THE HUNT FOR RED 'MICROBA': IDENTIFICATION OF MICROORGANISMS INVOLVED IN 'RED HEAT' CONTAMINATION OF SALT-CURED HIDES

S. M. Grace[1], M. L. Patchett[1] and G. E. Norris[1]

1 School of Fundamental Sciences, Massey University, Palmerston North 4410, New Zealand

a) Corresponding author: s.m.grace@massey.ac.nz

Abstract. 'Red heat' describes a specific and undesirable microbial contamination of salt-cured products, and is attributed to the occurrence of halophilic microorganisms in the curing salt. For this study, traditional microorganism cultivation is complemented by a cultivation-independent method to determine the taxonomic composition and diversity of microbial populations. Being compared are samples of untreated unaffected salt-cured, and red heat-affected salt-cured bovine hide, along with the curing salt products used. Marker gene sequencing is the primary method of identification for cultured isolates, with metagenomic amplicon sequencing or 'metabarcoding', planned to determine the bacterial, archaeal and fungal components of mixed microbial populations in these samples. This approach is predicted to reveal taxa that have escaped cultivation so far, which may be key to the onset of red heat contamination. This knowledge is expected to assist the leather industry by informing the design of a rapid screening method based on molecular techniques, to detect the occurrence of such taxa in curing salt.

1 Introduction

'Red heat' is an industry term that describes the appearance of brightly coloured patches, streaks or spots on salt-cured products, and is attributed to the occurrence of halophilic ('salt-loving'[1]) micro-organisms in the curing salt.[2-5] Red heat contamination is associated with damage and spoilage of cured hides and skins, understood to be the result of degradative, hydrolytic enzymes secreted by such organisms[2-7] often resulting in defective leather products[8-11] causing wastage and economic loss to industry participants.

Much of the historical work on the microorganisms of red heat has necessitated careful and laborious cultivation, limited to phenotypic analyses**.** Whilst such methods remain the gold standard of microbial diagnostics,[12] characterisation of slow-growing and difficult-to-culture microorganisms remains challenging.[13] Cultivation-independent, molecular techniques such as marker gene sequencing and metagenomic analysis can circumvent some of these difficulties.[14-15] Additionally, such techniques can directly sample a particular environment and begin to describe the *in situ* population of microorganisms.[16-17]

For this study, traditional cultivation was complemented by a cultivation-independent method to identify the composition and diversity of microorganism populations.[18] To control for between-animal variation, a single biological sample of cattle hide was treated with two different salt products. This was carried out to compare the effect of these treatments on the composition and diversity of the microorganism population within that hide. One of the curing salts is a minimally processed, unsterilised product, shown to produce discolouration characteristic of red heat; while the other has been subjected to a heat sterilisation (stoving) step during processing.

The cultivation-independent approach is predicted to show greater microbial diversity by uncovering taxa that are difficult to culture under conventional laboratory conditions. Such microorganisms may be key to the onset of red heat contamination in salt-cured hides and skins. To this end, both approaches primarily employ marker gene sequencing: the phylogenetically-informative 16S ribosomal

RNA gene is targeted to identify organisms of bacterial and archaeal origin,[19] while the ITS2 region of the eukaryotic ribosomal gene group is targeted for the identification of fungal organisms.[20]

In cultivation, marker gene sequencing represents a single genome per isolate. In contrast, cultivation-independent methods attempts to capture the microbial metagenome; that is, all of the microbial genomes of a mixed population. As a relatively cost-effective and less computationally-demanding alternative to whole-genome metagenomics, only the phylogenetic marker genes are targeted, as a proxy for the metagenome of a given sample. This is known as metagenomic amplicon sequencing, or 'metabarcoding.[21-22] A metabarcoding approach planned for this study, using the Illumina MiSeq high-throughput sequencing platform.

This paper describes the progress made thus far with the identification of cultured isolates and the preparation for the metabarcoding experiment. Once completed, it is anticipated that these results may provoke further investigation into whole-genome, functional metagenomics of salt-cured products, to better understand the microbial genes that influence contaminations such as red heat. For the leather and tanning industry, this knowledge may be useful by identifying new microbial targets for both prevention and control. On the basis of the methods used in this study, a PCR-based test could be developed, to allow rapid screening curing salts for microorganisms that either influence or cause red heat in salt-cured products, thus preventing the potential for red heat to occur.

2 Methods

2.1 Curing Salts Used

Two commercially solar-evaporated salt products, graded for agricultural use, were selected for comparison. Both were sourced from a New Zealand salt works. The salt referred to as 'unsterilised' is a raw, coarse-grained (10 mm) product with a typical moisture content of 1.8%. The salt referred to as 'sterilised' is a crushed (grain size <2.0 mm), washed, kiln-dried (stoved) product with a typical moisture content of 0.05%. Microorganisms were easily cultured from the unsterilised salt and produced discolouration characteristic of red heat when applied to pieces of bovine hide. Such contamination was not replicated when the sterilised salt was used. The same batch of each salt product was used to cure both of the cattle hides used in this study.

2.2 Hide Treatment & Sampling

Unshaved cattle hide from the OSP (official sampling position) was divided into two equal-sized pieces, in order to compare the effect of the different salt treatments on the microorganism populations within the same biological sample. Each piece had either sterilised or unsterilised salt applied to the flesh/hypodermal surface at a rate of 50 % w/w of the hide sample. Each salted piece was sealed separately inside a clear plastic container with salted side facing up/outwards, and left to cure at room temperature, in ambient light, on the laboratory bench. Hide from two different cattle animals were salt-cured independently in this manner; one hide was used for the cultivation of microorganisms, which was sampled at 90 days of cure. The other hide was used for the metabarcoding experiment, with samples of between 3-5 grams cut from the hide piece prior to being treated with salt (day 0), then at 24 hours after application of curing salt (day 1), then at 10, 20, 40, 50 and 60 days post-salt application (Table 1). Because of the potentially huge disparity in microbial populations between the hides of different animals, no biological replicates were used. However, to account for differences in extraction and processing, each sampling was done on three different areas of each hide at each time point.

Table 1. Sampling scheme for culture-independent, metabarcoding experiment.

	Number of Samples Taken From:					
	Controls: (no hide)	**Salt: Sterilised**	**Salt: Unsterilised**	**Hide: Untreated**	**Treated Hide: Sterilised Salt**	**Treated Hide: Unsterilised Salt**
Day 0	3	2	2	6	-	-
Day 1	1	-	-	-	3	3
Day 10	1	-	-	-	3	3
Day 20	1	-	-	-	3	3
Day 40	1	-	-	-	3	3
Day 50	1	-	-	-	3	3
Day 60	1	-	-	-	3	3

2.3 Enrichment, Culture and Isolation Media

2.3.1 Media for enrichment from hide samples

Three different media were used: for enrichment of fungal organisms ('Malt'; malt extract 30.0 g/L, glucose 10.0 g/L, peptone 5.0 g/L, yeast extract 1.25 g/L, pH 5.6-5.8); Modified Seghal & Gibbons for enrichment of fastidious organisms ('MSG': acid-hydrolysed casein 5.0 g/L, peptone 5.0 g/L, yeast extract 5.0 g/L, trisodium citrate 3.0 g/L, glucose 1.0 g/L, pH 7.2-7.5;) and lysogeny broth for enrichment of mesophilic bacteria ('LB'; tryptone 10.0 g/L, yeast extract 5.0 g/L, pH 7.0). Each of these media types was prepared with three different concentrations of salt, by diluting a concentrated salt water SW30 stock solution[23] with sterilised, ultrapure water to produce media with a final sodium chloride content of either 20%, 8% or 0.5% (w/v). These amounts were selected as salt concentration optima for enrichment of (extremely) halophilic, moderately halophilic and halotolerant, and non-halophilic microorganisms respectively,[24] resulting in nine different formulations altogether. Solid media was supplemented with 1.5% (w/v) bacteriological agar. All plates were sealed with paraffin film and incubated at 37 °C under a fluorescent bulb, while all liquid cultures were incubated at 37 °C in a table-top shaker in ambient light. Uninoculated controls were incubated to check for presence of environmental contaminants.

2.3.2 Media for enrichment from salt samples

A modified salt-rice-broth formulation was used[2] where SW30 stock solution was diluted to a final sodium chloride content of 12% (w/v) with sterilised ultrapure water, to which 10 g/L tryptic soy broth and 5 g/L acid-hydrolysed casein was added. Two parts of this solution was combined with one part of uncooked, short-grain white rice in a glass tube, then autoclaved to produce a solid, white-coloured growth medium that filled most of the tube.

2.3.3 Media for culture and isolation

Colonies selected from enrichment media were transferred to MSG media containing a similar sodium chloride component of either 16%, 8% or 0.5% (w/v) for isolation by streak plate technique. The sodium chloride content was reduced from 20% to 16% (w/v) to ease the preparation of solid media for cultivation of halophilic microorganisms. Aliquots of liquid culture from discrete colonies were frozen -80 °C in glycerol solution with a final concentration of 15% (w/v)

2.4 Collection of Microorganisms from Hides and Salts

2.4.1 Collection from hides for isolation and culture

Samples of approximately 1.5 cm^2 were cut and sterilised tweezers used to press each of the flesh and the hair sides of the sample onto the surface of solid media. The sample was then halved with a sterile blade, with each piece immediately transferred to a flask of sterile brine to wash out micro-organisms from within the hide tissue. One flask was prepared with undiluted SW-30 solution (pH 9.0) with a final sodium chloride content of 24% (w/v) and resultant pH of 9.0, to select for extremely halophilic and halotolerant microorganisms. The other flask was prepared by diluting SW-30 solution to achieve a final sodium chloride concentration of approximately 9.6% (w/v), with resultant pH of 8.0, to promote cultivation of halotolerant and slightly-halophilic microorganisms.[24] Flasks were incubated for 3 days at 37 °C with shaking and 120 µL of this liquid spread onto the surface of solid media.

2.4.2 Collection from salt for isolation and culture

To freshly-prepared rice-broth tubes, 2.5 g of salt sample was added, followed by 3 mL of sterilised water, to give an approximate sodium chloride concentration of 14.5% (w/v). Tubes, including uninoculated controls, were loosely capped and incubated at 37 °C under a fluorescent bulb. After ten weeks of incubation, [25] sterile loops were used to streak samples onto solid media for isolation.

2.4.3 Collection from hides for culture-independent, metabarcoding experiment

Approximately 3-5 g of hide sample was washed in 9.0 mL of 50 mM filter-sterilised ammonium bicarbonate solution (pH 8.0) for two hours at 37 °C on rotating arms. To remove particulate matter, the liquid mixture was passed through a nylon membrane (pre-soaked in 0.1% Tween-20) with 80 µm pore size, into a sterile collection tube.[26] The membrane was washed with a further 10.0 mL of the ammonium bicarbonate solution, with collected liquid lyophilised to powder. A 'reagents-only' (i.e. no hide sample) extraction was performed at each hide collection time point, as a control for environmental contaminants.

2.4.4 Collection from salt samples for culture-independent, metabarcoding experiment

Following the modified method of Henriet (2014),[27] brines of 25% (w/v) were made from 100 mL of sterilised, ultrapure water and 25 grams of salt sample and incubated in conical flasks at 37 °C with gentle shaking for 20-30 minutes. Brines were passed through 10 µm pore size nylon membranes to collect cells. A 'reagents-only' (i.e. no salt) collection was performed as a control for environmental contamination.

2.5 Genomic DNA Extraction

2.5.1 DNA extraction from pure cultures and from cell colonies.

Cell pellets from liquid cultures were subjected to mechanical homogenisation by bead-beating, using 106 um glass beads (Sigma). Homogenisation was carried out in a RiboLyser scientific chemical mixer (model FP120HY-230, Hybaid Ltd, UK) at 4 °C with the following procedure: 4.5 m/s for three cycles of 25 seconds, with 2-min rest interval in between each cycle. After removal of cell debris by centrifugation for 1 minute at 12,000 g, cell lysates were treated with RNase A (Sigma) for 20 minutes at 37 °C to break down contaminating RNA, and Promega Protein Precipitation Solution to remove contaminating protein. DNA was purified using ethanol precipitation[28] and resuspended in autoclaved, ultrapure water. Picked colonies were boiled for 8 minutes at 95 °C in buffer (10 mM Tris HCl pH 8.0, 0.1 mM EDTA, 0.1% Triton X-100). Tubes were centrifuged at 12,000 g for 1 minute to pellet cell debris. The supernatant containing the DNA was carefully removed and used immediately for PCR.

2.5.3 DNA extraction from powdered hide extracts & salt extracts

Including for reagents-only controls, the DNeasy Powersoil Kit (Qiagen) was used with the following changes to the manufacturer's protocol: PowerBead tubes were subjected to mechanical homogenisation with a RiboLyser using the aforementioned procedure, and DNA was eluted from MB Spin Columns using sterile, ultrapure water. All other steps were carried out according to the manufacturer's protocol.

2.6 Marker Gene Amplification using Polymerase Chain Reaction (PCR)

For DNA extracted from culture isolates and cell colonies, MyTaq Red Polymerase (BioLine) was used under thermocycling conditions recommended by the manufacturer. For metagenomic amplicon sequencing/metabarcoding sample preparation, Phusion HF Polymerase (New England Biolabs) was used as recommended by the manufacturer. Sequences for oligonucleotide primers are listed in Table 2. Primers used for metabarcoding included Illumina overhang-adapter sequences incorporated at the 5'-end of the marker gene-specific sequence (in 5'-3' direction: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG for forward primers; for reverse GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG). All PCR was carried out in 20 µL reaction volumes and included water-only controls for detection of contaminant DNA. Amplification products were visualised using agarose gel electrophoresis and ethidium bromide staining.

2.7 Sequencing & Analysis

Sequencing reactions were carried out by Massey Genome Service (Palmerston North, New Zealand) using dye-labelled, terminator cycle sequencing method.[35] Data was analysed using Geneious® v9.1.2 (Biomatters Ltd.).

Table 2. Oligonucleotide primers used for marker gene amplification and sequencing.

Application	Primer Name	Sequence (5'-3')	Target	Reference
Marker gene sequencing of culture isolates	344-F 915-R	ACGGGGYGCAGCAGGCGCGA GTGCTCCCCCGCCAATTCCT	Archaeal 16S rRNA gene	[29]
	27-F 1492-R	AGAGTTTGATCCTGGCTCAG GGTTACCTTGTTACGACTT	Bacterial 16S rRNA gene	[30]
	ITS86F ITS4-R	GTGAATCATCGAATCTTTGAA TCCTCCGCTTATTGATATGC	Fungal ITS2 region	[31] [32]
Metabarcoding/ metagenomic amplicon sequencing[1]	Arc344-F Arc806-R	ACGGGGYGCAGCAGGCGCGA GGACTACVSGGGTATCTAAT	Archaeal 16S rRNA gene	[29] [33]
	Bact-0341-F Bact-0785-R	CCTACGGGNGGCWGCAG GACTACHVGGGTATCTAATCC	Bacterial 16S rRNA gene	[34]
	5.8S-Fun ITS4-Fun	AACTTTYRRCAAYGGATCWCT AGCCTCCGCTTATTGATATGCTTAART	Fungal ITS2 region gene	[20]

1. Illumina overhang adapter sequences were added to the 5'- end of each of the locus-specific sequences: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG for forward primers and 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG for reverse primers.

The primary database queried was the '16S ribosomal RNA sequence (Bacteria and Archaea)' database from NCBI, using the BLASTn tool optimised for megablast with default settings (word size = 28).[36] Descriptions were assigned on the basis of fulfilling both the highest total alignment score and >97% similarity with reference sequences.[37]

3 Results & Discussion

3.1 Production of Red Heat

Both of the hide pieces cured with unsterilised salt developed discolouration characteristic of red heat, which first appeared as small, pale pink spots <1.0 mm diameter on the flesh surface, along with a faint pink colouration of the salt grains. This appeared at day 23 of cure on the hide used for microorganism cultivation. By day 46 of cure, the colour had deepened to a vivid, fuchsia-like pink and spots were distributed across the entire flesh surface (Fig. 1). At time of cultivation at day 90 of cure, the pink colouration had deepened and appeared quite dry.

Pink spots first appeared on the 40th day of cure on the hide piece sampled for metabarcoding. By day 60 of cure, about a quarter of the flesh surface displayed bright pink-coloured spots and small patches. Furthermore, most of the salt grains displayed a faint pink colouration. Interestingly, after being left for a total of 270 days, patches developed over the majority of the flesh surface and the most of the colour had changed from bright pink to a vivid pink-orange, with a glistening, semi-transparent appearance. Both of the discoloured pieces had a slight smell of rotting meat and urine, however it was not overpowering, nor reminiscent of ammonia.

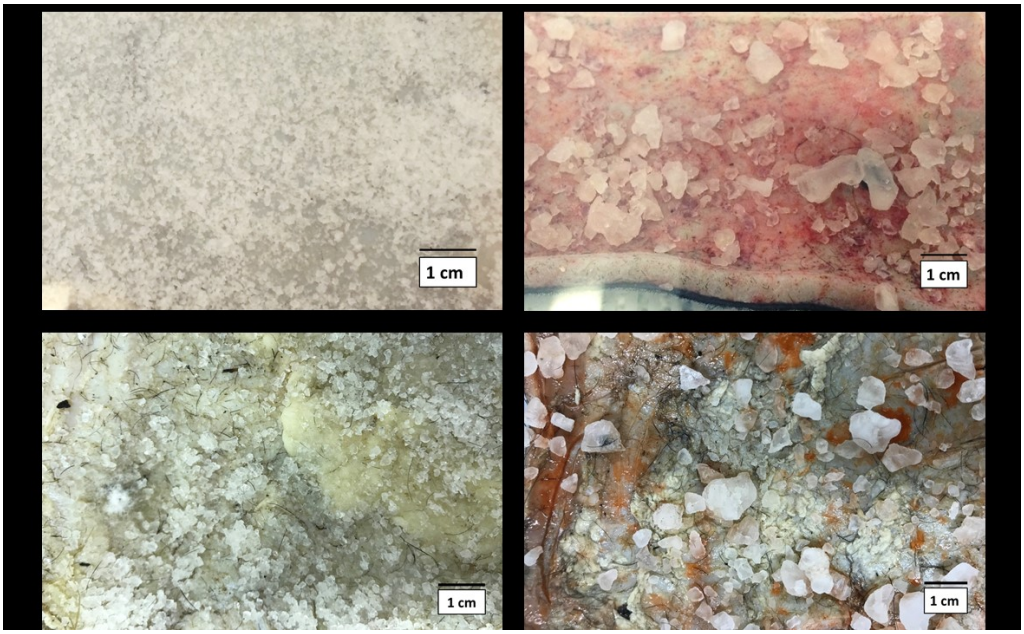

Fig. 1. 'Red heat' contamination of salt-cured products. Bovine hide pieces were dry-salted 50% (w/w) on the flesh side with either sterilised or unsterilised salt, sealed in clear plastic boxes and cured at ambient temperature and light. (A) Bovine hide treated with sterilised salt product and (B) unsterilised salt product at day 46 of cure, with both pieces sourced from the same animal. The hide of a different animal was used to provide both (C) hide treated with sterilised salt product and (D) hide treated with unsterilised salt product, both pictured at day 270 of cure. Hide pieces (B) and (D) show patchy blemishes of a bright pink and a pink-orange colour respectively, characteristic of red heat contamination.

Neither piece showed obvious liquefaction, though the pink-orange coloured piece had a slightly slippery, slimy feel when handling. Some hair was able to be pulled from both hides using tweezers.

The two hide pieces cured with sterilised salt did not develop any discolouration. Both had a very faint smell of rotten meat and urine. Neither piece showed any sign of liquefaction, appearing to be dry and relatively inflexible when handled, in comparison to the orange-pink contaminated piece. Hair slip was not apparent, but some hair was able to be pulled from the epidermal surface.

3.2 Isolation & Culture

A number of colony types were cultivated from the unsterilised-salted hide, with many successfully cultured, however, very few were captured on media containing 0.5% w/v sodium chloride (table 3). Coloured colonies were mostly of various shades of pink with some showing pink-orange pigmentation. All were glistening and semi-transparent. Shapes were either round or a 'fried egg' appearance; having an opaque, raised centre in an otherwise glistening, semi-transparent, irregular margin. These pink colony types occurred exclusively on the 20% and 8% sodium chloride containing plates. Some opaque, pale yellow colonies were evident on the plates with 8% sodium chloride or less. Many off white or beige types were isolated, mostly >3mm, with round to irregular margins, occurring most densely on plates containing 8% sodium chloride.

From the sterile-salted hide, a few large (20 mm) beige-pink, rugose growths with irregular margins growths were observed, as well as off-white colonies, and many yellow-pigmented colonies, mostly opaque with a glistening appearance. From this hide piece, a total of nine isolates were cultured (Table 3).

Many pale pink, glistening colonies were obtained from the unsterilised salt, along with one plate that developed deep red-pink, glossy, round colonies. However, efforts to culture the red colonies were largely unsuccessful, with only one isolate being cultured. No organisms were isolated from the sterilised salt using the rice-broth enrichment medium.

Table 3. Number of cultured isolates obtained in this study.

Sample	Enrichment Media	Total number of isolates	Total number of isolates cultured from enrichment media containing:		
			20% NaCl	8% NaCl	0.5% NaCl
Hide cured with unsterilised salt (red heat)	Malt	12	8	4	-
	MSG	13	10	3	-
	LB	11	3	5	3
Hide cured with sterilised salt (unaffected)	Malt	4	-	2	2
	MSG	3	-	2	1
	LB	2	-	1	1
Unsterilised salt	Malt	-	-	-	-
	MSG	1	1	-	-
	LB	-	-	-	-
Sterilised salt	Malt	-	-	-	-
	MSG	-	-	-	-
	LB	-	-	-	-

Additionally, to test the effectiveness of the rice-broth enrichment media, a variety of salt products were screened to detect the presence of coloured organisms (table 4). The aspect and onset of discolouration was markedly different between each of these salt samples, with sample 2547-2 producing a bright pink colour within 5 days of inoculation. The tube inoculated with unsterilised salt product developed colour more slowly, first appearing after 10 days, with others first showing obvious discolouration after 10-21 days of incubation. Most of those that produced discolouration of the enrichment media also produced slow-growing, pigmented, glossy, round to irregular shaped colonies on isolation media. However, non-pigmented colonies were also isolated, which were numerous but much smaller in size compared to the coloured isolates. No isolates were cultured from those tubes that remained white/uncoloured after the incubation period.

3.3 Marker Gene Amplification, Sequencing & Analysis

3.3.1 Marker gene sequencing analysis of cultivated microorganisms

A greater taxonomic diversity was cultivated from the hide samples cured with unsterilised salt, with 5 different bacterial and 3 archaeal genera identified by partial 16S rRNA marker gene sequencing (Table 5). In contrast, samples of hide cured with sterilised salt revealed only three different bacterial species across two genera. Only one species, *Staphylococcus equorum*, appears to be common to both hide samples, and has been identified in other cultivation studies of salted hides and skins that employ marker gene or partial 16S rRNA sequencing.[5]

Several colonies cultivated from the unsterilised salt were picked for colony PCR followed by marker gene sequencing, however, the required threshold of 97% reference similarity was not met (table 4). In all instances, one of several possible species of the genus *Haloarcula* was suggested as the closest phylogenetic relative. Additionally, the same followed for sequences obtained from a number of isolates cultivated from the unsterilised salted hide. In all but once instance, these sequences were unable to meet the similarity threshold due to a number mismatches arising from base-call ambiguities (more than one kind of nucleotide occupying the same sequence position), and all were suggested to be most closely related to *Haloarcula*. Intragenomic heterogeneity of the 16S rRNA gene is suggested as a possible reason for this observation. This is a particular feature of *Haloarcula*, which reportedly shows up to 5% sequence dissimilarity between the three copies of its 16S rRNA gene (designated as *rrsA, rrsB* and *rrsC*).[38] This was supported by multiple alignments of available *rrsA, rrsB and rrsC* reference sequences obtained from a number *Haloarcula* strains.

The resultant consensus data showed nucleotide ambiguities that aligned with those present in sequence data obtained from the isolates cultivated from unsterilised salt and hide (data not shown). Alternatively, these sequences could represent as yet uncharacterised strains of *Haloarcula*.

Table 4. Comparison of the microorganisms cultivated from different salt products.

Salt Sample Name	Enrichment Result	Colony Description	Sequence Description	Sequence Identity
Himalayan Rock Salt		Pale pink	*Halobacterium noricense*	99 %
Cornish Sea Salt		None	-	-
Tannery salt 2547-1		Bright pink Translucent white	*Halorubrum persicum* *Natrinema pellirubrum*	99 % 100 %
Tannery Salt 3387-9		Pink Off-white	*Halococcus* sp. *Chromohalobacter* sp.	99%
Unsterilised salt		Pink Red-pink	*Haloarcula* sp. (?)	92-93%
Sterilised Salt		None	-	-
AR-grade sodium chloride		None	-	-

Cultivated isolates were identified using phylogenetic marker gene sequencing. Sequence data was used to query the NCBI 16S ribosomal RNA sequence (Bacteria and Archaea) database using the BLASTn tool. Descriptions were assigned on the basis of the query sequence fulfilling both the highest total alignment score and >97% similarity with reference sequences.

Table 5. Phylogenetic identification of organisms cultivated from salt-cured bovine hide.

Sample	Organism domain	Description	Reference sequence accession	Number isolated from media containing:		
				20% NaCl	8% NaCl	0.5% NaCl
Cured with unsterilised salt	Bacteria	*Halomonas utahensis*	NR_117120.1	12	3	-
		Salicola marasensis	NR_043480.1	1	2	-
		Staphylococcus equorum	NR_027520.1	-	2	-
		Halobacillus locisalis	NR_025715.1	-	1	-
		Halobacillus sediminis	NR_145863.1	-	1	-
		Thalassobacillus cyri	NR_116915.1	-	1	-
	Archaea	*Halorubrum terrestre*	NR_113487.1	1	-	-
		Haloarcula argentinensis	NR_028218.1	1	-	-
		Haloarcula marismortui	NR_074201.1	1	-	-
		Halorubrum californiense	NR_113471.1	-	1	-
		Halovivax asiaticus	NR_042407.1	-	1	-
Cured with sterilised salt	Bacteria	*Staphylococcus equorum*	NR_027520.1	-	6	-
		Bacillus licheniformis	NR_118996.1	-	-	3
		Bacillus paralicheniformis	NR_137421.1	-	-	1

Bacterial and archaeal marker gene sequence data were used to query the NCBI 16S ribosomal RNA sequence (Bacteria and Archaea) database using the BLASTn tool. Descriptions were assigned on the basis of the query sequence fulfilling both the highest total alignment score and >97% similarity with reference sequences.

Three of the additionally-sampled salt products produced either colonies or cultured isolates that were able to be phylogenetically classified (Table 4). However, the small, transparent white/colourless colonies isolated from salt sample 2547-2 appears to be at-odds with the 100 % sequence similarity to 16S rRNA gene reference sequences (accession numbers NR_113528.1, NR_102444.1, NR_118137.1 & NR_112856.1) designated as *Natrinema pellirubrum*. The description of this microorganism includes formation of mostly orange-pigmented colonies due to the accumulation of carotenoid pigments and has been isolated from similarly-coloured patches appearing on salt-cured hides.[5, 39] This demonstrates the need for phenotypic analyses to complement genotypic methods, in order to properly classify these cultivates.

So far in this study, fungal organisms have not yet been identified by marker gene sequencing.

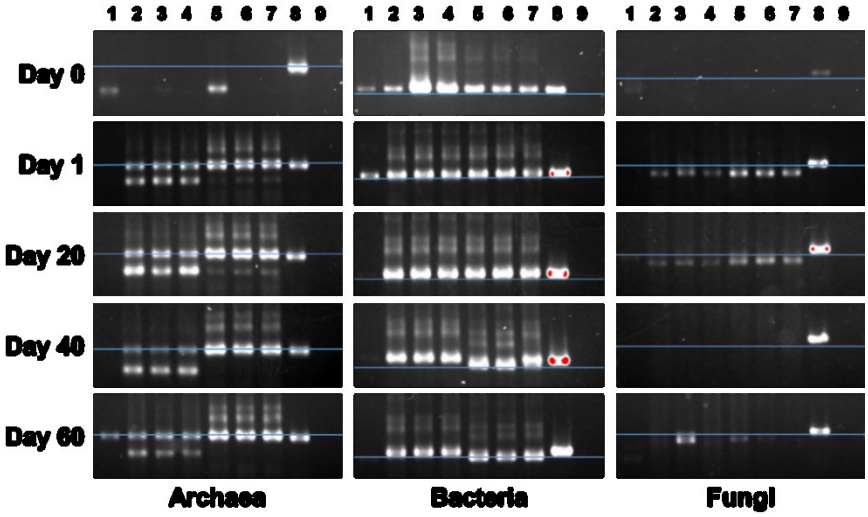

Fig. 2. Detection of microbial phylogenetic marker genes in salt-cured hide samples.

Genomic DNA was extracted from samples of salt-cured bovine hide and subjected to polymerase chain reaction (PCR) amplification targeting the following three marker genes: archaeal 16S rRNA; bacterial 16S rRNA and ITS2 region of fungal ribosome gene locus; with expected product sizes of 400-550 base pairs. Samples were taken prior to application of salt treatment (Day 0), at 24 hours after salt application (Day 1), then at 20, 40 and 60 days after salt application. Discolouration characteristic of 'red heat' contamination appeared on unsterilised salted hide samples at Day 40 onwards. PCR products were electrophoresed in 2% agarose gels and visualised using ethidium bromide stain. Blue lines show the position of the concurrently-run 500 base-pair size standard (not pictured). Red spots are artefacts of the gel imaging software used. Lane 1: Reagents-only (no hide) control extraction; Lanes 2-4: sterile-salted hide samples; Lanes 5-7: unsterilised salt-cured hide samples; Lane 8: known microbial strain (positive control for PCR); Lane 9: water-only reaction (negative control for PCR).

3.3.2 Visualisation of marker gene amplification from samples of cured hide using agarose gel electrophoresis and ethidium bromide staining

For the six samples taken from the hide pieces prior to being salted (day 0), bacterial marker gene amplification products were clearly detected in the untreated hide. However, amplification of archaeal marker genes across these six samples of untreated hide appeared inconsistent, and fungal marker gene amplification products were largely indistinguishable (Fig. 2).

Amplification of archaeal and bacterial marker genes was detected in all samples of cured hide taken from between day 1 and day 60 of cure, with clear differences in the DNA band profiles between the sterilised-salted hide and unsterilised-salted hide (Fig. 2).

In samples of hide cured with sterilised salt, the DNA band profile of archaeal marker gene amplification products shows one band approximately 500 base pairs (bp) in length, and another approximately 450 bp in length, and of similar staining intensity. This appears consistently within the three samples taken at each time point, and between each of the sampling time points. This contrasts with that shown for the unsterilised salted hide samples, where the staining intensity of the 500 bp band is greater compared to the faint, almost indistinguishable 450 bp band directly below it. The appearance of red heat discolouration on unsterilised salted hide from day 40 of cure did not appear to cause any change to the migration distances of these archaeal marker gene bands in agarose gel electrophoresis.

Interestingly, the appearance of red heat discolouration does coincide with a slight increase to the migration distance of the DNA band corresponding to bacterial marker gene amplification products, which is not seen in those obtained from samples of hide cured with the sterilised salt. In this case, the migration distance of this DNA band remains the same.

The 16S rRNA gene is reported to be approximately 1,500 base pairs in length for most bacterial and archaeal organisms.[19] Therefore, marker gene amplification is expected to produce DNA fragments of a mostly uniform length. Species-specific size variations in the 16S rRNA gene have been reported, due to accumulation of nucleotide substitutions and deletions, as well as gene truncations and intervening sequences.[40] Thus, variability of amplification product length for this marker gene can be indicative of distinct taxa in these samples.

The appearance of fungal marker gene amplification products showed as faint but clearly visible bands estimated at 480 bp in size, in all of the day 1 and day 10 hide samples. However, their visibility was inconsistent between all of the sampling time points. The appearance of red heat discolouration on the hide with unsterilised salt from day 40 of cure did not appear to cause any change to the migration distances of these DNA bands in agarose gel electrophoresis.

3.3.3 Visualisation of marker gene amplification from samples of salt using agarose gel electrophoresis and ethidium bromide staining

Amplification of archaeal phylogenetic marker genes was detected in both the unsterilised and sterilised curing salt, meeting the expected fragment size of around 500 bp (figure 3). The resultant DNA band profiles of the two different salts appear very similar to each other. Surprisingly, bacterial

marker genes were not amplified from either of these salt samples. This is in contrast to the work of Yilmaz & Birbir (2019),[41] who cultivated several *Bacillus* species directly from salt products from different leather factories, with 16S rRNA marker gene sequencing included in their method to characterise these isolates. Additional replicates will be screened in order to explain this result.

It is interesting to note that although archaeal organisms were (so far) unable to be cultured from the sterilised salt product, their presence (perhaps in a dormant form, or possibly non-viable) has been detected by marker-gene amplification.

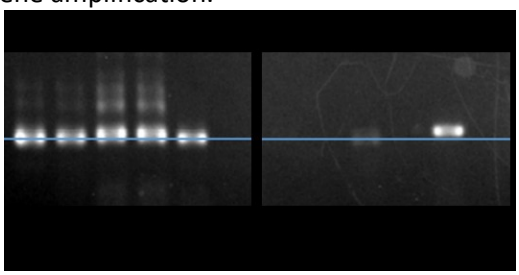

Fig. 3. Detection of microbial phylogenetic marker genes in curing salt products. Genomic DNA was extracted from salt samples and subjected to PCR amplification targeting the archaeal 16S rRNA and bacterial 16S rRNA marker genes, with expected product sizes of 400-550 base pairs. PCR products were electrophoresed in 2% agarose gels and visualised using ethidium bromide stain. Blue lines show the position of the concurrently-run 500 base-pair size standard (not pictured). Lanes 1-2: Sterilised salt; Lanes 3-4: unsterilised salt; Lane 5: known microbial strain (positive control for PCR); Lane 6: water-only reaction (negative control for PCR).

4 Conclusions

We have managed to induce red heat contamination in salt-cured cattle hide and cultivate microorganisms from samples of this hide and also from the salt product used to produce the contamination. We have also cultivated microorganisms from and unaffected hide samples. Further, in a cultivation-independent approach, red heat contamination was successfully reproduced in order to directly sample the *in situ* microorganism population of these hides, in preparation for analysis by high-throughput sequencing of marker gene amplification products. This work has produced a number of interesting results so far:

1. When treated with different salt products, the diversity and composition of both the cultivated and apparent *in situ* microorganism population differs markedly within single biological samples of bovine hide,
2. A number of different salt products harboured cultivable microorganisms that were phylogenetically distinct.
3. The marker gene sequences of cultivable microorganisms in the unsterilised salt product are so far quite dissimilar to known reference sequences, suggestive of an as-yet uncharacterised organisms. Analysis of high-throughput sequencing data is expected to clarify this observation.
4. A change in the composition of the bacterial population coincided with the appearance of red heat contamination, as detected by marker gene amplification, with no such changes apparent in the archaeal and fungal populations. We expect this to be explained by analysis of high-throughput sequencing data generated by these amplification products.
5. The salt product that was treated by heat-sterilisation did not yield cultivable organisms in this study. While microbial phylogenetic marker genes were detected, the viability of these organisms is not clear, indicating the importance of such treatments for the prevention of red heat.

5 Acknowledgements

The authors would like to thank the New Zealand Leather and Shoe Research Association (LASRA) for their financial and technical support. The kind contribution of a whole cattle hide to this study by M. Ahn and D. Allan is gratefully acknowledged.

6 References

1. *Oren, A.; Halophilic microorganisms and their environments, Springer, Dordrecht, 2002*
2. *Stuart, L. S., Frey, R. W., & James, L. H.: 'Microbiological studies of salt in relation to the reddening of salted hides', U.S. Dept Agric Tech Bull, 383, 1-23, 1933*
3. *Anderson, H.: 'The reddening of salted hides and fish', Appl Microbiol, 2(2), 64-69, 1954*
4. *Birbir, M., Kallenberger, W., Igaz, A., & Bailey, D. G.: 'Halophilic bacteria isolated from brine cured cattle hides', J Soc Leath Tech Ch, 80(3), 87-90, 1996*
5. *Akpolat, C., Ventosa, A., Birbir, M., Sanchez-Porro, C., & Caglayan, P.: 'Molecular identification of moderately halophilic bacteria and extremely halophilic archaea isolated form salted sheep skins containing red and yellow discolourations', J Am Leath Chem As, 110(7), 211-220, 2015*
6. *Lochhead, A. G.: 'Bacteriological studies on the red discoloration of salted hides', Can J Res, 10(3), 275-286, 1934*
7. *Bilgi, S. T., Yapici, B. M., & Karaboz, S.: 'Determination of hydrolytic enzyme capabilities of halophilic archaea isolated from hides and skins and their phenotypic and phylogenetic identification', J Am Leath Chem As, 110(2), 33-42, 2015*
8. *Birbir, M., & Ilgaz, A.: 'Isolation and identification of bacteria adversely affecting hide and leather quality', J Soc Leath Tech Ch, 80(5), 147-153, 1996*
9. *Bailey, D. & Birbir, M.: 'The impact of halophilic organisms on the grain quality of brine cured hides', J Am Leath Chem. As, 91, 47-51, 1996*
10. *Vreeland, R. H., Angelini, S., & Bailey, D. G.: 'Anatomy of halophile induced damage to brine-cured cattle hides', J Am Leath Chem As, 93, 121-131, 1998*
11. *Bitlisli, B.O., Karavana, H. A., Basaran, B., Sari, O., Yasa, I., & Birbir, M.: 'The effect of conservation defects on the suede quality of double-face', J Am Leath Chem As, 99(12), 494-501, 2004*
12. *Overmann, J., Abt, B., & Sikorski, J.: 'Present and Future of Culturing Bacteria', Annu Rev Microbiol, 71, 711-730, 2017*
13. *Burns, D. G., Camakaris, H. M., Janssen, P. H., & Dyall-Smith, M. L.: 'Combined use of cultivation-dependent and cultivation-independent methods indicates that members of most haloarchaeal groups in an Australian crystallizer pond are cultivable', Appl Environ Microbiol, 70(9), 5258-5265, 2004*
14. *Benlloch, S., Acinas, G., López-López, J. A., Luz, S. P., & Rodríguez-Valera, F.: 'Archaeal Biodiversity in Crystallizer Ponds from a Solar Saltern: Culture versus PCR', Microb Ecol, 41(1), 12-19, 2001*
15. *Quigley, L., O'Sullivan, D. J., Daly, D., O'Sullivan, O., Burdikova, Z., Vana, R., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., McSweeney, P. L. H., Giblin, L., Sheehan, J. J., & Cotter, P.D.: 'Thermus and the pink discoloration defect in cheese', mSystems, 1, e00023-16, 2016*
16. *Fernández, A. B., ,Blanca Vera-Gargallo, B., Sánchez-Porro, C., Ghai, R., Papke, R. T., Rodriguez-Valera, F., & Ventosa, A.: 'Comparison of prokaryotic community structure from Mediterranean and Atlantic saltern concentrator ponds by a metagenomic approach', Front Microbiol, 5, 196, 2014*
17. *Gibtan, A., Park, K., Woo, M., Shin, J. K., Lee, D. W., Sohn Jae Hak, Song, M., Roh, S. W., Lee, S. J., & Lee, H. S.: 'Diversity of Extremely Halophilic Archaeal and Bacterial Communities from Commercial Salts', Front Microbiol, 8, 799, 2017*
18. *Oren, A.: 'Halophilic microbial communities and their environments', Curr Opin Biotechnol, 33, 119-124, 2015*
19. *Clarridge J. E. III: (2004). 'Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases', Clin Microbiol Rev, 17(4), 840–862, 2004*
20. *Taylor, D. L., Walters, W. A., Lennon, N.J., Bochicchio, J., Krohn, A., Caporaso, J. G., & Pennanen, T.: 'Accurate estimation of fungal diversity and abundance through improved lineage-specific primers optimized for Illumina amplicon sequencing', Appl Environ Microbiol, 82, 7217–7226, 2016*
21. *Sharpton, T. J.: 'An introduction to the analysis of shotgun metagenomic data', Front Plant Sci, 5, 209, 2014*
22. *Walsh, A. M., Crispie, F.,Claesson, M. J., & Cotter, P. D.: 'Translating Omics to Food Microbiology', Annu Rev Food Sci Technol, 28(8), 113-134, 2017*
23. *Dyall-Smith, M.: The Halohandbook: protocols for haloarchaeal genetics, 2009, Retrieved from http://www.haloarchaea.com/resources/halohandbook/Halohandbook_2009_v7.2mds.pdf*
24. *Kushner D. J.: Microbial life in extreme environments, Academic Press, 1978*
25. *Cooper, S. & Crawford, E.: 'How long do salt samples need to be incubated for?' Paper presented at the Leather and Shoe Research Association Industry Technical Advisory Group meeting, Queenstown, New Zealand, 2015*
26. *Garcia-Garcerà, M., Garcia-Etxebarria, K., Coscollà, M., Latorre, A., Calafell, F.: 'A New Method for Extracting Skin Microbes Allows Metagenomic Analysis of Whole-Deep Skin', PLoS ONE 8(9), e74914, 2013*

27. *Henriet, O., Fourmentin, J., Delincé, B., & Mahillon, J., 'Exploring the diversity of extremely halophilic archaea in food-grade salts', Int J Food Microbiol, 191, 36-44, 2014*
28. *Green, M. R., & Sambrook, J.: Precipitation of DNA with ethanol, Cold Spring Harbour Protocols, 2016(12), pdb.prot093377, 2016*
29. *Raskin, L., Stromley, J. M., Rittmann, B. E., & Stahl, D. A.: 'Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens' Appl Environ Microbiol, 60(4), 1232–1240, 1994*
30. *Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J.: '16S ribosomal DNA amplification for phylogenetic study', J Bacteriol, 173, 697-703, 1991*
31. *Turenne, C. Y., Sanche, S. E., Hoban, D. J., Karlowsky, J. A., & Kabani, A. M.: 'Rapid Identification of Fungi by Using the ITS2 Genetic Region and an Automated Fluorescent Capillary Electrophoresis System' J Clin Microbiol, 37(6), 1846–1851, 1999*
32. *White, T., Bruns, T., Lee, S. & Taylor, J.: PCR protocols: a guide to methods and applications, Academic Press, 1990*
33. *Takai, K., & Horikoshi, K., 'Rapid Detection and Quantification of Members of the Archaeal Community by Quantitative PCR Using Fluorogenic Probes'. Appl Environ Microbiol, 66(11), 5066–5072, 2000*
34. *Herlemann, D. P. R., Labrenz, M., Jurgens, K., Bertilsson, S., Waniek, J. J., & Andersson, A. F.: 'Transitions in bacterial communities along the 2000km salinity gradient of the Baltic Sea', ISME J, 5, 1571–1579, 2011*
35. *Smith, L. M., Sanders, J. Z., Kaiser, R. J., Hughes, P., Dodd, C., Connell, C. R., Heiner, C., Kent, S. & Hood, L. E.: 'Fluorescence detection in automated DNA sequence analysis', Nature, 321, 674–679, 1986*
36. *Morgulis, A., Coulouris, G., Raytselis, Y., Madden, T. L., Agarwala, R., & Schäffer, A. A.: 'Database indexing for production MegaBLAST searches', Bioinformatics, 24(16), 1757–1764, 2008*
37. *Stackebrandt, E., & Goebel, B.: 'Taxonomic Note: A Place for DNA-DNA Reassociation and 16S rRNA Sequence Analysis in the Present Species Definition in Bacteriology', Int J Syst Evol Microbiol, 44(4), 846-849, 1994*
38. *Baliga, N. S., Bonneau, R., Facciotti, M. T., Pan, M., Glusman, G., Deutsch, E. W., … Ng, W. V.: 'Genome sequence of Haloarcula marismortui: a halophilic archaeon from the Dead Sea'. Genome Res, 14(11), 2221–2234, 2004*
39. *McGenity, T., Gemmell, R., & Grant, W.: 'Proposal of a new halobacterial genus Natrinema gen. nov., with two species Natrinema pellirubrum nom. nov. and Natrinema pallidum nom. Nov', Int J Syst Evol Microbiol, 48(4), 1187-1196, 1998*
40. *Pei, A. Y., Oberdorf, W. E., Nossa, C. W., Agarwal, A., Chokshi, P., Gerz, E. A., … Pei, Z.: 'Diversity of 16S rRNA genes within individual prokaryotic genomes', Appl Environ Microbiol, 76(12), 3886–3897, 2010*
41. *Yilmaz, E. & Birbir, M.: 'Characterization of Halotolerant Bacillus Species Isolated from Salt Samples Collected from Leather Factories in Turkey', J Am Leather Chem As, 114(4), 118-130, 2019*