Histological Analysis of the Skin Dermal Components in Bovine Hides Stored under Different Conditions

by

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Abstract

The leather industries are interested in avoiding *post-mortem* alterations of the skin components, since degeneration of the dermal structures composing raw hides decreases the quality of leather.

The goal of the present study is to realize a histological study of skin samples to assess the tissue alterations at different periods and under methods of conservation (salting and refrigeration) after the skinning of the animals at the slaughterhouse. The papillary region and the reticular dermis were both analyzed. The dermal components considered were the number of cell nuclei, the structure of the collagen and elastic fibers, and finally the presence of acidic polysaccharides. Results showed a progressive reduction of cellular nuclei and acid polysaccharides of the dermal layer during the passage of time in all the conditions considered. A moderate decay of collagen bundles was noted in salted hides whereas the elastic fiber networks maintained their organization over the time. No sign of accumulation of non-functional elements or other morphological alterations were observed in the dermis. These findings can be useful for the leather industry for choosing the desired curing and timing conditions to employ during refrigeration or salt-based treatment of the skins.

Introduction

Leather industries transform animal raw hide into leather, a stable material, which can be used to produce a broad range of products.[1] The leather is the result of a tanning processing which generates a final product with specific properties: stability, water and temperature resistance, elasticity and permeability to air.[2] The leather is obtained from the derma of different animal species, especially cattle, sheep and pigs. The dermis is the layer of skin between the epidermis and the hypodermis tissues. It consists of connective tissue divided in two parts: the superficial area called papillary region, and a deep area called reticular dermis.[3] The structural components of the dermis are collagen, elastic fibers, extra-fibrillar matrix and different types of cells (fibroblasts, immune cells, sensory and glandular cells). These dermal components provide a combination of flexibility and tensile strength to the skin.[4-6] The best quality of leather can be obtained from the upper part of the dermis, called also grain leather. It consists of a densely packed network of collagen and elastic fibers, embedded in polysaccharides. Conversely, the reticular dermis (called crust leather) is composed mainly of sparse fibrous collagen bundles with a scarce presence of elastin and it is used to produce low quality products.[7]

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Manuscript received September 4, 2014, accepted for publication October 16, 2014.

The bovine skin is an important byproduct of the meat industries and the tanners are interested in investigating the possible *post-mortem* alterations of the hides, to prevent leather damage and improve the quality of the products. A deepened knowledge of *post-mortem* dermal modifications may increase the quality of leather and improve the production processes reducing wastes and pollution. Indeed, previous studies revealed that 80% of the leather processing wastes are generated during pre-tanning processes.[8]

Data in literature are scarce and report that the decay of fresh dermal tissue's properties starts immediately after the machine-operated removal of the skin from the animals.[9] Tanners operate applying different curing methods to prevent the decay of dermal structures before the start of the leather processing.[10] Methods for short-term preservation (within a week) are the cooling using crushed ice or the storage in refrigerated room. The long-term preservation (up to six months) is carried out using salting, brining, drying and salt drying.[11-12] Decomposition damages the raw material and this is of particular concern when high specification leathers are being produced. However, if considerable efforts have been expended to evaluate the biodeterioration caused by microorganisms,[13] less attention has been dedicated to evaluate dermal tissue degradation of raw hides over the time and at the different conditions of storage[14-15] and a scientific approach to this issue is lacking.

Therefore, in this study we assess the damages caused by tissue degeneration in the time occurring between the slaughter of the animal and the start of the tanning process of bovine hides during the storage time in refrigerators or by salting. The papillary region and the reticular dermis were both separately analyzed and the histological analysis was performed by a light microscopy and using an expert grade assessment. Moreover, to obtain exhaustive statistical evaluation the data were analyzed using nonparametric statistical methods.

Materials and Methods

Animal and Specimen Collection

Bovine hides were obtained from commercial abattoirs. Animals were treated according to the European Community Council directive concerning animal welfare during the commercial slaughtering process (86/609/EEC), and were constantly monitored under mandatory official veterinary medical care. Skin samples were taken from a series of adult male bovine (total number 8) from the hips of each animal.

A detailed sampling plan was organized. We indexed the different sampling times in the following way:

T^0: four independent samples were collected from hides just after the skinning, and referred to as *fresh hide control samples*.

T^1: five hours after the bovine death (immediately after the trimming), four independent samples were collected, and referred to as *5h old fresh hides*.

T^2: five hours after the bovine death, four independent samples were stored for 11 days in a refrigerator at 4°C, then sampled, and referred to as *refrigerated hides*.

T^3: five hours after the bovine death, four independent samples were stored in salt for 19 days, then sampled, and referred to as *short time salted hides*.

T^4: five hours after the bovine death, four independent samples were stored in salt for 84 days, then sampled, and referred to as *long time salted hides*.

Histological Staining

Skin samples 10 cm^2 wide and 1cm thick were collected from the hips of the animals using a scalpel blade, and fixed for 24 h in 4% formaldehyde. Each sample was reduced in fragments of 1 cm^2, dehydrated and embedded in paraffin. From each sample, transverse sections of 4 μm thickness were obtained using a Leica RM2035 microtome. Slides were stained with conventional laboratory methods:[16] a) hematoxylin and eosin (H&E) for identification of general structure; b) Masson's trichrome technique, and c) orcein staining, to evaluate degeneration of collagen and elastic fibers; d) periodic acid-Schiff-positive technique (Alcian Blue - PAS) to detect neutral and acidic polysaccharides. The sections were then dehydrated and cover-slipped with balsam for light microscopy.

Expert Grader Assessment for Histological Analysis

Three histological experts randomly and blindly graded the stained slides representing hides at the sampling times previously described. For each stain, different features were considered: nuclei were counted using the H&E stain; the extent of the collagen-covered surface was determined using the Masson technique; the elastin-covered surface was calculated using the orcein stain; finally the quantity of acidic polysaccharides was estimated by using the Alcian-PAS method. Scores were assigned as follows: (–) the feature can be identified only at high magnification and with a careful scrutiny of the section (+) the feature is detected using at least 20× magnification; (++) the feature is clearly detectable in the section using 4× or 10× magnification; (+++) the feature is detectable at 2× magnification and distributed over large areas of the section. Each expert graded four samples for each one of the four hides analyzed per sampling time. Globally, forty eight data were considered to obtain each result reported in the tables. In the tables the mode of the judgments was reported.

Statistical Analysis

Since the expert assessments are actually ordered categorical scores, the statistical analysis was performed using suitable nonparametric tests, namely the permutation tests,[17] and as test

statistic we chose the Anderson-Darling.[18] More specifically, we associated the score 1, 2, 3 and 4 to the subjective assessment "-","+","++" and "+++" respectively. Note that, since there are three experts assigning ordered scores, the reference design of our statistical analysis was the randomized complete block design, which in this case represents an effective solution to accommodate the presence of confounding effects due to possible differences among both experts and hides. The pair-wise p-values related to the comparisons between the control level T^0 and the remaining four sampling times were adjusted by multiplicity using the Bonferroni method.[19] Suitable R codes implementing the permutation test routines were used for all analyses (all R codes are available upon request by authors). A significance level of 5% was used to reject the null hypothesis. In the graphs, values are reported as means ± SEM, which were calculated using as reference model a suitable multi-way ANOVA.[20]

Results and Discussion

We performed a histological evaluation of fresh bovine skin (T^0) *vs*. fresh hides sampled after the trimming (5 hours after the skinning, T^1), hides preserved at 4°C for 11 days after the trimming (T^2), and hides preserved in salt for 19 days (T^3), and 84 days (T^4) after the trimming. An expert grader assessment allows classifying and distinguishing the different traits developed by hides over the time. Scores reflect the opinion of three histological experts.

Reports concerning the degree of skin degeneration following death of the animals at the abattoirs are scarce. These studies highlighted that the action of abiotic and biotic agents induces a progressive corruption of the skin dermal layer. The effects of chilling methods on the quality of the leathers were tested comparing different temperatures of conservation (2, 4, 7 and 10°C) for a period of seven days.[15] Starting from the second decade of the 20th century, many studies were dedicated to the investigation of microbial biodeterioration of hides and leather by bacteria[21-22]. In recent years, other studies estimated the bacterial degradation of the collagen and non-collagenous proteins until 14 days after the slaughter on cow and goat hides maintained at different temperature (25, 30, 37 and 42°C). Tyrosine and hydroxyproline concentrations were used to estimate collagen's decomposition of the derma showing a temperature dependent degradation.[13-23]

However, only few studies were performed with the aim to evaluate the changes in the structure and composition of the skin.[24-25] In this study, our analysis contributes to provide new data about the degeneration of the dermal layer.

Grain Leather

Firstly, we evaluated the dermal morphology on H&E-stained skin slides. Comparisons of fresh dermis *vs* 5h old fresh hides brought out no alteration or degeneration of the morphology of the tissue. Conversely, comparisons of fresh samples with refrigerated and salted hides pointed out some variations in the grain leather. In these skins hair bulbs resulted smaller and round concurrently with the disappearance of dermal papillae. Moreover, no defective adhesion at the dermis to epidermis junction was remarked and no signs of cell necrosis or empty vacuoles were present (Figure 1A). These are two histologic *post-mortem* changes already observed in human skin between 4 and 7 days after death.[26] Also the remarked disappearance of the dermal papillae is in agreement with previous data of ultrastructural *post-mortem* skin evaluation.[27]

In literature data, skin variations have been studied mainly analyzing the innate and intrinsic human cutaneous aging. Old skin becomes wrinkled, lax and dry showing atrophy, loss of elasticity and firmness. Aged skin manifests disorganization of the collagen and elastic networks, elastosis (a peculiar defect consisting in aggregates of elastic fibers caused by sunlight), fragmentation of collagens and decreased thickness.[28] Other studies have analyzed human cutaneous aging caused by exposure to outdoor elements, primarily sunlight (UV irradiation),[29-30] or histological changes affecting human skin in order to estimate the *post-mortem* interval (PMI), an important issue because of its possible significance in legal investigations.[31]

A gradual reduction of cell nuclei was noted comparing fresh and refrigerated hides. As well, a further progressive nuclear loss was observed in salty skin. Salted hides showed the presence of few nuclei, identifiable only after high magnification careful scrutiny of the sections (Table I).

The trichrome's Masson staining pointed out that collagen bundles in fresh dermal skin were compact and densely packed without signs of fragmentation or degeneration over time (Figure 1B). In the refrigerated and salted skins, a slight collagen's atrophy was observed.

The Orcein staining was used to identify elastic fibers. In the fresh control samples, these fibers resulted thin, short and embedded in the extracellular matrix. The elastin networks were conserved and uniformly distributed at all the sampling times. No differences were noted between fresh, refrigerated and salted hides (Figure 1C, Table I). Notably, no signs of elastosis, a typical degenerative process described in photoaged skins, were present.

The Alcian-PAS staining was performed to highlight the neutral and acidic polysaccharides components of the skin: fresh hides and refrigerated were similar, while a decline of acidic polysaccharides in salted skins was observed over time (Figure 1D, Table I). Glycosaminoglycans are polysaccharide chains that fill space in the extracellular matrix and alterations of hyaluronic acids contribute to skin fragility of elderly

TABLE I

The table reports for each stain the subjective assessment at the different sampling times for the grain leather. In H&E stain nuclei were counted, in Masson stain, surface covered by collagen was considered, in orcein stain, surface covered by elastin was evaluated and in Alcian-PAS staining, the quantity of acidic polysaccharides was estimated. Scores reflect the opinion (the mode) of three histological experts.

Sampling times	Scoresheet (grain leather)			
	H&E	Masson	Orcein	Alcian PAS
	Nuclei	Collagen bundles	Elastic fibers	Acidic GAGs
T^0 (fresh)	+++	+++	+++	+++
T^1 (5h old fresh)	+++	+++	+++	+++
T^2 (refrigerated)	++	++	+++	+++
T^3 (short salted)	+	++	+++	+
T^4 (long salted)	-	++	+++	-

TABLE II

The table reports for each stain the subjective assessment at the different sampling times for the reticular dermis. In H&E stain nuclei were counted, in Masson stain, surface covered by collagen was considered, in orcein stain, surface covered by elastin was evaluated and in Alcian-PAS staining, the quantity of acidic polysaccharides was estimated. Scores reflect the opinion (the mode) of three histological experts.

Sampling times	Scoresheet (reticular dermis)			
	H&E	Masson	Orcein	Alcian PAS
	Nuclei	Collagen bundles	Elastic fibers	Acidic GAGs
T^0 (fresh)	+++	+++	+	-
T^1 (5h old fresh)	+++	+++	+	-
T^2 (refrigerated)	++	+++	+	-
T^3 (short salted)	+	+++	+	-
T^4 (long salted)	-	+++	+	-

people[32]. Our data are consistent with the finding that the acidic polysaccharides are crucial components regulating cutaneous aging and skin degeneration. Moreover, a reduction of hyaluronic acid in skin of aged people has already been described in a past work wherein these polyanionic macromolecules were related to the maintenance of cutaneous salt and water balance in skin.[33]

Reticular Dermis

The same evaluations performed on the grain leather, were carried out to evaluate the reticular dermis. The lower part of the dermis resulted less heterogeneous and no signs of degeneration were highlighted in the different samples over the time.

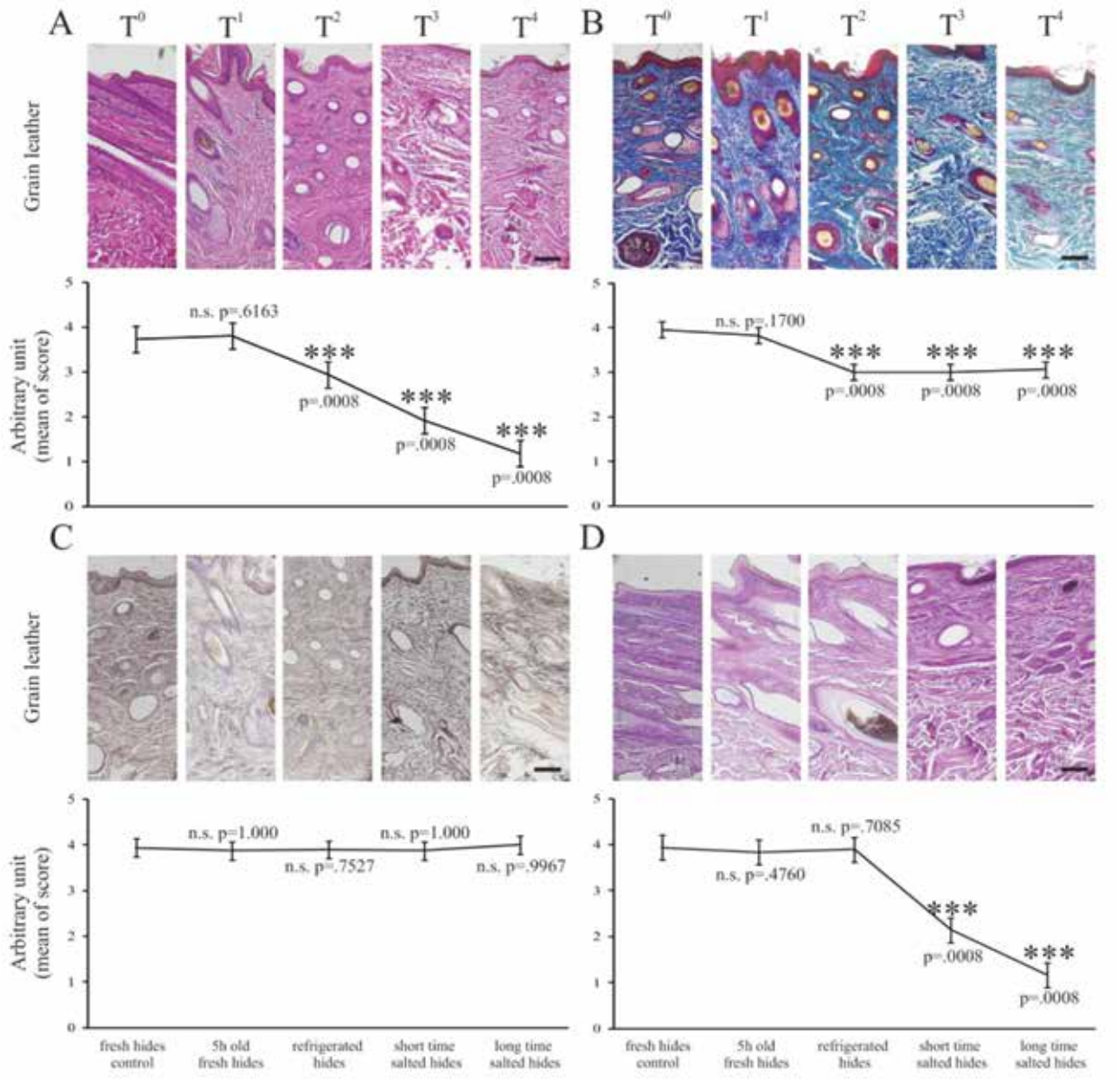

Figure 1. Histological analysis of the grain leather. Sample images representing slides of fresh (T^0), 5h old fresh (T^1), refrigerated (T^2), short time salted (T^3) and long time salted hides (T^4) stained with different routine methods. A) Hematoxylin and eosin (H&E). B) Masson's trichrome technique. C) Orcein staining. D) Alcian Blue - PAS (periodic acid-Schiff-positive technique) staining. Horizontal bars represent 500μm. Graphs below the figures showed the corresponding nonparametric statistical analysis. The average amounts of nuclei (A), collagen (B), elastin (C) and acidic polysaccharides (D) were estimated by the histological experts. We associated the score 1, 2, 3 and 4 to the subjective assessment "-","+","++" and "+++" respectively in order to transform subjective assessment into ordered numerical quantities. Average amounts are reported as means ± SEM, n.s.= not significant. *** P<0.001.

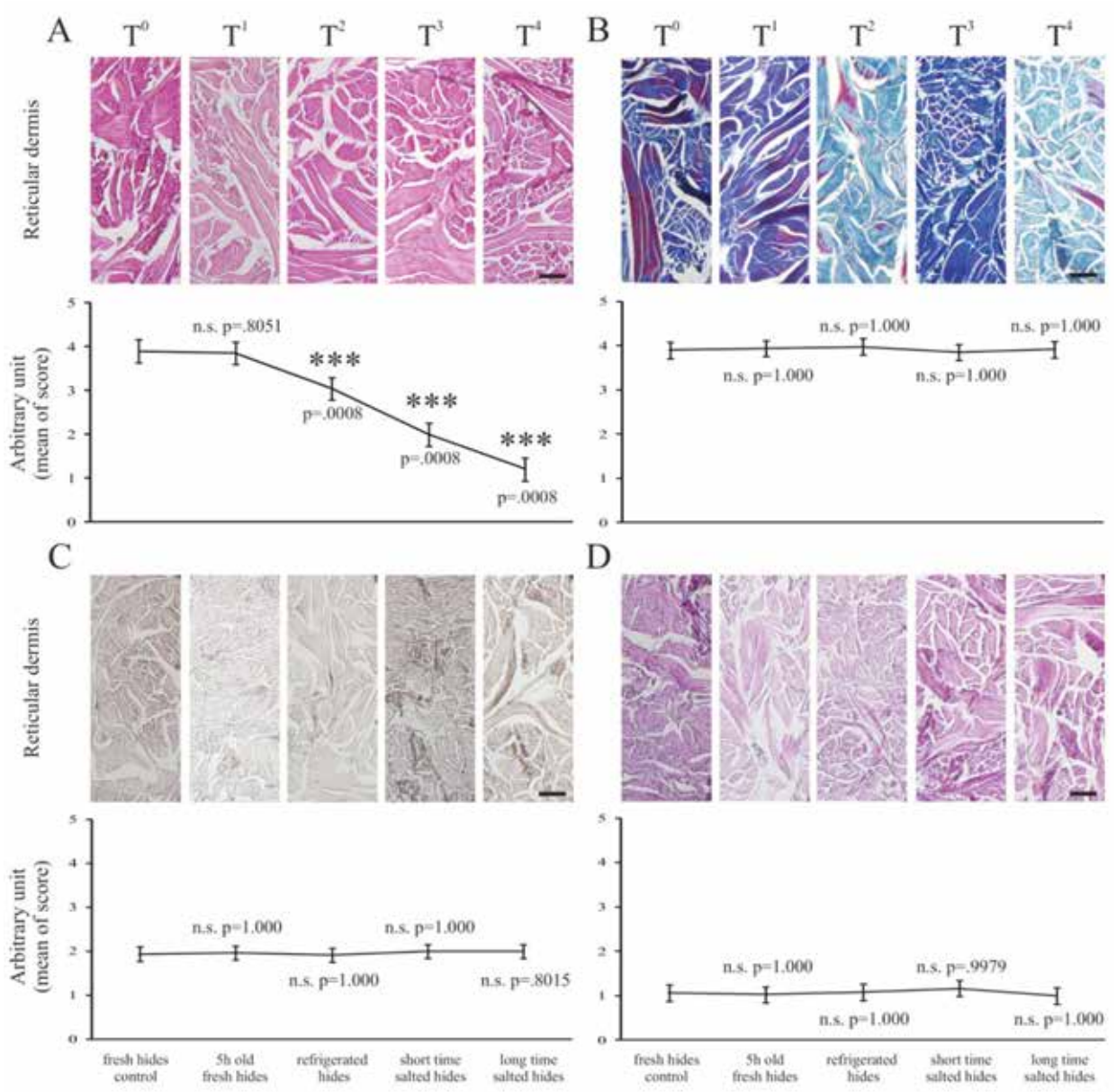

Figure 2. Histological analysis of the reticular dermis. Sample images representing slides of fresh (T^0), 5h old fresh (T^1), refrigerated (T^2), short time salted (T^3) and long time salted hides (T^4) stained with different routine methods. A) Hematoxylin and eosin (H&E). B) Masson's trichrome technique. C) Orcein staining. D) Alcian Blue - PAS (periodic acid-Schiff-positive technique) staining. Horizontal bars represent 500μm. Graphs below the figures showed the corresponding nonparametric statistical analysis. The average amounts of nuclei (A), collagen (B), elastin (C) and acidic polysaccharides (D) were estimated by the histological experts. We associated the score 1, 2, 3 and 4 to the subjective assessment "-","+","++" and "+++" respectively in order to transform subjective assessment into ordered numerical quantities. Average amounts are reported as means ± SEM, n.s.= not significant. *** P<0.001.

The general structure of the tissue was characterized by thicker bundles of collagen oriented in a less organized network (Figure 2A). The same gradual reduction of cells observed in the grain leather was pinpointed also in the reticular dermis (Table II).

Collagen debris were densely packed and distributed with variable orientation, often parallel to the surface. Neither degeneration nor atrophy over time was pointed out in skins (Figure 2B, Table II).

The elastic fibers network was strongly reduced (Figure 2C, Table II). Some fiber was detected using high magnification. The most amounts of fibers, oriented parallel to the surface, were identified near the subcutaneous tissue. The acidic polysaccharides components were almost totally absent in this part of the derma (Figure 2D, Table II).

Statistical Analysis

Statistical data confirmed a gradual decrease in number of dermal cells in refrigerated and salted hides both in the upper

and lower parts of the dermis (Figure 2A). Also the reduction of compactness of collagen bundles described in the grain leather of cured hides was significant (Figure 2B). The elastic fiber network was maintained during the time in fresh and salted hides, while a progressive time dependent decline of acidic GAGs resulted significant in the papillary region of the derma (Figure 2C and D). The processes of deterioration seem to affect principally the grain leather, whereas the crust leather undergoes only the disappearance of nucleic acids. Indeed, leathers obtained from our sampled skins resulted comparable to normal leather and fulfill all the trade requirements.

Conclusions

Because leather quality depends on the good preservation of the freshly slaughtered skins, we monitored by histological analysis the papillary region and the reticular dermis of the raw hide.

Two main alterations of the dermis were highlighted: a reduction of the number of cell nuclei during the time and a decline of acidic polysaccharides, regardless of the method of storage.

The bovine hides were subjected to an erosion of collagens filling the stromal dermal layer, but breakings of the collagen fibers were not found, as well as signs of cell necrosis and vacuoles.

Moreover, analyzing the structure of upper and deep dermis, we showed that the reticular dermis preserves its histological architecture and differences in the dermal composition were highlighted respect to the grain leather. It must be noted that leather industry is categorized as one of the most polluting industries. Since we observed no severe histological damages to the tissue in the skins preserved by salting and by refrigeration analyzed at different times, our results suggest the possibility of extending the storage time of raw hides by refrigeration. This will allow the industry to decrease costs due to the salting process and reduce the pollution caused by chlorides.

Practical indication reported in the document of the European Commission (Best Available Techniques (BAT) Reference Document for the Tanning of Hides and Skins) recommends the use of short-term preservation methods up to five days. Other studies pull this limit until seven days[15]. Our analysis suggests the possibility to keep hides till eleven days using refrigerated storage.

This study could have an important impact on quality control processes required by leather industries. Moreover, the analysis of the skin can improve manufacturing processing of leather goods. Despite the solutions for better environmental performance are frequently complex and have to be assessed with regard to their overall costs and benefits, our data may contribute to develop new techniques to curtail pollution levels according to the increasingly stringent environmental requirements.

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