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Microbial community on industrial salty bovine hides: From the slaughterhouse to the salting

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ABSTRACT

The leather-making industry is an age-old industry and desiccation with salt has been one of the most used methodologies for obtaining valuable skins. However, halophiles may proliferate and affect the integrity of the hide-collagen structure, as well as leading to undesirable red colorations or less-frequent purple stains. To understand the basis of these industrial hide contaminations, the microbial community from raw hide samples, salt-cured samples and four different industrial salts, was analyzed by 16S rRNA gene metabarcoding together with standard cultivation methods.

Comparison of raw hides and correctly cured hides revealed a core microbiome that was absent from contaminated hides. In addition, archaea were missing from well-cured hides, whereas *Psychrobacter* and *Acinetobacter* were highly represented (23 % and 17.4 %, respectively). In damaged hides, only a few operational taxonomic units (OTUs), from among the hundreds detected, were able to proliferate and, remarkably, a single *Halomonas* OTU represented 57.66 % of the reads. Halobacteria, mainly *Halovenus, Halorubrum* and *Halovivax,* increased by up to 36.24–39.5 % in the red- and purple-affected hides. The major contaminants were isolated and hide infections, together with collagenase activity, were evaluated. The results showed that hides enriched with the non-pigmented isolate *Halomonas utahensis* COIN160 damaged the collagen fibers similarly to *Halorubrum*, and together they were considered to be one of the major causes. Putative degrading inhibitors were also identified from among the *Alkalibacillus* isolates.

It was concluded that hide contaminations were driven by clonal outbreaks of a few specific microbes, which may have been non-pigmented collagen degraders. *Acinetobacter* and *Alkalibacillus*, members of the core microbiome of raw and well-cured salted hides, are suggested as hide contaminant inhibitors that need further analysis.

Introduction

Since the Paleolithic period, human beings have used the hides or skins of animals for many purposes and leather making is an age-old industry. Once separated from an animal's body, the skin's exposed flesh is highly susceptible to microbial attacks as it is rich in proteins and lipids. Nowadays, from among the different technologies used for curing hides, the addition of crude salt (NaCl) is one of the most profitable techniques in the leather industry for obtaining high-quality end products, due to its dehydrating ability and bacteriostatic effect (Bailey, 2003; Dassarma et al., 2009; Wu et al., 2017). In the past, several improvements in the salt-curing system have been proposed and a number of additional chemicals are currently added to industrial NaCl; for example, EDTA, silica gel, sodium metabisulfite or boric acid (Akpolat et al., 2015; Kanagaraj and Babu, 2002; Wu et al., 2017). The addition of salt prevents a raw hide from rotting due to the growth inhibition of most microbes. However, as salt absorbs the moisture of the skin, halophilic prokaryotes present in the salt granules may find the ideal scenario to proliferate, due to the high nutrient concentrations present on the surface of animal skin, such as those derived from proteins and lipids. Subsequently, this growth produces damage, due to degradation of collagen fibrils, which affects the quality of the leather and lowers its value (Birbir et al., 2002; Birbir et al., 2004; Birbir and Sesal, 2003; Bitlisli et al., 2004; Wu et al., 2017). In parallel to structural weakness, the appearance of undesirable colorations due to the presence of bacterial pigments are of great concern in the leather industry, since they cause large economic losses. In this regard, the most common

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contaminations in salt-cured hides are the so-called “red-heat” deterioration or the less-frequent purple stains.

Microbiological analyses of the reddening of cured salted hides have been undertaken for a very long time (Stuart, 1933). As a result, several primary causal agents, mainly pigmented halophiles, have been isolated by conducting laboratory cultures on salt-processed hides from different animals (mainly calves, cattle, sheep and goats) (Akpolat et al., 2015; Birbir et al., 2004; Caglayan et al., 2017; Enquahone et al., 2020). Within the Archaea domain, members of the *Halorubrum*, *Natrinema*, *Halococcus* and *Halobacterium* genera have been among the most common isolates from red-heat deteriorated leathers (Bilgi et al., 2015). In the case of purple-parchments, *Halobacterium salinarum* has been identified as the precursor of a long succession of contaminants (Perini et al., 2019). On the other hand, within Bacteria, members of the Gammaproteobacteria and Firmicutes groups were also correlated with a contaminant role, mainly due to species from the genera *Alkalibacillus*, *Pseudomonas*, *Acinetobacter*, *Halomonas*, *Salimicrobium*, *Nesterenkonia* and *Marinococcus* (Akpolat et al., 2015; Birbir and Bailey, 2000). Actinobacteria have also been described as playing an important contaminant role at the first treatment steps (Migliore et al., 2017), as well as being dominant in the degradation of already tanned ancient leather books (Strzelczyk et al., 1987).

In addition to culturing methods, metabarcoding 16S rRNA analysis, which is a more suitable methodology for performing microbial community analysis, has also been applied to describe the complexity in chrome-tanned damaged hides and historical purple-affected parchments (Migliore et al., 2019; Migliore et al., 2017; Perini et al., 2019). This approximation has allowed a parchment biodeterioration model to be proposed where several microbes succeed each other at an affected site according to a “facilitation” process. In other words, the modification introduced by the activity of the primary colonizers will make the environment more favorable to incoming species other than the colonizers themselves. However, apart from these studies focused on historical parchments, little or no attention has been paid to the analysis of the microbial community that leads to daily red-heat damage, despite being probably one of the most common affections in the leather industry for a number of centuries. Although extreme halophilic communities from saltern brines or other saline environments have been described in detail (Benlloch et al., 2002; Fernández et al., 2014; Ghai et al., 2011), only microbes present in certain clean commercial edible salts have been described (Satari et al., 2021). The microbial composition present in the dry salt granules used in the industrial curing process or, more importantly, the differences between the damaged and non-damaged industrial hides are not yet known.

Therefore, the aim of this study was to extend the knowledge of the prokaryotic communities that proliferate and damage cow hides during the preparatory stages of industrial curing, namely, the salting-process prior to tanning. In order to determine the microbial agents responsible for undesirable contamination, 16S rRNA amplicon sequencing of biomass recovered from red-heat and purple-colored NaCl-cured bovine hides was performed. Comparisons with the agents present in undamaged hides, raw hides and the salt used in the process were performed and are discussed. Major contaminants isolated in the laboratory were further used in order to perform laboratory-scale hide infections.

Materials and methods

NaCl-salt and hide samples

A commercial coarse-grained marine salt (Tsal; 3 mm grain) obtained from the marine Torrevieja salterns (Alicante, Spain) was used as a control. Industrial NaCl, Tsal.ad, was obtained from a private company. Rsal was salt recovered at the end of the salting process after being sieved through 1 mm mesh in order to eliminate organic residues. Msal was obtained by mixing 30 % (w/w) Rsal with Tsal.ad. When sterile salt was needed, four rounds of autoclaving following desiccation at 65 °C were performed. Hide specimens were obtained from the same company. Fragments of approximately 3 × 3 cm were cut from raw hides (RHid), recently (3 h) salted hides (SRHid.ad), apparently well-cured hides (Hid.ad), and purple and red-heat damaged specimens (HidPUR.ad, HidRED and HidRED.ad). When possible, hides were collected in two different periods, winter samples (February 2020) and summer samples (June 2020, July 2021, September 2021 and May 2022).

DNA extraction

Hide fragments, including the hair, were used to recover the DNA of the associated biomass. DNA was extracted from hides by cutting them into small pieces and adding 3 mL lysis buffer (40 mM EDTA, 50 mM Tris-HCl and 0.75 M sucrose, pH 8.3). Then, 1 mg mL^{-1} lysozyme, 0.2 mg mL^{-1} proteinase K and 10 % SDS at 0.1 mL/mL were added to the samples. Two steps of phenol–chloroform extraction and one of chloroform were performed in order to purify the DNA in the aqueous phase. Finally, DNA was precipitated using 3 M sodium acetate in a 1:10 ratio and absolute ethanol in a 1:2 ratio. After 15 min centrifugation at 4 °C, DNA was finally dissolved in 50 μL mQ water. For the salt samples, 1 L of distilled water was slowly added to 200 g of salt and filtrated using 0.22 μm filters (Millipore) in order to retain the associated microbes. DNA was recovered from the filters by cutting them into small pieces and using the same protocol as for the hide samples.

16S rRNA metabarcoding

The V3-V4 region of the 16S rRNA gene was amplified by a PCR reaction using universal primers modified by the addition of the Illumina-adapter sequences. The extracted genomic DNA was also used for PCR amplifications of the V3–V4 region of the 16S rRNA gene using the universal primers Pro341F (Takahashi et al., 2014) and Bact805R (Herlemann et al., 2011). The 16S rRNA amplicon sequencing was performed using a 2x300 bp paired-end run of Illumina MiSeq Nextera (Novogene, Cambridge, UK). The median read length was 417 bp and the main genomic features of the 16S rRNA libraries are described in Table 1.

QIIME2 analysis

Sequences were processed following the QIIME2 pipeline (Bolyen et al., 2019; Caporaso et al., 2010) for paired-end 16S rRNA libraries. Briefly, after trimming all reads using the *Cutadapt* integrated tool in QIIME2, low-quality sequences were removed. The final number of reads analyzed was 4,626,968 for all collections. Sequences were clustered into operational taxonomic units (OTUs) by the *Vsearch* algorithm (Rognes et al., 2016), followed by deletion of chimerical sequences using the same algorithm implemented in the QIIME2 pipeline. OTUs were defined at the level of 98.7 % similarity for the whole 16S rRNA gene, which is a threshold previously used to distinguish species (Stackebrandt and Ebers, 2006). The taxonomic classification of the OTUs was carried out with the classifier trained against the SILVA reference database (version 138) using the machine learning algorithm Scikit-learn in QIIME2 (Pedregosa et al., 2011).

Principal coordinate analysis

The frequency of the OTUs obtained by the 16S rRNA genes in each of the metagenomes was subjected to a PCoA analysis using the R package *ape* (Analyses of Phylogenetics and Evolution) with Bray-Curtis distances (Paradis and Schliep, 2019) obtained from the QIIME2 pipeline.

Table 1
Statistics of the 16S rRNA gene libraries constructed from the bovine hides and salts used in the salting process. (s), sample recovered in summer; (w), sample recovered in winter.

	SAMPLE	Sampling date	# Raw Reads	# OTUs at 98.75 %	# OTUs at 98.7 % (no singletons)	# OTUs at 98.7 % (mean)	# OTUs at 97 %	# OTU at 97 % (no singletons)	# OTU at 97 % (mean)	Shannon index (based on OTUs at 98.7 %)	Pielou's Evenness (based on OTUs at 98.7 %)
Hides	RHid-1 (s)	July 2021	108,691	75,313	2964	3436	77,834	2482	2911	6.96	0.649
	RHid-2 (s)	July 2021	134,753	81,867	4049		84,726	3462		7.3	0.658
	RHid-3 (s)	July 2021	112,908	77,241	3296		79,576	2788		7.03	0.647
	SRHid-1 (w)	February 2020	156,925	136,354	2731	2763	139,077	2115	2136	5.54	0.548
	SRHid-2 (w)	February 2020	146,233	130,774	2715		132,612	2072		5.72	0.560
	SRHid-3 (w)	February 2020	154,133	137,542	2843		139,348	2220		5.53	0.550
	Hid.ad-1 (s)	June 2020	163,156	88,586	2305	2680	99,151	1499	1922	5.12	0.502
	Hid.ad-2 (s)	June 2020	164,191	88,402	2333		100,229	1520		5.33	0.523
	Hid.ad-3 (s)	June 2020	148,812	74,392	2696		84,036	1795		5.68	0.537
	Hid.ad-4 (w)	February 2020	135,158	114,439	3123		115,739	2518		6.67	0.635
	Hid.ad-5 (w)	February 2020	150,489	127,613	2944		129,090	2278		6.4	0.621
	HidRED-1 (s)	June 2020	161,536	120,167	1429	1460	136,606	961	1043	3.5	0.377
	HidRED-2 (s)	June 2020	166,803	131,925	1981		136,586	1365		4.07	0.417
	HidRED-3 (s)	June 2020	152,456	109,771	859		126,187	551		3.34	0.388
	HidRED-4 (w)	February 2020	153,995	141,180	1706		142,700	1322		3.93	0.424
	HidRED-5 (w)	February 2020	147,576	132,270	1325		135,486	1016		3.73	0.421
	HidRED.ad-1 (s)	June 2020	155,174	141,636	1006	1174	143,272	655	796	4.07	0.468
	HidRED.ad-2 (s)	June 2020	155,069	143,118	1501		146,601	1031		2.87	0.319
	HidRED.ad-3 (s)	June 2020	166,518	153,627	999		157,125	715		2.79	0.330
	HidRED.ad-4 (w)	February 2020	158,555	126,577	1245		133,578	832		3.82	0.433
	HidRED.ad-5 (w)	February 2020	141,911	118,321	1142		124,216	793		3.66	0.418
	HidRED.ad-6 (w)	February 2020	149,052	124,525	1153		130,559	748		4.56	0.495
	HidPUR.ad-1 (w)	February 2020	146,304	98,125	2898	2989	106,719	2326	2415	5.57	0.528
	HidPUR.ad-2 (w)	February 2020	159,657	112,301	3080		121,652	2504		5.53	0.527
Salts	Tsal-1 (s)	June 2020	167,015	92,524	2599	2734	104,352	1979	2102	6.16	0.584
	Tsal-2 (s)	June 2020	157,356	96,600	2869		104,844	2225		6.69	0.631
	Tsal.ad-1 (s)	May 2022	107,060	58,423	4527	3435	70,268	3457	2536	7.64	0.664
	Tsal.ad-2 (s)	May 2022	158,409	66,849	2342		144,887	1614		2.92	0.298
	Msal-1 (s)	June 2020	85,544	73,306	5813	2623	75,227	4662	2126	7.89	0.666
	Msal-2 (s)	September 2021	94,765	67,859	1353		71,408	1170		5.35	0.543
	Msal-3 (s)	September 2021	49,816	33,996	702		36,060	545		5.83	0.627
	Rsal-1 (s)	June 2020	29,385	24,475	4153	4258	25,198	3226	3089	10.55	0.877
	Rsal-2 (s)	June 2020	31,734	26,197	3704		26,707	2780		9.84	0.835
	Rsal-3 (s)	June 2020	155,829	135,903	4917		139,142	3262		8.51	0.753

Note. “s”, sample recovered in summer; “w”, sample recovered in winter. Tsal, saltern salt; Tsal.ad, industrial salt; Msal, mixed salt; Rsal, used salt; RHid, raw hide; SRHid.ad, salted raw hide; Hid.ad, undamaged hide; HidRED, red hide salted with Tsal; HidRED.ad, red hide salted with Tsal.ad; HidPUR.ad, purple damaged hide.

Rarefaction curves

Species richness analyses have been carried out by the performance of rarefaction (interpolation) and prediction (extrapolation) curves based on the Hill numbers method (Chao et al., 2014; Colwell et al., 2012). In this analysis, diversity was measured by species richness, with all species having equal weight. Bootstrapping was performed in order to achieve 95 % confidence intervals for interpolated and extrapolated curves (Chao et al., 2014). Data analyses were carried out by using iNEXT in the R package (Hsieh et al., 2016).

MetaFast 1.3 analysis

In order to analyze the relationships between the 16S rRNA collections from this study and related published collections, MetaFast v.1.3 (METAgenome FAST analysis toolkit) was used to calculate and build a distance similarity matrix between all collections and plot them into a heatmap.

Growth of isolates

A total of 102 isolates were obtained with a single medium at 20 % salinity and containing 1 % w/w yeast extract. Sterile Tsal was used instead of laboratory pure NaCl to prepare the media and the pH was adjusted to 8–8.2. Three purifying steps were performed from the initial colonies.

Protease and lipase activities

Isolates were subjected to a screening of their protease and lipase activities. Protease activity was detected on agar plates using 1 % skimmed milk and a positive reaction was considered when a clear area was observed around the colonies. Lipase activity was tested using olive oil and Tween. Rhodamine-olive oil agar medium was prepared using 10 g L^{-1} yeast extract, 20 g L^{-1} agar, 200 g L^{-1} Tsal, 30 mL L^{-1} olive oil and 0.005 % rhodamine dye (1 mg mL^{-1}). Positive reactions were considered when an orange fluorescent halo (visible under UV light) was formed around the colonies. Tween 80 and Tween 20 agar media were prepared with 10 g L^{-1} yeast extract, 20 g L^{-1} agar, 200 g L^{-1} Tsal, 0.1 g L^{-1} $CaCl_2{\cdot}2H_2O$, and 10 mL L^{-1} Tween 80 or Tween 20. Positive reactions were considered when a white precipitate was formed around the colonies.

Hide salting assays on a laboratory scale

Two parts of the same hide were cut into 20x20 cm pieces, piled in a similar way as in the industrial process (hair-inner part), placed into a plastic bag and covered with 200 g of the salt to be tested. Small per-

forations were made in the bag in order to eliminate lixiviation of the liquids. Once this process was finished (48 h), microbial cells were added as needed. A total of 7 mL of liquid cultures at an optical density of 0.5 were poured onto the salt and mixed properly along the hide surfaces. Each type of sample was performed in quadruplicate. The strains used in this experiment were *Halomonas utahensis* COIN8, *Halomonas utahensis* COIN160, *Alkalibacillus salilacus* COIN54, *Alkalibacillus salilacus* COIN63, *Halorubrum* sp. COIN130, *Halorubrum* sp. COIN132, *Halococcus* sp. COIN80 and *Halococcus* sp. COIN124. Hides were incubated for 60 days at room temperature. Salted hides with Tsal, Tsal.ad and Tsal.sterile were used as controls.

Collagenase activity measurements

Hydroxyproline quantities were quantified according to the manufacturer's recommendations using the Hydroxyproline Assay Kit (Sigma-Aldrich, MAK008) to perform the analysis. Briefly, 10 mg of hide samples were collected and homogenized in 100 μL of water and hydrolyzed by adding 100 μL of 12 M hydrochloric acid at 120 °C for 3 h. A volume of 200 μL was used to measure the optical density at 580 nm.

Nucleotide sequence accession numbers

All 16S rRNA Illumina collections have been deposited in the SRA repository under BioProject number PRJNA899442. Biosample SAMN31656062 was used for the salt samples and SAMN31655887 for the hides. 16S rRNA sequences of the isolates have been published under the accession numbers OP942474-OP942575.

Results and discussion

The hides and industrial salts analyzed were obtained from a private company (in Spain) and hide samples from two periods of the year (summer and winter) were recovered. Briefly, after the slaughterhouse, hides were usually cooled immediately after flaying (0–4 °C) and arrived at the salting plant in 1–3 days (Fig. 1). After that, they were layered with salt and kept desiccating at room temperature for 21 days. At the end of the salting process, the hides were normally transported to tanneries to obtain the final leathers.

To determine the microbial agents responsible for hide degradation during the salting process, the V3-V4 region of the 16S rRNA gene was amplified and sequenced from samples of hides and salts recovered at three different stages: raw hides, freshly salted hides, and at the end of the salt-curing process (Fig. S1). Mimicking the industrial process, no hair or residual fat or feces were removed from the surfaces of the hides. Industrial NaCl, “Tsal.ad”, was obtained from the same Spanish company and coarse-grained marine salt “Tsal” was used as a control. During the industrial recycling process, the Tsal.ad was normally mixed at 30 % (w/w) with sieved residual salt from the salting process (“Rsal”). The final mixture, “Msal”, was used to cover the fresh raw hides (“RHid”). Samples recovered from recently salted hides were called “SRHid.ad”, and hides that evolved successfully were called “Hid.ad”. Damaged reddish hides treated with the industrial Tsal.ad were named “HidRED.ad”, and “HidRED” was used to designate those hides treated with the control Tsal. Finally, the nomenclature for purple-damaged hides was “HidPUR.ad” (Fig. 1).

Taxonomic profiling by 16S rRNA amplicon sequencing

One of the major methods for identifying the microbial community composition in environmental samples is high-throughput 16S rRNA amplicon sequencing in combination with bioinformatics analyses. A total of 34 libraries were sequenced from different bovine hide samples and also from salts of different origin (Table 1) (damaged red-heat hides HidRED.ad and HidRED, n = 11; damaged purple hides HidPUR.ad, n = 2; raw-fresh hides RHid, n = 3; recently salted hides SRHid.ad, n = 3; undamaged salt-cured hides Hid.ad, n = 5; marine salt Tsal, n = 2; industrial salt Tsal.ad, n = 2; residual salt recovered after curing-salt process Rsal, n = 3; and mixed salt used Msal, n = 3). For most of the samples, three replicates were obtained; however, it was recognized that for those with only two, the number of samples was small and that the results would require more extensive studies in order to be extrapolated.

OTUs and biodiversity in hides and salts

Sample yield varied from 105,571 ± 49,139 to 154,535 ± 14,746 sequences (reads) among the hides and salt samples, respectively. These sequences were classified against the SILVA database (v.138) using QIIME2, and OTUs were defined at 98.7 % identity (id). From a total of 3,427,441 reads, only 0.125 % remained unclassified. The results showed that the highest number of OTUs was for the raw hides (3,436 OTUs); meanwhile, the damaged hides had less than half, 1,460 and 1,170 for HidRED and HidRED.ad, respectively. From among the salt samples, the highest number of OTUs was observed for Rsal (4,258 OTUs) and the lowest for Tsal.ad, Tsal and Msal (3,435, 2,734 and 2,623 OTUs, respectively). Compared to the broad number of OTUs already described in saturated brines, between 650 and 5,200 OTUs (Mani et al., 2020; Zhong et al., 2016), the numbers in the current study were within the same range.

Uniformity among replicates was measured by pairwise Bray-Curtis distances and *Permanova* analysis (Tables S1 and S2). With the exception of one of the Msal samples, all the replicates were considered acceptable based on principal coordinate analysis (PCoA). PCoA based on OTU abundances was used to compare the composition of the hides and salt samples. For the hides, the plot showed damaged (HidRED.ad, HidPUR.ad and HidRED) and undamaged samples (Hid.ad) in two sets that were clearly differentiated according to their nature (Fig. 2a). With the exception of HidRED.ad samples, differences between seasons (summer or winter) were also evident. The fact that undamaged hides (Hid.ad), especially those from winter, were placed close to the fresh raw ones (RHid), may suggest that a significant fraction of the original animal-skin microbes was not displaced by the halophiles loaded into the salt. Viable or not, these cells (or DNA) persisted until the end of the salting stage and were detected among the Illumina sequences. In this regard, further metatranscriptomic analysis would help to clarify how active these cells were and, interestingly, if competitive interactions between microbes may be used advantageously to prevent hide alterations. In other words, whether “beneficial” microbes could be used as a greener alternative to the chemicals added to the salt. Regarding the salt samples (Fig. 2b), the PCoA plot showed Tsal to be remarkably different from the industrial (Tsal.ad) and the residual salt (Rsal). Accordingly, when Rsal was recycled, the resulting mixed Msal was more similar to Tsal.ad (with one exception). Divergence of the Rsal was expected due to the different microbial load of the animal skins (e.g. different farms and feeding of the animals, different slaughterhouses, etc.).

Common OTUs between the samples were identified and are shown in Fig. 3 (an OTU was considered common if it was present in 75 % of the replicates following the criteria published in Ainsworth et al. (Ainsworth et al., 2015) or Coleine et al. (Coleine et al., 2018). Remarkably, no OTUs were conserved among all of them and only nine OTUs were found to be shared among all the hides, which belonged to *Halomonadaceae* (*H. utahensis*, *H. jeotgali* and *Chromohalobacter*) and *Bacillaceae* family (*Alkalibacillus salilacus* and *Virgibacillus*). From among the salt samples, without considering the more divergent Msal salt, eight OTUs were shared, including members of *Salinibacter* and *Alkalibacillus* (Table S4).

To evaluate the species richness and depth of sampling, the Shannon index (alpha diversity) and rarefaction curves were calculated

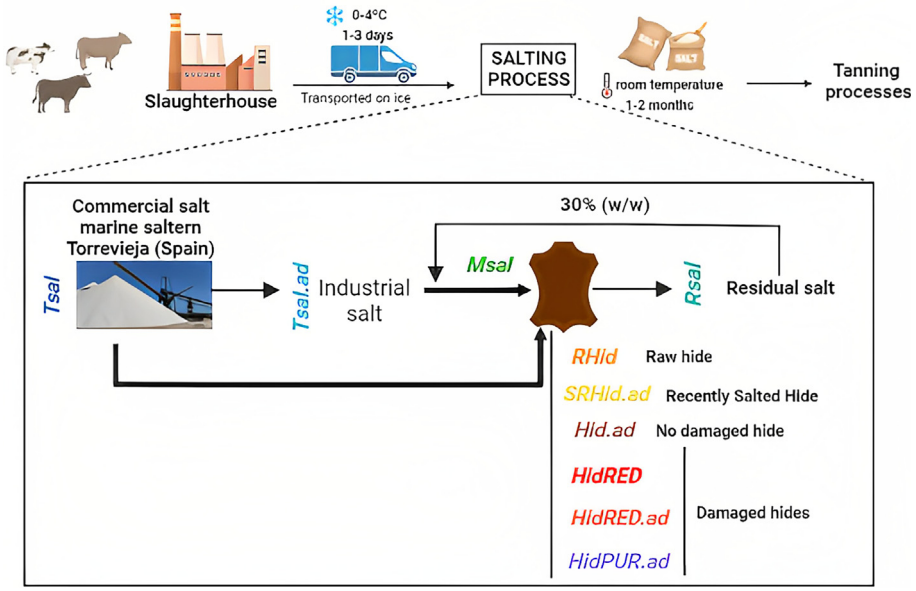

Fig. 1. Flow diagram of the steps of the bovine hide salting procedure and the salt samples used in the study.

based on 98.7 % OTU identities (Table 1, Fig. 2c–2d and Fig. S2). Supporting the previous OTU data, the higher diversity among hides was found for the fresh hides (RHid) (Shannon index, *Sin* = 7.10 ± 0.18) followed by undamaged hides from winter (Hid.ad; *Sin* = 6.53 ± 0.63) and the lower diversity for the red-damaged hides (HidRED and HidRED.ad, *Sin* = 3.71 ± 0.30 and 3.63 ± 0.69 respectively). Surprisingly, purple-damaged hides (HidPUR.ad; *Sin* = 5.55 ± 0.53) presented higher diversity than red-damaged ones (see the rarefaction curves, Fig. 2c). As seen below, this fact may be explained by a slightly higher percentage of under-represented OTUs (the black bars shown as "others" in Fig. 4a and 4b), together with the higher number of OTUs observed in this collection (Table 1). Conversely, a smaller number of clone-colonizers that proliferated extensively, decreasing the biodiversity, would be caused by red-heat contaminations. Rarefaction curves and *Sin* values were also calculated for salt samples and the highest value for the diversity was found in the residual salt (Rsal) recovered after the hide salting process, probably as a result of an additive effect of mixing salt from contaminated and non-contaminated hides (Rsal, *Sin* = 9.63 ± 1.03). This was followed by Tsal (*Sin* = 6.43 ± 0.37) , which mimicked the high diversity previously found in some saltern brines (Oren, 2008; Oren, 2020). Meanwhile, Tsal.ad and Msal showed uneven results between replicates that were difficult to explain from our data.

Microbial community on hides

As expected, the microbial community of the hides changed during the salt drying procedure. Regarding raw hides, previous studies reported different microbial composition in different skin regions, with the presence of different members of *Proteobacteria*, *Corynebacterium*, *Staphylococcus* spp., fungi, and also viruses (Grice and Segre, 2011). In the current study, raw hide samples were recovered randomly from different areas and hides (but from the same temporal batches) and the comparison of the bacterial communities did not show any significant divergence between them. OTUs were classified at the levels of phylum and genera, and the relative abundance was calculated (Fig. 4 and Fig. S3). Remarkably, 191 OTUs were found in both the raw hides (RHid) and the well-cured hides (Hid.ad), accounting for 65 % and 80 % of the total sequences, respectively. These conserved OTUs mainly consisted of members of the *Moraxellaceae* family. In particular, members of the genera *Acinetobacter* and *Psychrobacter* were highly represented on the undamaged hides (Hid.ad) (from 8 % to 22 % and 10 % to 16 % in raw and undamaged hides, respectively). *Alkalibacillus* (9 %), *Enterococcus* (6 %) and *Jeotgalibaca* (5 %) were also abundant in the well-cured hides (Hid.ad). The presence of *Acinetobacter* in the final salted hides (Hid.ad) was remarkable with up to 601 different OTUs. Despite the fact that 95 % of them could not be classified to the species level, two main OTUs (which represented a total of 76 % of the reads) were found to be similar by BLASTN comparisons to the water/soil non-pathogen isolate *Acinetobacter pseudolwoffii*. In general, *Acinetobacter* has been described as a fast-growing bacteria, with moderate or non-halophilic cells (Santhaseelan et al., 2022) but, interestingly, some strains have been described that have effective keratinases with low collagenase activities (Zhang et al., 2016). Therefore, although very speculative, it may be that the *Acinetobacter* present on the well-cured hides use the recalcitrant hair keratin as a carbon source and compete for other nutrients with the slower growing damaging Haloarchaea. Further metagenomics work in this area would clarify if this could be the case.

The recently salted hides (SRHid.ad) allowed the new microbiota added to the hides through the salt to be determined (Fig. S3a). As expected, *Haloferacaceae* members, commonly present in brines and salt granules, appeared in SRHid.ad for the first time (mainly *Halovenus*, 13.15 %; *Halorubrum*, 5.46 % and *Halobacterium*, 4.51 %). The final effect on the hide could be influenced by the selection of certain species, especially if enrichment led to a shift that favored these Archaea over time. Unexpectedly, one single OTU classified as *Halomonas utahensis* (previously *Pseudomonas halophila*) (Sorokin and Tindall, 2006) represented up to 99.3 % of the reads classified as *Halomonas* (17.67 % of the reads in SRHid.ad). Although *Halomonas* members have been described mainly as moderate halophiles, many are capable of growing at over 20 % NaCl w/v (Oren, 2010; Setati, 2010). Importantly, this OTU, together with the halophiles mentioned previously, increased to 38.41 % of the reads among the damaged hides (Fig. 4a), suggesting they had an important role as contaminants. Fecal debris attached to the surface of animal hides, which can be recovered at the end of the salting process through the recycled Rsal, would explain the presence of members of the *Enterococcaceae* family. These, which were barely detected in the fresh hides (0.06 %), represented up to 5.57 % of the reads in the recently salted hides (SRHid.ad). Other members of *Corynebacteriaceae* (*Corynebacterium*, 14.35 %) and *Carnobacteriaceae* (*Jeotgalibaca*, *Atopostipes*, 16.5 % and 4.44 %, respec-

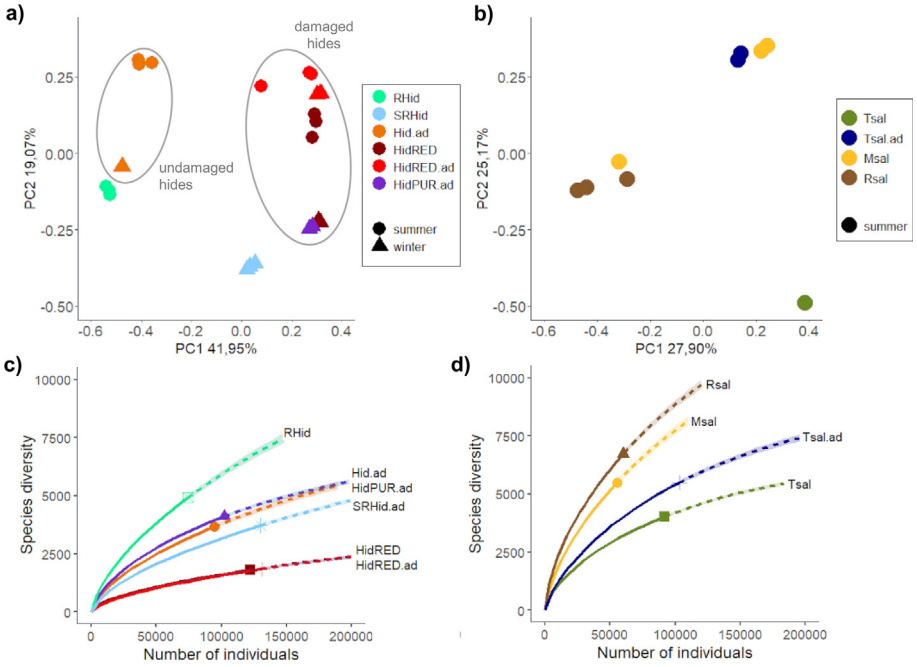

Fig. 2. PCoA plots of the bovine hides (a) and salt samples (b) of the 16S rRNA gene collections used in the study. Rarefactions curves for the hides (c) and salts (d) of the same datasets.

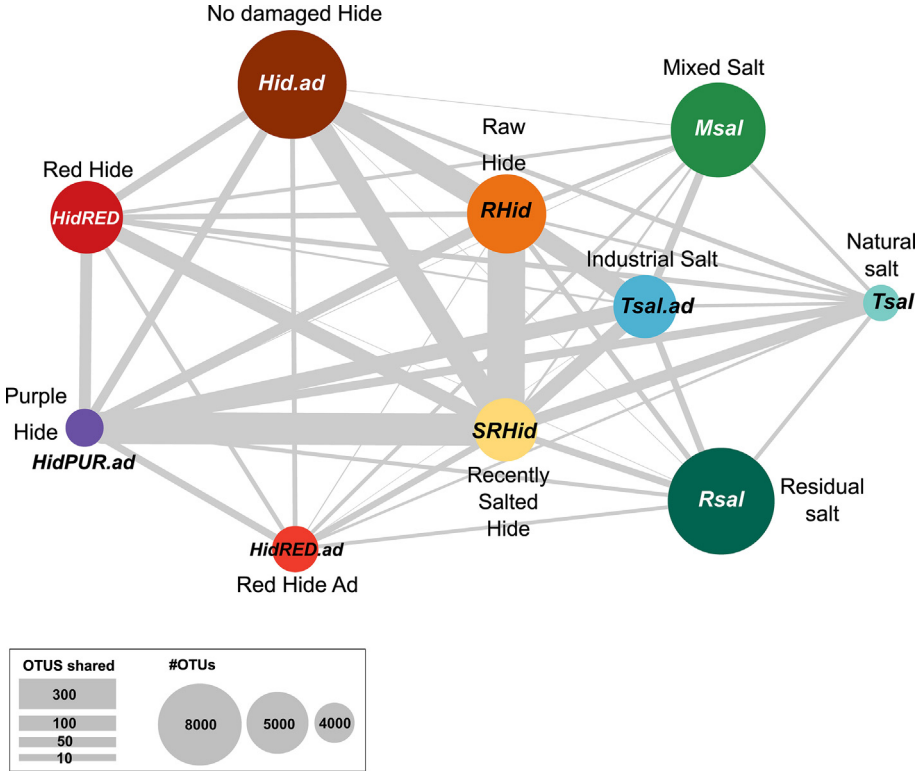

Fig. 3. Network of core bacterial operational taxonomic units (OTUs) on hides (RHid, SRHid.ad, Hid.ad, HidPUR.ad, HidRED and HidRED.ad) and in salts (Tsal, Tsal.ad, Msal and Rsal) during the salt drying procedure. The size of the nodes is analogous to the clustering coefficient for each OTU.

tively), as well as *Flavobacteriaceae* (0.81 %), decreased their abundance by 70 % to 90 % when the salt was added but were preserved in the final stage of the salting process.

The results showed Archaea members were barely detected on fresh hides (55 OTUs, 0.27 % of the reads) or on undamaged hides (428 OTUs, 1.36 %) (Fig. 4a and Fig. S3a). On the contrary, a total of 809 different archaeal OTUs were detected on the color-damaged hides, where *Halobacteria* stood out as the main cause of skin coloration, accounting for up to 36.2 % of the sequences in HidPUR.ad. The most frequent microbes were *Halovenus* (10.85 %), *Halorubrum*

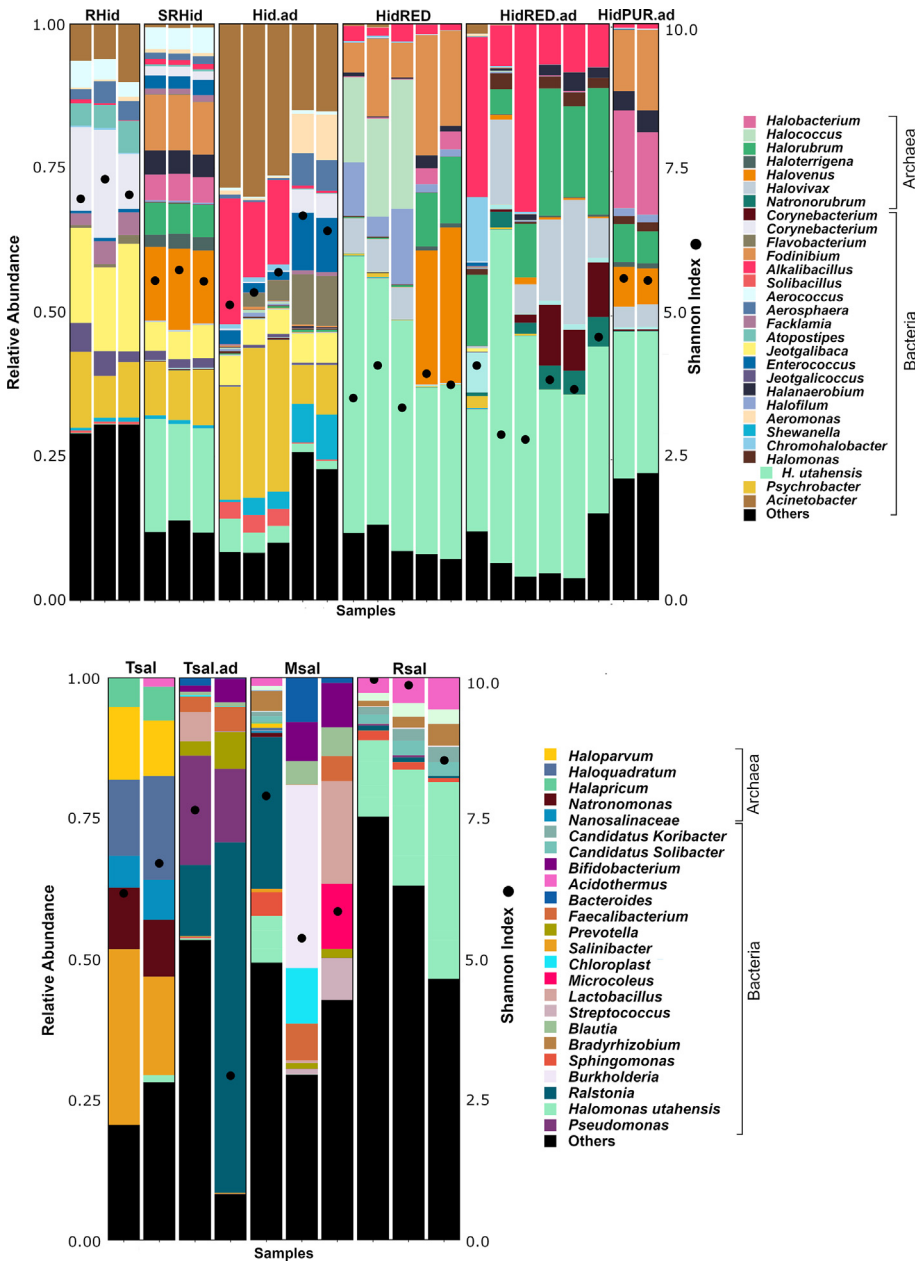

Fig. 4. Relative abundance of the microbial families found on hides and in salt samples used in the study. a) Fresh hides (RHid), recently fresh hides (SRHid), undamaged hides (Hid.ad) and damaged hides (HidRED, HidRED.ad and HidPUR.ad). b) Marine salt (Tsal), industrial salt (Tsal.ad), mixed salt (Msal) and residual salt at the end of the salting process (Rsal).

(4.61 %) and *Halovivax* (3.27 %) (*Haloferacaceae*). Also abundant were *Halococcus* (*Halococcaceae*), especially in the two summer HidRED samples. The presence of these archaea on the red-heat affected hides was not surprising as it has been reported several times that, for example, the *Halobacterium* and *Halorubrum* genera are mainly responsibility for skin colorations and degradation due to their proteolytic capabilities (Akpolat et al., 2015; Benmebarek et al., 2020; Perini et al., 2019).

In the red-damaged hides, Bacteria accounted for a total of 911,378 sequences assigned to 6,501 OTUs, which was eight times more than the Archaea OTUs (52.3 % of the reads and 3,855 OTUs in HidRED; 40.7 % and 3,745 OTUs in HidRED.ad). Notably, the family *Halomonadaceae* was prevalent with 40 % of the reads (553,783 sequences) and, as mentioned before in SRHid.ad, 92.6 % of them belonged to one single OTU classified phylogenetically as *H. utahensis* (data not shown). This OTU was barely found on the undamaged hide sequences (Hid.ad, only 2.8 %), and was also hardly found on raw hides (RHid, 0.073 %). Therefore, it was not only present on the reddish hides (35.87 % of the reads in HidRED.ad and 37.06 % in HidRED), but it was also found in the HidPUR.ad samples (24.9 % of the reads); therefore, *H. utahensis* could be considered one of the major contaminants of the hides in the salting process and may contribute actively to hide degradation. To our knowledge, the high ratio of *Halomonas* found in the salted hide samples has not been described previously. Its natural presence at a low level in Tsal (1.33 %) and Tsal.ad (0.33 %), and its persistence in Msal, suggested that despite being underrepresented in salt granules (or animal skin), it would proliferate more easily in a richer environment, such as the protein-rich hide surface, thus explaining the low diversity observed in the red-damaged hides (Fig. 4a and Table 1). Other putative causal isolates found among the damaged

hides were *Fodinibius* (Bacteriodetes), which increased one order of magnitude regarding RHid (0.3 % – 13 %), and members of *Bacillaceae*, mainly *Alkalibacillus*, which were present in HidRED.ad and HidPUR.ad (from 9 to 19 %). Previously, members of *Halomonas* were isolated from several skins (Enquahone et al., 2020) and were also present in tannery sludge contaminated soils (Kong et al., 2019) due to their resistance to harsh environments (alkaline and chromate resistance). Apart from this, several *Halomonas* and *Alkalibacillus* strains have been described as active collagen-degraders and bacteria that use amino acids as carbon sources (Caglayan et al., 2017; Caglayan et al., 2018). Similarly, a collagenase was described in *Halomonas stenophila* (Anithajothi et al., 2014; Hinrichsen et al., 1994; Sánchez-Porro et al., 2003) and another 153 putative collagenases are annotated among the 439 *Halomonas* genomes actually found in GenBank (in *H. utahensis*, none of the 21 proteases were annotated as collagenases).

Regarding the purple hides, the bacterial community structure varied slightly, with a decrease in the *Halomonadaceae* family (214 OTUs, 26.7 %) and an increase in *Halobacteriaceae* (188 OTUs, 15.6 %) (Fig. S3), compared to red-damaged hides. A total of 64 OTUs (31 Archaea and 33 Bacteria) were shared between HidPUR.ad and HidRED.ad, and the major contributors were *Halomonas* (26.4 %, mainly *H. utahensis*), *Halobacterium* (15.5 %), *Fodinibius* (12.3 %), *Halovenus* and *Halorubrum* (< 6.5 %).

Actinobacteriota, described as one of the most prominent degraders in the history of tanned leathers and parchments (Migliore et al., 2017), was fairly represented in the raw hides (RHid), SRHid.ad and Hid.ad (793, 348 and 340 OTUs, respectively) (Fig. 4a and Fig. S3). Despite being at a low proportion after the salting process (and even less probably after tanning), surviving or new Actinobacteria would grow again in the long term on leather surfaces. This suggests that Actinobacteria are common members of the microbial community from the first steps of hide processing until their transformation (Chao et al., 2014; Karbowska-Berent, 2000; Migliore et al., 2017). Therefore, the observations of Migliore et al. (Migliore et al., 2017) could be supported (but extrapolated to bovine hides) that "Actinobacteria are not only relevant in the last act of the parchments tragedy, but are also key players from the very first act of the drama".

Finally, it is interesting to note that, despite the high number of coexisting OTUs observed under the "contaminant" genera, only one (or very few) were capable of proliferating successfully on the hide surface where they caused the undesirable colorations. In spite of the obvious differences, these "clonal outbreaks" behaved similarly to clinical clonal pathogen infections (Fig. 5), which may allow the hide contamination problems to be treated with more concrete strategies against specific microorganisms. However, due to the ambiguity of the partial 16S rRNA genes, it is also possible that more than one microbial species could have been included under the same OTU and, therefore, deeper analysis must be performed to clarify these propositions.

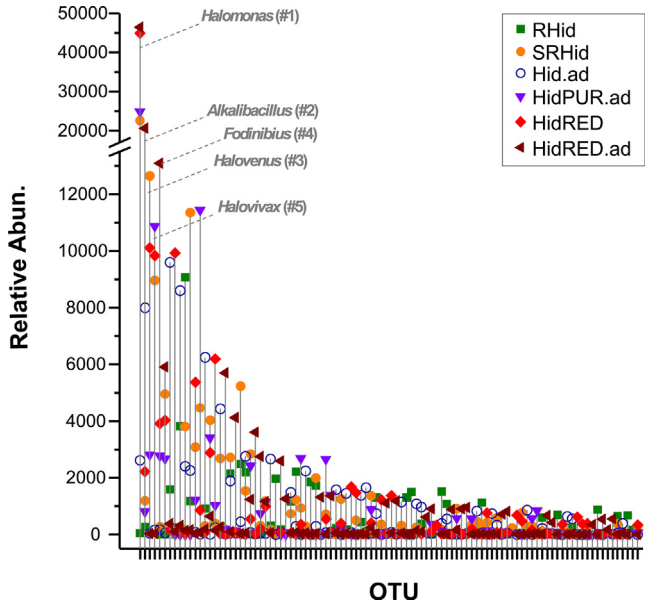

Fig. 5. Relative abundance of individual OTUs (x axis) obtained from the16S rRNA gene Illumina collections for the bovine hides used in the study.

Microbial community in salt

Stripped hides are normally covered in 35–40 % w/w NaCl to preserve them for one/two months during the salting process. To achieve a well-cured hide, it is recommended to cool them immediately after flaying, avoiding the flesh side uppermost as this can allow the flesh surface to dry out, making salt penetration difficult in the next step of the process. The flayed skin should be treated with clean NaCl (salt), which is normally coarse-grained salt. Ideally, salt crystals with a size of 2–3 mm should be used, since larger crystals are slower to dissolve and finer crystals can cause the leather to crack. In this respect, salt samples were completely dissolved in water to recover the biomass. Therefore, the microbes trapped within liquid inclusions throughout the growing crystals (Huby et al., 2020) were also included in the analysis.

When the taxonomic profiles of the salt samples were obtained, remarkable differences between the replicates of Msal and Tsal.ad were found depending on the sampling time, when *Ralstonia* sequences were present in the Tsal.ad and Msal obtained in summer 2021 and 2022, but they were absent in Msal from a previous salting process in 2020. When Tsal and Tsal.ad were compared, they were found to share a low number of species (31 OTUs) (Fig. 4b and Table S3). The best represented groups in Tsal, *Haloferacaceae* and *Nanosalinaceae*, decreased from 36 % to 0.15 % and 6.5 % to 0.004 %, respectively, in the industrial salt (Tsal.ad). The halophilic bacteria *Salinibacter ruber* and the square archaeon *Haloquadratum walsbyi* have been shown to be highly represented in multiple marine brines (Akpolat et al., 2015; Anton et al., 2000; Cray et al., 2013; Ghai et al., 2011; Oren and Hallsworth, 2014), but they almost vanished from the Tsal.ad (from 24 % to 0.1 % and 16 % to 0.05 %, respectively) and were displaced by human- and animal-associated bacteria, such as *Ralstonia*, *Pseudomonas* or *Prevotella*. Differences in the industrial salt samples were expected for many different reasons, such as a mixture of different salt batches (with different qualities and origins), different origins for hides with different microbial loads (which remain at the Rsal stage and, consequently, end up in the Msal), together with a plethora of external inputs, such as industrial mixers, human manipulation, etc. In any case, the halobacteria introduced with the salt that were noticeable in the recently salted hides (SRHid.ad), 0.021 % in RHid *vs* 29 % in SRHid.ad, suggested that the variability of the microbial community carried by different industrial batches of Msal was high and, in turn, the control of red-heat was a logistically difficult industrial task.

Comparison with other 16S rRNA Illumina collections

As previously shown, an important part of the microbial community present in the raw hides persisted in correctly cured hides, suggesting that initial microbial composition deserved further analysis. The microbiota found on the raw hides in this current study was compared with that found on living animals using 14 different 16S rRNA collections obtained from bovine guts (n = 8), bovine fecal samples (n = 3) and living bovine skins (n = 3) (Table S4). The resulting cladogram showed the libraries were placed in two main branches:

one containing damaged hides and the SRHid.ad samples together, and another for the remaining samples that were separated into several sub-clusters. One of them (marked with an asterisk in Fig. S4, top cluster), contained sequences from the gut, feces and live-animal skin datasets in one branch, while the other (marked with two asterisks, bottom cluster) comprised samples from raw and salted undamaged hides. These results agreed with previous data (damaged hide samples and recently salted ones that appeared together due to the high loads of the archaeal halophiles present in both) that indicated it was also reasonable to find the fecal and bovine-gut collections together (Cernicchiaro et al., 2019). In addition, it was logical that these microbes could overlap with those present on the skins, especially if the animals had been confined in small facilities. The clustering of external bovine hides (from very different origins and times) close to the Hid.ad and RHid samples, suggested the existence of a core microbiome that may constitute an underestimated array of biocontrollers against contaminant proliferation; however, further analysis in this direction would be valuable.

Contamination test of cultured isolates from bovine hides

In order to culture the major hide contaminants detected in the 16S rRNA Illumina collections, the isolation and identification of 102 colonies was conducted (Table S5). Their 16S rRNA gene determined that 83.5 % of them were bacteria and belonged to *Bacillaceae* (n = 44) and *Halomonadaceae* (n = 37). Non-pigmented bacteria from the genera *Halomonas*, *Alkalibacillus*, *Chromohalobacter*, *Marinococcus* or *Virgibacillus* also grew. From among Archaea (n = 17), isolates were identified as Halobacteria, mainly *Halococcus* (n = 13) and *Halorubrum* (n = 4). The abundance of their 16S rRNA sequences was evaluated using previously described Illumina libraries. Remarkably, 31 isolates recruited more than 5 % of the reads in at least one library (Fig. 6a) and from among these major contaminants, two isolates were related to the species *H. utahensis* (up to 33.27 % in HidRED.ad), 13 isolates were similar to *Alkalibacillus salilacus* (up to 12.93 % in HidRED.ad), 12 isolates were from genus *Halococcus* (up to 8.7 % in HidRED), three were *Halorubrum* (up to 7.65 % in HidRED.ad) and one was *Escherichia coli* (12.9 % in HidRED.ad) (Table S5). From among them, unevenly represented isolates from the same species were detected (in damaged hides), which supported the previous notion that hide damage occurred due to expansion of a few clonal or similar strains. Such population dynamics may have important applications and the discovery of key bacterial factors for these colonizers may lead to their use as "therapeutic" targets for solving the red-heat and purple coloration problems.

To analyze the *in situ* degradative/coloring capacity of these well-represented isolates, eight of them were selected (*H. utahensis* COIN8 and COIN160, *Alkalibacillus* sp. COIN54 and COIN63, *Halorubrum* sp. COIN130 and COIN132, and *Halococcus* sp. COIN80 and COIN124) and a laboratory-scale contamination test was performed. From liquid cultures, approximately 1.4×10^5 CFU were added to 200 g sterile salt (Tsal) in contact with raw hide pieces (20 × 20 cm) and incubated for 60 days at 25 ˚C. Hide samples were collected at intervals of 48 h, 21 days and at the end of incubation. Hides were frozen (-20 ˚C) until processing. Torrevieja saltern salt (Tsal), industrial salt (Tsal.ad) and autoclaved salt (Tsal.sterile) were used as controls. After 10 days, the hides treated with Tsal.sterile and those inoculated with *H. utahensis* COIN8, *H. utahensis* COIN160, *A. salilacus* COIN54 and *A. salilacus* COIN63 presented no visible signs of deterioration. However, hides treated with *Halorubrum* sp. COIN130 and *Halococcus* sp. COIN80 showed expansive reddish colorations on the flesh side. After 60 days incubation, samples with Tsal and those infected with archaea acquired an orange-red intensity (Fig. S5) and those treated with sterile salt, *A. salilacus* COIN54 and *H. utahensis* COIN160, developed a lighter pink coloration. Finally, samples with Tsal.ad did not develop any significant coloration. The fact that hides with sterile salt also developed coloration may be due to proliferation of halophilic or salt-resistant archaea present in very low concentrations on the original animal skin, as described by Ross et al. (Ross et al., 2018).

Quantification of free hydroxyproline (HPro) due to collagen degradation was measured in duplicate for each of the replicates (Fig. 6b). The results showed higher quantities of HPro in samples recovered at 48 h than those recovered at 21 days, which was probably due to the presence of this amino acid in other structural hide proteins (Ignat'eva et al., 2007). Once dehydration of the tissue takes place (leaching), the HPro concentration decreases, since collagen may also diminish (Wess and Orgel, 2000; Wess and Orgel, 2000) and, at the end of the experiment, the detected HPro was mainly due to collagen degradation. At 60 days, the highest HPro concentrations were found in Tsal and *Halorubrum*-infected hides (also in two samples with *Halococcus*). Interestingly, hides with an extra load of *H. utahensis* COIN160 cells presented similar values compared to hides infected with *Halorubrum*, despite showing the lighter pink coloration (comparable with those of Tsal.sterile). It was confirmed that this white colony-forming bacteria could degrade collagen *in situ* very efficiently without being visually noticed. Furthermore, following the "facilitation model" proposed by Migliore et al. (Migliore et al., 2017); it is likely that these *Halomonas* cells, which are faster growers than Haloarchaea, will contribute significantly at the outset of degradation, by acting as a no-return "declining point" for the hide and improving the expansion of the next slow growing Haloarchaea degraders.

Finally, the lowest final HPro concentrations were detected in *Alkalibacillus*-infected hides. The interpretation of these results may be twofold. First, low HPro concentrations and the fact that colorations where lighter than the controls, may suggest an interesting inhibitory effect over collagen degraders by this Bacilli that deserves wider investigation for clarification. Alternatively, collagen degradation may also take place, although hydroxylamine could be removed by endogenous dehydratases and hydroxyproline could be used as a carbon source, as previously described (Caglayan et al., 2018; Watanabe et al., 2017).

Conclusions

Red-heat (and purple) affected stock is undesirable as the resultant industrial hides often show grain damage, weakness, looseness and staining defects. For the first time, the results of this study allowed the highly complex community present on industrial hides to be unraveled during the salting process, as well as the microbial community added through the salt and that already present on the raw hides. This comparative study revealed a core microbiome that was shared by raw and well-cured, undamaged hides. As a result, the role of *Acinetobacter* members, among others, as antagonists of halobacterial damage must be further analyzed. A yet to be clarified imbalance of a hide's initial microbial community due to the extra load of salt-associated microbes, favoring the overgrowth of contaminant bacteria or haloarchaea, triggered a decrease of biodiversity and promoted red-heat expansion. On the damaged hides, besides the already suspected haloarchaea previously identified in several studies (*Halorubrum*, *Halococcus*, *Halovenus* and *Halobacterium*), major bacterial groups were even better represented than archaea (i.e. *Halomonas*, *Alkalibacillus* or *Fodinibius*). Interestingly, only a few OTUs from among the hundreds detected belonging to the same species were able to proliferate on the damaged hides. Due to different growth speeds, a synergistic cooperation between them could not be discarded, and it was possible that a single clone acting alone could not have led to the damage but rather it was due to a complex internal dependency, although with less abundant microbes.

A salt curing process on a laboratory scale allowed *in situ* collagenase activity by previously isolated major contaminants to be determined. From the hides in the current study, one non-pigmented

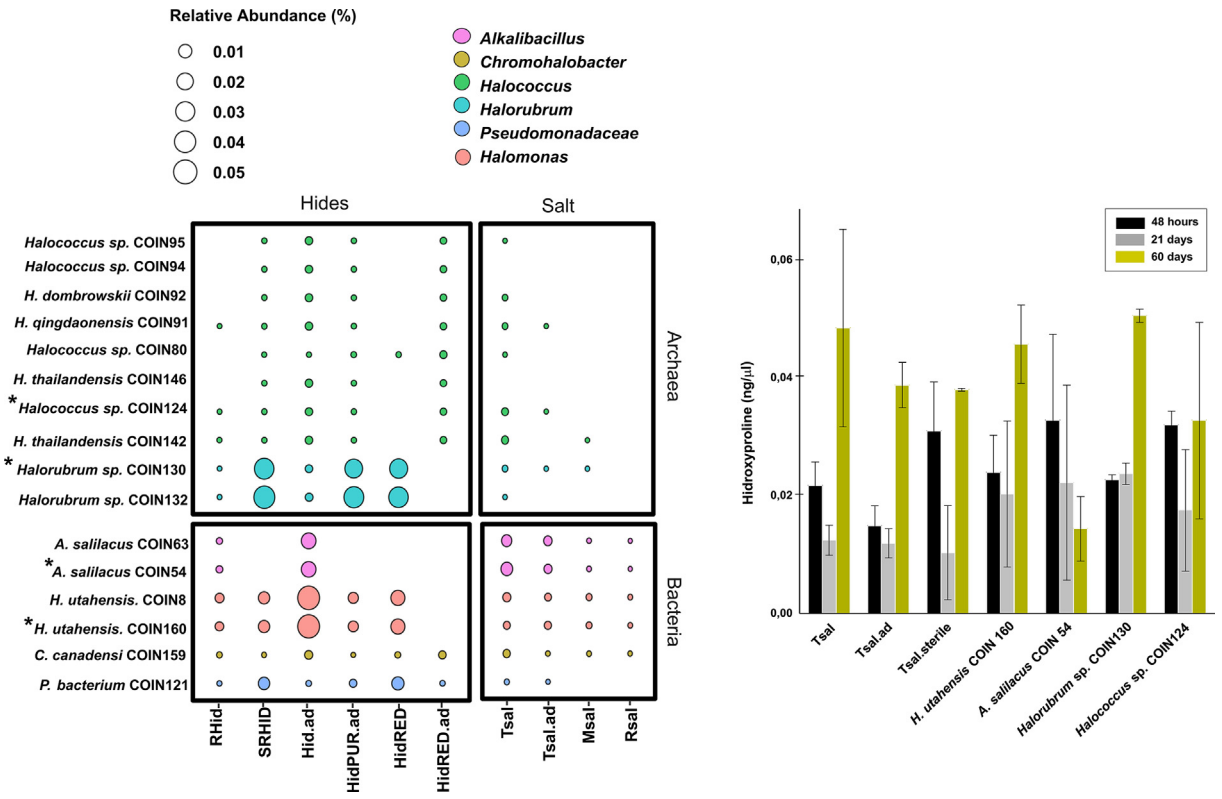

Fig. 6. A) Recruitment of the top 10 isolates among the 16S rRNA collection obtained from the libraries of hides (RHid; SRHid.ad; Hid.ad; HidPUR.ad; HidRED; HidRED.ad) and salts (Tsal; Tsal.ad; Msal; Rsal) sequenced in this study. (*) Isolates used in the laboratory-scale contamination tests. b) Hides infected with *Halomonas utahensis* COIN8, *Halomonas utahensis* COIN160, *Alkalibacillus salilacus* COIN54, *Alkalibacillus salilacus* COIN63, *Halorubrum* sp. COIN130, *Halorubrum* sp. COIN132, *Halococcus* sp. COIN80 and *Halococcus* sp. COIN124.

Halomonas was determined *in situ* as the major cause, together with *Halorubrum* or *Halococcus*. However, more research work should be undertaken in order to clarify better the involvement of non-pigmented microbes in hide degradation, since even no noticeable visual damage may still cause failure of subsequent processed hides and degradation in the very long term.

To prevent the negative impact of halophilic microbes on salted hides, it is important to identify the degradation-causing microbes. Further analysis should address the functionality and expression of important key degradative enzymes that require the use of both cultivation and cultivation-independent methods. These analyses should promote further studies on the intricate dynamics of the microbial community dynamics involved in hide biodeterioration, and will facilitate the prevention of hide colonization through bioremediation.

Authors' contribution

ABMC, FNM and ACL performed the bioinformatic analysis, ABMC and JT planned the experiments, FNM carried out the 16S rRNA amplicon analysis and obtained the DNA from the samples, ACL worked with the isolates, and ABMC wrote the manuscript.

Data availability

I have shared the link to my data in the attached file

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.syapm.2023.126421.

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