supported by the Scientific Research Project Commission of Marmara University, Project No. FEN C-DRP-040712-0281. We thank Scientific Research Project Commission of Marmara University. We also thank the tanneries in Tuzla and Corlu Leather Organized Industrial Zones, Turkey for helping during collection of skin samples.

References

1. Highberger, H.J.; The Chemical Structure and Macromolecular Organization of the Skin Proteins, The Chemistry and Technology of Leather, Volume 1, Reinhold Publishing Corporation, New York, pp. 65, 1956.
2. Hien, N.T.P.; A Rapid Quantitative Assay for Bacterial Protease Activity, Master Thesis, The Graduate Faculty of Texas Tech University, 2006.
3. Stuart, L.S., Frey, R.W., James, L.H.; Microbiological Studies of Salt in Relation to the Reddening of Salted Hides, Technical Bulletin 383, September, United States Department of Agriculture, Washington, 1933.
4. Kushner, D.J.; Life in High Salt and Solute Concentrations: Halophilic Bacteria, Microbial Life in Extreme Environments. Kushner, D.J. (Ed.), Academic Press, London, pp. 317-368, 1978.
5. Bailey, D.G., Birbir, M.; A Study of the Extremely Halophilic Microorganisms Found on Commercially Brine-cured Cattle Hides. *JALCA* **88**, 285-293, 1993.
6. Ventosa, A., Márquez, M.C., Garabito, M.J., Arahal, D.R.; Moderately Halophilic Gram-positive Bacterial Diversity in Hypersaline Environments. *Extremophiles* **2**, 297-304, 1998.
7. Llyod, D.J.; Red Heat in Salted Hides. *J. Soc. Leather Technol. Chem.* **13**, 538-569, 1929.
8. Kallenberger, W.E.; Halophilic Bacteria in Hide Curing. *Ph.D. Thesis*, Division of Graduate Studies and Research of the University of Cincinati, Department of Basic Science Tanning Research of The Collage of Arts and Science, 1985.
9. Birbir, M.; Examination of Halophilic Bacteria on Salt Cured France and Russian Hides. *J. Turkish Microbiol. Soc.* **1**, 68-73, 1997.
10. Bitlisli, B.O., Karavana, H.A., Basaran, B., Sarı O., Yasa, I., Birbir, M.; The Effect of Conservation Defects on the Suede Quality of Double-face. *JALCA* **99**, 494-501, 2004.
11. Akpolat, C., Ventosa, A., Birbir, M., Sánchez-Porro, C., Caglayan, P.; Molecular Identification of Moderately Halophilic Bacteria and Extremely Halophilic Archaea Isolated from Salted Sheep Skins Containing Red and Yellow Discoloratios. *JALCA* **110**(7), 211-220, 2015.
12. Caglayan, P., Birbir, M., Ventosa, A., Sánchez-Porro, C.; Characterization of Moderately Halophilic Bacteria from the Salt-pack Cured Hides. *J. Soc. Leather Technol.Chem.* **99**(5), 250-254, 2015.
13. Anderson, H.; The Reddening of Salted Hides and Fish. *Appl. Environ. Microbiol.* **2**, 64-69, 1954.
14. Haines, M.B.; Quality Rawstock. *JALCA* **66**, 164-173, 1984.
15. Birbir, M., Ilgaz, A.; Isolation and Identification of Bacteria Adversely Affecting Hide and Leather Quality. *J. Soc. Leather Technol. Chem.* **80**, 147-153, 1996.
16. Bailey, D.G, Birbir, M.; The Impact of Halophilic Organisms on the Grain Quality of Brine Cured Hides. *JALCA* **91**, 47-51, 1996.
17. Vreeland, R.H., Bailey, D.G.; Methods of Using Bile Salt to Inhibit Red Heat in Stored Brine Cured Hides and Skins. Patent Number 5945027, Docket number 22497, Serial Number 8906333, Agricultural Research Service, 1999.
18. Kanagaraj, J., Babu, N.K.C; Alternatives to Salt Curing Techniques: A Review. *J. Sci. Ind. Res.* **61**, 2002, 339-348.
19. Birbir, M., Bailey, D.G.; Controlling the Growth of Extremely Halophilic Bacteria on Brine-cured Cattle Hides. *J. Soc. Leather Technol. Chem.* **84**, 201-204, 2000.
20. Bailey D.G., DiMaio, G.L., Gehring, A.G., Ross, G.D.; Electron Beam Irradiation Preservation of Cattle Hides in a Commercial-scale Demonstration. *JALCA* **96,** 382-392, 2001.
21. Berber, D., Birbir, M., Hacıoğlu, H.; Efficacy Assesment of Bactericide Containing Didecyldimethylammonium Chloride on Bacteria Found in Soak Liquor at Different Exposure Times. *JALCA* **11**, 354-359, 2010.
22. Solaiman, D.K.Y., Ashby, R.D., Birbir, M., Caglayan, P.; Antibacterial Activity of Sophorolipids Produced by *Candida bombicola* on Gram-positive and Gram-negative Bacteria Isolated from Salted Hides. *JALCA* **111**, 358-364, 2016.
23. Birbir, Y., Birbir, M.; Inactivation of Extremely Halophilic Hide-damaging Bacteria via Low-level Direct Electric Current. *J. Electrostat.* **64**, 791-795, 2006.

24. Birbir, Y., Degirmenci, D., Birbir, M.; Direct Electric Current Utilization in Destruction of Extremely Halophilic Bacteria in Salt That is Used in Brine Curing of Hides, *J. Electrost.* **66**, 388-394, 2008.
25. Birbir, Y., Anik, S., Birbir, M., Caglayan, P.; Annihilation of Extremely Halophilic Archaea in Hide Preservation Salt Using Alternating Electric Current. *Johnson Matthey Technol. Rev.* **59**, 109-119, 2015.
26. Birbir, Y., Uğur, G., Birbir, M.; Inactivation of Bacterial Population in Hide-soak Liquors via Direct Electric Current. *J. Electrostat.* **66**, 355-360, 2008.
27. Birbir, Y., Dolek, N., Birbir, M., Caglayan, P.; Synergistic Effect of Direct and Alternating Electric Current Treatments and Bronopol to Inactivate Bacteria Found in Hide Soak Liquors. *JALCA* **108**, 373-385, 2013.
28. Ventosa, A., García, M.T., Kamekura, M., Onishi, H., Ruíz-Berraquero, F.; *Bacillus halophilus* sp. nov., a Moderately Halophilic *Bacillus* Species. *Syst. Appl. Microbiol.* **12**, 162-166, 1989.
29. Sánchez-Porro, C., Martin, S., Mellado, E., Ventosa, A.; Diversity of Moderately Halophilic Bacteria Producing Extracellular Hydrolytic Enzymes. *J. Appl. Microbiol.* **94**, 295-300, 2003.
30. Birbir, M., Ventosa, A., Caglayan, P.; Characterization of Moderately Halophilic Bacteria Found on the Sheep and Goat Skins, The Scientific Research Project Commission of Marmara University, Project number FEN-C-DRP-040712-0281, 2015.
31. Caglayan, P.; Characterization of Moderately Halophilic Bacteria Found on the Sheep and Goat Skins, *Ph.D. Thesis*, Marmara University, Istanbul, Turkey, 2015.
32. Harley, J.P., Prescott, L.M.; Laboratory Exercises in Microbiology, 5th ed., The McGraw-Hill Companies, New York, 2002.
33. Johnson, T.R., Case, C.L.; Laboratory Experiments in Microbiology, 9th ed., Pearson, pp. 118, 2010.
34. Bailey, D.G.; The Preservation of Hides and Skins. *JALCA* **98**, 308-319, 2003.
35. Park, J. C., Lee, M. S., Lee, D. H., Park, B. J., Han, D. W., Uzawa, M., Takatori, K.; Inactivation of Bacteria in Seawater by Low-amperage Electric Current. *Appl. Environ. Microbiol.* **69**(4), 2405-2408, 2003.
36. Kallenberger, W.E., Lollar, R.M.; Rapid Determination of Moisture in Cured Hides by Microwave Oven. *JALCA* **74**, 454, 1979.
37. Oren, A.; Diversity of Halophilic Microorganisms: Environments, Phylogeny, Physiology and Applications. *J. Ind. Microbiol. Biotechnol.* **28**, 56-63, 2002.
38. Szpak, P.; Fish Bone Chemistry and Ultrastructure: Implications for Taphonomy and Stable Isotope Analysis. *J. Archaeol. Sci.* **38**, 3358-3372, 2011.