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Selective Isolation and Identification of Microorganisms with Dual Capabilities: Leather Biodegradation and Heavy Metal Resistance for Industrial Applications

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Selective Isolation and Identification of Microorganisms with Dual Capabilities: Leather Biodegradation and Heavy Metal Resistance for Industrial Applications

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Abstract: Tanning, crucial for leather production, relies heavily on chromium, yet poses risks due to chromium's oxidative conversion, leading to significant wastewater and solid waste generation. Physico-chemical methods are typically used for heavy metal removal, but they have drawbacks, prompting interest in eco-friendly biological remediation techniques like biosorption, bioaccumulation, and biotransformation. The EU Directive (2018/850) mandates alternatives to landfilling or incineration for industrial textile waste management, highlighting the importance of environmentally conscious practices for leather products' end-of-life management, with composting being the most researched and viable option. This study aimed to isolate microorganisms from tannery wastewaters and identify those responsible for different types of tanned leather biodegradation. Using a leather biodegradation assay (ISO 20136) with tannery and municipal wastewaters as inoculum, bacterial shifts during leather biodegradation were observed. Over 10,000 bacterial species were identified in all analyzed samples, with 8 bacterial strains isolated from tannery wastewaters. Identification of bacterial genera like *Acinetobacter*, *Brevundimonas*, and *Mycolicibacterium* provides insights into potential microbial candidates for enhancing leather biodegradability, wastewater treatment, and heavy metal bioremediation in industrial applications.

Keywords: heavy metal reduction; leather biodegradation; microbial strain characterization; tannery wastewater

1. Introduction

The leather industry is vital in the global economy, providing high-quality fashion, automotive, and upholstery products [1]. Tanning is the chemical process by which collagen fibres are stabilized, preventing their putrefaction and ultimately forming durable leather [2]. For over 160 years, conventional chrome tanning has been employed in leather manufacturing [3], endowing leather with excellent shrinkage temperature (Ts) [4] and mechanical properties like flexibility, durability, and resistance to environmental factors [5]. Currently, 90% of hides are tanned using chrome [6], where chromium ions (Cr^{3+}) crosslink with the carboxyl and amino groups in the collagen fibres [7]. However, chrome tanning agents face significant limitations due to potential hazards, with the most concerning being the oxidative conversion of Cr(III) into hazardous and carcinogenic Cr(VI)[8]. This oxidation process could occur during tanning process due to elevated pH levels, temperature fluctuations, exposure to UV radiation, improper storage conditions, and the use of lubricants containing double bonds in their molecular structure[9]. At the micro-level, Cr(III) is an essential trace element for multiple physiological processes in the human body, such as glucose, fat, and protein metabolism, by enhancing insulin activity [10]. While Cr(III) complexes face challenges in penetrating cell membranes [11], Cr(VI) is highly soluble in water and toxic. It can pass rapidly through cell membranes, accumulating and, eventually, interacting with proteins and nucleic acids, ultimately damaging DNA [12].

The leather goods industry was worth USD 245 billion in 2022 [13] and stands out as one of the most environmentally impactful and resource-intensive sectors. From every 1,000 kg of raw material, 250 kg of leather is produced, leaving a substantial water footprint ranging from 15,000 to 120,000 cubic meters [14]. This process results in the generation of 15 to 50 metric tons of wastewater and 400 to 700 kg of solid waste, greenhouse gases (such as CO_2, H_2S, NH_3), as well as volatile organic compounds like amines, aldehydes, and hydrocarbons [12]. The emission of chemicals is strongly influenced by the treatment type and the technological processes employed in tanneries [15].

Various methods are employed to remove heavy metals from inorganic eluents [16]. Physico-chemical approaches include precipitation through the use of metal hydroxides, sulfides, carbonates, and phosphates [17]. Ion exchange utilizes solid resins for reversible ion exchange [18]. Membrane filtration methods include nanofiltration (NF) for molecules within the 300-500 Da molecular weight range [19] and reverse osmosis (RO) through a pressure-driven separation [20]. Other recent alternative techniques have also been employed, such as coagulation/flocculation, electrocoagulation, electro-floatation, and electro-deposition [21]. However, these methods may have drawbacks, such as incomplete metal removal, sludge generation, high reagent and energy requirements, and membrane fouling [22]. As a result, attention has turned towards biological remediation methods, such as biosorption, bioaccumulation and biotransformation, which offer cost-effective and environmentally friendly solutions for efficiently removing heavy metals like chromium from industrial waste [23]. Microorganisms eliminate heavy metals through enzyme-catalyzed metabolic pathways for toxic substance degradation, transforming them into carbon dioxide, methane, water, and biomass [24]. A wide range of microorganisms, bacteria [25], algae [26], fungi [27] and phyto species [28] have already been identified and isolated for potential heavy metal bioremediation and wastewater treatment.

On the other hand current Directive 1999/31/EC of April 26th 1999 [29] on the landfill of waste, as well as Directive 2008/98/EC [30] on garbage [16], allows the incineration or disposal in landfills of leather waste, chrome shavings, and solid waste. However, starting January 1st, 2025, the new Directive (EU) 2018/850 on landfills of waste [20] will prohibit landfilling or incineration as management methods for industrial textile waste. The European leather industry to increasingly focus on sustainability initiatives to reduce environmental impact and promote responsible sourcing [31]. Through the implementation of leather processing guides to improve and implement sustainable manufacturing practices [32], but most importantly by developmping more biodegradable leather products which could be composted. Leather biodegradability directly depends on the nature of the tanning agents and chemicals used in the manufacturing process, therefore the leather industry has also focused on developping more biodegradable leathers with the aim of giving their end products an sustainable, non-contaminating second life or feasable disposal. These include the use of chrome-free leather manufacturing using non-metal tanning materials such as vegetable tannins (polyphenolic agents known to bind to collagen) [33], aldehydes compounds (phenolic synthetic compounds bind to amine groups of collagens forming ionic bonds), calixarene [34], and other metal-free tanning agents [35]. Also the developpment of faux leather substitutes, mainly consist of polyvinyl chloride (PVC) or polyurethane (PU) layered onto a backing fabric (synthetic or natural, such as cotton or organic waste), undergo a surface coating procedure to boost their resilience and longevity. A recent study on the composting capacity of leathers[36] found that bovine leather treated with alginate derivatives degraded completely within 21 to 25 days, conventionally produced wet-blue leather degraded within 31 to 35 days, vegetable-tanned bovine leather showed initial signs of degradation after 60 days but did not fully disintegrate even after 90 days, whreas alternative materials, containing non-biodegradable components like PU and PVC, showed no degradation after 90 days.

Current compostability standard ISO 17088:2021[37] developped specifically for plastic compostability evaluation and has been commonly used to evaluate leather compostability. Leather and plastic have different physico-chemical characteristics and show different ways of degradation. Plastics degrades into smaller particles and poses a microplastic generation hazzard whereas leather posses chemical contanimation hazzard of the final compost. In this context International Standard ISO 20136:2020 "Leather. Determination of degradability by micro-organisms"[38] was developed specifically for leather. This methodology uses complex consortium of microorganisms from the tannery and urban wastewaters as inoculum in liquid medium to measure the biodegradation potential of leather as a meassure of CO_2 generated during leather degradation in 28 days.

This article aims to present recent research discoveries regarding various front lines; the identification and isolation of microorganisms from tannery wastewaters; identification of microbial composition in the starting inoculum used for a leather biodegradation assay according to ISO 20136 (tannery and municipal wastewater); and identifying microbial diversity shifts in initial inoculum during the process of different tanned leather degradation. The aim is to identify which genera is capable of biodegrading what type of tanning agent as well as which genera is acting at the different stages of degradation.

The focus is exploring their potential applications in enhancing leather biodegradability, wastewater treatment within the leather industry, and facilitating bioremediation processes for heavy metals. This study provides crucial insights into sustainable solutions for addressing environmental challenges within the leather industry, paving the way for developing efficient and environmentally friendly treatments for tannery wastewater. It will also contribute to optimizing leather composting processes, ultimately reducing the environmental impact of tannery operations and reinforcing the leather industry's commitment to responsible and sustainable production.

2. Materials and Methods

2.1. Species Identification from Tannery Wastewaters

2.1.1. Tannery Wastewater Collection and Preparation

A two-litre water sample was taken from an aeration pond at a wastewater treatment plant (Curtidos Serpiel S.A., Caudete, Spain) at a pH of 4.5 and estimated chromium concentration of ~100 ppm. The water sample was filtered through a paper filter to remove macroscopic precipitates. Part of the filtrate was supplemented with 0.5% yeast to promote growth, and bacteriological agar was added up to 1%. A set of dilutions was prepared from the remaining filtered water up to 10^{-4} to the initial concentration. 100 μL of each dilution was used to seed the solid media initially prepared. Two plates were seeded for each dilution and incubated at room temperature for 10 days.

2.1.2. Species Isolation

Colonies from the 10^{-3} dilution were resuspended in LB medium and reseeded on fresh plates at different dilutions. Up to 9 microorganisms were isolated and maintained through successive reseeding in standard LB medium.

2.1.3. Species Identification

Species identification was carried out by 16S/18S rRNA amplification and sequencing. Eight colonies were randomly selected and resuspended in 20 μL of ultrapure H_2O, incubated at 99 °C for 15 minutes to lyse the cells. The NZY Microbial gDNA Isolation kit (NZYtech, Lisboa, Portugal) [39] was used to extract the DNA sample for PCR with Taq DNA polymerase. Three pairs of degenerate universal oligonucleotides specific for the 16S/18S gene of bacteria, archaea, and eukaryotes were designed. - BACF: 5′-AGAGTTTGATCCTGGCTCAG-3′ - BACR: 5′-GGYTACCTTGTTACGACTT-3′ - ARCF: 5′-TCCGGTTGATCCYGCBRG-3′ - ARCR: 5′-TTMGGGGCATRCIKACCT-3′ - EUKF: 5′-GGTTGATYCTGCCAGTAG-3′ - EUKR: 5′-GTACACACCGCCCGTCGCT-3′.

The PCR product obtained was sequenced using an automated sequencer and bacterial oligonucleotides, following two strategies: cloning the PCR products into the pGEM-T easy vector (Promega, Madison, United States) and sequencing the PCR product directly after purifying the bands with the GFX PCR DNA and Gel Band Purification Kits (Cytiva, United States) [40].

2.2. Microorganism Identification from Leather Biodegradation Assay

2.2.1. ISO:20136:2020: Determination of Leather Degradability by Microorganisms' Assay

A leather biodegradation assay (method B) was performed as described in ISO20136:2020 [38]. The inoculum was a mixture of 50:50 (ratio) of tannery and municipal sewage wastewater. Municipal wastewaters were collected from the local area treatment plant and tannery wastewaters were collected from Curtidos Segorbe S.L. (Segorbe, Spain) [41] a different source of tannery waste water to that mention in 2.1.1. Pure collagen from bovine Achilles tendon (Sigma-Aldrich®, Missouri, United States) [42]was used as a positive control (control sample). The assay was run for approximately 800 hours (33 days). Five differently tanned leather samples were used for the assay; 0.5 g of each sample was placed in each Erlenmeyer flask containing minimal salts and the inoculum, as shown in Table 1. The total organic carbon content of the material being tested is determined by elemental analysis. This allows the theoretical maximum quantity of carbon dioxide evolution to be calculated as biodegradation.

Table 1. Leather samples (S1 to S4) and control (Pure Collagen) used in leather biodegradation assay.

Sample	Tanning agent	Carbon%	Weight (g)	Erlenmeyer Flask Ref
Control	None	50.60	0.5047	2
S1	Oxazolidine	44.76	0.5006	4
S2	Glutaraldehyde	47.76	0.5002	7
S3	Chromium	36.11	0.5012	10
S4	Aluminium	41.45	0.5036	14

2.2.2. Wastewater and Leather Biodegradation Assay Sample Collection

Table 2 shows the samples taken from each Erlenmeyer flask, the hour at which they were withdrawn after the start of the assay, and the leather biodegradation stage registered at that time. According to the standard either tannery or municipal wastewater can be used in the assay. Sample M1 was municipal residual wastewater, sample M2 was tannery wastewater, and sample M3 was a mixed inoculum (50:50) from M1 and M2. This was the inoculum used in the assay and microorganism profile identified in samples M4 to M29 shift from this sample (M3). The aim of microorganism identification in samples M1 and M2 was merely informative to confirm and compare the final microorganism profile in sample M3. The remaining samples were drawn from an ongoing leather biodegradation assay (ISO 20136 assay) [43] at different stages of the degradation process (initial, exponential, and final) using a syringe and 0.1 microlitre filters. All samples were stored and transported at -20 °C.

Table 2. Extracted samples from an ongoing leather biodegradation assay.

Sample	Time (h)[1]	E. Flask Ref	Leather Sample	Volume (ml)[2]	Biodegradation (%)[3]
M1	0	-	None	70	0
M2	0	-	None	50	0
M3	0	-	None	50	0
M4	52	2	Control	70	17
M5	75	2	Control	50	32

M6	75	4	S1	60	3.45
M7	75	14	S4	60	3.52
M8	117	2	Control	60	40.5
M9	117	4	S1	50	7.14
M10	117	7	S2	60	1.56
M11	117	10	S3	50	2
M12	117	14	S4	50	4.6
M13	144	4	S1	50	14.9
M14	240	2	Control	60	57
M15	240	4	S1	60	32.2
M16	240	14	S4	60	8.2
M17	263	4	S1	60	38.72
M18	263	7	S2	60	2.96
M19	263	10	S3	50	3.02
M20	263	14	S4	50	11.2
M21	263	7	S2	50	62.2
M22	335	14	S4	50	13.68
M23	335	7	S2	50	3.61
M24	335	10	S3	50	3.47
M25	747	2	Control	50	81.5
M26	747	4	S1	50	59.4
M27	747	14	S4	50	5.22
M28	747	10	S3	50	7.56
M29	747	14	S4	50	23.21

[1]Time (h) since time 0 of the assay. [2]Volume (ml) extracted from the Erlenmeyer Flask. [3]Biodegradation stage at which sample was extracted. M1: municipal wastewater, M2: tannery wastewater, M3: 50:50 mixture of M1 and M2.

2.2.3. DNA Extraction and Quality Control

Cells were recovered from filters followed by DNA isolation using the Qiagen QIAsymphony PowerFecal Pro DNA Kit (Qiagen, Hilden, Germany) [44]. That involved cellular lysis through mechanical disruption and enzymatic treatment. Subsequently, DNA purification from the sample was performed using a silica/gel column, allowing DNA isolation and removing contaminants and inhibitors for future reactions using the QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) [45]. Cellular lysis was carried out through mechanical disruption and enzymatic treatment. Subsequently, DNA purification from the sample was performed using a silica/gel column, which enables DNA isolation and removal of contaminants and inhibitors for future reactions using the Qiagen QIAamp DNA Micro Kit. The quality and concentration of the DNA were evaluated using Nanodrop.

2.2.4. Sequence Library Preparation

50 ng of extracted DNA was amplified using a two-stage PCR protocol, the 16S Metagenomic Sequencing Library Preparation protocol Illumina 15044223. Primers were designed with the following structure: 1) an amplicon consisting of a universal linker sequence that allows the incorporation of indexes and sequencing primers using the Nextera XT Index kit [46]; and 2) universal primers for the 16S rRNA gene [47].

2.2.5. Sequencing

The sequencing libraries were prepared and loaded onto the Illumina MiSeq platform following a 300 bp x 2 paired-end design. In the second and final step of the assay, amplification indexes were included. The resulting 16S libraries were quantified using fluorimetry with the Quant-iT™ PicoGreen™ dsDNA Assay kit (Thermo Fisher scientific, Waltham, Massachusetts, United States) ([48]. Libraries were pooled before sequencing on the MiSeq platform (Illumina) following 300 paired end-reads design cycles. The size and concentration of the pool were evaluated using Agilent Bioanalyzer 2100 (Agilent, California, United States) [49]. The PhiX Control library (v3) (Illumina) was combined with the amplicon library (with an expected percentage of 20%). The sequencing data became available in approximately 56 hours. Image analysis, base calling, and data quality control were performed on the MiSeq platform (MiSeq Control Software (MCS v3.1)). The raw sequences, forward (R1) and reverse (R2), were merged to obtain the complete sequence using the BBMerge package of the BBMap software V.38. With this approach, the ends of the sequences overlapped to obtain complete sequences. Sequencing adapters were searched for and removed using the Cutadapt program (v 1.8.1) to reduce bias in the subsequent annotation stage.

2.2.6. Bioinformatic Analysis

Identifying and removing non-genomic regions or those with poor quality were carried out. Initially, the BBMerge module of BBMap software V.38 was employed to merge each pair of sequences (R1 and R2) from the sequencing platform, ensuring a minimum overlap of 70 nts at each end. This process resulted in a unique and complete sequence. Subsequently, the Cutadapt v1.8.1 program was used to detect and eliminate sequencing adapters from both ends in each sample. Once adapter-free sequences were obtained, reads with quality below Q20 and lengths less than 200 bp were removed. The Reformat module of BBMap V.38 was utilized for this analysis, enabling the trimming of nucleotides with a quality value below Q20 from both ends.

The final step in quality processing involved eliminating potential chimaera sequences resulting from incomplete extension during PCR amplification. This step was performed using the cd-hit-dup module, part of the cd-hit 4.8.1 software, predicting these amplicons de novo from the sample. The resulting sequences were then used for annotation. Sequences sharing 99% similarity were grouped into a single sequence using the “cd-hit” program. The outcomes were applied to the sequence group represented by the analyzed one, referred to as the Operational Taxonomic Unit (OTU). Subsequently, each sequence group was compared against the RefSeq 16S rRNA gene database (NCBI) using the local alignment BLAST strategy to associate each group with a taxonomic group from the database.

3. Results

3.1. Species Identification from Tannery Wastewaters Treatment Plant (Curtidos Serpiel S.A., Caudete, Spain)

3.1.1. Species Identification

Agarose gel electrophoresis (1%) of PCR products shown in Figure 1 was obtained from eight colonies using oligonucleotides for bacteria, archaea, and eukaryotes. The bands that can be seen from B1 to B8 in Figure 1 are of the same size as those seen in Figure 2.

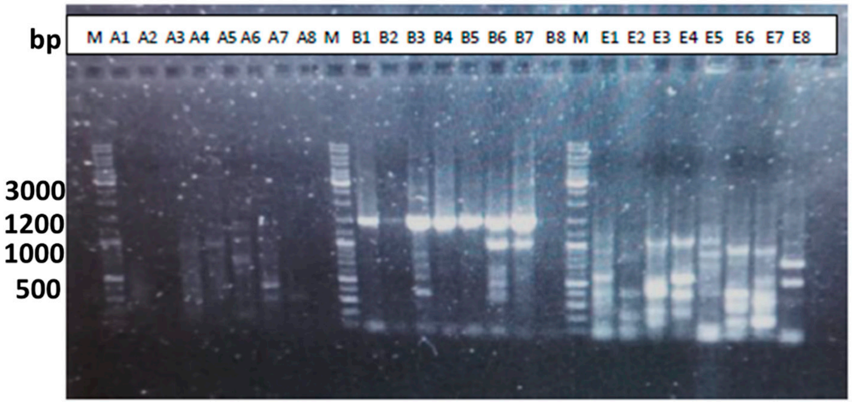

Figure 1. M: GeneRuler DNA Ladder Mix (Thermo Fisher scientific) [50]. A1-A8: PCR products using archaea oligonucleotides. B1-B8: PCR products using bacteria oligonucleotides. E1-E8: PCR products using eukaryotes oligonucleotides.

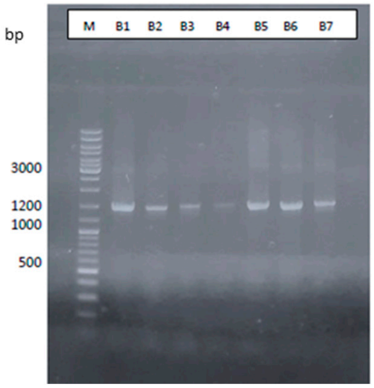

Figure 2. Agarose gel electrophoresis at 1% of the PCR products purified from PCR reactions performed using oligonucleotides for bacteria. Band sizes in bp are indicated. M: GeneRuler DNA Ladder Mix (Thermo Fisher scientific) [48].

All PCR products were sequenced with the corresponding oligonucleotides at the Genomics and Proteomics Service of the University of Alicante Technical Services. The sequences obtained in each case were compared in the NCBI databases and EZBioCloud. The identified bacterial strains are shown in Table 3. Strains have been identified by sequencing the whole bacterial 16S rRNA gene, therefore species indicating a similarity percentage over 99% are considered to be an exact match [51]. Other species would have to be identified by whole genome sequencing.

Table 3. Bacterial strains identified from tannery wastewater.

Name	Top-hit taxon	Similarity (%)	Completeness (%)	Length (bp)
Species 1	*Dietzia maris*	99.48	94.4	1355
Species 2	*Trichococcus pasteurii*	99.21	94.3	1396
Species 3	*Corynebacterium lubricantis*	97.86	97.7	1034
Species 4	*Microbacterium laevaniformans*	99.47	95.8	1370
Species 5	*Bacillus safensis*	99.36	96.2	1416
Species 6	*Proteiniphilum AB243818_s*	99.26	98	1419
Species 7	*Proteiniphilum AB243818_s*	95.80	97	1405

3.2. Microorganism Identification from Leather Biodegradation Assay Using ISO 20136:2020

3.2.1. ISO:20136: Leather- Determination of Degradability by Microorganisms

Biodegradation results for leather samples described in section 2.2.1 are shown in Figure 3. These biodegradation curves show the % of leather biodegradation throughout the assay (30 days); the times shown in Table 2 (section 2.2.2) correspond to the time (hours) in this graph. There is an initial, exponential, and final phase of leather biodegradation for all the samples at different levels of biodegradation percentage. Collagen is used as a positive control since it fully degrades in approximately 30 days [38]. At the final stage of the assay (747 h since time 0), when the last samples were taken, the biodegradation percentage for collagen was 81.5%, oxazolidine 59.4%, glutaraldehyde 5.2%, chromium 7.6% and aluminium 23.2%.

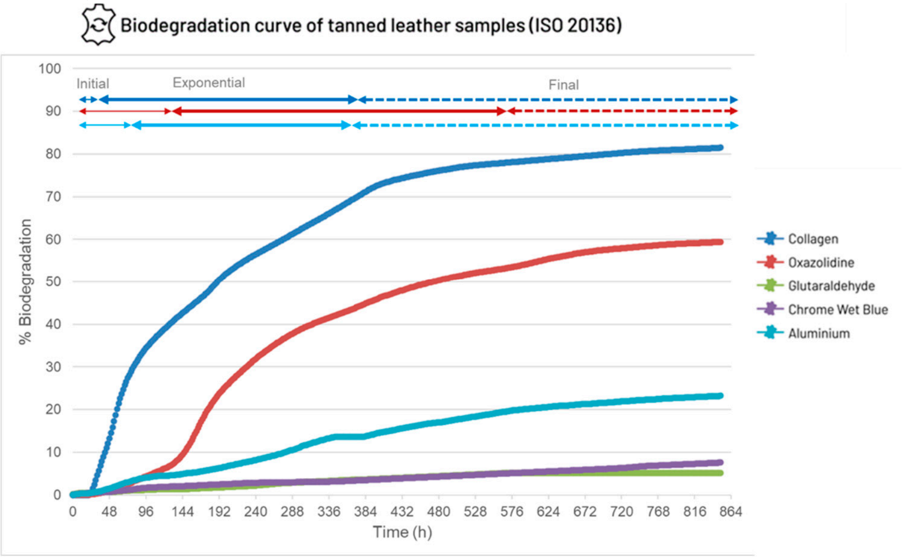

Figure 3. Biodegradation results of the ISO20136 assay performed with four different types of leather samples. The graph shows the biodegradation % vs time of each sample. Collagen is the positive control.

3.2.2. Sequencing

The concentration and purity of the extracted DNA reached enough levels to proceed with the preparation of sequencing libraries. The sequencing results, including the number of raw sequences obtained, average length, total sequenced bases measured in Megabases (Mb), and the mean quality for forward (R1) and reverse (R2) sequences, are shown in Table S1 within the supplementary materials. The read count exceeded 50,000 in all analysed samples, except for sample 210527A-M4, which yielded 43,447 reads.

3.2.3. Bioinformatics and Species Identification

Although this sample did not reach the desired read count during the sequencing process, this quantity proved optimal as it reached the plateau zone in the rarefaction curve. The profiles are studied at a maximum genus level, providing species-level information for discussion on potentially implicated species. However, due to the product size of around 400 nts for bacteria, precise species identification cannot be guaranteed.

It can be determined that it is close to the plateau zone with the inspected number of sequences and the microbial profile. As shown in the rarefaction curves (alpha-diversity index) in Figure 4, most samples are in the plateau zone, indicating that an increase in sequences would not yield a significant rise in the number of newly detected genera.

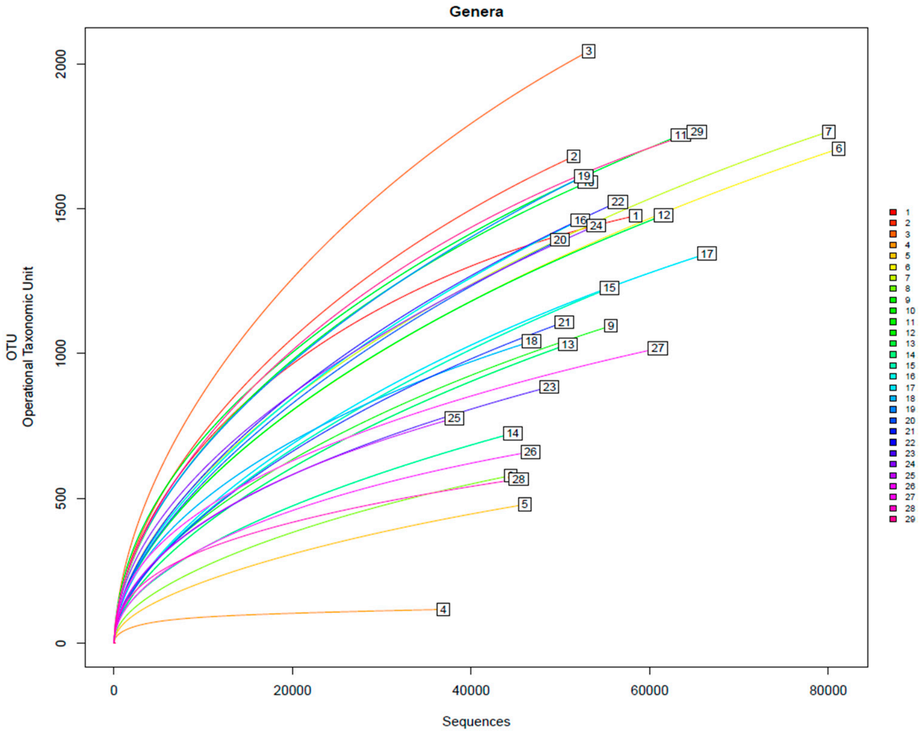

Figure 4. Rarefaction curves of the amplified samples for bacteria detection. Samples shown M1 to M29.

The statistical analysis employing the vegan package in R [52] allows for the examination of the organism-abundance relationship. These results are presented in the Table 4. The Shannon index estimates the specific biodiversity present in the sample, providing a positive numeric value starting from 0 (indicating a single species) and increasing as diversity increases [53]. The Chao 1 value estimates the total number of species that may be present in the sample based on the number of less represented species in the sample [54]. In taxon-based approaches, accurately estimating the number of microbial species in a sample is challenging due to the complexity of microbial diversity, therefore assessing species richness is crucial for understanding biological communities effectively [55]. Richness estimators like Chao 1 are employed to deduce the total richness of a microbial community based on observed Operational Taxonomic Units (OTUs). Unlike rarefaction, which compares observed richness among samples, richness estimators predict total richness from a single sample. For all the samples, Shannon index is around 4 and Chao 1 index was around 2500. Sample M4 has shown to be the only one that differs from other samples showing a Shannon index of 2.70 and a Chao 1 of 266.

Table 4. Bacterial diversity for each sample according to the Shannon and Chao 1 parameters.

Sample	Shannon	Chao 1
M1	4.56	2127
M2	4.62	2816
M3	5.05	3383
M4	2.70	266
M5	3.07	1205
M6	4.05	3296
M7	4.65	3177
M8	3.68	1344
M9	3.79	2232
M10	4.59	2817

M11	5.24	3295
M12	4.55	3053
M13	3.77	2284
M14	3.96	1821
M15	4.16	2818
M16	4.68	3068
M17	4.10	2553
M18	4.33	2040
M19	5.11	3406
M20	4.72	2972
M21	4.16	2198
M22	4.84	3049
M23	4.10	1837
M24	4.99	3031
M25	4.01	1506
M26	3.32	1217
M27	4.73	1903
M28	4.15	1105
M29	4.94	2727

Local BLAST alignment is conducted to associate each of the obtained reads (after cleaning and filtering) with an organism. Working with a well-curated database is crucial, as databases may be incomplete or lack certain organisms due to being sequenced or not being taxonomically assigned. In cases where a sequence is associated with multiple hits with the same e-value and identity, the first hit is considered the best. It's important to note that these taxonomic identifications represent the best outcome in local alignment, not guaranteeing the organism's presence in the sample.

Short sequences are excluded from the analysis to reduce the likelihood of duplicating sequences in the database and encountering false positives or misassignments. Sequencing errors that can alter assignments to a specific organism by modifying sequence similarity are also eliminated from the analysis. For each sequence, details such as percentage identity with the database hit, e-value, absolute number of identical positions in the alignment, relative number of identities considering the total number of alignment bases, absolute number of gaps in the alignment, and relative number of gaps considering the total number of alignment bases are provided.

Once each read is associated with an organism, various analyses and filters are applied. Reads are grouped based on family, genera, and species, with associations typically made at the family or genera level, but Species-level associations are also provided. The database is curated according to different taxonomic levels described in the NCBI database [56]. Some levels may remain null if they are not characterised, cultivated, or unknown. The presence of different taxonomic groups in the analysed samples is summarized in tables and graphs. A rarefaction curve is generated for each analysed sample, comparing the number of analysed sequences with the number of detected taxa at different levels, as shown in Figure 4. This curve helps determine if the detection has reached saturation, indicating that all organisms have been detected or if more sequences are needed to capture the total variability of the sample.

Sample M4 (collagen at 17% biodegradation at 52 h since start of the assay) has shown to be the only one that differs from other samples showing a Shannon index of 2.70 and a Chao 1 of 266, considerably lower to the rest of the reads. This is also shown in Figure 4, where the alpha-diversity index of M4 is considerably lower than the rest of the samples. The reduction of these is caused by the immense presence of *Acinetobacter* in the sample. This bacterium shows to be a great collagen degrader, as its presence grows exponentially within the first 48 hours within the assay. On the other hand, M3, being the mix of both wastewaters, shows the highest alpha-diversity index as well as one the highest Shannon index Chao parameters, 5.05 and 3383, respectively.

Bacterial abundance detected in the analysed samples at genera level is shown in Figure 5, M1-M29 samples refer to those shown previously in Table 2 and Table 4. Additionally, this diagram incorporates the LCBD beta diversity index, revealing diversity patterns. High LCBD values indicate that the bacterial composition of the sample significantly differs from the rest of the studied samples [57]. Bacterial strains identified in highest percentages in municipal wastewaters (sample M1) were *Nakamurella endophytica* (10.76%), *Clostridium saudiense* (9.87%), *Romboutsia timonensis* (9.21%) and *Mycolicibacterium peregrinum* (3.41%). On the other hand, the bacterial strains most present in tannery wastewaters (sample M2) were *Pseudonocardia rhizophila* (13.02%), *Variibacter gotjawalensis* (11.46%), *Pseudorhodoplanes sinuspersici* (6.42%), *Hyphomicrobium aestuarii* (3.46%) and *M. peregrinum* (3.38%). The inoculum used for the assay was sample M3 a 50:50 mixture of M1 and M2 and, as it would be expected, the composition in terms of bacterial presence is halfway between M1 and M2.

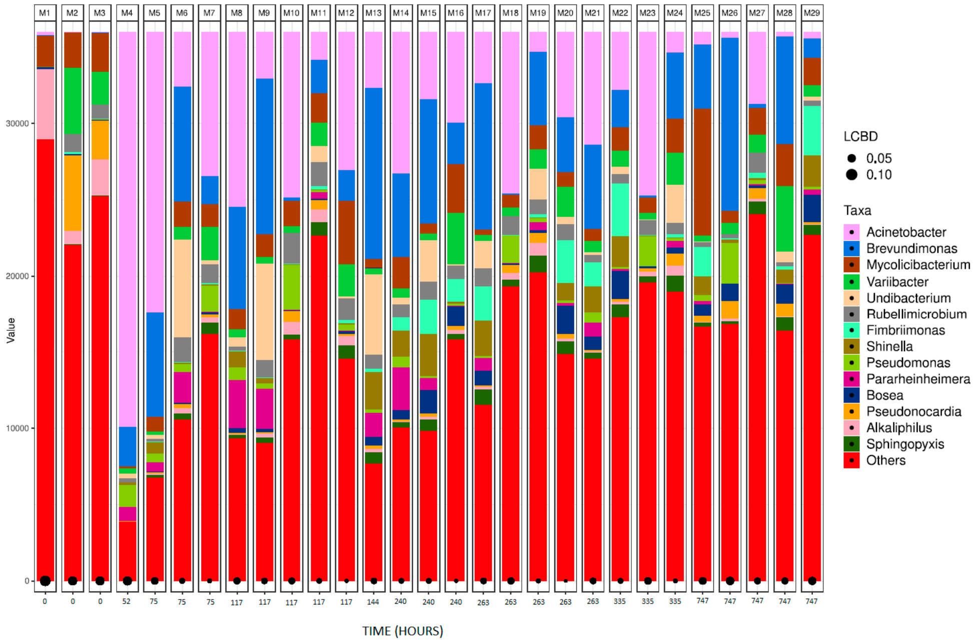

Figure 5. Bar plot figures representing the proportions of detected bacterial genera in the studied samples.

For comparison purposes, the results of bacterial identification have been categorized according to the different tanned leather samples. This allows for a comparison between different tanning agents, as well as between various stages of biodegradation within the same type of leather. M1, M2 and M3 have been left in all the following bar plot graphs since they represent the initial bacterial composition of the inoculum. M3 was the inoculum used in the assay, therefore all the bacterial shift in the following samples diverge from sample M3. Figure 6a shows the bacterial abundance at species level detected in the analysed samples for collagen (control sample). As collagen biodegradation takes place (samples M4 – M25) the most abundant species present are *Brevundimonas terrae*, *A. johnsonii* and *M. peregrinum.* Figure 6b shows a line graph representing bacterial presence shift in the sample as a percentage of the top six bacterial species identified at the different stages of collagen biodegradation. Within the first 52 hours after the start of tha assay the most predominant genius is Acinetobacter Some of the species, such as *Chryseobacterium indoltheticum*, start at very high concentrations, but quickly decrease as they phase no possibility to proliferate using collagen as the only carbon source.

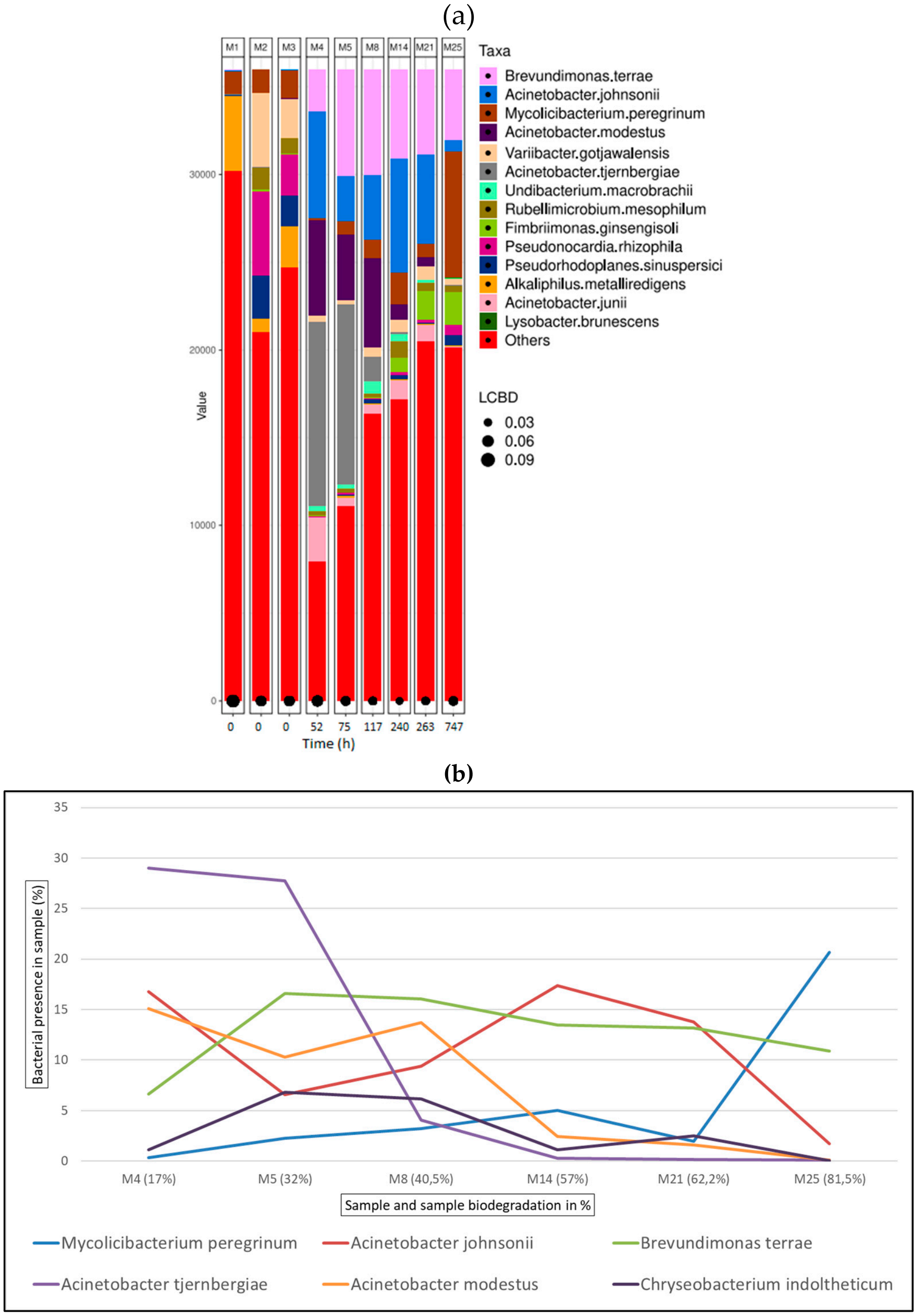

Figure 6. Bacterial species detected for collagen samples in ISO20136; (**a**) Bar plot figures representing the proportions of detected bacterial species in the studied samples (M4, M5, M8, M14, M21 and M25). M1 being municipal residual wastewater, sample M2 was tannery wastewater, and sample M3 was a mixed inoculum (50:50). (**b**) Line graph representing bacterial shift as bacterial presence in sample (%) of the top six bacterial species found for all collagen samples.

Figure 7a shows the bacterial abundance at species level detected in the analysed samples for chromium tanned leather. As leather biodegradation takes places (samples M4 – M25) the most abundant species present are *Brevundimonas terrae*, *Acinetobacter johnsonii* and *Mycolicibacterium peregrinum.* Figure 7b shows a line graph representing bacterial presence shift in the sample as a percentage of the top six bacterial species identified at the different stages of chromium leather biodegradation. In this case, Brevundimonas is the predominant bacterial genus, *Brevidumonas terrae* shows to be the bacterial strain most present in samples M11 to M4 shifting to *Brevundimonas kwangchunensis* which is present as a 15.9% in the sample.

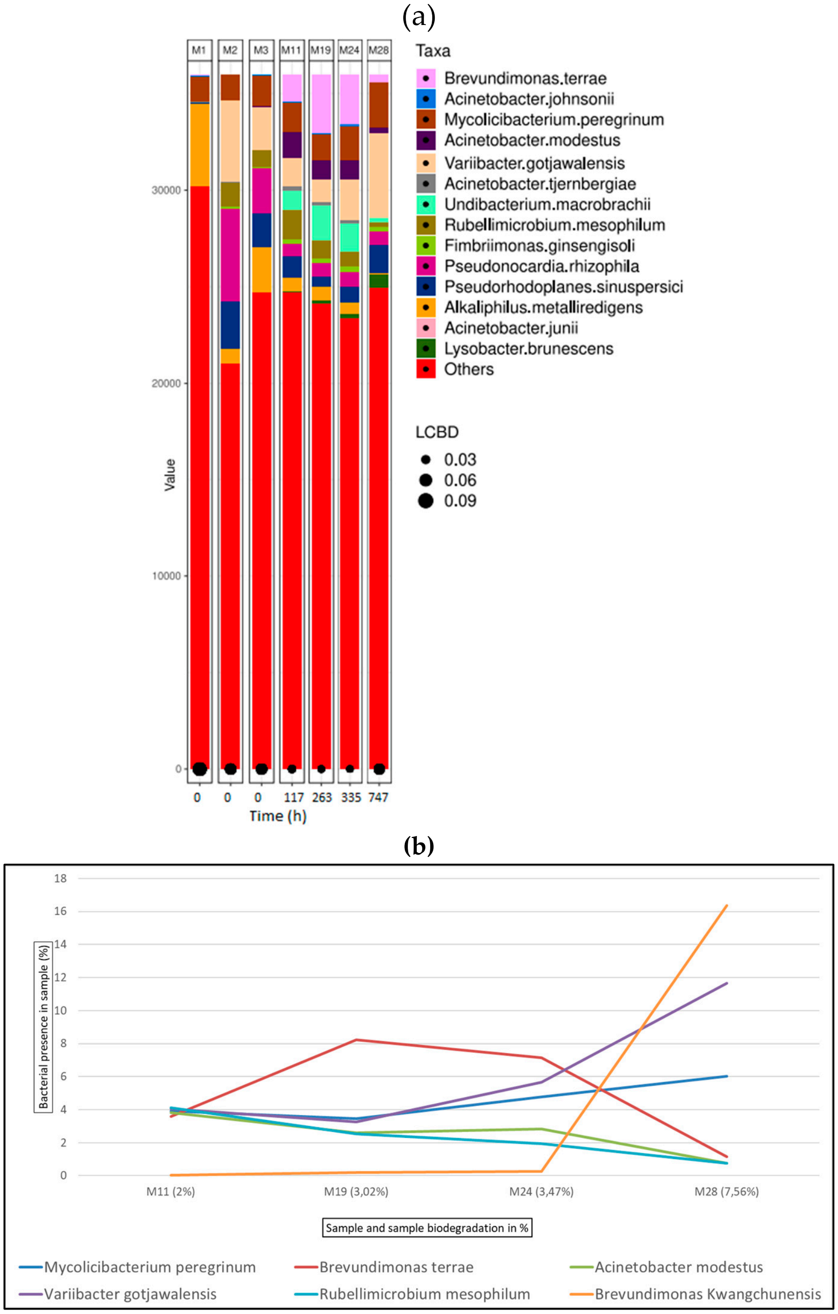

Figure 7. Bacterial species detected for chromium samples in ISO20136; (**a**) Bar plot figures representing the proportions of detected bacterial species in the studied samples (M11, M19, M24, M28). M1 being municipal residual wastewater, sample M2 was tannery wastewater, and sample M3 was a mixed inoculum (50:50). (**b**) Line graph representing bacterial shift as bacterial presence in sample (%) of the top 6 bacterial species found for all chromium samples.

Figure 8a shows the bacterial abundance at species level detected in the analysed samples for glutaraldehyde tanned leather. As leather biodegradation takes place (samples M10 – M27) the most abundant species present are *A. johnsonii*, *B. terrae* and *M. peregrinum*. Figure 8b shows a line graph representing bacterial presence shift in the sample as a percentage of the top six bacterial species identified at the different stages of glutaraldehyde leather biodegradation. In this case, *A. johnsonii* is the predominant bacterial strain being present in above 10% in all the samples M10 to M27.

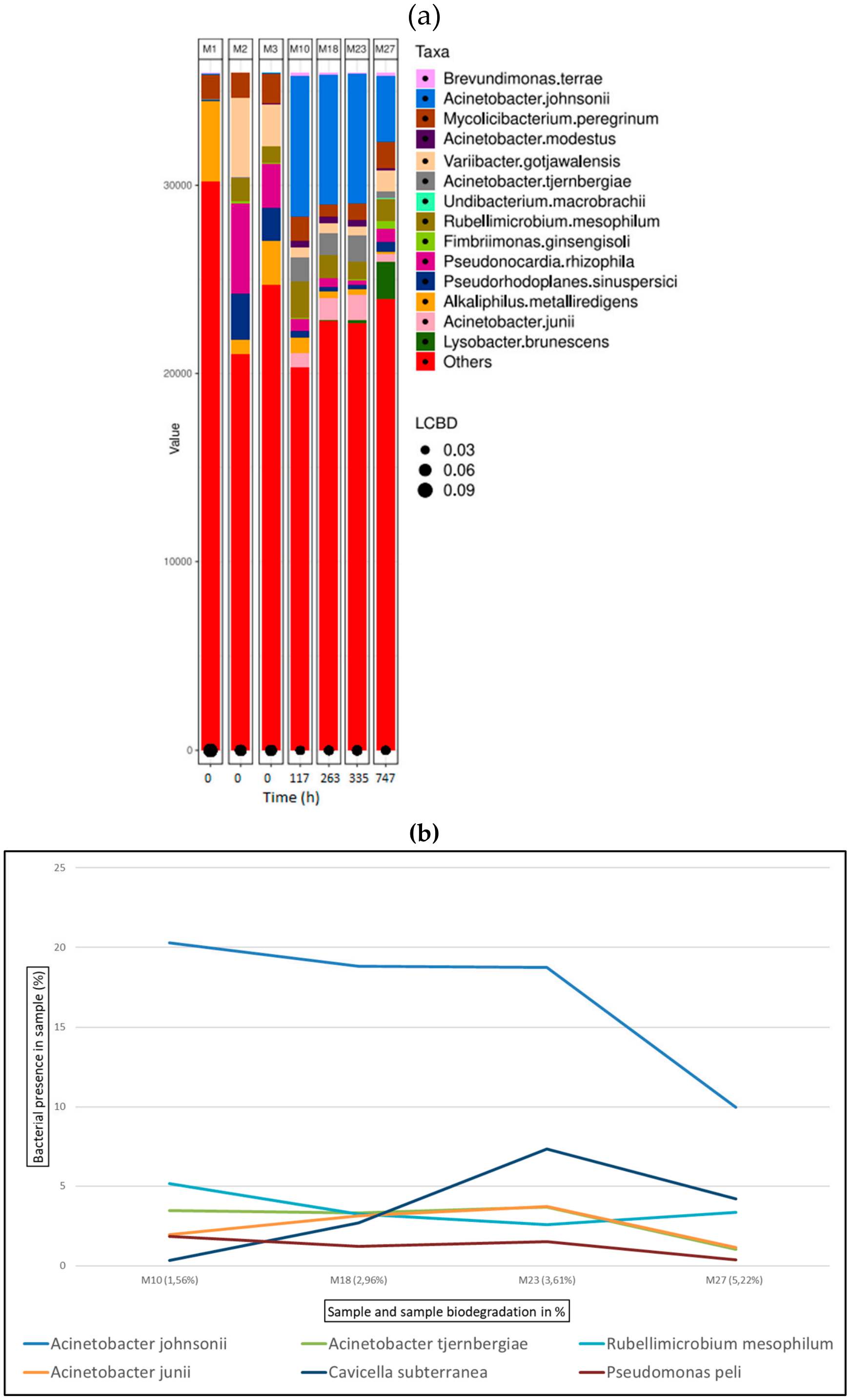

Figure 8. Bacterial species detected for glutaraldehyde samples in ISO20136; (**a**) Bar plot figures representing the proportions of detected bacterial species in the studied samples (M10, M18, M23, M27). M1 being municipal residual wastewater, sample M2 was tannery wastewater, and sample M3 was a mixed inoculum (50:50). (**b**) Line graph representing bacterial shift as bacterial presence in sample (%) of the top six bacterial species found for all glutaraldehyde samples.

Figure 9a shows the bacterial abundance at species level detected in the analysed samples for oxazolidine tanned leather. As leather biodegradation takes place (samples M6 – M26) the most abundant species present are *B. terrae, A. johnsonii* and *M. peregrinum*. Figure 9b shows a line graph representing bacterial presence shift in the sample as a percentage of the top six bacterial species identified at the different stages of oxazolidine leather biodegradation. In this case, *B. terrae* is the predominant bacterial strain in all samples M6 to M26, always present in around 20% in all the samples.

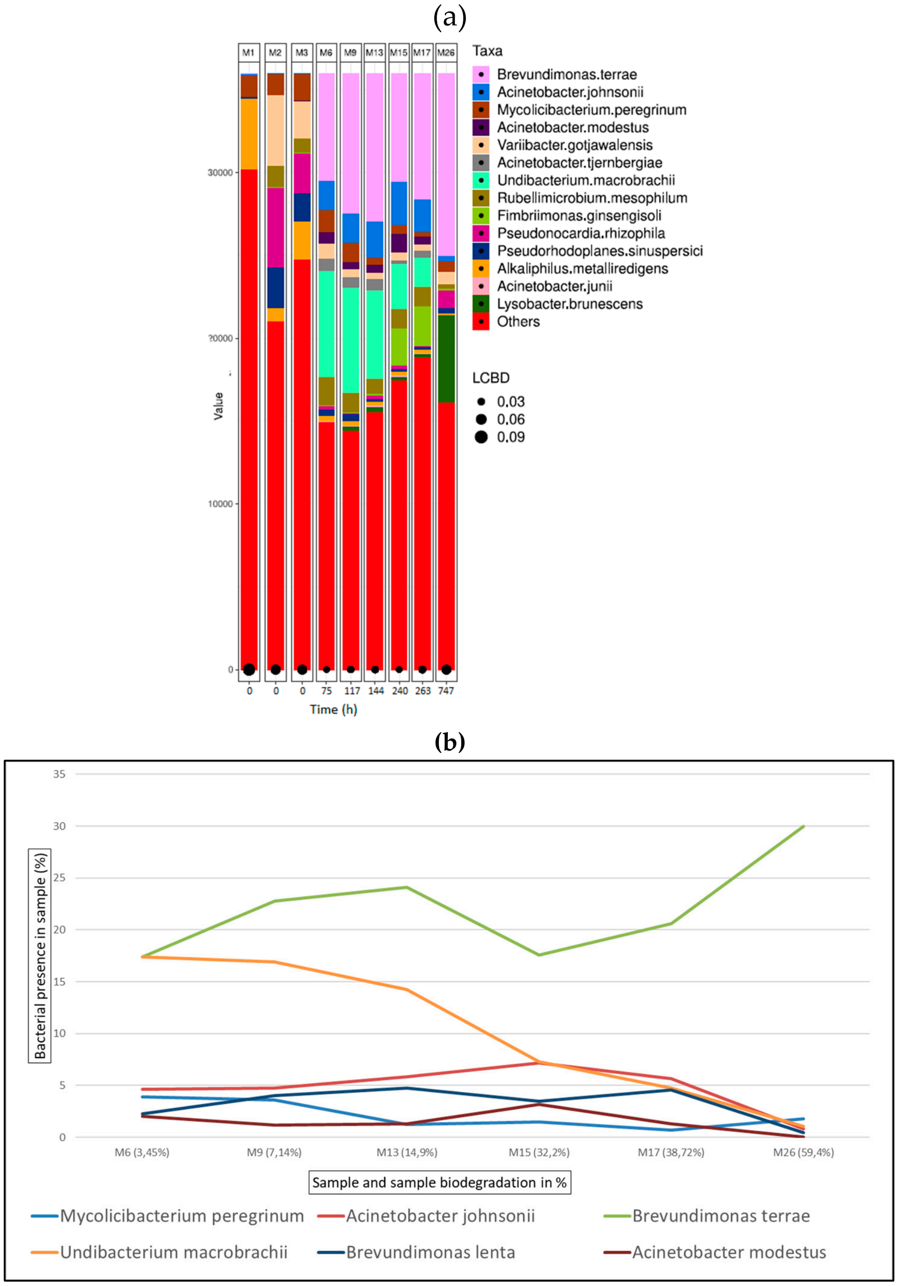

Figure 9. Bacterial species detected for oxazolidine samples in ISO20136; (**a**) Bar plot figures representing the proportions of detected bacterial species in the studied samples (M6, M9, M13, M15, M17, M26). M1 being municipal residual wastewater, sample M2 was tannery wastewater, and sample M3 was a mixed inoculum (50:50). (**b**) Line graph representing bacterial shift as bacterial presence in sample (%) of the top six bacterial species found for all oxazolidine samples.

Figure 10a shows the bacterial abundance at species level detected in the analysed samples for aluminium tanned leather. As leather biodegradation takes place (samples M7 – M29) the most abundant species present are *B. terrae, A. johnsonii* and *M. peregrinum.* Figure 10b shows a line graph representing bacterial presence shift in the sample as a percentage of the top six bacterial species identified at the different stages of aluminium leather biodegradation. In this case, *B.terrae* is the predominant bacterial strain in all samples M6 to M26, always present in around 20% in all the samples.

(a)

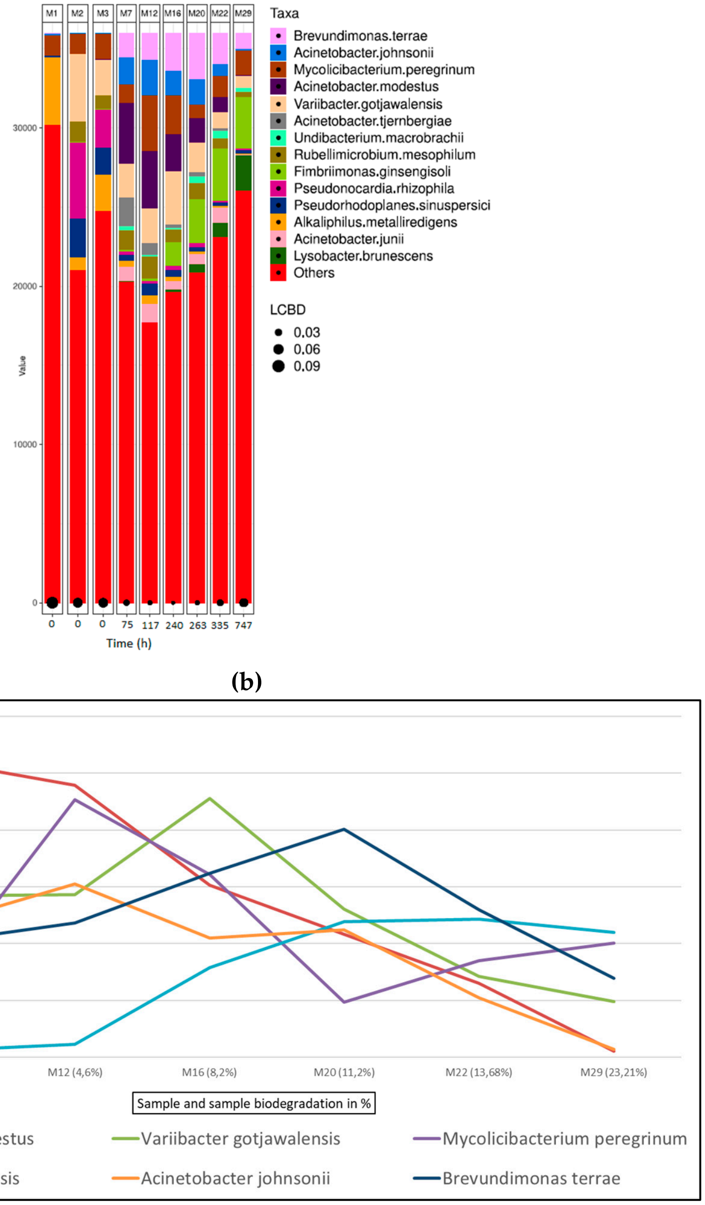

Figure 10. Bacterial species detected for aluminium samples in ISO20136; (**a**) Bar plot figures representing the proportions of detected bacterial species in the studied samples (M7, M12, M16, M20, M22, M29). M1 being municipal residual wastewater, sample M2 was tannery wastewater, and sample M3 was a mixed inoculum (50:50). (**b**) Line graph representing bacterial shift as bacterial presence in sample (%) of the top six bacterial species found for all aluminium samples.

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4. Discussion

4.1. Species Identification from Tannery Wastewaters

Though the years there has been different standards fixation for the use of 16S rRNA genes in taxonomy identification[58]. The latest cutoff value at the species level has been evaluated at 98.7%[59], however several authors have shown that these thresholds are not applicable to multiple genera[60]. Isolated species 1 *Dietzia maris* has been previously isolated from soil for zinc bioremediation [61], for petroleum hydrocarbons and crude oil degradation [62,63] and tolerance to heavy metals such as cadmium or cobalt [64] and multiple-extreme resistance [65]. Species 2 *T. pasteurii* has been previously identified for the reduction of hexavalent chromium [66], showing two potential pathways for Cr (VI) removal; sulfidogenesis-induced Cr (VI) reduction pathway, 90% Cr (VI) removal by sulfide generated from biological reduction of sulfate. The second being direct 10% Cr (VI) removal by bacteria as the electron acceptor [67]. *Corynebacterium lubricantis* has been previously isolated from chromite mine seepage of Odisha as a heavy metal tolerant and chromate reducing bacterium [68], as well as isolated from a chromium-polluted soil, tested for chromate reduction capability and multiple heavy metal tolerance up to a concentration of 22 mM [69]. *Microbacterium* strains have been previously studied for chromium waste biocementation [70] and isolated from tannery wastewaters for hexavalent chromium reduction [71]. *B. safensis* has been isolated from tannery effluent as a chromium (Cr) and tannic acid (TA) resistance bacterial strain [72], isolated from contaminated coal mining soil for chromium reduction [73], and isolated from rare-earth ore for hexavalent chromium conversion to trivalent chromium, where an gene *nfrA* is involved [74]. All these species represent good candidates for further investigation in which they are submitted to stress condition and evaluation of the functional groups in heavy metal bioremediation.

Identification of the specific functional groups responsible for metal ion binding to microbial biomass is crucial for understanding the biosorption mechanism in efficient methods for removing heavy metals from contaminated environments [75]. The type, structure, and arrangement of functional groups can vary significantly between microorganisms[76], with many of these groups primarily identified on microbial cell walls[77]. Functional groups such as aldehydes, alkyl chains, amides, amines, alcohols/phenols, carboxylic acids, esters, organic halides, phosphates, sulfoxides, and aliphatic organic chains of cellulose have been identified as key players in the biosorption of chromium[78]. Multiple spectroscopic and microscopic techniques, such as infrared and Raman spectroscopy, electron dispersive spectroscopy and nuclear magnetic resonance (NMR) have been identified for active sites involved in binding of heavy metal ions identification[79]. However, most studies employ Fourier-transform infrared spectroscopic (FT-IR) technique to identify and characterize certain functional groups present in microbial biomass for uptake of toxic heavy metals such as hexavalent chromium[80]. Specific functional groups and certain mechanisms for Cr biosorption already identified for certain species such as *Bacillus marisflavis* and *Bacillus arthrobacter* [81] as well *Klebsiella sp.*[82] include _OH, –NH acetamido group, amide bond, C=O of COO-, free phosphates, phosphate groups, –CN and NH2, O-H, -CONH-, -COOH, C = C, -CH2 (Freundlich adsorption isotherm) respectively.

4.2. Microorganism Identification from Leather Biodegradation Assay

4.2.1. ISO:20136:2020: Leather- Determination of Degradability by Microorganisms

Leather biodegradation process is heavily dependent on the tanning agent employed within the tanning process giving leather specific physical-chemical properties[1]. Pure collagen’s exponential degradation phase shows to be in the first 16 h of assay. Oxazolidines are cyclic condensation products of β-amino alcohols and aldehydes or ketone [64]. The ease of hydrolysis of oxazolidines in aqueous solution relates directly to their structural features, a cyclic ring structure that contains both a nitrogen atom and a carbonyl group [65]. These functional groups are susceptible to nucleophilic attack during hydrolysis, leading to the cleavage of the ring and the formation of the corresponding β-amino alcohol and aldehyde or ketone [66]. This leads into a higher biodegradation potential, like mineral based tanning methods such as zeolites, which have shown 81% biodegradation potential in 30 days [67]. Metal-tanned hides form stable cross-links between the collagen fibres, rendering the material less susceptible to enzymatic attack by microorganisms. These can exhibit toxicity to microorganisms, thereby inhibiting their ability to degrade the leather.[68] The presence of toxic metal ions can disrupt microbial activity, slowing down the biodegradation process [69].

4.2.2. Bioinformatics and Species Identification

More than 10,000 bacterial species have been identified to be present within all the analysed samples of the leather biodegradation assay and 8 bacterial strains have been isolated from tannery wastewaters. Bacterial genera identified and isolated from the first tannery wastewater sample, described in Table 3 have been identified in low percentages in sample M2 (tannery wasterwater) is as follows: *D. maris* 0% *T. pasteurii* 0.012%, *C. lubricantis* 0%, *M. laevaniformans* 0% and *B. safensis* 0%. Tannery wastewaters are generally characterised by its dark brown colour and high levels of pollutants total dissolved solids (TDS), chromium, phenolics, and high pH [83]. Bacterial diversity difference amongst these samples could be given due to changes in these conditions, which vary depending at which stage and time at which the sample is taken as well as the type of tanning that has been performed [84].

The most common identified microorganisms throughout the assay are the following: *Acinetobacter*, *Brevundimonas* and *Mycolicibacterium*. *Acinetobacter* genus is commonly found in aquatic environments such as wastewater and river waters [85]. Four *Acinetobacter* strains (*A. johnsonii, A. modestus, A. tjernbergiae, A. junii*) have been identified. These species have previously been identified and isolated from tannery and residual wastewater, characterised and evaluated for chromium bioremediation [86–89] and Cr^{6+} transformation to Cr^{3+} [90,91]. *Brevundimonas* has been mainly identified and isolated from sedimented waters [92], rivers [93] and soil samples [94–96]. *Brevundimonas* strains have also been identified and isolated for heavy metal bioremediation [97,98], cadmium and zinc bioremediation [99], and arsenic resistance [100,101]. *Mycolicibacterium* is a non-tuberculous identified and isolated in cotton fields [102], peat bog [103], sea coast [104] and mangrove sediment [105]. This strain has been previously identified and isolated for zinc-lead bioremediation [106].

Within the first 16 hours since the start of the assay, at the exponential phase of collagen biodegradation (M4 and M5) 60% and 37% of the inoculum consists of the genius *Acinetobacter* along with *Brevundimonas* present in 6.75% and 16% respectively. Within the bacterial profile for chromium samples, *Variibacter gotjawalensis* has been found to be present over or around 5% in all samples and 11.6% in samples M28 at the final stages of biodegradation assay (7.5% of leather biodegradation). This strain only appears significantly in samples from chromium tanned leather assay. This strain has been previously isolated from soil in a lava forest in Korea[107] and tested in Cd-Zn-Pb-contaminated soil for phytoextraction[108].

It must be noted that these identified genera (*Acinetobacter*, *Brevundimonas* and *Mycolicibacterium)* are merely present in initial inoculum (M3), *Mycolicibacterium* is around 5% and the other two do not reach 1%. There is an immense jump to such higher percentages as leather biodegradation takes places. The amount of leather sample and therefore of tanning agents are not significant to evaluate from these results the capability of the identified species to degrade and/or tolerate these molecules in higher concentrations. It is clear from the results that they have the capability of bond breakage between the tanning agent and the collagen fibres and biodegrade collagen itself. For certain tanning agents such as glutaraldehyde (Figure 8b) and oxazolidine (Figure 9b), *Acinetobacter johnsonii* and *Brevundimonas terrae* respectively, are the present species and therefore the most active species in terms of leather biodegradation. Further investigation would have to be carried out for a deeper understanding of species biodegradation mechanisms.

5. Conclusions

This study has contributed to the understanding of the microbial diversity and abundance in the context of leather biodegradation and heavy metal resistance. Despite the great abundance of

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identified bacterial species, the identification of bacterial genera such as *Acinetobacter*, *Brevundimonas*, and *Mycolicibacterium* in the samples has provided valuable insights into the potential microbial candidates showcasing their potential applications in enhancing leather biodegradability, wastewater treatment, and bioremediation processes for heavy metals.

Overall, the findings of this study underscore the importance of sustainable solutions for addressing environmental challenges within the leather industry. The selective isolation and identification of microorganisms with dual capabilities offer promising prospects for the development of efficient and environmentally friendly treatments for tannery wastewater, leather biodegradation, and heavy metal remediation, thereby contributing to the advancement of sustainable practices in the leather industry.

Author Contributions: Conceptualization, Marcelo Bertazzo and María-José Bonete; Data curation, Manuela Bonilla-Espadas, Basilio Zafrilla and Irene Lifante-Martínez; Formal analysis, Manuela Bonilla-Espadas; Funding acquisition, Elena Orgilés-Calpena and María-José Bonete; Investigation, Manuela Bonilla-Espadas and Basilio Zafrilla; Methodology, Manuela Bonilla-Espadas, Basilio Zafrilla and Irene Lifante-Martínez; Project administration, Elena Orgilés-Calpena, Francisca Arán-Aís and María-José Bonete; Resources, Mónica Camacho; Supervision, Elena Orgilés-Calpena, Francisca Arán-Aís, Marcelo Bertazzo and María-José Bonete; Validation, Manuela Bonilla-Espadas; Writing – original draft, Manuela Bonilla-Espadas; Writing – review & editing, Mónica Camacho, Marcelo Bertazzo and María-José Bonete.

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Data Availability Statement: The datasets presented in this study are available on request to the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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