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Microbial Activation of Wooden Vats Used for Traditional Cheese Production and Evolution of Neoformed Biofilms

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Three *Lactococcus lactis* subsp. *cremoris* strains were used to develop *ad hoc* biofilms on the surfaces of virgin wooden vats used for cheese production. Two vats (TZ) were tested under controlled conditions (pilot plant), and two vats (TA) were tested under uncontrolled conditions (industrial plant). In each plant, one vat (TA1 and TZ1) was used for the control, traditional production of PDO Vastedda della Valle del Belìce (Vastedda) cheese, and one (TA2 and TZ2) was used for experimental production performed after lactococcal biofilm activation and the daily addition of a natural whey starter culture (NWSC). Microbiological and scanning electron microscopy analyses showed differences in terms of microbial levels and composition of the neoformed biofilms. The levels of the microbial groups investigated during cheese production showed significant differences between the control trials and between the control and experimental trials, but the differences were not particularly marked between the TA2 and TZ2 productions, which showed the largest numbers of mesophilic lactic acid bacterium (LAB) cocci. LAB populations were characterized phenotypically and genotypically, and 44 dominant strains belonging to 10 species were identified. Direct comparison of the polymorphic profiles of the LAB collected during cheese making showed that the addition of the NWSC reduced their biodiversity. Sensory evaluation showed that the microbial activation of the wooden vats with the multistrain *Lactococcus* culture generated cheeses with sensory attributes comparable to those of commercial cheese. Thus, neoformed biofilms enable a reduction of microbial variability and stabilize the sensorial attributes of Vastedda cheese.

In Sicily (southern Italy), traditional cheese production from raw milk is often carried out in wooden vats without the inoculation of starter cultures (1), but animal rennet paste is added for curdling (2). Under these conditions, the desirable lactic acid bacteria (LAB) that transform curd into cheese (3) are provided only by the raw materials and/or the vat surfaces (4). The wooden vat surfaces host microbial biofilms that include dairy LAB (4–8).

The microbiological quality of raw milk depends on several factors; besides the milking conditions, storage parameters, such as cooling, holding temperatures, and the time needed for preservation before milk transformation, are particularly relevant (9–11). The presence of LAB in raw milk is variable in terms of species composition and levels reached at curdling, when most bacteria present in the milk are trapped in the curd (12). Furthermore, the LAB composition in raw milk is considered unstable, because the raw milk biota may be lost during transformation (13). Animal rennet pastes are coagulant agents that were recently demonstrated to contribute to LAB biodiversity (2); however, they cannot guarantee a constant supply of dairy LAB. In contrast, investigations carried out in Sicily showed that wooden vat surfaces provide all the dairy LAB species required for cheese production, both for fermentation (4, 5, 7, 8) and during ripening (14).

Vastedda della Valle del Belìce cheese is a stretched, raw ewes'-milk cheese produced in the homonymous valley located in western Sicily. Since the recognition of the protected designation of origin (PDO) by the European Union (GUE no. C 42/16 19.2.2010), there is a strong demand for this cheese (15), which does not undergo a ripening process but is sealed under vacuum after salting and stored refrigerated for a limited time after production (16). Vastedda cheese is traditionally produced only during the summer season. However, due to the increasing demand for this cheese, it is currently produced throughout the year. To minimize the differences that can arise during cheese productions, Gaglio et al. (17) selected a group of *Lactococcus lactis* subsp. *cremoris* strains that dominate during Vastedda cheese making. In that study, Gaglio et al. tested the added strains under different conditions, but with equipment made of stainless steel only.

Thus, the main aim of our study was to use the selected multistrain *Lactococcus* culture to develop stable biofilms on the surfaces of virgin wooden vats to keep the process traditional and the final product typical and consistent. The specific objectives of our study were as follows: (i) to characterize the microbial development during the activation of new vats; (ii) to evaluate the persistence of the added strains; and (iii) to evaluate the efficacy of conversion of the production process for PDO Vastedda della Valle del Belìce cheese from a production performed with raw milk without starters to a production carried out with raw milk and natural starters, and also to evaluate the sensory characteristics.

MATERIALS AND METHODS

Strains and development of natural whey starter culture (NWSC). *Lc. lactis* subsp. *cremoris* PON36, PON153, and PON203, isolated from PDO Vastedda della Valle del Belìce cheeses (16) and selected for their dairy

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performances *in vivo* during Vastedda-like cheese productions (17), were used in this study.

The cultures were grown individually overnight at 30°C in M17 broth (Oxoid, Milan, Italy) and then centrifuged at 5,000 × *g* for 5 min to separate the cells, which were washed in Ringer's solution (Oxoid). The cells were resuspended to an optical density at 600 nm (OD_{600}) of ca. 1.00 as determined spectrophotometrically (model 6400 spectrophotometer; Jenway Ltd., Felsted, Dunmow, United Kingdom), corresponding to a lactococcus concentration of about 10^9 CFU/ml. Each strain was inoculated to a final concentration of about 10^6 CFU/ml in a whey-based medium, prepared as reported by Settanni et al. (7). After incubation for 24 h at 30°C, the NWSC containing the multistrain culture was used for cheese making.

Wooden vat activation. Four chestnut wooden vats with a 100-liter volume were purchased from a local artisanal producer. The vats were used to transform milk under the following different conditions: (i) controlled conditions in a dairy pilot plant (Istituto Zooprofilattico Sperimentale della Sicilia Adelmo Mirri, Palermo, Italy), where the milk transformed was provided by a single farm selected for its high hygienic standards (low levels of microorganisms in milk at the time of delivery) and the wooden vats were designated TZ (the acronym from the Italian *tina-zooprofilattico*); and (ii) uncontrolled conditions in an industrial dairy (Il Cacio Siciliano, Belmonte Mezzagno, Palermo, Italy), wherein bulk milk quantities obtained from several farms were transformed in vats designated TA (*tina-azienda*). Two vats were used for each condition—one for control production (TA1 and TZ1) and one for experimental production (TA2 and TZ2).

In order to remove the tannin components from wood, all four vats were treated daily with hot water (75 to 80°C) for 30 days before biofilm activation. The biofilms of vats TA1 and TZ1 were activated as follows. The first step of cheese production was carried out with only the aim of keeping whey. The whey (60 liters) was then left overnight in contact with the vat, and the day after, the whey was removed for the further steps of cheese making. The biofilms of vats TA2 and TZ2 were activated with 60 liters of NWSC, prepared as reported above, which was left overnight and then removed to start cheese production.

Scanning electron microscopy. The biofilms of the wooden vats were analyzed by scanning electron microscopy (FEI Quanta 200F; FEI, Holland) of rectangular (50 × 35 mm) wood splinters (1- to 2-mm thickness) that were aseptically sampled before and after activation. The samples were dehydrated as reported by Mallia et al. (18) and dried as described by Lortal et al. (4). The vat splinters were mounted with the side that had been in contact with the leaf surface facing up on an aluminum holder. All specimens were then sputter coated with gold (20 mÅ; 300 s) (Edwards S150A sputter coater) and observed by scanning electron microscopy.

Cheese production and sample collection. The control cheese productions (in vats TA1 and TZ1) were carried out following the protocol for PDO Vastedda della Valle del Belìce cheese production, using raw ewes' milk exclusively, without starter culture inoculums. Cheese production in each vat was performed with 50 liters of milk (preheated at 38°C), which was left to rest in the wooden vat for almost 10 min, after which 15 g of animal rennet paste (Clerici Sacco International, Cadorago, Italy) was added. The experimental productions (in vats TA2 and TZ2) were performed with daily inoculation of the NWSC (800 ml) into the raw milk just before rennet addition. After curdling, the curds were supplemented with 5 liters of hot (60°C) water and cut until small, rice-size grains were observed. The curds were transferred to perforated molds, and when the pH value, measured using a Russell RL060P portable pH meter (Thermo Fisher Scientific, Beverly, MA), dropped into the range of 5.2 to 5.4, the curds were stretched in hot (85 to 90°C) water and molded into a round shape. Salting was performed in brine at 20% (wt/vol) NaCl for 30 min, and the resulting cheeses were air dried for 24 h, sealed under vacuum, and kept under refrigeration (7°C) for 15 days. Cheese productions were performed for five consecutive days during the first week of February 2014 and repeated at a 3-week interval for another five consecutive days to examine the stability of the biofilms.

The following samples were collected during each cheese production: samples from the vat surface, milk, whey, curd soon after transfer to the mold, acidified curd, cheese soon after salting, and cheese after 15 days of storage. Just before cheese production, each vat surface (400 cm^2) was sampled using UV-treated paper squares positioned halfway up the side and on the bottom of the vat as described by Didienne et al. (6). Each vat surface was sampled in duplicate at two diametrically opposite points.

Microbiological analyses and isolation of LAB. Microbiological analyses were carried out to evaluate the main microbial groups associated with cheese production and investigated for quality, hygiene, and safety aspects. Cell suspensions of the wooden vat surface and liquid (milk and whey) samples were subjected to decimal serial dilutions in Ringer's solution, while solid (curd and cheese) samples (15 g) were first homogenized in 135 ml of sodium citrate (2% [wt/vol]) solution in a stomacher (Bag-Mixer 400; Interscience, Saint Nom, France) for 2 min at the highest speed and then serially diluted. The inoculation, cultivation, and incubation of the different microbial groups were as follows. Total mesophilic microorganisms were spread plated on plate count agar supplemented with 1 g/liter skimmed milk and incubated aerobically at 30°C for 72 h. Total psychrotrophic microorganisms were plated as described for total mesophilic microorganisms, on plate count agar with skimmed milk, but incubation was performed at 7°C for 7 days. Mesophilic and thermophilic LAB cocci were pour plated on M17 agar and incubated anaerobically at 30°C and 44°C, respectively, for 48 h. Mesophilic LAB rods were pour plated on acidified (pH 5.4) MRS agar and incubated anaerobically at 30°C for 48 h. Thermophilic LAB rods were pour plated on whey-based agar medium prepared as described by Settanni et al. (7) by using nonacidified ewes'-milk whey, collected after curdling during the production of PDO Vastedda della Valle del Belìce cheese, and were incubated anaerobically at 44°C for 4 days. Enterococci were inoculated on kanamycin esculin azide agar and incubated aerobically at 37°C for 24 h. Members of the *Enterobacteriaceae* family were pour plated on double-layered violet red bile glucose agar and incubated aerobically at 37°C for 24 h. Pseudomonads were inoculated on *Pseudomonas* agar base supplemented with 10 mg/ml cetrimide-fusidic acid (Fucidin) and incubated aerobically at 20°C for 48 h. Clostridia were estimated by the most-probable-number technique as reported by Settanni et al. (7). Coagulase-positive staphylococci, *Salmonella* spp., *Listeria monocytogenes*, and *Escherichia coli* were analyzed as reported by Scatassa et al. (19). Microbiological counts were carried out in duplicate. All media were purchased from Oxoid.

Furthermore, the whey resulting from the first step of cheese making carried out in the control vats and the NWSC at the first step of its preparation (starting from sterile, whey-based medium inoculated with the three *Lactococcus* strains) were also analyzed for the initial level of each strain and for the concentration reached after incubation. Whey was analyzed for LAB content by plate counts on M17 agar at 30°C and 44°C and on MRS and whey-based agar medium, while the NWSC was analyzed only on M17 agar at 30°C for the presence of lactococci.

After incubation, five identical colonies (or fewer if five were not available) for each morphology detected, in terms of shape, surface, color, margin, and elevation, were randomly collected from MRS, M17, and whey-based agar medium plates inoculated with the highest dilutions of the cell suspensions and transferred to the corresponding broth media. The isolates were streaked by successive subculturing, and their purity was verified under an optical microscope. The isolates were subjected to the KOH test to determine the type of cell wall and to the catalase test, performed by addition of H_2O_2 (5% [wt/vol]) to the colonies. Only Gram-positive and catalase-negative cultures were stored in glycerol stocks at −80°C for further investigation.

Phenotypic grouping, strain differentiation, and identification of LAB. The presumptive LAB isolates were first subjected to phenotypic characterization based on morphological, physiological, and biochemical characteristics for their grouping. Cell morphology and cell disposition were determined microscopically. Growth at 15°C and 45°C, resistance to treatment at 60°C for 30 min, NH_3 production from arginine, esculin

TABLE 1 Microbiological characteristics of milk and whey used for activation of biofilms on the surfaces of wooden vats used for control cheese production[a,b]

Medium or organism	Plate count (log CFU/ml)		Statistical significance	Plate count (log CFU/ml)		Statistical significance
	Factory milk	Pilot plant milk		Factory whey	Pilot plant whey	
PCA-SkM 7°C	4.7 ± 0.2^{B}	1.7 ± 0.3^{A}	***	ND	ND	
PCA-SkM 30°C	5.9 ± 0.4^{B}	3.5 ± 0.2^{A}	***	7.4 ± 0.1^{A}	7.0 ± 0.2^{A}	*
M17 30°C	6.2 ± 0.1^{B}	3.8 ± 0.3^{A}	**	7.5 ± 0.4^{A}	7.1 ± 0.4^{A}	*
M17 44°C	5.5 ± 0.2^{B}	3.0 ± 0.1^{A}	***	7.4 ± 0.2^{B}	5.7 ± 0.2^{A}	***
MRS	5.9 ± 0.4^{B}	2.5 ± 0.2^{A}	***	7.7 ± 0.3^{A}	7.4 ± 0.1^{A}	*
WBAM	6.1 ± 0.3^{B}	2.4 ± 0.3^{A}	***	6.7 ± 0.1^{B}	6.2 ± 0.3^{A}	*
VRBGA	3.3 ± 0.4^{B}	1.7 ± 0.4^{A}	***	ND	ND	
KAA	3.3 ± 0.2^{B}	1.9 ± 0.2^{A}	**	ND	ND	
E. coli	1.5 ± 0.4^{B}	$<1^{A}$	***	ND	ND	
CPS	1.3 ± 0.3^{B}	$<1^{A}$	**	ND	ND	

[a] Plate count results are mean values ± standard deviations (SD) for two plate counts. Data within a line followed by the same letter are not significantly different according to Tukey's test. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

[b] Abbreviations: PCA-SkM 7°C, plate count agar with skimmed milk, incubated at 7°C for detection of total psychrotrophic microorganisms; PCA-SkM 30°C, plate count agar with skimmed milk, incubated at 30°C for detection of total mesophilic microorganisms; M17 30°C, medium 17 agar incubated at 30°C for detection of mesophilic coccus LAB; M17 44°C, medium 17 agar incubated at 44°C for detection of thermophilic coccus LAB; MRS, de Man-Rogosa-Sharpe agar for detection of mesophilic rod LAB; WBAM, whey-based agar medium for detection of thermophilic rod LAB; VRBGA, violet red bile glucose agar for detection of *Enterobacteriaceae*; KAA, kanamycin esculin azide agar for detection of enterococci; CPS, coagulase-positive staphylococci; ND, not determined.

hydrolysis, acid production from carbohydrates, and CO_2 production from glucose were determined as reported by Gaglio et al. (15). Cocci were also grouped according to their growth at pH 9.6 and in the presence of 6.5 g/liter NaCl in order to separate enterococci, which are able to grow under these conditions, from other dairy cocci that are sensitive to high pH and high salt concentrations.

DNAs from broth cultures, developed overnight at optimal temperatures in the media used for isolation, were extracted using InstaGene Matrix kits (Bio-Rad, Hercules, CA) following the manufacturer's instructions and then used for PCR.

Differentiation of the isolates collected during cheese making and monitoring of the lactococci used to activate the wooden vats were performed by random amplification of polymorphic DNA (RAPD)-PCR analysis. Strain typing was carried out in 25-μl reaction mixtures with the single primers M13, AB111, and AB106 as previously described by Settanni et al. (7). The PCR products and the GeneRuler 100-bp Plus DNA ladder (M Medical Srl, Milan, Italy) were separated by electrophoresis on 1.5% (wt/vol) agarose gels (Gibco BRL, Cergy Pontoise, France). The gels were stained with the SYBR Safe DNA gel stain (Molecular Probes, Eugene, OR) and visualized by UV transillumination. RAPD patterns were analyzed using GelCompar II software, version 6.5 (Applied-Maths, Sint-Marten-Latem, Belgium), and isolates with different profiles were considered to be different strains.

Genotypic characterization of LAB containing different RAPD-PCR profiles was carried out by 16S rRNA gene sequencing. PCR was performed as described by Weisburg et al. (20), using the primers rD1 (5′-AAGGAGGTGATCCAGCC-3′) and fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′). The PCR products were purified using 10 U of exonuclease I and 1 U of shrimp alkaline phosphatase (Thermo Fisher Scientific). DNA sequencing reactions were carried out using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Beverly, MA) with 5 μM (each) primers (the same as those used for PCR). Cycle sequencing reactions were performed according to the manufacturer's instructions following ethanol-EDTA-sodium acetate precipitation. Sequencing analyses were performed in an ABI Prism 3130xl genetic analyzer (Applied Biosystems). The sequences were compared with those available in the GenBank/EMBL/DDBJ (http://www.ncbi.nlm.nih.gov) and EzTaxon-e (http://eztaxon-e.ezbiocloud.net/) databases.

Sensory analysis. The effects of the wooden vat biofilms, the bulk milk samples, and the dairy factory environments on the sensory characteristics of the cheeses were evaluated by 11 judges (four men and seven women; 30 to 55 years old). For this purpose, the members of the descriptive panel, who were familiar with the sensory analysis of cheese, were trained in preliminary sessions using commercial PDO Vastedda della Valle del Belìce cheeses. The samples used for sensory evaluation were the cheeses sealed under vacuum and kept refrigerated for 15 days and were administered in small pieces (3 × 3 × 3 cm) after conditioning (about 60 min) at ambient temperature, with the pieces presented in a randomized order on white plates.

The evaluation of the sensory profiles of the experimental cheeses was performed following the ISO 13299 protocol (21), and the tasters were asked to score several parameters of the aspect (presence of eyes, uniformity of structure, color, and oil), smell (strength of odors and presence of pungent and pasture odors), taste (spicy, salty, bitter, and taste intensity), and consistency (soft/hard, dispersion, and saliva evoking). Each attribute was described extensively and explained to avoid any doubt.

The tasters evaluated four experimental cheeses and one commercial PDO Vastedda della Valle del Belìce cheese per day, for a total of 10 days of production (5 in the first week and 5 in the last week of February 2014).

Statistical analyses. Statistical analyses of microbiological counts were performed using Statistica software (StatSoft Inc., Tulsa, OK). Data from microbiological investigations and sensory analyses were analyzed using a generalized linear model that included the effects of the different productions. Data were converted to the log scale after statistical elaborations. Differences between means were determined by Tukey's multiple-range *post hoc* test. *P* values of <0.05 were deemed to be significant.

The scores from the sensory analysis were tested by a 2-factor analysis of variance, with judges ($i = 1...11$) and experimental cheeses ($j = 1...10$) as fixed factors. Least-square means were compared using Student's *t* test ($P < 0.05$). All statistical analyses were conducted using the software SAS 2004, version 9.1.2 (Statistical Analysis System Institute Inc., Cary, NC).

RESULTS

Biofilm activation. The *ad hoc* natural starter for Vastedda-like cheese production displayed initial levels of 5.9, 5.8, and 5.9 log CFU/ml for *Lc. lactis* subsp. *cremoris* PON36, PON153, and PON203, respectively, and a total inoculum of 6.4 log CFU/ml. After 24 h of incubation, the NWSC was characterized by a pH value of 3.7, and the concentration of lactococci was 8.6 CFU/ml.

The microbiological characteristics of the milk and the corresponding whey to be applied to vats TA1 and TZ1 are shown in Table 1. It is evident that the bulk milk delivered to the dairy factory was

TABLE 2 Microbial loads of biofilms activated on the surfaces of wooden vats after the first contact with whey[a,b]

Medium	Microbial load (log CFU/cm^2)				Statistical significance
	TA1	TA2	TZ1	TZ2	
PCA-SkM 7°C	3.1 ± 0.3^{A}	4.5 ± 0.3^{B}	2.7 ± 0.2^{A}	4.7 ± 0.3^{B}	***
PCA-SkM 30°C	4.8 ± 0.4^{B}	5.9 ± 0.2^{C}	4.1 ± 0.1^{A}	6.0 ± 0.4^{C}	***
M17 30°C	4.9 ± 0.1^{B}	6.1 ± 0.1^{C}	4.3 ± 0.3^{A}	6.1 ± 0.2^{C}	***
M17 44°C	3.7 ± 0.2^{B}	2.2 ± 0.4^{A}	3.5 ± 0.2^{B}	2.4 ± 0.1^{A}	***
MRS	4.0 ± 0.3^{A}	5.1 ± 0.2^{B}	3.7 ± 0.4^{A}	5.0 ± 0.3^{B}	**
WBAM	3.2 ± 0.1^{B}	2.0 ± 0.1^{A}	3.1 ± 0.3^{B}	2.1 ± 0.1^{A}	**
VRBGA	0.6 ± 0.2^{B}	$<1^{A}$	$<1^{A}$	$<1^{A}$	*
KAA	1.9 ± 0.3^{B}	$<1^{A}$	$<1^{A}$	$<1^{A}$	***

[a] Results indicate mean values ± SD for two plate counts. Data within a line followed by the same letter are not significantly different according to Tukey's test. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

[b] Abbreviations: TA1, control production at the dairy factory; TZ1, control production at the dairy pilot plant; TA2, experimental production at the dairy factory; TZ2, experimental production at the dairy pilot plant; PCA-SkM 7°C, plate count agar with skimmed milk, incubated at 7°C for detection of total psychrotrophic microorganisms; PCA-SkM 30°C, plate count agar with skimmed milk, incubated at 30°C for detection of total mesophilic microorganisms; M17 30°C, medium 17 agar incubated at 30°C for detection of mesophilic coccus LAB; M17 44°C, medium 17 agar incubated at 44°C for detection of thermophilic coccus LAB; MRS, de Man-Rogosa-Sharpe agar for detection of mesophilic rod LAB; WBAM, whey-based agar medium for detection of thermophilic rod LAB; VRBGA, violet red bile glucose agar for detection of *Enterobacteriaceae*; KAA, kanamycin esculin azide agar for detection of enterococci.

characterized by microbiological parameters that were higher than those of the milk used at the dairy pilot plant. In particular, TA milk showed the presence of total psychrotrophic microorganisms, total mesophilic microorganisms, and all LAB groups, except enterococci, at >2 log cycles higher than those in TZ milk. Furthermore, *E. coli* and coagulase-positive staphylococci were below the detection level in TZ milk. The resulting whey samples were analyzed only for total mesophilic microorganisms and LAB. Although the counts were statistically different for the two whey bulks, both matrices hosted mesophilic LAB cocci and rods at 10^7 CFU/ml.

Microbiological analysis and scanning electron microscopy of neoformed biofilms. The results of analysis of the cell suspensions from the wooden vat surfaces are shown in Table 2. Data regarding clostridia, coagulase-positive staphylococci, *E. coli*, *L. monocytogenes*, *Pseudomonas* spp., and *Salmonella* spp. are not shown in Table 2 because these microbial groups were not detected on any vat surface. The levels of total psychrotrophic microorganisms of vats TA2 and TZ2, activated with the NWSC, were almost 1.5 to 2 log cycles higher than those of the vats used for control cheese productions. No statistical differences were found between levels of total psychrotrophic microorganisms for vats TA1 and TZ1 as well as vats TA2 and TZ2. The total mesophilic microorganism levels were consistently higher in TA2 and TZ2 than in TA1 and TZ1. The levels of this microbial group in the vats activated with the NWSC were comparable. However, they were different between TA1 and TZ1, showing a difference between the whey contents of the two bulk quantities of milk in terms of biofilm activation, although the total numbers of mesophilic microorganisms in the whey samples were not statistically different according to Tukey's test (Table 1).

Among LAB, the highest cell counts registered were for mesophilic LAB cocci for all vats. The same level (6.1 log CFU/cm^2) was found for TA2 and TZ2. In contrast, the lowest levels recorded were for thermophilic LAB rods for all vats. In particular, the levels of these LAB in TA2 and TZ2 were almost 1 log cycle lower than those in TA1 and TZ1. *Enterobacteriaceae* and enterococci were found only in TA1, though at very low levels. The detection of both groups on TA1 vat surfaces could be a direct consequence of their presence at higher levels in TA milk (Table 1).

The results of the scanning electron microscopy analysis carried out on wood splinters collected from the vats kept under controlled conditions are shown in Fig. 1. The wood splinter sampled from chestnut vat TZ1 after hot water treatment did not show any microbial attachment (Fig. 1A). The same vat after overnight contact with the whey displayed the presence of rod and coccus bacteria (Fig. 1B). In contrast, overnight treatment of vat TZ2 with the NWSC

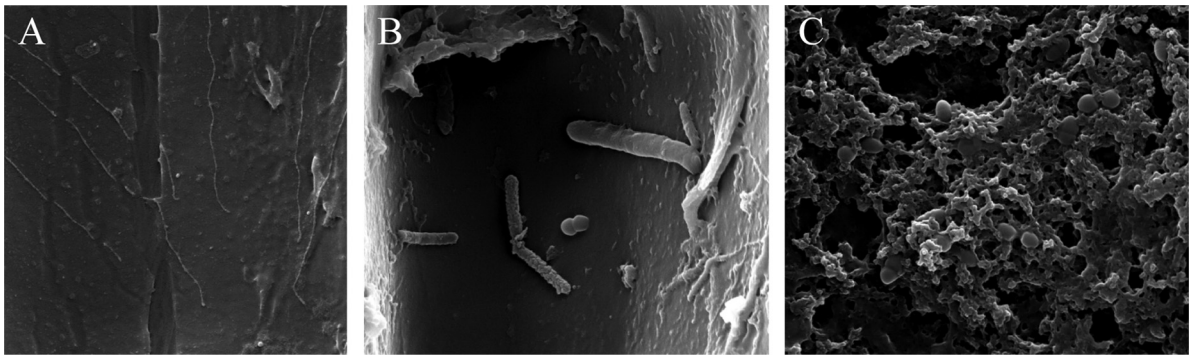

FIG 1 Scanning electron microscopy observations of wooden splinters. (A) Virgin vat after 30 days of hot water treatment. (B) Vat TZ1 after overnight contact with whey obtained from a traditional Vastedda cheese made with raw milk. (C) Vat TZ2 after overnight contact with the NWSC developed with the multistrain *Lactococcus* culture.

developed with the multistrain *Lactococcus* culture showed a defining prevalence of coccus-shaped bacteria (Fig. 1C). The marked differences observed visually between TZ1 and TZ2 in terms of cell concentration (Fig. 1B and C) may be the consequence of the differences in LAB counts estimated for the two whey bulks used for microbial activation. As reported above, the NWSC hosted lactococci at 10^8 CFU/ml, while the acidified whey resulting from the first control cheese production was characterized by a maximum LAB level of 10^7 CFU/ml (Table 1). A similar trend (results not shown) was registered for the TA1/TA2 vat couple.

Monitoring of cheese making. During the entire experiment, the room temperature of the dairy pilot plant ranged from 11.8°C (minimum registered in the night) to 18.9°C (maximum registered in the morning), while that of the dairy factory ranged from 9.8°C to 22.3°C (minimum during the night and maximum during the morning, respectively). On average, the characteristics of the NWSC at the time of inoculation into milk were as follows: pH 3.8 and 8.5 log CFU/ml of lactococci. All curds from wooden vats TA2 and TZ2 could be stretched within 24 h after transfer into perforated containers, but different observations were made for the curds produced in wooden vats TA1 and TZ1, which reached the desired pH value later than 24 h after milk coagulation, but within 48 h.

Microbiological characterization of cheese productions. The microbial loads of the samples collected during cheese making are reported in Table 3. The results for clostridia, *L. monocytogenes*, and *Salmonella* spp. are not reported because the levels of these bacteria were below the detection limit in all samples. Total psychrotrophic microorganisms were generally significantly less concentrated than total mesophilic microorganisms, even though the levels found on the surfaces of the TZ1 vat were not statistically different. Mesophilic LAB cocci clearly dominated the microbial populations of all vat surfaces. However, their levels detected in TZ1 were >1 log cycle lower than those registered in the other vats, which were about 10^6 CFU/cm^2. The levels of the total mesophilic microorganisms and, except for TZ2, those of mesophilic LAB cocci were almost superimposable with the loads registered soon after the activation of the microbial films (Table 2). The highest levels of thermophilic LAB cocci were found on the surfaces of vat TA1. Within the LAB group, the lowest cell densities were found for the thermophilic rods, which did not reach 2 log CFU/cm^2 in any vat, while the most variability was displayed by mesophilic rods, with levels ranging from 3.2 (TZ1) to 5.8 (TA2) log CFU/cm^2. Comparing the data registered during cheese production and those found after the activation of the vats, even though the levels of enterococci and members of the *Enterobacteriaceae* family were still under the detection limit for TZ2, they generally increased in the other vats during cheese production. *E. coli* and coagulase-positive staphylococci were never detected on the vat surfaces.

The bulk quantity of milk analyzed after contact with the vat surfaces in the dairy factory was characterized by microbial levels on the same orders of magnitude as those registered before contact. A different trend was found for the levels registered for the bulk milk quantities after resting in the wooden vats used in the pilot plant; all microbial groups increased, and the largest numbers were displayed by the mesophilic LAB cocci, which were detected at about 10^6 CFU/ml. Although the microbial loads of the bulk milk quantities before contact are not shown in Table 3, they were mostly on the same orders of magnitude as those registered for the milks used during biofilm formation (Table 1), except for the values for enterococci, *E. coli*, and coagulase-positive staphylococci in TZ milk. In general, the highest cell densities reached after contact with the vat surfaces were found for all microbial groups in TA2 bulk milk.

Except for the total psychrotrophic microorganisms, whose levels were on the same order of magnitude in all the vats, the levels of the other microbial groups increased in the curds by about 1 to 1.5 log cycles. However, the opposite trend was observed for *E. coli* in the curds obtained in vats TZ1 and TZ2—the *E. coli* disappeared completely. The levels of mesophilic LAB rods were particularly high, reaching 10^7 CFU/g, in the curds produced in the dairy factory. Regarding enterococci, a difference of 3 orders of magnitude was registered between the productions carried out at the dairy factory (4.7 log CFU/g) and those performed at the pilot plant (1.0 to 1.7 log CFU/g).

Soon after curdling, before acidification took place, the whey of each production was characterized microbiologically only for total microorganisms and different groups of LAB. It is evident that the daily addition of NWSC to the milk of TA2 and TZ2 resulted in higher levels of mesophilic LAB cocci than those of the other microorganisms under investigation. This population dominated all the acidified curds, which were analyzed when the pH value was in the range of 5.2 to 5.4, but the levels registered for the curds from TA2 and TZ2 were 10-fold higher than those for the curds from TA1 and TZ1. Both mesophilic LAB rods and thermophilic LAB cocci increased consistently, while thermophilic rods were present at approximately 10^4 CFU/g.

Acidification resulted in a reduction of *Enterobacteriaceae* of about 1 log cycle for all curds, except those obtained from TZ1, for which an increase of 2 log cycles was registered. Enterococci were present in large numbers, in the range of 5.5 to 5.9 log CFU/g, for acidified curds of TA1, TA2, and TZ1, while they were present at barely 2.8 log CFU/g for acidified curds of TZ2. *E. coli* and coagulase-positive staphylococci were detected only for the productions carried out without NWSC addition, though at low levels, reflecting the efficacy of the acidifying capacity of the starter lactococci. In these productions, mesophilic LAB cocci reached levels of little less than 10^{10} CFU/g.

The microbial level of total psychrotrophic microorganisms in the cheeses at time zero (T_0) was 10^6 CFU/g and, after 15 days, increased by 1 log cycle only for TA2 and TZ2 cheeses. The largest increases in numbers were also registered for total mesophilic microorganisms and mesophilic LAB cocci for the cheeses produced with NWSC. The increases in the levels of mesophilic LAB cocci were up to 9.0 and 9.1 log CFU/g for TZ2 and TA2 cheeses, respectively. In contrast, the cheese productions carried out without NWSC addition were characterized by the highest levels of thermophilic LAB cocci. In general, the cheeses produced with the addition of NWSC presented better hygienic conditions than those for cheeses made in TA1 and TZ1. However, although members of the *Enterobacteriaceae* family were still detectable in control cheeses after 15 days of refrigerated storage, *E. coli* was under the detection limit in all cheeses.

Phenotypic grouping, genotypic differentiation, identification, and monitoring of LAB. A total of 1,441 colonies were collected from the agar media used for LAB counts. All cultures were subjected to microscopic analysis and were separated into 1,256 cocci and 185 rods. Gram determination and the catalase test indicated that 1,183 cocci and 127 rods could be considered putative

TABLE 3 Microbial loads of samples collected during experimental cheese production[a]

Sample type	Production vat	Microbial load[b] PCA-SkM 7°C	PCA-SkM 30°C	M17 30°C	M17 44°C	MRS	WBAM	VRBGA	KAA	*E. coli*	CPS
VS	TA1	3.2 ± 1.0^{A}	5.7 ± 0.6^{B}	5.8 ± 0.7^{B}	4.0 ± 0.9^{B}	4.5 ± 0.5^{B}	1.9 ± 0.9^{B}	2.4 ± 1.0^{D}	2.5 ± 1.0^{D}	$<1^{A}$	$<1^{A}$
	TA2	4.4 ± 0.9^{B}	5.9 ± 0.9^{B}	6.1 ± 0.9^{B}	2.9 ± 0.9^{A}	5.8 ± 0.7^{C}	1.8 ± 0.8^{B}	1.2 ± 0.8^{B}	2.0 ± 0.8^{C}	$<1^{A}$	$<1^{A}$
	TZ1	3.5 ± 0.9^{A}	3.8 ± 0.9^{A}	4.6 ± 1.1^{A}	2.9 ± 0.8^{A}	3.2 ± 1.0^{A}	1.9 ± 0.4^{B}	1.7 ± 0.7^{C}	1.6 ± 0.9^{B}	$<1^{A}$	$<1^{A}$
	TZ2	4.3 ± 0.9^{B}	6.0 ± 1.0^{B}	5.7 ± 1.0^{B}	2.8 ± 0.9^{A}	4.5 ± 1.0^{B}	1.3 ± 0.6^{A}	$<1^{A}$	$<1^{A}$	$<1^{A}$	$<1^{A}$
Statistical significance		*	***	***	**	***	*	***	***		
BMAR	TA1	5.4 ± 0.6^{B}	5.8 ± 0.9^{B}	6.2 ± 0.5^{BC}	5.0 ± 0.6^{C}	5.1 ± 0.4^{B}	2.8 ± 1.0^{B}	3.9 ± 0.9^{C}	3.3 ± 0.7^{C}	2.5 ± 1.3^{B}	2.4 ± 1.7^{C}
	TA2	5.2 ± 0.4^{B}	6.4 ± 0.5^{C}	6.3 ± 0.6^{C}	5.2 ± 0.8^{C}	5.4 ± 0.6^{B}	2.4 ± 0.9^{AB}	3.2 ± 1.0^{B}	3.1 ± 0.4^{C}	2.4 ± 1.4^{B}	1.8 ± 1.1^{B}
	TZ1	4.6 ± 0.9^{A}	5.1 ± 0.6^{A}	5.5 ± 0.4^{A}	4.0 ± 0.7^{B}	3.8 ± 0.5^{A}	2.1 ± 0.8^{A}	2.2 ± 0.5^{A}	1.3 ± 0.5^{B}	1.4 ± 0.8^{A}	1.0 ± 0.6^{A}
	TZ2	4.5 ± 1.3^{A}	5.5 ± 0.8^{AB}	5.9 ± 0.8^{AB}	2.5 ± 0.9^{A}	3.5 ± 1.0^{A}	2.1 ± 0.9^{A}	1.9 ± 0.8^{A}	$<1^{A}$	1.3 ± 0.6^{A}	1.1 ± 0.5^{A}
Statistical significance		*	**	*	***	***	*	***	***	**	**
C	TA1	5.8 ± 0.5^{C}	7.0 ± 0.6^{B}	7.3 ± 1.2^{B}	6.3 ± 0.5^{B}	7.1 ± 0.9^{B}	4.1 ± 0.9^{B}	5.1 ± 0.8^{C}	4.7 ± 0.5^{C}	3.9 ± 0.9^{C}	2.3 ± 0.9^{B}
	TA2	5.3 ± 1.0^{B}	7.0 ± 0.5^{B}	7.2 ± 0.8^{B}	5.9 ± 0.9^{B}	7.4 ± 0.7^{B}	3.8 ± 0.8^{B}	4.6 ± 0.7^{B}	4.7 ± 0.8^{C}	2.5 ± 0.5^{B}	2.0 ± 1.0^{B}
	TZ1	4.8 ± 0.9^{A}	6.4 ± 0.8^{A}	6.7 ± 0.7^{A}	4.1 ± 0.7^{A}	4.5 ± 1.0^{A}	3.1 ± 0.9^{A}	2.8 ± 0.9^{A}	1.7 ± 0.7^{B}	$<1^{A}$	1.4 ± 1.1^{A}
	TZ2	4.5 ± 0.8^{A}	7.1 ± 0.8^{B}	7.5 ± 0.7^{B}	3.7 ± 0.6^{A}	4.6 ± 0.6^{A}	2.9 ± 0.8^{A}	2.9 ± 0.9^{A}	1.0 ± 0.9^{A}	$<1^{A}$	1.4 ± 1.2^{A}
Statistical significance		**	*	*	***	***	**	***	***	***	*
W	TA1	4.6 ± 0.8^{B}	5.8 ± 0.5^{A}	6.2 ± 1.2^{B}	4.4 ± 0.8^{BC}	5.8 ± 0.8^{A}	3.8 ± 0.9^{A}	ND	ND	ND	ND
	TA2	4.3 ± 0.7^{AB}	6.3 ± 0.6^{B}	7.0 ± 0.8^{C}	4.7 ± 0.8^{C}	5.8 ± 0.5^{A}	4.6 ± 0.8^{B}	ND	ND	ND	ND
	TZ1	4.6 ± 0.9^{B}	5.4 ± 0.9^{A}	5.6 ± 0.9^{A}	4.2 ± 0.8^{AB}	6.1 ± 1.0^{A}	3.9 ± 0.7^{A}	ND	ND	ND	ND
	TZ2	3.9 ± 0.8^{A}	6.7 ± 0.8^{B}	7.3 ± 0.9^{C}	3.8 ± 0.9^{A}	5.7 ± 0.9^{A}	4.1 ± 0.6^{A}	ND	ND	ND	ND
Statistical significance		*	**	***	*	NS	*				
AC	TA1	6.1 ± 0.6^{A}	8.1 ± 0.6^{A}	8.4 ± 0.9^{A}	6.0 ± 0.5^{A}	7.8 ± 0.7^{A}	4.5 ± 1.0^{B}	4.7 ± 0.7^{C}	5.9 ± 1.1^{B}	1.9 ± 0.7^{B}	1.3 ± 0.5^{B}
	TA2	6.2 ± 0.9^{A}	9.1 ± 0.8^{B}	9.7 ± 0.6^{B}	6.1 ± 0.9^{AB}	8.7 ± 1.0^{B}	4.6 ± 0.7^{B}	3.2 ± 0.8^{B}	5.8 ± 0.7^{B}	$<1^{A}$	$<2^{A}$
	TZ1	6.5 ± 0.7^{A}	7.9 ± 0.5^{A}	8.0 ± 0.5^{A}	6.3 ± 0.9^{AB}	7.5 ± 0.7^{A}	4.1 ± 0.8^{AB}	4.8 ± 0.9^{C}	5.5 ± 0.8^{B}	2.0 ± 0.9^{B}	1.8 ± 0.9^{C}
	TZ2	6.1 ± 0.9^{A}	9.3 ± 0.9^{B}	9.5 ± 0.7^{B}	6.5 ± 0.65^{B}	8.4 ± 0.9^{B}	3.9 ± 0.8^{A}	1.8 ± 0.9^{A}	2.8 ± 0.9^{A}	$<1^{A}$	$<2^{A}$
Statistical significance		NS	**	**	*	*	*	***	***	***	***
ChT_0	TA1	6.3 ± 0.8^{AB}	7.3 ± 1.0^{A}	7.4 ± 0.7^{A}	7.2 ± 0.6^{A}	8.2 ± 0.8^{A}	4.7 ± 0.9^{A}	2.4 ± 0.9^{C}	5.2 ± 0.6^{B}	1.8 ± 1.1^{B}	1.4 ± 1.2^{B}
	TA2	6.1 ± 1.2^{A}	8.1 ± 0.7^{BC}	8.4 ± 0.9^{B}	7.4 ± 0.8^{A}	8.2 ± 0.5^{A}	4.8 ± 0.7^{A}	$<1^{A}$	5.3 ± 0.8^{B}	$<1^{A}$	$<2^{A}$
	TZ1	6.6 ± 0.7^{B}	7.7 ± 0.5^{AB}	7.6 ± 0.8^{A}	7.6 ± 0.9^{A}	8.6 ± 0.9^{A}	4.8 ± 1.1^{A}	1.5 ± 0.8^{B}	5.6 ± 0.5^{B}	2.3 ± 1.1^{C}	2.6 ± 1.2^{C}
	TZ2	6.4 ± 0.9^{AB}	8.2 ± 0.8^{C}	8.5 ± 1.2^{B}	7.4 ± 0.7^{A}	8.5 ± 0.7^{A}	4.7 ± 0.8^{A}	$<1^{A}$	1.7 ± 0.7^{A}	$<1^{A}$	$<2^{A}$
Statistical significance		*	*	**	NS	NS	NS	***	***	***	***
ChT_{15}	TA1	6.8 ± 0.8^{AB}	8.1 ± 0.7^{AB}	8.2 ± 0.5^{A}	7.1 ± 0.9^{A}	7.4 ± 0.8^{A}	3.5 ± 0.7^{AB}	2.4 ± 0.8^{C}	6.4 ± 0.8^{C}	$<1^{A}$	$<2^{A}$
	TA2	7.2 ± 0.7^{BC}	8.8 ± 1.1^{C}	9.1 ± 0.8^{B}	6.8 ± 0.8^{A}	7.4 ± 0.6^{A}	3.1 ± 1.2^{A}	$<1^{A}$	4.9 ± 0.6^{B}	$<1^{A}$	$<2^{A}$
	TZ1	6.7 ± 0.6^{A}	7.8 ± 0.8^{A}	8.3 ± 0.9^{A}	7.8 ± 0.9^{B}	7.8 ± 1.0^{A}	4.7 ± 0.8^{C}	1.7 ± 0.8^{B}	6.3 ± 0.4^{C}	$<1^{A}$	$<2^{A}$
	TZ2	7.4 ± 0.9^{C}	8.4 ± 0.5^{BC}	9.0 ± 0.7^{B}	6.7 ± 0.6^{A}	7.7 ± 0.7^{A}	3.6 ± 0.9^{B}	$<1^{A}$	3.2 ± 0.8^{A}	$<1^{A}$	$<2^{A}$
Statistical significance		*	**	*	**	NS	***	***	***		

[a] Abbreviations: PCA-SkM 7°C, plate count agar with skimmed milk, incubated at 7°C for detection of total psychrotrophic microorganisms; PCA-SkM 30°C, plate count agar with skimmed milk, incubated at 30°C for detection of total mesophilic microorganisms; M17 30°C, medium 17 agar incubated at 30°C for detection of mesophilic coccus LAB; M17 44°C, medium 17 agar incubated at 44°C for detection of thermophilic coccus LAB; MRS, de Man-Rogosa-Sharpe agar for detection of mesophilic rod LAB; WBAM, whey-based agar medium for detection of thermophilic rod LAB; VRBGA, violet red bile glucose agar for detection of *Enterobacteriaceae*; KAA, kanamycin esculin azide agar for detection of enterococci; CPS, coagulase-positive staphylococci; TA1, control production at the dairy factory; TZ1, control production at the dairy pilot plant; TA2, experimental production at the dairy factory; TZ2, experimental production at the dairy pilot plant; VS, vat surfaces; BMAR, bulk milk after resting; C, curds; W, whey; AC, acidified curds; ChT_0, cheese at T_0; ChT_{15}, cheese at 15 days; ND, not determined; NS, not significant.

[b] Loads are reported as log CFU/cm^2 for vat surfaces, log CFU/ml for milk and whey samples, and log CFU/g for curds and cheeses. Results are mean values ± SD for 20 plate counts (carried out in duplicate for 10 days of production). Data within a line followed by the same letter are not significantly different according to Tukey's test. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

LAB cultures, as they were Gram positive and catalase negative. The combination of characteristics evaluated for the phenotypic differentiation of the isolates allowed the separation of 1,310 cultures into 13 groups (Table 4). Only two groups included rod-shaped isolates: group I represented obligate homofermentative cultures, while group II was characterized by facultative heterofermentative metabolism. The largest number of groups was observed for cocci in short chains. In particular, groups V and XII, which included the largest numbers of cultures (410 and 239, respectively), represented almost 50% of the total isolates. Thermophilic LAB were allotted into groups I, II, and XIII.

Applying the strategy described by De Angelis et al. (22), approximately 30% of the isolates representing each phenotypic group for each sample were processed by RAPD analysis with three primers. The 411 isolates subjected to genotypic differentiation revealed the presence of 44 distinct dominant strains (Fig. 2). The dendrogram reports only 80 isolates, which are those collected at least once in different samples; the other 331 isolates were not included in the figure because they shared the same RAPD profile with other cultures from the same sample. Three major clusters were obtained. Each cluster included one *Lc. lactis* subsp. *cremoris* strain of the NWSC. Examining the origins of the isolates in terms of production and sample, it is evident that the selected *Lc. lactis* subsp. *cremoris* strains, PON36, PON153, and PON203,

TABLE 4 Phenotypic grouping of LAB isolated from wooden vat surfaces and from samples collected during experimental cheese production[a]

Characteristic	Phenotype of cluster												
	I ($n = 96$)	II ($n = 31$)	III ($n = 119$)	IV ($n = 52$)	V ($n = 410$)	VI ($n = 33$)	VII ($n = 72$)	VIII ($n = 29$)	IX ($n = 81$)	X ($n = 14$)	XI ($n = 39$)	XII ($n = 239$)	XIII ($n = 95$)
Morphology	R	R	C	C	C	C	C	C	C	C	C	C	C
Cell disposition	sc	sc	sc	sc	sc	sc	sc	sc	sc	sc	sc	sc	lc
Growth at:													
15°C	−	−	+	+	+	+	+	+	+	+	+	+	−
45°C	+	+	−	−	−	−	+	+	−	−	+	+	+
pH 9.6	ND	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	+	+	+	+	+	+	+	+	+	+	+	+	−
Production of CO_2 from glucose	−	−	−	−	−	−	+	+	+	+	−	−	−

[a] Abbreviations: R, rods; C, cocci; sc, short chains; lc, long chains; ND, not determined.

were present at dominant levels in almost all samples from the TA2 and TZ2 productions, except in milk after resting in the vat and in whey from TZ2 vats.

All 44 strains were identified by sequencing of the 16S rRNA gene (see Table S1 in the supplemental material). Except for one strain (PT433) of *Lactobacillus casei*, whose gene shared a sequence similarity of 98% with those available within the BLAST and EzTaxon databases, all other strains' 16S rRNA genes had identities of at least 99% with some of the sequences included in both databases. The strains were allotted into 10 species within the genera *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Streptococcus*. Except for two *Lc. lactis* subsp. *lactis* strains (PT132 and PT513), all other strains clustered by species (Fig. 2).

Even though the subspecies encountered most was *Lc. lactis* subsp. *lactis* (Fig. 2), lactococci, including the species *Lactococcus raffinolactis*, were consistently also present within the dominant LAB of TA1 production. LAB strains characterizing the TZ1 production included *Lc. lactis* subsp. *lactis*, *Streptococcus thermophilus*, *Leuconostoc lactis*, *Leuconostoc mesenteroides*, and *Enterococcus faecium*, in comparable proportions.

Sensory evaluation. The cheeses were evaluated for their sensory characteristics after 15 days of refrigerated storage, and the results of the analysis are shown in Table 5. Except for the spicy descriptor, which was reported differently among the judges, and the saliva-evoking characteristic, which was different among the cheeses, all other sensory attributes were not significantly different for judges and cheeses. Furthermore, several descriptors of PDO cheese reached mean values identical to those registered for the experimental cheeses produced in this study with the new vats, in particular color, oil, strength of odors, and spiciness for TA2 cheese and oil, pasture odor, taste intensity, soft/hard texture, and dispersion for TZ2 cheese. Except for pasture odor for TZ1 cheese, no other attributes scored identically between PDO and control (TA1 and TZ1) cheeses.

DISCUSSION

The use of wooden vats is mandatory for some cheeses that have the PDO status, because wooden vats allow the development of a spontaneous microbiota on the inner surface (biofilm) that has been proved to be a very efficient delivery system for LAB (23).

In this study, an *ad hoc* natural starter for the production of Vastedda-like cheese was developed with three strains of *Lactococcus*. Following the daily practice applied by the cheese makers during the management of the wooden vats, the application of NWSC represents the best strategy for generating a microbial film on traditional dairy equipment. Generally, the NWSC is prepared by overnight incubation of the whey from the previous cheese production (24), which is acidified whey. Although the *Lactococcus* starter is commonly prepared in the form of "natural milk cultures" (25), strains PON36, PON153, and PON203 were able to develop in a whey-based medium, reaching the levels typically associated with growth of the starter *Lactobacillus* and *Streptococcus* strains in the NWSC used for raw cows'-milk cheese production (26, 27).

The biofilms of the four vats after the first contact with whey were recovered through brushing, a nondestructive technique

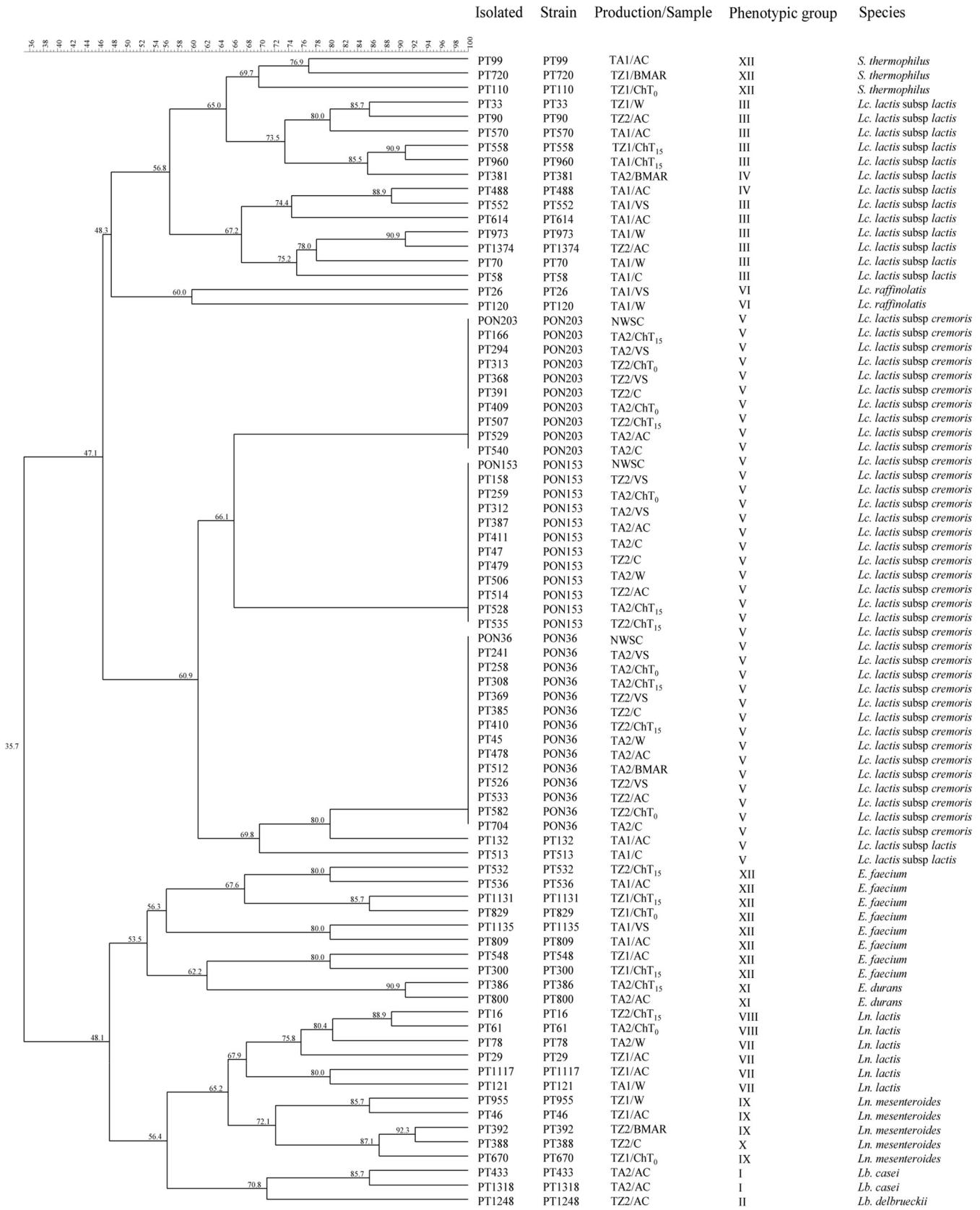

FIG 2 Dendrogram obtained with combined RAPD-PCR patterns generated with three primers for LAB strains from vats and samples collected during cheese productions. The line at the top indicates percentages of similarity. Abbreviations: *E.*, *Enterococcus*; *Lb.*, *Lactobacillus*; *Lc.*, *Lactococcus*; *Ln.*, *Leuconostoc*; *P.*, *Pediococcus*; *S.*, *Streptococcus*; TA1, control production at the dairy factory; TZ1, control production at the dairy pilot plant; TA2, experimental production at the dairy factory; TZ2, experimental production at the dairy pilot plant; VS, vat surfaces; BMAR, bulk milk after resting; C, curds; W, whey; AC, acidified curds; ChT_0, cheese at T_0; ChT_{15}, cheese at 15 days.

TABLE 5 Sensory evaluation of Vastedda-like cheeses[a]

Attribute	Mean score for cheese samples						Significance of differences[b]	
	PDO cheese	TZ1	TZ2	TA1	TA2	SEM	Judges	Cheese
Color	**3.45**	3.55	3.36	3.64	**3.45**	0.26	NS	NS
Oil	**1.09**	1.18	**1.09**	1.00	**1.09**	0.09	NS	NS
Eyes	1.64	1.73	1.55	1.91	1.45	0.23	NS	NS
Uniformity	1.45	1.82	1.27	1.73	1.36	0.20	NS	NS
Strength of odors	**2.36**	3.09	2.45	2.91	**2.36**	0.26	NS	NS
Pasture odor	**1.18**	**1.18**	**1.18**	1.36	1.00	0.12	NS	NS
Pungent odor	1.27	1.45	1.36	1.45	1.36	0.18	NS	NS
Taste intensity	**1.73**	2.00	**1.73**	2.18	1.91	0.29	NS	NS
Salty taste	1.64	1.73	1.45	1.73	1.55	0.22	NS	NS
Bitter taste	1.36	1.55	1.27	1.73	1.27	0.20	NS	NS
Spicy taste	**1.18**	1.00	1.09	1.36	**1.18**	0.12	*	NS
Soft/hard	**3.91**	3.73	**3.91**	4.18	4.00	0.27	NS	NS
Saliva evoking	1.27	1.36	1.18	2.36	1.36	0.19	NS	**
Dispersion	**3.09**	2.91	**3.09**	3.36	3.18	0.37	NS	NS

[a] Abbreviations: TA1, control production at the dairy factory; TZ1, control production at the dairy pilot plant; TA2, experimental production at the dairy factory; TZ2, experimental production at the dairy pilot plant; SEM, standard error of the mean; NS, not significant. Values reported in bold were identical for PDO cheese and experimental cheeses.

[b] *, $P \leq 0.05$; **, $P \leq 0.01$.

commonly applied for the microbiological investigation of wooden surfaces (28). The absence of clostridia, coagulase-positive staphylococci, *E. coli*, *L. monocytogenes*, *Pseudomonas* spp., and *Salmonella* spp. in the biofilms analyzed was probably due to the acidic conditions generated by the development of LAB in whey, which inhibit the adhesion and survival of several microorganisms (4, 7). The estimated levels of LAB in the neoformed biofilms were comparable to those reported for the wooden vats used for years to produce traditional cheeses (4–8, 19). Except for the vats activated with the NWSC, for which the dominance of LAB cocci was undoubtedly due to the added lactococci, a consistent dominance of LAB cocci over rods was also observed for the vats activated with the whey acidified spontaneously. Thus, it is possible to state that the common observation of a dominance of LAB cocci in the wooden vat biofilms used to produce several cheeses in different countries (6, 8, 19) starts during the first steps of their activation with whey. Furthermore, it is worth noting that the mixed *Lc. lactis* subsp. *cremoris* strain culture quickly formed the exopolysaccharide matrix typical of a biofilm structure (29).

The wooden vat activation and the cheese trials were carried out in February 2014 under an uncontrolled temperature regimen to mimic the dairy factory environmental conditions. The choice of carrying out the experimentation during one of the coldest months in Sicily was because temperatures in the warm seasons do not influence the production of Vastedda cheese, while the production can be negatively affected by low temperatures in winter (17).

The microbial concentrations in the milk in the wooden vats were on the same orders of magnitude as those reported during the production of other raw ewes'-milk cheeses (30). The levels of the microorganisms in the milk after contact with the wooden vat surfaces were affected by the microbial films only when their levels before contact were low. In particular, this observation confirmed the previous finding that the levels of LAB in milk are consistently influenced by wooden vat biofilms when their cell densities before contact with the vat surfaces are lower than 6 log CFU/ml (19).

The curds of all productions were acidified at ambient temperature until the pH value was in the range of 5.2 to 5.4, representing the level of acidity needed for curd stretching (31). All curds from wooden vats TA2 and TZ2 could be stretched within 24 h after transfer into perforated containers, a behavior compatible with the indications of the protocol for production.

The microbial increase registered in curds followed the general trend observed after curdling (7, 30). The presence of *E. coli* in the curds of the dairy factory reflected the hygienic quality of the bulk milk quantities used for cheese production. The microbial levels of total mesophilic microorganisms, mesophilic and thermophilic LAB cocci, and thermophilic LAB rods in the cheeses at T_0 and their evolution during refrigerated storage were comparable to the results reported by Gaglio et al. (17) when the multistrain inoculum composed of *Lc. lactis* subsp. *cremoris* PON36, PON153, and PON203 was selected as the starter culture. The increase in the levels of mesophilic LAB cocci registered during the refrigerated storage of the cheeses from TA2 and TZ2 reflected the ability of the selected lactococci added as starter strains to grow at low temperatures (17). The other microbial groups investigated in this study were not investigated in the work of Gaglio et al. (17). However, levels of mesophilic LAB rods and enterococci were on the same orders of magnitude as those reported for commercial Vastedda cheeses (15, 16). The presence of enterococci in raw ewes'-milk cheeses is quite common, and although these organisms are associated with cheese typicality (32), their presence in cheese is considered an indicator of a low hygienic quality of the dairy products (33), and for this reason, their number should be limited in the final cheese product. In our study, the levels of enterococci were much lower in the cheeses made in the TZ2 vat both at T_0 and at 15 days, but this finding has to be attributed to the milk quality rather than to the action of the NWSC. In light of Commission Regulation (EC) no. 2073/2005 (34), with further modifications in the microbiological criteria for foodstuffs, which are regarded as process hygiene criteria, *E. coli* and coagulase-positive staphylococci were not present in cheese at T_0, confirming that this was directly attributable to the presence of the starter lactococci.

RAPD analysis clearly demonstrated the dominance of the added *Lactococcus* strains during Vastedda-like cheese production. The genetic identification demonstrated that all species found belonged to the dairy LAB group (35). In particular, the majority of the strains characterized in this work represented mesophilic starter LAB (3). Since only three thermophilic strains (PT99, PT110, and PT720) were identified, this work confirmed previous observations that winter production of Vastedda cheeses is carried out mainly by mesophilic LAB species (15, 17).

The addition of the NWSC consistently reduced the biodiversity of the dominant LAB during cheese making under both production conditions (uncontrolled conditions, dairy factory plus low-quality milk; and controlled conditions, pilot plant plus high-quality milk). Thus, the strategy applied in this study might limit the variability during the production of cheeses due to the dominance of adventitious LAB, a phenomenon that cannot be controlled by cheese makers (36). The uncontrolled development of different strains may determine the differences among cheeses even when they are produced at the same factory in different vats on the same day (37, 38).

Sensory evaluation showed that the microbial activation of the wooden vat surfaces with the multistrain culture enabled the production of cheeses that had sensory characteristics similar to those available commercially. Thus, as in the previous work of Gaglio et al. (17), this study showed that the addition of *Lc. lactis* subsp. *cremoris* PON36, PON153, and PON203 during traditional Vastedda cheese production carried out in wooden vats enabled the production of cheese that was typical, even with the use of bulk milk quantities of different origins and with transformation in different dairy environments.

In conclusion, this study demonstrated that the addition of NWSC enables the rapid formation of biofilms incorporating the starter strains and that the neoformed biofilms reduce the microbiological variability of Vastedda cheeses. Hence, this strategy will be provided to the consortium for the production of PDO Vastedda della Valle del Belìce cheese in order to stabilize the sensorial attributes of cheese throughout the year.

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