Biochemical and Physical Changes in Goatskin during Bacterial Putrefaction

by

Vimudha Muralidharan,[1,3] Renganath Rao Ramesh,[2] Balaraman Madhan[1] and Saravanan Palanivel[2]*

[1]*Centre for Academic and Research Excellence (CARE), Central Leather Research Institute*, Adyar, Chennai - 20, Tamil Nadu.

[2]*Leather Process Technology Department, CSIR-Central Leather Research Institute*, Adyar, Chennai, Tamil Nadu, India.

[3]*Department of Leather Technology, A C Tech (Housed at CSIR-CLRI), Anna University,* Chennai – 25, Tamil Nadu, India

Abstract

The quality of the raw animal skin decides the final quality of leather. Preservation processes of raw animal skins until leather making predominantly uses salting as a popular method owing to the bacteriostatic effect provided by salt. The detrimental impact caused by the usage of salt from the leather processing is well established. This necessitates the quest for developing an economical, efficacious and environment-friendly preservation system. The present work investigates the effects on the physical and chemical characteristics of the animal skin caused by the putrefactive bacteria with respect to time. Physical changes were studied using visual examination, SEM analysis, and histological staining techniques where the structural deterioration was evidently established. Changes in biochemical aspects were studied by observing degradation in proteoglycan levels and collagen from the goat skin taken at various time intervals. Furthermore, the microorganisms that were responsible for the degradation of various skin components were isolated from the skin over the period of 36 hours from flaying. The occurrence of collagen-degrading organisms within 6 hours of initiation of putrefaction and increased number of proteolytic and collagenolytic bacteria at the end of 36-hours observation were indicative of tremendous skin spoilage leading to deteriorated quality of raw material.

Introduction

Leather manufacturing is one of the industrial activities globally widespread, which involves the use of animal hides and skins as raw material. Animal skins are non-edible by-products of the meat industry which, when accumulated, can cause serious pollution issue. Animal skin is a heterogeneous fibrous mass, which becomes a biological substrate for the microorganisms to proliferate when left unattended. Skin, which is the raw material for manufacturing of leather, is susceptible to growth of bacteria, mold and other microorganisms due to the fact that it is a rich source of protein.[1] The prime constituents of skin are proteins, fats and minerals. Fresh skin contains 65% water, 33% proteins, and rest is fats and minerals.[2] The inherent moisture and atmospheric physiological conditions are very much suitable for microorganisms to grow at a rapid rate. In order to arrest the growth of putrefactive microorganisms, the water content of the skin is principally decreased using several curing techniques.[3] Curing techniques that are based on decreasing the moisture content are drying, salting, dry-salting, chilling and freezing.[4,5] Methods that use common salt for curing are predominant in tanning industry. Although salt is beneficial in terms of preservation cost and does not lead to any quality loss in finished leathers, it has been identified as a serious environmental pollutant. The usage of salt increases the pollution load of the tannery effluent leading to higher levels of TDS (total dissolved solids) and chloride content.[6] The TDS remains high even in the treated effluent which comes out of the common effluent treatment plant (CETP).[7] This type of pollution problem needs to be addressed. Many alternatives to salt based preservation system have been researched over the past few decades yet salt still remains the most cost-effective option when preservation is concerned.[8-12]

Putrefactive activities by microorganisms' initiate just after skin/hide is flayed from the carcass.[13] Thereafter, a process called autolysis governs the onset of degradation, followed by microbial putrefaction. Autolysis is a process involving self-degradation initiated by lysozymes and an enzyme called cathepsin, that provides a nutrient-rich environment for other microorganisms to invade.[14] The process of putrefaction is a collective effort by several varieties of microorganisms. These microorganisms involved in the process of putrefaction proliferate by sourcing carbon and energy from the animal skin. Microorganisms breaks down larger molecules of proteins and other substrate components with the help of extracellular enzymes.[15] Once the core matrix protein collagen gets degraded by the collagenases, other proteolytic enzymes begin to further break down the fragments, collapsing the fibrous structure of the substrate. Several aerobic and anaerobic microorganisms that were involved in the degradation of fibrous protein collagen have been investigated so far. Clostridial hydrolytic enzymes from some *Clostridium* species have been known for their ability to degrade the extracellular matrix components of skin such as collagen, hyaluronan etc.[16] Certain anaerobic bacteria such as *Clostridium perfringens*, *C. histolyticum* and *C. capitovale* were found to cause considerable damage to hides.[17] There is evidence that states a correlation between the rawhide bacterial collagenolytic activity and leather decay.[18] And so, the time delay that could be exempted for curing based on the inception of the collagenolysis must be explored. By such means, the final leather quality could be preserved.

*Corresponding author email: saravananclri@gmail.com

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The leather is made mainly of interweaving collagen fibers (or fiber bundles), and the physical properties of the leather are the sum of the physical properties of the fibers and their interweaving that makes up the fiber structure of the leather.[19] During putrefaction, there occur several changes in the physical and chemical features in the fiber structure of the skin, which eventually degrades the final value of the leather. Therefore, it is possible to attribute the faulty leather to the physicochemical changes taken place in the fiber structure due to putrefaction or delayed/ underrated curing.

Detailed understanding of the putrefaction and its effect on skins is important from the point of view of protecting the skins from the microorganisms. The degradative microbial population causes damaging impacts on the morphological quality of the raw material. This has been established previously by several biochemical research efforts which studied the effect of putrefaction on unpreserved and salted goatskins kept at ambient conditions for several days. The current study is aimed to elucidate and consolidate the effects caused by putrefactive microorganisms on the physicochemical characteristics of the animal skin, within a short time span of 36 hours. Putrefactive bacteria were isolated from the putrefying skin within time intervals for a set time duration and characterized for their proteolytic, lipolytic and collagenolytic behavior. The physical changes in the skin were observed by visual examination, histological staining and using Scanning Electron Microscope (SEM). The chemical changes brought about by putrefaction was determined by performing elemental analysis, estimating proteoglycan loss and collagen loss from the putrefied skin.

Materials and Methods

Materials

All microbiological media were procured from Hi-Media laboratories. Other chemicals and reagents were of laboratory or analytical grade procured from M/s Merck. Fresh Indian goatskins were procured from local slaughterhouse located in Chennai, India.

Isolation and characterization of putrefactive microorganisms

Fresh goatskin was allowed to putrefy under ambient tannery conditions of temperature ranging between 27°C and 30°C and humidity ranging between 60 and 80%. The goatskin was visually examined for every 6 h and skin samples of size 2.5 cm x 2.5 cm were cut using a sterile surgical blade from the butt and belly regions of that goatskin. Sampling was done every six hours until 36 hours. The samples were labelled as S0, S1, S2, S3, S4, S5 and S6 corresponding to each 6-hour sample respectively (Fig.1). The samples were collected and transferred to a sterile centrifuge tube (50 mL) containing 10 mL of sterilized distilled water right after collection and shaken using a vortex mixer for 15 min. The skin extracts of all samples were serially diluted using sterile physiological saline (0.85% w/v). The suitably diluted suspension of each sample was then plated out using spread plate method on nutrient agar and incubated at 37°C for 24 – 48 h.

Sample Code	Time (h)	Sample Code	Time (h)
S0	0	S1	0
S1	6	S2	6
S2	12	S3	18
S3	18	S4	24
S4	24	S5	30
S5	30	S6	36
S6	36		

Figure 1. Schematic for sample collection from putrefied goatskin

The plates from several dilutions were observed for growth of the bacteria. The bacterial isolates were screened and selected based on its repetitive appearance on the plates from the subsequent dilutions of each sample.

The isolated strains of bacteria from each sample were Gram stained using Gram staining procedure and further tested on various selective and differential media. The isolates were morphologically characterized and screened for their proteolytic, lipolytic and collagenolytic activity on skim milk agar, tributyrin agar and gelatin liquefaction media respectively.

Studying the physical changes in the skin fiber network during putrefaction

Fresh goatskin was procured and left to putrefy for observation. Samples from the butt section of the skin were collected right from the initiation of experiment taken as 0^{th} h and labelled as S1. Further sampling was done at every 6 h and labelled as S2 up to S6 (Fig.1). The samples were examined for the effects of microbial action on the internal fiber network using histological staining and Scanning Electron Microscopy (SEM) analysis.

Histological staining of skin samples

Samples were thoroughly washed with distilled water and cut into small pieces prior to fixation and sectioning process. The samples were fixed in formalin solution prepared by adding 0.9 g sodium chloride in 100 mL of 10% formaldehyde solution.[20] After fixation, the samples were progressively dehydrated using 70% ethanol, 80% ethanol, 95% ethanol, and 100% ethanol with two changes for every one hour. Xylene was used to clear the alcohol, three changes for

every one hour. Xylene cleared samples were treated with paraffin wax (56-58°C), two changes, one and a half an hour each. Samples were embedded in paraffin blocks and trimmed to be cut using microtome. Sections of 4 μm were obtained and stained using various selective and differential stains – Masson's Trichrome stain, Sudan Black B stain, Safranin O stain and Hematoxylin & Eosin stain to examine the degradation in the levels of collagen, lipids, proteoglycans and the overall fiber network of putrefied skin respectively.

Scanning Electron Microscope technique to study physical changes

Washed skin samples were gradually dehydrated using absolute ethanol ranging from 10-100% v/v concentration. Dehydrated samples were then mounted on an aluminum specimen stub, and coated with a thin layer of gold by Edwards E306 sputter coater. The samples were viewed microscopically using Leica Cambridge Stereoscan 440 Scanning Electron Microscope to examine the degradation caused by bacterial putrefaction.

Studying chemical changes in the animal skin during putrefaction

Fresh goatskin was left to putrefy under ambient tannery conditions and sampling was done as mentioned above. The skin samples were cut in order to determine the elemental degradation and collagen degradation. The extracts from the same skin samples were used to estimate the proteoglycan loss caused due to putrefaction. The experiments were done in triplicates and the standard deviation was calculated to measure the dispersion of data.

Elemental analysis of skin samples

Skin samples, after removal of hair, were weighed to determine the moisture content. A known amount of sample was taken in a previously weighed porcelain dish and kept in a hot air oven at 105°C for about 5 hours. After drying, the china dish was cooled in a desiccator and weighed. The percentage of moisture content[21] was calculated using equation 1. Same samples were then lyophilized and analyzed for carbon, hydrogen, nitrogen and sulfur content (CHNS) using Elementar analyser (Vario Micro Cube – Elementar, Japan). For CHNS analysis, lyophilized samples were weighed (3-5 mg) and taken in a silver tin capsule, fed into a combustion zone, which was then combusted in a reactor at 1150°C.

$$\% \text{ Moisture} = \frac{(\text{Inital weight (g)} - \text{Final weight (g)})}{\text{Initial weight (g)}} \times 100 \qquad (1)$$

Determination of degradation in proteoglycan levels

The skin extracts of the putrefied skin samples were estimated for the presence of proteoglycan. The determination of proteoglycan was done using Periodic – Schiff's assay.[22] Briefly, 10 μL of 50% periodic acid was mixed with 10 mL of 7% acetic acid to prepare periodic acid mixture. To 1 mL of skin extract, 100 μL of freshly prepared periodic acid solution was added, mixed and incubated at 37°C for 120 min. For full-color development, 100 μL of decolorized Schiff's reagent was added, mixed and allowed to stand at room temperature for 30 minutes. Water was taken as blank. Samples were tested in duplicates. The absorbance of blank and sample was read at 555 nm using UV-Visible spectrophotometer (Jasco V-660 Spectrophotometer). The concentration of proteoglycan was calculated by taking mucin as a standard, calibrated at a range from 0 to 100 μg.

Determination of degradation in collagen levels

The extent of collagen degradation of putrefied skin samples was estimated by determining the hydroxyproline content, a key amino acid released during break-down of the collagen.[23] Putrefied skin samples were washed with physiological saline, unhaired and cut into small pieces weighing about 10 milligrams. Skin pieces were hydrolyzed with 5 mL of 6N hydrochloric acid at 110°C for overnight in sealed test tubes. After hydrolysis, the samples were evaporated to dryness at 80°C for 3-4 hours, the residue was dissolved in water and made up to known volume and used for hydroxyproline estimation. Hydroxyproline oxidation was initiated by adding 1 mL of Chloramine-T to each sample test tube. The contents of the tubes were mixed by shaking for few minutes and allowed to stand for 20 min at room temperature. To each test tube, 1 mL of perchloric acid was added and the contents were mixed and allowed to stand for 5 min. Finally, 1 mL of 20% p-Dimethylaminobenzaldehyde (PDAB) solution was added and the mixture was shaken. The tubes were placed in a 60°C water bath for 20 min and cooled for 5 minutes. The color developed was read using a spectrophotometer (Jasco V-660 Spectrophotometer) at 557 nm and the concentration of hydroxyproline was calculated using hydroxyproline standard ranging from 1-10 μg/mL. Collagen content was then calculated using equation 2

$$\text{Collagen} = 7.46 \times \text{hydroxyproline (μg/mL)} \qquad (2)$$

Results and Discussions

Observations during putrefaction

Goatskins left in ambient tannery conditions were observed visually during different time intervals, considering the initiation of observation to be 0^{th} hour and so on up to 36 hours. Visual observation at each time interval was done by carefully examining the flesh and grain side of the putrefying skin. During the first 3 hours from the initiation of visual examination, there was no perceptible odor. The following hour, a putrid odor that emanated from the skin was vividly observed. This obnoxious odor indicated the initiation of putrefaction. After 8 hours from the initial observation time, the flesh side of the skin was observed to be covered with a slimy coating on its surface. Around this hour, the skin's physical appearance and rigidity of the skin had started deteriorating. Around 10 hours, the loosening of hair follicles from within the surface of skin had started drastically, indicating the progress of putrefaction. Such loosening of hair is called hair slip and it is known as the first sign of putrefaction.[24] After 12 hours, complete hair loosening was observed.

The putrid odor at 12 hours was found to be strong. At 18 hours, the skin sample was found to be slippery due to release of protein degradation products. The structural integrity of the skin was found to be very poor. After 24 hours, the adipose (flesh) layer was found to be degraded significantly and loosened. The skin components such as hair, flesh and the grain surface had been worsened noticeably. Around 36 hours, the characteristic putrid odor was highly objectionable and the oozing fluids from the skin invited maggots (Fig. 1), marking the end of visual observation activity.

Characterization of putrefactive microorganisms

Animal skin is an outstanding organic substrate for microorganisms to grow. The present study aimed at isolating different kinds of microorganism that thrived between the time interval window of 0 to 36 hours observation period, on the surface of the putrefying skin. Varied microorganisms including few fungal species were observed to be grown on the nutrient agar plates. Amongst others, only the bacterial species were isolated at every 6 hours from 0 hours of the observation window until the 36th hour. The microbial isolates were characterized by their morphology, Gram stain response, proteolytic activity, lipolytic activity and collagenolytic activity. The invasion profile of bacterial isolates with time in Fig. 3 reveals that the onset of degradation of collagen and other protein has begun since the initial six hours. Presence of proteolytic and lipolytic bacteria was witnessed from the samples collected at zero hour of observation. This observation necessitates the need for a preservation technique that could curb the microbial growth within hours of flaying of skin.[25] At the outset, the organisms that could thrive on the globular proteins might have started growing. After 12 hours, the proteolytic bacteria increase in number feeding onto the degradation products of the globular proteins from the goatskin. After 18 hours, which marks the 4th stage of the observation window, lipolytic bacteria were found to be in abundance, which was found to be utilizing the lipid from the flesh side of the skin as their substrate for growth. During visual examination of the putrefied goatskin, after 24 hours the adipose layer was found to be heavily degraded. This could be explained owing to the action of lipolytic bacteria that degraded the lipid portions of the flesh side. Breakdown of inter-fibrillary globular proteins within the initial 24 hours, provided access to the lipid pockets for the lipolytic bacteria. At around 30 – 36 hours time scale, bacteria with multiple hydrolytic behavior (i.e. isolates exhibiting proteolytic, collagenolytic behavior) predominated the bacterial population (Table I). These consortia of bacteria synergistically degraded the collagen remaining in the skin. At this stage, collagen had deteriorated extensively, releasing several byproducts that caused the foul odor and invited the maggots. The microbiological aspect of the current study marked a certain set of organisms, which exhibited proteolytic activity without any collagenolytic activity identified at nine hours and thereafter might be primarily responsible for the degradation of globular proteins and proteoglycans. Similarly, the lipolytic organisms identified after nine hours might have been primarily responsible for degradation of lipids and adipose tissue. The bacterial profile corroborates well with the visual observation of the completely putrefied skin.

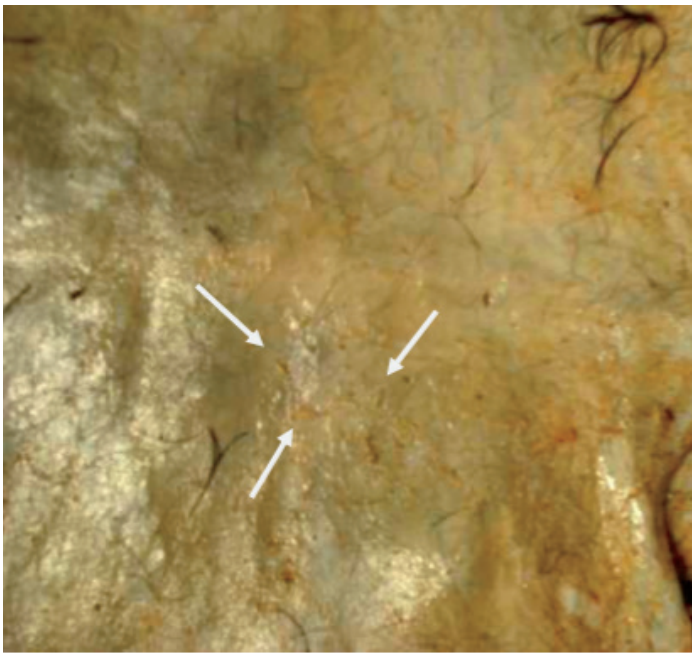

Figure 2. Flesh side of putrefied goatskin after 36 h (*Arrows indicating invasion of maggots*)

Similar results were observed when some of the leather making raw materials were assessed for bacterial abundance. Fresh hides prior to leather processing were found to be inhabited by a variety of microorganisms such as *Bacillus cereus*, *B. megaterium*, *B. sphaericus*, *B. subtilis*, *Kurthia variabilis*, *Micrococcus roseus* and *Staphylococcus aureus* that were proteolytic and collagenolytic in nature.[26] In a study conducted by Kayalvizhi *et al.* (2008),[27] almost 60% and 34% of total bacterial population isolated from fresh goat skins were found to be proteolytic and lipolytic, respectively. Unpreserved hide which was putrefied by the detrimental effects of several coccus-shaped species and rod-shaped species bacteria such as *Proteus vulgaris, B. subtilis* were also investigated.[28] The diversity of microorganisms that have degradative effects on raw cowhides and goatskins were studied for developing plant-based preservative formulation.[29] *Staphylococcus spp.*, *Micrococcus spp.*, *Corynebacterium spp.*, *Bacillus spp.*, *Escherichia coli* and *Pseudomonas spp.* were the predominant microorganisms isolated from Sudanese raw cattle hides and sheep skins that were obtained in unpreserved conditions in a tannery for leather processing.[30]

Physical changes observed during putrefaction - Histological staining of skin sections

Physical deterioration of the internal components of animal skin with time is one of the evident guides of microbial putrefaction. The skin samples collected were cut into sections and stained using four selective stains, to study the microscopic changes occurred in each of the key skin components such as collagen, proteoglycans, and lipids and the overall fiber network. The stained micrographs of the samples revealed the progress of degradation that occurred with time.

Table I

Characterization of bacterial isolates from putrefied goatskin

Isolate ID	Appearance on agar plate	Gram Reaction	Collagenolytic Activity[a]	Proteolytic Activity[b]	Lipolytic activity[c]
S0: 0th hour sample					
0.1	Dry flat white dots	Gram positive	_	+	_
0.2	Moist slimy smeared growth	Gram positive	_	+	+
0.3	Layered pale yellow growth	Gram positive	_	+	+
S1: 6th hour sample					
1.1	Creamy white shiny growth	Gram positive	_	_	+
1.2	Creamy yellowish small dots	Gram positive	+	_	_
1.3	Creamy flat moist dots	Gram positive	+	+	_
1.4	Dark creamy lustrous growth	Gram positive	_	+	_
1.5	Very small pale yellow dots	Gram positive	+	_	_
S2: 12th hour sample					
2.1	Swarming layered growth	Gram negative	+	+	_
2.2	Yellow dry growth	Gram positive	_	_	+
2.3	Very small creamy transparent dots	Gram positive	_	_	+
2.4	Orange lustrous viscous growth	Gram negative	_	+	_
S3: 18th hour sample					
3.1	Reddish orange viscous growth	Gram negative	+	+	_
3.2	Swarming streamlined growth	Gram negative	+	+	_
3.3	Bright yellow moist small dots	Gram positive	_	_	+
S4: 24th hour sample					
4.1	Swarming streamlined growth	Gram negative	+	+	_
4.2	Swarming streamlined growth	Gram negative	_	_	+
4.3	Filamentous growth	Gram positive	_	+	_
4.4	Transparent milky white growth	Gram negative	_	_	+
4.5	Creamish moist flat growth	Gram positive	+	+	_
S5: 30th hour sample					
5.1	Swarming growth	Gram negative	+	+	_
5.2	pale cream small transparent growth	Gram positive	+	+	+
5.3	transparent creamy slime like growth	Gram negative	+	+	_
S6: 36th hour sample					
6.1	Transparent creamy flower small dots	Gram negative	+	+	+
6.2	Creamish orange moist viscous growth	Gram negative	+	+	_

Masson's Trichrome stain for collagen

The Masson's Trichrome stain, that selectively stained collagen blue, revealed the collagen's degradation levels inside the matrix (Fig. 4). The connective tissue is stained blue, nuclei are stained purple and the cytoplasm is stained pink. The samples were viewed at two levels of magnification – 10x (Fig. 4 a-f) and 40x (Fig. 4 a'- f'). The collagen fiber bundle at the initial stage at 0th hours was found to be intact and tightly interwoven. It is evident from micrographs that at 6 hours, the collagen fiber bundle had started splitting. This could be due to the degradation of reticulin sheath that was responsible for the structural integrity of the collagen bundles. Also, the voids that appeared with brown rings indicated the details of hair follicles (Fig. 4b and Fig. 4b'). The samples from 18 hours showed that the collagen fibers were split up further. The voids pertaining to hair follicles were then seen to be stretched wide and empty, proving to be evidence of hair slip phenomenon as observed during the visual examination. At 24 hours, the structural integrity of collagen fibers was found to be impaired tremendously to the extent that the fibers were split up almost completely (Fig. 4d'). At 30 hours, the disintegration and degradation of collagen were witnessed clearly

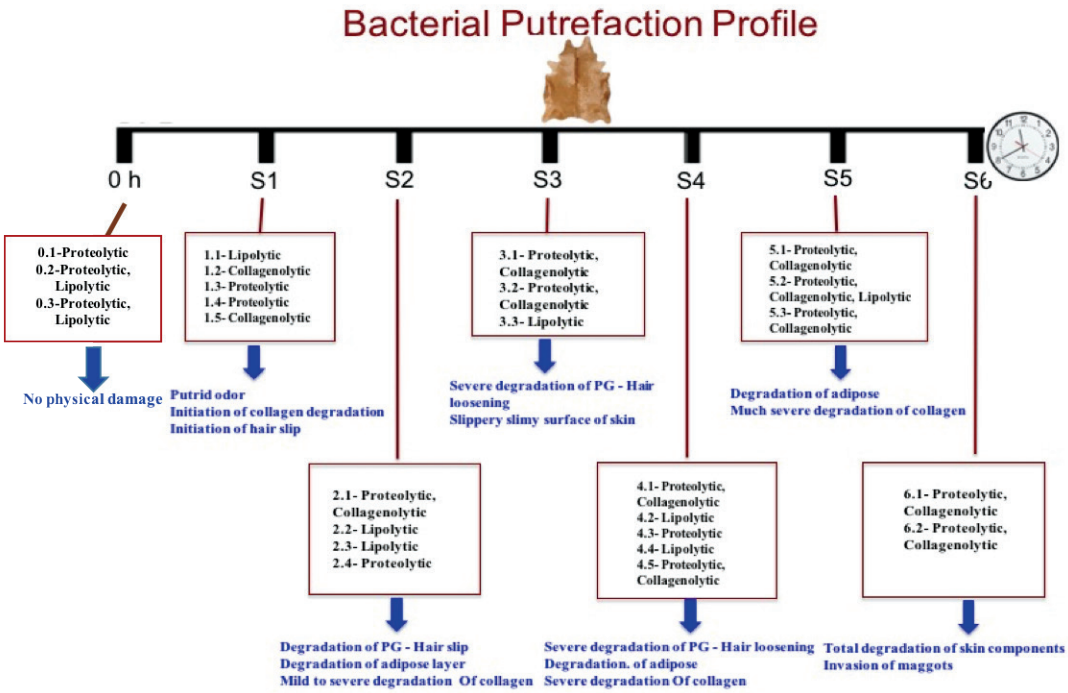

Figure 3. Putrefactive bacterial profile of putrefied skin samples collected over a period of 36 hours

by the disrupted fiber arrangement. The samples from 36th hour show that the fiber network was dilapidated and lost all the cellular network tightness leading to cellular atrophy. These results fall well in agreement with the visual observations. The degradation effects observed here could be linked to the results obtained while studying the bacterial invasion on the skin.

Safranin O stain for Proteoglycans

Proteoglycans, that form an important integral part of the skin's fiber network, were observed using Safranin O stain (Fig. 5 a-f). The micrographs indicated the physical changes in proteoglycans due to putrefaction. Proteoglycans are known to be present near the hair bulb and are responsible for holding the hair intact with the skin.[31] It is established that the presence of mucoid component such as proteoglycans play a very important role in tissue cohesion, stability and provide protection to the collagen.[32] Degradation of proteoglycan is associated with consequent loosening of hair and change in the cohesion of collagen fiber structure. This, in turn, brings about hair slip. The pictures clearly indicate the presence of proteoglycans around hair bulb during the initial hours of observation. The

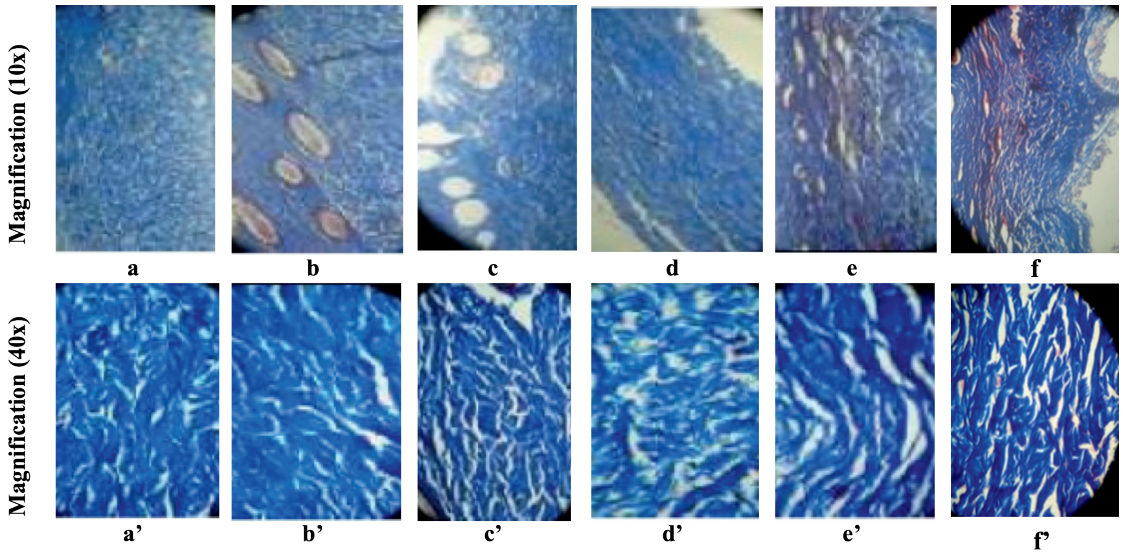

Figure 4. Masson's Trichrome Stained sections of putrefied goatskin for studying Collagen degradation of putrefied skin samples: **a, a'** – skin section showing intact and tightly interwoven fibers at 0th h; **b, b'** – details of the hair follicles visible during 6th h; **c, c'** – initiation of collagen fiber splitting at 18th h; **d, d'** – enhanced fiber splitting at 24 h; **e, e'** – disruption of fiber arrangement at 30th h; **f, f'** – major dilapidation of fiber arrangement causing cellular atrophy at 36th h

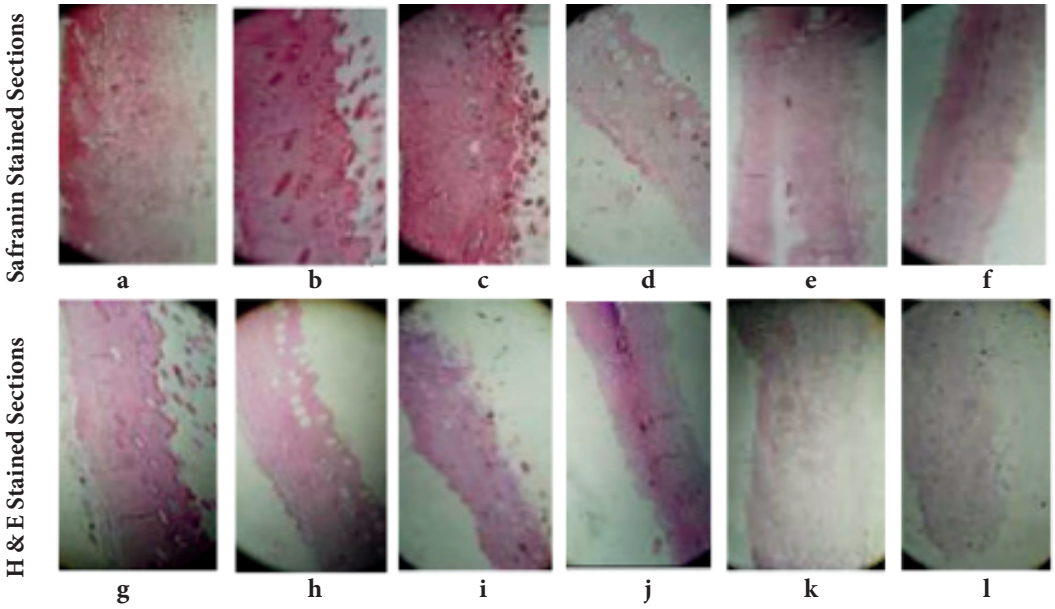

Figure 5. Safranin O stained sections from putrefied goatskin:
a) skin section dense red color indicating the presence of proteoglycan at 0th hour; **b)** details of the elliptical voids indicating hair follicles visible during 6th hour; **c)** elliptical voids being present at 18th hour; **d)** empty rings indicating lost hair follicles at 24 hours; **e)** loss of proteoglycan at 30th hour; **f)** very minimal levels of proteoglycan 36th hour

Hematoxylin and Eosin stained sections for studying changes in overall fiber network:
g) – i) entire fibrous matrix with elliptical hair follicle voids being seen until 18th hour;
j) – l) significant material loss visualized from 24 hours to 36 hours

elliptical dark red circles found in the micrographs from the samples of 6 hours and 18 hours are indicative of the presence of hair follicles. The reduction in the intensity of dark red color over the time indicated distinctly that the proteoglycan was degraded gradually with the progression of time. The empty rings visualized from the 24 hour sample implied that proteoglycans were immensely broken down which led to complete removal of hair from the surface. It was found that at around 30 – 36 hours, the presence of proteoglycan was very minimal. This was in accordance with the visual observations where hair slip and exudations of protein degradation products was observed at the similar time frame of observation.

Hematoxylin and Eosin stain for fiber network

Overall fiber network was visualized using Hematoxylin and Eosin stain that stained the collagen, elastin and erythrocytes (Fig. 5 g-l). It can be seen that the density of overall matrix that could be stained, had started decreasing from 18 hours and significant loss of skin network was witnessed at 30 hours (Fig. 5 k). The results from Hematoxylin and Eosin staining further confirmed the results from other staining results. Degradation of epidermis, dermis and hair follicle were observed to be falling in a similar trend when degradation of goatskins was histologically analyzed.[33] Distortion of grain and corium layers made of fibrous proteins of raw buffalo hide carried by enzymatic degradation was detected by Hematoxylin and Eosin staining method.[25] In a recent study involving sheep skins and cattle hides, histological examination of wet blue leathers made from putrefied raw material showed internally damaged tissue structures.[30]

Sudan Black B stain for lipid

The levels of lipid being physically transformed were visualized microscopically with Sudan Black B stain, that stained lipids blue-black (Fig. 6 a-f). Lipids are an important component that gives the skin handle and suppleness. Presence of lipids inside the network and in the adipose layer was found to be prominent till 18 hours. After 18 hours, the lipids started disappearing due to the action of lipolytic microorganisms, as evident from the discussions from the previous

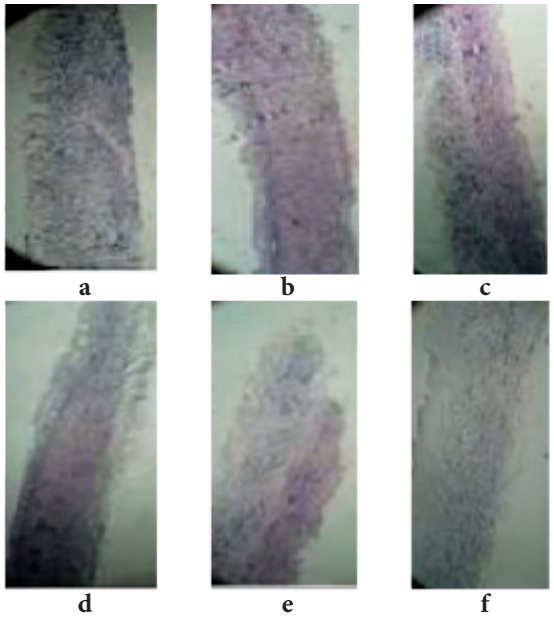

Figure 6. Sudan Black B stained sections of putrefied goatskin: **a) – c)** presence of lipids prominent till 0 to 18th hour; **d)** disrupted lipid layers at both sides of the skin viewed at 24 hours; **e) – f)** very little lipid presence at 36th hour

section. At 36 hours, there was very little evidence of presence of lipid in the skin sections. From the earlier results on investigations of microorganisms, it was found that the lipolytic bacteria were present from 9 hours of putrefaction and continued until 36 hours. Therefore, it could be expected that the degradation of lipids and the adipose layer had commenced from 9 hours and progressed further until the end. This trend was established from the gradual decrease in the intensity of pigmentation indicating the lack of lipid.

Scanning Electron Microscopy (SEM) study of putrefied skin samples

The structural details of the putrefied skin samples were pictured using scanning electron microscope in order to further ascertain the physical changes occurring in the fiber network of skin. The samples were viewed at a magnification of 3000× (Fig. 7 a-f). The micrographs revealed that the fiber network remains stable during the initial hour (Fig. 7a and Fig. 7b). Separation of fiber network had started occurring at around 18 hours (Fig. 7c). At 30 hours, significant level of degradation of fibers was witnessed. This was in congruence with the results from H & E stained samples. At 36 hours, the fiber network was found to be completely shattered. Similar nature of damaged fiber network was observed when the leathers made from unpreserved or insufficiently preserved raw materials were viewed using SEM, which revealed changes in structure and loss of protein leading to poor quality leather.[34]

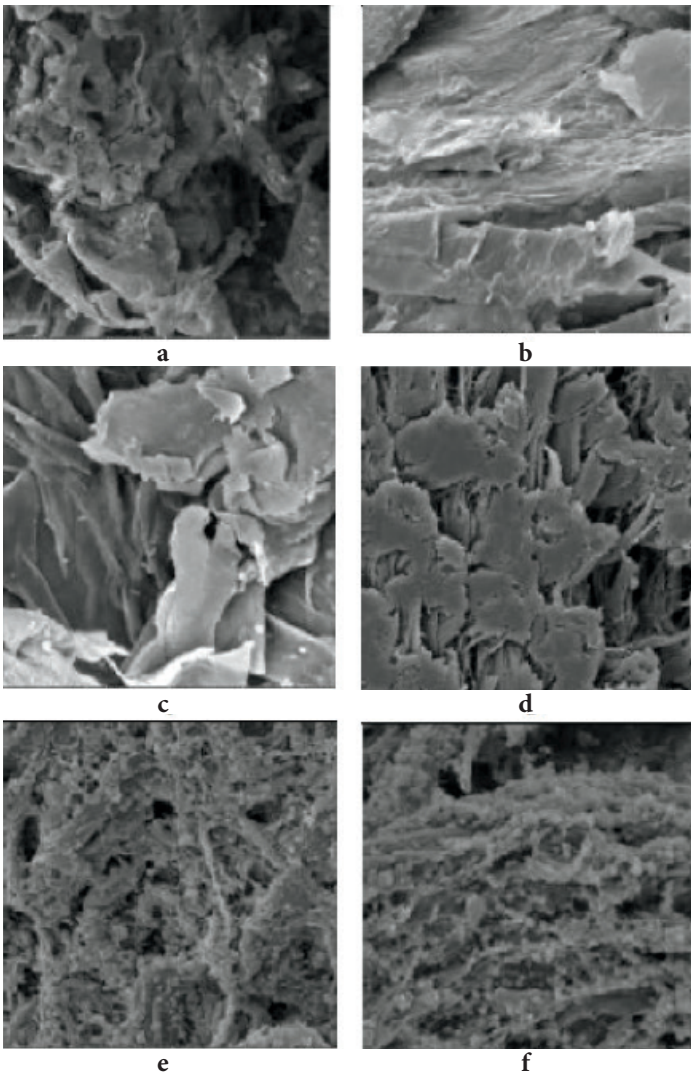

Figure 7. SEM micrographs of putrefied skin samples collected over the period of 36 hours: **a)** interwoven fiber network at initial period at 0th hour; **b)** 6th hour sample showing the breakage of fibers; **c)** fiber network getting separated at 18th hour; **d)** voids in the fiber network and fiber bundle seen at 24th hour; **e) – f)** completely shredded fibrous network working its way towards degradation viewed at around 30 – 36 hours

Chemical Changes Observed During Putrefaction

Elemental analysis of skin samples

The key changes in chemical composition of putrefied skin samples was monitored by estimating moisture content and other elemental components. The moisture content of each skin sample was analyzed. The moisture content was found to be sustained throughout the putrefaction. Moisture is the key growth factor required for the microorganisms to grow. The elemental composition (C, H, N and S) of the skin samples during the course of putrefaction was also estimated (Table II). It can be seen from the results that the total elemental composition did not change much within this time scale of observation. A declining trend could be perceived in the nitrogen levels, if the observation of degradation was extended for days. Evidently, Preethi et al. (2006)[33] observed that the total protein content of the degraded goatskins was found to be decreased over the period of 10 days when monitored in unpreserved condition.

Proteoglycan estimation based on Periodic acid – Schiff's assay

The proteoglycan released from the skin was estimated with respect to time. Fig. 8 gives the results of proteoglycan estimation. It is evident from the results that the proteoglycans release was not significant until 6 hours but increased rapidly within 18 hours. This exponentially increased release could be explained by correlating with the hair slip phenomenon that had started at around 10 hours and progressed to 12 hours leading to complete hair loss. After 18 hours, it was noted that the protein degradation products had made

Table II

Elemental analysis of skin samples

Sample ID	Elemental Analysis (%)						Moisture (%)
	N	C	H	S	C/N ratio	C/H ratio	
S1	16.38	51.89	7.54	0.41	3.16	6.87	72.61 ± 1.6
S2	15.30	45.03	6.68	0.48	2.94	6.73	69.88 ± 0.87
S3	15.70	46.42	7.09	0.44	2.95	6.54	70.21 ± 1.2
S4	15.15	46.09	7.23	0.40	2.98	6.36	67.22 ± 1.4
S5	15.89	47.84	7.24	0.48	3.01	6.60	70.15 ± 0.98
S6	15.48	45.24	7.11	0.36	2.92	6.35	69.12 ± 1.1

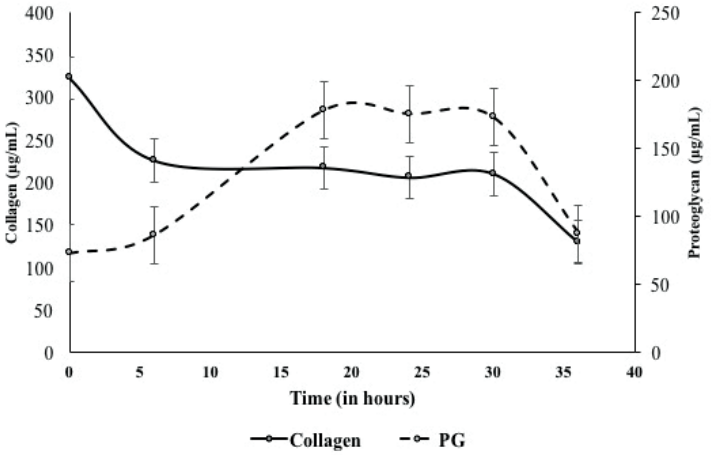

Figure 8. Collagen from putrefied skins and proteoglycan released from putrefied skin samples with respect to time (in hours)

the skin appear slippery and slimy. Such effect is validated by the results from proteoglycan estimation, as there occurs a levelled off proteoglycan release between 18 hours to 30 hours. Hair loosening was the result of the loss of proteoglycans that held the hair bulb. The observations from histological studies of proteoglycan were found to be in agreement with the proteoglycan release.

Hydroxyproline estimation of skin samples

Hydroxyproline is an amino acid very specific to the collagen protein, which becomes the marker component for this assay. The hydroxyproline released from the putrefied skin was estimated with respect to time. The content of hydroxyproline is the direct measure of level of collagen present in the sample. Hydroxyproline amounts were found to be gradually decreasing with respect to time from 6 hours and dropped down to a very low collagen content until the 36th hour of the observation (Fig. 8). These results are in congruence with the data from previous experiments pertaining to microorganisms, staining, electron microscopy and elemental analysis. Initiation of loss of hydroxyproline from the very early stage (within 6 hours) of putrefaction scale, establishes that the damage of the leather making protein starts within a few hours after flaying. This necessitates the need for the right kind of preservation to be done at the right time.

Insights into the cascade of events leading to a phenomenon called putrefaction

Microbial deterioration of an organic substrate such as raw animal skin takes place in several stages as seen in this study with help of several experiments. The initiation of putrefaction started right after flaying of the skin from the carcass by autolysis. The atmospheric temperature, humidity and the inherent moisture of the goatskin provided a conducive condition for several microorganisms to utilize the skin for its growth. The synergistic action of bacteria possessing multiple hydrolytic characteristics disintegrated each of the skin components starting from the easily accessible surface material. This detrimental activity by putrefactive bacteria caused the closely knit fiber network to collapse leading to indications such as hair slip and excretions of degradation products. The biodegradation also released gases such as ammonia from the skin that caused putrid odor indicating the degradation of the protein. The proteoglycan portion that held the hair bulb degraded by the proteolytic enzymes led to loosening of the hair along with the disruption of collagen stability. Since the collagen's protective sheath such as reticulin, elastin and other cementing mucoidal proteoglycan were destroyed by the action of proteolytic enzymes, the collagen became vulnerable to the action of bacterial collagenases that cleaved it into several fragments. The lipid portion also gets broken down releasing several slimy by-products that made the skin slippery and lacking physical solidarity. The complex integral components mineralized into simpler compounds such as amino acids, further inviting several other organisms such as maggots that resulted in the total deterioration of the raw material. The value of the raw material downgraded every hour after the flaying operation. Monitoring the effects of putrefaction with respect to time would provide a lead for devising a fool-proof and environment-friendly preservation technology. These results pave way for exploring the salt-free preservation alternatives that check microbial infestation with few hours of hide/skin flaying.

Conclusions

Goat skins were allowed to putrefy in ambient tannery conditions which indicated many physical as well as biochemical changes during spoilage. Physical observations helped in identifying the initiation of putrefaction that started at around six hours from flaying. Histological staining studies and scanning electron microscope studies were evident in indicating the significant level of collagen degradation that started occurring at the initial stage. The presence of collagenolytic bacteria from the six hours of initiation further confirmed that the collagen degradation started early. Therefore, on the grounds of conservation of quality of raw material, it can be concluded that the preservation should be started much earlier than six hours of flaying. However, it needs to be kept in mind that this phenomenon is dependent on the ambient temperature and other physiological conditions. In conclusion, this work provides evidence to understand the putrefaction processes with respect to time, which would aid in better comprehension of the factors that are involved in skin spoilage and would help in devising methods to avoid damage occurring due to bacterial putrefaction.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

References

1. Tancous, J.J. *Skin, hide and leather defects*. Washington, DC: Leather Industries of America Laboratory; 1986.
2. McLaughlin, G.D., and Theis, E.R. *Chemistry of leather manufacture*. Michigan: Reinhold Publishing Corporation; 1945.
3. Bienkiewicz, K. *Physical chemistry of leather making*. Florida: Robert E. Kreiger Publishing Company, 182-204, 1983.
4. Wilson, J.A. *The chemistry of leather manufacture*. The chemical catalog company, New York, USA, pp 133-139, 1923.
5. Lindner, W., Neuber, H.U. Preservation in the tannery. *International Biodeterioration*, **26**, 195-203, 1990.
6. Gudro, I., Valeika, V., Sirvaityte, J. Short term preservation of hide using vacuum: influence on properties of hide and processed leather, *PloS ONE* **9 (11)**:e112783, 2014
7. Iyappan, K., Ponrasu, T., Sangeethapriya, V.,Gayathri, V.S., Suguna, L. An eco-friendly method for short term preservation of skins/ hides using *Semacarpus anacardium* nut extract. *Environment Science and Pollution Research*, **20**, 6324-6330, 2013
8. Kanagaraj, J., Chandrababu, N.K. Alternatives to salt curing techniques – A review. *Journal of Scientific and Industrial Research*, **61**, 339-348, 2002.
9. Orlita, A. Microbial deterioration of leather and its control: a review. *International Biodeterioration and Biodegradation,* **53**, 157-163., 2004
10. Kanagaraj, J., Sastry, T.P., Rose, C. Effective preservation for raw goat skins for the reduction of total dissolved solids. *Journal of Cleaner Production,* **13**, 959-964, 2005.
11. Sivabalan, V., Jayanthi, A. A study to reduce salt usage in preservation of skins and hides with alternative use of plant extract. *Journal of Agricultural and Biological Science,* **4**, 43-48, 2009
12. Vijayalakshmi, K., Judith, R., Rajkumar, S. Novel plant based formulations for short term preservation of animal skins. *Journal of Scientific and Industrial Research*, **68**, 699-707, 2009.
13. Thorstensen, T.C. *Practical Leather Technology*. Florida: Kreiger Publishing Company, pp 84-117, 1993.
14. Tancous, J.J., Jayasimhulu, K. Changes in steer hide composition resulting from autolysis. Part II. Effect of enzyme inhibitors. *JALCA*, **68**, 132-147, 1973.
15. Didato, D., Bowen, J., Hurlow, E. Microorganism control during leather manufacture. In: Leafe MK (ed) Leather Technologists Pocket Book, Society of Leather Technologists and Chemists, East Yorkshire, UK, pp 339-352, 1999.
16. Matsushita, O., Okabe, A. (2001). Clostridial hydrolytic enzymes degrading extracellular components. *Toxicon,* **39**, 1769-1780, 2001.
17. Rao, R.S., Nandy, S.C., Santappa, M. Skin hydrolysis by two newly isolated facultatively anaerobic organisms. *Leather Science,* **24**, 1-6, 1977.
18. Woods, D.R., Rawlings, D.E., Cooper, D.R., Galloway, A.C. Collagenolytic activity of hide bacteria and leather decay. *Journal of Applied Bacteriology,* **36**, 289-295, 1973.
19. Dempsey, M. Part VI – The structure of the skin and the leather manufacture. *Journal of the Royal Microscopical Society,* **67**, 21-26, 1947.
20. Montelli, S., Corain, L., Cozzi, B., Peruffo, A. Histological analysis of the skin dermal components in bovine hides stored under different conditions. *JALCA*, **110**, 54-61, 2015.
21. APHA *Standard methods for examination of water and wastewater*, American Public Health Association, 21st Edition, Washington, DC, 2005.
22. Mantle, M., Allen, A. A colorimetric assay for glycoproteins based on the periodic acid/ Schiff's stain. *Biochemical Society Transactions,* **6**, 607-609, 1978.
23. Woessner, J.F. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Archives of Biochemistry and Biophysics*, **93**, 440-447, 1961.
24. Husen, A., Tilahun, A., Teshale, A., Gashaw, T. Review on pre and post- slaughter defects of hide and skin in Ethiopia. *Advances in Biological Research*, **10**, 154-161, 2016.
25. Shede, P.N., Kanekar, P.P., Polkade, A.V., Dhakephalkar, P.K., Sarnaik, S.S. Bacterial succession on raw buffalo hide and their degradative activities during ambient storage. *International Biodeterioration and Biodegradation*, **62,** 65-74, 2008.
26. Birbir, M., Ilgaz, A. (1996). Isolation and identification of bacteria adversely affecting hide and leather quality. *Journal of Society of Leather Technologists and Chemists,* **80**, 147-153, 1996.
27. Kayalvizhi, N., Anthony, T., Gunasekaran, P. Characterization of the predominant bacteria associated with sheep and goat skin. *JALCA*, **103**, 182-186, 2008.
28. Pekhtasheva, E., Neverov, A., Zaikov, G. (2012). Biodamages and protection of leather and fur. *Chemistry and Chemical Technology,* **6**, 327-337, 2012.
29. Tyagi, P.K., Anwar, M., Mukherjee, G. Nature and activities of different microorganism on raw hides / skin and their plant based preservation approach. *Journal of Indian Leather Technologists Association,* **62**, 319-328, 2012.
30. Mohamed, H.A.A., Klink, E.G.M., ElHassan, S.M. (2016) Damage caused by spoilage bacteria to the structure of cattle hides and sheep skins. *International Journal of Animal Health and Livestock Production Research*, **2,** 39-56, 2016.
31. Saravanan, P., Shiny, T.R., Gowthaman, M.K., Kamini, N.R. Understanding the chemical free enzyme based cleaner unhairing process in leather manufacturing. *Journal of Cleaner Production*, **79**, 258-264, 2014.
32. Burton, D., Reed, R. Mucoid material in hides and skins and its significance in tanning and dyeing. *Discussions of the Faraday Society*, **16,** 195-201, 1954.
33. Preethi, V., Jeyameenakshi, R., Kumar, M.S., Aishwarya, S., Sehgal, P.K. (2006) Degradation of goatskins – a detailed study on physiochemical, biochemical, microbial and histopathological changes at ambient conditions. *JALCA*, **101**, 51-57, 2006.
34. Fontura, F.T., Mariliz, G. Damage of pickle hides, wet blue leather and vegetable tanned leather due to biodeterioration. *JALCA,* **110**, 138-144, 2015.