A New Defect on Leather: Microbial Bio-Film

by

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Abstract

Hides and Skins are protein based materials containing high amounts of water, which makes them a nutritionally rich media ideal for bacterial growth. Being also the essential raw materials in leather manufacture, it is vital to carefully protect them against bacterial action especially in the early wet processing stages. It is well known that bacterial action can seriously damage the fiber structure of hides and skins. Hair slip, red discoloration and grain pilling are typical examples of bacterial action can lead to damage with subsequent defects on the tanned leather. The present study identifies a new defect caused by bacteria known as "biofilm" on hides and skins. For investigation of the observed defective areas, two techniques were used: Scanning Electron Microscopy (SEM), and Real-Time Polymerase Chain Reaction (RT-PCR). The latter technique, RT-PCR, is a novel rapid method for unique detection, identification and quantification of microorganisms. The results, obtained by applying these techniques, verified the presence of "microbial biofilm" on the leather.

Resumen

Cueros y pieles son materiales basados en proteínas que contienen grandes cantidades de agua, lo que los hace un medio nutritivo ideal para el crecimiento bacteriano. Siendo también la principal materia prima en la fabricación de cuero, es vital proteger cuidadosamente a la piel contra la acción bacteriana, especialmente en las primeras etapas de procesamiento húmedo. Es bien sabido que la acción bacteriana puede dañar seriamente la estructura de la fibra de los cueros y pieles. Desprendimiento del pelo, decoloración rojiza y separación de la flor son ejemplos típicos de que la acción bacteriana puede causar daños con defectos posteriores sobre el cuero curtido. El presente estudio identifica un nuevo defecto causado por una bacteria conocida como "biofilm" en cueros y pieles. Para el estudio de las áreas defectuosas observadas, se utilizaron dos técnicas: Microscopía de Barrido Electrónico (SEM), y Reacción en Cadena de la Polimerasa en tiempo real (RT-PCR). Esta última técnica, RT-PCR, es un método rápido y novedoso para la detección, identificación y cuantificación de microorganismos. Los resultados, obtenidos mediante la aplicación de estas técnicas, verificaron la presencia de "biofilm microbiano" en el cuero.

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INTRODUCTION

Hides and skins, which are the raw materials for the leather industry, are by-products of the meat industry. The leather industry constitutes a large market in the world with the capacity of processing millions of hides/skins. The quality of a leather product determines its value and price. The first provision is need of quality hide/skin for a high quality final product, besides a successful leather processing. However; hides/skins, which are the most important cost items for leather production, are exposed to many defects during the life of animal, slaughtering, within the period until they are taken into processing or even during leather making. These defects on the leathers cause serious loss of quality and economical value. Today, it is almost impossible to find a perfect animal skin; this is due in part to carelessness and indifference in breeding, feeding, handling, diseases and parasite control, all of which result in leather defects.[1] The leather industry has various complaints about the quality of raw materials, and resulting defects on finished leathers. For example, a company from Turkey has asked consultancy about an unidentified defect which they had observed on their upper leathers in wet-blue stage and subsequently on dyed leathers. Green stains in the form of unevenly distributed spots on the surface of wet-blue leathers, forming unevenness and roughness, and bigger circular areas in greenish color which remained undyed on the black crust leather sample, were observed from the visual examination of the samples (Figure 1).

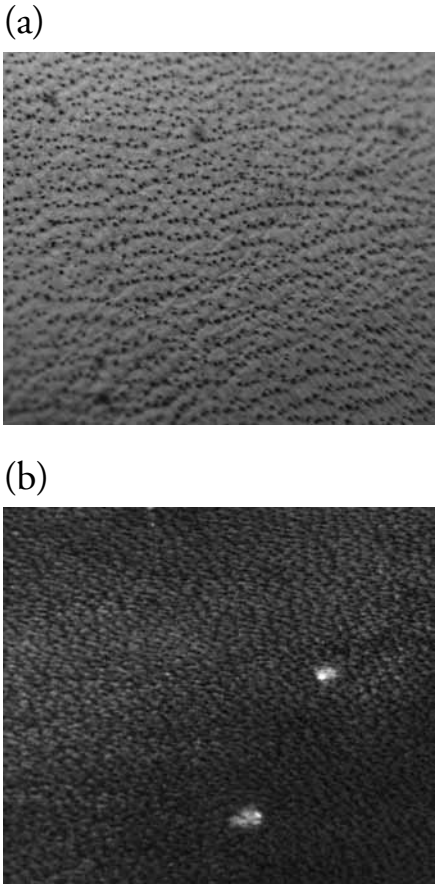

Figure 1. – Photographic images showing the defects on the leather samples: (a) wet-blue (b) black crust leather.

Upon the first microscopical investigations on the defected areas, and comparing them with the images of previous studies on biofilm, we have got the impression that these structures might be biofilms. A biofilm is composed of a single or multiple species of bacteria, embedded in polyanionic extracellular polymeric substances anchored to a surface.[2,3] The extracellular matrix is made from water (97%), secreted polymers, absorbed nutrients and metabolites, products from cell lysis and even particulate material and detritus from the immediate surrounding environment.[4] Thus, all major classes of macromolecule–proteins, polysaccharides, DNA and RNA can be present in addition to peptidoglycan, lipids, phospholipids and other cell components.[5] Biofilm formation consists of initial attachment, micro-colonization and EPS (extracellular polymeric substances) production, followed by maturation.[6] Attachment often occurs within 20 min to a maximum of 4 h by van der Waals and electrostatic forces, dipole-dipole interaction, hydrogen bonds, hydrophobic, and ionic covalent bonding.[7-12] Biofilms in nature can have a high level of organization, as they may exist in single or multiple species communities, form a single layer or 3 dimensional structures, or take the form of aggregates such as flocs or granules.[13-15]

Biofilms are also formed in drainage pipes for undersea tunnels, drainpipes, water-cooled sides of metal surfaces in heat exchangers, valves for oil pipes etc.[16] Bacterial biofilm formation in industrial water lines results in various problems, like drain-stopping, metal corrosion, obstacles to valve-handling, etc. Also in food industry, poor sanitation of food contact surfaces, equipment and processing environments has been a contributing factor in food borne disease outbreaks.[17] Biofilm is also a significant medical problem in human health because of its formation on orthopedic prostheses and other implanted foreign materials and in dentistry.[18,19] The present study deals with microscopical and molecular biological investigations to identify the cause of this defect which has not been mentioned before in the literatures for leather.

MATERIALS AND METHODS

Material

A wet-blue and a black upper leather sample having the defect described above.

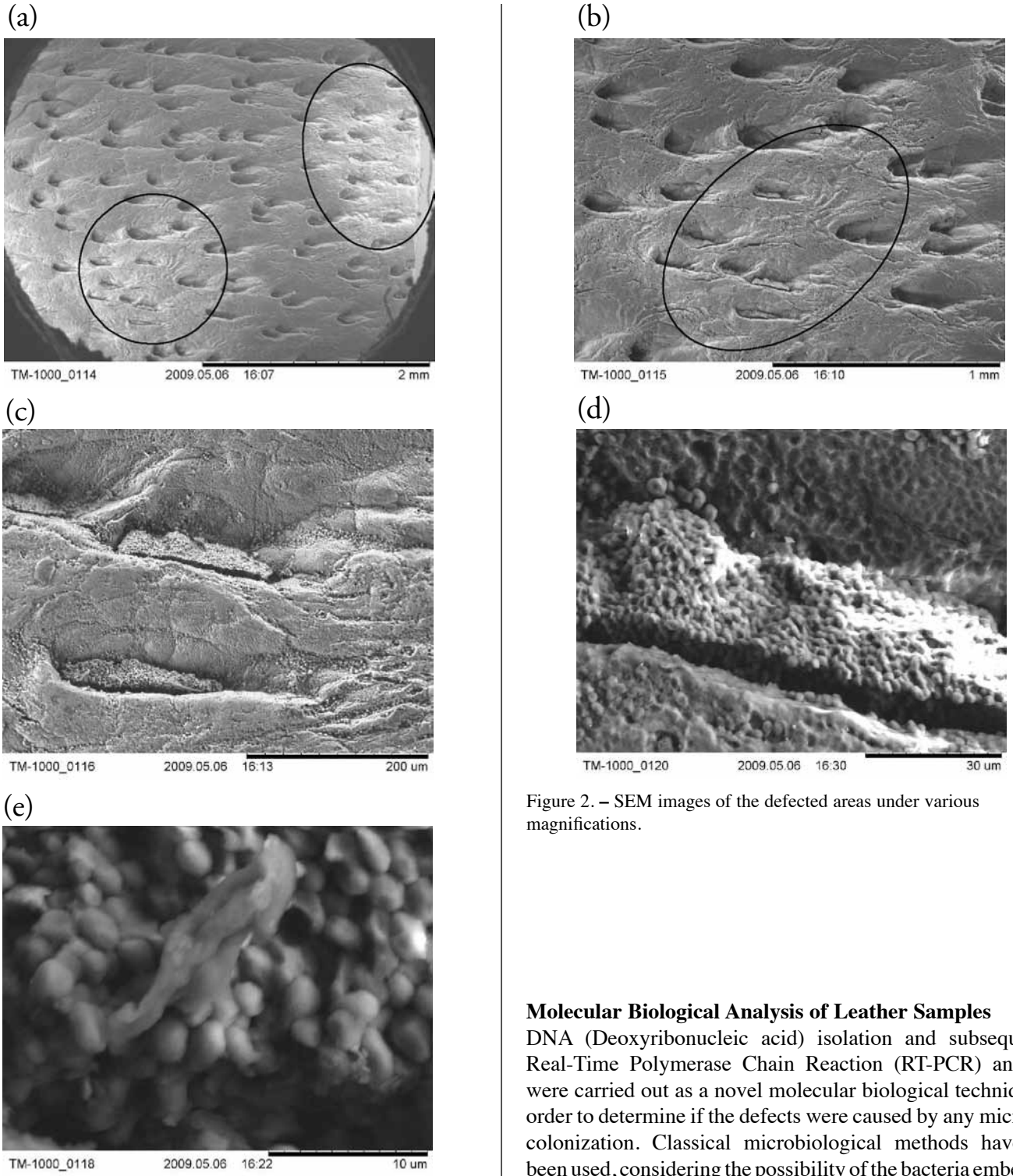

Figure 2. – SEM images of the defected areas under various magnifications.

Methods

Imaging the Leather Samples

Microscopic investigations were performed by using Hitachi TM-1000 table top scanning electron microscope (SEM) with the magnification power 20 – 10.000 and requiring no special sample preparation technique.

Molecular Biological Analysis of Leather Samples

DNA (Deoxyribonucleic acid) isolation and subsequently Real-Time Polymerase Chain Reaction (RT-PCR) analysis were carried out as a novel molecular biological technique in order to determine if the defects were caused by any microbial colonization. Classical microbiological methods have not been used, considering the possibility of the bacteria embedded in the matrix was not alive due to the chemical processes of leather making.

Total Bacterial DNA Isolation: Two different defected areas (D1, D2; 1mm^2) and a control area without defect (C; 1mm^2) were taken as samples from the leather surface. DNA extraction of the bacteria from the samples was carried out by using High Pure PCR (polymerase chain reaction) Template Preparation Kit (Roche Diagnostics, Germany) according to the manufacture's instructions.

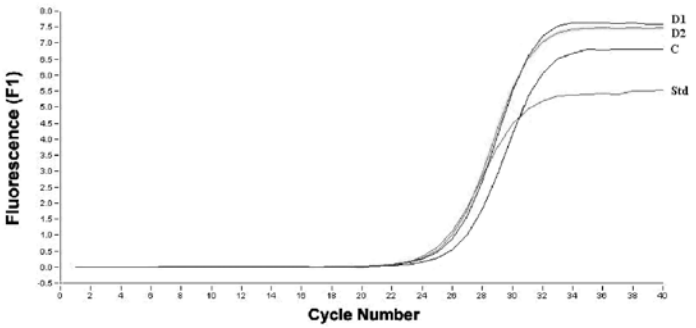

Figure 3. – Fluorescence emission of DNA samples related to the cycle numbers.

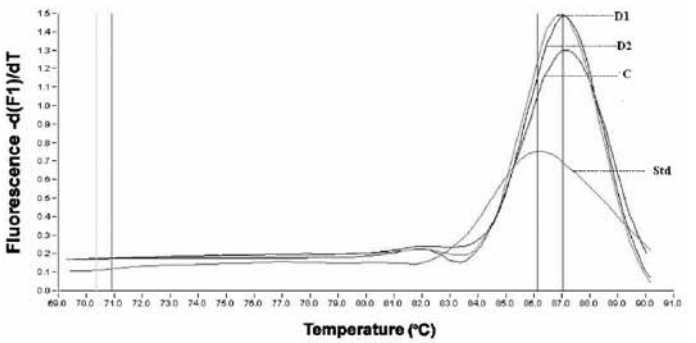

Figure 4. – Fluorescence emission of DNA samples related to the melting peaks.

Real-time PCR: Quantitative RT-PCR is a novel rapid method for the unique detection, identification and quantification of microorganisms. Individual bacterial species can be detected using with species-specific primers in Real-time PCR. This method is reliable and can provide accurate and sensitive bacterial quantification[20].

This assay was performed with Light Cycler 1.5 system (Roche Diagnostics, Germany) and the dsDNA-binding dye SYBR Green I® using universal bacterial primers. The total number of bacteria in samples was determined with a bacteria standard using the universal primers: TotalF (5'- GTG STG CAY GGY TGT CGT CA -3') and TotalR: (5'- ACG TCR TCC MCA CCT TCC TC -3')[21].

Using the Light-Cycler system is considerably rapid for detection of unknown samples. Briefly, amplification was performed in a 10μl of final volume containing 2.5μl template DNA, 1μl Light-Cycler – Fast-Start DNA Master SYBR Green I®, 1μl forward and reverse primers and 0.8μl $MgCl_2$, 3.7μl dH_2O. The protocol included the initial denaturation step at 20 ºCs^{-1} to 95 ºC with a 5-min hold was followed by 40 cycles for amplification step that comprised heating at 20 ºCs^{-1} to 95 ºC with a 10s hold and then cooling at 20 ºCs^{-1} to 61 ºC with a 1s hold, heating was continued at 20 ºCs^{-1} to 72 ºC with a 30s hold, melting step comprised heating at 20 ºCs^{-1} to 95 ºC with a 0s hold, cooling at 20 ºCs^{-1} to 65 ºC with a 15s hold, and heating at 0.1 ºCs^{-1} to 95 ºC with a 0s hold. Cooling step was performed at 20 ºCs^{-1} to 40 ºC with a 30s hold. Fluorescent products were detected at the last step of each cycle and melting curves were used to determine the specificity of the PCR.

Data acquisition and subsequent analysis were performed using Light-Cycler Software 3.5, Melting curve analysis was performed to determine the melting point of the amplification products for assessing the reaction specificity. Unknown sample curves were evaluated according to the curve of the standard total bacterial DNA. Thus, any possible primer-dimer interference was avoided. Quantification was performed using the automated (default) algorithm which calculates the crossing point as the first maximum of the second derivative of the amplification curve.

Results And Discussion

Firstly, leather samples were investigated under a light microscope and then the defected areas were further investigated with a scanning electron microscope under various magnifications. The defected areas indicated themselves with lighter color, as seen clearly from the images (Figure 2a-b). In higher magnifications these regions were observed distinct from the other areas as the follicles were filled with a cement-like material (Figure 2c-d).

In the case of more magnification: the matter which looked like cement, was found to be looking like bacteria colonies in the form of clusters as a bunch of grapes (Figure 2e). In order to confirm this foresight, the DNA was amplified by bacteria specific primers and RT-PCR analysis was applied to the samples. The results are given in Figure 3 and 4.

In the Figure 3, the cycle numbers of D1, D2, C and standard DNA were proliferated at the minute that the logarithmic phases increase. According to the initiation of the logarithmic phases of the cycle numbers that refers the amount of DNA; the defected regions have more DNA of bacteria than the non-defected area of the leather samples. This indicates that there is a bacterial colonization in the defected areas.

Melting points of the samples were investigated in order to confirm the results obtained from the Figure 3 (Figure 4). DNA amounts of the samples were calculated automatically, depending on the total DNA standard curve, by using Light-Cycler Software. Accordingly, analysis results of the two defected samples, D1 and D2 were not different from each other. The DNA amounts of D1 and D2 were calculated as $2.1x10^7$ and $2.4x10^7$ cfu/mm^2 , respectively. And the DNA amount of control sample was found to be $1.1x10^6$ cfu/mm^2.

These data confirm that the melting peaks of the D1 and D2 which represent DNA of the defected areas are higher than the Std (total bacteria standard) and C (Control) peaks which mean that the organisms present on the samples are bacteria and the population of the bacteria in the defected areas are higher than the others.

Considering the results obtained from SEM displays and RT-PCR analyses, the cause of the defects, seen as the cement like material filling the hair follicles on the leather, is concluded as a microbial biofilm.

CONCLUSIONS

When the results of visual displays and molecular biological analysis were evaluated, a new kind of bacterial defect, different from well known bacteria-borne defects[1,22,23] (like hair slip, red discoloration and grain pilling), on hide/skin and leather has been identified as biofilm. The defected areas on the examined leather samples present examples to these biofilms. The green spots observed on the leathers were possibly occurred due to the precipitation of chromium on the biofilm matrix accumulated in follicles. Similar findings regarding to the accumulation of chromium on biofilms were reported by Coleman and Paran.[24] The rigid, dissolvable and anionic nature of biofilm matrix has caused the defected areas to remain undyed in the black crust leathers even after dyeing process. It has been considered that the most suitable conditions for formation of biofilms on hides/skins would be in the period before (conservation and storage) or in early stages of beamhouse processes.

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