Survival and Growth of *Bacillus cereus*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* in Extreme Environments Present During the Early Stages of a Conventional Leather Making Process

by

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Abstract

Raw hides/skins used as a raw material for manufacturing leather contain various microorganisms including potential pathogens. The presence of hazardous chemicals during a conventional leather-making process may create extreme environmental conditions for the growth of the bacteria present on hides/skins. Bacteria, however, are known to survive harsh environmental conditions. This study was undertaken in order to determine the survival and growth of certain bacteria during a conventional pre-tanning and chromium-tanning process. Calfskin pieces were decontaminated and inoculated with log8 (10^8) to log10 (10^{10}) colony forming units (cfu) of *Bacillus cereus* ATCC11778, *Pseudomonas aeruginosa* ATCC10145 and *Staphylococcus aureus* ATCC25923, and the survival was monitored following a conventional pre-tanning and chromium-tanning process. The presence of inoculated bacteria in effluent and on skins was determined. A higher bacterial growth was observed during the pre-soaking process, followed by a significant reduction ($p \leq 0.05$) during the main-soaking, unhairing and reliming processes. Although limited, bacterial growth was observed during the subsequent deliming, bating, pickling and tanning processes. The study showed that these bacterial species are not only capable of surviving a conventional leather-making process, but also showed the ability to proliferate if provided with suitable environmental conditions.

Resumen

Pieles crudas se emplean como materia prima en la manufactura del cuero contienen varios micro-organismos incluyendo potenciales patógenos. La presencia de agentes químicos peligrosos durante el proceso tradicional de la fabricación del cuero podría crear medio-ambientes con condiciones extremas para el crecimiento de las bacterias presentes en las pieles. Bacterias, sin embargo, han sido reconocidas como sobrevivientes de condiciones ambientales extremamente difíciles. Este estudio se emprendió con el objeto de determinar la sobrevivencia y crecimiento de ciertas bacterias durante los procesos convencionales de precurtido y curtido al cromo convencionales. Pedazos de pieles de ternero fueron descontaminados y luego inoculados con log8 (10^8) hasta log10 (10^{10}) unidades de formación de colonias (cfu) de *Bacillus cereus* ATCC11778, *Pseudomonas aeruginosa* ATCC10145, y *Staphylococcus aureus* ATCC25923, y sus supervivencias se monitorearon siguiendo los procesos convencionales de precurtido y curtido al cromo. La presencia de la bacteria inoculada en los efluentes y en las pieles fue determinada. Una mayor tasa de crecimiento bacterial fue observada en los procesos de preremojo, seguida por una significante reducción ($p \leq 0{,}05$) durante el transcurso del remojo principal, pelambre y re-encalado. Aun que un limitado crecimiento bacterial fue observado durante los siguientes procesos de desencale, rendido, piquelado y curtido. El estudio demostró que estas especies bacterianas ~~son~~ no solo son capaces de sobrevivir un proceso convencional de fabricación de cuero, sino demostraron la habilidad de proliferarse cuando se les presentan unas condiciones ambientales favorables.

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Introduction

Raw hides/skins, which are by-products of the meat industry, are used to manufacture leather.[1] The conversion of putrescible raw hides/skins to non-putrescible leather involves various chemical and mechanical processes and is generally categorized into four groups: pre-tanning, tanning, post-tanning and finishing. In this research, the early stages of a conventional leather manufacturing process that includes pre-soaking, main-soaking, unhairing, reliming, deliming, bating, pickling and tanning using chromium were studied.

Animal skins naturally contain a variety of microorganisms that are either natural inhabitants or acquired from the environment. Bacterial species have been isolated from raw hides/skins as well as hides/skins at various stages of leather processing.[2-8] The majority of the microbial species isolated from hides/skins are non-pathogenic to humans,[9,10] however, a number of species, viz., *B. cereus*,[3,5,11] *S. aureus*,[2,3,5] and *P. aeruginosa*[3] are considered to be pathogens or potential pathogens.[10] There is a possibility that these pathogens present on hides/skins and their subsequent presence in tannery effluent may infect tannery personnel and contaminate the environment. It is not evident whether bacteria found on hides/skins and leather, survive the environmental conditions of the leather manufacturing processes or are a result of contamination during leather processing.

Theoretically, the extreme environmental conditions present during a conventional leather making process, particularly the unhairing, pickling and tanning are thought to destroy most of the microorganisms on hides/skins.[12] This study was carried out to investigate the survivability of a pathogenic (*B. cereus*) and potential pathogenic (*P. aeruginosa* and *S. aureus*) bacteria at various stages of a conventional pre-tanning and chromium-tanning process.

Experimental

Calfskins (Latco Ltd., UK) were used as a source of raw material for this study. Calfskins were soaked overnight in water (300% w/w) and bactericide (0.2% w/w). The adipose tissue was removed by fleshing and the skins were preserved using sodium chloride (NaCl).

A preliminary experiment was carried out to determine the presence of bacteria on calfskins. Fleshed and re-salted calfskins were cut into 5 pieces. Each calfskin piece was placed in a separate trial drum. The trial drums were washed well with commercial bleach (sodium hypochlorite-based disinfectant) and rinsed with water 3 times and processed as shown in the Appendix except for the reliming process. A reliming stage was subsequently added during the main experiments for reproducibility of the data. Once the experiment is completed, effluent was collected using a 15 ml sterilised centrifuge tube (Fisher Scientific, UK) promptly and aseptically at the end of the pre-soaking, main-soaking, liming, deliming, bating, pickling and tanning process, and stored in a refrigerator at 4°C. The collected samples were inoculated on a solid media Tryptone soya agar (TSA) for bacterial growth using a spiral-plater as stated under subheading "Sample Plating," within 24 hours of collection. TSA is a non-selective media and therefore used for general purpose to encourage growth of the culturable bacteria present in the effluent following a conventional pre-tanning and chromium tanning process. The inoculated plates were incubated for 24 hours at 37°C. The following day the plates were examined, colonies present on the plates were counted and recorded. Based on the results obtained during the preliminary experiment, the experiments were modified and are described as follows:

Soaked, fleshed and sodium chloride-treated calfskin pieces each weighing 100±10 g were decontaminated using sodium hypochlorite (NaOCl) followed by inoculation with a known pure strain of bacterial species stated below. A conventional pre-tanning and chromium-tanning process were carried out with the inoculated calfskin pieces. The experiment with each bacterial species was repeated 5 times to ensure reproducibility and consistency of data.

Sodium Hypochlorite Treatment

The decontamination procedure was conducted in a category II cabinet to limit the risk of microbial contamination. A stock NaClO solution, 1.25 g/ml (Fisher Scientific, UK) was diluted using deionised water to 0.125 g/ml NaClO solution. Calfskin pieces were washed twice with sterilized tap water (tap water was sterilized by autoclaving at 121°C for 15 minutes) to remove salt and extraneous matters, placed in 250 ml NaOCl (0.125 g/ml) and agitated manually for 3 minutes. The NaOCl-treatment was repeated three times. The treated skin pieces were then washed with sterilized tap water for 15-20 times in order to reduce the total chlorine concentration in the water after washing, to below 0.2 mg/l as this concentration was found to allow the inoculated bacterial species to proliferate. A chlorine test kit (Chlor-test Reflectroquant®Plus 00109888/462, Merck, France) was used to measure the total chlorine concentration. Water from the final washing cycle was collected and the flesh side of the decontaminated calfskin pieces was swabbed after the final washing cycle, in order to determine the presence of viable microbial colonies on the skins and water.

Bacterial Species

B. cereus ATCC11778, *P. aeruginosa* ATCC10145, and *S. aureus* ATCC25923 (Oxoid, UK) were used for this study. The growth media (Oxoid, UK) and the required incubation temperature used for the growth of the above-mentioned bacterial species are given in Table 1.

TABLE 1
Growth media and incubation temperature used for each bacterial species studied.[13-15]

Bacterial species	Inoculated media	Incubation temperature (°C)
B. cereus	Polymyxin pyruvate egg yolk mannitol agar (PEMBA) (CM0617, SR0099, SR0047)	30
P. aeruginosa	*Pseudomonas* CN media, containing glycerol, cetrimide and nalidixic (CM0559, SR0102)	37
S. aureus	Baird-Parker media with egg yolk emulsion and tellurite (CM0275, SR0054)	37

Bacterial Inoculation
Bacterial inoculums were collected from the mid-exponential growth phase, since bacterial cells in the exponential phase are the most active as well as the most stable in terms of its physical and chemical properties.[16,17] The fiber structure of the outermost layer of a hide/skin is compact and dense while the fiber structure of the flesh side is comparatively less compact.[18] Additionally raw hides/skins contain hair and epidermis, which are removed during the unhairing processes. The bacterial cells were therefore inoculated onto the flesh side to maximise the growth of inoculated bacterial species. The average number of inoculated *B. cereus*, *P. aeruginosa* and *S. aureus* on the decontaminated calfskin pieces were log8.61±0.15 cfu, log10.02±0.05 cfu and log8.37±0.10 cfu respectively. The inoculated calfskin pieces were incubated overnight at the required temperature (see Table 1) to allow the bacterial species to adapt to the new environment. The following day bacterial cells were collected from the flesh side of each of the inoculated skins using a 10 μl sterilized loop, streaked on corresponding media (Table 1) and incubated overnight. This was conducted to verify the growth of the inoculated bacteria on skins.

Leather-Making Process
The processes were conducted in a closed cabinet containing 6 removable and autoclavable trial drums (see the Appendix for the detailed process). Before processing, the drums were removed, washed thoroughly and sterilized by autoclaving at 121°C for 15 minutes. Each of the calfskin pieces was placed in separate drums. Autoclaved tap water was used for processing. The control experiment contained the same constituents used in conventional processing with the omission of the calfskin pieces and will be referred to as the control sample for the purpose of this study.

Sample Collection
Effluent was immediately collected using 15 ml sterilized centrifuge tubes using aseptic techniques at the end of the pre-soaking, main-soaking, unhairing, reliming, deliming, bating, pickling and tanning process in duplicate. Effluent was also collected from the control samples. Centrifuge tubes were completely filled with the effluent to limit air contact with the collected samples and so bacterial contamination.

The flesh sides of calfskin pieces were swabbed (in duplicate) following the collection of effluent. Swabs were collected aseptically by rubbing a sterilized swab (SWA3018, Scientific Laboratory Supplies Ltd., UK) on the flesh side of the skin 50 times to ensure consistency throughout the experiments. The swabs were placed in charcoal-based microbial media to preserve bacterial samples during transport and storage. This was undertaken to determine the presence of the inoculated bacteria on the flesh side during processing. Swab samples were not collected from the control experiments, as no calfskins were added. Effluent samples and swabs were stored immediately in a refrigerator at 4°C, tightly sealed to avoid contamination, and analyzed within 24 hours of collection.

Bacterial Enumeration in Effluent and Swabs
Effluent collected from the pre-soaking and soaking processes were diluted to 10^{-2} or 10^{-4}, based on the initial concentrations of bacterial colonies in the effluent, using sterilized phosphate-buffered saline (PBS (pH 7.4), Oxoid, UK).[15] The diluted samples were vortexed at a high speed for 10 seconds and inoculated on the respective selective media (see Table 1) in duplicate using a spiral-plater. The unhairing effluents (10 ml) were centrifuged at 4°C, 5000 rpm for 5 minutes to separate hair from the effluent. The centrifuged supernatant was inoculated without dilution on the selective media. Effluent collected from the reliming, deliming, bating, pickling and tanning processes were also inoculated on the selective media without dilution. Swabs were placed in a sterilized 5 ml bottle containing 2.5 ml PBS solution and agitated using a vortex at high speed for 20 seconds followed by inoculation of the PBS solution on the selective media in duplicate. The inoculated plates were incubated at a required temperature (see Table 1) for 24 hours. The plates were examined; bacterial colonies present on the plates were enumerated; and recorded.

Sample Plating
Samples (50 μl) were inoculated on a minimum of two petri-dishes containing respective solid media (Table 1) using "Whitley automated spiral-plater" (WASP2, Don Whitley Scientific Ltd. UK).

Confirmatory Tests
Various confirmatory tests according to the National Standard Method,[13-15] along with a Biolog® system (Biolog Inc., USA), were followed for identification of *B. cereus*, *S. aureus* and *P. aeruginosa*. The confirmatory tests were applied for a pure

culture of *B. cereus* ATCC11778, *P. aeruginosa* ATCC10145, and *S. aureus* ATCC25923 to evaluate the accuracy of the selected methods for identification of the bacterial species. Upon obtaining satisfactory results the presumptive *B. cereus*, *S. aureus* and *P. aeruginosa* isolated from various stages of the leather-making processes were then subjected to the confirmatory tests. Typical and atypical bacterial colonies were collected at various stages of the leather manufacturing processes and purified to a single colony by streaking. The purified single colony was re-streaked in order to produce more colonies and to obtain sufficient bacterial colonies to perform the confirmatory tests. Pure strain of *B. cereus* ATCC11778, *P. aeruginosa* ATCC10145, and *S. aureus* ATCC25923 were used as a positive control while undertaking these tests.

Statistical Analysis

Total number of bacterial colonies were expressed as cfu/ml of the samples and transformed to $\log_{10}$ values, and these values were used to determine the average values and standard deviation. The values for standard deviation are expressed by ±. The total number of calfskin pieces used for each experiment will be referred to as n. Statistical software (SPSS) was used for data analysis. One sample Kolmogorov-Smirnov test was used to determine whether the data distribution is normal. An independent T-test and Mann-Whitney test were carried out to compare means between groups if the data were found to have normal or skewed distribution respectively. Values of $p \leq 0.05$ were accepted as statistically significant.

Results

Preliminary Experiments

Figure 1 illustrates the average number of total bacterial colonies enumerated at various stages of the pre-tanning and tanning process. The results showed that the highest bacterial growth occurred during the pre-soaking and main-soaking processes, which were log5.08 (±0.10, n=5) cfu/ml and log5.31 (±0.10, n=5) cfu/ml respectively, followed by a large reduction to log0.45 (±0.74, n=5) cfu/ml during the unhairing process. An increase in the number of total bacterial colonies from log0.45 to log1.94 (±1.15, n=5) cfu/ml was observed during the deliming process followed by a further increase to log2.98 (±0.55, n=5) cfu/ml during the bating process. However, the number of bacterial cells decreased during the pickling and tanning process. The average number of isolated colonies in the pickling and tanning effluent was log0.92 (±0.80, n=5) cfu/ml and log0.09 (±0.34, n=5) cfu/ml respectively.

Bacterial Isolation from Effluent Prior to and During Processing of Calfskins

The average number of isolated bacteria from the effluent and swabs, after decontamination of calfskin pieces that were subsequently used to inoculate *B. cereus*, was log 0.17 (±0.46, n=5) cfu/ml and log 0.09 (±0.34, n=5) cfu/ml respectively. The isolated bacterial colonies were not identified as *B. cereus* in this instance. Sodium hypochlorite may not cause total elimination of microorganisms, but often found to reduce the viable microbial cells to log1 or below.[19] No bacterial growth was observed in the water and skin samples following the decontamination of calfskin pieces that were subsequently inoculated with *P. aeruginosa* and *S. aureus*.

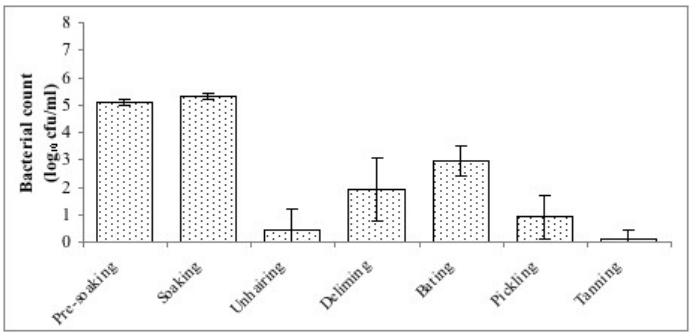

Figure 1. Total bacterial count (cfu/ml) in effluent during a conventional pre-tanning and chromium-tanning process. The error bars represent the standard deviation.

Analysis of the effluent collected from the control samples during processing of calfskin pieces showed the absence of bacterial colonies indicating a non-appearance of microbial contaminants during the processing.

Bacillus cereus

Figure 2 shows the average number of isolated colonies from effluent for each of the processing steps. An average of log4.75 (±0.45, n=5) cfu/ml was isolated from the pre-soaking process followed by a significant reduction ($p<<0.001$) during the main-soaking process to log3.56 (±0.62, n=5) cfu/ml. A further significant reduction ($p<<0.001$) from log3.56 to log0.48 (±0.69, n=5) cfu/ml was observed during the unhairing process. The average number of isolated *B. cereus* colonies from the reliming effluent was log0.42 (±0.86, n=5) cfu/ml followed by a significant increase ($p=0.021$) to log1.16 (±1.08, n=5) cfu/ml during the deliming process. However, a significantly ($p<<0.001$) reduced number of *B. cereus* from log1.22 (±0.94, n=5) cfu/ml (isolated from the bating effluent) to log0.07 (±0.29, n=5) cfu/ml was observed during the pickling process followed by no growth during the tanning process.

Pseudomonas aeruginosa

The average number of isolated *P. aeruginosa* colonies from effluent is shown in Figure 3. An average of log7.79 (±0.05, n=5) cfu/ml and log7.64 (±0.07, n=5) cfu/ml was isolated from the pre-soaking and main-soaking processes, followed by a significant reduction ($p<<0.001$) during the unhairing process. Although, no bacterial growth was observed in both the unhairing as well as the reliming processes, however, a

significant (p=0.02) increased number of *P. aeruginosa* from nil to log0.79 (±1.09, n=5) cfu/ml was isolated from the subsequent deliming process. A lower number of *P. aeruginosa*, from log0.79 to log0.66 (±0.93, n=5) cfu/ml was enumerated from the bating process; this was statistically not significant (p=0.756). No bacterial growth was observed during the pickling and tanning processes.

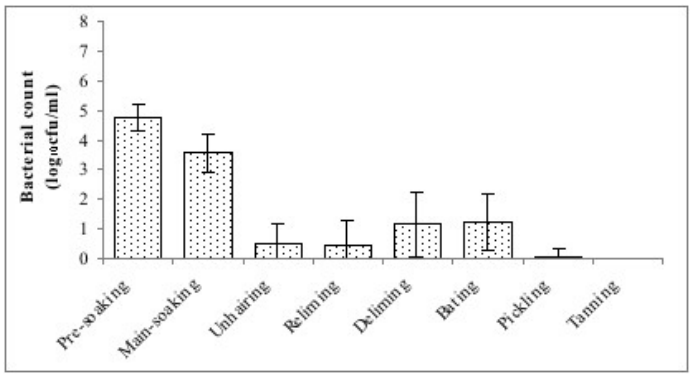

Figure 2. Number of isolated ***B. cereus*** (cfu/ml) colonies from effluent during the pre-tanning and chromium-tanning process. The error bars represent the standard deviation.

Staphylococcus aureus

The confirmatory tests showed that only 43% of the isolated species were *S. aureus*. However, presence of *S. aureus* was noted during the tanning process, indicating presence of viable *S. aureus* colonies. Other isolated species were identified as *S. epidermidis*, *S. arlettae* and *Brochothrix campestris* using the Biolog® identification system. These bacterial species were isolated from the unhairing, reliming, deliming and bating processes. As 86% of the isolated colonies were identified as *Staphylococcus* spp. (including, *S. aureus*) in this study, the isolated colonies will therefore be referred to as *Staphylococcus* spp. Figure 4 shows the number of isolated colonies from effluent following the pre-tanning and tanning processes. The average number of isolated *Staphylococcus* spp. from the pre-soaking process was log5.21 (±1.82, n=5) cfu/ml followed by a significant reduction (p<<0.001) to log2.38 (±1.84, n=5) cfu/ml during the main-soaking process. No bacterial colonies were isolated from the unhairing process. An average of log0.72 (±0.76, n=5) cfu/ml, log0.45 (±0.82, n=5) cfu/ml and log0.74 (±1.04, n=5) cfu/ml of *Staphylococcus* spp. was isolated from the reliming, deliming and bating processes respectively, followed by no growth during the pickling process. Nominal growth of *Staphylococcus* spp. (log0.09 (±0.40, n=5) cfu/ml) was observed during the tanning process.

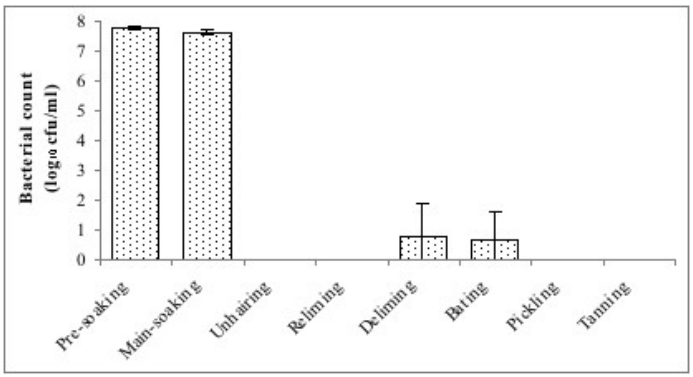

Figure 3. Number of isolated ***P. aeruginosa*** colonies (cfu/ml) from effluent during the pre-tanning and chromium-tanning process. The error bars represent the standard deviation.

Bacterial Isolation from Swabs

The number of isolated bacterial colonies from swabs is given in Table 2.

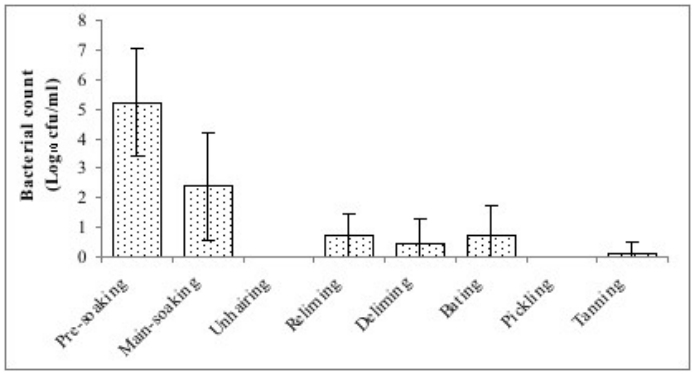

Figure 4. Number of isolated ***Staphylococcus*** colonies (cfu/ml) from effluent during the pre-tanning and chromium-tanning process. The error bars represent the standard deviation.

Discussion

During the preliminary experiments, due to the growth of a high number of bacterial colonies, particularly during the pre-soaking and main-soaking process, difficulties arose in isolation and identification of individual species present on each calfskin piece. Moreover, the calfskin pieces used may not be the sole source of isolated bacterial colonies. Bacterial contamination may also occur during flaying, storage, transportation and during processing. Species and quantities of microbial flora may vary from animal to animal and within the same skin. As a consequence too many variations may affect the validity of the results obtained, and therefore the method followed during the preliminary experiments was evaluated and modified. Alternative methods, such as decontamination of the calfskin pieces followed by inoculation of known pure bacterial culture were studied and established to standardize the procedure by eliminating the possibility of having variable bacterial species on the skin samples. This facilitated the monitoring of inoculated bacterial species throughout the leather manufacturing process and identification of the principle colonies in order to obtain data that was reliable and reproducible. The confirmation tests

TABLE 2
Number of bacterial colonies isolated from swabs. Swabs samples were collected from the flesh side of each of the calfskin pieces following collection of the effluent.

Process	Bacterial count (log_{10} cfu/ml) in effluent		
	B. cereus	*P. aeruginosa*	*Staphylococcus spp.*
Pre-soaking	3.61 (±0.54, n=5)	5.83 (±0.07, n=5)	4.19 (±1.31, n=5)
Main-soaking	2.00 (±0.91, n=5)	5.82 (±0.07, n=5)	2.63 (±1.61, n=5)
Unhairing/ Liming	1.17 (±0.89, n=5)	No growth	0.46 (±0.84, n=5)
Reliming	0.13 (±0.40, n=5)	No growth	0.51 (±0.0.69, n=5)
Deliming	No growth	No growth	0.15 (±0.48, n=5)
Bating	No growth	No growth	0.08 (±0.36, n=5)
Pickling	No growth	No growth	0.07 (±0.30, n=5)
Tanning	No growth	No growth	0.46 (±0.75, n=5)

showed that although decontaminated calfskin pieces were inoculated with a pure culture of *S. aureus* ATCC25923 before leather processing, however, not all the species isolated during the pre-tanning process were *S. aureus*. The isolated *Staphylococcus* spp., which were not *S. aureus*, but *S. epidermidis* and *S. arlettae,* maybe indigenous flora of calfskins. Sodium hypochlorite is a chlorine-based disinfectant that is effective against a wide range of microorganisms including fungi, viruses, bacteria and bacterial spores[10,20,21]. However, dissimilar to the autoclaving technique, NaClO may not cause total elimination of microorganisms.[20] There is a possibility[22,23] that some bacterial species presents on the calfskin may survive the decontamination process in a non-culturable state and reappeared when provided with suitable environmental conditions. *S. epidermidis* have been isolated from hides,[3] goat and sheep skins.[5] *S. arlettae* has also been isolated from animal origins[24] such as bovine skins,[25] and goats milk.[26] *S. aureus* is a weak competitor in a mixed microbial culture[27] reducing the possibility of survival when exposed to an environment containing various microorganisms. Alternatively, reduction of the number of inoculated bacterial colonies, that is, *S. aureus*, after the soaking process may provide the indigenous bacterial flora of the calfskin an opportunity to proliferate.

B. cereus and *S. aureus* have been isolated from hides,[3] goat and sheep skins.[5] Presence of *B. cereus, P. aeruginosa* and *S. aureus* were also found by Birbir and Ilgaz[3] on NaCl-preserved hides. *B. cereus* has been isolated from limed and chromium-tanned hides, re-tanned and finished leather.[3] Factors such as, available nutrition, moisture content, pH and environmental temperatures are important for bacterial growth. Microorganisms are able to use organic compounds to derive essential elements, such as carbon, hydrogen, oxygen, nitrogen and sulfur, for microbial metabolism.[20,21] Hides/skins can provide an essential nutritional source for bacterial growth and proliferation.[28,29]

Bactericides are generally added during the soaking process to control bacterial growth as the presence of a high number of bacterial colonies cause protein deterioration and hence reduce the quality of the leather production.[5] Since, bactericides are designed to control or destroy bacteria,[30] an inhibitory effect on bacterial growth may be observed. However, bactericides may not cause total removal of the microorganisms present on hides/skins. It has been suggested by Berber and Birbir[8] that a soaking process may assist bacterial proliferation on hides due to the presence of moisture and organic components. The authors also showed that the used bactericides are not sufficient to prevent growth and proliferation of mesophilic and halophillic bacteria during the soaking process. The bacterial species (*B. cereus*, *P. aeruginosa* and *S. aureus*) used in this study are all categorized as mesophiles (required a temperature range between 20-40°C for growth).[20] Similar to the research carried out by Berber and Birbir[8], the bacterial species used in this study showed the capability to grow and proliferate in effluent and on skins during the soaking process.

Presence of a surfactant may also have a negative impact on bacterial growth as surfactants used during leather-processing are found to interrupt microbial activity.[30,31] During processing, due to the mechanical agitation, microorganisms on hides/skins may be transferred to the effluent reducing the number of bacterial cells on the hides/skins.[3] This could also be a reason for the isolation of a lower number bacterial colonies from the main-soaking effluent than the pre-soaking effluent (see Figures 2 to 4). The pH of the soaking effluent was 7.6-8.1, and was within the pH range required for bacterial growth.[20]

The results obtained show that both the unhairing and reliming processes did not provide suitable environmental conditions for bacterial growth and proliferation. Lime[32,33] and Na_2S[34-36] have antimicrobial activity mainly due to the strong alkaline properties. Additionally, Na_2S is a reducing agent, reducing oxygen levels in water,[34,36,37] this may adversely affect the growth of aerobic microorganisms. Due to the presence of alkalis the pH of the unhairing and reliming effluent was 11.9±1.1 and 12.5±0.1 respectively, and was higher than the pH range required for bacterial growth.[20]

The deliming and bating processes on the other hand may have provided suitable conditions for the recovery of the inoculated bacterial species. Bacterial colonies may change to a non-culturable but viable state due to environmental stress.[22,23] Absence of *P. aeruginosa*, in the unhairing and reliming effluent and their consequent presence in the deliming and bating effluent, suggests that the bacterial cell may become dormant or non-culturable during the unhairing and reliming processes, but still viable. Therefore, the absence of bacterial colonies in effluent or skins does not necessarily indicate total elimination of bacterial species. Recovery of the dormant bacterial cells is possible when provided with suitable growth conditions.[22,23] The number of *Staphylococcus* spp. colonies did not increase, but the presence of the bacterial species was still observed. This indicates that although, proliferation did not occur, bacterial cells were still viable. In this study, the pH of the deliming and bating effluent was 8.2-8.8 and 8.0-8.7 respectively. Based on the results obtained in this study it may be suggested that a reduction in alkalinity assists bacterial growth. Ammonium salts may also promote bacterial growth and proliferation, since many bacteria are able to utilise ammonium salts as a nitrogen source, which is an essential element for microbial metabolism.[20, 21]

Low growth or no growth of the bacterial species during the deliming and bating process on the skins may be due to the following reasons:

1. Transfer of bacterial colonies to the effluent due to the mechanical agitation[3]
2. Absence of bacterial colonies on the swabbed area
3. Presence of non-culturable bacterial colonies

The presence of a high level of NaCl, and a low pH during the pickling (2.3±1.1) and tanning (4.5±0.6) processes, may hinder bacterial growth.[20,38,39] Research have shown heavy metals such as chromium, cadmium, lead and copper exert a toxic effect on microorganisms.[40,41] Therefore, the presence of high concentrations of chromium during the tanning process may prevent bacterial growth in the effluent and on hides/skins. Research conducted by Sawai and Yoshikawa,[42] and Sawai *et al*.[43] also showed that magnesium oxide (MgO) powder shows antimicrobial activity, and the addition of MgO during the processing may also contribute towards a reduction in bacterial growth.

The study showed that *B. cereus, P. aeruginosa* and various *Staphylococcus* species have the capability to recover and regenerate when the conditions are favourable, such as during the deliming and bating processes. *B. cereus* produce spores under environmental stress such as a lack of nutrition. The spores are capable of withstanding adverse environmental conditions, such as high temperatures, irradiation, strong acids and disinfectants for a long period of time.[16] The ability of *B. cereus* to produce spores may aid the survival of *B. cereus* cells[3] in the harsh environmental conditions during the conventional unhairing process. *P. aeruginosa* has minimal nutritional requirements[44] and is able to utilise a wide range of organic compounds as a carbon source, including a variety of sugars, fatty acids, amino acids, amines and various organic compounds.[20,21] The minimal nutritional requirement along with ability to withstand a wide range of antibiotics and chemicals may assist the survival of *P. aeruginosa* in the extreme environmental conditions such as those found in leather manufacturing processes. Although, *S. aureus* was found to be suppressed by other *Staphylococcus* species, *S. aureus* have been able to withstand high sodium chloride concentrations and a wide pH range (4-10),[21,27] and may even capable to grow in the presence of chromium.[45]

Conclusion

The study showed that bacteria are capable of withstanding the early stages of a conventional leather-making process. The absence of bacterial colonies in effluent or on skins does not necessarily indicate total elimination of bacterial species, as the study showed that bacterial cells have the capability to recover and regenerate when environmental conditions are favorable. Therefore, the presence of pathogens on hides and skins and their consequent presence in effluent are required to be monitored. Prolonged deliming and bating processes may enable the bacterial species (including potential pathogens) to recover and proliferate. Alternatively addition of a reliming process after the unhairing process may be beneficial to suppress growth and proliferation of bacteria on hides and skins as well as in effluent.

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APPENDIX
Conventional leather-making process.

Process	Chemicals	Amount (% w/w)*	Time	pH	Temperature (°C)
Pre-soaking	Water Truposept BA (sodium dimethyldithiocarbamate -based bactericide; Trumpler, UK)	300 0.2	60 mins	7.6 – 8.0	21.1 - 25.8
Main-soaking	Water CorileneW385 (sodium salt of alkyletherphospha; STAHL Europe, The Netherlands) Truposept BA	300 0.2 0.2	120 mins and left overnight	7.6 – 8.1	21.5 – 25.6
Unhairing	Water Sodium sulfide Lime	200 3 2	20 - 24 hrs	11.1 - 12.7	21.2 – 27.8
Washing	Water	300	10 mins		
Reliming	Water Lime	200 2	72 hrs	12.5 -12.6	20.6 – 25.8
Washing (three times)	Water	300	10 mins		
Deliming	Zero float Ammonium chloride Water	2 100	15 mins 30-45 mins	8.2 – 8.8	24.5 – 26.8
Bating	Oropon ON 2 (Pancreatic - based enzyme, TFL Germany)	0.1	30 mins	8.0 – 8.7	24.9 – 26.8
Washing (twice)	Water	300	10 mins		
Pickling	Water Sodium chloride Sulfuric acid Formic acid	100 8 1.2 1.0	3-4 hrs and left overnight	1.5 – 3.1	20.4 – 26.6
Tanning and Basification	Chromium (III) sulphate Feliderm®MGO (Clariant, Switzerland)	8 0.4	90 mins 4 hrs	4.1 - 4.9	22.8 – 27.7° C

*Note: Percentage of the added chemicals was based on the weight of the salted calfskin pieces; mins=minutes and hrs=hours.