AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology®

FOOD MICROBIOLOGY

Formation and Characterization of Early Bacterial Biofilms on Different Wood Typologies Applied in Dairy Production

Margherita Cruciata,[a] Raimondo Gaglio,[a,b] Maria Luisa Scatassa,[b] Giovanna Sala,[a] Cinzia Cardamone,[b] Marisa Palmeri,[b] Giancarlo Moschetti,[a] Tommaso La Mantia,[a] Luca Settanni[a]

[a]Department of Agricultural, Food and Forest Sciences, University of Palermo, Palermo, Italy
[b]Istituto Zooprofilattico Sperimentale della Sicilia Adelmo Mirri, Palermo, Italy

ABSTRACT The main hypothesis of this work was that Sicilian forestry resources are suitable for the production of equipment to be used in cheese making and indigenous milk lactic acid bacteria (LAB) are able to develop stable biofilms providing starter and nonstarter cultures necessary for curd fermentation and cheese ripening, respectively. Hence, the present work was carried out with deproteinized whey to evaluate LAB biofilm formation on different woods derived from tree species grown in Sicily. Microbiological and scanning electron microscopy analyses showed minimal differences in microbial levels and compositions for the neoformed biofilms. The specific investigation of *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli*, coagulase-positive staphylococci (CPS), and sulfite-reducing anaerobes did not generate any colony for all vats before and after bacterial adhesion. LAB populations dominated all vat surfaces. The highest levels (7.63 log CFU/cm^2) were registered for thermophilic cocci. Different colonies were characterized physiologically, biochemically, and genetically (at strain and species levels). Six species within the genera *Enterococcus*, *Lactobacillus*, *Lactococcus*, and *Streptococcus* were identified. The species most frequently present were *Lactobacillus fermentum* and *Lactococcus lactis*. LAB found on the surfaces of the wooden vats in this study showed interesting characteristics important for dairy manufacture. To thoroughly investigate the safety of the wooden vat, a test of artificial contamination on new Calabrian chestnut (control wood) vats was carried out. The results showed that LAB represent efficient barriers to the adhesion of the main dairy pathogens, probably due to their acidity and bacteriocin generation.

IMPORTANCE This study highlights the importance of using wooden vats for traditional cheese production and provides evidence for the valorization of the Sicilian forest wood resources via the production of dairy equipment.

KEYWORDS biofilm formation, lactic acid bacteria, technological screening, tree species, wooden vats

The Mediterranean area represents an important site of plant diversity (1). Sicily (south Italy) is a Central Mediterranean region characterized by a high concentration of autochthonous and allochthonous species with approximatively more than 300,000 ha covered by forestland (2). Forest management in Sicily dates back to the Roman Empire, and this activity includes wood collection for firewood, charcoal, and timber for local handicrafts (3). Forest wood is also used to produce equipment for food manufacture (4).

In Europe, wood as a food contact material is subject to regulation (EC) no. 1935/2004 of the European Parliament and of the Council of 27 October 2004 on materials and articles intended to come into contact with food (5). This regulation refers to different materials that may be subject to specific measures and have been harmo-

Received 22 September 2017 **Accepted** 22 November 2017

Accepted manuscript posted online 27 November 2017

Citation Cruciata M, Gaglio R, Scatassa ML, Sala G, Cardamone C, Palmeri M, Moschetti G, La Mantia T, Settanni L. 2018. Formation and characterization of early bacterial biofilms on different wood typologies applied in dairy production. Appl Environ Microbiol 84:e02107-17. https://doi.org/10.1128/AEM.02107-17.

Editor Johanna Björkroth, University of Helsinki

Address correspondence to Raimondo Gaglio, raimondo.gaglio@unipa.it.

nized and adopted at the European level, including plastics, epoxy derivatives, active and intelligent materials, regenerated cellulose, and ceramics, but not yet for wood. However, wooden equipment is still used to produce several traditional cheeses, especially in France and in Sicily, as a result of the commission regulation (EC) no. 2074/2005, which allows derogation from regulation (EC) no. 852/2004 for foods with traditional characteristics "as regards the type of materials of which the instruments and the equipment used specifically for the preparation, packaging, and wrapping of these products are made" (6).

The production of typical cheeses in Sicily has remained almost unchanged through centuries. These productions are carried out from raw milk coagulated and transformed in wooden vats without the addition of exogenous microorganisms (7). In the last 10 years, several works carried out by French and Italian groups (7–12) have demonstrated that the wooden vat represents a safe system to produce cheeses. In particular, all investigations conducted on the wooden vat biofilms evidenced the presence of desired dairy lactic acid bacteria (LAB) and the absence of pathogenic species such as *Listeria monocytogenes* and *Salmonella* spp. To transform milk into cheese, the presence of LAB is needed (13). For this purpose, the biofilms of the wooden vat surfaces and the raw materials (milk and animal rennet) represent the main sources of desirable dairy LAB involved in traditional cheese productions (7, 11, 14).

The bacterial biofilms are primarily formed on the surfaces of virgin wooden vats due to the colonization of LAB transferred by whey (9). A biofilm is an aggregate of microorganisms in which cells that are frequently embedded within a self-produced matrix of extracellular polymeric substances (EPS) adhere to each other and/or to a surface (15). Lortal et al. (16) proved that the biofilms investigated on the wooden vats used in cheese making represent efficient delivery systems for dairy LAB.

The technological characterization and genotypic identification of the LAB biofilms associated with the wooden vats used to produce different cheeses revealed the presence of several dairy LAB, including starter LAB (SLAB), responsible for the acidification of curd, and nonstarter LAB (NSLAB), implicated in the maturation (7).

In Sicily, the use of wooden vats is mandatory for the production of all Protected Designation of Origin (PDO) Sicilian cheeses, such as Ragusano, Pecorino Siciliano, Piacentinu Ennese, and Vastedda della valle del Belìce. Nowadays, the tree species most used for this purpose are Douglas fir and chestnut imported from other regions. Although little is known about the use of other tree species in cheese making, there is an ancient tradition of the use of wood in Sicily (3). Indeed, the use of wood from tree species grown in this region to produce wooden vats intended for cheese making would represent a valuable strategy to valorize the Sicilian forestry resources.

With this in mind, the aim of this study was to evaluate the neoformed biofilms on the surfaces of wooden vats produced from seven different Sicilian woods. The specific aims of the work were to determine the numbers of LAB present during the bacterial adhesion to virgin vats and the strains and species dominating the biofilms and their technological dairy traits *in vitro*.

RESULTS

Evaluation of the inhibitory activity of water-soluble extracts from wood and scanning electron microscopy of the wooden biofilms. None of the water-soluble extracts (WSE) obtained from the hot water treatment of the wooden vats showed inhibitory properties against the main protechnological and pathogenic bacteria relevant for dairy productions. This test was conducted only on the WSE collected after the first overnight treatment, since the subsequent treatments generated visibly clearer solutions with less components extracted from the woods over time. Thus, all wood types displayed no drawbacks during the lactic acid fermentation due to their inability to inhibit all 13 starter and nonstarter LAB associated with cheese production and ripening. The four pathogenic species, including two that were Gram negative (*Escherichia coli* ATCC 35150 and *Salmonella enteritidis* ATCC 13076) and two that were Gram positive (*L. monocytogenes* ATCC 7644 and *Staphylococcus aureus* ATCC 33862), were

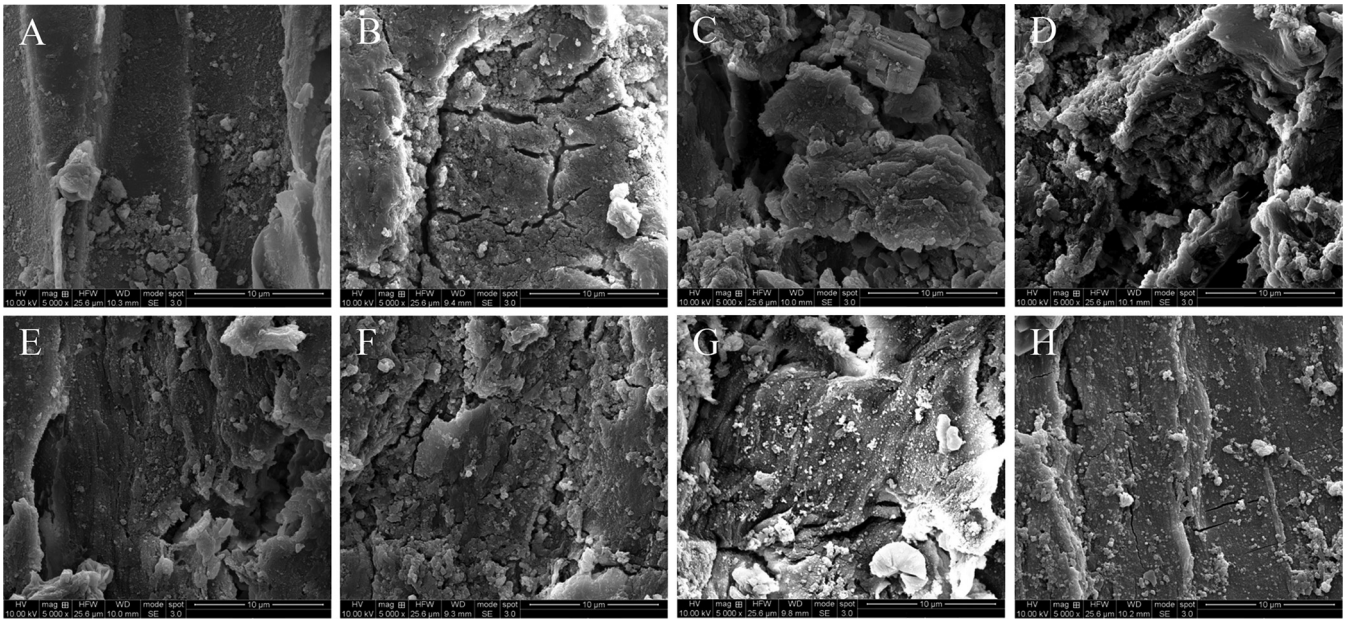

FIG 1 Scanning electron microscopy observations of wooden splinters after 30 days of hot water treatment of vats made from Calabrian chestnut (A), Sicilian chestnut (B), cedar (C), cherry (D), ash (E), walnut (F), black pine (G), and poplar (H) woods.

not negatively affected by the WSE, indicating that the seven types of wood can be susceptible to colonization by undesirable organisms.

The results of scanning electron microscopy (SEM) carried out on wood splinters collected from the eight vats after 30 days of hot water treatment and after 7 days of contact with whey are shown in Fig. 1 and 2, respectively. The treatment with water prevented microbial development on all of the vats analyzed (Fig. 1A to H). On the contrary, the treatment with hot whey facilitated the attachment of different bacteria, which generated a visible exopolysaccharide matrix typical of biofilm structures on the

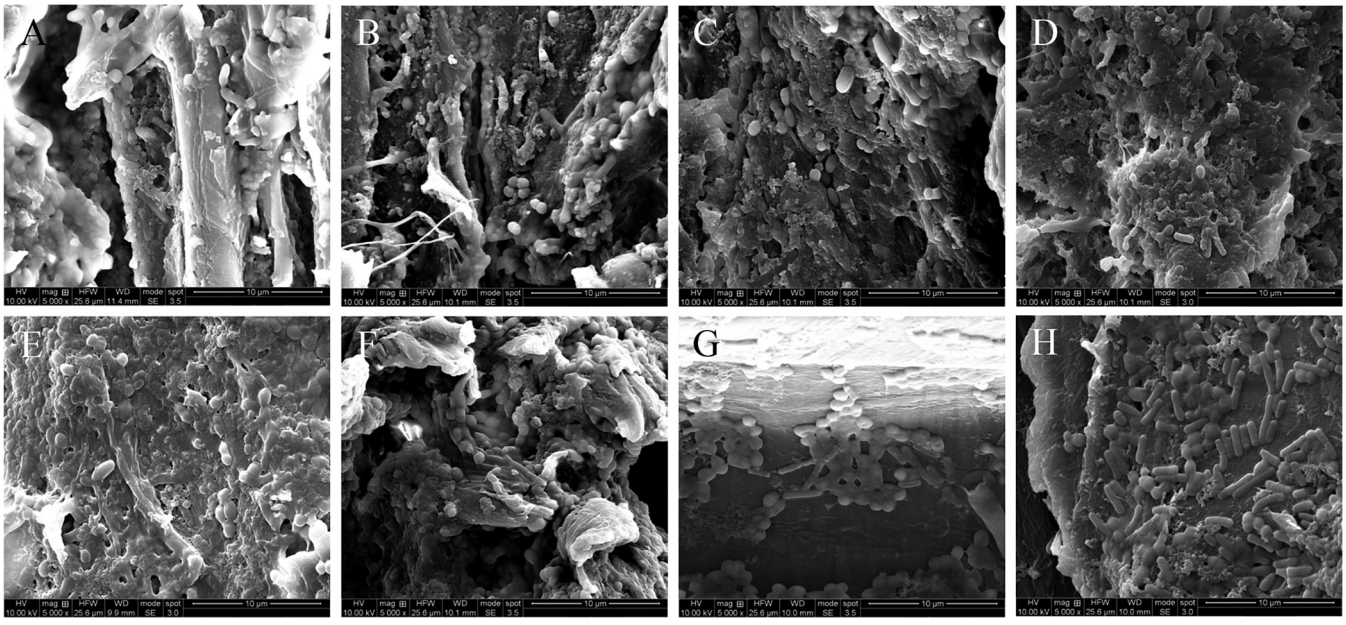

FIG 2 Scanning electron microscopy observations of wooden splinters after activation with whey in vats made from Calabrian chestnut (A), Sicilian chestnut (B), cedar (C), cherry (D), ash (E), walnut (F), black pine (G), and poplar (H) woods.

internal surfaces of all vats (Fig. 2A to H). In particular, the microscopic inspection showed the presence of both rod and coccus bacteria.

Microbial levels. The viable counts registered for the 14 microbial groups harbored on the wooden vat surfaces after tannin removal (30 days of hot water treatment) and after adhesion of bacteria transferred by whey (7 days of whey treatment) are reported in Table 1. No colonies of *Salmonella* spp., *L. monocytogenes*, *E. coli*, coagulase-positive staphylococci (CPS), or sulfite-reducing anaerobes (SRA) were detected in any of the vats before or after whey treatment.

As shown by Table 1, before whey treatment, no mesophilic microorganisms (TMM), total psychrotrophic microorganisms (TPM), pseudomonads, enterococci, LAB, or members of the family *Enterobacteriaceae* developed on the surfaces of the wooden vats. An opposite trend was registered after the adhesion of bacteria transferred by whey. TMM were in the range of 3.79 log to 6.18 log CFU/cm^2. No bacterium able to grow at 7°C was found. *Pseudomonas* spp. were found only in the vats of trial W2 that was also positive for the presence of enterobacteria. The last group was detected also in trials W3, W4, W6, and W8, while enterococci were present only in W3 and W4. All vats displayed high levels of rod, coccus, mesophilic, and thermophilic LAB. The levels of LAB cocci and LAB rods were comparable for almost all vats with the exception of W1 for both thermal groups and W8 for the thermophilic group. The highest level of LAB (7.63 log CFU/cm^2) was registered for thermophilic LAB cocci.

Composition of bacterial populations of wooden vat biofilms. Four hundred eleven colonies of presumptive LAB were collected from the eight wooden vat surfaces in the first replicate trials. After purification and microscopic inspection, 237 rods and 174 cocci were found. After Gram and catalase tests, 213 rods and 156 cocci were still considered presumptive LAB cultures, being Gram positive and catalase negative. The combination of the phenotypic characteristics determined the separation of the 369 cultures into six groups, two for rods and four for cocci (Table 2). The largest groups, including almost 33% and 31% of the total isolates, were groups 1 and 3, respectively. Rods included obligately heterofermentative cultures (group 1) and obligately homofermentative bacteria (group 2). Furthermore, the isolates of the groups 1, 2, 5, and 6 showed the ability to grow at 45°C but not at 15°C and were classified as thermophilic LAB.

One hundred twenty cultures, representative of the different wooden vat surfaces analyzed, were selected from each phenotypic group and subjected to randomly amplified polymorphic DNA (RAPD) analysis. This genotypic differentiation revealed the presence of 10 distinct strains (data not shown). The strains were identified by 16S rRNA gene sequencing, and the sequence comparison within two distinct databases identified six species: *Enterococcus faecium* ($n = 3$), *Lactobacillus delbrueckii* ($n = 1$), *Lactobacillus fermentum* ($n = 2$), *Lactococcus lactis* ($n = 1$), *Streptococcus gallolyticus* subsp. *macedonicus* ($n = 2$), and *Streptococcus parauberis* ($n = 1$). *Lactobacillus fermentum* was isolated from all the wooden vat surfaces of this study, *L. lactis* was not found in W7, while the other species were found associated with one or two vats. In particular, *E. faecium* was isolated from W2 and W3, *L. delbrueckii* from W6 and W8, *S. gallolyticus* subsp. *macedonicus* from W1 and W3, and *S. parauberis* only from W4.

Technological traits of LAB. The results of the technological characterization of the 10 strains are reported in Table 3. The acidification kinetics showed that the fastest decrease in the milk pH was observed in the presence of *L. delbrueckii* TV199, *L. fermentum* TV187, and *L. lactis* TV70, which displayed values in the range of 3.76 to 4.05, after 24 h of fermentation. *L. lactis* was the best acidifier, since the milk inoculated with this strain reached a pH of 5.44 after 8 h. Except for *L. fermentum* TV173, all the strains produced a drop in pH below 6.00 only after 24 h.

Strains *L. lactis* TV147 and *S. gallolyticus* subsp. *macedonicus* TV69 were the only ones showing a relevant decrease in the optical density at 600 nm (OD_{600}) after 24 h of incubation, reaching values of 0.564 and 0.585, respectively. Diacetyl production was scored positive only for *E. faecium* TV 92 and *S. gallolyticus* subsp. *macedonicus* TV69.

TABLE 1 Microbial loads of wooden vat surfaces after 30 days of hot water treatment and after the activation

Sample	Bacterial counts (log CFU/cm²)[a]								
	TMM[b]	TPC[c]	*Pseudomonas*	*Enterobacteriaceae*	Enterococci	Rod LAB MRS 30°C	Rod LAB WBAM 44°C	Coccus LAB M17 30°C	Coccus LAB M17 44°C
Wooden vat after hot water treatment									
W1BA	<1 A	<1 A	<1 A	0	<1 A	0	0	0	0
W2BA	<1 A	<1 A	<1 A	0	<1 A	0	0	0	0
W3BA	<1 A	<1 A	<1 A	0	<1 A	0	0	0	0
W4BA	<1 A	<1 A	<1 A	0	<1 A	0	0	0	0
W5BA	<1 A	<1 A	<1 A	0	<1 A	0	0	0	0
W6BA	<1 A	<1 A	<1 A	0	<1 A	0	0	0	0
W7BA	<1 A	<1 A	<1 A	0	<1 A	0	0	0	0
W8BA	<1 A	<1 A	<1 A	0	<1 A	0	0	0	0
Wooden vat after activation									
W1AA	4.95 ± 0.31 B	<1 A	<1 B	<1 D	<1 C	5.28 ± 0.43 B	4.46 ± 0.29 D	5.79 ± 0.41 BC	4.94 ± 0.36 B
W2AA	3.95 ± 0.27 C	<1 A	1.38 ± 0.24 A	1.77 ± 0.12 B	<1 C	5.22 ± 0.37 B	5.24 ± 0.49 CD	5.37 ± 0.35 C	5.91 ± 0.45 B
W3AA	6.08 ± 0.33 A	<1 A	<1 B	2.95 ± 0.25 A	2.62 ± 0.18 A	7.55 ± 0.39 A	7.48 ± 0.31 A	7.37 ± 0.27 A	7.39 ± 0.30 A
W4AA	4.85 ± 0.24 B	<1 A	<1 B	1.85 ± 0.16 B	2.18 ± 0.21 B	6.66 ± 0.47 A	6.23 ± 0.21 BC	5.89 ± 0.24 BC	5.64 ± 0.45 B
W5AA	5.88 ± 0.21 A	<1 A	<1 B	<1 D	<1 C	7.46 ± 0.32 A	7.41 ± 0.40 A	7.26 ± 0.26 A	7.36 ± 0.30 A
W6AA	5.91 ± 0.25 A	<1 A	<1 B	0.85 ± 0.08 C	<1 C	7.24 ± 0.42 A	7.18 ± 0.41 AB	7.16 ± 0.37 A	7.17 ± 0.49 A
W7AA	3.79 ± 0.18 C	<1 A	<1 B	<1 D	<1 C	5.08 ± 0.41 B	5.04 ± 0.45 D	4.99 ± 0.45 C	5.08 ± 0.47 B
W8AA	6.18 ± 0.24 A	<1 A	<1 B	1.88 ± 0.21 B	<1 C	7.08 ± 0.45 A	7.13 ± 0.47 AB	6.69 ± 0.35 AB	7.63 ± 0.22 A
P value	≤0.001		≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001

[a]Results are means ± SDs from four plate counts (carried out in duplicates for two independent productions). Data within a column followed by the same uppercase letter are not significantly different according to Tukey's tests.

[b]TMM, total mesophilic microorganisms.

[c]TPC, total psychrotrophic count.

TABLE 2 Phenotypic grouping of the LAB isolated from the wooden vat surfaces after activation with whey

Characteristic	Cluster					
	1 (*n* = 121)	2 (*n* = 31)	3 (*n* = 114)	4 (*n* = 67)	5 (*n* = 29)	6 (*n* = 7)
Morphology	R[a]	R	C[b]	C	C	C
Cell disposition	sc[c]	sc	sc	sc	lc[d]	lc
Growth at						
15°C	−	−	+	+	−	−
45°C	+	+	−	+	+	+
pH 9.6	ND[e]	ND	+	+	−	−
6.5% NaCl	ND	ND	−	+	−	−
Resistance to 60°C	+	+	+	+	+	+
Hydrolysis of						
Arginine	+	−	+	+	−	+
Esculin	+	−	+	+	−	+
Acid production from						
Arabinose	+	−	−	+	−	+
Ribose	+	−	+	+	+	+
Xylose	+	−	−	+	−	+
Fructose	+	+	+	+	+	+
Galactose	+	+	+	+	+	+
Lactose	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+
Glycerol	+	+	+	+	+	−
CO_2 from glucose	+	−	−	−	−	−

[a]R, rod.
[b]C, coccus.
[c]sc, short chain.
[d]lc, long chain.
[e]ND, not determined.

The antibacterial activity was observed only for *L. fermentum* TV173 and TV187, which were able to inhibit the growth of *Lactobacillus sakei* LMG2313, *Listeria innocua* 4202, and *L. monocytogenes* ATCC 19114. The treatment with proteolytic enzymes revealed a loss of the inhibitory power.

Artificial contamination test. The surfaces of wooden vats and milk samples collected during the simulation of cheese productions in the presence of dairy pathogenic bacteria were also analyzed (Table 4). As expected, wooden vat surfaces sampled after milk coagulation during both the control production (CP) and the experimental production (EP) hosted high numbers of LAB. Their levels were above 6 log CFU/ml. The bulk milk showed a mesophilic and thermophilic coccus LAB count of approximately 4 log CFU/ml, while the mesophilic and thermophilic rod LAB count was approximately 3 log CFU/ml. The levels of LAB after contact with the wooden containers showed an increase of 1 log CFU/ml. Pathogenic bacteria were found in milk used in EP at levels almost the same as those for inoculation, while in CP, only *S. aureus* was detected. The specific investigation of *E. coli*, *L. monocytogenes*, *S. enteritidis*, and CPS did not reveal any colony in either of the wooden vats.

DISCUSSION

The use of wooden vats in Sicily is mandatory for the production of all four regional PDO cheeses: Ragusano, Pecorino Siciliano, Piacentinu Ennese, and Vastedda della valle del Belìce. Nowadays, the tree species most used for this purpose are Douglas fir and Calabrian chestnut. Regarding these tree species, chestnut wood shows excellent technological features (17, 18), but the cultivated areas have decreased notably in the last years (19). The use of wood from black pine and cedar would provide an economical significance for the reforestation. Black poplar, although widespread along the hedges in the past, was almost completely destroyed by fire (20). Thus, the revival of

TABLE 3 Technological characteristics of LAB isolated from wooden vat surfaces

Species	Strain	pH/autolysis at time (h)[a]								Diacetyl production	Bacteriocin-like inhibitory activity of indicator strains (inhibition zone size [mm])[b]		
		0	2	4	6	8	24	48	72		19114[c]	4202[d]	2313[e]
E. faecium	TV92	6.74/0.995	6.74/0.972	6.67/0.887	6.62/0.880	6.55/0.874	5.09/0.863	4.49/0.846	4.37/0.819	+			
	TV99	6.71/0.999	6.68/0.987	6.55/0.975	6.47/0.968	6.41/0.966	5.31/0.933	4.38/0.861	4.36/0.786	−			
	TV121	6.75/0.999	6.66/0.986	6.56/0.983	6.44/0.983	6.40/0.977	5.83/0.977	5.12/0.864	4.82/0.864	−			
L. delbrueckii	TV199	6.76/0.991	6.74/0.991	6.72/0.958	6.69/0.958	6.68/0.945	4.45/0.945	4.06/0.945	3.97/0.910	−			
L. fermentum	TV173	6.75/0.999	6.64/0.943	6.57/0.903	6.57/0.880	6.53/0.868	6.19/0.786	5.64/0.494	4.70/0.451	−	1.5 ± 0.12	1.6 ± 0.17	1.6 ± 0.00
	TV187	6.70/0.999	6.60/0.943	6.55/0.865	6.52/0.86	6.50/0.836	5.95/0.778	4.21/0.693	3.76/0.621	−	1.5 ± 0.00	1.6 ± 0.10	1.7 ± 0.17
L. lactis	TV70	6.76/0.998	6.68/0.983	6.44/0.873	5.94/0.855	5.44/0.801	4.34/0.567	4.31/0.409	4.05/0.396	−			
S. gallolyticus subsp. *macedonicus*	TV69	6.74/0.996	6.66/0.949	6.59/0.870	6.46/0.861	6.41/0.849	5.56/0.585	4.90/0.407	4.25/0.402	+			
	TV103	6.71/0.999	6.64/0.980	6.53/0.980	6.46/0.973	6.39/0.973	5.73/0.973	4.99/0.855	4.89/0.855	−			
S. parauberis	TV125	6.74/0.998	6.66/0.758	6.59/0.758	6.50/0.756	6.44/0.756	5.83/0.756	5.01/0.643	4.79/0.643	−			

[a]Results are means from two independent experiments.
[b]Results are means ± SDs from three independent experiments.
[c]*Listeria monocytogenes* ATCC 19114.
[d]*Listeria innocua* 4202.
[e]*Lactobacillus sakei* 2313.

TABLE 4 Microbial loads of pathogenic bacteria during the artificial contamination test

Pathogenic bacteria	Microbial load (log CFU/ml)							
	First production				Second production			
	Milk CP[a]	Milk EP[b]	Vat CP	Vat EP	Milk CP	Milk EP	Vat CP	Vat EP
E. coli	<1	2.96 ± 0.26	<1	<1	<1	3.06 ± 0.20	<1	<1
L. monocytogenes	<1	1.59 ± 0.19	<1	<1	<1	1.49 ± 0.15	<1	<1
S. enteritidis	<1	1.65 ± 0.21	<1	<1	<1	1.45 ± 0.24	<1	<1
CPS	1.82 ± 0.21	3.03 ± 0.22	<1	<1	1.75 ± 0.19	3.09 ± 0.23	<1	<1

[a]CP, control production.
[b]EP, experimental production.

this crop could serve to restore its habitats. The extensive distribution of ash in Sicily could enable a renewed use of this species.

In this study, we evaluated the early stage of the formation of lactic acid bacterial biofilms on eight different wooden vat typologies. SEM inspection indicated the absence of bacterial cells on the surfaces of the virgin wooden vats and the presence of bacterial aggregates in EPS matrices after whey treatment. The aims were to expand the characterization of the LAB populations at the strain level and to investigate their technological traits in order to evaluate their potential in cheese manufacture and ripening. The eight wooden vats applied for cheese production were made from seven Sicilian tree species that have never been used for dairy purposes and one control made with Calabrian chestnut.

The bacteria on the internal surfaces of the wooden vats were analyzed after 30 days of hot water treatment or 7 days of contact with whey by using the brushing method commonly applied for the microbiological investigation of wooden surfaces (21). No sample before or after activation harbored pathogenic bacteria such as *Salmonella* spp., *L. monocytogenes*, *E. coli*, and CPS. The absence of these bacteria after activation was mainly due to the acidic conditions resulting from LAB that ferment lactose from whey and inhibit the adhesion and survival of several microorganisms (11, 12). Plate counts confirmed the SEM analysis, since viable bacterial cells were not detected before whey treatment. The comparison between LAB and TMM levels unequivocally indicated that all vat biofilms were dominated by LAB which showed levels above 4 log cycles per square centimeter for all samples. These findings confirmed those from previous inspections on aged vats (7, 8, 10–12).

Enterococci were detected only on the wooden vat surfaces of cedar and cherry at levels lower than those registered for the other LAB. Previously, enterococci were found on the surfaces of the wooden vats made from Douglas fir or Calabrian chestnut used for traditional cheese making in western Sicily (7, 9, 12, 22). These works demonstrated the influence of the equipment during the production of traditional cheeses and highlighted the importance of this bacterial group for conferring cheese typicality.

The presumptive LAB were isolated from the eight wooden vat surfaces, and after the investigation of several phenotypic (morphological, physiological, and biochemical) characteristics, they were divided into 6 groups. A representative percentage of the isolates of each group was examined by RAPD-PCR that is commonly used, alone or in combination with other techniques, to discriminate LAB strains associated with food matrices (12). With this approach, 10 strains were successfully recognized, indicating that RAPD is a valid technique for differentiating bacteria of food interest associated with the equipment. A total of six species belonging to four LAB genera (*Enterococcus*, *Lactobacillus*, *Lactococcus*, and *Streptococcus*) commonly found in raw milk, traditional Sicilian cheeses, and wooden vats analyzed in Italy and France (7–10, 22–24) were identified.

With the objective to investigate the technological potential of the LAB associated with the biofilms of the wooden vats, the 10 strains were subjected to a general characterization of dairy traits. With this in mind, the first parameters evaluated were the acidification kinetics, to determine the rapid pH drop necessary to inhibit the

TABLE 5 Typology and origin of the woods used for wooden vat production

Trial	Wood type	Species	Origin
W1	Calabrian chestnut	*Castanea sativa* Miller	Cosenza, CS, Calabria
W2	Sicilian chestnut	*Castanea sativa* Miller	Petralia Sottana, PA, Sicily
W3	Cedar	*Cedrus libani* A. Rich.	Polizzi Generosa, PA, Sicily
W4	Cherry	*Prunus avium* L.	Castelbuono, PA, Sicily
W5	Ash	*Fraxinus ornus* L.	Castelbuono, PA, Sicily
W6	Walnut	*Juglans regia* L.	Castelbuono, PA, Sicily
W7	Black pine	*Pinus nigra* J.F. Arnold	Polizzi Generosa, PA, Sicily
W8	Poplar	*Populus nigra* L.	Castelbuono, PA, Sicily

undesired microbiota present in raw milk (25), and the autolysis capacity, which correlates positively with the final flavor development of several cheeses due to the release of cell peptidases (26). In general, optimal SLAB are characterized by a fast and appropriate acidification and a rapid autolysis, whereas optimal NSLAB show opposite performances (27, 28). The fastest acidifiers included the strains *L. delbrueckii* TV199, *L. fermentum* TV187, and *L. lactis* TV147, while enterococci showed a slow-acidifying aptitude and a slow autolysis.

Diacetyl is a flavor compound generated from citrate important in cheese production (29), but only two LAB studied in this work showed this capacity. All LAB were also investigated for their antimicrobial activity; two strains produced antibacterial compounds which were recognized as proteins and, for this reason, are referred to as bacteriocin-like inhibitory substances (30). The production of bacteriocins is of great technological importance in cheese manufacture when LAB act as starter cultures and/or cocultures, as they can inhibit pathogenic or spoilage bacteria (31). The presence of strains showing characteristics typical of SLAB and NSLAB indicates that the wooden vat is responsible for the colonization of the bacteria and is necessary for the production and ripening of cheese.

In eight trials, no pathogenic bacteria commonly connected with cheese manufacture were detected. Thus, an artificial contamination was performed (only on chestnut vats) to test the efficacy of the wooden vat LAB to inhibit the adhesion of the undesired bacteria of dairy interest. After the contamination test, the foodborne pathogens were not detected in the vat biofilms. Their absence could be due to the acidic conditions generated by the development of LAB in whey. Furthermore, an additional hypothesis to explain the absence of foodborne pathogens in the wooden vat biofilms involves the presence of bacteriocin producers (11).

In conclusion, although the duration of this study was not sufficient to exclude the possibility that changes in the surface roughness and/or biofilm composition evolution compromise the safety of the wooden vat system, the repeated daily treatment at 80°C with deproteinized whey represents a hurdle to the survival of pathogenic bacteria, as confirmed by several works performed on aged wooden vats. The levels of the different LAB groups on a given wood type were almost comparable. However, the wood type influenced the levels of LAB; high levels were registered on the surfaces of cedar, ash, walnut, and poplar vats. Within this bacterial group, enterococci were only detected on cedar and cherry woods. The LAB strains isolated from the eight biofilms analyzed were identified as species commonly associated with the traditional dairy products. The technological characterization of the LAB found at high numbers on the surfaces of the wooden vats of this study showed interesting dairy properties. These observations strengthen the importance of using the wooden vats for traditional cheese production and provide evidence to valorize Sicilian forest wood resources via the production of traditional dairy equipment. For this purpose, further studies are being prepared to better investigate the influence of the different wooden vats on the characteristics of the final cheeses.

MATERIALS AND METHODS

Wood types and vat production. The wooden vats applied in cheese production were made from seven tree species (Table 5). Chestnut (*Castanea sativa* Miller) wood was collected from Petralia Sottana,

Italy, one of the areas where this species was largely cultivated in the past (19). Chestnut wood from the Calabria region was used as a control, since it represents the most common wood species for wooden dairy equipment used in western Sicily (3). Cedar (*Cedrus libani* A. Rich.) is an allochthonous species widely diffused in Italy for reforestation (32) but is no longer cultivated for its wood. Black pine (*Pinus nigra* J.F. Arnold) is present in an autochthonous population on Mount Etna and is widely used in Sicily for afforestation purposes. Ash (*Fraxinus ornus* L.) represents the forest tree species most found in Sicily, used in the past for multiple purposes by farmers and shepherds (3). Black poplar (*Populus nigra* L.) is spread throughout the areas around streams but at present, is barely cultivated in a restricted area (33). In this work, some tree species grown for fruit production were also included, in particular, walnut (*Juglans regia* L.), which is the most used species in Sicily for furniture production, and cherry (*Prunus avium* L.), which has been used for this purpose in the past (3).

Eight trials were performed, seven of which (W2 to W8) were carried out in the vats produced from the wood of trees grown in Sicily that have never been used for dairy purposes before, and one trial (W1 [control]) was carried out in Calabrian chestnut vats. A total of 16 wooden vats (two replicates for each wood type) with 15-liter volumes were made by a local artisanal producer. A treatment with hot water (80°C) was applied for 30 consecutive days to remove the tannins from the wood. After it was added, the hot water was left cooling at room temperature and remained in contact with the wood for approximately 24 h. The last washing step was followed by a vigorous brushing of coarse salt on the internal surfaces of the vats.

Evaluation of the dairy suitability of the different wood types. To verify the absence of drawbacks for using different wood types in cheese making, the wood components extracted during the water treatment performed to remove tannins were tested for their potential inhibitory activity on the main LAB species and main dairy pathogens: *Enterococcus durans* DSM 20633^T, *E. faecium* DSM 20477^T, *Lactobacillus buchneri* LMG 6852^T, *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842^T, *Lactobacillus delbrueckii* subsp. *lactis* ATCC 12315^T, *Lactobacillus casei* LMG 6904^T, *Lactobacillus paracasei* subsp. *tolerans* LMG 9191^T, *Lactobacillus rhamnosus* LMG 6400^T, *Lactococcus lactis* subsp. *cremoris* DSM 20069^T, *Lactococcus lactis* subsp. *lactis* DSM 20481^T, *Leuconostoc mesenteroides* DSM 20343^T, *Pediococcus acidilactici* LMG 11384^T, *Streptococcus thermophilus* DSM 20617^T, *Escherichia coli* ATCC 35150, *L. monocytogenes* ATCC 7644, *Salmonella enteritidis* ATCC 13076, and *S. aureus* ATCC 33862. The aqueous solutions (1.2 liters) obtained after the first overnight contact between the hot water and each wooden vat were collected in disposable aluminum trays and frozen at −80°C for 48 h. The water-soluble extracts were then freeze-dried using a Scanvac Coolsafe 55-9 (Denmark) apparatus. Each WSE was rehydrated with distilled water to a final concentration of 500 mg/ml, corresponding to their complete dissolution. The inhibitory activity of WSEs was tested by applying the paper disc diffusion method as previously reported (34). Briefly, an agar base support (2% [wt/vol] water agar) was overlaid with 7 ml of the optimal soft agar (0.7% [wt/vol]) medium, as indicated by the respective culture collection for each strain, previously inoculated with approximately 10^7 CFU/ml of a given test organism. Sterile filter paper discs (Whatman no. 1) of a 6-mm diameter were placed on the surface of the double agar layer and soaked with 10 μl of each WSE. Sterile water and streptomycin (10% [wt/vol]) were used as negative and positive controls, respectively. Plates were incubated at the optimal growth temperatures indicated by the culture collection for 24 h, and the inhibitory activity was evaluated as positive if a definite clear area was detected around the paper discs.

Biofilm formation. The biofilms were formed on the wooden surfaces with hot (approximately 80°C) deproteinized whey, which is the residual whey from ricotta cheese production, left in contact with the vats for 24 h. The treatment with whey was repeated for seven consecutive days in an artisanal dairy farm (Ovini e Natura Società Agricola di Firpo F. & C. s.a.s., Santa Margherita di Belìce, Italy) selected from a previous survey (35) for its high hygienic standards.

The development of microbial populations on the internal surfaces of the wooden vats was analyzed by SEM as previously reported (9). Parallelepiped wood splinters (approximately 40 mm by 20 mm by 2 mm) were aseptically collected from each vat with a stainless steel scalpel on the 30th day of hot water treatment (before bacterial adhesion) and after the 7 days of contact with whey (after bacterial adhesion). The samples were analyzed with an FEI Quanta 200F microscope (FEI, Holland) at ×5,000 magnification. SEM analyses were conducted at the Department of Industrial and Digital Innovation, University of Palermo, Italy.

Viable cell counts. The culture-dependent analyses of the biofilms of the wooden vats were performed on the surface samples (100 cm^2) collected at the same time as the wood splinters. The biofilms were collected by the brushing recovery method as previously described (8) using sterile plastic squares (Biogenetics s.r.l., Padua, Italy) positioned halfway up the sides and on the bottoms of the vats. The cell suspensions of wooden vat surface samples were subjected to decimal serial dilutions in Ringer's solution (Sigma-Aldrich, Milan, Italy). Several microbial groups were investigated: TMM on plate count agar (PCA) supplemented with 1 g/liter skimmed milk (SkM) incubated aerobically at 30°C for 72 h; TPM as described for TMM, but the incubation occurred at 7°C for 7 days; mesophilic and thermophilic LAB cocci on M17 agar incubated anaerobically at 30°C and 44°C, respectively, for 48 h; mesophilic rod LAB on de Man-Rogosa-Sharpe (MRS) agar, acidified to pH 5.4 with lactic acid (5 M) and incubated anaerobically at 30°C for 48 h; thermophilic rod LAB on whey-based agar medium (WBAM) as reported by Settanni et al. (12); enterococci on kanamycin esculin azide (KAA) agar, incubated aerobically at 37°C for 24 h; members of the *Enterobacteriaceae* family on violet red bile glucose agar (VRGBA), incubated aerobically at 37°C for 24 h; coagulase-positive staphylococci (CPS) on Baird-Parker (BP) agar supplemented with rabbit plasma fibrinogen (RPF), incubated aerobically at 37°C for 48 h; pseudomonads on *Pseudomonas* agar base (PAB) supplemented with cephaloridine sodium fusidate cetrimide (CFC),

incubated aerobically at 25°C for 48 h; *E. coli* on tryptone bile glucuronide (TBG) agar, incubated aerobically for 24 h at 44°C; *E. coli* O157 by the method described in AFNOR BIO 12/25-05/09 (36); enzyme-linked fluorescence assays (ELFAs) performed with the automated system VIDAS (bioMérieux, Marcy l'Etoile, France) were used for *Salmonella* spp. with the screening method described in AFNOR BIO 12/32-10/11 (37) and for *L. monocytogenes* with the method described in AFNOR BIO 12/11-03/04 (38), while for sulfite-reducing anaerobes (SRA), the ISO 15213:2003 technique was followed (39). Microbiological counts were carried out in triplicates for all samples at each collection time. Anaerobiosis occurred in hermetically sealed jars with the AnaeroGen AN25 system (Oxoid, Milan, Italy). All media were purchased from Oxoid.

Isolation, phenotypic grouping, and genetic identification of LAB. The presumptive colonies of LAB were collected after growth on all agar media used for their plate counts (M17, MRS, and WBAM). At least five colonies per each morphology detected were isolated from the agar plates and purified to homogeneity after several subculturing steps on the same medium used for the viable cell count determination. After microscopic inspection, the pure cultures were subjected to the KOH method (40) to determine Gram type and to the catalase test (5% [wt/vol] H_2O_2) to exclude bacteria that produce energy from respiration. Only Gram-positive and catalase-negative cultures were considered putative LAB and were stored in glycerol stocks at −80°C until further investigations.

The presumptive LAB isolates were grouped as previously described (23) on the basis of cell morphology, cell disposition, growth at 15 and 45°C, heat resistance (60°C for 30 min), NH_3 production from arginine, esculin hydrolysis, acid production from carbohydrates, and CO_2 production from glucose. The coccus-shaped isolates were further grouped according to their growth at pH 9.6 and in the presence of 6.5 g/liter NaCl to separate enterococci able to grow under these conditions from other LAB.

All phenotypic groups were subjected to the genetic characterization performed initially to differentiate the isolates at strain level and then to identify the LAB species. The genetic investigation was carried out on DNA extracted from the cultures grown overnight in the optimal conditions in broth media (M17, MRS, and WBAM) using the InstaGene Matrix kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Crude cell extracts were used as the templates for the PCRs.

The differentiation of the LAB strains was performed by RAPD-PCR with individual applications of the primers M13 (41), AB111, and AB106 (42). RAPD-PCR profiles were analyzed with the pattern analysis software package GelCompar II version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium), and the isolates with different profiles were considered different strains.

All LAB with different RAPD-PCR profiles were identified by 16S rRNA gene sequencing. PCRs were performed as previously described (43), and the resulting DNA amplicons were sequenced at the Istituto Zooprofilattico Sperimentale della Sicilia A. Mirri (Palermo, Italy). DNA sequences were determined by an ABI PRISM 3500 genetic analyzer (Applied Biosystems, Carlsbad, CA, USA). The identities of the sequences were determined by a blastn search against the NCBI nonredundant sequence database and by comparison with the sequences of the sole type strains within the EZTaxon database (https://www.ezbiocloud.net/taxonomy).

Dairy properties of wooden vat LAB. The LAB isolated from the wooden vat biofilms were tested for the main technological traits useful in cheese production: acidification, diacetyl formation, autolytic kinetics and generation of antimicrobial compounds.

The acidifying capacity of each LAB was assayed in 100 ml of full fat ultrahigh temperature (UHT) milk inoculated with a 1% (vol/vol) cell suspension and incubated at the optimal growth temperature. The pH was measured in aliquots of 4 ml, aseptically collected from the flasks, at 2-h intervals for the first 8 h, and then at 24, 48, and 72 h after inoculation.

Diacetyl production was determined by inoculating each strain in UHT milk as described above. After 24 h of incubation, aliquots of 1 ml were added to 0.5 ml of α-naphthol (1% [wt/vol]) and KOH (16% [wt/vol]) and kept at 30°C for 10 min. Diacetyl generation was indicated by the formation of a red ring at the top of the tube (44).

Autolysis of whole cells was determined in buffer solution (0.5 M potassium phosphate, pH 6.5) following the method of Mora et al. (45) using a 6400 spectrophotometer (Jenway Ltd., Felsted Dunmow, UK) at 600 nm wavelength. Optical density (OD) was measured at 2-h intervals for the first 8 h and then at 24, 48, and 72 h after inoculation.

The antimicrobial activity of each LAB was determined against strains that are highly sensitive to bacteriocins, such as *L. sakei* LMG2313, *L. innocua* 4202, and *L. monocytogenes* ATCC 19114 (30). The inhibitory activity was first evaluated with the agar-spot deferred method, and only the strains displaying a clear inhibition of the indicator bacteria were further analyzed by the well diffusion assay (WDA) (46) as modified by Settanni et al. (47). All tests were carried out in triplicates. The supernatants showing inhibitory properties were treated with proteinase K (12.5 U/mg), protease B (45 U/mg), and trypsin (10.6 U/mg) diluted to 1 mg/ml in phosphate buffer (pH 7.0). After incubating at 37°C for 2 h, the remaining activity was measured by a second WDA (47). All enzymes were purchased from Sigma-Aldrich (St. Louis, MO).

Wooden vat contamination. To thoroughly investigate the safety of the wooden vat system in dairy production, tests of artificial contamination were carried out. Four additional new Calabrian chestnut wooden vats were activated with hot deproteinized whey as reported above. All vats were filled with 12 liters of milk provided by the same farm where the whey treatment was carried out. After 15 min under manual agitation, which represents the time that commonly occurs before rennet addition, two vats were artificially contaminated with a cocktail of the four main dairy pathogenic bacteria (*E. coli*, *S. enteritidis*, *L. monocytogenes*, and *S. aureus*) suspended in Ringer's solution (40 ml) (experimental production [EP]), while the other two vats were kept as control systems (the same volume of Ringer's

solution was added without pathogens) (control productions [CP]). The vats were used to transform contaminated and uncontaminated milk into cheese under controlled conditions in a dairy pilot plant (IZS, Palermo, Italy) in order to investigate the ability of the dairy pathogenic bacteria to grow or survive on the wooden vat surfaces during the cheese-making process. Cheeses were produced as reported by Gaglio et al. (48) with the addition of a commercial rennet paste (Clerici Sacco International, Cadorago, Italy). Cheese trials were carried out in duplicates in two consecutive weeks.

Pathogenic cultures were grown overnight in the optimal conditions and centrifuged at 5,000 $\times$ *g* for 5 min. The cells were washed twice in Ringer's solution (Sigma-Aldrich, Milan, Italy) and resuspended in the same solution until reaching an OD of ca. 1.00, determined by means of a 6400 spectrophotometer (Jenway Ltd., Felsted Dunmow, UK) at 600 nm wavelength, which approximately corresponds to a concentration of 10^9 CFU/ml, as verified by plate counts. The final cell densities in milk were 10^3 CFU/ml for *E. coli* O157 ATCC 35150 and *S. aureus* ATCC 33862, and 30 CFU/ml for *L. monocytogenes* ATCC 7644 and *S. enteritidis* ATCC 13076, which simulated a massive contamination (49, 50).

The wooden vat surfaces and milk samples were subjected to microbiological analysis.

Statistical analyses. Microbiological data were subjected to one-way analyses of variance (ANOVAs). Pair comparisons of treatment means were achieved by using Tukey's procedure at a *P* value of <0.05. Differences between the different woods were evaluated with the generalized linear model (GLM) procedure. The statistical analysis was conducted with SAS 9.2 software (Statistical Analysis System Institute Inc., Cary, NC, USA).

Accession number(s). All sequences determined in this study were deposited in GenBank database under the accession numbers MF575838 to MF575847.

ACKNOWLEDGMENTS

This work was financially supported by the Italian Ministry of Health Research Project RC 07/15 Analisi metagenomica per la caratterizzazione del biofilm presente sulle attrezzature in legno utilizzate per la produzione di formaggi tradizionali e valutazione del rischio microbiologico (CUP, H76J16000580001).

The authors declare no competing financial interests.

REFERENCES

1. Heywood VH. 1999. The Mediterranean region a major centre of plant diversity, p 5–13. *In* Heywood VH, Skoula M (ed), Wild food and non-food plants: information networking, vol 38. CIHEAM-IAMC, Chania, Greece.
2. Gasparini P, Tabacchi G, L'Inventario Nazionale delle Foreste e dei serbatoi forestali di Carbonio (INFC). 2005. The second Italian national forest inventory. Methods and results. Ministero delle Politiche Agricole, Alimentari e Forestali; Corpo Forestale dello Stato. Consiglio per la Ricerca e la Sperimentazione in Agricoltura, Unità di ricerca per il Monitoraggio e la Pianificazione Forestale. Edagricole–Il Sole 24 ore, Bologna, Italy. (In Italian.)
3. La Mantia T. 2009. La biodiversità delle formazioni naturali e seminaturali in Sicilia: cambiamenti e ipotesi di gestione, p 199–204. Proceedings of the 3rd National Congress of Forestry, Taormina, Italy.
4. Sala G, Maggiore V, La Mantia T. L'uso tradizionale del legno in Sicilia. Parte I, in press. Naturalista siciliano, II. Sicilian Society of Natural Sciences, Palermo, Italy. (In Italian.)
5. European Parliament, Council of the European Union. 2004. Commission regulation no. 1935/2004 of 27 October 2004 on materials and articles intended to come into contact with food and repealing directives 80/590/EEC and 89/109/EEC. Off J Eur Union 338:4–17. http://data.europa.eu/eli/reg/2004/1935/oj.
6. European Parliament, Council of the European Union. 2005. Commission regulation no. 2074/2005 of 5 December 2005 laying down implementing measures for certain products under regulation (EC) no. 853/2004 of the European Parliament and of the Council and for the organisation of official controls under regulation (EC) no. 854/2004 of the European Parliament and of the Council and regulation (EC) no. 882/2004 of the European Parliament and of the Council, derogating from regulation (EC) no. 852/2004 of the European Parliament and of the Council and amending regulations (EC) no. 853/2004 and (EC) no. 854/2004. Off J Eur Union 338:27–59. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2005:338:0027:0059:EN:PDF.
7. Scatassa ML, Gaglio R, Macaluso G, Francesca N, Randazzo W, Cardamone C, Di Grigoli A, Moschetti G, Settanni L. 2015. Transfer, composition and technological characterization of the lactic acid bacterial populations of the wooden vats used to produce traditional stretched cheeses. Food Microbiol 52:31–41. https://doi.org/10.1016/j.fm.2015.06.008.
8. Didienne R, Defargues C, Callon C, Meylheuc T, Hulin S, Montel MC. 2012. Characteristics of microbial biofilm on wooden vats ('gerles') in PDO Salers cheese. Int J Food Microbiol 156:91–101. https://doi.org/10.1016/j.ijfoodmicro.2012.03.007.
9. Gaglio R, Cruciata M, Di Gerlando R, Scatassa ML, Cardamone C, Mancuso I, Sardina MT, Moschetti G, Portolano B, Settanni L. 2016. Microbial activation of wooden vats used for traditional cheese production and evolution of neoformed biofilms. Appl Environ Microbiol 82:585–595. https://doi.org/10.1128/AEM.02868-15.
10. Licitra G, Ogier JC, Parayre S, Pediliggieri C, Carnemolla TM, Falentin H, Madec MN, Carpino S, Lortal S. 2007. Variability of the bacterial biofilms of the "tina" wood vat used in the Ragusano cheese-making process. Appl Environ Microbiol 73:6980–6987. https://doi.org/10.1128/AEM.00835-07.
11. Lortal S, Di Blasi A, Madec MN, Pediliggieri C, Tuminello L, Tanguy G, Fauquant J, Lecuona Y, Campo P, Carpino S, Licitra G. 2009. Tina wooden vat biofilm: a safe and highly efficient lactic acid bacteria delivering system in PDO Ragusano cheese making. Int J Food Microbiol 132:1–8. https://doi.org/10.1016/j.ijfoodmicro.2009.02.026.
12. Settanni L, Di Grigoli A, Tornambé G, Bellina V, Francesca N, Moschetti G, Bonanno A. 2012. Persistence of wild *Streptococcus thermophilus* strains on wooden vat and during the manufacture of a Caciocavallo type cheese. Int J Food Microbiol 155:73–81. https://doi.org/10.1016/j.ijfoodmicro.2012.01.022.
13. Gatti M, Bottari B, Lazzi C, Neviani E, Mucchetti G. 2014. Invited review: microbial evolution in raw-milk, long-ripened cheeses produced using undefined natural whey starters. J Dairy Sci 97:573–591. https://doi.org/10.3168/jds.2013-7187.
14. Cruciata M, Sannino C, Ercolini D, Scatassa ML, De Filippis F, Mancuso I, La Storia A, Moschetti G, Settanni L. 2014. Animal rennets as sources of dairy lactic acid bacteria. Appl Environ Microbiol 80:2050–2061. https://doi.org/10.1128/AEM.03837-13.
15. Vert M, Doi Y, Hellwich KH, Hess M, Hodge P, Kubisa P, Rinaudo M, Schué F. 2012. Terminology for biorelated polymers and applications (IUPAC recommendations 2012). Pure Appl Chem 84:377–410. https://doi.org/10.1351/PAC-REC-10-12-04.
16. Lortal S, Licitra G, Valence F. 2014. Wooden tools: reservoirs of microbial biodiversity in traditional cheesemaking. Microbiol Spectr 2:CM-0008-2012. https://doi.org/10.1128/microbiolspec.CM-0008-2012.
17. La Mantia T, Cutino I, Fioravanti M, Maggiore C, Origlio A, Spampinato

RG. 2006. Lo status della castanicoltura da legno e possibili interventi per la salvaguardia: il caso studio dell'Etna, p 318–320. Proceedings of the 4th National Conference Del Castagno. Montella, Italy.
18. Maggiore C, Fioravanti M, La Mantia T. 2006. Prime valutazioni delle caratteristiche tecnologiche del legno di castagno siciliano, p 302–304. Proceedings of the 4th National Conference Del Castagno. Montella, Italy.
19. La Mantia T, La Mela Veca DS, Gherardi L. 1999. Chestnut woods on Madonie mountains (Sicily, Italy): reasons for abandonment and possibilities of recovery. Acta Hortic 494:89–91. https://doi.org/10.17660/ActaHortic.1999.494.12.
20. Massa B, La Mantia T. 2007. Forestry, pasture, agriculture and fauna correlated to recent changes in Sicily. Forest@ 4:418–438. https://doi.org/10.3832/efor0495-0040418.
21. Ismaïl R, Le Bayon I, Michel V, Jequel M, Kutnik M, Aviat F, Fédérighi M. 2015. Comparative study of three methods for recovering microorganisms from wooden surfaces in the food industry. Food Anal Methods 8:1238–1247. https://doi.org/10.1007/s12161-014-0008-3.
22. Di Grigoli A, Francesca N, Gaglio R, Guarrasi V, Moschetti M, Scatassa ML, Settanni L, Bonanno A. 2015. The influence of the wooden equipment employed for cheese manufacture on the characteristics of a traditional stretched cheese during ripening. Food Microbiol 46:81–91. https://doi.org/10.1016/j.fm.2014.07.008.
23. Gaglio R, Francesca N, Di Gerlando R, Cruciata M, Guarcello R, Portolano B, Moschetti G, Settanni L. 2014. Identification, typing, and investigation of the dairy characteristics of lactic acid bacteria isolated from "Vastedda della valle del Belìce" cheese. Dairy Sci Technol 94:157–180. https://doi.org/10.1007/s13594-013-0150-5.
24. Guarcello R, Carpino S, Gaglio R, Pino A, Rapisarda T, Caggia C, Marino G, Randazzo CL, Settanni L, Todaro M. 2016. A large factory-scale application of selected autochthonous lactic acid bacteria for PDO Pecorino Siciliano cheese production. Food Microbiol 59:66–75. https://doi.org/10.1016/j.fm.2016.05.011.
25. Akabanda F, Owusu-Kwarteng J, Tano-Debrah K, Parkouda C, Jespersen L. 2014. The use of lactic acid bacteria starter culture in the production of Nunu, a spontaneously fermented milk product in Ghana. Int J Food Sci 2014:721067. https://doi.org/10.1155/2014/721067.
26. Lortal S, Lemée R, Valence F. 1997. Autolysis of thermophilic lactobacilli and dairy propionibacteria: a review. Lait 77:133–150. https://doi.org/10.1051/lait:199719.
27. Franciosi E, Settanni L, Cavazza A, Poznanski E. 2009. Biodiversity and technological potential of wild lactic acid bacteria from raw cows' milk. Int Dairy J 19:3–11. https://doi.org/10.1016/j.idairyj.2008.07.008.
28. Settanni L, Gaglio R, Guarcello R, Francesca N, Carpino S, Sannino C, Todaro M. 2013. Selected lactic acid bacteria as a hurdle to the microbial spoilage of cheese: application on a traditional raw ewes' milk cheese. Int Dairy J 32:126–132. https://doi.org/10.1016/j.idairyj.2013.04.010.
29. Marilley L, Casey MG. 2004. Flavours of cheese products: metabolic pathways, analytical tools and identification of producing strains. Int J Food Microbiol 90:139–159. https://doi.org/10.1016/S0168-1605(03)00304-0.
30. Corsetti A, Settanni L, Braga TM, De Fatima Silva Lopes M, Suzzi G. 2008. An investigation on the bacteriocinogenic potential of lactic acid bacteria associated with wheat (*Triticum durum*) kernels and non-conventional flours. LWT—Food Part Sci Technol 41:1173–1182. https://doi.org/10.1016/j.lwt.2007.07.022.
31. Sarantinopoulos P, Leroy F, Leontopoulou E, Georgalakia MD, Kalantzopoulos G, Tsakalidoua E, Vuyst DL. 2002. Bacteriocin production by *Enterococcus faecium* FAIR-E 198 in view of its application as adjunct starter in Greek feta cheese making. Int J Food Microbiol 72:125–136. https://doi.org/10.1016/S0168-1605(01)00633-X.
32. La Mela Veca DS, Maetzke FG, Ferrara S. 2013. Les reboisements en cèdre de l'Atlas en Sicile. Forêt Méditerr 1:13–24.
33. La Mantia T, Spoto M, Massa B. 2002. The colonisation of the Great Spotted Woodpecker (*Picoides major* L.) in Eucalypt woods and popular cultivations in Sicily. Ecol Mediterr 28:65–73.
34. Gaglio R, Barbera M, Aleo A, Lommatzsch I, La Mantia T, Settanni L. 2017. Inhibitory activity and chemical characterization of *Daucus carota* subsp. *maximus* essential oils. Chem Biodivers 14:e1600477. https://doi.org/10.1002/cbdv.201600477.
35. Todaro M, Francesca N, Reale S, Moschetti G, Vitale F, Settanni L. 2011. Effect of different salting technologies on the chemical and microbiological characteristics of PDO Pecorino Siciliano cheese. Eur Food Res Technol 233:931–940. https://doi.org/10.1007/s00217-011-1593-7.
36. Agence Francaise de Normalisation. 2009. Certification BIO 12/25-05/09. VIDAS Up *E.coli* O157 including H7 (ECPT). Agence Francaise de Normalisation, La Plaine Saint-Denis, France.
37. Agence Francaise de Normalisation. 2011. Certification BIO 12/32-10/11. VIDAS UP *Salmonella* SPT. Agence Francaise de Normalisation, La Plaine Saint-Denis, France.
38. Agence Francaise de Normalisation. 2004. Certification BIO 12/11-03/04. VIDAS *Listeria Monocytogenes* II (VIDAS LMO2) with an enrichment stage at 37°C. Agence Française de Normalisation, Saint-Denis La Plaine, France.
39. International Organization for Standardization. 2003. ISO 15213. Microbiology of food and animal feeding stuffs a horizontal method for the enumeration of sulfite-reducing bacteria growing under anaerobic conditions. International Organization for Standardization, Geneva, Switzerland.
40. Gregersen T. 1978. Rapid method for distinction of gram-negative from gram-positive bacteria. Appl Microbiol Biotechnol 5:123–127. https://doi.org/10.1007/BF00498806.
41. Stenlid J, Karlsson JO, Hogberg N. 1994. Intraspecific genetic variation in *Heterobasidion annosum* revealed by amplification of minisatellite DNA. Mycol Res 98:57–63. https://doi.org/10.1016/S0953-7562(09)80337-7.
42. van den Braak N, Power E, Anthony R, Endtz HP, Verbrugh HA, van Belkum A. 2000. Random amplification of polymorphic DNA versus pulsed field gel electrophoresis of SmaI DNA macrorestriction fragments for typing strains of vancomycin-resistant enterococci. FEMS Microbiol Lett 192:45–52. https://doi.org/10.1111/j.1574-6968.2000.tb09357.x.
43. Weisburg W, Barns SM, Pelletier DA, Lane DJ. 1991. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697–703. https://doi.org/10.1128/jb.173.2.697-703.1991.
44. King N. 1948. Modification of Voges-Proskauer test for rapid colorimetric determination of acetyl methyl carbimol plus diacetyl in butter. Dairy Ind 13:860–866.
45. Mora D, Musacchio F, Fortina MG, Senini L, Manachini PL. 2003. Autolytic activity and pediocin-induced lysis in *Pediococcus acidilactici* and *Pediococcus pentosaceus* strains. J Appl Microbiol 94:561–570. https://doi.org/10.1046/j.1365-2672.2003.01868.x.
46. Schillinger U, Lücke FK. 1989. Antibacterial activity of *Lactobacillus sake* isolated from meat. Appl Environ Microbiol 55:1901–1906.
47. Settanni L, Massitti O, Van Sinderen D, Corsetti A. 2005. *In situ* activity of a bacteriocin producing *Lactococcus lactis* strain. Influence on the interactions between lactic acid bacteria during sourdough fermentation. J Appl Microbiol 99:670–681. https://doi.org/10.1111/j.1365-2672.2005.02647.x.
48. Gaglio R, Scatassa ML, Cruciata M, Miraglia V, Corona O, Di Gerlando R, Portolano B, Moschetti G, Settanni L. 2014. *In vivo* application and dynamics of lactic acid bacteria for the four-season production of Vastedda-like cheese. Int J Food Microbiol 177:37–48. https://doi.org/10.1016/j.ijfoodmicro.2014.02.007.
49. Chatelard-Chauvin C, Pelissier F, Hulin S, Montel MC. 2015. Behaviour of *Listeria monocytogenes* in raw milk Cantal type cheeses during cheese making, ripening and storage in different packaging conditions. Food Control 54:53–65. https://doi.org/10.1016/j.foodcont.2015.01.007.
50. European Parliament, Council of the European Union. 2005. Commission regulation no. 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Off J Eur Union 338:1–26. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2005:338:0001:0026:EN:PDF.