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Polyphasic Characterization of Microbiota of "Mastredda", a Traditional Wooden Tool Used during the Production of PDO Provola dei Nebrodi Cheese

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Citation: Busetta, G.; Garofalo, G.; Mangione, G.; Botta, L.; Franciosi, E.; Di Gerlando, R.; Todaro, M.; Licitra, G.; Scatassa, M.L.; Gaglio, R.; et al. Polyphasic Characterization of Microbiota of "Mastredda", a Traditional Wooden Tool Used during the Production of PDO Provola dei Nebrodi Cheese. *Appl. Sci.* **2021**, *11*, 8647. https://doi.org/10.3390/app11188647

Academic Editor: Athina S. Tzora

Received: 30 July 2021
Accepted: 12 September 2021
Published: 17 September 2021

Abstract: The biofilms of the wooden tables used for the acidification of the curd were investigated for PDO Provola dei Nebrodi cheese, a traditional stretched cheese made in eastern Sicily (southern Italy) from raw cows' milk. To this purpose the wooden tables of four dairy facilities were analysed for their microbiota by scanning electron microscopy (SEM) analysis and a combined culture-independent and -dependent microbiological approach. SEM inspection showed an almost continuous biofilm formation. MiSeq Illumina analysis identified 8 phyla, 16 classes, 25 orders, 47 families and 50 genera. *Corynebacterium*, *Bifidobacterium* and lactic acid bacteria (LAB) were detected in all samples. In particular, the LAB genera detected on all wooden tables were *Lactobacillus*, *Streptococcus* and *Lactococcus*. LAB dominated the surfaces of all wooden tables with levels higher than 7.0 Log CFU/cm^2. In particular, the LAB found at the highest levels were mesophilic cocci. Coagulase positive staphylococci, *Salmonella* spp., *Listeria monocytogenes* and Shiga-toxigenic *Escherichia coli* were never detected. Twenty-seven dominating LAB strains were identified within the genera *Enterococcus*, *Lactobacillus*, *Lacticaseibacillus*, *Lactiplantibacillus*, *Levilactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus*. This work showed that the wooden table used during the production of PDO Provola dei Nebrodi cheese is a safe system and a microbiologically active tool.

Keywords: dairy wooden tool; lactic acid bacteria; Miseq Illumina; scanning electron microscopy; traditional cheese

1. Introduction

Provola dei Nebrodi is a stretched cheese produced in the Nebrodi Mountains area located in eastern Sicily (Southern Italy). This cheese is made from raw cows' milk without the addition of starter cultures [1] and, in 2020, it gained the protected denomination of origin (PDO) recognition by the European Union [2]. PDO Provola dei Nebrodi cheese has a typical pear-shape with a smooth, uniform and pale yellow crust. Five categories of these cheeses can be produced within PDO protocol of production: fresh (up to 1 month), semi-hard (ripened for 3–4 months), hard (ripened for 4–5 months), *sfoglia* (flaky; ripened

more than 5 months) and with *limone verde* (green lemon; ripened more than 3 months). The entire production process of PDO Provola dei Nebrodi cheese is performed with wooden equipment [3], whose use is allowed in Europe by the commission regulation (EC) no. 2074/2005, which allows derogation from regulation EC no. 852/2004 for foods with traditional characteristics [4].

Wood is a traditional and natural material with a porous structure [5] able to absorb and retain microbial cells within a self-produced matrix of extracellular polymeric substance (EPS) known as "biofilms" [6]. In light of the alert issued from the US Food and Drug Administration on the potential presence of pathogenic bacteria about Italian and French cheeses ripened on wooden shelves [7], different research groups have focused their efforts to the microbial characterization of wooden equipment used in traditional dairy productions such as wooden vats [5,8–12] and wooden shelves [13–16]. Galinari et al. [15] also evaluated the wooden tables and forms used for moulding the Brazilian Minas cheese. All these studies detected the desired dairy lactic acid bacteria (LAB) on the wooden surfaces of all tools and equipment and reported the absence of the typical dairy pathogenic bacteria *Listeria monocytogenes* and *Salmonella* spp.

PDO Provola dei Nebrodi cheese, like all stretched cheeses, is made through a 2-step production: the first step consists of curdling and acidification, while the second step is aimed to stretch the acidified curd into the final cheese shape [17]. The acidification of the curd is performed by LAB. Since starter cultures are not deliberately added during production, the acidification process relies on the presence of LAB in the raw milk, on the surfaces of the wooden equipment and even in the animal rennet [11,18,19]. These LAB convert milk lactose into lactic acid very rapidly and, for this reason, the species of this group are classified as starter LAB (SLAB). The same sources can also transfer to the curd non starter LAB (NSLAB) which are mainly involved in the ripening process and influence several biochemical events necessary to provide the final cheeses with the typical aromatic profile [20].

The acidification process is particularly important for stretched cheeses because a pH drop in the range 5.2–5.4 is necessary for stretching [21,22], since dicalcium paracaseinate is converted into monocalcium paracaseinate determining the formation of fibres that can be reorganized to give shape and sheen to the cheese [23]. A pH value > 5.4 does not allow stretching of the curd, while at pH below 5.2 the acidified curd is too tough due to the excessive fat loss [24]. After draining, the curd for PDO Provola dei Nebrodi cheese production is traditionally left to acidify at room temperature for about 24 h on a wooden open-topped table, namely "mastredda" [3] (Figure 1).

Figure 1. "Mastredda", a wooden table used for curd acidification during PDO Provola dei Nebrodi cheese production.

So far, no study on the microbiological characterization of "mastredda" is available in the literature.

The purpose of the present research was to fill the gap in knowledge on the microbial biofilms of an important wooden tool used for traditional dairy productions. In particular, wooden tables of different dairy facilities producing PDO Provola dei Nebrodi cheese were investigated for their microbial biofilms through several approaches: scanning electron microscopy (SEM) inspection; culture-independent strategy applied to the bacterial community; plate counts of the main dairy-involved microbial groups; and isolation, characterization and identification of LAB.

2. Materials and Methods

2.1. Collection of Wooden Table Biofilms

The wooden tables used for curd acidification during PDO Provola dei Nebrodi cheese production were investigated from four dairy facilities of eastern Sicily (Italy) all located within Catania province (Table 1).

Table 1. Characteristics of the wooden tables used for curd acidification of PDO Provola dei Nebrodi cheese production.

Wooden Table	City of Dairy Factory (Province) [1]	Age of Table (Years)	Type of Wood [2]
WTA	Randazzo (CT)	5	silver fir
WTB	Maniace (CT)	2	silver fir
WTC	Randazzo (CT)	8	douglas fir
WTD	Randazzo (CT)	10	chestnut

[1] Province codes: CT, Catania. [2] Tree species: silver fir, *Abies alba* L.; chestnut, *Castanea sativa* Miller; douglas fir, *Pseudotsuga menziesii* (Mirb.) Franco. Abbreviation: WT, wooden table; A–D, dairy factory A–dairy factory D.

Each wooden table surface (100 cm^2) was delimited using a sterile paper square (Area Space 100, VWR International PBI s.r.l., Milan, Italy) to perform the biofilm collection by a non-destructive brushing method. To this purpose, the method described by Didienne et al. [9] was applied. Briefly, a sterile toothbrush was rubbed onto the wooden surface and a sterile cotton gauze was pressed onto the brushed area. Subsequently, the gauze was transferred into a sterile Durham bottle containing 100 mL of Ringer's solution (Sigma-Aldrich, Milan, Italy) where the toothbrush was previously washed. Wooden table surface collection was performed in two technical repeats (two adjacent areas) and repeated after one month for a total of four collections in two independent sampling days. The wooden tables are cleaned from time to time by brushing with the hot (70–80 °C) deproteinized whey that results from Ricotta cheese production [25]. All samples were transported with a portable fridge to the Agricultural Microbiology laboratory (Department of Agricultural, Food and Forestry Science, University of Palermo, Palermo, Italy).

2.2. Scanning Electron Microscopy

The presence of the biofilms on the surface of the wooden tables was also analysed by a destructive method to perform the scanning electron microscopic (SEM) investigation. Square wood splinters (approximately 10 mm by 10 mm by 2 mm) were aseptically collected from each wooden table with a sterile stainless steel scalpel [26]. Wooden samples were analysed using the FEI ESEM Quanta 200 apparatus (FEI Company, Hillsboro, OR, USA) at the Department of Engineering of the University of Palermo (Italy). Before being mounted on the aluminium holder, the splinters were dehydrated [27] and dried [5]. A thin layer of gold (20 mÅ; 300 s) (Edwards S150A sputter coater) under argon atmosphere for 90 s (Scancoat Six Ed-wards, Crawley, UK) was spattered on the wooden samples to avoid electrostatic charging under electron beam.

2.3. DNA Extraction, Miseq Library Preparation and Illumina Sequencing

Cell suspensions (10 mL) of each wooden table biofilm were centrifuged at 3200× *g* for 15 min at 4 °C [28]. Cell pellets (10 mg) were harvested and used to extract total genomic DNA using the QIAamp® DNA Investigator Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The DNA quality and concentrations were determined by NanoDrop™ 8000 Microvolume UV-Vis spectrophotometer (ThermoFisher Scientific, Inc., Wilmington, DE, USA). Amplicon library preparation, quality and quantification of pooled libraries, and pair-end sequencing using the Illumina MiSeq system were carried out at the Sequencing Platform in Fondazione Edmund Mach (FEM, San Michele a/Adige, Italy). Briefly, total genomic DNA was amplified using primers specific to the bacterial 16S rRNA gene [29,30]. Each sample was amplified by PCR using 25 µL reaction with 1µM of each primer. PCR reactions were executed by GeneAmp PCR System 9700 (Thermo Fisher Scientific, Waltham, MA, USA). The amplification products were checked on 1.5% agarose gel and purified using the Agencourt AMPure XP system (Beckman Coulter, Brea, CA, USA), following the manufacturer's instructions. Afterward, a second PCR was used to apply dual indices and Illumina sequencing adapters Nextera XT Index Primer (Illumina, San Diego, CA, USA). The amplicon libraries were purified using Agencourt AMPure XP system (Beckman), and the quality control was performed on a Typestation 2200 platform (Agilent Technologies, Santa Clara, CA, USA). Finally, all barcoded libraries were pooled in an equimolar ratio and sequenced on an Illumina® MiSeq (PE300) platform (MiSeq Control Software 2.5.0.5 and Real-Time Analysis software 1.18.54.0).

2.4. Illumina Data Analysis and Sequences Identification by QIIME2

Raw paired-end FASTQ files were demultiplexed using idemp (https://github.com/yhwu/idemp/blob/master/idemp.cpp, last accessed: 29 July 2021) and imported into Quantitative Insights into Microbial Ecology (Qiime2, version 2020.8). Sequences were de-noised and merged using DADA2 [31]. DADA2 was run as described in https://benjjneb.github.io/dada2/tutorial.html (last accessed: 29 July 2021) using the default parameters. In order to improve the overall quality of the sequences, the reads were quality filtered and trimmed using the Filter and Trim functions implemented in DADA2. Chimeric sequences had been identified and removed via the consensus method in DADA2. The taxonomic assignment and compositional analyses were carried on by using plugins feature-classifier (https://github.com/qiime2/q2-feature-classifier, last accessed: 29 July 2021)) and performed using a pre-trained naïve Bayesian classifier method based on the Greengenes 13_8 99% Operational Taxonomic Units (OTUs) database which had been previously trimmed to the V4 region of 16S rDNA, bound by the 341F/805R primer.

The FASTQ files generated by MiSeq Illumina sequencing were deposited in the NCBI Sequence Read Archive (SRA) and are available under Ac. Number PRJNA750672 (https://www.ncbi.nlm.nih.gov/bioproject/750672, last accessed: 29 July 2021).

2.5. Classical Microbiological Analyses

Biofilm cell suspensions (1 mL) were also serially diluted (1:10) in Ringer's solution (Sigma-Aldrich, Milan, Italy). The consecutive dilutions were homogenized by vortexing the test tubes and plated on agar media for the following microbial groups: total mesophilic aerobic microorganisms (TMM) by spread plating on Plate Count Agar (PCA), incubated at 30 °C for 48 h; mesophilic and thermophilic LAB cocci on M17 agar incubated anaerobically for 72 h at 30 °C and 44 °C, respectively; mesophilic and thermophilic LAB rods on MRS agar adjusted to pH 5.4 with 5 Mol lactic acid, incubated anaerobically for 72 h at 30 °C and 44 °C, respectively; enterococci on kanamycin esculin azide (KEA) agar, incubated aerobically for 24 h at 37 °C. *Pseudomonas* spp. on *Pseudomonas* Agar Base (PAB) supplemented with 10 mg/mL cetrimide-fusidic acid, incubated at 25 °C for 72 h; Enterobacteriaceae on Violet red bile glucose agar (VRBGA), incubated at 37 °C for 24 h. In ad-

dition, all samples were also analyzed for the presence of the main pathogenic microorganisms: coagulase-positive staphylococci (CPS) on Baird Parker (BP) supplemented with rabbit plasma fibrinogen (RPF); *Listeria* spp. and *Listeria monocytogenes* on *Listeria* selective agar base with SR0140E supplement; *Salmonella* spp. and *E. coli* on Hektoen Enteric agar (HEA). All pathogens were incubated at 37 °C for 24 h. All media and supplements were purchased from Oxoid Microbiology Products (Thermo-Scientific, Milan, Italy), except HEA provided by Microbiol Diagnostici (Uta, Italy). Plate counts were performed in duplicate.

2.6. Isolation, Grouping, Genotypic Differentiation and Identification of LAB

After growth on MRS and M17 agar media, the colonies of presumptive mesophilic and thermophilic LAB with different morphologies were picked up from the plate. In order to cover the entire variability of viable LAB, at least five colonies sharing the same appearance (shape, size, margin edge, elevation, colour and opacity of surface) were collected for all morphologies distinguished. All isolates were purified by several streaking steps onto the same agar media used for plate count and tested for Gram type by treatment with 3% (*w*/*v*) KOH method [32], and for catalase test performed by the addition of H_2O_2 at 3% (*v*/*v*) to the colonies [33]. Pure cultures were first subjected to a grouping based on cell morphology and arrangement of the cells [34], and then analysed for their physiological and biochemical traits as described by Gaglio et al. [35]. LAB cocci were further analysed for their growth at pH 9.2 and in the presence of NaCl (6.5 g/L) to discriminate enterococci from other dairy LAB cocci.

In order to restrict the number of presumptive LAB to be subjected to the genetic identification, all cultures were subjected to strain typing by random amplification of polymorphic DNA (RAPD)-PCR analysis. Genomic DNAs were extracted from overnight grown cultures using the DNA-SORB-B kit (Sacace Biotechnologies Srl, Como, Italy) according to the manufacturer's instructions. Crude cell extracts were amplified using singly the primers M13, AB111, and AB106 as reported by Gaglio et al. [36]. RAPD patterns were then analysed by GelCompar II software version 6.5 (Applied-Maths, Saint-Marten-Latem, Belgium) to obtain a dendrogram and evaluate the similarity among the LAB community. Genotypic identification of all different strains was performed by amplification and sequencing of the 16S rRNA gene [26]. The sequences obtained were compared to those available in the GenBank/EMBL/DDBJ (http://www.ncbi.nlm.nih.gov, last accessed: 12 July 2021) and EzTaxon-e (http://eztaxon-e.ezbiocloud.net/, last accessed: 12 July 2021) databases.

2.7. Shiga-Toxigenic E. coli Detection

In case of detection of *E. coli* colonies, they were analysed for their Shiga-toxigenic *E. coli* (STEC) genes. The multiplex PCR described by Osek [37] and designed on the genes for Shiga toxins 1 and 2 (*stx1* and *stx2*) was applied on the *E. coli* isolates developed at the highest dilutions of the biofilms.

2.8. Statistical Analyses

Plate count data were subjected to one-way variance analysis (ANOVA) using XLStat software version 7.5.2 for Excel (Addinsoft, New York, NY, USA). The Tukey's test was applied for pairwise comparison between the different wooden table biofilms analysed. Statistical significance was attributed to p values of $p < 0.01$.

3. Results and Discussion

3.1. Scanning Electron Microscopy of Wooden Table Biofilms

The wooden splinters collected from the four tables, used for the curd acidification of PDO Provola dei Nebrodi, were evaluated for the presence of microbial biofilms by SEM, and the results of the analysis are shown in Figure 2.

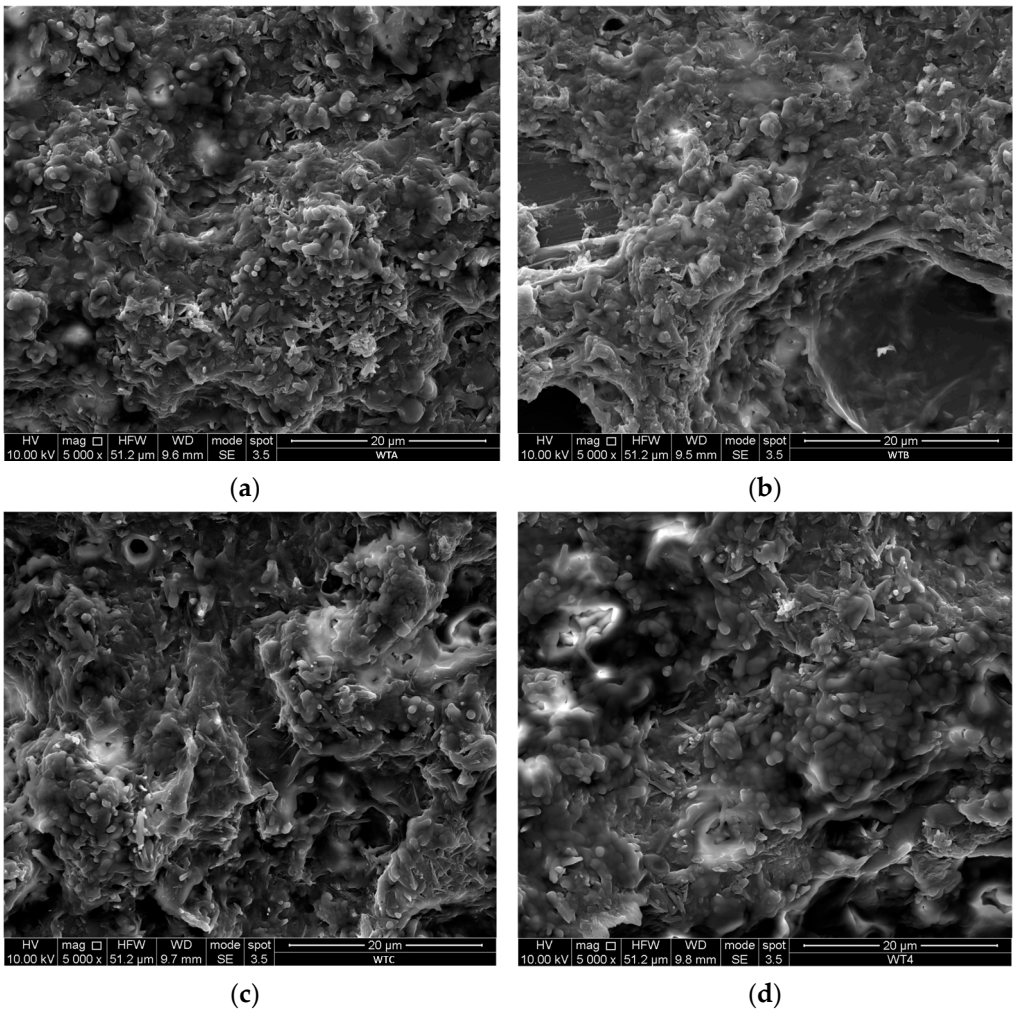

Figure 2. Scanning electron microscopy (SEM) observations of wooden splinters from wooden tables used during PDO Provola dei Nebrodi cheese production. Pictures of the wooden splinters from dairy factories A–D (**a**–**d**).

The images acquired by SEM clearly showed that the biofilm cover the wood surfaces of the tables entirely. As per the wooden vats used for milk curdling to produce other PDO and traditional cheeses [5,12,26], the exopolysaccharide matrix typical of microbial biofilms is also clearly visible on the wooden tables used for the acidification of curds of PDO Provola dei Nebrodi cheese. However, the wood surface is rarely still recognizable (WTB). This inspection showed a consistent presence of coccus-shaped bacterial populations, while short rod bacteria were detected at lower densities.

3.2. Taxonomic Distribution of Wooden Table Bacteria

In recent years, the Illumina technology has been widely applied to reveal the entire bacterial composition of raw materials used in food production in order to also determine the dormant and/or viable but not cultivable bacterial community before processing [38–40]. In this study the DNA extracted from the biofilms collected in replicate collections from the four wooden table samples was successfully amplified in the bacterial V3-V4 16S

rRNA gene region. A total of 320,396 paired-end sequences were obtained. The taxonomy classification allowed the identification of eight phyla, 16 classes, 25 orders, 47 families and 50 genera. The relative abundance (%) of the operational taxonomy units (OTUs) identified from the biofilms is reported in Figure 3.

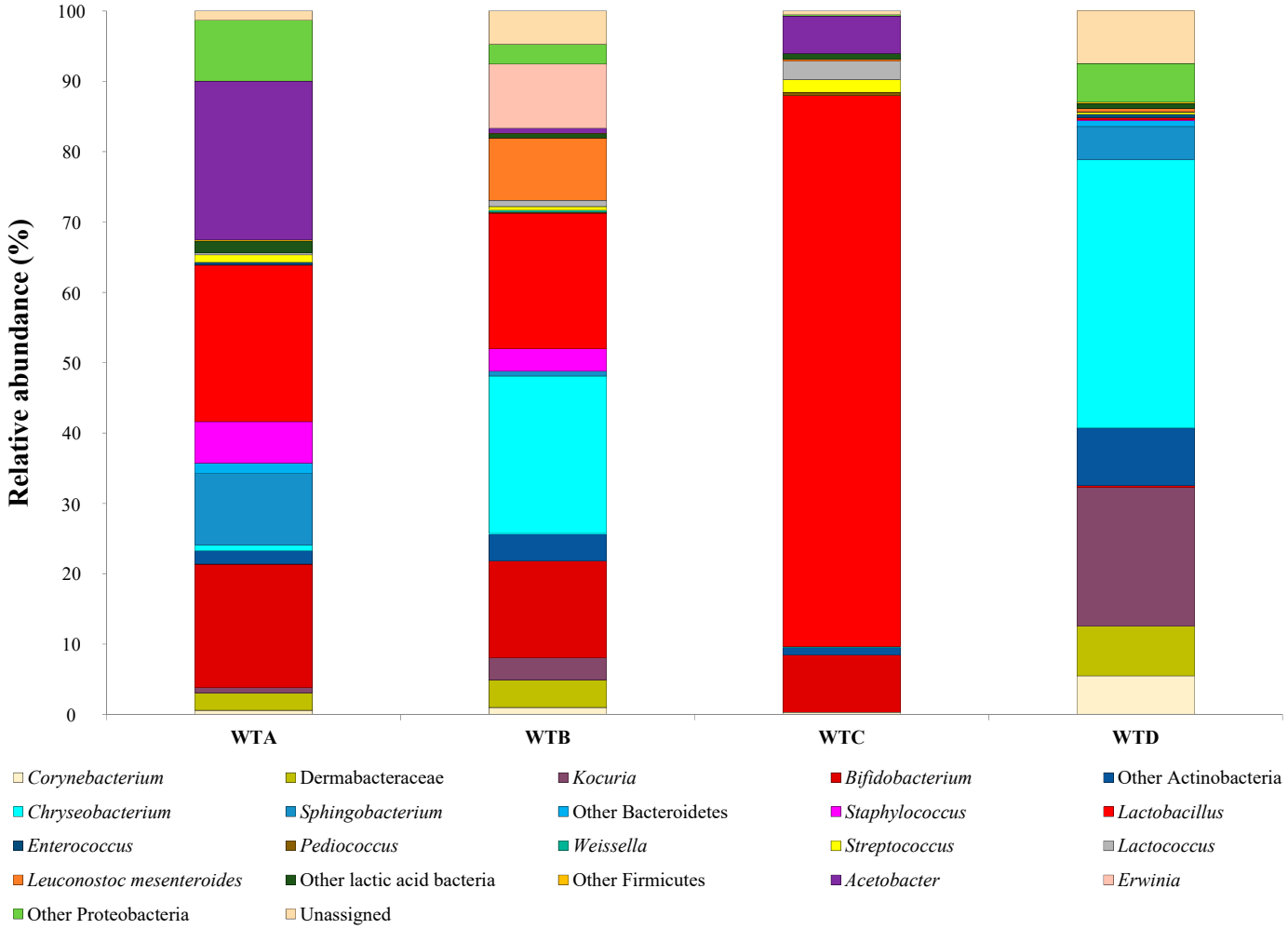

Figure 3. Relative abundances (%) of bacteria identified by MiSeq Illumina in wooden table biofilms. Abbreviations: WT, wooden table; A–D, dairy factory A–dairy factory D.

The OTUs with an individual relative abundance < 0.1% were not considered since this is the threshold indicated for abundant communities [41].

All wooden tables were characterized for the presence of *Corynebacterium*, *Bifidobacterium* and LAB. In particular, the LAB genera detected on all four wooden tables were *Lactobacillus*, *Streptococcus* and *Lactococcus*. The abundance of these groups were greatly variable with *Corynebacterium* ranging from 0.29 to 5.80%, *Bifidobacterium* from 0.29 to 17.69, *Streptococcus* from 0.22 to 1.84%, *Lactococcus* from 0.10 to 2.65% and, especially, *Lactobacillus* from barely 0.29 in WTD to 78.61% in WTC. The LAB group also included other genera found only in one sample like *Weissella* in WTB, or two samples like *Enterococcus* in WTA and WTD and *Pediococcus* in WTB and WTC or three samples as per *Leuconostoc* (with the species *Leuconostoc mesenteroides*) in WTB–WTD.

In dairy environments, corynebacteria represent part of the surface community of smear cheeses [42] and are also found on the wooden shelves used for cheese ripening [13,16]. Since they are not involved in curd acidification they colonize the wooden surfaces of the tables analysed as a result of an environmental contamination or they are transferred from the raw milk [43]. The presence of bifidobacteria on dairy equipment is not

negative, since this bacteria are common human probiotics [44], but they do not have to interfere with the activities of LAB during cheese production [45]. It is not surprising that LAB constituted the dominant populations of wooden table biofilms. In fact, LAB form stable biofilms on the surfaces of the wooden vats used for cheese curdling [5,8–10,46–48] and a similar LAB development was even more expected on the surfaces of the tables used for the overnight curd acidification.

Dermabacteraceae, *Kocuria*, *Chryseobacterium*, *Sphingobacterium* and S*taphylococcus* were not found in sample WTC. Among these, only *Kocuria* in WTD and *Chryseobacterium* in WTB were detected at very consistent levels (20.84 and 22.58%, respectively). Dermabacteriaceae and Staphylococcaceae have been recently detected in raw milk used to process Italian hard cheeses [49]. In particular, *Staphylococcus* is part of the raw cow's milk bacterial community [50]. *Kocuria* is also found in raw cow's milk [51] and is even selected as adjunct culture to enhance flavour and sensory attributes of traditional cheeses [52]. Several *Chryseobacterium* species (*C. joostei*, *C. oranimense* and *C. haifense*) have been recently identified from raw milk [53–55]. These bacteria are generally psychrotolerant, lipolytic and proteolytic and, for these reasons, undesirable because they might cause alteration of cheeses. Regarding sphingobacteria, they are also psychrothropic and are involved in cheese lipolysis and proteolysis [56].

Acetobacter were identified in the samples WTA–WTC with a particularly high relative abundance (22.66%) in WTA, while 9.18% of WTB biofilm OTUs were allotted into *Erwinia* genus. *Acetobacter* are not involved in cheese production, but associated with fermented milks [57,58]. Thus, its presence on the wooden tables used for curd acidification is not particularly singular. *Erwinia* were detected only in one sample and this is quite obvious, since bacteria belonging to this genus are plant pathogens [59] and play no role in dairy production. Except other *Actinobacteria*, in the range 1.08–8.68%, and other *Proteobacteria*, in the range 0.25–8.75%, found in all four samples, several OTUs from three of the four wooden tables could not be assigned to a hierarchical level below phylum and were grouped as other *Bacteroidetes* and other *Firmicutes*.

3.3. Levels of Viable Microorganisms

Plate counts of the bacterial groups harbored onto the surfaces of the wooden tables object of investigation are reported in Table 2.

Table 2. Microbial load [1] of the biofilms of the wooden tables used for curd acidification during PDO Provola dei Nebrodi cheese production.

Bacterial Counts	Samples				*p* Value
	WTA	WTB	WTC	WTD	
TMM	7.34 ± 0.26	7.43 ± 0.25	7.55 ± 0.15	7.21 ± 0.21	0.352
Mesophilic rod LAB	5.36 ± 0.16	5.82 ± 0.21	5.57 ± 0.23	5.55 ± 0.21	0.128
Thermophilic rod LAB	5.73 ± 0.20	5.35 ± 0.17	5.59 ± 0.16	5.39 ± 0.13	0.074
Mesophilic coccus LAB	7.39 ± 0.13	7.34 ± 0.20	7.25 ± 0.18	7.39 ± 0.12	0.690
Thermophilic coccus LAB	5.87 ± 0.20	5.79 ± 0.21	5.63 ± 0.14	5.39 ± 0.13	0.057
Enterococci	2.35 ± 0.17 ^{C}	2.44 ± 0.15 ^{BC}	2.78 ± 0.15 ^{AB}	3.05 ± 0.17 ^{AB}	0.0024
Pseudomonas	<1 ^{B}	2.97 ± 0.12 ^{A}	<1 ^{B}	2.84 ± 0.12 ^{A}	0.0001
Enterobacteriaceae	1.44 ± 0.15 ^{D}	3.54 ± 0.19 ^{A}	1.87 ± 0.12 ^{C}	2.81 ± 0.10 ^{B}	0.0001
E. coli	1.24 ± 0.15 ^{C}	3.44 ± 0.16 ^{A}	<1 ^{D}	2.39 ± 0.13 ^{B}	0.0001

[1] Units are log CFU/cm^2 for area samples. Results indicate mean values ± standard deviation (SD) of n = 8 plate counts (carried out in duplicates for two independent sampling). Abbreviation: WT, wooden table; A–D, dairy factory A–dairy factory D; TMM, total mesophilic microorganisms; LAB, lactic acid bacteria; *E.*, *Escherichia*. On the row A, B, C, D: $p < 0.01$.

According to Tukey's test, no statistically significant differences ($p > 0.05$) between the wooden table biofilms analysed were found for the levels of TMM and all LAB groups.

TMM cell densities were higher than 7.0 Log CFU/cm^2 on all four wooden tables investigated. Lower levels of TMM were reported by Galinari et al. [15] on the wooden table used for the molding of artisanal Minas cheese made in the Serro and Serra da Canastra regions (4.29 and 4.58 log CFU/cm^2, respectively). In this case, the curd was in contact with the tables for a shorter time compared to the Provola dei Nebrodi curd. All wooden table surfaces displayed high levels of LAB (mesophilic and thermophilic rods and cocci). These findings were somewhat expected considering previous studies focused on wooden vat biofilm characterization [5,8–11,60]. The highest cell densities of LAB were registered for mesophilic LAB cocci. The levels of this LAB group were superimposable to those of TMM indicating that these bacteria dominated all wooden table biofilms. The dominance of cocci over rods was previously reported in wooden vats used for the production of PDO bovine [9–11] and ovine [11,12,61] cheeses. Enterococci were detected in all wooden table samples in the range 2.35–3.05 Log CFU/cm^2. Similar levels of enterococci were found on the surfaces of the wooden vats used for making Caciocavallo Palermitano and PDO Vastedda della valle del Belìce cheeses [11] and on wooden shelves used in the ripening of a traditional French raw milk smear cheese [14]. However, high throughput analysis detected *Enterococcus* only in WTA and WTD biofilms, probably because *Enterococcus* were part of the unassigned OTUs of total bacterial community or they were below 0.1% of abundance or their DNAs were rendered inaccessible by nucleases. Regarding *Pseudomonas* spp., known agents of food spoilage [62], only the samples WTB and WTD were characterized by the presence of these bacteria with concentrations of about 3 Log CFU/cm^2. Generally *Pseudomonas* spp. are absent or present at very low levels onto the surfaces of wooden equipment used in dairy facilities [10].

3.4. Microbiological and Hygiene Criteria for Foodstuffs

In light of the Commission Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs [63], *Salmonella* spp. and *L. monocytogenes* were analysed as food safety criteria, while *E. coli* and CPS as process hygiene criteria.

In general, the surfaces of the tables hosted low numbers of members of the Enterobacteriaceae family, except WTB that was also characterized for the highest cell density of *E. coli* (3.44 Log CFU/cm^2). This bacterium was also found on the surfaces of the tables WTA and WTD. Similar levels of *E. coli* have been previously observed on the surfaces of the wooden vats used for making PDO Salers cheese [9] and on those analysed by Lortal et al. [5] in eastern Sicily. Considering that, after *Campylobacter* and *Salmonella*, STEC was the third most frequent bacterial agent detected in food-borne outbreaks in the EU in 2019 [64], the presence of STEC genes was specifically investigated in this study, but they were not detected in any of *E.coli* isolated at the highest numbers from the wooden table biofilms. The specific search for CPS, *L. monocytogenes* and *Salmonella* spp. did not generate any colony (for this reason these results are not reported in Table 2), showing the hygienic suitability of the "mastredda" tool for the curd acidification of PDO Provola dei Nebrodi cheese. The absence of these bacteria is undoubtedly due to their inability to adhere or to survive in wooden table biofilms in the presence of acidic conditions generated by LAB [5,10].

3.5. Differentiation and Identification of Viable LAB

After enumeration, 312 Gram positive and catalase negative colonies (putative LAB) were isolated from the agar media used for LAB counts. After microscopic inspection, the cultures were separated into 225 cocci and 89 rods. The combination of the morphological/physiological/biochemical traits, separated all LAB into 11 groups (Table 3).

Table 3. Phenotypic grouping of the LAB forming biofilms on the wooden table surfaces.

Characters	Clusters										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
	(n = 10)	**(n = 51)**	**(n = 9)**	**(n = 19)**	**(n = 85)**	**(n = 36)**	**(n = 13)**	**(n = 29)**	**(n = 14)**	**(n = 11)**	**(n = 37)**
Morphology [1]	R	R	R	R	C	C	C	C	C	C	C
Cell arrangement [2]	sc	sc	sc	sc	sc	sc	sc	sc	t	t	lc
Growth:											
15 °C	-	+	+	+	+	+	+	+	+	+	-
45 °C	+	+	+	-	-	+	-	+	+	+	+
pH 9.2	n.d.	n.d.	n.d.	n.d.	-	-	+	+	+	+	-
6.5% NaCl	n.d.	n.d.	n.d.	n.d.	+	+	-	+	+	+	-
Resistance to 60 °C	+	+	+	-	-	+	+	+	+	+	+
Hydrolysis of:											
arginine	-	-	+	-	-	+	+	+	-	+	+
aesculin	+	+	+	+	-	+	+	+	-	+	-
Acid production from:											
arabinose	-	+	+	+	+	+	-	+	+	+	+
ribose	-	+	+	+	+	+	+	+	+	+	+
xylose	-	+	+	+	+	+	-	+	+	+	+
fructose	+	+	+	+	+	+	+	+	+	+	+
galactose	+	+	+	+	+	+	+	+	+	+	+
lactose	+	+	+	+	+	+	+	+	+	+	+
sucrose	+	+	+	+	+	+	+	+	+	+	+
glycerol	+	+	+	+	+	+	+	+	-	+	+
CO_2 from glucose	-	-	-	-	+	+	-	-	-	-	-

[1] R, rod; C, coccus. [2] sc, short chain; t, tetrads; lc, long chain. Abbreviation: n.d., not determined.

The highest number of groups (seven) was observed for cocci which presented three main cell arrangements: short chains (four groups), tetrads (two groups), and long chains (one group). Among these, two groups (5–6) were able to generate CO_2 from glucose, displaying an obligate hetero-fermentative metabolism. The community of rod isolates included four groups and all of them showed a homo-fermentative metabolism. About 50% of the isolates representing each phenotypic group was analyzed by RAPD-PCR, a genotypic approach commonly applied, to perform the strain typing of the LAB associated with food products [35]. With this approach, 27 distinct RAPD profiles representing 27 strains were recognized (Figure 4), indicating the validity of this technique for differentiating LAB associated with the wooden equipment as previously reported by Cruciata et al. [12]. The sequencing of the 16S rRNA gene confirmed that all 27 strains belonged to the LAB group and they were allotted into nine genera (*Enterococcus*, *Lactobacillus*, *Lacticaseibacillus*, *Lactiplantibacillus*, *Levilactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus*). The 11 species identified were deposited to GenBank as follows: *Enterococcus casseliflavus* (Ac. No. MZ669935); *Enterococcus gallinarum* (Ac. No. MZ669936); *Lactobacillus delbrueckii* (Ac. No. MZ669937); *Lactococcus lactis* (Ac. No. MZ669938); *Lacticaseibacillus rhamnosus* (Ac. No. MZ669939-MZ669943); *Lactiplantibacillus plantarum* (Ac. No. MZ669944-MZ669945) and *Levilactobacillus brevis* (Ac. No. MZ669946); *Leuconostoc mesenteroides* (Ac. No. MZ669947-MZ669953); *Leuconostoc pseudomesenteroides* (Ac. No. MZ669954-MZ669956); *Pediococcus acidilactici* (Ac. No. MZ669957-MZ669958); *Steptococcus thermophilus* (Ac. No. MZ669959-MZ669961). All these species are generally associated to dairy environments such as raw milk [50], cheeses [35,46], animal rennets [19] and wooden vats [11], considering that *Lcb. rhamnosus*, *Lpb. plantarum* and *Lvb. brevis* were formerly *Lactobacillus rhamnosus*, *Lactobacillus plantarum* and *Lactobacillus brevis*, respectively [65]. In particular, *Lcb. rhamnosus* are typically associated to the ripening of PDO Provola

dei Nebrodi cheese and have been recently object of selection for non starter LAB addition due to their desirable technological and enzymatic activities [66]. Pediococci are also generally isolated from semi-ripened PDO Provola dei Nebrodi cheeses [67].

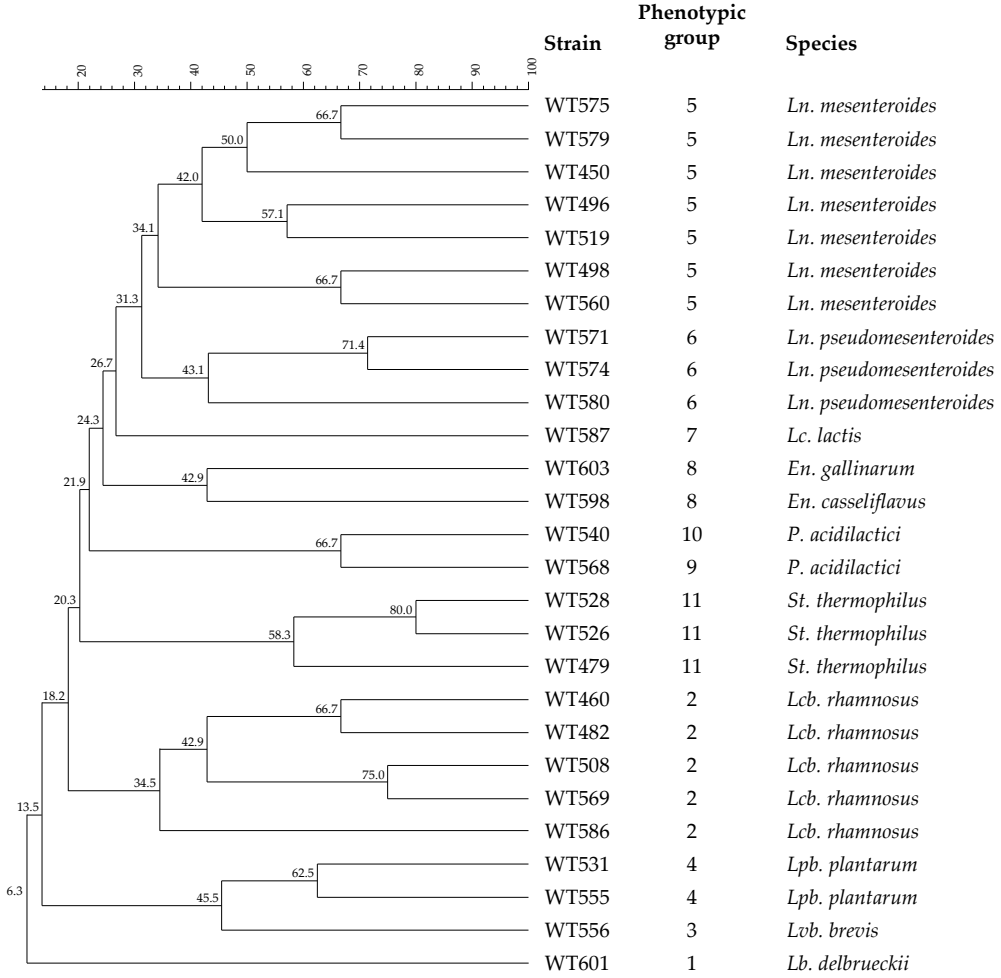

Figure 4. Dendogram obtained from combined RAPD-PCR patterns generated with three primers of the LAB strains identified. Abbreviations: *En., Enterococcus*; *Lb., Lactobacillus*; *Lcb., Lacticaseibacillus*; *Lc., Lactococcus*; *Lpb., Lactiplantibacillus*; *Ln., Leuconostoc*; *Lvb., Levilactobacillus*; *P., Pediococcus*; *St., Streptococcus*.

3.6. Species Distribution

The distribution of LAB species among the wooden tables used for the curd acidification of PDO Provola dei Nebrodi cheese is reported in Table 4.

Table 4. Distribution of LAB species among wooden tables.

LAB species	Wooden Tables			
	WTA	**WTB**	**WTC**	**WTD**
En. casseliflavus				■
En. gallinarum				■
Lb. delbrueckii				■
Lc. lactis				■
Lcb. rhamnosus	■	■	■	
Lpb. plantarum			■	
Lvb. brevis			■	
Ln. mesenteroides	■	■	■	■

Ln. pseudomesenteroides				■
P. acidilactici			■	
St. thermophilus	■	■		

Symbols: black square indicate the presence of a given species on wooden table biofilm. Abbreviations: WT, wooden table; A–D, dairy factory A–dairy factory D; *En.*, *Enterococcus*; *Lb.*, *Lactobacillus*; *Lc.*, *Lactococcus*; *Lcb.*, *Lacticaseibacillus*; *Lpb.*, *Lactiplantibacillus*; *Ln.*, *Leuconostoc*; *Lvb.*, *Levilactobacillus*; *P.*, *Pediococcus*; *St.*, *Streptococcus*.

Ln. mesenteroides were the only LAB species found in all four wooden table surfaces. *En. casseliflavus*, *En. gallinarum*, *Lb. delbruekii*, *Lc. lactis* and *Ln. pseudomesenteroide*s were found associated only to the WTD sample, as well as *Lpb. plantarum*, *Lvb. brevis* and *P. acidilactici* to the WTC sample. *St. thermophilus* was isolated only from the WTA and WTB samples. Additionally, in this case, the discrepancy between the culture-dependent and culture-independent survey might be imputable, as reported above, to the fact that some species were not detected by Illumina analysis because those species were part of the unassigned OTUs of total bacterial community or they were below 0.1% of abundance or their DNAs were rendered inaccessible by nucleases.

4. Conclusions

This work provided, for the first time, an in-depth microbiological characterization of the wooden tables, namely "mastredda", used for the acidification of the curd of PDO Provola dei Nebrodi cheese. The combined approach based on high throughput DNA analysis, enumeration, isolation and identification of LAB and evaluation of food safety and process hygiene criteria undoubtedly demonstrated the absence of risk factors associated to the wooden tables. The dominance of *Lactobacillus*, *Streptococcus* and *Lactococcus* proved the positive role of this traditional tool to transfer the typical LAB that favour the acidification process, which is of paramount importance for cheese productions carried out with raw milk without the addition of commercial starter cultures. On the whole, this study contributed to valorize a typical niche dairy product unrevealing the microbiota of the wooden tables in contact with cheese curd and provided further evidence on the suitability of wooden equipment in traditional cheese making.

Author Contributions: Conceptualization, G.L., R.G. and L.S.; methodology, R.G. and L.S.; software, E.F. and R.G.; validation, L.B., E.F., R.D.G., M.L.S. and R.G.; formal analysis, R.G. and L.S.; investigation, G.B., G.G., G.M., L.B., E.F. and R.D.G.; resources, M.T.; data curation, R.G.; writing—original draft preparation, R.G. and L.S.; writing—review and editing, R.G. and L.S.; project administration, M.T.; funding acquisition, M.T. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by AGER 2 Project, grant no. 2017-1144.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data included in this study are available upon request by contacting the corresponding author.

Conflicts of Interest: The authors declare that they have no conflict of interest.

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