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Transfer, composition and technological characterization of the lactic acid bacterial populations of the wooden vats used to produce traditional stretched cheeses

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ABSTRACT

The biofilms of 12 wooden vats used for the production of the traditional stretched cheeses Caciocavallo Palermitano and PDO Vastedda della valle del Belìce were investigated. *Salmonella* spp. and *Listeria monocytogenes* were never detected. Total coliforms were at low numbers with *Escherichia coli* found only in three vats. Coagulase-positive staphylococci (CPS) were below the enumeration limit, whereas lactic acid bacteria (LAB) dominated the surfaces of all vats. In general, the dominance was showed by coccus LAB. Enterococci were estimated at high numbers, but usually between 1 and 2 Log cycles lower than other LAB. LAB populations were investigated at species and strain level and for their technological properties relevant in cheese production. Eighty-five strains were analysed by a polyphasic genetic approach and allotted into 16 species within the genera *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus*. *Enterococcus faecium* was found in all wooden vats and the species most frequently isolated were *Enterococcus faecalis*, *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Pediococcus acidilactici* and *Streptococcus thermophilus*. The study of the quantitative data on acidification rate, autolysis kinetics, diacetyl production, antibacterial compound generation and proteolysis by cluster and principal component analysis led to the identification of some strains with promising dairy characteristics. Interestingly, a consistent percentage of LAB was bacteriocin-like inhibitory substances (BLIS) producer. Thus, the microbial biofilms of the wooden vats analysed in this study might contribute actively to the stability of the final cheeses.

1. Introduction

Traditional Italian cheeses are often manufactured with raw milk without the addition of commercial or natural starter cultures using wooden equipment. In order to transform milk into cheese, the presence of lactic acid bacteria (LAB) is needed (Settanni and Moschetti, 2010). During cheese production, LAB are distinct in two groups: starter LAB (SLAB), responsible for the acidification of curd; non starter LAB (NSLAB), implicated in the maturation.

When selected starter cultures are not inoculated in milk, the main sources of desirable LAB are generally the milk, the equipment used during processing and the dairy environment (Beresford et al., 2001; Franciosi et al., 2008). Lortal et al. (2009) assessed that lactic acid is produced both by the natural milk microbiota and that from the biofilms of the wooden vat surfaces.

The US Food and Drug Administration declared that "the structure of the wood as porous, would absorb and trap bacteria that may contaminate food products", during the presentation of the advice about Italian and French cheeses ripened on wooden planks (Cutini, 2014). The Commission Regulation (EC) No 2074/2005 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" (Commission Regulation, 2005a). Furthermore, it has been demonstrated that the aging on wooden planks reduces

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the presence of bacteria dangerous for humans, such as *Listeria monocytogenes*, showing instead a potential of wood for bio-protection against food pathogens (Mariani et al., 2011). Since several traditional cheeses are made in wooden vats, the microbial characterization of these recipients has been the object of different research groups, mainly Italian and French, in the last few years (Licitra et al., 2007; Lortal et al., 2009; Didienne et al., 2012; Settanni et al., 2012; Scatassa et al., 2015). They found LAB at dominant levels, while the undesired microorganisms, including coliforms and coagulase-positive staphylococci (CPS), were at very low densities and, overall, the presence of the pathogenic bacteria *L. monocytogenes* and *Salmonella* spp. was never revealed.

The wooden vats used to produce different raw cow's milk pasta-filata cheeses in Sicily (southern Italy) have been found to host high levels of LAB, mainly belonging to the species *Streptococcus thermophilus* (Licitra et al., 2007; Settanni et al., 2012; Scatassa et al., 2015). Pasta-filata technology consists of a first acidification step and a subsequent scalding of the acidified curd to be moulded into the final shape (Salvadori del Prato, 1998). The dominant role of wooden vat SLAB over the indigenous LAB hosted in the bulk milk has been demonstrated for cheeses manufactured with this technology (Lortal et al., 2009; Settanni et al., 2012).

The analysis of the composition of the biofilms associated with the wooden vats used to produce different cheeses revealed the presence of several dairy LAB, including the SLAB species *Lactobacillus helveticus*, *Lactococcus lactis* and *Leuconostoc mesenteroides* and different NSLAB such as *Lactobacillus plantarum* and *Lactobacillus casei* (Didienne et al., 2012; Scatassa et al., 2015). Settanni et al. (2012) also evidenced the presence of other NSLAB in the wooden vat biofilms, in particular enterococci, that are considered important for the typicality of traditional cheeses (Foulquié Moreno et al., 2006), even though the safety status of some species of the *Enterococcus* genus must be considered. Recently, enterococci of wooden vat origin were detected in ripened cheeses, demonstrating the ability to follow the different stages of cheese making and to persist during ripening, thus influencing the characteristics of the final products (Di Grigoli et al., 2015).

Although the dominance and the role of the SLAB of wooden vat origin has been verified for pasta-filata cheeses made with raw cow's milk (Settanni et al., 2012), the contribution of the wooden vat SLAB during the manufacture of raw ewe's milk cheeses has not been investigated yet. Furthermore, very little is known on the potential of the wooden vat NSLAB during cheese ripening. Since the wooden vat biofilms are living systems whose activities are defining during cheese production, an investigation of the characteristics of SLAB and NSLAB of these ecosystems deserves attention.

In a previous work of ours (Scatassa et al., 2015), carried out mainly to investigate on the safety and hygiene aspects of several wooden vats used in western Sicily, the dominant communities of the microbial biofilms were identified as members of LAB. Based on the main findings of this preliminary study (Scatassa et al., 2015) and in order to better investigate the wooden vats for the presence of LAB useful for cheese fermentation as well as during ripening, we sampled a different set of wooden vats used to produce two different pasta-filata cheeses in the same area. In addition, we examined the levels of the transfer of the microbial populations from the wooden vats to milk, the numbers of LAB present on the wooden vats, the strains and the species dominating the biofilms, and their technological dairy traits *in vitro*.

2. Materials and methods

2.1. Biofilm collection

Twelve wooden vats (Table 1) used in 11 dairy factories located in western Sicily (Italy) producing two different pasta-filata (stretched) cheese typologies, Caciocavallo Palermitano cheese and PDO Vastedda della valle del Belìce cheese, obtained with raw cows' and raw ewes' milk, respectively, without the addition of starter cultures, were microbiologically investigated. Vat surfaces (400 cm^2) were sampled, just before cheese production took place, as reported by Didienne et al. (2012) using UV-treated paper squares positioned halfway up the side and on the bottom. Milk samples were collected to evaluate the transfer of the major populations of the vat biofilms to milk. To this purpose, milks were sampled before (milk before contact, MBC) and after 5 min of contact with the vat surfaces (milk after contact, MAC), as reported by Didienne et al. (2012). MBC and MAC bulks were subjected to agitation before sampling. The samples were collected in duplicate at two week intervals in the spring season 2012. All samples were transported to the laboratory, under refrigeration with a portable fridge, where they were immediately analysed.

2.2. Microbiological analyses

Cell suspensions of wooden vat surface and milk samples were subjected to decimal serial dilutions in Ringer's solution (Sigma–Aldrich, Milan, Italy) and then plated and incubated as follows: total mesophilic count (TMC) bacteria were plated on plate-count agar (PCA) supplemented with 1 g L^{-1} skimmed milk (SkM) and incubated aerobically for 72 h at 30 °C; enterococci on kanamycin aesculin azide (KAA) agar, incubated aerobically at 37 °C for 24 h; mesophilic and thermophilic rod-shaped LAB on de Man-Rogosa-Sharpe (MRS) agar, acidified to pH 5.4 with lactic acid (5 mol L^{-1}) and incubated anaerobically for 48 h at 30 and 44 °C, respectively; mesophilic and thermophilic coccus-shaped LAB on M17 agar, and incubated anaerobically for 48 h at 30 and 44 °C, respectively.

In light of the Commission Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs (Commission Regulation, 2005b), *Salmonella* spp. and *L. monocytogenes* were analysed as food safety criteria, while *Escherichia coli* and CPS as process hygiene criteria. For milk samples, total coliforms were analysed applying the ISO 4832 (2006), *E. coli* the ISO 16649–2 (2001), CPS the ISO 6888-2 (1999) and Amend 1 (2003), *Salmonella* spp. with the screening method AFNOR BIO 12/22-05/07 (2007), an enzyme immunoassay Enzyme Linked Fluorescent Assay (ELFA) performed with the automated system VIDAS (bioMérieux, Marcy l'Etoile, France), and *L. monocytogenes* by the method ELFA AFNOR BIO 12/11-03/04 (2004). Vat surfaces were analysed for total coliforms, *L. monocytogenes* and *Salmonella* spp. as reported above, while *E. coli* was investigated on tryptone bile glucuronide (TBG) agar, incubated for 24 h at 44 °C, and CPS on Baird Parker supplemented with RPF, incubated for 48 h at 37 °C. All media were purchased from Oxoid (Milan, Italy). Microbiological counts were performed in triplicate.

2.3. Isolation and phenotypic grouping of LAB

Four colonies of presumptive LAB (Gram-positive, determined by KOH method, and catalase negative, determined by transferring fresh colonies from a Petri dish to a glass slide and adding 5%, w/v, H_2O_2) for each morphology (colour, margin, surface and elevation) were isolated from Petri dishes inoculated with the highest plated dilutions that generated a number of colonies in the range 30–300 on MRS and M17 and 20–200 on KAA. All different morphologies were considered in order to evaluate the total LAB diversity. The colonies were transferred into the corresponding broth media, except for the isolates from KAA which were inoculated in M17 broth. The isolates were then purified with successive sub-

Table 1
Characteristics of the wooden vats.

Wooden vat	City of dairy factory (province)[a]	Age of vat (years)	Type of wood	Cheese	Milk processed	Milk volume (L)	Type of washing
1	Godrano (PA)	28	chestnut	Caciocavallo Palermitano	Bovine	160	HDW (in winter); CW (in summer)
2	Godrano (PA)	10	chestnut	Caciocavallo Palermitano	Bovine	400	HDW
3	Santa Margherita del Belìce (AG)	5	douglas	Vastedda della valle del Belìce	Ovine	200	HDW
4	Menfi (AG)	5	douglas	Vastedda della valle del Belìce	Ovine	250	CW
5	Terrasini (PA)	10	chestnut	Caciocavallo Palermitano	Bovine	170	HDW (in winter); CW (in summer)
6	Cinisi (PA)	10	chestnut	Caciocavallo Palermitano	Bovine	300	HDW
7	Godrano (PA)	10	chestnut	Caciocavallo Palermitano	Bovine	250	HDW
8	Terrasini (PA)	10	chestnut	Caciocavallo Palermitano	Bovine	300	HDW (in winter); CW (in summer)
9	Salemi (TP)	5	douglas	Vastedda della valle del Belìce	Ovine	190	CW
10	Salemi (TP)	7	douglas	Vastedda della valle del Belìce	Ovine	190	CW
11	Partanna (TP)	5	douglas	Vastedda della valle del Belìce	Ovine	150	HDW
12	Godrano (PA)	20	chestnut	Caciocavallo Palermitano	Bovine	220	HDW (in winter); CW (in summer)

Abbreviations: HDW, Hot deproteinized whey (whey resulting after separation of whey proteins coagulated by thermal treatment); CW, cold water.
[a] AG, Agrigento; PA, Palermo; TP, Trapani.

culturing and stored in broth media containing 20% glycerol (v/v) at −80 °C until further analysis.

Phenotypic characterization was carried out to obtain an initial grouping of the isolates as reported by Gaglio et al. (2014) based on cell morphology, growth at 15 and 45 °C, resistance at 60 °C for 30 min, NH_3 production from arginine, aesculin hydrolysis, acid production from the carbohydrates reported by Di Grigoli et al. (2015), and CO_2 production from glucose following the method described by Settanni et al. (2012). The coccus-shaped isolates were further grouped by their ability to grow at pH 9.6 and in the presence of NaCl (6.5 g/L) to separate enterococci, which are able to grow in both conditions, from other LAB.

2.4. Strain differentiation and identification of LAB

Genomic DNA for PCR assays was prepared from LAB isolates after their overnight growth in broth media at 30 °C. Cells were harvested and DNA was extracted using an InstaGene Matrix kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Crude cell extracts were used as templates for PCR.

Strain differentiation was performed with random amplification of polymorphic DNA (RAPD)-PCR analysis as previously described by Settanni et al. (2012) using single primers M13 (Stenlid et al., 1994), AB111, and AB106 (van den Braak et al., 2000). Genotypic identification of the different LAB strains was carried out by 16S rRNA gene sequencing as reported by Di Grigoli et al. (2015). The resulting DNA was sequenced by Istituto Zooprofilattico Sperimentale della Sicilia "A. Mirri" (IZS – Palermo, Italy) using the same primers employed for the PCR amplifications. The sequences were compared with those available in the GenBank/EMBL/DDBJ (http://www.ncbi.nlm.nih.gov) (Altschul et al., 1997) and EzTaxon-e (http://eztaxon-e.ezbiocloud.net/) (Chun et al., 2007) databases. The last database compares a given sequence to those of type strains only. The isolates were considered to represent the species in question if 97% or higher similarity was detected (Stackebrandt and Goebel, 1994). However, the multiplex PCR assay based on the *sodA* gene reported by Jackson et al. (2004) was applied to better classify *Enterococcus* species when a discrepancy between the two databases was found or when a homology very closed to 97%, in at least one database, was observed. Similarly, lactobacilli were analysed by the *rrn* operon based multiplex PCR reported by Settanni et al. (2005).

2.5. Characterization of the technological properties of LAB

The different LAB strains were tested for some of the technological traits useful during cheese production: acidification capacity; diacetyl formation; autolytic properties; proteolytic activities; and production of antimicrobial compounds.

The acidifying capacity was assayed at the optimal growth temperature for each strain in 100 mL of full fat, ultra-high temperature (UHT) milk inoculated with a 1% (v/v) cell suspension obtained by growing the cultures overnight in their optimal medium, centrifuging at 5000 × g for 5 min, washing and re-suspending in Ringer's solution. To standardise bacterial inocula, the cells were re-suspended in Ringer's solution to an optical density at 600 nm of 1.00, corresponding to approximately 10^9 CFU mL^{-1}, as measured with a 6400 Spectrophotometer (Jenway Ltd., Felsted, Dunmow, UK) and confirmed by plate count with the optimal media. The incubations were at 30 or 44 °C for mesophilic and thermophilic strains, respectively. The pH was measured on aliquots of 4 mL, aseptically collected from each test flask, at 2-h intervals for the first 8 h, and then at 24, 48 and 72 h after inoculation and incubation at 30 and 44 °C.

Diacetyl production was determined as described by King (1948). LAB were inoculated in UHT milk and after the incubation at 30 °C for 24 h, aliquots of 1 mL were added to 0.5 mL of α-naphthol (1%, w/v) and KOH (16%, w/v) and maintained at 30 °C for 10 min. Diacetyl generation was indicated by the formation of a red ring at the top of the tube.

The autolysis of whole cells was determined applying the method described by Mora et al. (2003). Overnight cultures developed in their optimal media were washed as reported above and re-suspended into potassium phosphate buffer (50 mmol L^{-1}, pH 6.5). OD_{600} was measured at 2-h intervals for the first 8 h and then 24, 48, and 72 h after inoculation.

The extracellular protease activity of LAB was determined with the method described by Vermelho et al. (1996). Bovine serum albumin (BSA) and gelatine (Sigma–Aldrich) were incorporated into agar plates (MRS or M17, depending on the medium of growth and isolation of the strains to be tested) at 1% (w/v) and used as protease substrates.

The antimicrobial activity of LAB was first detected by the

agar-spot deferred method using bacterial colonies, and the strains displaying positive results were subsequently tested with the well diffusion assay (WDA) using their cell-free supernatants, adjusted to pH 6.5 with 1 mol L^{-1} NaOH and treated with catalase (1 mg mL^{-1}) to eliminate the inhibitory effect of lactic acid and/or H_2O_2 (Schillinger and Lücke, 1989). Both assays (in triplicate) were performed as modified by Corsetti et al. (2008). *Listeria innocua* 4202, *L. monocytogenes* ATCC 19114 and *Lactobacillus sakei* 2313 were used as indicator strains. The supernatants showing inhibitory properties were subjected to the action of proteolytic enzymes: proteinase K (12.5 U/mg); protease B (45 U/mg); and trypsin (10.6 U/mg) at a final concentration of 1 mg mL^{-1} in phosphate buffer (pH 7.0). The tests for residual activities were performed as reported by Corsetti et al. (2004) by WDA after 2 h at 37 °C. All enzymes were purchased from Sigma–Aldrich.

2.6. Statistical and multivariate analyses

Statistical analyses of microbiological counts were conducted using STATISTICA software (StatSoft Inc., Tulsa, OK, USA). Microbial data were analysed using a generalised linear model (GLM) that included the effects of the wooden vat. Data were converted to the Log scale after statistical elaborations. Differences between means were determined by the *post-hoc* Tukey's multiple-range test. A P-value <0.05 was deemed significant.

Data from the acidification rate, autolysis kinetics, production of diacetyl, antibacterial assay and proteolytic activities of LAB were subjected to multivariate analysis to evaluate the relationship among LAB strains, as well as their dairy potential.

To this purpose, a hierarchical cluster analysis (HCA) (joining, dendrogram clustering) was carried out for grouping the LAB strains according to their similarity, measured by Euclidean distances, whereas cluster aggregation was based on the single linkage method (Todeschini, 1998).

The input matrix used for HCA was subjected also to a principal component analysis (PCA) for grouping the strains into homogeneous groups according to their activities. The analysis was performed with the average quantitative activity data of the 85 LAB strains assayed in this work. The number of principal factors was selected according to the Kaiser criterion (Jolliffe, 1986) and only factors with eigen-values higher than 1.00 were retained. Cases introduced in the analysis were the 85 strains, while explanatory variables were the five technological traits considered. Statistical data processing and graphic construction were achieved by using STATISTICA software.

3. Results and discussion

3.1. Microbiological analyses

In this study, the brushing recovery method (Didienne et al., 2012) was applied to investigate the microbial biofilms of the wooden vats. Among non-destructive techniques, brushing is being commonly applied for the microbiological analyses of the wooden surfaces in direct contact with raw materials and food products (Ismaïl et al., 2015).

Table 2 shows the viable counts of the microbial groups harboured on the vat surfaces before milk was added and those present in milk before and after contact with the wooden containers. The results of *Salmonella* spp. and *L. monocytogenes* are not reported in table, because no surface and milk sample was scored positive for their presence. Lortal et al. (2009) assessed that the inability of pathogens to adhere or to survive in wooden vat biofilms is mainly due to the acidic conditions, determined by LAB that ferment lactose from the residual whey (Settanni et al., 2012), the competition for nutrients and the high temperatures applied for cheese cooking.

Average TMC of wooden vats was in the range 4.3–6.4 Log CFU cm^{-2}. In general, the surfaces of the vats hosted low numbers of total coliforms, except WV4 for which this bacterial group was registered at the same level of TMC. WV4 was also characterized for the highest cell density of *E. coli* (3.2 Log CFU cm^{-2}). This bacterium was also found in measurable populations in the inner surfaces of the vats WV5 and WV12, but, in general, low levels of *E. coli* were registered in this study. A similar observation was reported by Didienne et al. (2012). In all vats, CPS counts were below the enumeration limit. A low frequency of this microbial group was reported for the wooden vat biofilms analysed for pasta-filata cheeses (Lortal et al., 2009; Didienne et al., 2012; Scatassa et al., 2015).

All the wooden vat surfaces harboured high numbers of LAB. The majority of biofilms showed the presence of coccus LAB at dominant levels, while the sample WV9 showed mesophilic and thermophilic rod LAB at almost 1 Log cycle higher than cocci. Sample WV7 displayed the highest cell density of LAB, since mesophilic cocci were detected at 6.9 Log CFU cm^{-2}. High numbers of LAB were already reported in previous wooden vat biofilm inspections (Licitra et al., 2007; Lortal et al., 2009; Didienne et al., 2012; Settanni et al., 2012; Scatassa et al., 2015) and the dominance of cocci over rods is common to other studies. E.g. Didienne et al. (2012) detected lactococci at higher levels than lactobacilli for the majority of the vats analysed. Within the coccus LAB community, thermophilic bacteria were found at dominant levels onto the surfaces of the vats used for raw cows' milk stretched cheese production in Sicily (Lortal et al., 2009; Settanni et al., 2012; Scatassa et al., 2015).

Enterococci, detected in all wooden vat samples, were generally enumerated at lower levels than the LAB counted on M17 and MRS and the highest numbers were found for WV4, which was filled in with ewe's milk. No big differences, on average, were registered for the levels of enterococci detected in the wooden vats used to transform different types of milk, even though some of the lower concentrations were found for raw cow's milk. The levels of enterococci found in this study were in the same order of magnitude of only one of the wooden vats used for making Ragusano cheese (Lortal et al., 2009).

Milk samples before contact with the wooden vat surfaces were all dominated by LAB. In fact, the levels of TMC and LAB were almost comparable. Interestingly, four samples (MBC1, MBC4, MBC5 and MBC11) showed enterococci at the same level of rod LAB and the samples MBC6 and MBC9 hosted almost the same cell densities of enterococci and both rod and coccus LAB. Total coliforms, *E. coli* and CPS were investigated in milk only after resting in the wooden vats. After contact, total coliforms were in the range 1.5–5.9 Log CFU mL^{-1} and the highest levels were reached for sample MAC4, for which also the density of *E. coli* was the highest. *E. coli* was below the enumeration limit of plate count method in samples MAC1, MAC2, MAC6, MAC9 and MAC10. CPS, undetectable in all wooden vat biofilms were, instead, present in all milks with the highest level (4.2 Log CFU mL^{-1}) registered for the sample MAC4. The comparison with TMC indicated the dominance of LAB, including enterococci, over the other microbial groups investigated. A general trend was observed after contact with the wooden vat surfaces: the levels of LAB increased for all those samples of milk characterized by levels lower than 6 Log CFU mL^{-1} at delivery. The best example was provided by the sample MAC9 whose levels of mesophilic and thermophilic rod LAB were barely 3.6 and 3.9, respectively, before contact, but increased strongly (at 5.9 and 5.6 Log CFU mL^{-1}, respectively) after resting in the wooden

Table 2
LAB concentrations[a] in biofilms of wooden vats and milk before and after contact with the vat surfaces.

Sample	Bacterial counts								
	TMC	Total coliforms	*E. coli*	CPS	Enterococci	Rod LAB MRS-30 °C	Rod LAB MRS-44 °C	Coccus LAB M17-30 °C	Coccus LAB M17-44 °C
Wooden vat:									
WV1	4.9 ± 0.2^{AB}	0.3 ± 0.0^{A}	$<1^{A}$	$<1^{A}$	2.3 ± 0.2^{A}	4.7 ± 0.2^{B}	4.9 ± 0.2^{CD}	5.3 ± 0.3^{B}	5.9 ± 0.1^{C}
WV2	5.7 ± 0.2^{C}	2.3 ± 0.1^{CD}	$<1^{A}$	$<1^{A}$	3.2 ± 0.1^{BC}	3.9 ± 0.2^{A}	5.4 ± 0.2^{D}	5.4 ± 0.2^{BC}	5.7 ± 0.2^{C}
WV3	4.5 ± 0.3^{AB}	0.9 ± 0.1^{B}	$<1^{A}$	$<1^{A}$	3.2 ± 0.2^{BC}	3.6 ± 0.1^{A}	3.0 ± 0.1^{A}	4.9 ± 0.1^{AB}	5.2 ± 0.1^{BC}
WV4	6.1 ± 0.2^{CD}	6.0 ± 0.1^{E}	3.2 ± 0.2^{C}	$<1^{A}$	4.4 ± 0.1^{D}	5.8 ± 0.2^{C}	5.9 ± 0.1^{DE}	6.2 ± 0.2^{C}	6.3 ± 0.1^{CD}
WV5	6.1 ± 0.1^{CD}	3.2 ± 0.1^{D}	2.5 ± 0.2^{B}	$<1^{A}$	2.9 ± 0.1^{B}	4.8 ± 0.2^{B}	4.7 ± 0.2^{C}	6.2 ± 0.2^{C}	5.8 ± 0.1^{C}
WV6	5.0 ± 0.1^{B}	0.7 ± 0.0^{AB}	$<1^{A}$	$<1^{A}$	4.2 ± 0.2^{D}	4.8 ± 0.2^{B}	4.9 ± 0.1^{CD}	5.8 ± 0.2^{C}	5.6 ± 0.1^{BC}
WV7	6.4 ± 0.1^{D}	0.7 ± 0.1^{AB}	$<1^{A}$	$<1^{A}$	4.0 ± 0.0^{CD}	4.1 ± 0.1^{A}	5.8 ± 0.2^{DE}	6.9 ± 0.1^{D}	6.5 ± 0.2^{D}
WV8	5.4 ± 0.1^{BC}	2.0 ± 0.2^{C}	$<1^{A}$	$<1^{A}$	2.2 ± 0.2^{A}	5.1 ± 0.1^{B}	5.1 ± 0.1^{CD}	5.7 ± 0.1^{BC}	5.5 ± 0.1^{BC}
WV9	5.7 ± 0.1^{C}	1.8 ± 0.1^{C}	$<1^{A}$	$<1^{A}$	3.5 ± 0.1^{C}	6.0 ± 0.1^{C}	6.1 ± 0.1^{E}	4.6 ± 0.2^{A}	5.3 ± 0.1^{BC}
WV10	4.3 ± 0.1^{A}	0.6 ± 0.1^{AB}	$<1^{A}$	$<1^{A}$	3.4 ± 0.1^{BC}	4.6 ± 0.1^{B}	4.9 ± 0.1^{CD}	4.5 ± 0.2^{A}	3.6 ± 0.1^{A}
WV11	5.2 ± 0.1^{BC}	1.4 ± 0.1^{BC}	$<1^{A}$	$<1^{A}$	2.5 ± 0.1^{AB}	3.8 ± 0.1^{A}	4.0 ± 0.1^{B}	4.9 ± 0.1^{AB}	5.1 ± 0.2^{B}
WV12	5.4 ± 0.1^{BC}	2.8 ± 0.2^{D}	2.0 ± 0.1^{B}	$<1^{A}$	3.3 ± 0.1^{BC}	5.2 ± 0.1^{B}	5.0 ± 0.1^{CD}	5.4 ± 0.2^{BC}	5.5 ± 0.2^{BC}
Statistical significance	***	***	***		***	***	***	***	***
Milk before contact:									
MBC1	5.4 ± 0.2^{CD}	nd	nd	nd	4.4 ± 0.1^{CD}	4.9 ± 0.1^{C}	4.8 ± 0.1^{B}	5.0 ± 0.2^{B}	5.1 ± 0.1^{BC}
MBC2	6.5 ± 0.2^{E}	nd	nd	nd	6.8 ± 0.3^{F}	5.9 ± 0.2^{DE}	6.2 ± 0.2^{D}	6.5 ± 0.3^{DE}	6.5 ± 0.1^{DE}
MBC3	7.1 ± 0.1^{F}	nd	nd	nd	4.1 ± 0.2^{C}	7.2 ± 0.1^{F}	7.1 ± 0.2^{E}	7.0 ± 0.1^{F}	7.1 ± 0.1^{F}
MBC4	6.2 ± 0.1^{DE}	nd	nd	nd	5.1 ± 0.1^{DE}	5.5 ± 0.1^{D}	5.4 ± 0.2^{C}	6.1 ± 0.3^{CD}	6.1 ± 0.2^{D}
MBC5	5.4 ± 0.1^{C}	nd	nd	nd	3.7 ± 0.3^{BC}	3.7 ± 0.2^{AB}	4.1 ± 0.1^{A}	5.6 ± 0.1^{C}	4.8 ± 0.2^{B}
MBC6	4.8 ± 0.1^{B}	nd	nd	nd	4.7 ± 0.1^{D}	4.2 ± 0.1^{B}	4.3 ± 0.1^{AB}	4.5 ± 0.2^{B}	4.4 ± 0.1^{AB}
MBC7	5.8 ± 0.2^{CD}	nd	nd	nd	5.6 ± 0.2^{E}	6.1 ± 0.2^{E}	6.1 ± 0.1^{D}	6.2 ± 0.1^{D}	6.0 ± 0.3^{D}
MBC8	7.1 ± 0.2^{F}	nd	nd	nd	4.4 ± 0.1^{CD}	6.9 ± 0.2^{F}	6.9 ± 0.2^{E}	6.8 ± 0.1^{EF}	6.8 ± 0.1^{EF}
MBC9[b]	4.0 ± 0.2^{A}	nd	nd	nd	3.4 ± 0.2^{AB}	3.6 ± 0.3^{A}	3.9 ± 0.2^{A}	3.7 ± 0.1^{A}	3.9 ± 0.2^{A}
MBC10[b]	4.0 ± 0.2^{A}	nd	nd	nd	3.4 ± 0.2^{AB}	3.6 ± 0.3^{A}	3.9 ± 0.2^{A}	3.7 ± 0.1^{A}	3.9 ± 0.2^{A}
MBC11	5.0 ± 0.1^{BC}	nd	nd	nd	3.5 ± 0.1^{B}	3.7 ± 0.2^{AB}	3.8 ± 0.2^{A}	4.9 ± 0.1^{B}	5.0 ± 0.2^{BC}
MBC12	5.6 ± 0.2^{C}	nd	nd	nd	3.1 ± 0.1^{A}	5.4 ± 0.1^{CD}	5.6 ± 0.2^{C}	5.6 ± 0.1^{C}	5.4 ± 0.1^{C}
Statistical significance	***				***	***	***	***	***
Milk after contact:									
MAC1	5.5 ± 0.2^{B}	1.5 ± 0.1^{A}	$<1^{A}$	3.2 ± 0.2^{C}	5.0 ± 0.2^{BC}	5.6 ± 0.2^{C}	5.6 ± 0.2^{C}	6.1 ± 0.3^{C}	6.2 ± 0.2^{BC}
MAC2	6.2 ± 0.1^{C}	3.4 ± 0.1^{C}	$<1^{A}$	2.3 ± 0.1^{B}	6.5 ± 0.1^{D}	5.7 ± 0.1^{CD}	6.2 ± 0.3^{D}	6.5 ± 0.2^{CD}	6.5 ± 0.1^{C}
MAC3	7.4 ± 0.1^{E}	3.1 ± 0.1^{BC}	2.7 ± 0.2^{C}	3.7 ± 0.1^{CD}	5.2 ± 0.3^{BC}	6.2 ± 0.2^{D}	6.8 ± 0.1^{E}	7.5 ± 0.2^{E}	7.5 ± 0.2^{D}
MAC4	7.1 ± 0.3^{DE}	5.9 ± 0.1^{E}	4.1 ± 0.2^{E}	4.2 ± 0.2^{D}	5.3 ± 0.3^{C}	7.2 ± 0.2^{E}	6.1 ± 0.1^{CD}	7.3 ± 0.2^{E}	7.2 ± 0.3^{D}
MAC5	5.7 ± 0.1^{BC}	2.7 ± 0.2^{B}	1.2 ± 0.1^{B}	2.5 ± 0.3^{B}	4.7 ± 0.2^{B}	4.5 ± 0.3^{AB}	4.3 ± 0.2^{B}	5.4 ± 0.1^{BC}	4.8 ± 0.2^{A}
MAC6	5.8 ± 0.2^{BC}	1.7 ± 0.1^{A}	$<1^{A}$	3.8 ± 0.2^{D}	5.0 ± 0.3^{BC}	4.1 ± 0.1^{A}	3.7 ± 0.1^{A}	5.4 ± 0.2^{BC}	5.2 ± 0.2^{AB}
MAC7	6.8 ± 0.2^{D}	3.2 ± 0.2^{BC}	1.2 ± 0.2^{B}	2.5 ± 0.2^{B}	6.2 ± 0.2^{D}	6.4 ± 0.1^{D}	6.3 ± 0.2^{DE}	6.3 ± 0.3^{CD}	6.4 ± 0.1^{C}
MAC8	7.0 ± 0.2^{DE}	3.7 ± 0.1^{CD}	2.2 ± 0.3^{C}	3.8 ± 0.2^{D}	5.2 ± 0.1^{BC}	6.7 ± 0.3^{DE}	6.6 ± 0.2^{DE}	6.7 ± 0.2^{D}	6.7 ± 0.2^{CD}
MAC9	4.5 ± 0.2^{A}	2.0 ± 0.2^{A}	$<1^{A}$	2.5 ± 0.1^{B}	4.1 ± 0.2^{A}	5.9 ± 0.1^{CD}	5.6 ± 0.2^{C}	4.7 ± 0.3^{A}	5.1 ± 0.1^{A}
MAC10	4.9 ± 0.1^{A}	1.6 ± 0.1^{A}	$<1^{A}$	2.6 ± 0.1^{B}	4.3 ± 0.1^{AB}	4.6 ± 0.3^{AB}	4.5 ± 0.1^{B}	5.3 ± 0.3^{B}	5.7 ± 0.1^{B}
MAC11	5.5 ± 0.2^{B}	3.6 ± 0.2^{CD}	2.5 ± 0.2^{C}	3.7 ± 0.2^{CD}	4.5 ± 0.2^{AB}	4.8 ± 0.1^{B}	4.6 ± 0.2^{B}	5.5 ± 0.2^{BC}	6.6 ± 0.2^{C}
MAC12	6.3 ± 0.2^{CD}	4.1 ± 0.3^{D}	3.5 ± 0.3^{D}	1.3 ± 0.2^{A}	4.1 ± 0.3^{A}	5.9 ± 0.2^{CD}	5.9 ± 0.1^{CD}	5.9 ± 0.2^{C}	6.0 ± 0.1^{BC}
Statistical significance	***	***	***	***	***	***	***	***	***

Results indicate mean values ± S.D. of six plate counts (carried out in triplicate for two collection times).
Data within a column followed by the same letter are not significantly different according to Tukey's test.
Abbreviations: MBC, milk before contact; MAC, milk after contact; TMC, total mesophilic counts; CPS, coagulase-positive staphylococci; nd, not determined.
P value: ***, $P \leq 0.001$.
[a] Log CFU cm^{-2} for surfaces; Log CFU mL^{-1} for milk samples.
[b] Originating from the same bulk milk before contact with two different wooden vats.

vat (WV9) that hosted 6.0 Log CFU cm^{-2} of mesophilic rod LAB and 6.1 Log CFU cm^{-2} of thermophilic rod LAB. On the contrary, MAC10, which originated from the same bulk milk of MBC9, did not show a high increase in mesophilic and thermophilic rod LAB number, after contact with WV10 that hosted lower levels of these bacterial groups than WV9. This phenomenon could be explained with the observation of Lortal et al. (2009) who registered until a level of 10^6 CFU mL^{-1} of thermophilic LAB released into milk, after contact with wooden vats. Thus, the increase of LAB number is particularly evident for milks characterized by low levels of LAB before contact with the vat surfaces.

3.2. Isolation and grouping of LAB

A total of 713 colonies were collected from the 12 wooden vats. All cultures were inspected microscopically and classified as 542 cocci and 171 rods. After Gram determination and catalase test, 492 coccus-shaped and 165 rod-shaped Gram-positive and catalase-negative cultures were further examined.

Based on the combination of the phenotypic features evaluated, the 657 LAB cultures were separated into 24 groups (Table 3). The highest number of groups was observed for cocci. Almost 46% of the total isolates was in group XVI. More than the half of the groups comprised a few isolates. Only group I was found to have an obligate homo-fermentative metabolism for the lack of growth in the presence of pentose carbohydrates. The ability to grow at 45 °C but not at 15 °C resulted in the classification of the groups I, II, VII, XXII-XXIV as thermophilic LAB.

3.3. Genetic differentiation and identification of LAB

The isolates representative of each phenotypic group, for all wooden vats, were subjected to RAPD analysis. The reproducibility of this technique was verified by comparing the PCR products

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

Characters	Clusters																							
	I (n = 8)	II (n = 6)	III (n = 16)	IV (n = 8)	V (n = 9)	VI (n = 8)	VII (n = 16)	VIII (n = 35)	IX (n = 23)	X (n = 8)	XI (n = 6)	XII (n = 10)	XIII (n = 30)	XIV (n = 61)	XV (n = 7)	XVI (n = 301)	XVII (n = 24)	XVIII (n = 17)	XIX (n = 7)	XX (n = 10)	XXI (n = 6)	XXII (n = 5)	XXIII (n = 20)	XXIV (n = 16)
Morphology[a]	R	R	R	R	R	R	R	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
Cell disposition[b]	sc	sc	sc	sc	sc	sc	sc	sc	sc	sc	sc	sc	sc	sc	sc	sc	t	t	t	t	t	lc	lc	lc
Growth:																								
15 °C	−	−	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
45 °C	+	+	−	−	+	−	+	−	−	−	+	+	+	+	+	+	+	+	+	+	−	+	+	+
pH 9.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	+	−	−	−	−	+	+	+	+	+	+	+	+	−	−	−	−
6.5% NaCl	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	−	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+
Resistance to 60 °C	+	+	−	−	+	−	+	+	+	−	+	−	+	+	+	+	+	+	+	+	−	+	+	+
Hydrolysis of:																								
arginine	−	+	−	−	+	+	+	+	+	−	+	−	+	+	−	+	−	+	−	+	−	+	+	+
aesculin	+	+	−	+	+	+	−	+	+	−	+	+	+	+	+	+	−	+	+	+	−	+	−	+
Acid production[c] from:																								
arabinose	−	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ribose	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
xylose	−	+	+	+	+	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+
glycerol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+
CO_2 from glucose	−	+	+	+	−	−	+	−	+	+	+	+	−	−	−	−	−	−	−	+	+	+	−	−

n.d., not determined.
[a] R, rod; C, coccus.
[b] sc, short chain; t, tetrads; lc long chain.
[c] Fructose, galactose, lactose and sucrose were fermented by all isolates.

obtained with the primers M13, AB106 and AB111 using DNA extracted from three separate cultures of two strains per morphology. RAPD profiles were analysed separately for each cell morphology resulting in four dendrograms (Fig. 1) for 85 dominant strains. As expected, the most numerous dendrogram included LAB cocci in short chains.

The 85 strains were subjected to the 16S rRNA gene sequencing. The sequences were compared with those available in two distinct databases; all strains were clearly identified as members of the LAB community, since sequence similarity was higher than 97% in both databases (Table 1S) with species within the genera *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus*. Five strains, allotted into the *Enterococcus* genus, could not be identified at species level. Due to the different results of BLAST and EzTaxon search, these strains were further analysed by a species-specific multiplex PCR strategy which identified one *Enterococcus faecium* (strain 395) and four *Enterococcus durans* (strains 23, 87, 113 and 275). The highest number of strains (n = 39) belonged to *E. faecium*. Also some *E. faecium* (strains 1, 45, 106, 108, 143, 215, 238 and 264) and one *Lb. fermentum* (strain 354) were processed by multiplex PCRs for species confirmation, because their homology percentage was very closed to 97% in EzTaxon-e database.

All LAB included in the dendrograms of Fig. 1A,C,D, belonged to a single genus: *Lactobacillus* for LAB rods; *Streptococcus* for LAB cocci in long chains; *Pediococcus* for LAB cocci in tetrads. All strains belonging to a given species clustered closely, specifically all *Lactobacillus brevis*, *Lactobacillus reuteri* (Fig. 1A), *S. thermophilus* (Fig. 1C), *Pediococcus acidilactici* and *Pediococcus lolii* (Fig. 1D). Regarding LAB cocci in short chains, basically the majority of strains clustered per species; one major cluster was showed by *L. lactis*, *L. mesenteroides*, *E. durans* and *Enterococcus faecalis*, while basically five main clusters were obtained for *E. faecium* (Fig. 1B). However, some strains clustered with different species, e.g. *E. durans* strains which resulted mixed within the *E. faecalis* group, *Lc. lactis*, *Ln. mesenteroides* and *Ln. pseudomesenteroides* that clustered together with *E. faecium*. A mixed species clustering for LAB of dairy environments was also observed by other authors (De Angelis et al., 2001; Franciosi et al., 2009; Hazma et al., 2009).

All the species identified are commonly associated with raw milk and cheeses (Wouters et al., 2002; Settanni and Moschetti, 2010; Franciosi et al., 2011), including stretched cheeses (Morea et al., 2007; Piraino et al., 2008; Settanni et al., 2012; Gaglio et al., 2014) and several of them were also found associated with wooden vats used for cheese making in Italy and France (Licitra et al., 2007; Didienne et al., 2012; Settanni et al., 2012; Scatassa et al., 2015).

3.4. Species distribution

A total of 16 species of LAB were identified at dominating levels in the biofilms associated with the wooden vats used for the production of Vastedda della valle del Belìce and Caciocavallo Palermitano cheeses (Table 4). The species isolated from all wooden vat surfaces was *E. faecium* found at cell densities among 10^3–10^6 CFU cm^{-2}. *Lb. brevis*, *Lb. delbrueckii*, *Lb. fermentum*, *Lb. paracasei*, *Lb. plantarum*, *Ln. mesenteroides*, *Lb. reuteri*, and *Streptococcus lutetiensis* were found associated only to a single wooden vat, with the last 2 species detected at 10^6 CFU cm^{-2}. Although *Lb. reuteri* was found at the same level of the other species (*E. faecium*, *Ln. pseudomesenteroides* and *P. lolii*) isolated from the highest dilution plates of the gauzes used to collect the biofilms from WV7, *S. lutetiensis* was 2 Log cycles higher than *E. durans* and *E. faecium* present in WV4. *S. thermophilus* was isolated only from the vats used for Caciocavallo Palermitano cheese making, always at

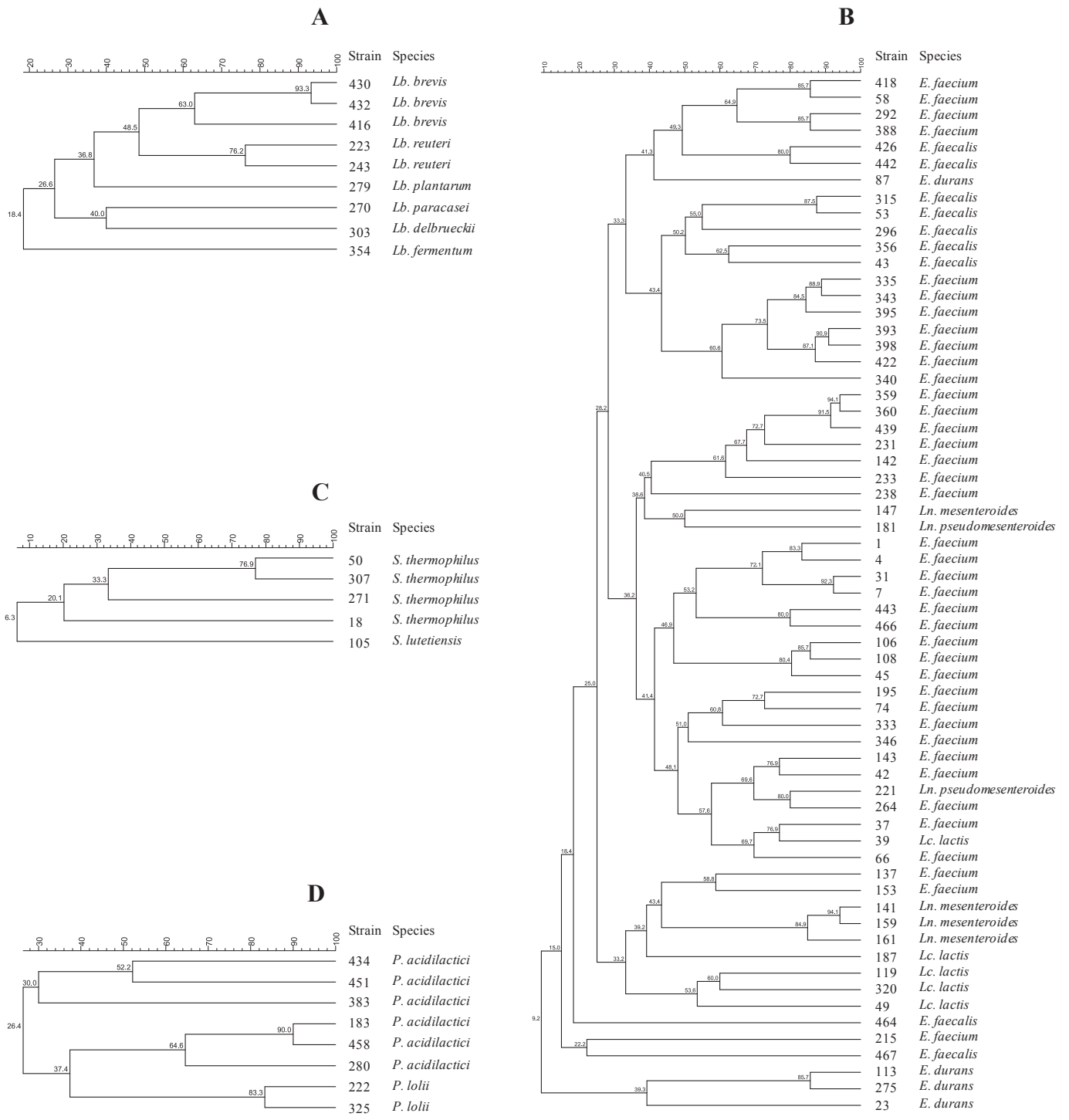

Fig. 1. Dendrogram obtained from combined RAPD-PCR patterns of LAB strains from wooden vats generated with the primers M13, AB106 and AB111. A, LAB rods; B, LAB cocci in short chains; C, LAB cocci in long chains; D, LAB cocci in tetrads. Upper line indicate the percentage of similarity. Abbreviations: *E.*, *Enterococcus*; *Lb.*, *Lactobacillus*; *Lc.*, *Lactococcus*; *Ln.*, *Leuconostoc*; *P.*, *Pediococcus*; *S.*, *Streptococcus*.

10^5 CFU cm^{-2}. *P. acidilactici* and *E. faecalis* were largely distributed among the LAB biofilms analysed, both in the range 10^3–10^5 CFU cm^{-2}. The highest biodiversity in terms of enterococci was determined for WV1 which was the oldest vat used in cheese manufacture (28 years).

In general, a very low complexity of the lactobacilli community was found in the biofilms. In particular, the vats made with douglas wood, except WV9, were characterized by the absence of *Lactobacillus* isolates. An explanation to these observations could be that typical dairy *Lactobacillus* strains are present as dormant (non-cultivable) flora and cannot be detected by classical cultural methods. When detected, *S. thermophilus* was associated with the vats made with chestnut wood. All bovine milk cheese productions were performed in chestnut wooden vats. The vats (WV1, WV2, WV6, WV7, WV8 and WV11) for which a co-dominance of several species (4–6) was found were all subjected to the washing with hot

Table 4
Distribution[a] of LAB species among wooden vats.

LAB species	Wooden vats											
	WV1	WV2	WV3	WV4	WV5	WV6	WV7	WV8	WV9	WV10	WV11	WV12
E. durans	■ (4)			■ (4)				■ (4)				
E. faecium	■ (4)	■ (3)	■ (3)	■ (4)	■ (4)	■ (3)	■ (6)	■ (5)	■ (4)	■ (4)	■ (3)	■ (5)
E. faecalis	■ (4)	■ (3)							■ (5)		■ (5)	■ (5)
Lb. brevis											■ (4)	
Lb. delbrueckii	■ (4)											
Lb. fermentum									■ (6)			
Lb. paracasei								■ (5)				
Lb. plantarum								■ (5)				
Lb. reuteri							■ (6)					
Lc. lactis		■ (5)	■ (5)			■ (5)						
Ln. mesenteroides					■ (5)							
Ln. pseudomesenteroides						■ (4)	■ (6)					
P. acidilactici						■ (3)		■ (5)		■ (4)	■ (3)	■ (5)
P. lolii		■ (5)					■ (6)					
S. lutetiensis				■ (6)								
S. thermophilus	■ (5)	■ (5)						■ (5)				

Abbreviations: *E.*, *Enterococcus*; *Lb.*, *Lactobacillus*; *Lc.*, *Lactococcus*; *Ln.*, *Leuconostoc*; *P.*, *Pediococcus*; *S.*, *Streptococcus*.
[a] The number reported between brackets refers to the highest concentration (Log cycle) of detection.

deproteinized whey after cheese making, even though the vats WV1, WV8 and WV12 were washed with cold water in the period June–September. Surprisingly, despite the large number of strains detected, the number of *Enterococcus* species displayed by the surfaces of the vats (WV3, WV4, WV9, WV10 and WV11) used to transform raw ewe's milk was quite limited.

The high number of strains identified as members of the genus *Enterococcus*, especially those belonging to the species *E. faecium*, was not surprising for the vats filled in daily with ewe's milk. In fact, the species *E. faecium*, *E. faecalis* and *E. durans* comprise the enterococci most prevalent in artisanal European raw ewe's cheeses (Prodromou et al., 2001; Todaro et al., 2011). Even though enterococci were not found at consistent levels in French wooden vats (Didienne et al., 2012), their presence was already reported for those used in Sicily (Licitra et al., 2007; Settanni et al., 2012; Scatassa et al., 2015). A recent work by Di Grigoli et al. (2015) showed that three enterococci belonging to three species (*E. faecalis*, *Enterococcus casseliflavus* and *Enterococcus gallinarum*), isolated from the wooden vat surface, were able to persist at different times of ripening of Caciocavallo Palermitano cheese, demonstrating the influence of the equipment during the production of traditional cheeses and highlighting the importance of this group of LAB to confer typicality. In that work, *E. faecalis* persisted longer than other species during ripening. This species, together with *E. faecium*, *E. durans*, *Enterococcus mundtii* and *E. casseliflavus*, is commonly found in many raw materials and foods (Corsetti et al., 2007; Franciosi et al., 2009; Settanni et al., 2014).

3.5. Technological characteristics of LAB

The results of the technological characterization based on acidification, autolysis, proteolysis, production of diacetyl and synthesis of antibacterial compounds were simultaneously considered by applying a multivariate data analysis consisting of hierarchical clustering analysis (HCA) and principal components analysis (PCA). This statistical approach had been widely applied in food processes as reported by Berrueta et al. (2007) and Rodríguez-Gómez et al. (2012).

The HCA is a graphical representation of a matrix of distances such as the dendrogram where the objects (strains) are joined together in a hierarchical ascendant analysis from the closest one, that is the most similar, to the furthest apart, that is the most different. HCA mainly classified the strains into two mega-cluster at around 75% of their mutual dissimilarity (Fig. 2A). Furthermore, within each mega-cluster, many groups at different level of dissimilarity were found. The strains 279 and 303 isolated from the samples WV8 and WV1, respectively, were clearly separated from the others. Several strains isolated from WV1 clustered with 50% of relative linkage distance. Furthermore, the strains from the vats WV2, WV4, WV5, WV8, WV9 and WV12 were mainly grouped into a single mega-cluster with a relative linkage distance lower than 35%. In general, the strains isolated from a given vat did not group closely to each other, even though four of the seven strains of the vat WV12, representing different species, clustered together below 16%. This analysis indicated that most of the strains tested had fairly similar activities and only some of them showed important dairy characteristics.

Two Factors with eigen-values higher than 1.00 were found for PCA (Fig. 2B and C). This means that the variability of the technological traits is mostly expressed as linear combination of only two Factors accounting for a 63.78% of the total variance. As shown in Fig. 2B, the components of the PCA were correlated to the variables considered. Factor 1, representing 42.54% of the total variance, was positively related to autolysis, proteolysis and diacetyl production and negatively to acidification and antibacterial compound production. Except for diacetyl production, the Factor 2, accounting for 21.24% of the total variance, was positively related to all variables. The discrimination of the wooden vats can be visualized in the plot of the scores (Fig. 2C). Although the highest loading values were associated to acidification and autolysis variables, vats were significantly separated along both Factors 1 and 2. The longest distance along Factor 1 was found among WV3 and WV7. Along Factor 2, wooden vats were mostly differentiated for the antagonistic activities.

In general, optimal SLAB are characterized by a fast and appropriate acidification and a rapid autolysis, whereas optimal NSLAB show opposite performances (Franciosi et al., 2009; Settanni et al., 2013). The group of the fastest acidifiers included some strains of *Lc. lactis* and *S. thermophilus* which were also positive for diacetyl production, thus proving that the wooden vats might act as sources of cultures useful in cheese making.

Wooden vat LAB strains were particularly active in hydrolysing gelatine and BSA, since after incubation, a clear halo was detected for 74 strains in presence of both substrates. On the contrary, *E. faecalis* 464, *Lb. delbrueckii* 303, *Lb. reuteri* 243 and *P. acidilactici* 393 did not hydrolyse gelatine or BSA. Three *E. faecium* and two

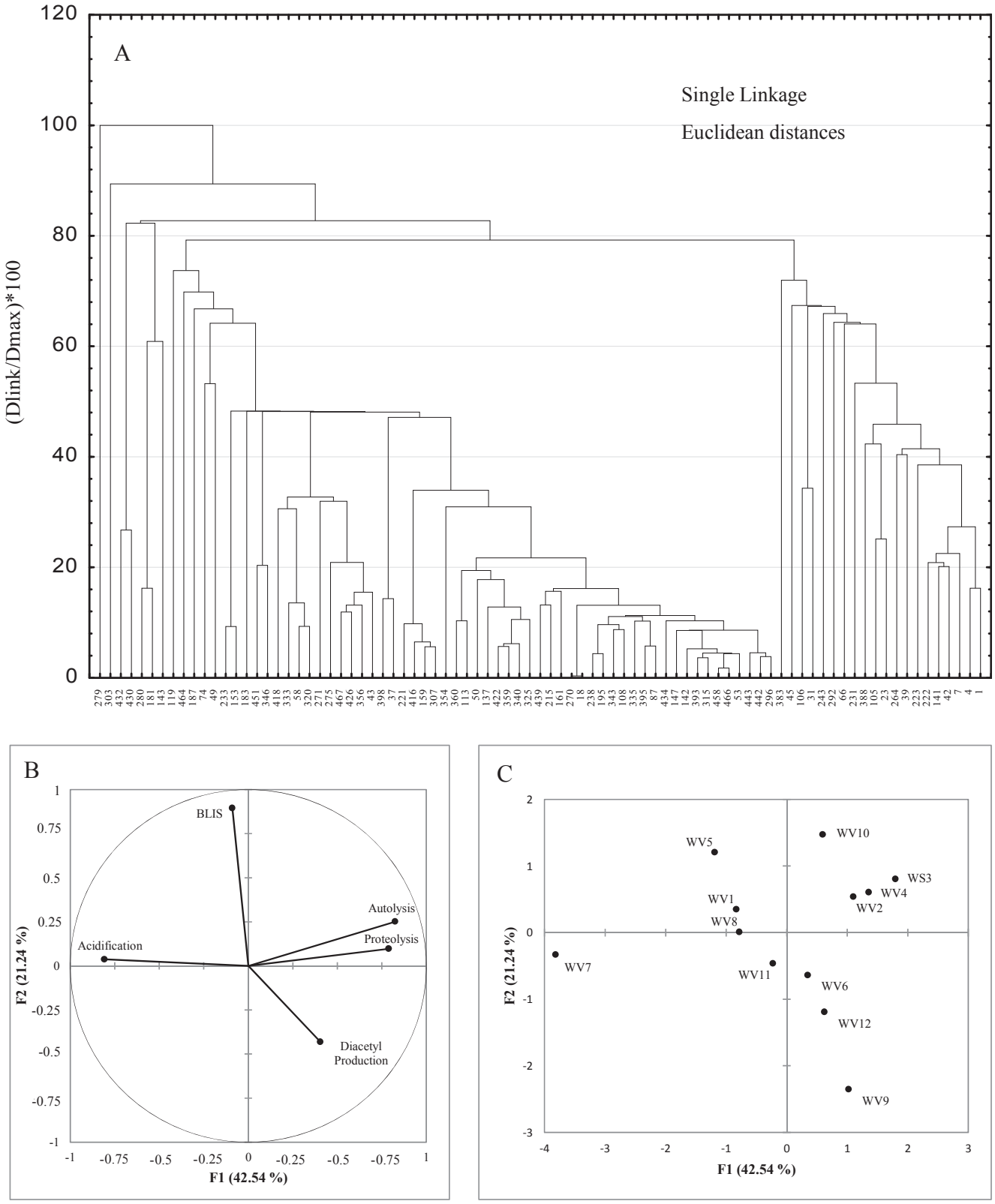

Fig. 2. Multivariate analysis of the technological traits (acidification rate, autolysis kinetics, diacetyl production, antimicrobial activities and proteolysis) of the wooden vat LAB. A, hierarchical clustering analysis; B, loading plot of principal component analysis (PCA); C, score plot of PCA. BLIS, bacteriocin-like inhibitory substances; WV, wooden vat.

P. acidilactici strains were active only on BSA, while barely one strain per each of these two species hydrolysed only gelatine.

Lortal et al. (2009) supposed that an additional hypothesis to explain the absence of foodborne pathogens in the wooden vat biofilms is due to the presence of bacteriocin producers. For this reason, all LAB were also investigated for this character. The antimicrobial activity was registered for 31 strains. The highest activity in terms of number of indicators inhibited was displayed by

E. faecium 66, *Lb. plantarum* 279 and *P. acidilactici* 383 which inhibited all three sensitive organisms, but the highest inhibitory power in terms of diameter of the clear area in plate was registered for *E. faecium* 45 and 292 and *Lb. plantarum* 279. These attributes confer competitive advantages to the producing strains. Furthermore, the anti-*Listeria* effect found in some LAB, might contribute to the safety of the microbial biofilms during cheese production. All antibacterial compounds lost their activities after treatment with proteolytic enzymes. Thus, they were proven to be proteins and, for this reason, indicated as bacteriocin-like inhibitory substances (Corsetti et al., 2008). This study, together with previous studies performed on other food chains (Francesca et al., 2013) demonstrated that high percentages of LAB present also onto the surfaces of the equipment used for food production are found to be BLIS producers.

4. Conclusions

The levels of LAB in milk is consistently influenced by the wooden vat biofilms when their cell densities before contact with the vat surfaces are lower than 6 Log CFU mL^{-1}. Species and strain composition of LAB associated with the wooden vats used to produce Caciocavallo Palermitano and Vastedda della valle del Belìce cheeses were not particularly affected by the origin of milk and confirmed previous investigations performed in other dairy factories. The microbial biofilms of the vats analysed were dominated by cocci LAB. A high percentage of these strains were enterococci, which are strictly linked to the cheese typicality. The technological characterization of the LAB found at the highest numbers showed interesting dairy properties useful during curd fermentation or cheese ripening. The multivariate analysis was proved to be a useful tool to manage the large amount of data generated in this study in relation to the diverse biochemical activities of LAB. Several strains showed the capacity of inhibiting undesired bacteria highlighting their active contribution to the safety of the final cheeses.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2015.06.008.

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