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THE USE OF NATURAL PRODUCTS IN THE LEATHER INDUSTRY: DEPILATION WITHOUT DAMAGE

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Abstract. Sheepskin, a by-product of the meat industry, is often processed to leather and used for fashion items including jackets, coats and gloves. Where the tanneries are distant from the abattoirs and freezing works, the raw skins have to be transported long distances to be processed and in warm weather, there is the potential for putrefaction of the skins which are then of no commercial value. Before they can be tanned, the wool is removed by a process that traditionally uses strong alkali and sulfides both of which are environmentally unfriendly. We have found a natural product that prevents putrefaction, preserving the skin for days at room temperature. In addition, it allows easy removal of the wool from the skin, eliminating a need for most of the beamhouse processes that produce toxic waste.

1 Introduction

The chemicals used in the pre-tanning processes are significant contributors to environmental pollution.[1] Large quantities of both solid and liquid waste are produced during various pre-tanning processes, with one-third of the pollution produced from the leather industry being due to the sulfide and alkaline water waste from the depilation process.[2-3] Conventional depilation and collagen fibre-opening processes for sheep skins require painting a thick solution containing calcium or sodium hydroxide and sodium sulfide, on the flesh side of the pelt which allows the wool to be mechanically removed from the skin.[4] The chemicals are removed by washing the depilated skins with copious volumes of water which then has to be treated before it can be fed into the waste stream. As environmental compliance becomes more demanding such processes will place a significant financial burden on tanneries and the industry.[5]

To address this problem, research efforts have been aimed to depilate skins using enzymes as these are recyclable and environmentally friendly. Various enzymes, such as keratinases, proteases and lipases have been shown to successfully remove hair from skin, but usually damage it.[6-9] Furthermore, in some cases, the addition of sulfide to the enzyme mixture is necessary to provide depilation efficiency.[10] Hence, at present, although significant advances have been made, the use of enzymes has not been effective on an industrial scale.

We have found a natural product that when incubated with fresh sheepskin prevents putrefaction and preserves the skin for up to five days at room temperature (20 °C). In addition, it allows easy removal of the wool from the skin through gentle thumb pressure. Microscopic examination of the depilated product showed no sign of damage to the surface of the skin. This innovative procedure not only depilates, but also preserves the skins over the time required for transport or processing. This paper describes the progress that has been made to understand the science behind this phenomenon and to compare the properties of skins depilated using this method with those depilated using the traditional beamhouse process.

2 Materials and Methods

2.1 Natural product survey

All fresh sheepskin was obtained with the help of New Zealand Leather and Shoe Research Association (LASRA). Unwashed skins were cut into 20 cm x 6 cm sections using a sterile scalpel blade. These were then placed in sterilised sealed containers before being submerged in sufficient volume of sterilised natural product to ensure the wool was completely covered. The process of depilation was followed by monitoring the pH of the liquid, the smell, the condition of the skin and the ease of depilation twice a day until the wool could be removed from the skin with gentle thumb pressure. Controls included sterile water, water at pH 4.0 and water that was maintained at pH 4.0 during the experiment.

2.2 Identification of microorganisms that could contribute to the depilation process

To assess whether the microbiome of the depilation liquid was changing throughout the process, samples were taken for identification of the organisms present before and after depilation. It should be noted that in all these experiments, the skin samples were not washed or treated in any way before the experiment.

2.2.1 Isolation of the microorganisms after successful depilation with natural products

One hundred μL of the natural product that was used to incubate and depilate sheepskin were taken and plated on five different nutrient agar plates: Tryptone soya broth (TSB), Luria broth (LB), *Lactobacilli* MRS broth (MRS), malt and fungal minimal growth media (Wilson's media)[11] agars. Distinctive colonies were isolated and re-plated on their respective nutrient agar plates.

2.2.2 Identification of the microorganisms isolated from depilation trials

Standard procedures were used to extract microbial genomic DNA[12] from cultures grown from each single colony, which was then subjected to the colony polymerase chain reaction (PCR) using the primers listed in Table 1. The PCR products were purified using ethanol precipitation[13], then sequenced using a capillary ABI3730 DNA Analyser (Thermofisher; USA) with the BigDye Sequencing Ready Reaction Mix (ThermoFisher; USA). The results were analysed using the Nucleotide Basic Local Alignment Search Tool (BLASTn); http://blast.ncbi.nlm.nih.gov/Blast.cgi online search engine.

Table 1. PCR primer sets for the amplification of the bacterial 16s and fungal 18s rRNA.

Primer name	Sequence	Gene to be amplified	Reference
fD1 rD1	AGAGTTTGATCCTGGCTCAG AAGGAGGTGATCCAGCC	16S rRNA	14
Eub338F Eub518R	ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	16S rRNA	15
nu-SSU-0817 nu-SSU-1196	TTAGCATGGA ATAATRRAATAGGA TCTGGACCTGGTGAGTTTCC	18S rRNA	16

2.3 Fractionation experiment

To detect any compound that may contribute to the preservation of the skins, the liquid used for depilation was subjected to size fractionation and screened for differences in protein content by denaturing gel electrophoresis (SDS-PAGE). The fractions were also tested for antimicrobial properties using traditional plate assays and ability to depilate sheepskin.

2.3.1 Fractionation of the natural product

The natural product used to successfully depilate skin samples was first passed through a 0.2 μm filter to remove any particulate material, then subjected to sequential ultrafiltration using different molecular weight cut-off (MWCO) membranes (100, 30, 10 and 3 kDa (Millipore; USA)), in a pre-sterilised pressure-based stirred cell (Amicon 8400; USA). Both the filtrate and retentate of each fraction were collected in sterilised bottles and stored at 4 °C before they were analysed by tricine SDS-PAGE. Samples were also tested for antimicrobial activity against a number of different microorganisms. This involved making indicator plates with different bacterial species embedded in the agar forming wells in the plate with a hole cutter, then adding 50 μL of each filtrate or retentate into each well and incubating at 37 °C overnight. Fresh skins were also incubated in each filtrate and retentate fraction to assess their ability to depilate.

2.4 Chemical analysis of the depilated sheepskin

Collagen crosslink and glycosaminoglycan (GAG) analyses were done on sheepskin before and after depilation with product A. Extraction and quantitation of the collagen crosslinks were done as described by Naffa *et al.* (2016).[17] Glycosaminoglycan analyses were done as described by Naffa (2017).[18]

2.5 Scanning electron microscopy (SEM) analysis of the depilated sheepskin

Sheepskin samples that were depilated with the natural product were analysed with the FEI Quanta 200 Environmental Scanning Electron Microscope (ThermoFisher; USA) at an accelerating voltage of 20 kV. Samples were dried to critical point using liquid CO_2 as the critical point fluid and absolute ethanol as the intermediary with the Polaron E3000 series II (Quorum Technologies; UK) critical point drying apparatus. Samples were then mounted onto aluminium stubs using double sided tape and sputter coated with approximately 100 nm of gold with a Baltec SCD 050 sputter coater (Capovani Brothers Inc.; USA). The surface of the samples was then examined.

3 Results and Discussion

3.1 Natural product A depilates and preserves sheepskin

The results of the depilation experiment with 8 natural products showed that only one sample, named product A, could successfully depilate sheepskin within 3 – 5 days. The skins treated with product A appeared pink in colour, were plump and smelled slightly fermented after depilation, whereas the skin treated with other solutions, appeared grey and had an unpleasant odour due to the onset of putrefaction. A change in the pH of the media during the process was also observed. Product A had an initial pH of 7.0 that continued to drop as the incubation progressed and stabilised when it reached 4.5 at which point, the wool could be easily removed. In contrast, the pH of the other samples increased over the course of the experiment, eventually reaching 7.5 – 8.0.

3.2 Four main microorganisms identified in the media after depilation

The change in the pH and the smell during the process of depilation was indicative of the success of the process (*i.e.* if the pH of the media dropped to 4.5 and smelled slightly fermented and musty, the sheepskin could be successfully depilated). It has been shown that an increased pH in meat is a sign of putrefactive bacterial growth and subsequent product spoilage.[19] This was also seen in our

depilation trials as the increased pH of the media after depilation was concomitant with a rotten smell. The question therefore arises, what is in product A that enables depilation to occur, and at the same time prevents the skins from putrefying. Control experiments showed that skins on their own in acidified water or in pure water putrefy quickly, even at low temperatures. Product A also deteriorates quickly if exposed to the air at room temperature. It is therefore possible that compounds and/or microorganisms in product A combined with those on the skins create an environment that suppresses the growth of putrefying microorganisms while encouraging the growth of others. It is feasible that both produce enzymes responsible for depilation and perhaps other antimicrobial compounds that control the microbiome.

To identify the microorganisms from depilation trials, the liquid after depilation was plated and differences in the colony morphologies of the microorganisms grown on nutrient agar plates were observed. It was not surprising to see a large variety of microorganisms present on the plates swabbed with fresh sheepskin or samples from water incubated with the skin (**Fig. 1**). Product A was also plated before and after the incubation with fresh sheepskin (**Fig.2**). After skin had been incubated with product A, the number of different colonies was drastically reduced to three to four types. The common morphologies are shown in **Fig. 3**.

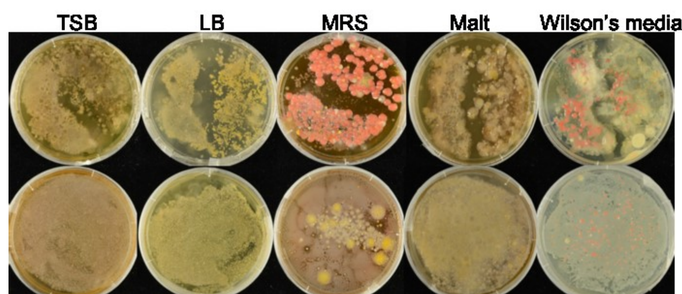

Figure 1. Agar plates that were swabbed with fresh sheepskin (top row) and the H_2O that was incubated with sheepskin (bottom row).

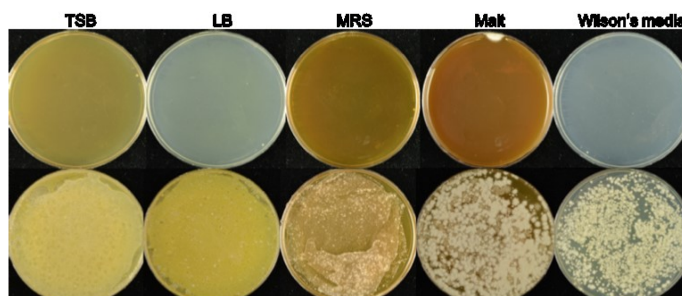

Figure 2. Agar plates spread with sterilised product A (top row) and the liquid that was incubated with sheepskin after depilation (bottom row).

After treatment with product A (Fig. 1-3) the number of colony types decreased significantly, compared to those cultured form the fresh sheepskin. The reason for the apparent survival of only a few species is not yet understood and is part of the investigation of this study. To identify the microorganisms, DNA from the individual colonies were isolated, and used as template to amplify phylogenetic markers encoding 16S rRNA and 18S rRNA genes. The amplicons were subsequently sequenced and analysed using the standard bioinformatic tool NCBI BLASTn. Two dominant fungal and two dominant bacterial species were identified. Out of these, three are known to produce antimicrobial substances, including bacteriocins. It is, therefore, possible that they are responsible for the reduction in the number of microbial species that occurs during depilation.

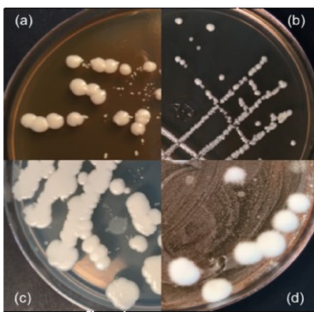

Figure 3. Examples of the common morphologies of microorganisms seen on nutrient agar plates after depilation with product A. (a) cream-coloured large circular colonies (b) cream-coloured small circular colonies (c) cream-coloured irregular-shaped colonies (d) large white fluffy colonies.

Using culture-based methods only the species that grow rapidly under standard laboratory conditions (*i.e*. incubation temperature, types of nutrients in the growth media) can be identified. Hence, it is possible that some of the microorganisms that are involved in this complex interplay between the sheepskin microbiome and product A remain unidentified using these methods. Therefore we used metabarcoding as a culture-independent method to obtain microbial community profiles of the sheepskin before and after depilation alongside that of product A *post* depilation. The sequencing data is currently being analysed through a bioinformatic pipeline.[20]

3.3 Fractionation of sterilised product A and its depilation effect

All retentate and filtrate fractions were able to depilate sheepskin within 4-5 days without obvious damage to the skin. The pattern of depilation was identical to that observed when unfractionated product A was used; skins depilated in all fractions smelled slightly fermented, and had a pH of 4.5. Furthermore, tricine SDS-PAGE analysis showed that the peptide/protein concentration of all fractions was low and their profile was identical (**Fig. 4**). The protein concentrations of all fractions were around 0.03 to 0.08 mg/mL. As all fractions of retentates and filtrates of product A were able to depilate and preserve sheepskin, it is likely that the antimicrobial substance is contributed by one or more metabolites produced by the bacterial population. A metabolomics analysis of the liquid pre and post depilation will further identify metabolites that have the potential for antimicrobial action.

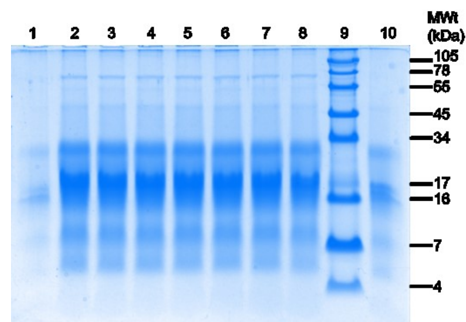

Figure 4. Tricine-SDS gel (16.5 %) of sterilised product A fractions. Lane 1, 100 kDa MWCO retentate; lane 2, 100 kDa MWCO filtrate; lane 3, 30 kDa MWCO retentate; lane 4, 30 kDa MWCO filtrate; lane 5, 10 kDa MWCO retentate; lane 6, 10 kDa MWCO filtrate; lane 7, 3 kDa MWCO retentate; lane 8, 3 kDa MWCO filtrate; lane 9, molecular weight marker; lane 10, sterilised product A filtered through 0.8 um filter.

3.4 Biochemical analysis of the depilated sheepskin reveals differences in the skin molecular composition after depilation with our method

3.4.1 Glycosaminoglycans (GAGs) analysis

Glycosaminoglycans were extracted from three biological samples of raw and sterilised product A depilated sheepskin. Skin 3 appeared to have a significantly higher concentration of GAGs compared to skins 1 and 2, although this difference was attenuated in the depilated skins (**Fig. 5**). The raw sheepskin GAG concentrations were also similar to previous reports.[21] Although the GAG content of the skins decreased by half after depilation by sterilised product A, they were still 20 times higher than those measured in pickled sheepskin.[21] Many reports have shown through the processing steps of liming, deliming, bating and then pickling, significant amounts of GAGs are removed.[22] Further experiments will show whether this higher concentration of GAGs remaining in the skins has any effect, either beneficial or detrimental on the physical properties of the final leather product.

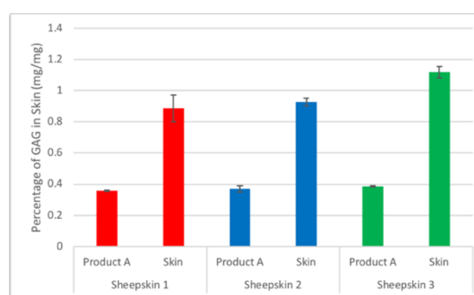

Figure 5. Percentage of sulfated glycosaminoglycan in raw and sterilised product A depilated sheepskin. Error bars represent standard deviation.

3.4.2 Collagen crosslink analysis

Collagen crosslinks were extracted from three biological samples of raw and sterilised product A depilated sheepskin. All skin samples were shown to contain mature collagen crosslinks histidine-hydroxylysinonorleucine (HHL) and histidinohydroxymerodesmosine (HHMD), and immature crosslinks hydroxylysinonorleucine (HLNL) and dihydroxylysinonorleucine (DHLNL). The ratio between the mature to immature crosslinks was calculated (**Fig. 6**) and decresed two to three fold after depilation with product A. Preliminary results showed that the process of soaking raw sheepskin in product A to depilate removed three to five folds of the skins' total crosslinks. Future experiments will compare the crosslink concentration of product A depilated sheepskin with conventionally picked skin. It has been reported that there is a relationship between the total crosslink concentration and the strength of skin. Sheepskin, is a relatively weak skin, compared to cow skins, and already has the lowest crosslink content.[21] Reducing it further may not provide a good outcome. Further testing on leather made from skins depilated with product A will be carried out to determine the effect of this reduction.

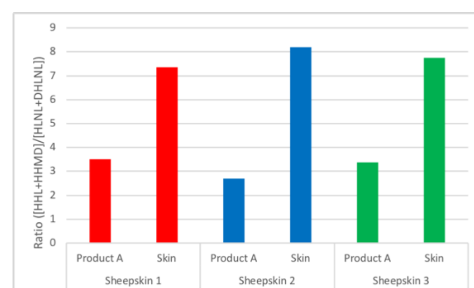

Figure 6. The ratio of mature crosslinks to immature crosslinks in sheepskin before and after depilation with product A.

3.5 Microscopy analysis of the depilated skins showed no signs of damage

SEM was used to examine the surface of the skin after depilation with product A. The depilation treatment did not appear to damage the surface of the skin, and the wool was cleanly removed without damage to the follicle (**Fig. 7**). Bacterial species could be seen on the surface of the skin and the hair follicle, which was not unexpected as two dominant bacterial and a few other bacterial species could be cultured from product A *post* depilation.

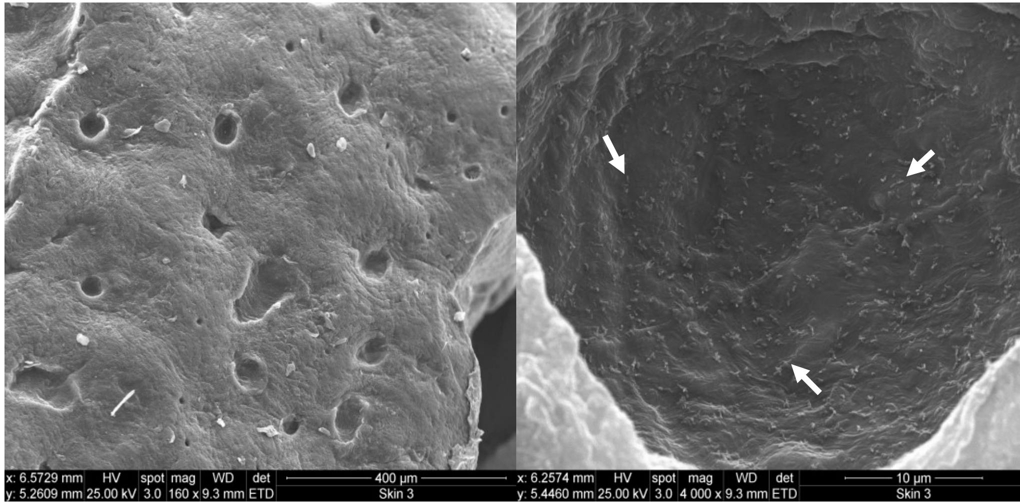

Figure 7. SEM images of the sheepskin that was incubated with sterilised product A and was successfully depilated. (a) The surface of the skin (b) the empty hair follicle; white arrows indicate the presence of bacteria.

Conclusions

We found a natural product that not only depilates sheepskin but also prevents putrefaction of the skin for a significant period of time. The investigation into understanding this phenomena has produced a number of interesting and unpredictable results.

1) Skins exposed to product A, have a pH of approximately 4.5 after depilation. Hence, it is possible they could be tanned without further treatment (*i.e.* bate and pickle).
2) Four main microbial species were identified from the liquid after depilation was complete. It is possible that they secrete metabolites that are responsible for preserving the skins as well as enzymes responsible for the depilation.
3) Microscopy analysis of the depilated skins showed no signs of damage. A full biochemical analysis of the skin components is being carried out to compare the molecular differences between skins depilated with product A and skins depilated with lime-sulfide.
4) We have developed a method for depilating sheepskin that avoids the production of toxic waste and is environmentally friendly.

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