Examination of Gram-negative Bacteria on Salt- pack Cured Hides

by

E. Aslan[1] And M. Birbir[2]*
[1]*University of Waterloo, Department of Biology, 200 University Avenue West,*
Waterloo, Ontario, Canada N2l 3G1
[2]*Marmara University, Faculty of Science and Letters, Department of Biology*
34722 Goztepe, Istanbul, Turkey

Abstract

Salt-pack curing is the most widely used method to preserve hides. However, inadequately applied salt-pack curing method supports the growth of proteolytic and lipolytic bacterial and archaeal flora which may reduce the quality of leather. Therefore, to examine the efficacy of salt-pack curing method applied to hides and to determine harmful microorganisms of the hides, Gram-negative bacteria on the salt-pack cured hides were isolated and identified and their proteolytic and lipolytic activities were investigated in the present study. Salt-pack cured hides examined were collected from different tanneries in Leather Organized Tannery Region, Tuzla-Istanbul, Turkey and 40% of the hides were imported from abroad. A total of 256 Gram-negative bacterial isolates containing 21 different genera (*Acinetobacter, Aeromonas, Alcaligenes, Burkholderia, Citrobacter, Comamonas, Edwardsiella, Enterobacter, Escherichia, Hafnia, Klebsiella, Mannheimia, Pasteurella, Proteus, Pseudomonas, Salmonella, Serratia, Sphingomonas, Stenotrophomonas, Vibrio* and *Yersinia)* and 46 different bacterial species were isolated and identified from the hide samples. The percentage of proteolytic, lipolytic and both proteolytic and lipolytic Gram-negative isolates on the hides were found as 68%, 52% and 43%, respectively. The most common Gram-negative genera on the salt-pack cured hides were *Enterobacter* (66), *Pseudomonas* (59) and *Vibrio* (32). These isolates showed both proteolytic and lipolytic activities in the highest number on the hides. As a conclusion, the hides contained a wide variety of destructive Gram-negative bacterial species originating from different environmental sources and traditional salt-pack curing method was not sufficient to inactivate these Gram-negative bacteria.

Resumen

Curado por apilamiento con sal es el método más común de preservación de pieles. Sin embargo este método de salazón mal aplicado engendra la actividad bacteriana proteolítica y lipolítica así como por flora halofílica [monocelular muy primitiva], que pueden reducir la calidad del cuero resultante. Es así que para determinar la eficacia de la salazón cuando se aplica a pieles y para identificar micro-organismos dañinos a las pieles, bacterias Gram-negativas en las pilas de pieles ya curadas fueron aisladas e identificadas y sus actividades proteolíticas y lipolíticas fueron investigadas en el presente estudio. Pieles apiladas con sal fueron recolectadas de diferentes tenerías de la región de curtiembres organizadas de Tuzla-Estambul, Turquía y hasta 40% de las pieles fueron de las importadas del extranjero. Un total de 256 cepas bacterianas Gram-negativas fueron aisladas que contenían 21 géneros (*Acinetobacter, Aeromonas, Alcaligenes, Burkholderia, Citrobacter, Comamonas, Edwardsiella, Enterobacter, Escherichia, Hafnia, Klebsiella, Mannheimia, Pasteurella, Proteus, Pseudomonas, Salmonella, Serratia Sphingomonas, Stenotrophomonas, Vibrio,* y *Yersinia)* y 46 especies de bacterias fueron aisladas y identificadas de las pieles muestreadas. El porcentaje de bacterias proteolíticas, lipolíticas y ambas proteoíticas y lipolíticas Gram-negativas aisladas de las pieles, fueron 68%, 52%, y 43%, respectivamente. Los géneros más comunes Gram-negativos en las pieles saladas apiladas fueron *Enterobacter* (66), *Pseudomonas* (59) y *Vibro* (32). Estos aislados demostraron ambos actividades proteolíticas y lipolíticas en un número más grande de pieles. Como conclusión, las pieles presentaron una amplia variedad de destructivas bacterias Gram-negativas originadas de diferentes fuentes medio ambientales y el método tradicional de curado por salazón en pilas no fue lo suficiente efectivo para inhibir la actividad de estas bacterias Gram-negativas.

*Corresponding Author e-mail: mbirbir@marmara.edu.tr
Manuscript Received October 2, 2011, accepted for publication December 17, 2011

INTRODUCTION

Insufficient preserved hides usually harbor a wide variety of microorganisms including Gram-positive and Gram-negative *Bacteria* and *Archaea*.[1-4] Previous studies showed that these microorganisms may cause important quality defects on the leather.[1,3,5] Identification of destructive microorganisms on the salted hides will supply an important clue to prevent bacterial damage on the hides. It is known that Gram-positive and Gram-negative *Bacteria* and *Archaea* have different cell structures. To inactivate these different types of microorganisms, we should know all bacterial populations on the salted hides by isolating and identifying all Gram-positive and Gram-negative *Bacteria* on the hides. In our previous study, we identified all Gram-positive bacterial populations on these hides and in this study we focused on Gram-negative bacterial flora. Gram-negative *Bacteria* comprise the largest group of microorganisms such as cocci, coccobacilli, facultatively anaerobic bacilli and aerobic bacilli that live in a wide variety of habitats.[6] The cell walls of Gram-negative bacteria composed of outer membrane covering a thin layer of peptidoglycan. Outer membrane of Gram-negative bacteria contains Lipid A, core polysaccharide, O-specific polysaccharide, phospholipids and proteins. Lipid A (endotoxin) of Gram-negative bacteria is toxic to animals and causes gas, diarrhea and vomiting in humans. Outer membrane that has structural role may help Gram-negative bacteria to survive better in harsh environments such as high concentration of salt. *Enterobacteriaceae,* also called enteric bacteria, is one large family of Gram-negative bacteria. Although many members of this group are inhabitants of the intestine of humans and animals, they also found in soil, water and decaying matter and vegetation.[7] Enteric bacteria which have industrial importance show pathogenic potential for man, animals, plants and insects.[8] Although some species of *Enterobacteriaceae* are opportunistic pathogens in intestine, the others may cause infections on skin, lung, urinary tract, prostate gland, bone and meninges.[9] Hides may be contaminated by Gram-negative enteric bacteria from feces.[10] The total number of Gram-negative bacteria in animal feces is 10^6 c.f.u./g[11] Some of these bacteria may be pathogenic strains and adhere to epithelial cells. Major adherence factors such as capsule or slime layer, adherence proteins, lipoteichoic acid, flagella and fimbriae may facilitate attachment of microbial pathogens to hide. Moreover, enzymes enhance pathogen colonization and growth on the salted hides.[12,13] After slaughtering and flaying processes of animals, pathogens more readily colonize on hide and begin the invasion and damage process. Especially, pathogens producing protease break down the tissue-supporting collagen network and enable these pathogens to diffuse through hide. Although generation time of bacteria may change according to temperature, composition of the medium, genetic characteristics of the bacteria itself, many bacteria may multiply in 1-3h under the optimum growth conditions.[6] Thus, cell numbers of actively growing bacteria on or in the raw hide may reach to high population in a short period. Oppong and his colleagues isolated 10^8 c.f.u./g of bacteria from fresh hides.[14] When the population density of bacterial species become sufficiently high on the hides, a regulatory mechanism called Quorum sensing is activated by these bacteria.[12,15,16] Quorum sensing can take place both within a single bacterial species and between diverse species. Quorum sensing also allow the bacteria to act as multicellular organisms and help communication of bacteria with the same and other species.[12,15,16] Thus, mix populations of bacteria act synergistically for metabolic and other processes. According to researchers' reports we may conclude that populations of Gram negative *Bacteria* on the salted hides may act synergistically to give damage.

Therefore, Gram-negative bacterial numbers on the hides are very important in point of bacterial defects on leather. Researchers emphasized that bacteria on raw hides are major cause of deterioration and damages of hides and numbers and species of bacteria on hides may play an important role in decomposition of hides. Especially, rich nutrient content of freshly flayed hide offer an ideal medium for bacteria to grow. During the exponential growth, the bacteria produce several enzymes such as proteases and lipases and these enzymes may reduce the quality of leather. Decomposition of hide by bacteria and autolysis is prevented by effectively applied hide curing process. Salt-pack and brine curing methods which dehyrate hides are widely used in the preservation of hides. However, using inadequate concentration of bactericide and salt during hide curing, hide contamination with dirt, blood and bacteria, utilizing contaminate salt for hide preservation, low penetration of the salt due to heavy fat or flesh deposits may cause putrefaction of hides.

Our previous studies verified that presence of high bacterial numbers on the salt-pack cured hides were related with poor preservation method.[4,17,18] The bacteria (10^4-10^8 c.f.u./g) and extremely halophilic archaea (10^3-10^8 c.f.u./g) were observed in considerably high numbers on the 36 salt-pack cured hides. It was found that 97% of the hide samples contained proteolytic (10^2-10^6 c.f.u./g) and lipolytic bacteria (10^3-10^7 c.f.u./g). It was determined that 94% and 81% of the samples had proteolytic (10^2-10^6 c.f.u./g) and lipolytic extremely halophilic archaea (10^2-10^6 c.f.u./g), respectively.[17]

The bacterial activity on the hides is prevented by antibacterial agents or the other inactivation methods but these treatment systems may not kill effectively different genera of bacteria. Cell surface structures, biochemical pathways, the composition of cell walls and cytoplasmic membrane in microorganisms directly influence the efficiencies of antibacterial agents or the other inactivation methods. To solve

deterioration problems on the hide, the effect of commercial bactericides and inactivation methods on the different species of bacteria isolated from salted hides should be examined. Although there are several studies on the non-halophile and halophile microbial population on the salted hides, the efficacy and inactivation power of salt curing process in terms of Gram-negative bacterial flora and their enzymatic activities on the salt-pack cured hides samples have not been examined yet. Hence, this study was designed to determine the species of Gram-negative *Bacteria* found on the salt-pack cured hides, to examine their proteolytic and lipolytic activities and evaluate the efficiency of salt-pack curing methods applied in different countries in this study.

EXPERIMENTAL

In the present study, 10 salt-pack cured hides were obtained from different tanneries in Leather Organized Tannery Region, Tuzla-Istanbul, Turkiye. Four hide samples were imported from England (HS1-2) and Australia (HS3-4). Six hide samples were obtained from different tanneries in Leather Organized Tannery Region, Tuzla-İstanbul, Turkiye (HS5-10). The temperatures of the tanneries where the hide samples were collected were between 14 and 30 °C. Then, these samples were immediately placed into the sterilized bags and containers and they were carried on ice during the transportation. Before the experiments, all of the salt–pack cured hide samples were cleaned thoroughly of hair, fat and dirt. Then, the hide samples were cut into small pieces. Ten gr of each salt-pack cured hide was separately placed in a flask containing 90 ml 0.85% sterilized physiological saline solution. The flasks were placed in a shaking incubator (Edmund Bühler, Germany) for half an hour at 25°C in 100 rpm and later 0.1 ml of direct and serial dilutions (10^{-2}, 10^{-4} and 10^{-6}) of the bacterial suspension were performed for isolation and identification of all of the isolates. All experiments were conducted in duplicate. Positive and negative controls were used in all tests performed.

Isolation and identification of species in the family of *Enterobacteriaceae*

A 0.1 ml aliquot of direct and serial dilutions (10^{-2}, 10^{-4}, 10^{-6}) of the bacterial suspension were spreaded onto the agar plates containing Eosin-Methylene Blue Agar and Blood Agar. After the incubation at 37°C for 48 hours, different colonies were picked up and restreaked several times onto Blood Agar to obtain pure culture. Gram staining of different pure cultures were performed. When Gram-negative rods were detected, oxidase test was applied to this pure cultures. All biochemical tests were accomplished according to earlier described procedures.[19] The API 20E test kit (Biomèrieux, France) was used to identify the isolates belonging to family *Enterobacteriaceae*. The isolates were grown on Eosin-Methylene Blue Agar at 37°C for 24 hours and suspended in sterilized saline solution (0.85 % NaCl) to adjust the density of the bacterial cultures to McFarland No. 0.5 as described in the manufacturers' instructions. The culture dilutions were then loaded to the test strips. These test strips were incubated at 37°C for 24-48 hours. The results of all biochemical tests were read and evaluated after incubation period.[19]

Isolation and identification of *Pseudomonas* species

0.1 ml aliquots of direct and serial dilutions (10^{-2}, 10^{-4}, 10^{-6}) of the bacterial suspension were spreaded onto the agar plates containing Cetrimide Agar (Biomèrieux, France). After the incubation at 37°C for 48 hours, characteristic colonies of the bacteria belonging to genus *Pseudomonas* were picked up and restreaked several times onto Cetrimide Agar to obtain pure culture. Gram staining of different pure cultures were performed. When Gram-negative rods were detected, oxidase test was applied to this pure cultures. Oxidase positive isolates were streaked onto Cetrimide Agar and incubated at 37°C for 24 hours. The API 20NE test kit (Biomèrieux, France) was used to determine the isolates belonging to genus *Pseudomonas*. The isolates were grown on Cetrimide Agar at 37°C for 24 hours and suspended in sterilized saline solution (0.85% NaCl) to adjust the density of the bacterial cultures to McFarland No. 0.5 as described in the manufacturers' instructions. The culture dilutions were then loaded to the test strips. These test strips were incubated at 37°C for 48 hours. The results of all biochemical tests were read and evaluated after incubation period.[20] All biochemical tests were accomplished according to earlier described procedures.[19]

Determination of protease activity

Proteolytic activity of the isolates was screened on gelatine agar medium containing triptone, 10 g; yeast extract, 2.5 g; D-mannit,10 g; K_2HP0_4, 5 g; gelatine, 30 g; ammonium sulfate 75 g; sodium hydroxide (10%), 6 cc; sodium chloride, 5 g; agar 20 g and distilled water, 1000 ml. After the incubation at 37°C for 24-48 hours, clear zones around the colonies were taken as evidence of protease activity.[19]

Determination of lipase activity

Lipolytic activity of the isolates was examined on the agar medium containing peptone, 10 g; sodium chloride, 5 g; $CaCl_2$, 0.1 g; Tween 80, 10 g; agar, 15 g and distilled water, 1000 ml. After the incubation at 37°C for 24-48 hours, clear zones around the colonies were taken as evidence of lipase activity.[19]

RESULTS AND DISCUSSION

The number and frequency of Gram-negative bacteria on the hides were found to be highly variable. Total numbers of different species of Gram-negative bacteria on the hides were between 10 and 19. While the hide sample 3 contained 19

different species of Gram-negative bacteria, the hide sample 2 contained 10 different species of Gram-negative bacteria. In addition, the hide sample 9 contained the highest number of the bacterial isolates (35) (Table I). Overall, 21 genera and 46 species containing of 256 Gram-negative bacteria were isolated and identified from the salt-pack cured hide samples (Table I). *Enterobacter* (66), *Pseudomonas* (59) and *Vibrio* (32) were found as the most common genera of Gram-negative bacteria on the hide samples.

TABLE I
Frequencies of various species of Gram-negative bacteria isolated from the salt-pack cured hides

Hide samples											
Genera and species of Gram-negative bacteria	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Total**
Genus *Acinetobacter*											**16**
Acinetobacter baumannii			1					2			3
Acinetobacter calcoaceticus		1				1					2
Acinetobacter haemolyticus			1								1
Acinetobacter junii ssp. johnsonii			1			1		3			5
Acinetobacter lwoffii								2	2	1	5
Genus *Aeromonas*											**2**
Aeromonas caviae			1								1
Aeromonas hydrophila						1					1
Genus *Alcaligenes*											**3**
Alcaligenes faecalis				1	1	1					3
Genus *Burkholderia*											7
Burkholderia gladioli							3	2	1	1	**7**
Genus *Citrobacter*											**4**
Citrobacter amalonaticus		1									1
Citrobacter ferundii	1		1	1							3
Genus *Comamonas*											**2**
Comamonas testesteroni	1					1					2
Genus *Edwardsiella*											**1**
Edwardsiella tarda			1								1
Genus *Enterobacter*											**66**
Enterobacter aerogenes	1						1	2			4
Enterobacter agglomerans	1		1				1			1	4
Enterobacter amnigenus	1	1	2	2	2	2	1	1			12
Enterobacter cloacae	1	3	1	1	2	3	3	2	4	2	22
Enterobacter gergoviae	1	1	1	1	1		1	3			9
Enterobacter intermedius				1		1		2			4
Enterobacter liquefaciens	1		1				1				3
Enterobacter sakazakii			1	1	1			2	2	1	8
Genus *Escherichia*											**9**
Escherichia coli				2	4		2	1			9
Genus *Hafnia*											**14**

Hafnia alvei			1	3	3	3	2	2			14
Genus *Klebsiella*											**2**
Klebsiella oxytoca		1									1
Klebsiella pneumoniae ssp. ozanae			1								1
Genus *Mannheimia*											**3**
Mannheimia haemolytica									2	1	3
Genus *Pasteurella*											**9**
Pasteurella multocida									2	2	4
Pasteurella pneumotropica									3	2	5
Genus *Proteus*											**1**
Proteus mirabilis			1								1
Genus *Pseudomonas*											**59**
Pseudomonas aeruginosa								1			1
Pseudomonas fluorescens				1		2		2	2	2	9
Pseudomonas luteola	2	2	2	2	2	2	4	2	7	4	29
Pseudomonas maltophila		1	1								2
Pseudomonas paucimobilis				1							1
Pseudomonas pseudoalcaligenes	1										1
Pseudomonas putida	2	2		1	2	1	2	1	2	3	16
Genus *Salmonella*											**3**
Salmonella choleraesuis ssp. arizonae				1							1
Salmonella paratyphi A				1							1
Salmonella typhimurium				1							1
Genus *Serratia*											**1**
Serratia marcescens			1								1
Genus *Sphingomonas*											**5**
Sphingomonas paucimobilis	2	2				1					5
Genus *Stenotrophomonas*											**12**
Stenotrophomonas maltophila					2	5			3	2	12
Genus *Vibrio*											**32**
Vibrio fluvialis			3	4	3	3	3	2	2	7	27
Vibrio vulnificus						2		1		2	5
Genus *Yersinia*											**5**
Yersinia pseudotuberculosis	1										1
Yersinia ruckeri									3	1	4
Total number of Gram-negative isolates	**16**	**15**	**23**	**25**	**23**	**30**	**24**	**33**	**35**	**32**	**256**
Total number of Gram-negative species	**13**	10	19	17	11	16	12	18	13	15	144

Enterobacter was found as the most common genera on the hides in the present study (Table I). *Enterobacter* species are commonly found in humans, animals, water, sewage, vegetables and soil.[21] Some species of this organism may be found on skin, meat and in intestinal canals of humans and animals.[21] *Enterobacter cloacae* and *Enterobacter aerogenes* can cause urinary tract infections.[22] Presence of high number of *Enterobacter* isolates on the salted hides may be related with fecal contamination.

A total of 8 different species of genus *Enterobacter* containing 66 isolates were isolated and identified from the hide samples. It was seen that *Enterobacter cloacae* (22 isolates), *Enterobacter amnigenus* (12 isolates), *Enterobacter gergoviae* (9 isolates) and *Enterobacter sakazakii* (8 isolates) were commonly found species of genus *Enterobacter* on the salted hides examined. Although *Enterobacter intermedius* (4 isolates), *Enterobacter aerogenes* (4 isolates), *Enterobacter liquefaciens* (3 isolates) and *Enterobacter agglomerans* (4 isolates) were isolated from a few hide (3-4) samples, *Enterobacter cloacae, Enterobacter amnigenus, Enterobacter gergoviae* and *Enterobacter sakazakii* isolated from 10, 8, 7 and 6 hide samples, respectively. A total of 7 different species of genus *Pseudomonas* containing 59 isolates were isolated and identified from the hide samples. It has been explained that motile *Pseudomonas* species which have very simple nutritional requirements are commonly found in soil, seawater, fresh water, decaying organic matter, swimming pool, hot tubs, washcloths and contact lens solutions and the other natural environments.[6,12,23] Deterioration of meats, poultry and fish caused by *Pseudomonas* species were reported.[24]

A few number of *Pseudomonas* species are pathogenic to humans and animals and some species are plant pathogen.[23] The most important characteristic of *Pseudomonas* species is to produce an unusually large number of enzymes such as protease, amylase, pectinase and cellulase. [12,22,23] In addition, *Pseudomonas* species are resistant to antibacterial agents, detergents, heavy metals, organic solvents and antibiotics. [6,23] In this study, presence of high number of *Pseudomonas* isolates on the hides may be related with these characteristic features of this genus.

Pseudomonas luteola (10 hides) and *Pseudomonas putida* (9 hides) were commonly found species of genus *Pseudomonas* on the hides, while *Pseudomonas aeruginosa, Pseudomonas paucimobilis* and *Pseudomonas pseudoalcaligenes* were isolated from the only 1 hide sample examined. Although *Vibrio* was found as the third common genus on the hides, only 2 different species of genus *Vibrio* containing 32 isolates were isolated and identified from the hide samples. *Vibrio fluvialis* (8 hide samples) was the most common species on the hides while *Vibrio vulnificus* was only seen on the 3 hide samples. Most species of genus *Vibrio* are found in marine, brackish or freshwater habitats.[12,25] Researchers explained that halophilic *Vibrio fluvialis* is cause of gastroenteritis.[26] It has been reported that *Vibrio vulnificus* may cause blistering skin lesion.[27] According to researchers' reports, it was concluded that the presence of *Vibrio* species on the hides may be related with salt and fecal contamination.

Although species numbers of genera *Acinetobacter* (5 species) and *Hafnia* (1 species) were low, these genera were isolated from the 6 hide samples. It has been explained that species of *Acinetobacter* are common soil, water, sewage, food and skins of patients and sometimes they are found as parasites of animals.[28] Genera *Aeromonas, Alcaligenes, Burkholderia, Citrobacter, Comamonas, Escherichia, Mannheimia, Pasteurella, Sphingomonas* and *Yersinia* were isolated from a few hide samples (2-4 hide samples) and their isolate numbers were low. Additionally, genera *Edwardsiella, Proteus, Salmonella* and *Serratia* were isolated from only 1 hide sample. It was observed that these genera were not commonly found on the salt-pack cured hides.

Pseudomonas luteola (29 isolates), *Vibrio fluvialis* (27 isolates) and *Enterobacter cloacae* (22 isolates) were the commonly found Gram-negative bacterial species on the hides. Although *Enterobacter cloacae* and *Pseudomonas luteola* were isolated from 10 salt-pack cured hide samples, *Vibrio fluvialis* was found at 8 hide samples, respectively. Our results were similar to the previous studies reporting Gram-negative bacterial flora found on the fresh hides. It was mentioned that Gram-negative bacteria such as *Aeromonas hydrophila*, *Aeromonas punctata caviae, Acinetobacter baumannii, Acinetobacter radioresistans, Acinetobacter calcoaceticus, Citrobacter ferundii, Delftia acidovorans, Escherichia coli, Klebsiella pneumonia, Pantoea agglomerans, Proteus mirabilis, Proteus vulgaris, Pseudomonas aeruginosa, Pseudomonas cannabina, Pseudomonas fulgida* and *Shigella boydii* were isolated and identified from the hides.[14,29]

Polkade reported isolation of 13 genera containing 26 species immediately after slaughtering and before processing of raw buffalo hides. Researcher explained that 50% of isolates from buffalo were found to be highly degradative and harmful bacteria which reduce leather quality may come from many sources including the animal skin itself, slaughterhouse and the leather tanning and processing environment.[29] Shede and his colleagues isolated and identified *Acinetobacter, Aeromonas, Escherichia, Proteus, Myroides* and *Weeksella* from raw buffola hides.[30]

In this study, a lot of different species of enteric bacteria were isolated from the hides. Presence of enteric bacteria on the salt-pack cured hides shows contamination of the hides with feces. It was thought that enterics have fimbria which may adhere to hide surface and decompose hide substances. Everett and Cordon explained that the bacteria found on the hides may originate from soil and intestine.[5] Determining enzymatic activities of the bacteria isolated from the salt-pack cured hides will give us important clues on the bacteria causing deterioration of hide. Therefore, proteolytic, lipolytic and both proteolytic and lipolytic activities of the Gram-negative bacteria isolated from the hides were examined in this study.

In our study, the total numbers and percentages of proteolytic, lipolytic and both proteolytic and lipolytic Gram-negative bacteria isolated from the hides were 175 (68.36%), 133(51.95%) and 109 (42.58%), respectively (Tables II-III). The numbers of proteolytic isolates among Gram-negative bacteria isolated from the hide samples were found higher than that of lipolytic and both proteolytic and lipolytic isolates (Table II). Although all isolates of *Enterobacter agglomerans, Enterobacter aerogenes* and *Enterobacter cloacae* showed both protease and lipase activities, all isolates of *Enterobacter gergoviae*, *Enterobacter amnigenus*

and *Enterobacter intermedius* were protease and lipase negative. The number of proteolytic isolates of genus *Enterobacter* were almost found similar to that of lipolytic isolates.

All isolates of *Pseudomonas aeruginosa, Pseudomonas putida, Pseudomonas maltophila* and *Pseudomonas fluorescens* showed both protease and lipase activities while all isolates of *Pseudomonas luteola* were found as protease positive and lipase negative. The number of proteolytic isolates of *Pseudomonas* was found higher than that of the lipolytic isolates (Table II) while the number of the isolates that show both proteolytic and lipolytic activities was found similar to that of the lipolytic isolates. The highest number of proteolytic isolates was detected at *Pseudomonas* species and 98% of the *Pseudomonas* isolates showed proteolytic activity. Accordingly, it is thought that *Pseudomonas* isolates may play an important role in the decomposition of salt-pack cured hides. In our study, all isolates of genera *Aeromonas, Alcaligenes, Proteus, Serratia, Sphingomonas* and *Vibrio* showed proteolytic, lipolytic and both proteolytic and lipolytic activities while genera *Comamonas, Edwardsiella, Escherichia, Hafnia, Mannheimia* and *Pasteurella* did not show any hydrolytic activities (Table II).

The genera of *Aeromonas*, *Acinetobacter*, *Pseudomonas*, *Proteus* and *Salmonella* which were also isolated from our study have been explained as common spoilage bacteria of fresh meat.[12,23,28] We found that the isolates belong to these genera produce hydrolytic enzymes. It is clear that these bacteria on the salt-pack cured hides may cause reduction of hide quality. Proteolytic activity was detected in all isolates of genera *Burkholderia, Salmonella* and *Stenotrophomonas* while lipolytic activity was not detected. Although all isolates of genus *Citrobacter* showed lipolytic activities, proteolytic activity was not detected in the isolates of this genus.

TABLE II
Total numbers of proteolytic, lipolytic, both proteolytic and lipolytic genera of Gram-negative bacteria isolated from the salt-pack cured hides

Genera of Gram-negative Bacteria	Total numbers of isolates	Total numbers of proteolytic isolates	Total numbers of lipolytic isolates	Total numbers of both proteolytic and lipolytic isolates
Acinetobacter	16	10	12	6
Aeromonas	2	2	2	2
Alcaligenes	3	3	3	3
Burkholderia	7	7	0	0
Citrobacter	4	0	4	0
Comamonas	2	0	0	0
Edwardsiella	1	0	0	0
Enterobacter	66	39	40	30
Escherichia	9	0	0	0
Hafnia	14	0	0	0
Klebsiella	2	1	1	1
Mannheimia	3	0	0	0
Pasteurella	9	0	0	0
Proteus	1	1	1	1
Pseudomonas	59	58	28	28
Salmonella	3	3	0	0
Serratia	1	1	1	1
Sphingomonas	5	5	5	5
Stenotrophomonas	12	12	0	0
Vibrio	32	32	32	32
Yersinia	5	1	4	0
Total	**256**	**175**	**133**	**109**

TABLE III
The percentages of proteolytic, lipolytic, both proteolytic and lipolytic genera of Gram-negative bacteria isolated from the salt-pack cured hides

Genera of Gram-negative bacteria	Proteolytic activity (%)	Lipolytic activity (%)	Both proteolytic and lipolytic activities (%)
Acinetobacter	62.5	75	37.5
Aeromonas	100	100	100
Alcaligenes	100	100	100
Burkholderia	100	0	0
Citrobacter	0	100	0
Comamonas	0	0	0
Edwardsiella	0	0	0
Enterobacter	59.1	60.6	45.45
Escherichia coli	0	0	0
Hafnia	0	0	0
Klebsiella	50	50	50
Mannheimia	0	0	0
Pasteurella	0	0	0
Proteus	100	100	100
Pseudomonas	98.30	47.46	47.46
Salmonella	100	0	0
Serratia	100	100	100
Sphingomonas	100	100	100
Stenotrophomonas	100	0	0
Vibrio	100	100	100
Yersinia	20	80	0

The present study results showed that almost half of the Gram-negative isolates had ability to damage to the hides.

Other researchers also examined the proteolytic and lipolytic activities of bacteria found on the hides. Woods and his collegues reported the presence proteolytic bacteria in high numbers on the salted hides collected from South Africa. [31] Polkade stated that protease producing *Acinetobacter*, *Proteus*, *Escherichia* and *Bacillus* are the dominant genera whereas major genera showing lipolytic activity were *Stenotrophomonas, Bacillus* and *Acinetobacter* on the raw buffalo hides.[29] Researcher proved that *Acinetobacter sp*. removed epidermis layer leaving behind few patches adhered to corium and emphasized that species of genera *Acinetobacter*, *Proteus* and *Pseudomonas* were responsible for hide degradation process.

CONCLUSION

This studies results proved that Gram-negative bacterial population on the salt pack cured hides may change from hide to hide. It was found that the salt-pack cured hides contained a wide variety of Gram-negative bacteria and presence of these bacteria on the hides was thought related with feces and external sources. The numbers of proteolytic, lipolytic and both proteolytic and lipolytic Gram-negative isolates on the hides were enough to give damage to the hides. This and our previous studies proved that salt curing method applied in the different countries was not enough to prevent bacterial activity on the hides. To reduce bacterial numbers on animal hide, the live animal should be cleaned effectively with water and antiseptics just before slaugthering, temperature of hides should be reduced after flaying and effective bactericides,

boric acid and salt should be applied together to the hides. Due to presence of extremely halophilic archaea in curing salt, the salt should be treated with electric current to inactivate the halophilic archaea.[32,33] The efficiency of bactericides which will be used in hide preservation should be tested on the Gram-positive and Gram-negative bacteria isolated from the hides. After curing process, the hides should be stored in cold and dry rooms in the tannery. When bacterial activity was detected on the hides during storage, effective bactericides should be applied to the hides again. In addition, hide stores should be cleaned with bleach to reduce contamination.

ACKNOWLEDGEMENT

This work was supported by the Scientific Research Project Commission of Marmara University, Project No. FEN-C-YLP-030408-0080. We thank the Scientific Research Project Commission, Mrs.Pinar Yilmaz-Caglayan, Mr.Can Akpolat and the tanneries in Leather Organized Tannery Region, Tuzla-Istanbul for valuable support,technical assistance and providing salt-pack cured hide samples for testing.

REFERENCES

1. Dahl, S.; Prevention of Microbiological Deterioration of Leather. *JALCA* **51,** 103-117, 1956.
2. Kallenberger, E.W.; Halophilic Bacteria in Hide Curing. Division of Graduate Studies and Research of the University of Cincinati, *Ph.D. Thesis*, Department of Basic Science Tanning Research of The Collage of Arts and Science, 1-79, 1985.
3. Bailey, D.G. and Birbir, M.; A Study of the Extremely Halophilic Microorganisms Found on Commercially Brine-Cured Cattle Hides. *JALCA* **88**, 285-293, 1993.
4. Birbir, M. and Ilgaz, A.; Isolation and Identification of Bacteria Adversely Affecting Hide and Leather Quality. *JSLTC* **80,** 147-153, 1996.
5. Everett, A.L. and Cordon, T.C.; Resistance of Purified Collagen to Degradation by Salt Tolerant Bacteria in Pure Culture. *JALCA* **51** (2), 59–66, 1956.
6. Bauman, R.W.;Microbiology with Diseases by Taxonomy, 3th Edition, Pearson Education, Benjamin Cummings, 2011.
7. Talaro, K.P. and Talaro, A.; Foundations in Microbiology, 4th Ed., The McGraw Hill Companies, pp.58-120, 2002.
8. Brenner, D.J. and Farmer III, J.J.; Order XIII *Enterobacteriales*, *In* Garrity, G.M., Brenner,D. J., Krieg, N.R.and Staley, J.T.(Eds.), Bergey's Manual of Systematic Bacteriology, 2nd Ed., Vol. 2, Proteobacteria, Part C, The Alfa-, Beta-, Delta-,and Epsilon proteobacteria, Part B The G*ammaproteobacteria,* Springer, pp.587-849, 2005.
9. Farmer III, J.J. and Kelly, M.T.; *Enterobacteriaceae,* Chapter 36, *In* Balows, A., Hausler, W.J., Herrmann, K.L., Isenberg, H.D., Shadomy, H.J. (Eds.), Manual of Clinical Microbiology 5th Ed. American Society for Microbiology, Washington, D.C., pp. 360-383, 1991.
10. Elder, R.O., Keen, J.E., Siragusa, G.R., Barkocy-Gallagher, G.A., Koohmaraie, M.; Correlation of Enterohemorrhagic *Escherichia coli* 0157 Prevalence in Feces, Hides, and Carcasses of Beef Cattle during Processing. *J APPL BIOL SCI* **97** (7), 2999-3003, 2000.
11. Sawant, A. A., Hegde, N.V., Straley, B. A., Donaldson, S.C., Love, B.C., Knabel, S.J., Jayarao, B.M.; Antimicrobial-Resistant Enteric Bacteria from Dairy Cattle. *Appl. Environ. Microb.* **73**, 156-163, 2006.
12. Madigan, M.T., Martinko, J.M., Dunlop, P.V. and Clarck, D.P.; Brock Biology of Microorganisms, 13th Ed., Pearson Education, Benjamin Cummings, 2012.
13. Finlay, B.B. and Falkow, S.; Common Themes in Microbial Pathogenicity Revisited. MICROBIOL MOL BIOL R, *sic*136–169, 1997.
14. Oppong, D., Bryant, S., Rangarajan, R., Steele, S., Radwell, D. and Hyllengren, L.; Application of Molecular Techniques to Identify Bacteria Isolated from the Leather Industry. *JALCA* **101**, 140-145, 2006.
15. Whitehead, N.A., Barnard, A.M.L., Slater, H., Simpson, N.J.L., Salmond, G.P.C.; Quorum-Sensing in Gram-negative Bacteria, *Fems. Microbiol. Rev.* **25**, 365-404, 2001.
16. Bassler, B.L. and Miller, W.B., Quorum Sensing, *In* Dworkin, M., Rosenberg, S., Schleifer, K.H., Stackebrant, E.(Eds.), The Prokaryotes, 3rd Ed.A Handbook on the Biology of Bacteria:Ecophysiology and Biochemistry.,Vol.2, Springer, pp.336-353, 2006.
17. Berber, D. and Birbir M.; Examination of Bacterial Populations in Salt, Salted Hides, Soaked Hides and Soak Liquors. *JALCA* **105** (10), 320-326, 2010.
18. Aslan, E. and Birbir, M.; Examination of Efficiency and Sufficiency of Salt-Pack Curing Method. *JSLTC* **95** (3), 98-103, 2011.
19. Harley, J.P. and Prescott, L.M.; Laboratory Exercises in Microbiology, 5th ed., The McGraw-Hill Companies, New York, 2002.
20. Palleroni, N.J.; Family I. *Pseudomonadaceae*, *In* Krieg, N.R. and Holt J.G.(Eds.), Bergey's Manual of Systematic Bacteriology, Vol. 2, Williams &Wilkins, Baltimore, pp.323-328, 1984.
21. Grimont, F. and Grimont, P.A.D.; The Genus *Enterobacter*, *In* Dworkin, M., Rosenberg, S., Schleifer, K.H., Stackebrant, E. (Eds.), The Prokaryotes, 3rd Ed. A Handbook on the Biology of Bacteria, Vol.6, Proteobacteria: Gamma Subclass, Springer, pp.197-214, 2006.
22. Tortora, G.J., Funke, B.R. and Case, C.L.; Microbiology: An Introduction, 10th Edition, Pearson Education, Benjamin Cummings, 2009.

23. Moore, E. R.B.,Tindall, B.J., Santos, V.A.P.M.D, Pieper, D.H., Ramos, J.L., Palleroni, N.J.; Nonmedical: *Pseudomonas, In* Dworkin, M., Rosenberg, S., Schleifer, K.H.,Stackebrant, E.(Eds.), The Prokaryotes, 3rd Ed. A Handbook on the Biology of Bacteria, Vol.6, Proteobacteria: Gamma Subclass, Springer, pp.646–703, 2006.
24. Barrett, E. L., Solanes, R. E., Tang, J. S. and Palleroni, N. J.; *P. fluorescens* biovarf V: Its Resolution into Distinct Component Groups and the Relationship of These Groups to Other *P. fluorescens* biovars, to *P. putida*, and to Psychrotrophic *Pseudomonads* Associated with Food Spoilage. *J. Gen. Microbio.* **60**, 2709–2721, 1986.
25. Farmer III, J.J.,The Family *Vibrionaceae., In* Dworkin, M., Rosenberg, S., Schleifer, K.H., Stackebrant, E.(Eds.), The Prokaryotes, 3rd Ed. A Handbook on the Biology of Bacteria, Vol.6, Proteobacteria: Gamma Subclass, Springer, pp. 495–507, 2006.
26. Lee, J.Y., Park, J.S., Oh, S.H., Kim, H.R., Lee, J.N., Shin, J.H.; Acute Infectious Peritonitis Caused by *Vibrio fluvialis*. *Diag. J. Micr. Infec. Dis.,* **62**, 216–218, 2008.
27. Oliver, J.D. and Kaper, J.; *Vibrio vulnificus. In* Oceans and Health: Pathogens in the Marine Environment. Belken, S.S., Colwell, R.R,, (Eds.), 2nd Ed., Springer, 2005.
28. Towner, K.; The Genus *Acinetobacter, In* Dworkin, M., Rosenberg, S., Schleifer, K.H., Stackebrant, E. (Eds.), The Prokaryotes, 3rd Ed. A Handbook on the Biology of Bacteria, Vol.6, Proteobacteria: Gamma Subclass, Springer, pp.746-758, 2006.
29. Polkade, A.V.; Molecular and Phylogenetic Studies on Microbial Diversity of Raw Buffalo Hide to be Used in Leather Manufacturing. *Doctorate Thesis*, University of Pune, Faculty of Science, India, Pune 104, 2007.
30. Shede, P.N., Kanekar, P.P., Polkade, A.V., Dhakephalkar, P.K. and Sarnaik, S.S.; Bacterial Succession On Raw Buffalo Hide and Their Degradative Activities During Ambient Storage. *Int. Biodrterior. Biodegrad.,* **62**, 65-74, 2008.
31. Woods, D.R., Welton, R.L, Thomson, J.A. and Cooper, D.R.; Collagenolytic Activity of Cured Hide Bacteria. *J. Appl. Bact.* **35**, 123–128, 1972.
32. Birbir, Y. and Birbir, M.; Inactivation of Extremely Halophilic Hide-Damaging Bacteria via Low-Level Direct Electric Current. *J. Electrostat.,* **64**, 791-795, 2006.
33. Birbir, Y., Degirmenci, D. and Birbir, M.; Direct Electric Current Utilization in Destruction of Extremely Halophilic Bacteria in Salt That is Used in Brine Curing of Hides. *J. Electrostat.,* **92** (2) **53-58,** 2008.