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Milk provides the basis for an eco-friendly shorter process for skin preservation and leather manufacture

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ABSTRACT

The traditional beamhouse method for tanning skins to produce leather uses copious amounts of water, large quantities of chemicals deemed to be dangerous by the public and produces large quantities of liquid waste (90 kg/ton of raw hide) that have become a significant problem due to environmental concerns. In this work, we chart the development of the use of a milk product that both preserves unwashed sheepskins and depilates them, removing the need for salt, sulfide and lime and reduces the copious amounts of water used in the traditional process. This method can completely replace salting and the first five steps of the traditional beamhouse process and the depilated skins can be taken straight to degreasing and tanning. Evaluation of the depilated skins by both eye and high-resolution microscopy showed no apparent damage to the grain or fraying of the collagen bundles. The leather produced from them had physical properties that were either identical or better than those of leather made using the traditional methods. To try and understand the process, microbial culturing of the depilation liquid routinely identified two major bacterial species (*Lactococcus lactis* and *Lactiplantibacillus plantarum*) and two major fungal species (*Geotrichum candidum* and *Yarrowia lipolytica*) among other minor species. We predict that the secretion of an unidentified mix of antimicrobial substances and extracellular enzymes from these microorganisms are responsible for the preservation and depilation of sheepskin. This procedure shows potential for further development and could result in a sustainable green beamhouse operation for the leather industry.

1. Introduction

Leather products are manufactured from animal skins that have been subjected to various chemical procedures to form a product with high tensile and tear strengths and is both insulating and water resistant. Because of these properties, leather has been used to make clothing, shelters, tools, and other objects since 5000 BCE, and it has been estimated that by 2027, the global leather industry will be worth US $306.1 billion to the world economy (GIA, 2021). However, the industry is facing the negative impact of environmental pollution caused by the chemicals used, and the solid and liquid wastes produced during the manufacturing process. Sheepskins make up 6% of the raw material used by the world leather industry (Food and Agriculture Organization of the United Nations, 2016). They are a by-product of the meat industry that must be disposed of if not used, incurring a significant cost to farmers. When processed, the environmental pollution caused by the current fellmongering process incurs significant costs to the industry because of changes in legislation that are being introduced to control environmental pollution. Another consideration is the copious volumes of water used in the process, (a precious resource in these times of climate change). Therefore, it is imperative that leather technologists and scientists worldwide find environmentally friendly alternatives for skin/-hide preservation and leather production.

Animal skin is made up of three main layers: the epidermis, dermis, and hypodermis. The epidermis is the outer-most protective layer of skin and consists of keratinocytes (keratin-producing cells) and parts of the hair shaft (fibre). Beneath the epidermis lies the epidermis-dermis junction, that contains the coarse basement membrane, a special structure made up from types IV and VII collagens, as well as proteoglycans (heavily glycosylated proteins) (Breitkreutz et al., 2013). The dermis of sheepskin contains water (53%), protein (27.5%), fat (18.3%) as well as trace minerals. The major structural components of dermis are collagen types I and III, which make up approximately 60–70% of the dry weight of skin (Frantz et al., 2010). The uppermost layer of the dermis, also

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termed the enamel, is responsible for the outward appearance of the final leather product which is thought to be due to the presence of the beaded microfilament network formed by collagen VI (Edmonds et al., 2008). The bottom layer of skin, the hypodermis, is made up muscle and fat. Both the epidermis and hypodermis are removed during leather making.

Once the skin has been removed from the animal, it needs to be preserved, especially in warm weather, or when stacks of skins are plastic wrapped for transport to the tannery. The conventional method is the application of salt (30–40%, w/w), which then has to be removed and the skins rehydrated, which uses large volumes of water (Covington and Wise, 2019). The first step of making sheepskin into leather, known as depilation, is removal of the wool, which is achieved by painting a thick strong alkaline sulfide-lime solution (Na_2S or NaHS, NaOH, $Ca(OH)_2$) on the flesh side of the pelt and allowing it to soak through to the dermis. The alkaline conditions open up the major protein network formed by the collagen fibres, allowing the chemicals to reach the hair shaft where they act to degrade the keratins in the hair follicle, and the wool to be removed either mechanically or by hand. Na_2S is, however, a strong reducing agent, that interferes with the oxidation of organic wastes (*i.e.*, hair/wool and proteins), and as a result, contributes to the high chemical oxygen demand (COD) and biological oxygen demand (BOD) of the effluent. In fact, 70% of the total COD and BOD load from leather making is due to the beamhouse process. Even worse, if the pH of the effluent drops below 9.5, H_2S, a colourless, flammable gas that is poisonous when inhaled, is released. (Madhavi et al., 2011). After depilation, the skin is washed several times in water (delimed) before being incubated with an ammonium salt solution (to reduce) the pH, then enzymes such as trypsin or chymotrypsin that degrade the non-collagenous proteins in the skins in a process called bating. After washing again in cool water, the skins are immersed in a solution of sulfuric acid and salt at pH 0.9–1.1 that both preserves and prepares them for tanning, a process called pickling. Due to their high fat content, sheepskins in New Zealand undergo a process called degreasing right after pickling to remove the fat layer in the skin before it is tanned (Covington and Wise, 2019). The purpose of this is to allow the uniform penetration of tanning reagents into the skin matrix and through the pelt cross section during tanning. After degreasing, the skins are then tanned and the raw skin is permanently turned into a stable substance.

Much effort has gone into the search for 'greener' depilation agents. Interest has largely been focused on the use of proteolytic enzymes as depilation agents because they are safer to handle and biodegradable. An early experiment carried out by Schlosser et al. (1986) using an undisclosed *Lactobacillus* culture to preserve hides was based on the fact that many lactic acid bacteria were being used in the food industry for the preservation of food. They incubated hides in this culture, to find that not only were the hides preserved, but they were also depilated. However, no further research was carried out to understand the mechanisms of either preservation or depilation, and an extensive search of the literature has not found any examples of the use of bacterial cultures used for dehairing. In contrast, many enzymes of bacterial origin have been trialled as dehairing agents on a laboratory scale and have been shown to successfully remove hair from skins as reviewed by Khambhaty (2020). For example, Dettmer et al. (2013) used an enzyme preparation produced by a *Bacillus* species to successfully depilate hides in a process that reduced COD, BOD and the total nitrogen and sulfides in the waste water. However, most also damaged the skin surface, and sulfide had to be added to the enzyme(s) used to increase the depilation efficiency of enzymatic dehairing of goatskins using a cell-free supernatant of a *Pseudomonas fluorescens* culture (Kandasamy et al., 2012), of bovine hides using a number of commercial enzyme preparations (de Souza and Guterres, 2012), a semi purified enzyme secreted by *Bacillus megaterium* (Wahyuntari and Hendrawati 2012), and latex peptidases from *Calotropis procera* and *Cryptostegia grandiflora* (Lopéz et al., 2017). Furthermore, the use of enzymes requires stringent control of both pH and temperature to produce consistent depilation. Although recombinant protein technology has seen a decrease in the purchase price of enzymes, they are still expensive in comparison to the traditional chemical methods, and although significant advances have been made, the use of enzymes to depilate skins has not been widely adopted by the leather industry (Sujitha et al., 2018).

We report here the development of a process that removes wool from sheepskins using whey or whey permeate, both by-products of the cheese-making industry. This process is environmentally friendly as it not only omits the use of sulfide and alkali for depilation, but also prevents microbial degradation of skins for up to a week at ambient temperature, reducing the need for washing soaking and the use of NaCl and biocides for preservation. Sheepskins immersed directly in whey or permeate without washing could be both preserved and depilated during transport to the tannery and then be taken straight to degreasing, skipping the lime, delime, bate and pickle pre-tanning steps. The quality of the skins depilated by this method was assessed using scanning electron microscopy, transmission electron microscopy, tear strength, tensile strength, and shrinkage temperature and found to be either better or the same as those processed using the traditional sulfide method. Lastly, the possible

Mechanisms of both preservation and depilation of sheepskin using whey/permeate is discussed.

2. Materials and methods

2.1. Materials

All sheepskins were collected from a local freezing works (Ovation Ltd., Fielding, New Zealand). The unwashed skins were transported to New Zealand Leather and Shoe Research Association (LASRA) where the flesh was removed using a flesher (Rizzi, Italy). Depilation trials with milk products and by-products were carried out on approximately 15 × 8 cm pieces of defleshed sheepskin cut from the official sampling position (OSP). Milk products, including homogenised milk, UHT milk, and milk powder (Anchor, Auckland, New Zealand), were commercially sourced from the supermarket. Fresh liquid whey was obtained from the Cartwheel Creamery, Pohangina, New Zealand. Fresh liquid whey was sterilised at 121 °C for 15 min. The resulting precipitate was centrifuged at 10,000×*g* for 20 min, leaving a straw-coloured liquid. Whey protein powder (Red8, Auckland, New Zealand) and milk powder were made up to 6.25% (w/v), and 12.5% (w/v) with H_2O, respectively. For simplicity, artificial whey permeate solution (4.5% (w/v) lactose (Sigma Aldrich ≥99%), 0.0035% (w/v) $CaCl_2$ (Merck, ≥99.99%), 0.0045% (w/v) NaCl (Sigma Aldrich ≥99%), 0.14% (w/v) KCl (Sigma Aldrich ≥99%), and 0.05% lactic acid (v/v) (Sigma Aldrich ≥85%), autoclaved at 121 °C for 15 min. This will be referred to as permeate solution in this paper. A list of chemicals used for tanning, and their manufacturers is given in the supplementary information.

2.2. Depilation trials using milk products and permeate solution

Ten pieces of unwashed sheepskin (15 × 8 cm) were cut from the skin of a single animal, placed in sterilised glass containers then submerged in enough liquid (milk, UHT milk, milk powder, whey, sterilised whey, whey powder and permeate solution, all bovine in origin) to ensure the wool was completely covered. All the following procedures were carried out in a biohazard hood. The process of depilation was followed by monitoring the pH of the liquid, and its smell until the wool could be easily removed from the skin using gentle thumb pressure. Controls of H_2O, acetic acid, and lactic acid solution with an initial pH of 4.5 were included to ensure that the antimicrobial and depilation effects were not due to pH alone, or to lactic acid which is the acid produced by many microorganisms cultured in milk (Oikonomou et al., 2020). After the samples were depilated, they were subjected to TEM and SEM, and samples of the depilation liquid at completion of the process were taken for microbiological analysis. In order to test reproducibility, the

experiment was repeated using a second animal.

2.3. Scaled up trials using permeate

Three pieces of sheepskin, each from a different animal (nine in total) were used in this study. Each was cut in half, and one half depilated using the conventional sulfide method, while the other was treated with permeate. Due to the different sizes of each piece of sheepskin, the volume of permeate solution used varied. Each piece of skin was placed in a sterile plastic container large enough to let it lie flat, then submerged in enough sterile permeate solution to completely cover all the wool. After the liquid was added, a lid was used to seal the container, which was then left at room temperature until depilation was complete. To check progress, the lids were removed every 12 h and the skins visually inspected and tested for ease of depilation using gentle thumb pressure. A sample of the liquid was also taken and its pH measured using a pH strip. After three to four days (d), depending on the ambient temperature, when the wool was fully loosened, the skins were removed from the permeate solution and depilated. Part of the skin was taken for tear and tensile strength analysis (pre-tanning samples), and the rest processed to leather using the LASRA standard protocol from the degreasing stage (Table S1). The other half of the pelt was both depilated and processed using the standard LASRA protocol (Table S1).

2.4. Scanning electron microscopy (SEM) evaluation of permeate-depilated sheepskin

SEM samples were prepared by the Manawatu Microscopy Imaging Centre (MMIC), Massey University, Palmerston North, New Zealand, using the protocol detailed in the supplementary information.

2.5. Transmission electron microscopy (TEM) evaluation of the permeate-depilated sheepskin

Permeate-depilated skin samples were cut into 1 mm thin slices using a sterile scalpel blade and fixed overnight using a fixative containing 2.5% (v/v) glutaraldehyde (Merk, NJ, USA) and 0.06% (w/v) cuprolinic blue (Polysciences, PA, USA) in 50 mM acetate buffer (pH 5.6) supplemented with 0.3 M $MgCl_2$. After fixing, the samples were rinsed three times with the same fixative solution omitting cuprolinic blue. Samples were treated for 1 h with 0.5% sodium tungstate in 50 mM acetate buffer (pH 5.6), and then overnight with 0.5% sodium tungstate in 30% ethanol. The samples were then processed by the MMIC according to the protocol described in the supplementary information.

2.6. Identification of the viable microorganisms in the milk media at the completion of depilation

Five culture media (TSB, LB, MRS, malt, and Wilson's media (Wilson et al., 1969)) were used to culture viable microorganisms from the milk, UHT milk, milk powder, whey, sterilised whey, and permeate solutions at the completion of depilation. TSB and LB are general high-nutrient culture media for bacteria; MRS is a media specifically designed to culture lactobacilli; malt and Wilson's media are general media with high and low-nutrient contents respectively, that are optimised for fungi. Sterile agar plates (1.5%) were made for each sample replicate using the five different media to comprise a set of plates for each depilation solution. One hundred (100) μL of the liquid from each completed depilation was pipetted onto each set of sterile nutrient plates, then spread over the surface using a sterilised glass spreader. The TSB, LB, and MRS plates were incubated overnight at 37 °C, while the malt and Wilson's media plates were incubated at 30 °C. Morphologically distinctive colonies were picked at random and re-streaked onto new sterile plates. Microbial genomic DNA (gDNA) was extracted from cells using the standard procedure of Gussow and Clackson (1989). The crude gDNA was used as a template to amplify the 16S- and 18S rRNA gene-encoding regions with primers 27f/1492r (Weisburg et al., 1991), Eub338/Eub518 (Lane, 1991; Muyzer et al., 1993), and nu-SSU-0817-5'/nu-SSU-1196-3' (Borneman and Hartin, 2000) (Table 1) and PCR conditions described in Table S2. The resulting amplicons were purified by ethanol precipitation (Sambrook et al., 2012) and sent to Massey Genome Service (MGS, Massey University, Palmerston North) to be sequenced (Capillary ABI3730 DNA analyser, Thermo Fisher Scientific, USA). Sequences were analysed using the blastn algorithm against NCBI (Altschul et al., 1990) nucleotide collection (nr/nt) database. Species identified were those that had at least 97.5% sequence identity. The process was repeated a second time with the solutions obtained using a different animal.

Table 1
PCR primer sets for bacteria 16S and fungal 18S ribosomal ribonucleic acid (rRNA) encoding gene amplification.

Primer name	Sequence (5' → 3')	Target gene	Amplicon length (bp)
27f	AGAGTTTGATCCTGGCTCAG	Full length 16S rRNA	1400
1492r	GGTTACCTTGTTACGACTT		
Eub338	ACTCCTACGGGAGGCAGGAG	V3 region of 16S rRNA	200
Eub518	ATTACCGCGGCTGCTGCTGG		
nu-SSU-0817-5'	TTAGCATGGAATAATRRAATAGGA	Nuclear small subunit of 18S rRNA	420
nu-SSU-1196-3'	TCTGGACCTGGTGAGTTTCC		

2.7. Tear strength analysis of depilated skins and the final leather product

Tear strength measurements were carried out using the TA.XT Plus Texture Analyser (Stable Micro Systems, Surrey, UK) according to the international standard ISO 3377–2:2016 (Williams, 2000). The method used is detailed in the supplementary information.

2.8. Tensile strength analysis of depilated skins and final leather product

Tensile strength measurements were carried out using the TA.XT Plus Texture Analyzer (Stable Micro Systems, Surrey, UK) at room temperature. Test samples were prepared at the same time as the tear strength samples except they were cut into a 'dog bone' shape of 100 mm × 20 mm pieces parallel to the backbone. The process used is detailed in the supplementary information.

2.9. Leather shrinkage temperature measurements

The thermal stability of leather was measured by its shrinkage temperature (^{0}C). The shrinkage temperatures of chrome-tanned leather made from both permeate- and chemically depilated sheepskins were measured according to the ISO 3380:2015(E) standard protocol with modifications (ISO 3380, 2015, supplementary information).

3. Results and discussion

3.1. Sheepskin depilation using milk products and by-products

We repeated the experiment of Schlosser using commercially sourced yoghurt made from bovine milk and found it prevented putrefaction and

Table 2
Depilation trials with milk products and by-products.

	Initial pH of solution	Final pH	Days to depilate	Smell	Depilated skin condition
Milk	6.8	4.5	3	Fermented/ sour milk	Pink, shiny, plump, and very smooth
UHT milk	6.7	4.5	3		
12.5% (w/v) milk powder solution	6.8	4.5	3		
Whey	6.2	4.5	3		
Sterilised whey	6.0	4.5	3		
Sterilised permeate	6.0	4.5	3		
6.25% (w/v) whey protein solution	6.5	6.0	5	Putrefied and rotten	Grey, easily broken into pieces, and rigid
H_2O	7.0	7.5	5		
H_2O with acetic acid	4.5	7.5	3		
H_2O with lactic acid	4.5	8.0	3		

depilated unwashed sheepskins after 3 to 4 d of incubation (unpublished result). As yoghurt is a fermented milk product, further trials were carried out using other bovine milk products, such as, milk, UHT milk, milk reconstituted from milk powder, whey, sterilised whey, and whey protein solutions. Controls of H_2O, and water acidified to pH 4.5 with both acetic and lactic acids were included in the experiment and the course of the depilation was monitored through pH, and smell (Table 2).

Although all media tested eventually depilated skins, including the H_2O and acid controls, two major differences were observed. Sheepskin that was depilated with milk products except for whey protein solution, smelled like fermented milk, whereas the others (H_2O, acid controls and the solution made from powdered whey protein) had the unpleasant smell of putrefaction. The other difference was the change in pH during depilation. All media that smelled like fermented milk at the end of the depilation trial had an initial pH around 6.5–6.8, that dropped to approximately 4.5 as the incubation continued. At this stage, wool could easily be removed from the skin samples, and the skin appeared to be pink, plump, and shiny. In contrast, in the other group of samples the pH increased over the course of depilation, eventually plateauing between 7.5 and 8.0. Surface damage was obvious, the skin was grey and smelt rotten. Interestingly, all the milk products trialled were able to depilate sheepskin without any obvious damage, with the exception of whey protein powder. Whey powder is manufactured by ultrafiltration of liquid whey, which retains the proteins but removes lactose and other minerals, suggesting that it was not an antimicrobial milk peptide or protein that was responsible for the preservation of the skins. Because it was impossible to source commercially prepared permeate, an artificial permeate solution containing only lactose and traces of metal ions was made, and when this was used to incubate the skins, they were preserved and depilated with the same efficiency seen for milk and whey.

3.2. Physical appearance of the permeate-depilated sheepskins

After incubating the sheepskins in permeate for three to 4 d, the wool was easily removed by scraping with the thumb. Fig. 1a shows that despite leaving the unwashed piece of skin in permeate at room temperature for three to 4 d, the skin did not go rotten, and the surface showed no visible damage. To test whether this process was effective on a larger scale, one-quarter of the sheepskin was processed using permeate while the other was processed using the traditional beamhouse method. The wool was easily removed from the permeate treated samples after 3 d and they were degreased and chrome tanned using the standard LASRA process (Table S1, Fig. 1b and c). Although not tested in any way, the wool consistently had a silky feel and did not appear to be damaged during removal. To visualise any damage that was less obvious to the eyes, SEM images of the surface of the depilated skin were taken. These showed that the grain appeared to be smoother than that of the conventional sulfide depilated bated and pickled skins (Fig. 2a and b), and the wool was cleanly removed from the follicle without damage to the skin (Fig. 2c and d). Interestingly, bacteria could be seen on the surface of the skin and in the hair follicle (Fig. 2e), which was not unexpected due to the fact that various bacteria, especially lactococci and lactobacilli, could be cultured from the permeate that depilated sheepskin (Section 3.3). TEM was used to visualise the arrangement of collagen fibrils in permeate-depilated sheepskin and showed that individual collagen fibrils were densely packed and nicely aligned, although no proteoglycans, which should appear as small rods linking the collagen fibres, could be seen (similar to the results obtained by Naffa, 2017) (Fig. 3).

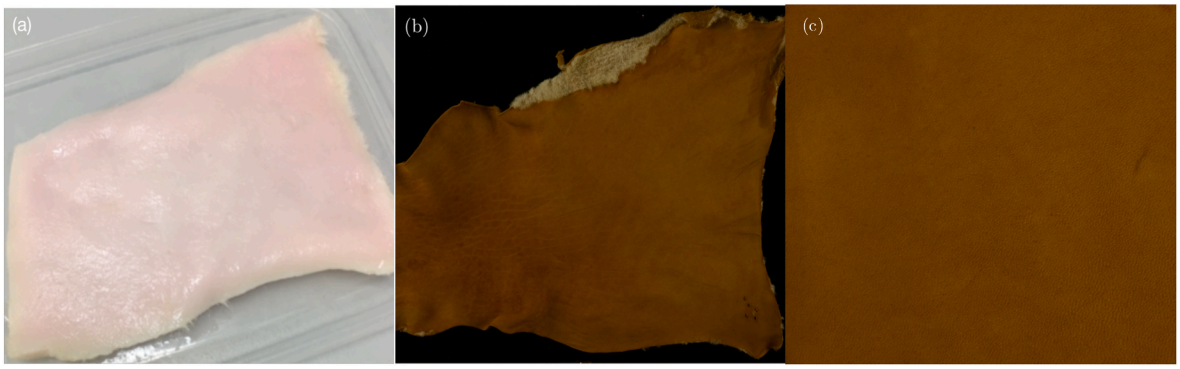

Fig. 1. Images of the sheepskin depilated with permeate and the final leather product. (a) The OSP region after depilation with permeate. (b) One quarter of a sheepskin depilated using permeate, degreased then chrome tanned. (c) A closer look of the grain of the leather in (b).

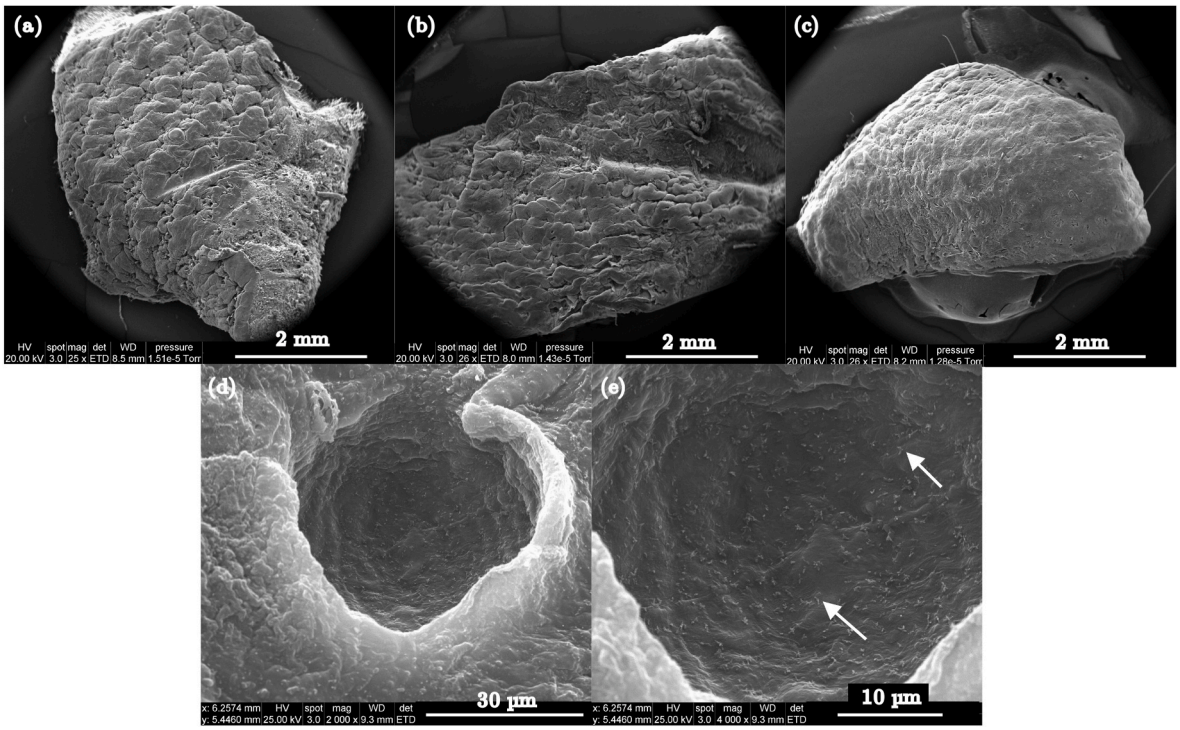

Fig. 2. SEM images of the sheepskin depilated using (a) the conventional sulfide method, (b) the conventional sulfide method then processed to bate, (c) permeate. (d) higher magnification of sheepskin depilated using permeate showing the empty wool follicle, and (e) higher magnification of the empty wool follicle, showing microorganisms residing on the wall (white arrows). The magnification is indicated by the bar at the base of each image.

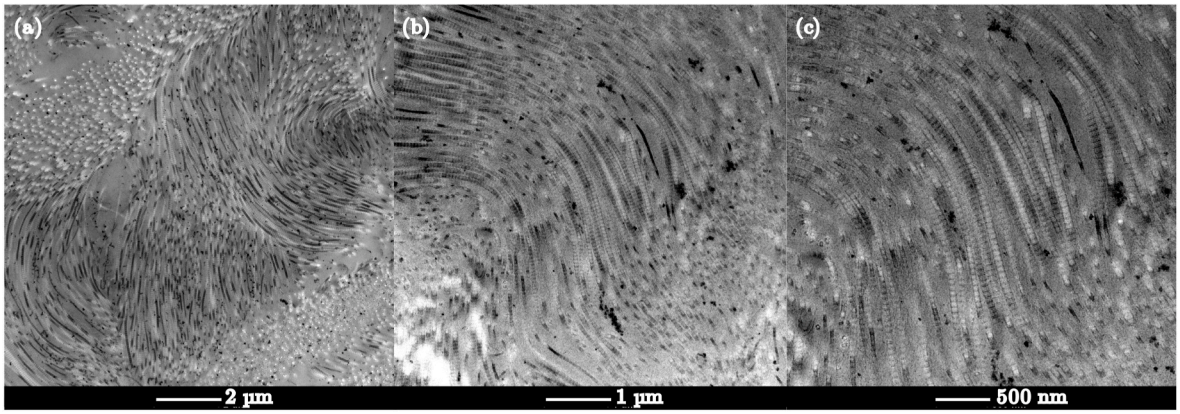

Fig. 3. TEM images of a section of the corium of permeate-depilated sheepskins. The collagen fibrils are stained with lead citrate. (a), (b), and (c) are at different magnifications as shown by the bar at the base of each image. The characteristic D-banding of collagen fibrils can clearly be seen in (b) and (c).

3.3. Identification of microorganisms isolated from depilation trials with various milk products

Conventional microbial culturing methods were used to isolate and identify the microorganisms present in the depilation solutions (*i.e.*, sheepskin immersed in a specific milk product) at the end of each depilation trial. Three biological replicates were tested for each milk product. Differences in the colony morphologies of the microorganisms grown on nutrient agar plates were observed (Fig. S3), and a total of 75 colonies were randomly selected for colony PCR and their full length or partial 16S- and 18S rRNA genes amplified and sequenced. Out of these, 21 bacterial and six fungal species were identified (Tables 3 and 4). Bacteria were classified into three phyla (Bacteroidetes, Firmicutes and Proteobacteria), four classes, six orders, 12 families, 17 genera, and 22 species (Table 3). The most frequently identified genera were: *Lactococcus*, *Lactobacillus* (now known as *Lactiplantibacillus/Levilactobacillus/Latilactobacillus* and 22 other genera (Zheng et al., 2020)), and *Hafnia*. Fungi were classified into two phyla (Ascomycota and Basidiomycota), three classes (Microbotryomycetes, Saccharomycetes and Tremellomycetes), three orders, four families, five genera (*Galactomyces candidum* is the teleomorph of *Geotrichum candidum*) and five species (Table 4). *Geotrichum candidum* (*Galactomyces candidum*) and *Yarrowia lipolytica* were the two most frequently isolated and identified fungi.

Two of the most frequently isolated and identified bacterial genera, Lactococcus and Lactobacillus, are lactic acid bacteria (LAB). LABs are known to produce antimicrobial metabolites (*e.g.*, lactic acid and acetic acid) and bacteriocins (proteinaceous or peptidic toxins) and are widely used in the food industry for preservation purposes (Collins et al., 2010). LABs are able to use lactose in their energy production pathway, unlike other bacteria, and produce lactic acid as a major metabolite. *Lactobacillus plantarum* (Duan et al., 2019), *Geotrichum candidum* (Boutrou et al., 2006) and *Yarrowia lipolytica* (Gonzalez-Lopez et al., 2002) have all been

Table 3
Bacteria species identified in the sheepskin depilation trials using various milk products and by-products.

NCBI BLAST identification	Accession	Primer used	% Identity	Culturing media	Depilation media
Bacteroides xylanolyticus	MT192666.1	27f/1492r	98.6%	Malt	Sterilised permeate
	MT459291.1	Eub338/Eub518	100.0%		
Citrobacter europaeus	NR_156052.1	Eub338/Eub518	98.8%	Malt	Whey
Enterobacter sp.	MH477686.1	Eub338/Eub518	99.4%	Wilson's media	Sterilised permeate
Enterococcus faecalis	MT158867.1	Eub338/Eub518	100.0%	Wilson's media	Whey
Empedobacter falsenii	MN198120.1	Eub338/Eub518	98.7%	MRS	Sterilised permeate
Escherichia fergusonii	NR_074902.1	27f/1492r	99.1%	LB	Whey
	MT645516.1		100.0%	MRS	Sterilised permeate
Escherichia coli	CP066366.1	27f/1492r	100.0%	LB	Whey
	MW846276.1	Eub338/Eub518	100.0%	MRS	Sterilised permeate
Hafnia alvei	LR699008.1	27f/1492r	98.9%	TSB	Whey
	KC210872.1		99.1%	Wilson's media	Sterilised permeate
			98.3%		Raw milk
	KX674363.1		99.4%		Milk
Hafnia paralvei	MT470952.1	27f/1492r	99.2%	TSB	Whey
	NR_116898.1		99.7%	MRS	Sterilised permeate
			98.8%	Wilson's media	Milk
	NR_025334.1		99.2%		Milk powder
	MT470952.1	Eub338/Eub518	99.4%		Milk
	MN868256.1		99.3%	Malt	Sterilised permeate
Klebsiella aerogenes	MW784626.1	Eub338/Eub518	99.3%	Wilson's media	Sterilised permeate
Kurthia gibsonii	MN966854.1	27f/1492r	99.5%	Malt	Sterilised whey
	MK898830.1	Eub338/Eub518	100.0%		
Lactobacillus brevis	NR_116238.1	27f/1492r	99.5%	MRS	Whey
	MG722900.1	Eub338/Eub518	99.4%		
			100.0%	Malt	Sterilised permeate
Lactobacillus curvatus	MT645312.1	Eub338/Eub518	99.4%	MRS	Sterilised permeate
Lactobacillus graminis	MN640858.1	Eub338/Eub518	99.3%	MRS	Milk
Lactobacillus plantarum	MF623219.1	27f/1492r	97.9%	MRS	Raw milk
	MK652787.1	Eub338/Eub518	100.0%		
	EU931245.1		99.4%		Milk
	KT626385.1		100.0%		Sterilised permeate
			99.4%	Malt	Sterilised whey
Lactococcus lactis	NR_113960.1	27f/1492r	100.0%	TSB	Whey
			99.2%	Malt	Milk
	GQ337875.1		98.5%		Sterilised whey
			98.2%		Sterilised permeate
	MT545096.1		99.5%	MRS	
	MT597705.1		99.9%		Raw milk
			99.9%	Wilson's media	Sterilised whey
	MH666046.1	Eub338/Eub518	100.0%	MRS	Raw milk
			98.6%		Sterilised permeate
			99.3%	Wilson's media	
Lactococcus lactis subsp. *cremoris*	NR_040954.1	27f/1492r	99.3%	Wilson's media	Milk powder
Lactococcus lactis subsp. *lactis*	MF108188.1	27f/1492r	99.7%	Malt	Sterilised permeate
Leuconostoc holzapfelii	NR_042620.1	Eub338/Eub518	98.8%	Malt	Milk
Lysinibacillus macroides	NR_114920.1	27f/1492r	97.9%	MRS	UHT milk
Pseudomonas sp.	KJ496054.1	27f/1492r	100.0%	Malt	Sterilised permeate
	MW844014.1	Eub338/Eub518	100.0%		
Proteus vulgaris	KX867797.1	27f/1492r	98.1%	MRS	Sterilised whey

Table 4
Fungal species identified in the sheepskin depilation trials using various milk products and by-products.

NCBI BLAST identification	NCBI Accession	% Identity	Culturing media	Depilation media
Galactomyces candidum	KY457577.1	99.7	Wilson's media	Milk
		99.5		UHT milk
		99.7	TSB/LB/MRS/Malt/ Wilson's media	Fresh whey
Geotrichum candidum	KY977411.1	99.7	MRS/Malt	Sterilised permeate
		99.7	Malt	Sterilised whey
Pichia insulana	NG_063091.1	99.5	Malt	Sterilised whey
Rhodotorula spp.	MT569975.1	99.5	Malt	UHT milk
Trichosporon lactis	NG_070852.1	99.7	Wilson's media	Milk powder
Yarrowia lipolytica	MH545931.1	99.2	Wilson's media	Sterilised permeate
		99.1	Malt	Sterilised whey
	NG_013120.1	99.7		Milk

reported to secrete extracellular enzymes (proteases, lipases, and glycosidases) and are commonly used in the cheese-making process . We therefore speculate that enzymes secreted by these microorganisms specifically degrade the structural components at hair root, facilitating the loosening of wool from the hair follicles and allowing it to be easily removed. Spoilage bacteria such as *Pseudomonas* and *Shewanella* were rarely identified.

3.4. Tear and tensile strength analysis of permeate-depilated sheepskins

To confirm that processing sheepskins with permeate resulted in a product as least as good as that produced using traditional methods, two sets of tear and tensile strength measurements were made for skins from the same animal that had been processed using both methods: (a) skin depilated using permeate against pickled skin that had been sulfide depilated, (b) the final leather product made from permeate-depilated skin *vs*. skin depilated with sulfide. Although tear and tensile strengths are related, they are not identical measurements. Both, however, are indicative of the strength of a material. The tear strength of leather

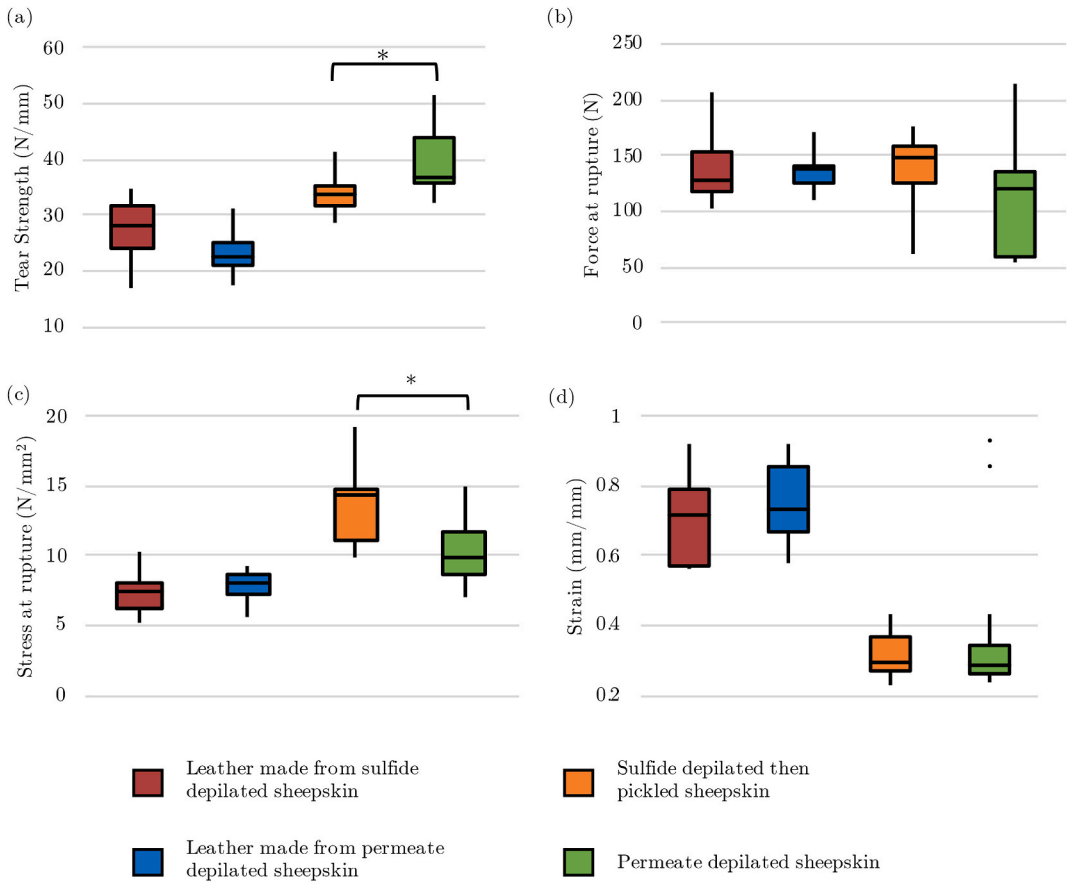

Fig. 4. Whisker plots showing the tear (a) and tensile strengths ((b) force at rupture, (c) stress at rupture and (d) strain) of tanned leather and pre-tanned skins. (*, p-value < 0.01. Dots represent outliers.)

reflects the 'toughness' of the material and its ability to resist rupture. As a result, it is commonly used as the industry standard for strength as it relates closely to physical performance. Tensile strength on the other hand, measures the mechanical properties of leather more precisely, as it is related to the strength of the collagen fibres in the skin (Sizeland et al., 2013). Both tear and tensile strengths were measured on three pieces of sheepskin from three different animals taken parallel to the backbone, then normalised to the thickness of the skins. There were no significant differences in the tear strengths of the final leather products produced from skins depilated using the two different methods, despite permeate-depilated skin having a slightly higher tear strength compared to that of pickled skin (Fig. 4a, one-way ANOVA, p-value = 0.001). Although pickled skin had a slightly higher stress value at rupture compared to permeate-depilated skins (Fig. 4c, p-value = 0.001), it did not appear to affect the tensile strength of the tanned leather products which had similar tensile strengths regardless of the process (Fig. 4b, c and d). Table 5 shows the force, stress and strain at rupture for tanned-leather and pre-tanned skin. A stress-strain curve for skin can be drawn from the tensile strength results, and typically shows three parts, the toe, heel, and the linear regions. In the toe region, at low strain, the skin behaves elastically, reflecting the removal of macroscopic crimps in the collagen fibres as force is applied. As the force is increased, the heel region of the curve is seen at medium strain, where the kinks in the collagen molecules are straightened out. Under high force the curve becomes linear as the collagen molecules are stretched out and glide against each other, uniformly aligning. Eventually, the skin ruptures and curls back (Yang et al., 2015). All leather and skin samples showed typical stress-strain curves in these three regions (Fig. 5).

Table 5
Comparison of tensile strength of tanned leather and pre-tanned skins. Each measurement was done on three different sheepskins, with four technical repeats on each skin. The values in the table represent the average of each measurement.

Sample type		Thickness (mm)	Force at rupture (N)	Stress at rupture (N/mm^2)	Strain at rupture (mm/mm)
Leather made from	Sulfide depilated skin	1.9	140.5	7.3	0.7
	Permeate depilated skin	1.7	134.3	7.7	0.8
Pre-tanned skin	Pickled	1.0	138.3	14.1	0.3
	Permeate depilated	1.0	116.7	10.4	0.3

To visualise the linear and non-linear ranges in the stress-strain curve, the first derivative (the slope) of the stress-strain curve was calculated and shown in Fig. 5. Typically, a linear region has a constant slope, therefore, its first derivative would be a relatively flat line with a constant value (*i.e.*, the slope is unchanged). In a non-linear region, the slope constantly changes, generating a curved line in the first derivative plot. There were no significant differences in the first derivatives of the

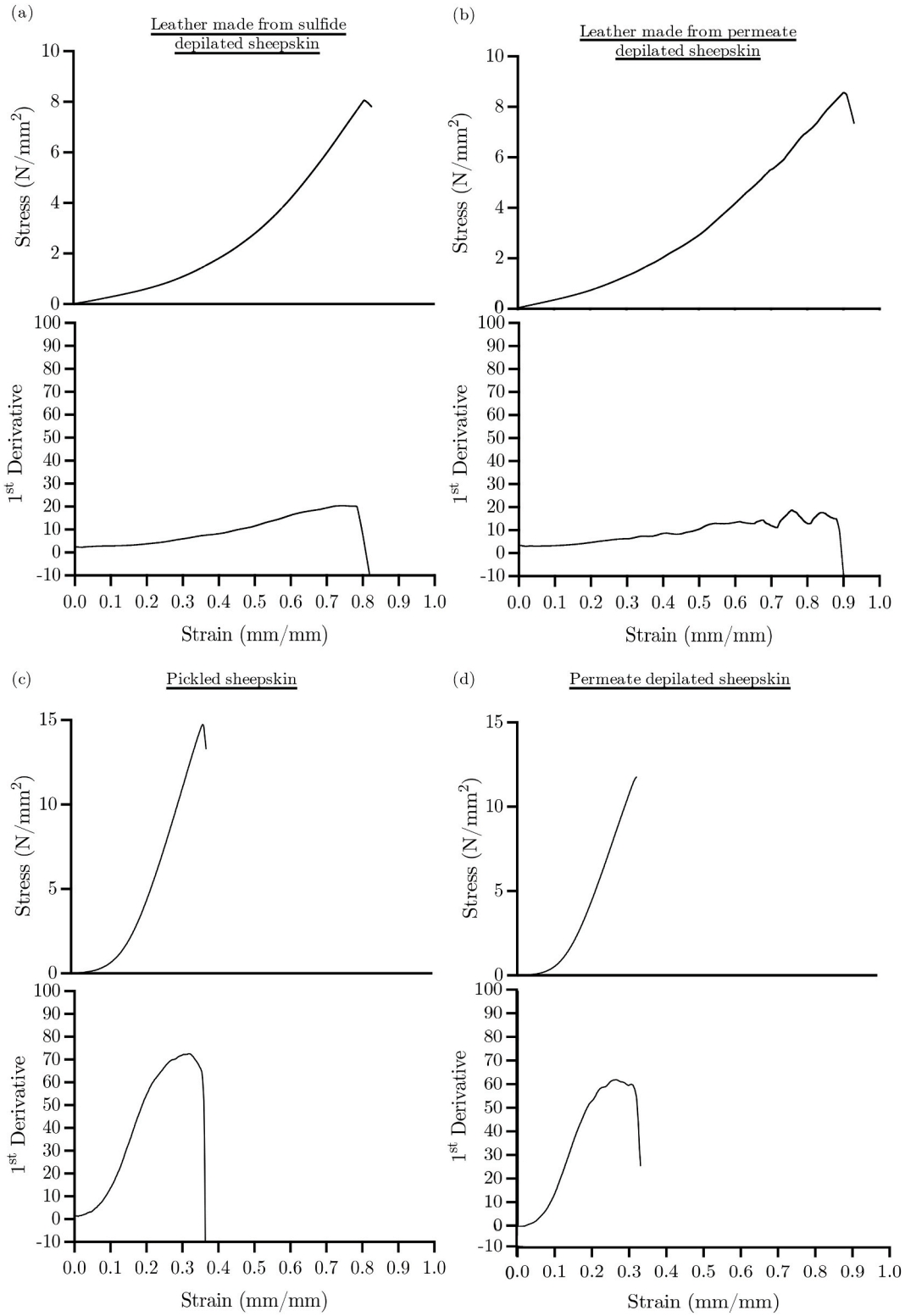

Fig. 5. Representative stress-strain curves and their first derivative (slope) curves of leather produced from sheepskin depilated using sulfide (a) and permeate (b), and their respective pickled (c) and permeate depilated (d) pre-tanned skins.

final leather products made from the two processes. They were both flat with similar slopes (between strain 0.1 and 0.5, slope of permeate-depilated skin leather product 25.2 *cf*. Slope of pickled skin leather product 21.6) strongly indicating there is no difference in the tensile strengths of these leathers (Fig. 5a and b). Although no significant difference was observed between the pre-tanned skins (*i.e.*, pickled skins and permeate-depilated skins, Fig. 5c and d) there are, however, obvious differences in the ranges of both the linear and non-linear regions of the curves for leather and pre-tanned skin. Pre-tanned skin samples in general, had a steeper slope in the linear region compared to leather samples, which is characteristic of a brittle substance (Diamant et al., 1972). This suggests that after tanning, the leather samples are more

ductile, as shown by their ability to stretch. In other words, both pre-tanned skin samples were more brittle (or stiff), as evident from the low strain (0.37 *cf*. 0.8 for leather) and the steeper slope of the first derivative plot (Fig. 5) for the pre-tanned skin samples. Even although the pre-tanned skin samples (pickled and permeate-depilated skins) were more brittle, they were able to withstand a higher stress than their matched leather samples before rupture (Fig. 5c and d).

3.5. Leather shrinkage temperature

The hydrothermal stability of skin, whether in its natural state, or chemically modified, is routinely measured by leather technologists as shrinkage temperature. The shrinkage temperature is defined as the effect of wet heat on the integrity of the material, and it can vary greatly depending on the tanning efficiency and structure of the collagen fibre network of the material (Covington and Wise, 2019). The shrinkage temperature of skin, including leather, is reported to be correlated to the thermal transition of the tropocollagen helical structure to random coil upon heat denaturation (Covington and Wise, 2019). In the case of native collagen, the shrinkage temperature is 65–70 °C, while for skin tanned using the most common protocol, it can reach as high as 120 °C (Covington and Wise, 2019).

The shrinkage temperatures of the leather depilated with permeate compared to conventional sodium sulfide is shown in Fig. 6. Although no significant difference was found between the shrinkage temperatures of leather depilated using the two different methods, that of the permeate depilated skin showed smaller differences from sample to sample. The leather made from chemically depilated sheepskins underwent three extra steps, delime, bate and pickle, to remove all non-collagenous proteins before the skin was degreased and tanned using the standard LASRA process (Table S1). In contrast, permeate-depilated leather samples went straight from depilation to degreasing then tanning, and on the basis of biochemical analyses retained more non-collagenous proteins (as found in the amino acid and proteomic analyses of sheepskin (not reported) in this publication). It has been long thought by leather chemists and technologists that the removal of non-collagenous proteins is essential for the efficient stabilization of the collagen network by chromium salts (Covington and Wise, 2019). The whisker plot suggests that the structure of the permeate processed leather is more uniform than the leather produced by the fellmongering process. The tanning procedure used in this work includes disodium phthalate, which has been reported to increase the shrinkage temperatures of wet blue sheepskin to 115–121 °C (Covington and Wise, 2019). While the shrinkage temperature of leather made from permeate depilated sheepskins appeared to be higher than this, they are no higher than those obtained for wet blue sheepskins exposed to other anions, where shrinkage temperatures of as high as 133 °C have been reported (Covington and Wise, 2019). Whether this increase is due to the incomplete removal of non-collagenous proteins, such as proteoglycans is not known and requires further research.

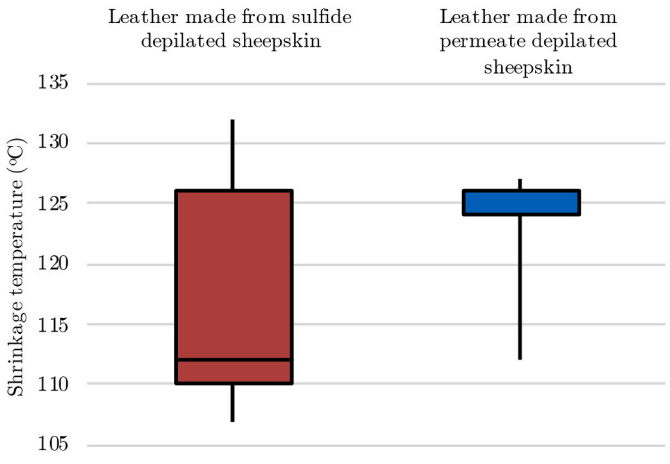

Fig. 6. Whisker plot showing the shrinkage temperature of leather samples made from sheepskin depilated using sulfide and permeate. p-value = 0.08

4. Conclusion

In this work, we have shown on a laboratory scale that a novel and environmentally friendly process for depilating and tanning sheepskin produces leather that is indistinguishable from or better than that produced using the traditional beamhouse methods. The process eliminates the need for NaCl and biocides to preserve the skins, and Na_2S, NaSH, NaOH, $Ca(SH)_2$, and $Ca(OH)_2$ to depilate them, and uses far less water than is currently used in the pre-tanning steps of current processes. Although they were not analysed, the waste products from permeate depilation are most likely to be comprised of water, a small amount of protein, other metabolites generated by the microorganisms, and microorganisms that are already prevalent in the environment. Others who have used a combination of enzymes with sulfides and alkali have shown there is a reduction in BOD, COD, total suspended solids, and total dissolved solids compared to the beamhouse process (Khambhaty, 2020). On this basis we would predict an even greater reduction in these parameters using permeate depilation. We predict that the lactose will have been exhausted by the lactic acid bacteria that predominate the culture at the point of depilation. Using this method to process a large number of randomly selected skins showed they were consistently depilated without damage using whey or whey permeate, both by-products of the dairy industry. It does not require careful temperature or pH control, and as long as the skin was left submerged in permeate, it could be preserved up to a week without putrefaction. We also showed that the skin depilated using permeate is equivalent to pickled sheepskin, and can be degreased and tanned, eliminating four pre-tanning steps. It also raised the question of whether it is necessary to completely remove all non-collagenous proteins from the sheepskin matrix to produce good leather.

Our experiments have allowed us to propose a mechanism for the observed preservation and depilation of the raw skins. It became obvious that the prevention of putrefaction was due to the high concentrations of lactose at the initial stages of the process. We speculate that both restricting the carbon source at the early stages of depilation combined with the gradual reduction in the pH of the depilation liquid, most likely due to the production of lactic acid, initially controlled the growth of the bacteria on the unwashed skin and wool. *Lactobacillus* and *Lactococcus* species are also known to produce many antimicrobial substances. As depilation proceeded and protein and other macromolecules were released from the skin, such substances were able to control the number of microorganisms now able to grow especially those associated with putrefaction. *Lactococcus lactis*, in particular, is known for its production of nisin, a wide spectrum bacteriocin that is often used as a preservative by the food industry. However, when fresh sheepskin was incubated in phosphate buffered saline (PBS) supplemented with nisin, putrefaction still occurred (results not shown) indicating the process of preservation is complex, and not due to a single compound.

Bacteria and fungi are also known to secret proteases and glycosidases, and those found to be present in this environment are known to secrete enzymes that could preferentially attack the structure around the hair follicle, loosening it to allow easy removal, but without disrupting

the collagen structure of the skin. These claims require further research that was carried out as part of this project and will be reported elsewhere. The use of whey or permeate, both by-products and in some cases waste products of the dairy industry to incubate sheepskins appears to be a technically simple and cost-effective process for removing wool and pre-tanning and has the potential to provide the leather industry with a cost-effective option to reduce its waste load on the environment. While a comprehensive study of its environmental COD and BOD loads was not carried out, this should be done to strengthen the claim that it is, for sheepskin at least, a 'green' process for the leather industry. Lastly, the observations about the quality of the wool removed using whey or permeate depilation warrants further investigation. It is possible that it has gained some desirable properties that could be exploited by an industry that is trying to find new high-value products.

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CRediT statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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