An Examination of Inactivation Efficacy of NaCl and Boric Acid on Bacteria Isolated from Salted Hides

by

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Abstract

Due to isolation of bacteria in high number on the salt-pack cured hides in our recent studies, the inactivation efficacy of different concentrations of NaCl and Boric acid, used as curing agents, on Gram-positive (*Staphylococcus epidermidis* and *Enterococcus faecium*), Gram-positive endospore forming bacteria (*Bacillus pumilus* and *Bacillus licheniformis*), Gram-negative bacteria (*Pseudomonas aeruginosa* and *Pseudomonas fluorescens*) and the mixed culture of these isolates was investigated. These proteolytic and lipolytic bacteria were isolated from the salted hides. Inactivation efficacy of NaCl and Boric acid on the bacteria was examined in Nutrient Broth and Nutrient Agar media containing 5%, 10%, 15%, 20%, 25%, 30% NaCl; 1%, 2%, 3%, 4%, 5%, 7% Boric acid; and both 5%, 10%, 15% NaCl and 1% Boric acid. In addition, the test bacteria were inoculated in Nutrient Broth containing 20%, 25%, 30% NaCl and 2%, 3%, 4%, 5%, 7% Boric acid and incubated for 24 hours at 35°C. Afterwards, viable bacterial cell counts were determined on NaCl-free Nutrient Agar and boric acid-free Nutrient Agar. The data obtained from this study showed that bacterial growth was prevented by high concentrations of NaCl and boric acid, but the bacteria were still alive and complete destruction of the test bacteria was not accomplished. When NaCl and Boric acid were removed from the hides with the first soaking process, bacteria on the hides may damage the hides during a long main soaking process. As a conclusion, more effective antibacterial treatments should be applied to fresh hides to completely destroy proteolytic and lipolytic bacteria on hides.

Resumen

Debido al aislamiento de un alto número de bacterias en un paquete de pieles conservadas con sal en nuestros recientes estudios, la eficacia de la inactivación de diferentes concentraciones de NaCL y ácido bórico, utilizados como agentes de curado, sobre bacterias Gram-positivas (Staphylococcus epidermidis y Enterococcus faecium), Gram-positivas bacterias formadoras de endosporas (Bacillus pumilus y Bacillus licheniformis), bacterias Gram-negativas (Pseudomonas aeruginosa y Pseudomonas fluorescens) y el cultivo mixto de estas cepas fue investigada. Estas bacterias proteolíticas y lipolíticas fueron aisladas de las pieles saladas. La eficacia de inactivación de ácido bórico y NaCl sobre las bacterias fueron examinadas en caldo nutriente y nutrientes de agar conteniendo 5%, 10%, 15%, 20%, 25%, 30% NaCl; 1%, 2%, 3%, 4% , 5%, 7% de ácido bórico, y ambos juntos 5%, 10%, 15% de NaCl y 1% de ácido bórico. Además, las bacterias de prueba se inocularon en caldo nutriente conteniendo 20%, 25%, 30% de NaCl y 2%, 3%, 4%, 5%, 7% de ácido bórico y se incubaron durante 24 horas a 35°C. Luego, el recuento de células bacterianas viables fueron determinadas en NaCl libre de nutriente de agar y ácido bórico libre de nutriente de agar. Los datos obtenidos de este estudio mostraron que el crecimiento bacteriano fue impedido por las altas concentraciones de ácido bórico y NaCl, pero las bacterias continúan vivas y la destrucción completa de las bacterias de prueba no se ha obtenido. Cuando NaCl y ácido bórico fueron retirados de las pieles con el primer proceso de remojo, las bacterias pueden dañar las pieles durante el largo proceso del remojo principal. Como conclusión, los tratamientos antibacterianos más eficaces deben aplicarse a los cueros frescos para destruir completamente las bacterias proteolíticas y lipolíticas de las pieles.

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Introduction

The most important problem in the leather industry is poor quality, which results from inadequately applied preservation methods. If the hides are not preserved effectively, bacterial populations on the hides may proliferate readily and damage the hides. Bacterial populations on hides originate from the animal itself or the animal's preslaughter environments and may be related with water, manure, soil and feed. In addition to normal bacterial flora on hides, some bacteria can easily be transferred to hide from the gastrointestinal and respiratory tracks of the animal.[1,2] Once bacterial contamination has occurred on the hides, bacterial attack and damage to the hides is inevitable. Hides containing high moisture, protein and fats offer ideal environments for bacterial growth, resulting in an explosive increase in bacterial population. Subsequently, bacteria produce enzymes that digest proteins and fats on the hides. Furthermore, some of the bacteria may penetrate the inner part of hide and damage its collagen fibers. Thus, hides must be adequately preserved after slaughtering to control bacterial damage. Some researchers have recommended that washing with sanitizer, organic acid solutions, chlorinated water or hot water might effectively reduce bacterial contamination on the hides.[2,3] Moreover, different applications such as potassium chloride,[4] boric acid,[5] electron beam irradiation[6] and electric current treatment[7] have been suggested by researchers to control bacterial growth on hides. The most common method of hide preservation is based on the reduction of moisture content in hides, and therefore NaCl curing is routinely applied in leather industry. Also, salt curing greatly reduces water activity and prevents the bacterial growth.[8] Boric acid is usually applied together with NaCl for hide preservation. NaCl and Boric acid absorb water from hide to control bacterial activity on hides.[5] Despite curing of hides with NaCl and Boric acid, proteolytic and lipolytic bacteria and extremely halophilic archaea (10^5-10^6 CFU/g) were isolated in considerably high numbers from the hides.[9] In our previous studies, 10^4-10^8 CFU/g of bacteria were found on the cured hides treated with NaCl and Boric acid.[10]

A total of 396 Gram-positive bacteria comprising 12 different genera and 47 bacterial species were isolated and identified from the salted hides cured with NaCl and Boric acid. The percentage of Gram-positive proteolytic bacteria, lipolytic bacteria, and Gram-positive bacteria that have both proteolytic and lipolytic activities on these hides were 70%, 69%, and 57%, respectively.[11] A total of 256 Gram-negative bacterial isolates containing 21 different genera and 46 different bacterial species were isolated and identified from these hides. The percentage of Gram-negative proteolytic bacteria, lipolytic bacteria, and both proteolytic and lipolytic isolates on the hides were 68%, 52%, and 43%, respectively.[12] These studies showed that the curing methods which were commonly applied to the hides did not completely destroy bacteria on these hides. In addition, it was observed that inadequately preserved hides had bad odor, hair slip, sticky layer on the hide surface, purple and yellow discolorations because of bacterial growth.

Due to the presence of bacteria in high numbers on the hides cured with NaCl and Boric acid in our previous studies, the inactivation efficacy of different concentrations of NaCl and Boric acid as curing agents on *Staphylococcus epidermidis, Enterococcus faecium*, *Bacillus pumilus, Bacillus licheniformis, Pseudomonas aeruginosa, Pseudomonas fluorescens* and mixed culture of these bacteria was examined both separately and together. The bacteria used in this study were isolated from the salted hides cured in different countries such as Australia, England and Turkey.

Experimental

Test Bacteria

*Pseudomonas aeuroginosa (*API 20 NE)*, Pseudomonas fluorescens (*API 20 NE)*, Bacillus pumilus* (API® 50CH)*, Bacillus licheniformis* (API® 50CH)*, Staphylococcus epidermidis* (API Staph) and *Enterococcus faecium* (API 20 Strep) were isolated from the salted hides and identified by the API test kits (BioMériux, France) in the previous studies. These isolates were used as test isolates. The test isolates belong to the most common genera on the salted hides in our previous study. [10,11]

Chemicals

The commercial form of sodium chloride (Merck, Germany) and Boric acid (Tekkim, Turkiye) applied in leather industry were used in this study.

0.5 Mc Farland Standard

The basic 0.5 Mc Farland standard was prepared with 0.048 M $BaCl_2$ (0.05mL) and 0.18M H_2SO_4 (9.95 mL).[13]

Determination of Protease Activity

Proteolytic activity of the isolates was screened on Tryptic Soy Agar medium containing 15 g peptone from casein, 5 g peptone from soymeal, 5 g sodium chloride, 40 g gelatine**,** 15 g agar and 1000 mL distilled water. After the incubation at 37°C for 48 hours, the plates were flooded with saturated ammonium sulphate solution. Clear zones around the colonies were taken as evidence of positive protease activity.[14]

Determination of Lipase Activity

Lipolytic activity of the isolates was screened on Tween 80 Agar medium containing 10 g peptone, 5 g sodium chloride, 0.1 g calcium chloride, 10 g Tween 80, 20 g agar and 1000 mL distilled water. After the incubation at 37°C for 48 hours, opaque zones around the colonies were taken as evidence of positive lipase activity.[15]

Examination of Growth of the Test Isolates and Mixed Culture of These Isolates in Test Media Containing Different NaCl Concentrations

Each test isolate was adjusted to McFarland 0.5 (10^8 CFU/mL). In addition, the mixed culture of these isolates was prepared. To determine growth of isolates and the mixed culture in Nutrient Broth (NB) media containing different NaCl concentrations, 0.5 mL of Nutrient Broth (NB) media containing 5%, 10%, 15%, 20%, 25% and 30% NaCl were prepared. Afterwards, 0.5 mL of each test isolate, and 0.5 mL of the mixed culture, all adjusted to McFarland 0.5, were separately added into these test tubes and incubated for 24 hours at 35°C. After the incubation, the tubes were controlled for turbidity in the NB media. In addition, to determine reduction factors of each isolate and the mixed culture in the NB media containing different NaCl concentrations after the incubation period, serial dilutions (10^{-2}, 10^{-4} and 10^{-6}) of each test isolate and the mixed cultures were prepared. Then, 100 μL aliquots of the direct and serial dilutions of the bacterial suspensions were spread over NA plates containing different NaCl concentrations. The plates were incubated at 35°C for 24 hours. At the end of the incubation period, the colonies on NA were counted and the number of viable cells in 1 mL was calculated.[15] The results of these experiments were evaluated according to two common measures of bacterial cell growth: turbidity (visible growth) in NB and viable bacterial cell counts (colonies) on NA. The $\log_{10}$-reduction factors (RF) for each test isolate and the mixed culture were determined according to the following formula:

$$RF = \log_{10}n_b - \log_{10}n_a$$

where n_b is the initial number of viable cells (CFU) in NB before the experiment, and n_a is the number of viable cells (CFU) in NB after the incubation period.

Examination of Growth of the Test Isolates and Mixed Culture in the Test Media Containing Different Boric Acid Concentrations

Each test isolate was adjusted to McFarland 0.5 (10^8 CFU/mL). In addition, the mixed culture of these isolates was prepared. To determine growth of isolates and the mixed culture in Nutrient Broth (NB) media containing different Boric acid concentrations, 0.5 mL of Nutrient Broth (NB) media containing 1%, 2%, 3%, 4%, 5% and 7% Boric acid were prepared. Afterwards, 0.5 mL of each test isolate, and 0.5 mL of the mixed culture, all adjusted to McFarland 0.5, were separately added into these test tubes and incubated for 24 hours at 35°C. After the incubation, the tubes were controlled for turbidity in the NB media. In addition, to determine reduction factors of the isolates and the mixed culture in the NB media containing different Boric acid concentrations after the incubation period, serial dilutions (10^{-2}, 10^{-4} and 10^{-6}) of each test isolate and the mixed cultures were prepared. Then, 100 μL aliquots of the direct and serial dilutions of the bacterial suspensions were spread over NA plates containing different Boric acid concentrations. The plates were incubated at 35°C for 24 hours. At the end of the incubation period, the colonies on NA were counted and the number of viable cells in 1 mL was calculated.[15] The results of these experiments were evaluated according to two common measures of bacterial cell growth: turbidity (visible growth) in NB and viable bacterial cell counts (colonies) on NA. The $\log_{10}$-reduction factors (RF) for each test isolate and the mixed culture were determined according to the formula as described.

Examination of Growth of the Test Isolates and Mixed Culture in the Test Media Containing 1% Boric Acid Containing 5%, 10% and 15% NaCl

NB media, each containing 1% Boric acid combined with different NaCl concentrations (5%, 10% and 15%), in which the growth of the test isolates was detected, were separately prepared. Then, 0.5 mL of each test isolate and their mixed culture, all adjusted to McFarland 0.5, were separately inoculated into the NB media containing 1% Boric acid combined with different NaCl concentrations (5%, 10% and 15%) and incubated at 35°C for 24 hours. After the incubation, the tubes were controlled for turbidity in the NB media. In addition, to determine reduction factors of the isolates and the mixed culture in the NB media after the incubation period, serial dilutions (10^{-2}, 10^{-4} and 10^{-6}) of each test isolate and the mixed culture were prepared. Afterwards, 100 μL aliquots of the direct and serial dilutions of the bacterial suspensions were spread over NA plates containing 1% Boric acid combined with different NaCl concentrations (5%, 10% and 15%) and incubated at 35°C for 24 hours. At the end of the incubation period, the colonies on the plate surfaces were counted, and viable bacterial cell counts in 1 mL were calculated.[15] The $\log_{10}$-reduction factors (RF) for each test isolate and the mixed culture were determined.

Determination of Viable Bacterial Cell Counts in NB Media Containing 20%, 25% and 30% NaCl

Aliquots of 0.5 mL of NB media containing 20%, 25% and 30% NaCl concentrations, that show no visible growth (turbidity), were separately prepared. 0.5 mL of the test isolates and their mixed culture, all adjusted to McFarland 0.5, were placed into these NB media and incubated at 35°C for 24 hours. To determine inactivation effect of 20%, 25% and 30% NaCl concentrations on the test isolates and their mixed culture after the incubation period, serial dilutions (10^{-2}, 10^{-4} and 10^{-6}) of each test isolate and the mixed culture were prepared. Then, 100 μL aliquots of the direct and serial dilutions of the bacterial suspensions were spread over NaCl-free NA plates and incubated at 35°C for 24 hours. At the end of the incubation period, the colonies on the plate surfaces were counted, and viable bacterial cell counts in 1 mL were calculated.[15] The $\log_{10}$-reduction factors (RF) for each test bacteria and the mixed culture were determined.

Determination of Viable Bacterial Cell Counts in NB Media Containing 2%, 3%, 4%, 5%, and 7% Boric Acid

NB media containing 2%, 3%, 4%, 5% and 7% Boric acid concentrations, that show no visible growth (turbidity), were separately prepared. 0.5 mL of the test isolates and their mixed culture adjusted to McFarland 0.5 were placed into these NB media and incubated at 35°C for 24 hours. To determine inactivation effect of 2%, 3%, 4%, 5% and 7% Boric acid concentrations on the test isolates and their mixed culture after the incubation period, serial dilutions (10^{-2}, 10^{-4} and 10^{-6}) of each test isolate and the mixed culture were prepared. Then, 100 µL aliquots of the direct and serial dilutions of the bacterial suspensions were spread over Boric acid-free NA plates and incubated at 35°C for 24 hours. At the end of the incubation period, the colonies on the plate surfaces were counted, and viable bacterial cell counts in 1 mL were calculated.[15] The $\log_{10}$-reduction factors (RF) for each test bacteria and the mixed culture were determined.

RESULTS AND DISCUSSION

All isolates used in the present study showed both proteolytic and lipolytic activities as reported by other investigators. The proteolytic and lipolytic activities of *Bacillus licheniformis, Bacillus pumilus* and *Pseudomonas species* isolated from different sources were also reported by researchers.[16-18] It is well known that proteolytic and lipolytic bacteria play an important role in the damage of hides.[1,19,20] When the growth of all isolates and the mixed culture was examined at different NaCl concentrations, it was observed that all bacteria showed turbidity in NB and formed colonies on NA containing 5%-10% NaCl. Although *Pseudomonas fluorescens*, *Bacillus licheniformis*, *Bacillus pumilus* and *Staphylococcus epidermidis* and the mixed culture showed turbidity in NB and formed colonies on NA containing 15% NaCl, *Pseudomonas aeruginosa* and *Enterococcus faecium* did not. Neither turbidity caused by bacteria in NB nor colonies caused by bacteria on NA were observed in the media containing 20, 25 and 30% NaCl. After the incubation period of the bacteria in NB, when the isolates and the mixed culture were grown on NA containing 5% NaCl, insignificant log reduction factors in the viable bacterial cell counts were observed (Table I). While log reduction factors of *Bacillus licheniformis*, *Bacillus pumilus*, *Staphylococcus epidermidis, Enterococcus faecium*, and the mixed culture were found to be low (0.9-2.4 log RF) on NA containing 10% NaCl, log reduction factors of *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* were high (5.3-6.5 log RF). When the bacteria were grown on NA containing 15% NaCl, a significant decrease in the viable bacterial cell counts was observed. Especially, *Pseudomonas aeruginosa* and *Enterococcus faecium* did not form colonies at NA containing 15% NaCl (Table I). All isolates and the mixed culture did not form colonies on NA in the presence of 20% or higher NaCl concentrations (Table I). These results do not mean that all test bacteria were completely killed by NaCl. It was observed that some bacteria remained alive (Table IV).

TABLE I
Log Reduction factors (RF) of each test isolate and the mixed culture incubated for 24 h in NB media and then spread over NA media containing different NaCl concentrations.

Test Isolates	NaCl Concentrations						
	Log_{10}*	**5%**	**10%**	**15%**	**20%**	**25%**	**30%**
Pseudomonas aeruginosa ($4x10^8$ CFU/mL)	8.6	0.1	6.5	**8.6**	**8.6**	**8.6**	**8.6**
Pseudomonas fluorescens ($1x10^8$ CFU/mL)	8.0	0.1	5.3	6.7	**8.0**	**8.0**	**8.0**
Bacillus licheniformis ($2x10^8$ CFU/mL)	8.3	0.1	0.9	7.0	**8.3**	**8.3**	**8.3**
Bacillus pumilus ($4x10^8$ CFU/mL)	8.6	0.1	1.2	6.9	**8.6**	**8.6**	**8.6**
Staphylococcus epidermidis ($3x10^8$ CFU/mL)	8.5	0.04	2.2	8.2	**8.5**	**8.5**	**8.5**
Enterococcus faecium ($2x10^8$ CFU/mL)	8.3	0.1	2.2	**8.3**	**8.3**	**8.3**	**8.3**
Mixed culture ($3x10^8$ CFU/mL)	8.5	0.2	2.4	7.7	**8.5**	8.5	8.5

*Log_{10} values of the test bacteria before the experiment

Similar results for NaCl tolerance of bacteria were reported by other researchers. Lawrence and colleagues[21] found that some species of *Staphylococcus* grew at high NaCl concentrations. Researchers stated that the species of the genus *Bacillus* could survive at high-salt concentrations (7%, 10%, and 12% NaCl), and reported the growth of *Bacillus licheniformis* at 7% and 10% NaCl.[22] In the present study, we observed that *Bacillus licheniformis* slightly grew in a medium containing 15% NaCl (Table I).

Another study reported that *Pseudomonas* species may be salt-tolerant. Some species of the genus *Pseudomonas* are able to grow in media containing 18% NaCl.[23] According to the result of our study, *Pseudomonas fluorescens* was able to grow in a medium containing 15% NaCl, and *Pseudomonas aeruginosa* could grow in a medium containing 10% NaCl (Table I). Ahmad and his colleagues[24] found that *Enterococcus faecium* and *Enterococcus faecalis* could grow in Brine Heart Broth containing 6.5% NaCl. The growth of *Enterococcus faecium* in 5% and 10% NaCl was detected in our study (Table I). Aslan and Birbir[10,11] stated that *Pseudomonas aeruginosa, Pseudomonas fluorescens, Bacillus pumilus*, *Bacillus licheniformis*, *Enterococcus faecium*, and *Staphylococcus epidermidis* were isolated from one, five, nine, nine, ten, and one, respectively, out of ten hide samples obtained from Australia, England, and Turkey. Researchers demonstrated that *Staphylococcus aureus* can grow in a medium containing 15% NaCl.[25] In the present study, *Staphylococcus epidermidis* isolated from salted hides grew in the medium containing 15% NaCl, but the viable bacterial cell counts were extremely low (Table I).

Woods and Atkinson[26] pointed out that the high concentration of NaCl reduces the number of bacteria. However, large numbers of bacteria were isolated from the salted hides by the researchers. Our research results were similar to the findings of Woods and Atkinson.[26] In our study, the reductions in the viable bacterial cell counts were observed as the salt concentrations were increased in the media, but the bacteria were not completely killed (Table IV). When inactivation efficacy of different concentrations of Boric acid on the test isolates and the mixed culture was examined, it was seen that all isolates and the mixed culture showed turbidity in NB and formed colonies on NA containing 1% Boric acid. Fairly low log reduction factors ranging from 0.6 to 2.3 were observed on NA containing 1% Boric acid for all isolates and the mixed culture. However, the bacteria did not form colonies on NA containing 2%, 3%, 4%, 5% and 7% Boric acid (Table II).

The applications of Boric acid (1%) in combination with NaCl (5% and 10%) did not prevent the growth of the isolates and mixed culture (Table III). Although we observed colonies formed by all isolates and the mixed culture on NA media containing 1% Boric acid and 5% NaCl, and 1% Boric acid and 10% NaCl, the combination of 1% Boric acid and 15% NaCl prevented only growth of *Pseudomonas aeruginosa* and *Enterococcus faecium* (Table III). These combinatory applications resulted in more reductions in the number of viable cells than the applications of 5%, 10% and 15% NaCl applied by themselves (Tables I and III.) Similar results had been observed by Kanagaraj and colleagues.[5] When the hides were preserved with 1% Boric acid and 5% NaCl, loose hair and bad smell were detected within 48 hours.

TABLE II
Reduction factors (RF) of each test bacteria and the mixed culture incubated for 24 h in NB media and then spread over NA media containing different Boric acid concentrations.

Test Isolates	Boric Acid Concentrations						
	Log_{10}	1%	2%	3%	4%	5%	7%
Pseudomonas aeruginosa ($4x10^8$ CFU/mL)	8.6	1.9	**8.6**	**8.6**	8.6	8.6	8.6
Pseudomonas fluorescens ($1x10^8$ CFU/mL)	8.0	2.3	**8.0**	**8.0**	**8.0**	**8.0**	**8.0**
Bacillus licheniformis ($2x10^8$ CFU/mL)	8.3	0.6	**8.3**	**8.3**	**8.3**	**8.3**	8.3
Bacillus pumilus ($4x10^8$ CFU/mL)	8.6	1.4	**8.6**	**8.6**	**8.6**	**8.6**	**8.6**
Staphylococcus epidermidis ($3x10^8$ CFU/mL)	8.5	0.8	**8.5**	**8.5**	**8.5**	**8.5**	**8.5**
Enterococcus faecium ($2x10^8$ CFU/mL)	8.3	0.8	8.3	8.3	8.3	8.3	8.3
Mixed culture ($3x10^8$ CFU/mL)	8.5	2.0	**8.5**	**8.5**	**8.5**	**8.5**	**8.5**

TABLE III
Reduction factors of each test isolate and the mixed culture incubated in NB media for 24 hours and then spread over NA media containing NaCl and Boric acid concentrations in which the test isolates were grown.

Test Isolates	Log_{10}	1% Boric Acid and 5% NaCl	1% Boric Acid and 10% NaCl	1% Boric Acid and 15% NaCl
Pseudomonas aeruginosa ($4x10^8$ CFU/mL)	8.6	2.1	6.6	**8.6**
Pseudomonas fluorescens ($1x10^8$ CFU/mL)	8.0	3.5	6.4	6.9
Bacillus licheniformis ($2x10^8$ CFU/mL)	8.3	0.7	1.8	7.5
Bacillus pumilus ($4x10^8$ CFU/mL)	8.6	2.2	2.3	7.4
Staphylococcus epidermidis ($3x10^8$ CFU/mL)	8.5	1.1	3.0	8.2
Enterococcus faecium ($2x10^8$ CFU/mL)	8.3	0.9	2.9	**8.3**
Mixed culture ($3x10^8$ CFU/mL)	8.5	2.3	4.9	7.8

Researchers reported that the effect of Boric acid against bacteria was bacteriostatic. It was also stated that when hides were preserved in combination with Boric acid, naphthalene, and salt, red heat and deterioration of hides could be prevented.[27,28]

As stated before, the visible growth (turbidity) of the test bacteria and the mixed culture were not observed in NB media containing 20%, 25%, and 30% NaCl. To determine inactivation efficacy of NaCl against the isolates and the mixed culture, 100 μL aliquots of the isolates and the mixed culture were taken from NB media containing 20%, 25%, and 30% NaCl after the incubation period. Afterwards, serial dilutions (10^{-2}, 10^{-4} and 10^{-6}) of each test isolate and the mixed culture were prepared. Then, 100 μL aliquots of the direct and serial dilutions of the bacterial suspensions were spread over NaCl-free NA plates and incubated at 35°C for 24 hours. After the incubation period, the colonies were observed on NaCl-free NA plates, but viable bacterial cell count for each isolate and the mixed culture slightly declined in comparison with the initial counts of the isolates (Table IV). These results showed that the viable bacterial cell count related to each test isolate did not increase during incubation period at 35°C in the presence of 20%, 25% and 30% NaCl in the NB media. Therefore, we did not see any visible growth (turbidity) in the test tubes but we detected some bacteria still alive in these liquid media (Table IV). After the incubation period of the test bacteria in NB media containing 20%, 25% and 30% NaCl, the viable bacterial cell counts slightly decreased but the cell counts in these test tubes were 10^7, 10^6 and 10^5 CFU/mL, respectively. Almost 1 log, 2 log and 3 log reduction factors of *Pseudomonas aeruginosa, Staphylococcus epidermidis, Enterococcus faecium* and the mixed culture were detected at 20%, 25% and 30% NaCl, respectively. Log reduction factors of *Pseudomonas fluorescens, Bacillus licheniformis* and *Bacillus pumilus* were found to be low (Table IV). The survival ability and salt tolerance of our isolates at high salt concentrations explain the dominance of *Pseudomonas, Staphylococcus*, *Bacillus*, and *Enterococcus* on salt-pack cured hides in our previous studies.[10,11]

In our previous study, the survival of *Bacillus cereus*, *Bacillus firmus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus,* and *Bacillus subtilis* was detected in brine solution containing 30% NaCl.[1]

In the present study, similar results were obtained from 2%, 3%, 4%, 5% and 7% Boric acid concentrations. These concentrations show no visible growth in NB. When the isolates and mixed culture, which show no visible growth in NB containing 2%, 3%, 4%, 5% and 7% Boric acid, were spread over Boric acid-free NA plates, the colonies were observed on Boric acid-free NA plates after the incubation period (Table V). As the concentration of Boric acid was increased in NB medium, the viable bacterial cell counts of the isolates and mixed culture on Boric acid-free NA plate decreased in proportion to the concentration of Boric acid (Table V). The Log reduction factors for the isolates and mixed culture were quite low at the concentrations of 2%, 3%

TABLE IV
Reduction factors of bacteria count on NaCl-free NA media of the test isolates and mixed culture incubated in the NB Media containing 20%, 25% and 30% of NaCl concentrations that show no visible growth of the test isolates.

Test Isolates	NaCl Concentrations			
	Log_{10}	**20%**	25%	30%
Pseudomonas aeruginosa ($4x10^8$ CFU/mL)	8.6	1.5	1.8	2.9
Pseudomonas fluorescens ($1x10^8$ CFU/mL)	8.0	0.7	1.1	2.8
Bacillus licheniformis ($2x10^8$ CFU/mL)	8.3	0.2	0.6	1.2
Bacillus pumilus ($4x10^8$ CFU/mL)	8.6	0.9	1.2	1.8
Staphylococcus epidermidis ($3x10^8$ CFU/mL)	8.5	1.2	2.1	2.9
Enterococcus faecium ($2x10^8$ CFU/mL)	8.3	1.0	2.0	2.8
Mixed culture ($3x10^8$ CFU/mL)	8.5	1.1	1.9	2.9

TABLE V
Reduction factors of bacteria count on Boric acid-free NA media of the test isolates and mixed culture incubated in NB media containing different Boric acid concentrations that show no visible growth of test isolates.

Test Isolates	Boric Acid Concentrations					
	Log_{10}	**2% Boric Acid**	**3% Boric Acid**	**4% Boric Acid**	**5% Boric Acid**	**7% Boric Acid**
Pseudomonas aeruginosa ($4x10^8$ CFU/mL)	8.6	0.8	1.9	1.8	2.0	4.4
Pseudomonas fluorescens ($1x10^8$ CFU/mL)	8.0	0.5	1.1	2.2	2.3	3.9
Bacillus licheniformis ($2x10^8$ CFU/mL)	8.3	0.2	1.4	1.4	2.8	3.8
Bacillus pumilus ($4x10^8$ CFU/mL)	8.6	0.4	0.8	1.7	1.7	3.1
Staphylococcus epidermidis ($3x10^8$ CFU/mL)	8.5	1.8	2.2	1.9	2.6	4.9
Enterococcus faecium ($2x10^8$ CFU/mL)	8.3	0.9	1.4	1.5	3.6	4.2
Mixed culture ($3x10^8$ CFU/mL)	8.5	1.4	1.8	1.9	2.7	4.0

and 4% Boric acid. High log reduction factors (2-4 log) for the isolates were achieved at the concentrations of 5% and 7% Boric acid (Table V). According to results of these experiments, Boric acid concentrations prevented growth of the test isolates and the mixed culture but did not kill them. Thus, our results confirmed that Boric acid has bacteriostatic effect.

Researchers reported that loose hair was not detected on the hides preserved with 2-4% Boric acid, but bad odor was caused by bacteria.[27,28] It was found that 5% Boric acid prevented bad odor, hair slip, and bacterial activity on hides. The research results demonstrated that when 5%-10% Boric acid was used for preservation of hides, bad odor diminished.[27,28]

Conclusion

It is believed that salt curing treatment is enough to prevent destructive bacterial activities on salt-packed cured hides, but the data obtained from the present study showed that a concentration of ≥20% NaCl or ≥ 2% Boric acid resulted in no visible growth of test bacteria in NB. However, NaCl and Boric acid have bacteriostatic effect on the isolates and these bacteria are still alive in the presence of these chemicals. As a result, Gram-negative bacteria, Gram-positive bacteria and Gram-positive endospore forming bacteria were not completely killed with NaCl and Boric acid. In addition, bacteriostatic effect of NaCl and Boric acid changed according to the species of bacteria. After application of different concentrations of NaCl and Boric acid, the viable bacterial cell counts were still high. These results explained why the salt-pack cured hides contained different bacteria in high numbers in our previous studies.[9-12] It is clearly seen that when NaCl and Boric acid are removed from hides through the soaking process, bacteria found on salted hides will start to multiply rapidly and damage the hides during the long soaking process. Moreover, depending on data obtained from our previous study, we concluded that it would be difficult to decrease high numbers of different bacterial populations on hides using antibacterial agent during soaking process. Ultimately, it should be focused on inactivation of bacteria on hides via effective alternative treatments.

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References

1. Birbir, M. and Ilgaz, A.; Isolation and Identification of Bacteria Adversely Affecting Hide and Leather Quality. *J. Soc. Leather Technol. Chem.* **80,** 147-153, 1996.
2. Bell, R.G.; Distribution and Sources of Microbial Contamination on Beef Carcasses. *J. Appl. Microbiol.* **82**, 292-300, 1997.
3. Dickson, J.E. and Anderson, M.E.; Microbial Decontamination of Food Animal Carcasses by Washing and Sanitary Sytem: A Review. *J. Food Prot.* **55**, 133-140, 1992.
4. Bailey, D.G. and Gosselin, J.; The Preservation of Animal Hides with Potassium Chloride. *JALCA* **91**, 317-333,1996.
5. Kanagaraj, J.; Sundar, V.J.; Muralidharan, C. and Sadulla, S.; Alternatives to Sodium Chloride in Prevention of Skin Protein Degradation-a Case Study. *J. Clean Prod.* **13**, 825-831, 2005.
6. 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.
7. 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, **53-58,** 2008.
8. Bailey, D.G.; The Preservation of Hides and Skins. *JALCA* **98**, 308-319, 2003.
9. Berber, D. and Birbir M.; Examination of Bacterial Populations in Salt, Salted Hides, Soaked Hides and Soak Liquors. *JALCA* **105**, 320-326, 2010.
10. Aslan, E. and Birbir, M.; Examination of Efficiency and Sufficiency of Salt-Pack Curing Method. *J. Soc. Leather Technol. Chem.* **95**, 98-103, 2011.
11. Aslan, E. and Birbir, M.; Examination of Gram Positive Bacteria on Salt-Pack Cured Hides. *JALCA* **106**, 372-380, 2011.
12. Aslan, E. and Birbir, M.; Examination of Gram Negative Bacteria on Salt-Pack Cured Hides. *JALCA* **107**, 106-115, 2012.
13. Bilgehan, H.; Clinical Microbial Identification, 4th Edition, Baris Publishers, Ankara, 133, 2004.
14. Barnett, M.E. and Venghaus, J.D.; Microbiology Laboratory Exercises. *Wm.C. Brown Publishers* Dubuque, Iowa, 292, 1988.
15. Harley, J.P. and Prescott, L.M.; Laboratory Exercises in Microbiology. 5th Edition. *The McGraw- Hill Companies*, New York, 93, 2002.
16. Kunitate, A., Okamoto, M. and Ohmori, I.; Purification and Characterization of a Thermostable Serine Protease from *Bacillus thuringiensis*. *Agric. Biol. Chem.* **53**, 3251-3256, 1989.

17. Parvathi, A.; Krishna, K.; Jose, J.; Josph, N. and Nair, S.; Biochemical and Molecular Characterization of *Bacillus pumilis* Isolated from Coastal Environment in Cochin, India. *Braz. J. Microbiol*. **40**, 269-275, 2009.
18. Abouseoud M., Maachi R. and Amrane A.; Biosurfactant Production from Olive Oil by *Pseudomonas fluorescens*. Communicating Current Research and Educational Topics and Trends in Applied Microbiology, Microbiology Book Series- Number 2, *Formatex, Spain* A. Méndez-Vilas (Ed.), pp.340-347, 2007.
19. Bailey, D.G. and Birbir, M.; The Impact of Halophilic Organisms on the Grain Quality of Brine Cured Hides. *JALCA* **91**, 47-51, 1996.
20. Polkade, A.V.; Molecular and Phylogenetic Studies on Microbial Diversity of Raw Buffalo Hide to be Used in Leather Manufacturing. *Ph.D. Thesis*, University of Pune, Faculty of Science, India, Pune, 104, 2007.
21. Smolka, L.R; Nelson, F.E. and Kelley, L.M.; Interaction of pH and NaCl on Enumeration of Heat-Stressed *Staphylococcus aureus*. *Appl. Microbiol*. **27**, 443-447, 1974.
22. Jadhav, G.G.; Salunkhe, D.S.; Nerkar, D.P. and Bhadekar, R.K.; Isolation and Characterization of Salt-Tolerant Nitrogen-Fixing Microorganisms from Food. *Eur. Asia J. BioSci*. **4**, 33-40, 2010.
23. Kanlayakrit, W.; Ikeda, T.; Tojai, S.; Rodprapakorn, M. and Sirisansaneeyakul, S.; Isolation and Characterization of Extracellular Halophilic Ribonuclease from Halotolerant *Pseudomonas* species. *Kasetsart J. (Nat Sci)* **35**, 179-187, 2001.
24. Ahmad, M.; Smith, D.G.and Mahboob, S.; Effect of NaCl on Heat Tolerance of *Enterococcus faecium* and *Enterococcus faecalis*. *OJBS* **2**, 483-484, 2002.
25. Loir, Y.L.; Baron, F.; Gautier, M.; *Staphylococcus aureus* and Food Poisoning. *Genet. Mol. Res*. **2**, 63-76, 2003.
26. Wood, J.T.; The Bacteriology of the Leather Industry. *JALCA* **5**, 360, 1910.
27. Woods, D.R.; Atkinson, P.; Cooper, D.R. and Galloway, A.C.; The Microbiology of Curing and Tanning Processes. Part II. Analysis of Aerobic Bacteria in Static Hide Brining. *JALCA* **65,** 164, 1970.
28. Woods, D.R.; Atkinson, P. and Cooper, D.R.; The Microbiology of Curing and Tanning Processes. Part III: Analysis of Aerobic Bacteria in Agitated Hide Brining. *JALCA* **65**, 410-418, 1970.