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Activation, transfer, distribution, composition, evolution and characterization of the lactic acid bacterial biofilms for the valorization of Sicilian autochthonous dairy and wood resources

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RIASSUNTO TESI

Il legno è il principale materiale utilizzato per la fabbricazione delle attrezzature comunemente impiegate per la produzione di formaggi Siciliani tipici e tradizionali. Le attrezzature in legno principalmente impiegate in caseificazione sono rappresentate dalle "*tine"* utilizzate per la coagulazione del latte, maturazione della cagliata e cottura delle forme, la "*ruotula"* impiegata per la rottura del coagulo e il "*piddiature"* usato per la filatura, altre attrezzature impiegate includono i tavolieri, gli scaffali, i ripiani o le pertiche per le fasi di formatura, affinatura e stagionatura dei formaggi.

A causa della sua natura porosa l'utilizzo del legno è oggetto di numerose controversie benché non vi sia alcuna prova scientifica che attesti la diretta correlazione tra l'impiego delle attrezzature in legno e le malattie di origine alimentare sono sorte di recente implicazioni relative a problematiche igienico-sanitarie. Peraltro, nessuna normativa vigente vieta l'uso del legno in caseificazione. In realtà, sulla base di un'ampia recensione di pubblicazioni scientifiche internazionali, relative a studi condotti sulla sicurezza microbiologica del legno nell'industria alimentare, è stato evidenziato il contributo positivo di questo materiale alla qualità finale e proprio alla sicurezza igienico-sanitaria di molti prodotti alimentari. Ciò è garantito dai biofilm microbici associati a tali attrezzature che rappresentano una fonte di batteri lattici (BL) pro-tecnologici responsabili della corretta acidificazione della cagliata e della successiva trasformazione in formaggio così come della sicurezza microbiologica dei prodotti finiti. Pertanto, i recipienti in legno e le altre attrezzature impiegate in caseificazione rappresentano dei veri e propri "sistemi viventi" utili e sono dei serbatoi di biodiversità microbica per i formaggi tradizionali. I biofilm presenti sulle superfici delle tine non solo influenzano positivamente lo sviluppo della microflora lattica, ma esercitano anche un effetto inibente nei confronti di vari patogeni. Inoltre, i batteri presenti sulla superficie delle tine contribuiscono in maniera decisiva anche alla tipicità del prodotto finale e alla caratterizzazione organolettica di ciascun formaggio, esaltandone la forte identità territoriale e storica.

Tali attrezzature sono tutt'ora in uso nelle piccole e medie aziende del territorio siciliano per la produzione di vari formaggi della tradizione regionale. La Vastedda della valle del Belìce DOP, il cui disciplinare di produzione prevede l'utilizzo della tina in legno per la coagulazione del latte (Reg. UE n. 971 del 28.10.10), è un formaggio che ha acquisito una certa rilevanza per il territorio di produzione. In passato, la produzione di questo formaggio era limitata soltanto al periodo estivo, ma in seguito alla cresciuta e continua richiesta da parte dei consumatori la produzione è stata estesa a tutto l'anno. Questo determina enormi variazioni della qualità del prodotto finale. Tali differenze possono essere dovute alle

diverse caratteristiche microbiologiche delle materie prime, come risultato delle diverse temperature medie stagionali.

Sulla base delle suddette motivazioni è sorta l'esigenza di sviluppare un'attività di ricerca atta a minimizzare le differenze microbiologiche stagionali tra le diverse produzioni di formaggio. A tal proposito è stata impiegata una miscela di batteri lattici autoctoni appartenenti alla specie *Lactococcus lactis* subsp. *cremoris* (PON36, PON153, PON203) per lo sviluppo di un biofilm *ad hoc* sulla superficie di tine in legno di castagno vergini (nuove) destinate alla produzione di formaggi della tipologia Vastedda. Due tine sono state testate in condizioni controllate in un impianto pilota (TZ1 e TZ2), e due tine sono state testate in condizioni non controllate in un impianto industriale (TA1 e TA2). In entrambi i casi, una delle tine è stata utilizzata per la produzione controllo (TA1 e TZ1) applicando il disciplinare produttivo della Vastedda della Valle del Belìce DOP dopo l'attivazione microbiologica con il siero residuo della lavorazione della ricotta. L'altra tina di ciascun ambiente produttivo (TA2 e TZ2) è stata impiegata per la produzione sperimentale effettuata dopo l'attivazione (neoformazione) del biofilm con la miscela selezionata di lattococchi e l'aggiunta giornaliera di un siero innesto naturale, anch'esso costituito dalla miscela dei tre lattococchi utilizzati per sviluppare i biofilm sulle tine. Le analisi microbiologiche e l'osservazione al microscopio elettronico a scansione (SEM) hanno mostrato delle differenze in termini di livelli microbiologici e composizione dei biofilm neoformati tra le tine controllo e quelle delle produzioni sperimentali, in entrambe le condizioni. Le differenze non sono state particolarmente marcate tra le due produzioni sperimentali TA2 e TZ2 per le quali sono stati rilevati elevati livelli di BL mesofili di forma coccica. I presunti BL provenienti dalla superficie delle tine e dai formaggi prodotti sono stati isolati, purificati, raggruppati fenotipicamente e genotipicamente. Tale procedimento ha portato all'identificazione di 44 ceppi dominanti appartenenti a 10 specie (*Streptococcus thermophilus*, *Lactococcus lactis* subsp *lactis*, *Lactococcus raffinolactis*, *Lc. lactis* subsp *cremoris*, *Enterococcus faecium*, *Enterococcus durans*, *Leuconostoc lactis*, *Leuconostoc mesenteroides*, *Lactobacillus casei* e *Lactobacillus delbrueckii*) tutte di interesse caseario. Questo lavoro conferma la dominanza dei BL mesofili nelle produzioni invernali ed una generale riduzione della loro biodiversità a vantaggio dei ceppi starter aggiunti nelle produzioni sperimentali. Il risultato ottenuto è importante, in quanto la strategia attuata potrebbe limitare la variabilità microbiologica dei formaggi prodotti nelle diverse stagioni. Lo sviluppo incontrollato di diversi ceppi a livelli dominanti può giocare un ruolo determinante per le differenze tra i formaggi anche quando sono prodotti nello stesso caseificio lo stesso giorno ma con tine diverse.

Infine, la valutazione sensoriale ha mostrato che l'attivazione microbica delle tine con la miscela multiceppo di *Lc. lactis* subsp *cremoris* ha permesso l'ottenimento di prodotti con attributi sensoriali paragonabili a quelli dei formaggi Vastedda della valle del Belìce DOP prodotti con il protocollo tradizionale.

Pertanto, l'attivazione delle tine con BL selezionati e l'aggiunta del siero innesto naturale sviluppato con gli stessi ceppi durante il processo di produzione rappresentano una strategia vincente per ridurre la variabilità microbiologica e stabilizzare gli attributi sensoriali del formaggio tipo Vastedda nelle diverse stagioni.

Successivamente, il progetto di dottorato ha previsto la valutazione dell'idoneità microbiologica di diverse tipologie di legno provenienti da ambienti boschivi e agrari siciliani impiegate nella fabbricazione delle attrezzature da utilizzare per le produzioni lattiero-casearie al fine di valorizzare le risorse legnose siciliane. A tal proposito, sette diverse specie (*Castanea sativa* Miller, *Cedrus libani* A. Rich., *Prunus avium* L., *Fraxinus ornus* L., *Juglans regia* L., *Pinus nigra* J.F. Arnold e *Populus nigra* L.) sono state impiegate per la fabbricazione di altrettante tine da usare in caseificazioni sperimentali. Una tina controllo è stata realizzata con legno di castagno calabrese comunemente utilizzato per la fabbricazione di tali attrezzature in Sicilia. L'obiettivo è stato quello di valutare la capacità di formazione di biofilm stabili sulla superficie dei legni in grado di fornire BL autoctoni sia starter che non starter, rispettivamente, necessari per la fermentazione della cagliata e la maturazione dei formaggi. La formazione del biofilm di BL sulla superficie delle tine è stata realizzata mediante la scotta, proveniente da una lavorazione tradizionale, lasciata in contatto con il legno. Le analisi microbiologiche e le indagini SEM della superficie delle tine hanno confermato l'iniziale assenza di microrganismi nelle tine vergini e la formazione del biofilm dopo il contatto con la scotta. In particolare, in tutte le tine, prima e dopo l'attivazione microbiologica, non sono stati rinvenuti microrganismi indesiderati appartenenti ai seguenti gruppi microbici: *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli*, staphylococci coagulasi positivi e batteri solfito riduttori anaerobici, generalmente ricercati nei prodotti caseari. L'assenza di questi microrganismi nei biofilm è principalmente dovuta alle condizioni acide determinate dai BL che ne inibiscono l'adesione e la sopravvivenza e che rappresentano in tutti i casi la popolazione dominante, i livelli più alti sono stati registrati per i cocci termofili. La caratterizzazione fenotipica, biochimica e genetica delle diverse colonie purificate dalle conte in piastra delle sospensioni cellulari di biofilm ha permesso l'identificazione di sei specie appartenenti ai generi *Enterococcus*, *Lactobacillus*, *Lactococcus* e *Streptococcus* comunemente identificati nel latte crudo, nei formaggi tradizionali siciliani e sulle tine in legno. Le specie presenti con maggiore frequenza sono risultate *Lactobacillus fermentum* e

Lactococcus lactis. La caratterizzazione tecnologica dei BL ha permesso di valutare il potenziale tecnologico dei biofilm. Sulla superficie delle tine sono stati individuati potenziali ceppi starter (BLS) (elevata capacità acidificante e rapida autolisi) e non starter (BLNS) (bassa capacità acidificante e lenta autolisi), nonché due ceppi produttori di composti antimicrobici che possono contribuire all'assenza di batteri patogeni. La presenza di BLS e BLNS nei biofilm determina l'inoculo dei batteri necessari alla produzione e alla maturazione del formaggio. Al fine di comprendere le controversie alla base della sicurezza delle tine in legno, sono stati eseguiti dei test di contaminazione artificiale del latte con i principali patogeni caseari (*L. monocytogenes, E. coli*, *Salmonella enteritidis* e *Staphilococcus aureus*). Questa strategia ha permesso di rilevare la totale assenza dei ceppi aggiunti sulla superficie delle tine dimostrando, così, come l'acidità e la produzione di composti antimicrobici da parte dei BL dei biofilm costituiscano delle efficaci barriere per l'adesione dei patogeni. I risultati di tali indagini rafforzano l'importanza dell'uso delle tine di legno per la produzione di formaggi tradizionali e forniscono evidenze per valorizzare le risorse boschive siciliane attraverso la produzione di attrezzature tradizionali ad uso caseario.

L'influenza delle diverse tipologie di legno impiegate è stata valutata anche in relazione alle caratteristiche microbiologiche, chimiche, fisiche e sensoriali dei formaggi Vastedda della valle del Belìce sperimentali, dopo 15 giorni di conservazione refrigerata. In tutti i campioni, la comunità microbica è stata dominata dalle popolazioni di BL. I ceppi più frequentemente identificati appartenevano alla specie *Lc. lactis* tra i BLS e *Lactobacillus paracasei*, *Lactobacillus rhamnosus* e *Lactobacillus fermentum* tra i BLNS. La composizione della comunità microbica di ciascun formaggio è stata anche approcciata mediante una metodologia coltura indipendente. Tali analisi sono attualmente in fase di elaborazione. Generalmente, l'uso di diverse tipologie di legno non ha influenzato negativamente la composizione chimico-fisica dei formaggi. Tuttavia, il colore dei formaggi è risultato diverso per il parametro luminosità. Tra i parametri chimici, le variazioni significative sono state osservate per l'attività dell'acqua (a_w), lo stato ossidativo, il contenuto in polifenoli e in composti organici volatili (VOCs). Le differenze rilevate tra i VOCs emessi dai formaggi non sono state percepite dagli assaggiatori che hanno indicato tutti i formaggi delle diverse prove sperimentali come simili. Questo studio fornisce ulteriori evidenze sull'idoneità di impiego delle diverse specie arboree siciliane per la fabbricazione di tine da impiegare nelle produzione casearie tradizionali regionali.

CHAPTER I

Introduction

Part of the present chapter has been submitted to the journal

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CHAPTER I

1.1. INTRODUCTION

1.1.1. Origin of cheese production in Sicily

Sicily is a southern Italian region characterized by an ancient history of cheese production. The dairy tradition is dated back to the presence of the Phoenician community in the island (OESAAS, 2007). According to one legend, cheese was born in Sicily (in the city of Pergusa within Enna province) thanks to the shepherd Aristeo, the son of Apollo and the nymph Cerere, who taught men how to transform milk into cheese (Betta and Cantarelli, 2000). The first written description of a cheese production in Sicily was reported by Homer in the IX book of Odyssey, when the activities of the Cyclope Polyphemus in the Etna Volcano were described (Ballarini, 1999). Recent archaeological discoveries of an oval hut and fragments of sieves, small colanders and perforated vessels (Fig. 1) within Troina area (Enna province) support these legends and indicate that the dairy activity in Sicily was conducted daily during the Eneolithic age (end of third – begin of second millennium b.C.) (Ricci, 2017).

Fig. 1. The ancient ceramic fragments discovered in Sicily (Reference: Mario Ricci, 2017).

In recent years, the food style evolved very fast towards a renewed request of typical products, including cheeses that are produced applying traditional transformation

processes. These products, often processed with procedures respectful of the environment, are perceived as "natural" and preferred by consumers because do not contain chemical preservatives (Settanni and Moschetti, 2010). These requirements are satisfied by the "recognition of quality" [protected designation of origin (PDO), protected geographical indication (PGI), and traditional specialty guaranteed (TSG),] conferred by the European Community to promote and protect the names of quality of agricultural products and foodstuffs (EU Regulation No 1151/2012).

Table 1. Traditional Sicilian cheeses

Sicilian cheeses	Milk	Type	Ripening	Quality certificate
 Ragusano DOP	Raw cow's milk	Semi-hard Stretched	Least 30 days	PDO, Slow Food and PAT
 Piacentinu Ennese DOP	Raw sheep's milk	Hard Pressed	About 60 day	PDO, Slow Food
 Pecorino siciliano DOP	Raw sheep's milk	Hard Pressed	More than 4 months	PDO
 Vastedda della valle Belice DOP	Raw sheep's milk	Fresh Stretched	-	PDO, Slow Food

Abbreviation: PDO, protected designation of origin; PAT, Traditional Agri-Food Products.

Several Sicilian cheeses are strongly linked to the territory of origin and are made from the raw milk of indigenous breeds, curdled with animal rennet pastes, processed in traditional wooden equipment without the addition of starter cultures (Scatassa et al., 2015a). Among these cheeses, Pecorino Siciliano, Ragusano, Vastedda della valle Belìce (VdB) and Piacentinu Ennese enjoy a PDO status (Table 1). Due to the production conditions of these non-started cheeses, lactic acid bacteria (LAB) necessary to transform curd in cheese

(Settanni and Moschetti, 2010) derive from raw milk (Franciosi et al., 2009; Guarcello et al., 2016), animal rennet (Cruciata et al., 2014), traditional wood equipment (Licitra et al., 2007), and the transformation environment (Scatassa et al., 2015b) (Fig. 2).

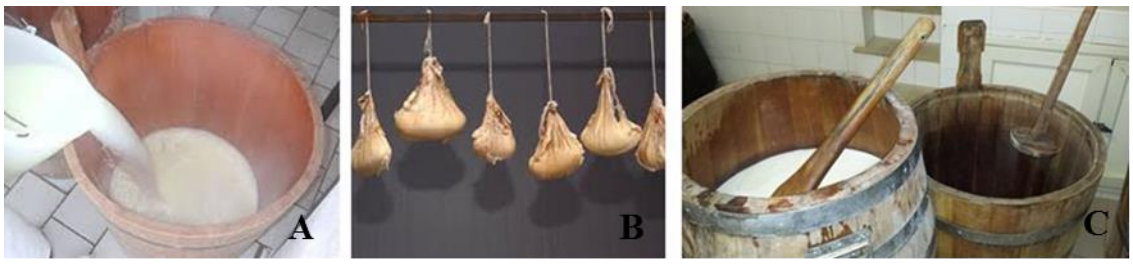

Fig. 2. Main sources of lactic acid bacteria for the traditional cheese production without additional starter. **A**, raw milk; **B**, animal rennet; **C**, traditional wood equipment.

1.1.2. The use of wood equipment in traditional cheese making

The production of artisanal Sicilian cheeses is traditionally based on the use of wooden equipment. In general, Italy has a wide varieties of cheeses and many of them were originally produced in wooden vats. In the 1859, the characteristics of the vats for the transformation of milk in different Countries were described in the Volume III of "Enciclopedia Agraria" (Bruni, 1859), where it was reported that vats made of wood were commonly used also in Holland and England. Several Italian cheeses, especially in central and southern regions, are produced applying the stretching technology. Some original documents of the beginning of the 19th Century (De Caprariis, 1912) report the different phases of production of the typical Caciocavallo, Scamorza and Manteca cheeses and show that the wooden vat was used until stretching of the acidified curd (Fig. 3). Besides the wooden vat, most of the tools employed in cheese making are made of wood and are used during different phases of the production process, from transformation of milk in curd until ripening (Galinari et al., 2014; Di Grigoli et al., 2015; Scatassa et al., 2015a-b).

Fig. 3. Front page of the article reporting the protocols for production of Caciocavallo, Scamorza and Manteca cheese published in 1912 in Italy. Focus on the wooden vat.

The use of equipment made of wood is regulated by the Commission Regulation (EC) No 2074/2005 which allows derogation from the 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). Regarding the materials in contact with cheese, the rule CE n. 1935/2004 reports the "principle of no contamination". However, no limitations to the use of wooden equipment are specifically indicated (Della Ciana, 2015).

Traditionally, wood was the main material used for the production of tools to be employed in cheese making due to its territorial availability, resistance over time and low cost. However, nowadays, the wood used to this purpose is imported from other regions. In general, the tree species employed to produce dairy equipment in Sicily are chestnut and Douglas-fir, genus *Pseudotsuga*.

The main wooden equipment (Fig. 4) used for the production of the majority of typical Sicilian cheeses are as follows: *tina*, vat for milk coagulation; *rotula*, stick for curd breaking; *cisca*, bowl for curd pressing; *cannara*, a cane plan from residual whey loss by pressing; *appizziatuma*, horizontal stick for curd acidification; *fuscelle*, rattan baskets for curd acidification*; piddiaturi*, conical vat for stretching curd; *maciliatuma*, stick used for stretching curd; *tavuleri*, for moulding; shelve for ripening.

Fig. 4. Traditional wooden equipments.

The vat made of wood contribute to the syneresis of cheese and affect the flavor and the texture of cheeses (Mariani et al., 2011; Galinari et al., 2014; Scatassa et al., 2015b), without the addition of commercial starter cultures. This phenomenon is due to the development of biofilms of LAB onto the internal surfaces of the wooden vats, making the wood itself responsible for the typicality of the final cheeses (Settanni and Moschetti 2014).

1.1.3. Dairy microorganisms

Lactic acid bacteria are the main microorganisms of dairy interest. They are responsible for the acidification process, the degradation of milk compounds and the ripening process (Salvadori del Prato, 1998). LAB constitute a highly heterogeneous group characterized by the production of L(+) and/or D(-) lactic acid from the fermentation of carbohydrates. LAB are Gram-positive, catalase-negative, anaerobic aerotolerant, acid-tolerant, non-spore-forming, mainly nonmotile rods or cocci. They may develop in a wide range of

temperatures, but are mainly distinguished in mesophilic (optimum temperature about 30°C) and thermophilic (optimum temperature about 44°C) species. LAB are nutritionally fastidious and require several nutritional factors. For this reason, they are able to grow in complex foods (Jay et al., 2009). Due to the lack of the respiratory chain, LAB obtain energy for the anabolic functions exclusively from fermentation, through phosphorylation at the substrate level (Kandler and Weiss, 1986). LAB genera and species can be differentiated based on the glucose fermentation and fermentation of carbohydrates other than glucose (e.g. carbohydrates pentose) (Fig. 5).

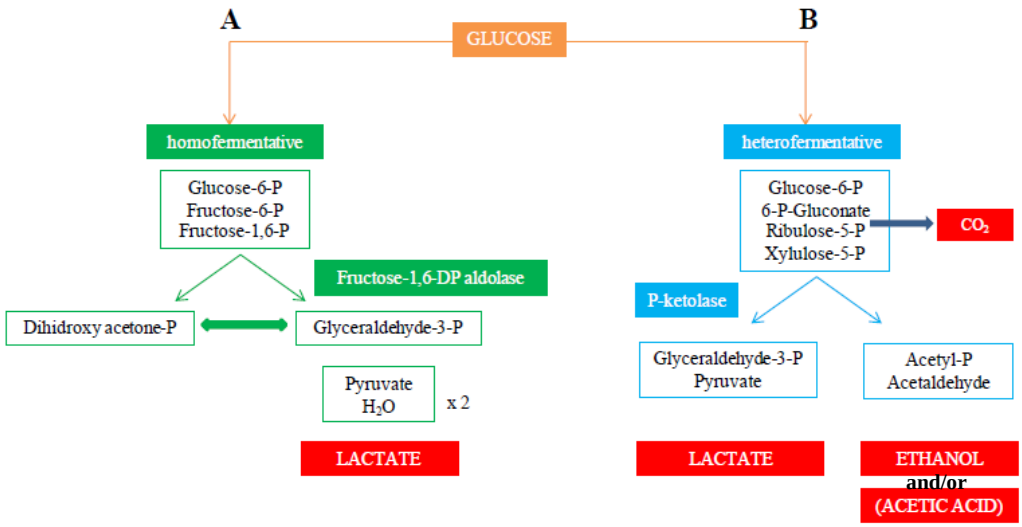

Fig. 5. Lactic acid fermentation. **A**, homolactic fermentation; **B**, heterolactic fermentation.

The homofermentative species produce mainly lactic acid from the fermentation of hexose carbohydrates through the Embden-Meyerhof (glycolysis) pathway, and cannot ferment pentose carbohydrates; the heterofermentative species produce, besides lactic acid, acetic acid and/or ethanol (depending on the presence of additional substrates acting as electron acceptors) and carbon dioxide through the 6-phosphogluconate/phosphoketolase (6PG/PK) pathway from the hexoses, while CO_2 is not produced from pentose carbohydrates. However, a third group of LAB (facultative heterofermentative) is able to perform both metabolic pathways, glycolysis in presence of hexoses and 6PG/PK in presence of

pentoses, because the aldolase is a constitutive enzyme, while phosphoketolase is inducible (Axelsson, 1998).

Typical dairy LAB are ascribable to the genera *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Leuconostoc*, *Pediococcus* and *Enterococcus*. Due to the main role during cheese production, dairy LAB are generally distinguished in starter LAB (SLAB) that are responsible for the rapid acidification of the curd, and non-starter LAB (NSLAB) which drive the ripening process (Settanni and Moschetti, 2010). The general fermentation pathway adopted by dairy LAB are reported in Table 2. SLAB and NSLAB show an opposite evolution trend (Fig. 6); SLAB are high in number (about 10^8-10^9 CFU/g) at the beginning of the transformation process and decrease regularly during ageing. On the contrary, NSLAB are present at low concentrations soon after curdling and increase of about four e five orders of magnitude within a few months (Fox et al., 2004).

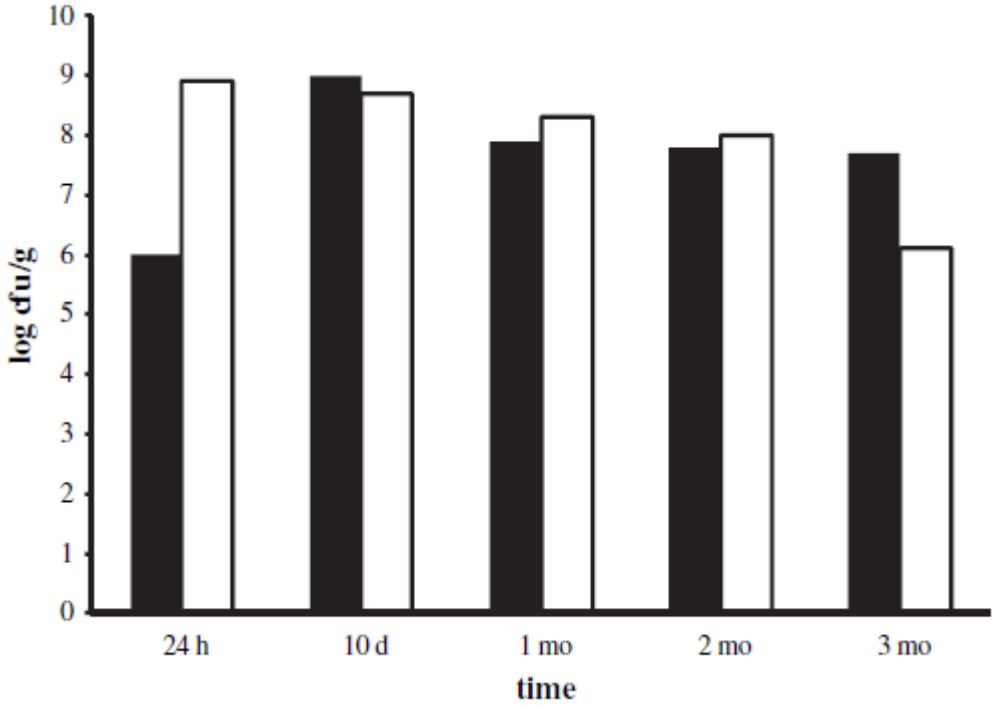

Fig. 6. Evolution of SLAB (empty columns) and NSLAB (full columns) during ripening of Puzzone di Moena cheese (Reference: Franciosi et al., 2008).

Table 2. Fermentative class of LAB genera in dairy production

Genera	Fermentative classes	Sugar	Pathway	Main products
Lactococcus	Obligate homofermentative	hexoses	EMP	lactic acid
Streptococcus	Obligate homofermentative	hexoses	EMP	lactic acid
Pediococcus	Facoltative heterofermentative	hexoses	EMP	lactic acid
		pentose	PP	lactic acid Acetate/ethanol (1:1)
Leuconostoc	Obligate heterofermentative	hexoses	HMS	lactic acid CO_2 Acetate/ethanol (1:1:1)
		pentose	PP	lactic acid Acetate/ethanol (1:1)
Lactobacillus				
group 1	Obligate homofermentative	hexoses	EMP	lactic acid
group 2	Facoltative heterofermentative	hexoses	EMP	lactic acid
		pentose	PP	lactic acid Acetate/ethanol (1:1)
group 3	Obligate heterofermentative	hexoses	HMS	lactic acid CO2 Acetate/ethanol (1:1:1)
		pentose	PP	lactic acid Acetate/ethanol (1:1)
Enterococcus	Obligate homofermentative	hexoses	EMP	lactic acid

Abbreviation: EMP, Embden-Meyerhoff-Parnas; HMS, Hexose Monophosphate Shunt (phosphogluconate-phosphoketolase); PP, Pentose Phosphate.

The different timing for LAB evolution is due to their roles. SLAB participate to the fermentation process converting rapidly lactose into lactic acid that promotes the acidification of the curd, favour the removal of whey, creates an environment suitable for a long preservation, contrasts the action of spoilage microorganisms and, in case of stretched cheese, provide an appropriate plasticity degree to the paste due to a demineralization of the curd (Salvadori Del Prato, 1998). Regarding stretching (pasta filata) technology, it consists of two distinct steps: the first step involves an acidification of the curd, which results in it assuming a plastic consistency; after that, the curd is heated to a scalding temperature (80 - 90°C), allowing it be moulded into the final shape and it is then left to ripen (Salvadori del Prato 1998). The microorganisms involved in fermented dairy products are mesophilic lactococci, as *Lactococcus lactis*, and thermophilic rod and cocci, as *Streptococcus thermophilus* and *Lactobacillus delbrueckii*, generally considered SLAB (Wouters et al., 2002).

NSLAB have the unique ability to grow under the highly selective condition and for these play their major roles during ripening. In the conditions of typical Sicilian cheese production, they are present in milk, but recently they have become object of selection (Franciosi et al., 2008; Settanni et al., 2011, 2013; Guarcello et al., 2016). NSLAB group is very complex and include microorganism belonging to the genera *Lactobacillus*, *Pediococcus*, *Enterococcus* and *Streptococcus*. NSLAB group is particularly heterogeneous and include different species such as *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus curvatus*, *Lactobacillus rhamnosus*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Enterococcus durans*, *Enterococcus faecalis*, *Enterococcus faecium* (Settanni and Moschetti, 2010).

1.1.3.1. Raw milk microorganisms

Milk is a nutritionally rich medium; it contains proteins, fats, carbohydrates, vitamins, minerals and essential amino acids, all at an almost neutral pH. Thanks to its high water activity, milk provides an optimal environment for the growth of several microorganisms. These nutrients can be directly available or can be released from other components through the metabolism of certain microorganisms (Frank, 1997). Raw milk is commonly colonized by microorganisms that come from a variety of sources including the teat apex, milking and storage equipment, air, water, feed, grass, soil and breeding environment (Fig. 7) (Coorevits et al., 2008; Lejeune and Rajala-Schultz, 2009; Vacheyrou et al., 2011).

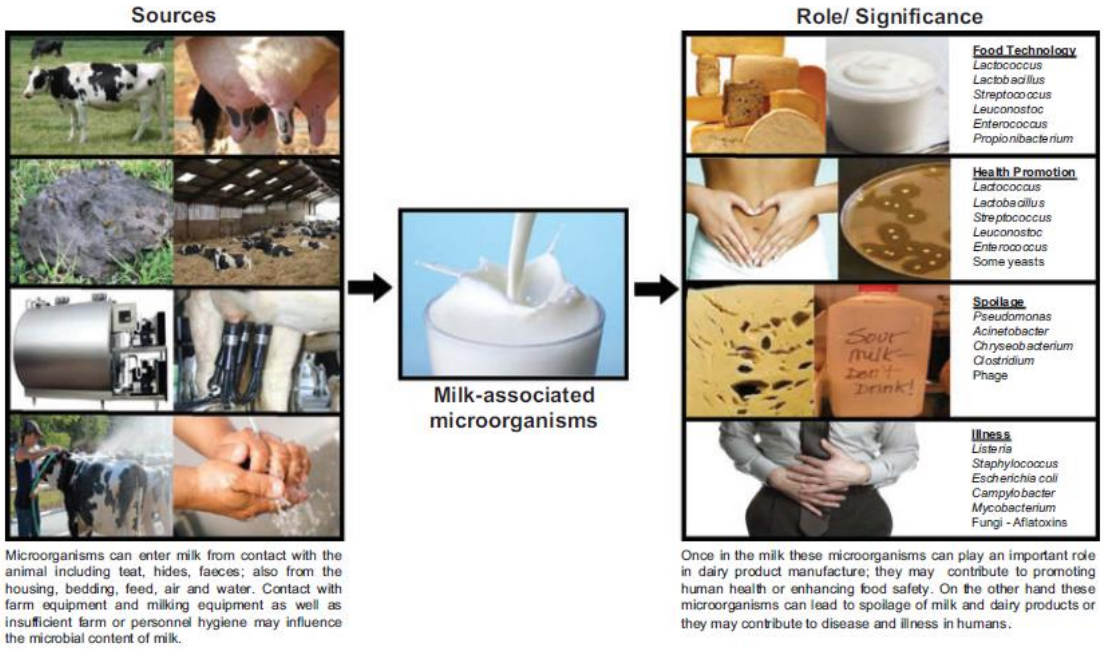

Fig. 7. The potential sources of the microorganisms that are present in raw milk and the role/significance that some of these have when present in milk (Reference: Quigley et al., 2013).

Raw bovine, goat, sheep and buffalo milk are dominated by LAB population. The most common LAB genera in milk include *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus* and *Enterococcus* (Quigley et al., 2013). These microorganisms impact

differently the sensory, texture, flavour and organoleptic properties of the final dairy products (Wouters et al., 2002).

Milk hosts also psychrotrophic microorganisms such as *Pseudomonas*, *Flavobacterium* and *Acinetobacter* spp.. that proliferate during the refrigerated storage (Notiziario ERSA 2/2006). These bacteria exert a negative impact on milk quality and shelf life due to the production of extracellular lipases and proteases responsible for spoilage of dairy products (Desmasures and Gueguen, 1997; Hantsis-Zacharov and Halpern, 2007). Furthermore, *Clostridium*, *Bacillus* and other spore-forming or thermoduric microorganisms are considered milk spoilage bacteria. Due to the high availability of nutrients, milk hosts several pathogenic species with *Listeria monocytogenes*, *Salmonella enterica* and *Escherichia coli* as being the most dangerous (Quigley et al., 2013) (Fig. 7).

1.1.3.2. Animal rennet microorganisms

Animal rennet represents the coagulant agent mostly used in cheese making. This agent is extracted from the abomasum of young ruminants, such as milk-fed calves, lamb and kid (Salvadori del Prato, 1998). Artisan animal rennets can also provide microorganisms that generate defects in cheese or participating to the fermentation process. Voidarou et al. (2011) revealed of lamb a wide biodiversity of microorganisms such as Lactobacilli, Lactococci, Leuconostoc, Pediococci, Streptococci, Bifidobacteria, Enterococci, Clostridia and coliforms in artisan Greece rennet.

Cruciata et al. (2014) investigated on the microbial composition of artisan and industrial Italian animal rennets commonly used for the production of traditional Sicilian cheeses. All rennet samples contained mesophilic cocci LAB, while mesophilic and thermophilic rods and thermophilic cocci were cultivable only in a few samples. The cultivable LAB species detected at high levels were mainly *Enterococcus casseliflavus*, *E. faecium*, *E. faecalis*, *Enterococcus lactis*, *L. delbrueckii* and *S. thermophilus*. The investigation of the dairy

aptitudes of the several LAB strains indicated their potential to act as starter cultures as showing acidifying capacity, rapid autolysis and diacetyl formation.

The Italian animal rennet were also analysed by a culture-independent approach, in order to investigate the dormant/viable but not cultivable (VBNC) bacterial community. High levels of *Lactobacillaceae* and *Streptococcaceae* were found. However, *Lactobacillus crispatus* and *Lactobacillus reuteri* dominated artisan rennet, while *S. thermophilus* was mainly found in industrial rennets (Fig. 8). Flórez et al. (2006) analized samples rennet extracts preparated "from dried kid stomachs cut strips and left to steep in acidified cheese whey for at least 24 h. The last authors have also adopted a culture-independent method and identified, between LAB group, mainly *L. lactis* and lactobacilli. Therefore, all these works proved that animal rennet represents a source of LAB.

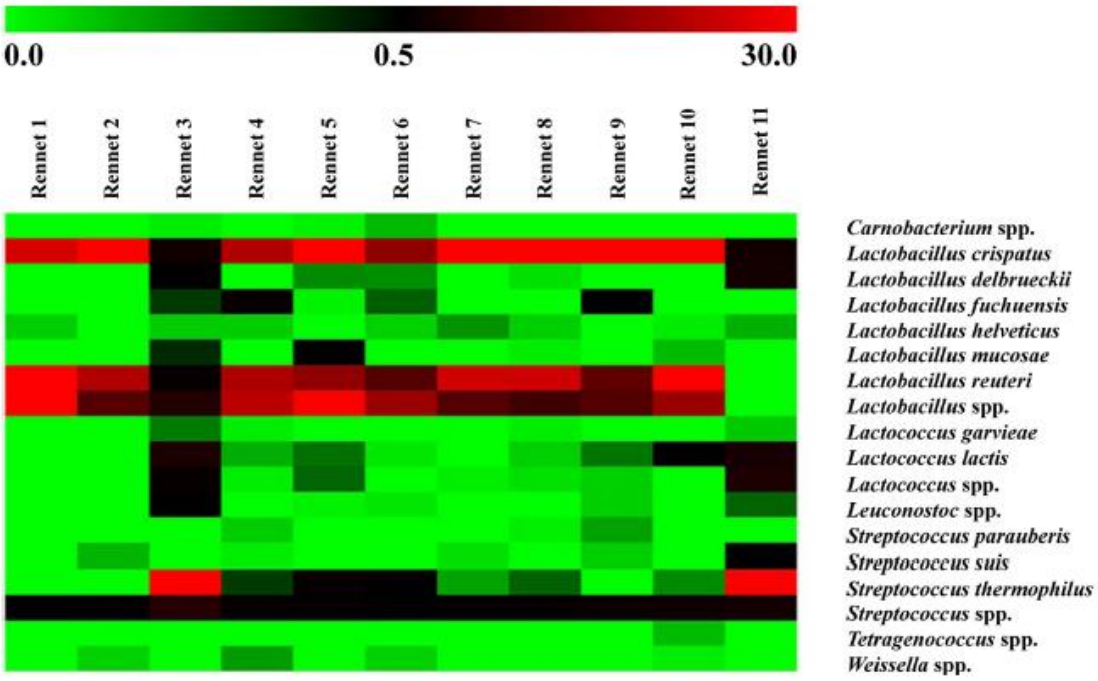

Fig. 8. Distribution of bacterial genera and species as identified by pyrosequencing in animal rennet pastes. Only OTUs belonging to the lactic acid bacteria group and occurring at > 0.1% abundance in at least one sample were included. The color scale indicates the relative abundance of each OTU within the samples (Reference: Cruciata et al., 2014).

1.1.3.3. Biofilm on wood equipment

In nature, microorganisms often form multicellular aggregates that usually accumulate at a solid–liquid interface and are imbedded in a self-produced extracellular polymeric matrix (EPS) highly hydrated, mainly formed by exopolysaccharides, protein and DNA, namely biofilm (Vert et al., 2012). Biofilms can develop to an inert or living surface and can have positive or negative ruole. 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 wood equipment in contact with raw milk and cheeses are rapidly covered by a microbial biofilm. Figure 9 shows image at scanning electon microscope (SEM) of the wooden surface covered by a milk microbial biofilm.

Due to their importance during cheese production, the microbial composition of the wooden vat biofilms used for traditional cheese making have been object of several studies, mainly carried out by Italian and French researchers (Didienne et al., 2012; Licitra et al., 2007; Lortal et al., 2009 Scatassa et al., 2015, Di Grigoli et al., 2015; Gaglio et al., 2015; Scatassa et al., 2015; Settanni et al., 2012). This because both Italy and France are in derogation from the EC No 852/2004 thanks to the EC No 2074/2005 with regards to the use of wooden tools.

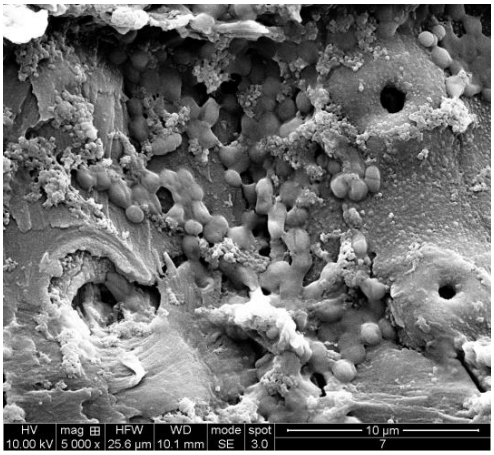

Fig. 9. Scanning electron microscopy image of wooden vat microbial biofilm.

The works performed to analyse the microbiology of the wooden vat biofilms showed a very high biodiversity highlighting the strong influence of the territorial origin of the products. Indeed, a biofilm is mainly formed by LAB that spontaneously evolved through time to a stable consortium, that persist and dominate over the lactic acid and non-lactic acid bacterial populations of raw milk (Lortal et al., 2009; Settanni et al., 2012). Vat LAB biofilms influence deeply the characteristics of the final products since they can lead the microbiology of the ripening process and are defining for their typicality (Di Grigoli et al., 2015). As a matter of fact, the wooden vat biofilm is a very efficient delivery system for LAB, releasing in a few minutes 10^5 to 10^6 CFU of LAB per ml of milk poured into the vat (Lortal et al., 2009). Lactic acid is produced by a combination of the natural raw milk ecosystem and its inoculation by the lactic flora from the vat biofilm.

The predominant species of LAB found in the biofilms used for Ragusano PDO cheese production was *S. thermophilus*. Other codominant species (from 2 to 10 co-dominant species for each vat) were *L. lactis, Lacotobacillus delbrueckii* subsp *lactis*, and *Lactobacillus acidophilus* (Licitra et al., 2007). Interestingly, some streptococci isolated during ripening were identical to those of the corresponding vat. A similar finding was revealed for Caciocavallo palermitano cheese making process (raw cow milk pasta filata cheese produced in Sicily). Vat biofilm was dominated by *S. thermophilus*, but other LAB species belonging to genera *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus* and *Weissella* were also detected (Settanni et al., 2012).

Scatassa et al. (2015b) investigated the ecology of biofilms from several wooden vats used for the production of the traditional stretched cheeses Caciocavallo Palermitano and PDO Vastedda della valle del Belìce (pasta filata cheese from raw sheep's milk produced in Sicily). They observed the predominance of *Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Streptococcus* genera. Dominance LAB were cocci shape,

and unlike to the Ragusano biofilm, with a high percentage of enterococci, which are linked to the cheese typicality.

Regarding the contribution of the biofilm microflora to the cheese ripening some study confirmed the persistence of some non starter LAB from the wooden vat surface to cheese ripening. In particular, 3 strains belonging to the species *E. faecalis*, *E. casseliflavus* and *Enterococcus gallinarum* were found to dominate the *Enterococcus* populations of Caciocavallo palermitano during ripening (Fig. 10) (Di Grigoli et al., 2015).

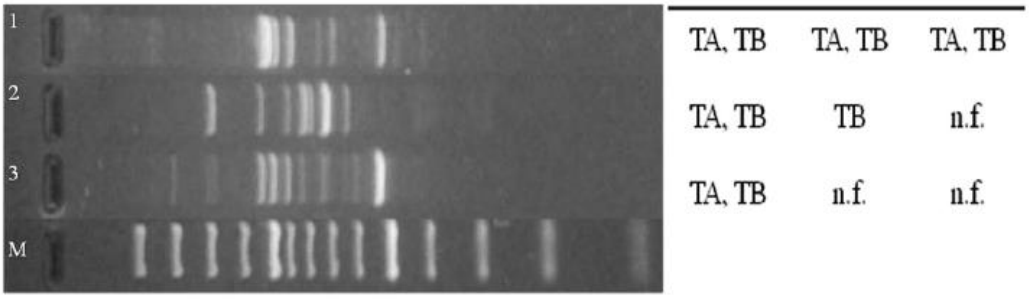

Fig. 10. Persistence of LAB [carried out by RAPD (with primer M13) profile comparison of wooden vat origin during the ripening of traditional Caciocavallo Palermitano cheeses. Lines M, 1-kb DNA molecular size markers (Invitrogen). Lines: 1, *E. faecalis* FMA721; 2, *E. gallinarum* FMA288; 3, *E. casseliflavus* FMA108. n.f., not found (Reference: Di Grigoli et al., 2015).

Some studies (Didienne et al., 2012; Licitra et al., 2007; Lortal et al., 2009; Settanni et al., 2012; Scatassa et al., 2015b) also investigated on the presence of pathogenic bacteria. In particular, these works demonstrated that the wooden vats biofilm do not host *Salmonella* spp., *L. monocytogenes* and other undesired microorganisms and are respectful of the EC No 2073/2005 concerning the microbiological criteria for foodstuffs both for food safety and process hygiene criteria (Commission Regulation, 2005b).

The high biodiversity of LAB associated to the wooden equipment biofilms make them a reservoir of autochthons bacteria including SLAB and NSLAB. Thus, these biofilms strongly contribute to the curd acidification and cheese ripening influencing deeply the

characteristics of the final products and the organoleptic properties of each cheese, highlight the strong territoriality and the historical identity of the traditional products (Galinari et al., 2014; Di Grigoli et al., 2015; Carpino et al., 2017).

1.1.4. Vastedda della valle del Belìce PDO, a cheese typicality to valorise

PDO VdB cheese is produced following the general traditional Sicilian cheese making in the western part of the region using exclusively the raw milk of the autochthonous sheep breed Valle del Belìce (OJC no. C 42/16 19.2.2010). This cheese is produced applying the stretching ("*pasta filata*") technology consisting of an acidification followed by the scalding of the acidified curd (Salvadori del Prato, 1998). Briefly, the production process of VdB (Fig. 11) requires the heating of milk at 40°C, its coagulation with lamb rennet paste, obtained from the abomasum of suckling lambs of Valle del Belìce breed, breaking of the curd to rice dimensions, transfer of the mass into rattan baskets to allow whey drain, acidification of the curd and then stretching in hot (80-90°C) water. After production, VdB cheese is sealed under vacuum, refrigerated and sold fresh in local markets (Mucchetti et al., 2008). Up to date, VdB is the only PDO raw ewes' milk cheese that does not undergo to the ripening and, as a matter of fact, is consumed fresh.

The name "Vastedda" originates from its characteristic shape, resembling a local kind of bread loaf, round and flat, called "vastedda". It might also be originated from the dialect word "*vasta*" meaning spoiled. This because it is said that the first production of the cheese was performed with the attempt to recover a spoiled raw ewes' milk cheese; the cheese maker cut the acidified curd, put the small pieces in hot water but when the mass started to stretch it was put in a soup plate acquiring the typical vastedda bread shape.

The production area of VdB includes the whole homonymous Valle del Belìce area that takes its name from the river that flows through it and comprises three provinces (Agrigento, Trapani and Palermo) located in western Sicily. VdB cheese is strongly linked to its production area, since the animals are fed only with fresh forage on grazed grass, hays and straw produced in the same area (Salvadori del Prato, 1998). Furthermore, a specific nutritional model applied to the sheep breed Valle del Belìce, that resulted from the cross between Pinzirita with Comisana and then with Sarda, officially recognized in 1998, enhances the quality of the milk (OEASAAS, 2007).

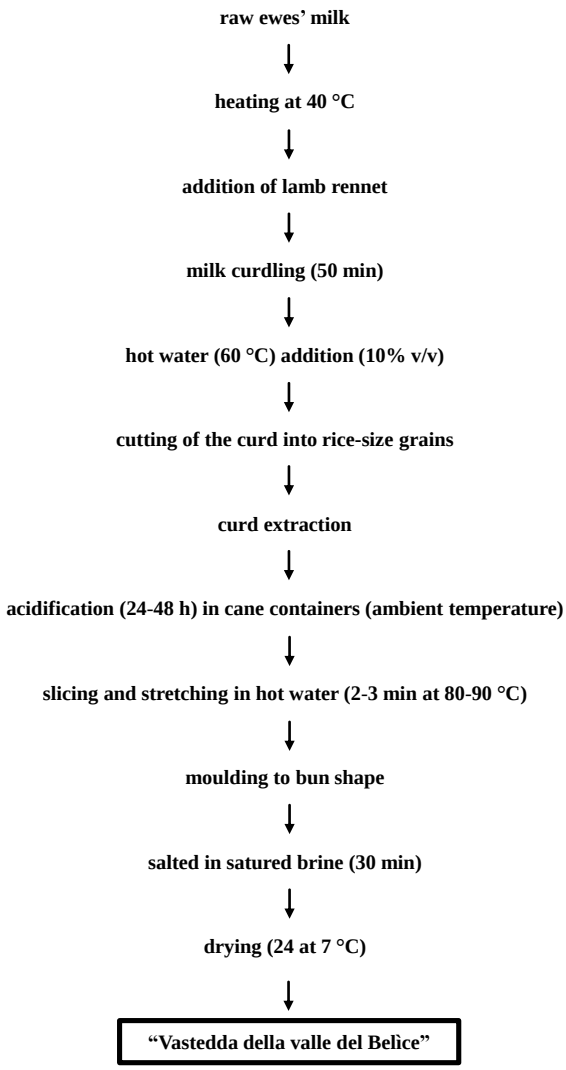

Fig. 11. Flow diagram of "Vastedda della valle del Belìce" cheese production (Reference: Gaglio et al., 2014a).

The final characteristics of VdB cheese are strongly influenced by the use of the traditional wooden equipment (Fig. 12) mainly made of chestnut wood. Among these equipment, the wooden vat (“*tina”*) used for milk curdling assumes a role of paramount importance to inoculate the desired LAB responsible for the acidification of the curd (Settanni et al., 2012; Scatassa et al., 2015a-b). Due to the contact with milk and whey, the internal surface of the wooden vats are covered by aggregates of microorganisms in which cells that are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS) adhere to each other and/or to a surface and these microbial structures are known as “biofilms” (Vert et al., 2012). The wooden vat biofilms play beneficial roles during the cheese production processes, especially because they strongly contribute to enrich milk with LAB responsible for curd fermentation and cheese ripening (Scatassa et al., 2015b; Cruciata et al., *in press*).

Fig. 12. Main traditional wooden equipment used for PDO Vastedda della valle del Belìce cheese production. **A,** “*tina”,* wooden vat used for milk coagulation; **B***, “rotula”,* stick for curd breaking; **C**, “*fuscella*”, rattan basket for curd acidification; **D**, “*piddiaturi”*, wooden vat used for curd stretching.

In past, VdB cheese was traditionally produced only during the summer season. However, due to an increased demand of this fresh product, mostly appreciated than hard ewes’ milk cheeses by several consumers, its production occurs year round. This phenomenon determined a great variability among the production seasons, especially in terms of quality of the final products, as a direct consequence of the different characteristics of the bulk

milks (Verzera et al., 2010) and the different dominating LAB populations, mostly represented by mesophilic species during winter and thermophilic species during warmer seasons (Gaglio et al., 2014).

1.1.4.1. Microbiota of PDO Vastedda della valle del Belìce cheese

Due to the relevance of VdB for the production area, this cheese has been object of several research projects aimed to valorise and ameliorate the production system and the final quality. With this in mind, recent studies analysed the microbial composition of VdB (Reale et al., 2007; Scatassa et al., 2007; Mucchetti et al., 2008; Gaglio et al., 2014a, Todaro et al., 2014, Todaro et al., 2017). LAB resulted always the dominant microbial group, although the growth of this population can be affected in composition and cell density by the geographical location of the dairy factories and the season of production. In general, LAB found in VdB belong to the group of mesophilic cocci (Gaglio et al., 2014a), despite the fact that stretched cheeses are mainly started by streptococci that are thermophilic (Parente et al., 1998). Gaglio et al. (2014b) monitored the behaviour of the different groups of LAB in VdB produced under controlled conditions (pasteurized milk in and experimental dairy plant with stainless steel equipment) and registered an increase of the mesophilic populations, especially lactococci from milk to acidified curd, followed by a decrease during stretching and an increase during refrigerated storage evidencing their potential contribution to maturation even at low temperatures.

The LAB found in VdB showed a certain biodiversity at species level and they were ascribable to the following five main genera: *Enterococcus, Lactobacillus, Lactococcus, Leuconostoc* and *Streptococcus*. The species mostly represented were *L. lactis* and *Leuconostoc mesenteroides* among mesophilic LAB, while *S. thermophilus* and *Streptococcus gallolyticus* subsp. *macedonicus* among the thermophilic ones (Gaglio et al., 2014a). Although the last species is not typically reported as dairy starter shows interesting

dairy aptitudes during ripening and is being considered as adjunct culture in cheese making (Settanni et al., 2011; Guarcello et al., 2016). The dominating VdB LAB showed inhibitory factors against undesired microorganisms; strains of *L. lactis* were able to produce bacteriocin-like inhibitory substances (BLIS) active against *L. monocytogenes* showing their contribution to the biopreservation of these cheese.

Enterococci are also considered as components of cheese adjunct cultures (Foulquié Moreno et al., 2006), but due to some undesired features, such as virulence factors and antibiotic resistance, their harmlessness has to be proven before deliberate addition during cheese making (Gaglio et al., 2016). Enterococci constitute part of the common LAB community present in raw milk (Franciosi et al., 2009) and they were also found in wooden vat biofilms (Settanni et al., 2012) and animal rennet pastes (Cruciata et al., 2014) and are often found after stretching and during refrigerated storage (Todaro et al., 2017). *Enterococcus* species frequently found in VdB were *E. durans*, *E. faecium*, *E. faecalis* and *E. gallinarum* (Gaglio et al., 2014a). Harmless enterococci take part to the definition of the sensory profile of the cheeses and contribute to extend their shelf-life through bacteriocins production (Foulquié Moreno et al., 2006). Gaglio et al. (2016) investigated deeply the antimicrobial resistance and virulence of enterococci collected along the entire production chains of traditional cheeses made in Sicily, including VdB, confirming that dairy enterococci might be a potential source for dissemination of antimicrobial resistances and virulence among bacteria, but also that some harmless strains that did not carry the genes for virulence and antibiotic resistance were found at dominating levels indicating a possible competition with the *Enterococcus* strains expressing the undesired traits.

VdB cheeses have been also extensively investigated for the presence of the four main dairy pathogens *Salmonella* spp., *L. monocytogenes*, β-glucuronidase positive *E. coli* and coagulase positive staphylococci. In general, *Salmonella* spp. and *L. monocytogenes* were

never found in any final cheese and any step of production, *E. coli* and *S. aureus* were detected sometimes at low levels in raw milk, but never in cheese (Scatassa et al., 2007, 2009; Todaro et al., 2017). These studies evidenced that the process conditions determine the high microbiological quality of VdB cheese although it is processed from raw milk and does not undergo a ripening stage.

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PhD OBJECTIVES

PDO Vastedda della valle del Belìce is a stretched cheese produced in the homonymous valley in Sicily (South Italy). This cheese is made from raw milk of the autochthonous sheep breed Valle del Belìce curdled with artisanal animal rennet and transformed into traditional wooden equipment without the addition of starter cultures. This cheese was traditionally produced only during the summer season when the microbiological quality of raw milk could not allow the common dairy transformation into "Pecorino" cheese and the stretching phase was necessary to reduce the levels of the undesired microbial populations, especially that of enterobacteria. However, due to the increasing demand for this cheese throughout the year, it is currently produced year round. The extended production process generated marked differences among the production seasons, especially between summer and winter as a direct consequence of the different characteristics of the bulk milks and the different dominating LAB populations, mostly represented by mesophilic species during winter and thermophilic species during warmer seasons. In order to minimize the differences that can arise during cheese productions, the research group of Agricultural Microbiology – University of Palermo, selected a blend of *Lactococcus lactis* subsp. *cremoris* strains able to dominate during Vastedda cheese making performed under controlled conditions with stainless steel equipment.

Thus, the main aim of the present PhD project was the development of a strategy *ad hoc* for the traditional cheese making performed in Sicily to apply the selected multi-strain *Lactococcus* culture in order to keep the transformation processes traditional and the final products typical. The experimental plan included the development of stable biofilms onto the surfaces of virgin wooden vats with the selected strains. The specific objectives were: to follow and characterize the microbial development during the activation of virgin (new) vats; to evaluate the persistence of the added strains; and 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.

This project pursued also a second general aim focused on the valorization of the local Sicilian forest wood resources *via* production of dairy equipment. This because the use of wooden vats is mandatory for the production of all PDO Sicilian cheeses (Ragusano, Pecorino Siciliano, Piacentinu Ennese, and Vastedda della valle del Belìce), but the tree species most used for this purpose, Douglas fir and chestnut, are imported from other regions. Thus, the use of wood from tree species grown in this region to produce wooden vats intended for cheese making would represent a valuable strategy to valorize the Sicilian forestry resources. To this purpose, biofilms were let to develop on several wood types and the neoformed biofilms were characterized. In particular, the dominating strains were investigated for their technological dairy traits and the corresponding cheese productions were followed in order to evaluate the influence of the different woods on the final characteristics of cheeses, including the organoleptic properties and the sensory attributes using VdB as model cheese.

CHAPTER II

Microbial activation of wooden vats used for traditional cheese production and evolution of neoformed biofilms

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ABSTRACT

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. Lactic acid bacterium 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.

2.1. INTRODUCTION

In Sicily (southern Italy), traditional cheese production from raw milk is often carried out in wooden vats without the inoculation of starter cultures (Settanni et al., 2014), but animal rennet paste is added for curdling (Cruciata et al., 2014). Under these conditions, the desirable lactic acid bacteria (LAB) that transform curd into cheese (Settanni and Moschetti, 2010) are provided only by the raw materials and/or the vat surfaces (Lortal et al., 2009). The wooden vat surfaces host microbial biofilms that include dairy LAB (Lortal et al., 2009; Licitra et al., 2007; Didienne et al., 2012; Settanni et al., 2012; Scatassa et al., 2015). The microbiological quality of raw milk depends on several factors; besides the milking conditions, storage parameters, such as cooling and holding temperatures and the time needed for preservation before milk transformation, are particularly relevant (Heeschen WH, 1996; Murphy and Boor, 2000; Franciosi et al., 2011). 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 (Ernstrom and Woing, 1974). Furthermore, the LAB composition in raw milk is considered unstable, because the raw milk biota may be lost during transformation (Wouters et al., 2002). Animal rennet pastes are coagulant agents that were recently demonstrated to contribute to LAB biodiversity (Cruciata et al., 2014); 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 (Lortal et al., 2009; Licitra et al., 2007; Settanni et al., 2012; Scatassa et al., 2015a) and during ripening (Di Grigoli ey al., 2015). 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

(Gaglio et al., 2014a), which does not undergo a ripening process but is sealed under vacuum after salting and stored refrigerated for a limited time after production (Mucchetti et al., 2008). 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. (2014b) 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.

2.2. MATERIALS AND METHODS

2.2.1. Strains and development of natural whey starter culture (NWSC)

Lactococcus lactis subsp. cremoris PON36, PON153, and PON203, isolated from PDO Vastedda della Valle del Belìce cheeses (Mucchetti et al., 2008) and selected for their dairy performances *in vivo* during Vastedda-like cheese productions (Gaglio et al., 2014b), 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 600nm (OD600) of ca. 1.00 as determined spectrophotometrically (model 6400 spectrophotometer; Jenway Ltd., Felsted, Dunmow, United Kingdom), corresponding to a lactococcus concentration of about 109 CFU/ml. Each strain was inoculated to a final concentration of about 106 CFU/ml in a whey-based medium, prepared as reported by Settanni et al. (2012). After incubation for 24 h at 30°C, the NWSC containing the multistrain culture was used for cheese making.

2.2.2. 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), where in 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.

2.2.3. 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. (2005) and dried as described by Lortal et al. (2009). 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.

2.2.4. 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. (2012). Each vat surface was sampled in duplicate at two diametrically opposite points.

2.2.5. 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. (2012) 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. (2012). Coagulase-positive staphylococci, *Salmonella* spp., *Listeria monocytogenes*, and *Escherichia coli* were analyzed as reported by Scatassa et al. (2015b). 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 H2O2 (5% [wt/vol]) to the colonies. Only Gram positive and catalase-negative cultures were stored in glycerol stocks at -80°C for further investigation.

2.2.6. 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 hydrolysis, acid production from carbohydrates, and CO_2 production from glucose were determined as reported by Gaglio et al. (2014a). Cocci were also grouped according to their growth atpH9.6 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. (2012). 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. (1991), 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 M (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.

2.2.7. 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, 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).

2.2.8. 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).

2.3. RESULTS

2.3.1. 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 *L. 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 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.

2.3.2. 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.

Table 1. Microbiological characteristics of milk and whey bulks used for the activation of the biofilms onto the surfaces of the wooden vats for control cheese productions[ab]

Medium or organism	Plate count (Log/ml)		Statistical significance	Plate count (Log/ml)		Statistical significance
	Milk TA	Milk TZ		Whey TA	Whey TZ	
PCA-SkM 7°C	4.7 ± 0.2^{B}	1.7 ± 0.3^{A}	***	n.d.	n.d.	
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}	***	n.d.	n.d.	
KAA	3.3 ± 0.2^{B}	1.9 ± 0.2^{A}	**	n.d.	n.d.	
E.coli	1.5 ± 0.4^{B}	$<1^{A}$	***	n.d.	n.d.	
CPS	1.3 ± 0.3^{B}	$<1^{A}$	**	n.d.	n.d.	

[a] Results indicate mean values ± S.D. of two plate counts.Data within a line followed by the same letter are not significantly different according to Tukey's test. P value: *, P≤0.05; **, P≤0.01; ***, P≤0.001***.
[b]Abbreviations: TA, production at the dairy factory; TZ, production at the dairy pilot plant; PCA-SkM 7°C, plate count agar added with skimmed milk incubated at 7°C for total psychrotrophic counts; PCA-SkM 30°C, plate count agar added with skimmed milk incubated at 30°C for total mesophilic counts; M17 30°C, medium 17 agar incubated at 30°C for mesophilic coccus LAB; M17 44°C, medium 17 agar incubated at 44°C for thermophilic coccus LAB; MRS, de Man-Rogosa-Sharpe agar for mesophilic rod LAB; WBAM, whey-based agar medium for thermophilic rod LAB; VRBGA, violet red bile glucose agar for *Enterobacteriaceae*; KAA, kanamycin aesculin azide agar for enterococci; CPS, coagulase-positive staphylococci; n.d., not determined.

Table 2. Microbial loads of the biofilms activated onto the surfaces of the wooden vats after the first contact with whey[ab]

Medium	Microbial load (Log CFU/cm2)				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 ± S.D. of two plate counts. Data within a line followed by the same letter are not significantly different according to Tukey's test. P value: *, P≤0.05; **, P≤0.01; ***, P≤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 added with skimmed milk incubated at 7°C for total psychrotrophic counts; PCA-SkM 30°C, plate count agar added with skimmed milk incubated at 30°C for total mesophilic counts; M17 30°C, medium 17 agar incubated at 30°C for mesophilic coccus LAB; M17 44°C, medium 17 agar incubated at 44°C for thermophilic coccus LAB; MRS, de Man-Rogosa-Sharpe agar for mesophilic rod LAB; WBAM, whey-based agar medium for thermophilic rod LAB; VRBGA, violet red bile glucose agar for *Enterobacteriaceae*; KAA, kanamycin aesculin azide agar for enterococci.

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 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.

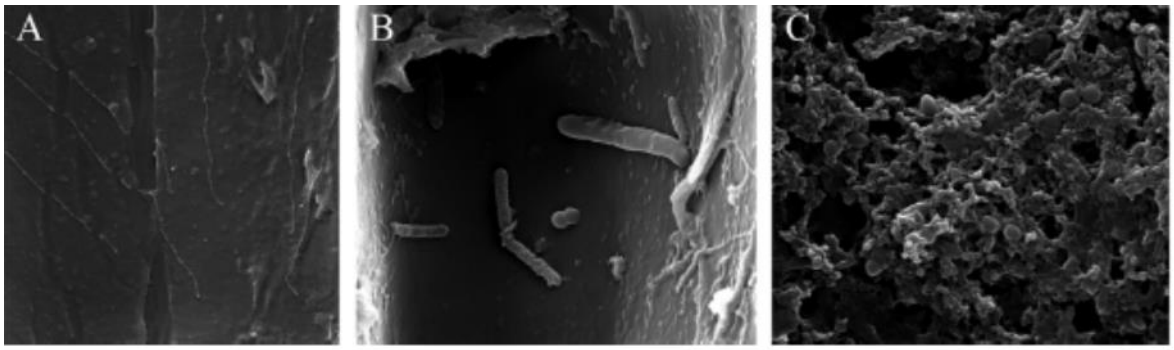

Fig 1. Scanning electron microscopy observations of wooden splinters. (A), Virgin vat after 30 days of hot water treatment; (B), Vat TZ1 after overnight contact with whey obtained from a traditional Vastedda cheese made with raw milk; (C), Vat TZ2 after overnight contact with the NWSC developed with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

2.3.3. 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 theNWSCat 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.

2.3.4. 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

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}	8.4 ± 0.7^{C}	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}	n.d.	n.d.	n.d.	n.d.
	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}	n.d.	n.d.	n.d.	n.d.
	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}	n.d.	n.d.	n.d.	n.d.
	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}	n.d.	n.d.	n.d.	n.d.
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 ± 0.8A	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 ± 0.6A	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 ± 1.0A	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 ± 0.7A	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 added with skimmed milk incubated at 7°C for total psychrotrophic counts; PCA-SkM 30°C, plate count agar added with skimmed milk incubated at 30°C for total mesophilic counts; M17 30°C, medium 17 agar incubated at 30°C for mesophilic coccus LAB; M17 44°C, medium 17 agar incubated at 44°C for thermophilic coccus LAB; MRS, de Man-Rogosa-Sharpe agar for mesophilic rod LAB; WBAM, whey-based agar medium for thermophilic rod LAB; VRBGA, violet red bile glucose agar for *Enterobacteriaceae*; KAA, kanamycin aesculin azide agar for 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, curd; W, whey; AC, acidified curd; ChT_0, cheese at T_0; ChT_{15}, cheese at 15 d.; n.d., not determined.
P value: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.
[b] Log CFU/cm^2 for vat surfaces; Log CFU/ml for milk and whey samples; Log CFU/g for curd and cheeses. Results indicate mean values ± S.D. of 20 plate counts (carried out in duplicate for 10 d of production). Data within a line followed by the same letter are not significantly different according to Tukey's test.

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 ofNWSCto 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.

2.3.5. Phenotyping 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 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. (2001), 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 *L. lactis* subsp. *cremoris* strain of the NWSC. Examining the origins of the

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

Characters	Clusters												
	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:													
15°C	-	-	+	+	+	+	+	+	+	+	+	+	-
45°C	+	+	-	-	-	-	+	+	-	-	+	+	+
pH 9.6	n.d.	n.d.	+	+	-	+	-	-	-	-	+	+	-
6.5% NaCl	n.d.	n.d.	-	-	-	-	+	+	+	+	+	+	-
Resistance to 60°C	+	-	+	-	+	+	-	+	+	+	+	+	+
Hydrolysis of:													
arginine	-	-	+	+	-	+	-	-	+	-	+	+	-
aesculin	-	-	+	+	+	+	-	-	+	-	+	+	-
Acid production from:													
arabinose	+	-	-	-	-	+	-	-	+	+	-	+	-
ribose	+	-	+	+	-	+	-	+	+	+	+	+	+
xylose	+	-	-	-	-	+	-	-	+	+	+	+	-
fructose	+	+	+	+	+	+	+	+	+	+	+	+	+
galactose	+	+	+	+	+	+	+	+	+	+	+	+	+
lactose	+	+	+	+	+	+	+	+	+	+	+	+	+
sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+
glycerol	+	+	+	+	+	+	+	+	+	+	+	+	-
CO_2 from glucose	-	-	-	-	-	-	+	+	+	+	-	-	-

[a] Abbreviations: R, rods; C, coccus; sc, short chain; lc, long chain; n.d., not determined.

isolates in terms of production and sample, it is evident that the selected *L. lactis* subsp. *cremoris* strains, PON36, PON153, and PON203, were present at dominant levels in almost all samples from the TA2 and TZ2 productions, except in milk after resting in thevat and in whey from TZ2 vats. All 44 strains were identified by sequencing of the 16S rRNA gene. 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 *L. lactis* subsp. *lactis* strains (PT132 and PT513), all other strains clustered by species (Fig. 2).

Table 5. Sensory evaluation of Vastedda-like cheeses[a]

	Means score for cheese samples						Significance of differences[b]	
Attribute	PDO cheese	TZ1	TZ2	TA1	TA2	SEM	Judges	Cheese
colour	**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 odours	**2.36**	3.09	2.45	2.91	**2.36**	0.26	NS	NS
pasture	**1.18**	**1.18**	**1.18**	1.36	1.00	0.12	NS	NS
pungent odour	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
salt	1.64	1.73	1.45	1.73	1.55	0.22	NS	NS
bitter	1.36	1.55	1.27	1.73	1.27	0.20	NS	NS
spicy	**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; LSM, least square means; SEM, standard error of means; NS, not significant.
Results indicate mean values. Values reported in bold were identical for PDO cheese and experimental cheeses.
[b] P value: *, P≤0.05; **, P≤0.01.

Even though the subspecies encountered most was *L. 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 *L. lactis* subsp. *lactis*, *Streptococcus thermophilus*, *Leuconostoc lactis*, *Leuconostoc mesenteroides*, and *Enterococcus faecium*, in comparable proportions.

2.3.6. 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.

2.4. 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 (Lortal et al., 2014). 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 (Neviani e Carini, 1994), which is acidified whey. Although the *Lactococcus* starter is commonly prepared in the form of "natural milk cultures" (Parente and Cogan, 2004), 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 (Rossetti et al., 2008; Monfredini et al., 2012).

The biofilms of the four vats after the first contact with whey were recovered through brushing, a nondestructive technique commonly applied for the microbiological investigation of wooden surfaces (Ismaïl et al., 2015). 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 (Lortal et al., 2009; Settanni et al., 2012). 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 (Lortal et al., 2009; Licitra et al.,2007; Didienne et al., 2012; Settanni et al., 2012; Scatassa et al., 2015ab). Except for the vats activated with the

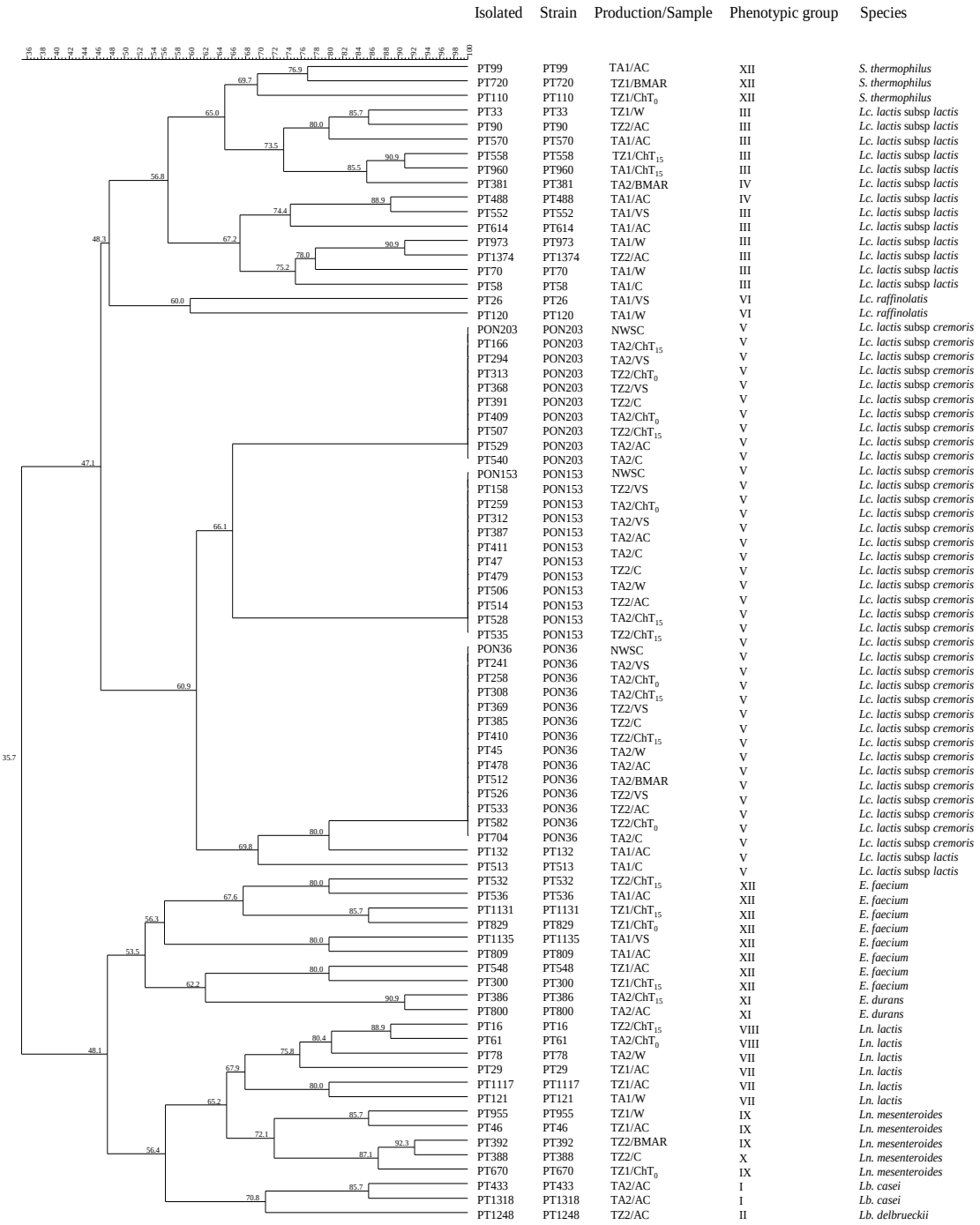

Fig. 2. Dendrogram obtained from combined RAPD-PCR patterns generated with three primers of LAB strains from vats and samples collected during cheese productions. Upper line indicates the percentage of similarity. Abbreviations: *E.*, *Enterococcus*; *Lb.*, *Lactobacillus*; *Lc.*, *Lactococcus*; *Ln.*, *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, curd; W, whey; AC, acidified curd; ChT_0, cheese at T_0; ChT_{15}, cheese at 15 d.

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 (Didienne et al., 2012; Scatassa et al., 2015a-b) starts during the first steps of their activation with whey. Furthermore, it is worth noting that the mixed *L. lactis* subsp. *cremoris* strain culture quickly formed the exopolysaccharide matrix typical of a biofilm structure (Costerton et al., 1994). 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 (Gaglio et al., 2014b).

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 (Settanni et al., 2013). 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 (Scatassa et al., 2015b).

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 (Niro, 2011). 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 (Settanni et al., 2012, 2013). 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. (Gaglio et al., 2014b) when the multistrain inoculum composed of *L. 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 (Gaglio et al., 2014b). The other microbial groups investigated in this study were not investigated in the work of Gaglio et al. (2014b). However, levels of mesophilic LAB rods and enterococci were on the same orders of magnitude as those reported for commercial Vastedda cheeses (Mucchetti et al., 2008; Gaglio et al., 2014b). The presence of enterococci in raw ewes'-milk cheeses is quite common, and although these organisms are associated with cheese typicality (Folquié Moreno et al., 2006), their presence in cheese is considered an indicator of a low hygienic quality of the dairy products (Giraffa, 2003), 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 (Commission Regulation 2005), 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 (Fox et al., 2004). In particular, the majority of the strains characterized in this work represented mesophilic starter LAB (Settanni and Moschetti, 2010). 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 (Gaglio et al., 2014a, b).

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 highquality 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 (Franciosi et al., 2008). 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 (Fitzsimons et al., 1999; Williams et al., 2002).

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. (2014b), this study showed that the addition of *L. 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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CHAPTER III

Formation and characterization of early bacterial biofilms on different wood typologies applied in dairy production

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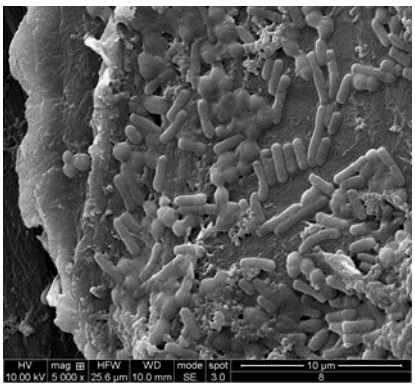

CHAPTER III

Formation and characterization of early bacterial biofilms on different wood typologies applied in dairy production

ABSTRACT

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

IMPORTANCE

This study highlights the importance to use the wooden vats for traditional cheese production and provides evidences for the valorisation of the Sicilian forest wood resources through the production of dairy equipment.

3.1. INTRODUCTION

The Mediterranean area represents an important site of plant diversity (Heywood, 1999). Sicily (south Italy) is a Central Mediterranean region characterized by a high concentration of autochthonous and allochthonous species with approximatively more than 300,000 ha covered by forestland (Gasparini et al., 2005). Forest management in Sicily dates back to the Roman Empire, and this activity includes wood collection for firewood, charcoal, and timber for local handicrafts (La Mantia, 2009). Forest wood is also used to produce equipment for food manufacture (Sala et al.).

In Europe, wood as a food contact material is subject to regulation (EC) no. 1935/2004 of the European Parliament and of the Council of 27 October 2004 on materials and articles intended to come into contact with food (European Parliaments, Council of the European Union. 2004). This regulation refers to different materials that may be subject to specific measures and have been harmonized and adopted at the European level, including plastics, epoxy derivatives, active and intelligent materials, regenerated cellulose, and ceramics, but not yet for wood.

However, wooden equipment is still used to produce several traditional cheeses, especially in France and in Sicily, as a result of the commission regulation (EC) no. 2074/2005, which allows derogation from regulation (EC) no. 852/2004 for foods with traditional characteristics “as regards the type of materials of which the instruments and the equipment used specifically for the preparation, packaging, and wrapping of these products are made” (European Parliaments, Council of the European Union. 2005).

The production of typical cheeses in Sicily has remained almost unchanged through centuries. These productions are carried out from raw milk coagulated and transformed in wooden vats without the addition of exogenous microorganisms (Scatassa et al., 2015a). In the last 10 years, several works carried out by French and Italian groups (Scatassa et al.,

2015a; Didienne et al., 2012; Gaglio et al.,2016; Licitra et al., 2007 Lortal et al., 2009; Settanni et al., 2012) have demonstrated that the wooden vat represents a safe system to produce cheeses. In particular, all investigations conducted on the wooden vat biofilms evidenced the presence of desired dairy lactic acid bacteria (LAB) and the absence of pathogenic species such as *Listeria monocytogenes* and *Salmonella* spp. To transform milk into cheese, the presence of LAB is needed (Gatti et al., 2014). For this purpose, the biofilms of the wooden vat surfaces and the raw materials (milk and animal rennet) represent the main sources of desirable dairy LAB involved in traditional cheese productions (Scatassa et al., 2015a, Lortal et al., 2009; Cruciata et al., 2014).

The bacterial biofilms are primarily formed on the surfaces of virgin wooden vats due to the colonization of LAB transferred by whey (Gaglio et al., 2016). A biofilm is an aggregate of microorganisms in which cells that are frequently embedded within a self-produced matrix of extracellular polymeric substances (EPS) adhere to each other and/or to a surface (Vert et al., 2012). Lortal et al. (2014) proved that the biofilms investigated on the wooden vats used in cheese making represent efficient delivery systems for dairy LAB.

The technological characterization and genotypic identification of the LAB biofilms associated with the wooden vats used to produce different cheeses revealed the presence of several dairy LAB, including starter LAB (SLAB), responsible for the acidification of curd, and nonstarter LAB (NSLAB), implicated in the maturation (Scatassa et al., 2015a).

In Sicily, the use of wooden vats is mandatory for the production of all PDO Sicilian cheeses, such as Ragusano, Pecorino Siciliano, Piacentinu Ennese, and Vastedda della valle del Belìce. Nowadays, the tree species most used for this purpose are Douglas fir and chestnut imported from other regions. Although little is known about the use of other tree species in cheese making, there is an ancient tradition of the use of wood in Sicily (La Mantia, 2009). Indeed, the use of wood from tree species grown in this region to produce

wooden vats intended for cheese making would represent a valuable strategy to valorize the Sicilian forestry resources.

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

3.2. MATERIALS AND METHODS

3.2.1. Wood types and vat production

The wooden vats applied in cheese production were made from seven tree species (Table 1). Chestnut (*Castanea sativa* Miller) wood was collected from Petralia Sottana, Italy, one of the areas where this species was largely cultivated in past (La Mantia et al., 1999). Chestnut wood from the Calabria region was used as a control, since it represents the most common wood species for wooden dairy equipment used in western Sicily (La Mantia, 2009). Cedar (*Cedrus libani* A. Rich.) is an allochthonous species widely diffused in Italy for reforestation (La Mela Veca et al., 2013) but is no longer cultivated for its wood. Black pine (*Pinus nigra* J.F. Arnold) is present in an autochthonous population on Mount Etna and is widely used in Sicily for afforestation purposes. Ash (*Fraxinus ornus* L.) represents the forest tree species most found in Sicily, used in the past for multiple purposes by farmers and shepherds (La Mantia, 2009). Black poplar (*Populus nigra* L.) is spread throughout the areas around streams but at present, is barely cultivated in a restricted area (La Mantia et al., 2002). In this work, some tree species grown for fruit production were also included, in particular, walnut (*Juglans regia* L.), which is the most used species in Sicily for furniture production, and cherry (*Prunus avium* L.), which has been used for this purpose in the past (La Mantia, 2009). Eight trials were performed, seven of which (W2 to W8) were carried out in the vats produced from the wood of trees grown in Sicily that have never been used for dairy purposes before, and one trial (W1, control) was carried out in Calabrian chestnut vats. A total of 16 wooden vats (two replicates for each wood type) with 15 liter volumes were made by a local artisanal producer. A treatment with hot water (80°C) was applied for 30 consecutive days to remove the tannins from the wood. After it was added, the hot water was left cooling at room temperature and remained in contact

with the wood for approximately 24 h. The last washing step was followed by a vigorous brushing of coarse salt on the internal surfaces of the vats.

Table 1. Typology and origin of the woods used for wooden vats production

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

3.2.2. Evaluation of the dairy suitability of the different wood types

To verify the absence of drawbacks for using different wood types in cheese making, the wood components extracted during the water treatment performed to remove tannins were tested for their potential inhibitory activity on the main LAB species and main dairy pathogens: *Enterococcus durans* DSM 20633T, *Enterococcus faecium* DSM 20477T, *Lactobacillus buchneri* LMG 6852T, *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842T, *Lactobacillus delbrueckii* subsp. *lactis* ATCC 12315T, *Lactobacillus casei* LMG 6904T, *Lactobacillus paracasei* subsp. *tolerans* LMG 9191T, *Lactobacillus rhamnosus* LMG 6400T, *Lactococcus lactis* subsp. *cremoris* DSM 20069T, *Lactococcus lactis* subsp. *lactis* DSM 20481T, *Leuconostoc mesenteroides* DSM 20343T, *Pediococcus acidilactici* LMG 11384T, *Streptococcus thermophilus* DSM 20617T, *Escherichia coli* ATCC 35150, *L. monocytogenes* ATCC 7644, *Salmonella enteritidis* ATCC 13076, and *Stafilococcus aureus* ATCC 33862. The aqueous solutions (1.2 liters) obtained after the first overnight contact between the hot water and each wooden vat were collected in disposable aluminum trays and frozen at - 80°C for 48 h. The water-soluble extracts were then freeze-dried using a Scanvac Coolsafe 55-9 (Denmark) apparatus. Each WSE was rehydrated with distilled water to a final concentration of 500 mg/ml, corresponding to their complete dissolution.

The inhibitory activity of WSEs was tested by applying the paper disc diffusion method as previously reported (Gaglio et al., 2017). Briefly, an agar base support (2% wt/vol water agar) was overlaid with 7 ml of the optimal soft agar (0.7% wt/vol) medium, as indicated by the respective culture collection for each strain, previously inoculated with approximately 10^7 CFU/ml of a given test organism. Sterile filter paper discs (Whatman no. 1) of a 6-mm diameter were placed on the surface of the double agar layer and soaked with 10 l of each WSE. Sterile water and streptomycin (10% wt/vol) were used as negative and positive controls, respectively. Plates were incubated at the optimal growth temperatures indicated by the culture collection for 24 h, and the inhibitory activity was evaluated as positive if a definite clear area was detected around the paper discs.

3.2.3. Biofilm formation

The biofilms were formed on the wooden surfaces with hot (approximately 80°C) deproteinized whey, which is the residual whey from ricotta cheese production, left in contact with the vats for 24 h. The treatment with whey was repeated for seven consecutive days in an artisanal dairy farm (Ovini e Natura Società Agricola di Firpo F. & C. s.a.s., Santa Margherita di Belìce, Italy) selected from a previous survey (Todaro et al., 2011) for its high hygienic standards. The development of microbial populations on the internal surfaces of the wooden vats was analyzed by SEM as previously reported (Gaglio et al., 2016). Parallelepiped wood splinters (approximately 40 mm by 20 mm by 2 mm) were aseptically collected from each vat with a stainless steel scalpel on the 30th day of hot water treatment (before bacterial adhesion) and after the 7 days of contact with whey (after bacterial adhesion). The samples were analyzed with an FEI Quanta 200F microscope (FEI, Holland) at 5,000 magnification. SEM analyses were conducted at the Department of Industrial and Digital Innovation, University of Palermo, Italy.

3.2.4. Viable cell counts

The culture-dependent analyses of the biofilms of the wooden vats were performed on the surface samples (100 cm^2) collected at the same time as the wood splinters. The biofilms were collected by the brushing recovery method as previously described (Didienne et al., 2012) using sterile plastic squares (Biogenetics s.r.l., Padua, Italy) positioned halfway up the sides and on the bottoms of the vats. The cell suspensions of wooden vat surface samples were subjected to a decimal serial dilutions in Ringer's solution (Sigma-Aldrich, Milan, Italy). Several microbial groups were investigated: TMM on plate count agar (PCA) supplemented with 1 g/liter skimmed milk (SkM) incubated aerobically at 30°C for 72 h; TPM as described for TMM, but the incubation occurred at 7°C for 7 days; mesophilic and thermophilic LAB cocci on M17 agar incubated anaerobically at 30°C and 44°C, respectively, for 48 h; mesophilic rod LAB on de Man-Rogosa-Sharpe (MRS) agar, acidified to pH 5.4 with lactic acid (5 M) and incubated anaerobically at 30°C for 48 h; thermophilic rod LAB on whey-based agar medium (WBAM) as reported by Settanni et al. (2012); enterococci on kanamycin esculin azide (KAA) agar, incubated aerobically at 37°C for 24 h; members of the *Enterobacteriaceae* family on violet red bile glucose agar (VRGBA), incubated aerobically at 37°C for 24 h; coagulase-positive staphylococci (CPS) on Baird-Parker (BP) agar supplemented with rabbit plasma fibrinogen (RPF), incubated aerobically at 37°C for 48 h; pseudomonads on Pseudomonas agar base (PAB) supplemented with cephaloridine sodium fusidate cetrimide (CFC), incubated aerobically at 25°C for 48 h; *E. coli* on tryptone bile glucuronide (TBG) agar, incubated aerobically for 24 h at 44°C; *E. coli* O157 by the method described in AFNOR BIO 12/25-05/09 (36); enzyme-linked fluorescence assays (ELFAs) performed with the automated system VIDAS (bioMérieux, Marcy l'Etoile, France) were used for *Salmonella* spp. with the screening method described in AFNOR BIO 12/32-10/11 (Agence Francaise de Normalisation.

2011.) and for *L. monocytogenes* with the method described in AFNOR BIO 12/11-03/04 (Agence Francaise de Normalisation. 2004), while for sulfite-reducing anaerobes (SRA), the ISO 15213:2003 technique was followed (International Organization for Standardization. 2003.). Microbiological counts were carried out in triplicates for all samples at each collection time. Anaerobiosis occurred in hermetically sealed jars with the AnaeroGen AN25 system (Oxoid, Milan, Italy). All media were purchased from Oxoid.

3.2.5. Isolation, phenotypic grouping, and genetic identification of LAB

The presumptive colonies of LAB were collected after growth on all agar media used for their plate counts (M17, MRS, and WBAM). At least five colonies per each morphology detected were isolated from the agar plates and purified to homogeneity after several subculturing steps on the same medium used for the viable cell count determination. After microscopic inspection, the pure cultures were subjected to the KOH method (Gregersen, 1978) to determine Gram type and to the catalase test (5% wt/vol H_2O_2) to exclude bacteria that produce energy from respiration. Only Gram-positive and catalase-negative cultures were considered putative LAB and were stored in glycerol stocks at -80°C until further investigations. The presumptive LAB isolates were grouped as previously described (Gaglio et al., 2014a) on the basis of cell morphology, cell disposition, growth at 15 and 45°C, heat resistance (60°C for 30 min), NH_3 production from arginine, esculin hydrolysis, acid production from carbohydrates, and CO_2 production from glucose. The coccus-shaped isolates were further grouped according to their growth at pH 9.6 and in the presence of 6.5 g/liter NaCl to separate enterococci able to grow under these conditions from other LAB. All phenotypic groups were subjected to the genetic characterization performed initially to differentiate the isolates at strain level and then to identify the LAB species. The genetic investigation was carried out on DNA extracted from the cultures grown overnight in the optimal conditions in broth media (M17, MRS, and WBAM) using the InstaGene Matrix

kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Crude cell extracts were used as the templates for the PCRs. The differentiation of the LAB strains was performed by RAPD-PCR with individual applications of the primers M13 (Settanni et al., 2012), AB111, and AB106 (van den Braak et al., 2000). RAPD-PCR profiles were analyzed with the pattern analysis software package GelCompar II version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium), and the isolates with different profiles were considered different strains. All LAB with different RAPD-PCR profiles were identified by 16S rRNA gene sequencing. PCRs were performed as previously described (Wheisburg et al., 1991), and the resulting DNA amplicons were sequenced at the Istituto Zooprofilattico Sperimentale della Sicilia A. Mirri (Palermo, Italy). DNA sequences were determined by an ABI PRISM 3500 genetic analyzer (Applied Biosystems, Carlsbad, CA, USA). The identities of the sequences were determined by a blastn search against the NCBI nonredundant sequence database and by comparison with the sequences of the sole type strains within the EZTaxon database (https://www .ezbiocloud.net/taxonomy).

3.2.6. Dairy properties of wooden vat LAB

The LAB isolated from the wooden vat biofilms were tested for the main technological traits useful in cheese production: acidification, diacetyl formation, autolytic kinetics and generation of antimicrobial compounds. The acidifying capacity of each LAB was assayed in 100 ml of full fat ultrahigh temperature (UHT) milk inoculated with a 1% (vol/vol) cell suspension and incubated at the optimal growth temperature. The pH was measured in aliquots of 4 ml, aseptically collected from the flasks, at 2 h intervals for the first 8 h, and then at 24, 48, and 72 h after inoculation. Diacetyl production was determined by inoculating each strain in UHT milk as described above. After 24 h of incubation, aliquots of 1 ml were added to 0.5 ml of α-naphthol (1% wt/vol) and KOH (16% wt/vol) and kept at

30°C for 10 min. Diacetyl generation was indicated by the formation of a red ring at the top of the tube (King, 1948). Autolysis of whole cells was determined in buffer solution (0.5 M potassium phosphate, pH 6.5) following the method of Mora et al. (2003) using a 6400 spectrophotometer (Jenway Ltd., Felsted Dunmow, UK) at 600 nm wavelength. Optical density (OD) was measured at 2-h intervals for the first 8 h and then at 24, 48, and 72 h after inoculation. The antimicrobial activity of each LAB was determined against strains that are highly sensitive to bacteriocins, such as *L. sakei* LMG2313, *L. innocua* 4202, and *L. monocytogenes* ATCC 19114 (Corsetti et al., 2008). The inhibitory activity was first evaluated with the agar-spot deferred method, and only the strains displaying a clear inhibition of the indicator bacteria were further analyzed by the well diffusion assay (WDA) (Schillinger and Lücke, 1989.) as modified by Corsetti et al. (2005). All tests were carried out in triplicates. The supernatants showing inhibitory properties were treated with proteinase K (12.5 U/mg), protease B (45 U/mg), and trypsin (10.6 U/mg) diluted to 1 mg/ml in phosphate buffer (pH 7.0). After incubating at 37°C for 2 h, the remaining activity was measured by a second WDA (Settanni et al., 2005). All enzymes were purchased from Sigma-Aldrich (St. Louis, MO).

3.2.7. Wooden vat contamination

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

vats were kept as control systems (the same volume of Ringer's solution was added without pathogens) (control productions, CP). The vats were used to transform contaminated and uncontaminated milk into cheese under controlled conditions in a dairy pilot plant (IZS, Palermo, Italy) in order to investigate the ability of the dairy pathogenic bacteria to grow or survive on the wooden vat surfaces during the cheese-making process. Cheeses were produced as reported by Gaglio et al. (2014) with the addition of a commercial rennet paste (Clerici Sacco International, Cadorago, Italy). Cheese trials were carried out in duplicates in two consecutive weeks. Pathogenic cultures were grown overnight in the optimal conditions and centrifuged at 5,000 × g for 5 min. The cells were washed twice in Ringer's solution (Sigma-Aldrich, Milan, Italy) and resuspended in the same solution until reaching an OD of ca. 1.00, determined by means of a 6400 spectrophotometer (Jenway Ltd., Felsted Dunmow, UK) at 600 nm wavelength, which approximately corresponds to a concentration of 10^9 CFU/ml, as verified by plate counts. The final cell densities in milk were 10^3 CFU/ml for *E. coli* O157 ATCC 35150 and *S. aureus* ATCC 33862, and 30 CFU/ml for *L. monocytogenes* ATCC 7644 and *S. enteritidis* ATCC 13076, which simulated a massive contamination (Chatelard-Chauvin et al., 2015, European Parliament, Council of the European Union. 2005).

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

3.2.8. Statistical analyses

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

3.2.9. Accession number(s)

All sequences determined in this study were deposited in GenBank database under the accession numbers MF575838 to MF575847.

3.3. RESULTS

3.3.1. Evaluation of the inhibitory activity of wood water soluble extracts and scanning electron microscopy of the wooden biofilms

None of the water soluble extracts (WSE) obtained from the hot water treatment of the wooden vats showed inhibitory properties against the main protechnological and pathogenic bacteria relevant for dairy productions. This test was conducted only on the WSE collected after the first overnight treatment, since the subsequent treatments generated visibly clearer solutions with less components extracted from the woods over time. Thus, all wood types displayed no drawbacks during the lactic acid fermentation due to their inability to inhibit all 13 starter and nonstarter LAB associated with cheese production and ripening. The four pathogenic species, including two that were Gram negative (*E. coli* ATCC 35150 and *S. enteritidis* ATCC 13076) and two that were Gram positive (*L. monocytogenes* ATCC 7644 and *S. aureus* ATCC 33862), were not negatively affected by the WSE, indicating that the seven types of wood can be susceptible to colonization by undesirable organisms. The results of scanning electron microscopy (SEM) carried out on wood splinters collected from the eight vats after 30 days of hot water treatment and after 7 days of contact with whey are shown in Fig. 1 and 2, respectively.

The treatment with water prevented microbial development on all of the vats analyzed (Fig. 1A to H). On the contrary, the treatment with hot whey facilitated the attachment of different bacteria, which generated a visible exopolysaccharide matrix typical of biofilm structures on the internal surfaces of all vats (Fig. 2A to H). In particular, the microscopic inspection showed the presence of both rod and coccus bacteria.

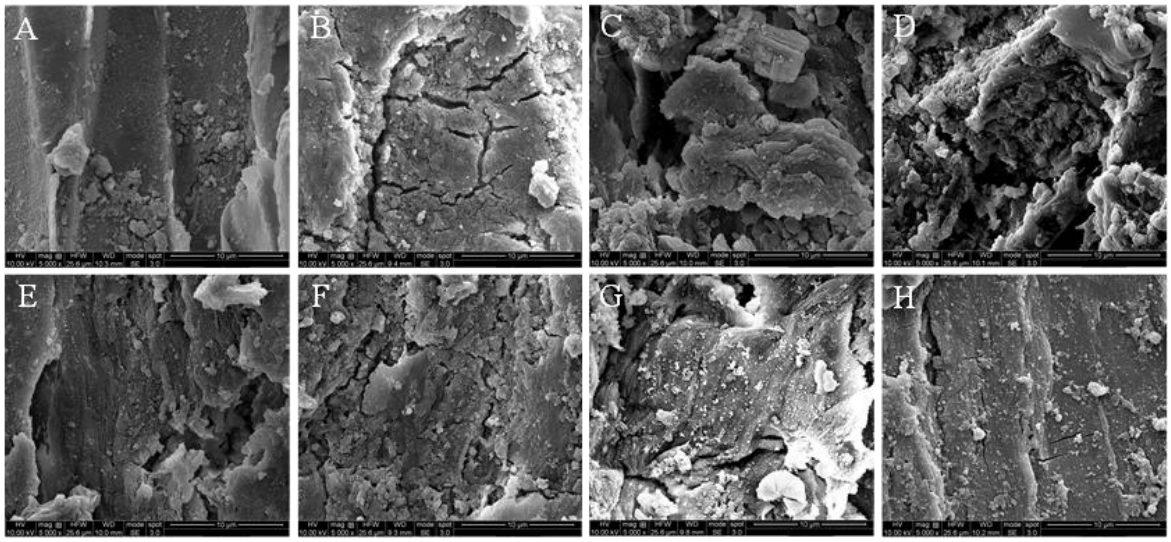

Fig. 1. Scanning electron microscopy observations of wooden splinters after 30 days of hot water treatment. (A) Calabrian chestnut; (B) Sicilian chestnut; (C) Cedar; (D) Cherry; (E) Ash; (F) Walnut; (G) Black pine; (H) Poplar.

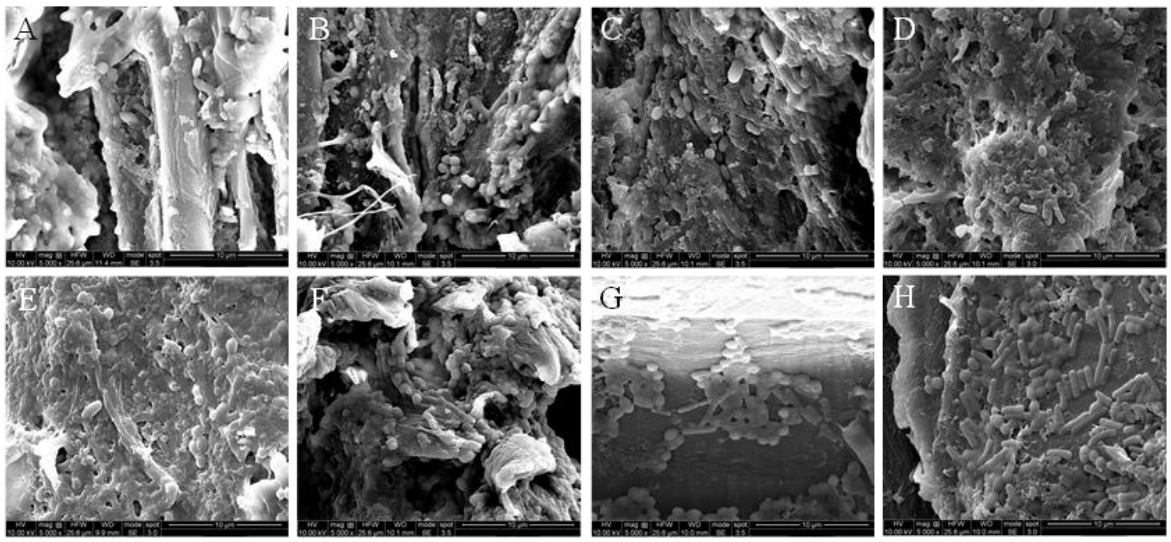

Fig. 2. Scanning electron microscopy observations of wooden splinters after activation with whey. (A) Calabrian chestnut; (B) Sicilian chestnut; (C) Cedar; (D) Cherry; (E) Ash; (F) Walnut; (G) Black pine; (H) Poplar.

3.3.2. Microbial levels

The viable counts registered for the 14 microbial groups harbored on the wooden vat surfaces after tannin removal (30 days of hot water treatment) and after adhesion of bacteria transferred by whey (7 days of whey treatment) are reported in Table 2. No

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

3.3.3. Composition of bacterial populations of wooden vat biofilms

Four hundred eleven colonies of presumptive LAB were collected from the eight wooden vat surfaces in the first replicate trials. After purification and microscopic inspection, 237 rods and 174 cocci were found. After Gram and catalase tests, 213 rods and 156 cocci were still considered presumptive LAB cultures, being Gram positive and catalase negative. The combination of the phenotypic characteristics determined the separation of the 369 cultures into six groups, two for rods and four for cocci (Table 3).

The largest groups, including almost 33% and 31% of the total isolates, were groups 1 and 3, respectively. Rods included obligately heterofermentative cultures (group 1) and obligately homofermentative bacteria (group 2).

Table 2. Microbial loads of wooden vat surfaces after 30 days of hot water treatment and after the activation[a].

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

[a] Units are log CFU/cm^2. Results indicate mean values ± S.D. of four plate counts (carried out in duplicate for two independent productions). Data within a column followed by the same letter are not significantly different according to Tukey's test. P value: ***$P \leq 0.001$.
Abbreviations: TMM, total mesophilic microorganisms; TPC, total psychrotrophic count

Table 3. Phenotypic grouping of the LAB isolated from the wooden vat surfaces after activation with whey.

Characters	Clusters					
	1 (n=121)	2 (n=31)	3 (n=114)	4 (n=67)	5 (n=29)	6 (n=7)
Morphology	R[a]	R	C[c]	C	C	C
Cell disposition	Scc	sc	sc	sc	lc[d]	lc
Growth:						
15°C	-	-	+	+	-	-
45°C	+	+	-	+	+	+
pH 9.6	ND[e]	ND	+	+	-	-
6.5% NaCl	ND	ND	-	+	-	-
Resistance to 60°C	+	+	+	+	+	+
Hydrolysis of:						
arginine	+	-	+	+	-	+
aesculin	+	-	+	+	-	+
Acid production from:						
arabinose	+	-	-	+	-	+
ribose	+	-	+	+	+	+
xylose	+	-	-	+	-	+
fructose	+	+	+	+	+	+
galactose	+	+	+	+	+	+
lactose	+	+	+	+	+	+
sucrose	+	+	+	+	+	+
glycerol	+	+	+	+	+	-
CO_2 from glucose	+	-	-	-	-	-

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

Furthermore, the isolates of the groups 1, 2, 5, and 6 showed the ability to grow at 45°C but not at 15°C and were classified as thermophilic LAB. One hundred twenty cultures, representative of the different wooden vat surfaces analyzed, were selected from each phenotypic group and subjected to randomly amplified polymorphic DNA (RAPD) analysis. This genotypic differentiation revealed the presence of 10 distinct strains (data not shown). The strains were identified by 16S rRNA gene sequencing, and the sequence comparison within two distinct databases identified six species: *E. faecium* (n 3), *Lactobacillus delbrueckii* (n 1), *Lactobacillus fermentum* (n 2), *Lactococcus lactis* (n 1), *Streptococcus gallolyticus* subsp. *macedonicus* (n 2), and *Streptococcus parauberis* (n 1). *L. fermentum* was isolated from all the wooden vat surfaces of this study, *L. lactis* was not found in W7, while the other species were found associated with one or two vats. In particular, *E. faecium* was isolated from W2 and W3, *L. delbrueckii* from W6 and W8, *S. gallolyticus* subsp. *macedonicus* from W1 and W3, and *S. parauberis* only from W4.

Table 4. Technological characteristics of LAB isolated from wooden vat surfaces

Strains	Species	pH / autolysis[a]								Diacetyl production	Bacteriocin-like inhibitory activity[a]		
											Indicator strains[b]		
		T_0	2h	4h	6h	8h	24h	48h	72h		19114	4202	2313
TV92	*E. faecium*	6.74 / 0.995	6.74 / 0.972	6.67 / 0.887	6.62 / 0.880	6.55 / 0.874	5.09 / 0.863	4.49 / 0.846	4.37 / 0.819	+	-	-	-
TV99	*E. faecium*	6.71 / 0.999	6.68 / 0.987	6.55 / 0.975	6.47 / 0.968	6.41 / 0.966	5.31 / 0.933	4.38 / 0.861	4.36 / 0.786	-	-	-	-
TV121	*E. faecium*	6.75 / 0.999	6.66 / 0.986	6.56 / 0.983	6.44 / 0.983	6.40 / 0.977	5.83 / 0.977	5.12 / 0.864	4.82 / 0.864	-	-	-	-
TV199	*L. delbrueckii*	6.76 / 0.991	6.74 / 0.991	6.72 / 0.958	6.69 / 0.958	6.68 / 0.945	4.45 / 0.945	4.06 / 0.945	3.97 / 0.910	-	-	-	-
TV173	*L. fermentum*	6.75 / 0.999	6.64 / 0.943	6.57 / 0.903	6.57 / 0.880	6.53 / 0.868	6.19 / 0.786	5.64 / 0.494	4.70 / 0.451	-	1.5 ± 0.12	1.6 ± 0.17	1.6 ± 0.00
TV187	*L. fermentum*	6.70 / 0.999	6.60 / 0.943	6.55 / 0.865	6.52 / 0.86	6.50 / 0.836	5.95 / 0.778	4.21 / 0.693	3.76 / 0.621	-	1.5 ± 0.00	1.6 ± 0.10	1.7 ± 0.17
TV70	*L. lactis*	6.76 / 0.998	6.68 / 0.983	6.44 / 0.873	5.94 / 0.855	5.44 / 0.801	4.34 / 0.567	4.31 / 0.409	4.05 / 0.396	-	-	-	-
TV69	*S. gallolyticus* subsp. *macedonicus*	6.74 / 0.996	6.66 / 0.949	6.59 / 0.870	6.46 / 0.861	6.41 / 0.849	5.56 / 0.585	4.90 / 0.407	4.25 / 0.402	+	-	-	-
TV103	*S. gallolyticus* subsp. *macedonicus*	6.71 / 0.999	6.64 / 0.980	6.53 / 0.980	6.46 / 0.973	6.39 / 0.973	5.73 / 0.973	4.99 / 0.855	4.89 / 0.855	-	-	-	-
TV125	*S. parauberis*	6.74 / 0.998	6.66 / 0.758	6.59 / 0.758	6.50 / 0.756	6.44 / 0.756	5.83 / 0.756	5.01 / 0.643	4.79 / 0.643	-	-	-	-

Abbreviations: *E.*. *Enterococcus*; *L.*. *Lactobacillus*; *L.*. *Lactococcus*; *S.*. *Streptococcus*.

[a] Results of pH and autolysis indicate mean values of two independent experiments.

[b] With of the inhibition zone (millimeters). Results indicate mean ± SD of three independent experiments.

[c] Bacterial species: *Listeria monocytogenes* ATCC 19114; *Listeria innocua* 4202; *Lactobacillus sakei* 2313

3.3.4. Technological traits of LAB

The results of the technological characterization of the 10 strains are reported in Table 4. The acidification kinetics showed that the fastest decrease in the milk pH was observed in the presence of *L. delbrueckii* TV199, *L. fermentum* TV187, and *L. lactis* TV70, which displayed values in the range of 3.76 to 4.05, after 24 h of fermentation. *L. lactis* was the best acidifier, since the milk inoculated with this strain reached a pH of 5.44 after 8 h. Except for *L. fermentum* TV173, all the strains produced a drop in pH below 6.00 only after 24 h. Strains *L. lactis* TV70 and *S. gallolyticus* subsp. *macedonicus* TV69 were the only ones showing a relevant decrease in the optical density at 600 nm (OD600) after 24 h of incubation, reaching values of 0.564 and 0.585, respectively. Diacetyl production was scored positive only for *E. faecium* TV 92 and *S. gallolyticus* subsp. *macedonicus* TV69. The antibacterial activity was observed only for *L. fermentum* TV173 and TV187, which were able to inhibit the growth of *Lactobacillus sakei* LMG2313, *Listeria innocua* 4202 and *L. monocytogenes* ATCC19144. The treatment with proteolytic enzymes revealed a loss of the inhibitory power.

Table 5. Microbial loads of pathogenic bacteria during the artificial contamination test

First production				**Second production**			
Milk CP	Milk EP	Vat CP	Vat EP	Milk CP	Milk EP	Vat CP	**Vat EP**
<1	2.96 ± 0.26	<1	<1	<1	3.06 ± 0.20	<1	**<1**
<1	1.59 ± 0.19	<1	<1	<1	1.49 ± 0.15	<1	**<1**
<1	1.65 ± 0.21	<1	<1	<1	1.45 ± 0.24	<1	**<1**
1.82 ± 0.21	**3.03 ± 0.22**	**<1**	**<1**	**1.75 ± 0.19**	**3.09 ± 0.23**	**<1**	**<1**

3.4. DISCUSSION

The use of wooden vats in Sicily is mandatory for the production of all four regional PDO cheeses: Ragusano, Pecorino Siciliano, Piacentinu Ennese, and Vastedda della valle del Belìce. Nowadays, the tree species most used for this purpose are Douglas fir and Calabrian chestnut. Regarding these tree species, chestnut wood shows excellent technological features (La Mantia et al., 2006; Maggiore et al., 2006), but the cultivated areas have decreased notably in the last years (La Mantia et al., 1999). The use of wood from black pine and cedar would provide an economical significance for the reforestation. Black poplar, although widespread along the hedges in the past, was almost completely destroyed by fire (Massa and La Mantia, 2007). Thus, the revival of this crop could serve to restore its habitats. The extensive distribution of ash in Sicily could enable a renewed use of this species.

In this study, we evaluated the early stage of the formation of lactic acid bacterial biofilms on eight different wooden vat typologies. SEM inspection indicated the absence of bacterial cells on the surfaces of the virgin wooden vats and the presence of bacterial aggregates in EPS matrices after whey treatment. The aims were to expand the characterization of the LAB populations at the strain level and to investigate their technological traits in order to evaluate their potential in cheese manufacture and ripening. The eight wooden vats applied for cheese production were made from seven Sicilian tree species that have never been used for dairy purposes and one control made with Calabrian chestnut. The bacteria on the internal surfaces of the wooden vats were analyzed after 30 d of hot water treatment or 7 d of contact with whey by using the brushing method commonly applied for the microbiological investigation of wooden surfaces (Ismaïl et al., 2015). No sample before or after activation harbored pathogenic bacteria such as

Salmonella spp., *L. monocytogenes*, *E. coli*, and CPS. The absence of these bacteria after activation was mainly due to the acidic conditions resulting from LAB that ferment lactose from whey and inhibit the adhesion and survival of several microorganisms (Lortal et al., 2009; Settanni et al., 2012). Plate counts confirmed the SEM analysis, since viable bacterial cells were not detected before whey treatment. The comparison between LAB and TMM levels unequivocally indicated that all vat biofilms were dominated by LAB which showed levels above 4 log cycles per square centimeter for all samples. These findings confirmed those from previous inspections on aged vats (Licitra et al., 2007; Lortal et al., 2009; Diedienne et al., 2012; Settanni et al., 2012; Scatassa et al., 2015a). Enterococci were detected only on the wooden vat surfaces of cedar and cherry at levels lower than those registered for the other LAB. Previously, enterococci were found on the surfaces of the wooden vats made from Douglas fir or Calabrian chestnut used for traditional cheese making in western Sicily (Settanni et al., 2012; Di Grigoli et al., 2015; Scatassa et al., 2015a; Gaglio et al., 2016). These works demonstrated the influence of the equipment during the production of traditional cheeses and highlighted the importance of this bacterial group for conferring cheese typicality. The presumptive LAB were isolated from the eight wooden vat surfaces, and after the investigation of several phenotypic (morphological, physiological, and biochemical) characteristics, they were divided into 6 groups. A representative percentage of the isolates of each group was examined by RAPD-PCR that is commonly used, alone or incombination with other techniques, to discriminate LAB strains associated with food matrices (Settanni et al., 2012). With this approach, 10 strains were successfully recognized, indicating that RAPD is a valid technique for differentiating bacteria of food interest associated with the equipment. A total of six species belonging to four LAB genera (*Enterococcus*, *Lactobacillus*, *Lactococcus*, and *Streptococcus*)

commonly found in raw milk, traditional Sicilian cheeses, and wooden vats analyzed in Italy and France (Licitra et al., 2007; Diedienne et al.,2012; Gaglio et al., 2014; Di Grigoli et al., 2015; Scatassa et al., 2015; Gaglio et al., 2016; Guarcello et al., 2016) were identified. With the objective to investigate the technological potential of the LAB associated with the biofilms of the wooden vats, the 10 strains were subjected to a general characterization of dairy traits. With this in mind, the first parameters evaluated were the acidification kinetics, to determine the rapid pH drop necessary to inhibit the undesired microbiota present in raw milk (Akabanda et al., 2014), and the autolysis capacity, which correlates positively with the final flavor development of several cheeses due to the release of cell peptidases (Lortal et al., 1997).

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 fastest acidifiers included the strains *L. delbrueckii* TV199, *L. fermentum* TV187, and *L. lactis* TV70, while enterococci showed a slow-acidifying aptitude and a slow autolysis. Diacetyl is a flavor compound generated from citrate important in cheese production (Marilley and Casey, 2004), but only two LAB studied in this work showed this capacity. All LAB were also investigated for their antimicrobial activity; two strains produced antibacterial compounds which were recognized as proteins and, for this reason, are referred to as bacteriocin-like inhibitory substances (Corsetti et al., 2008). The production of bacteriocins is of great technological importance in cheese manufacture when LAB act as starter cultures and/or cocultures, as they can inhibit pathogenic or spoilage bacteria (Sarantinopoulos et al., 2002). The presence of strains showing characteristics typical of SLAB and NSLAB indicate that the wooden vat is responsible for the colonization of the bacteria and is necessary for the

production and ripening of cheese. In eight trials, no pathogenic bacteria commonly connected with cheese manufacture were detected. Thus, an artificial contamination was performed (only on chestnut vats) to test the efficacy of the wooden vat LAB to inhibit the adhesion of the undesired bacteria of dairy interest. After the contamination test, the foodborne pathogens were not detected in the vat biofilms. Their absence could be due to the acidic conditions generated by the development of LAB in whey. Furthermore, an additional hypothesis to explain the absence of foodborne pathogens in the wooden vat biofilms involves the presence of bacteriocin producers (Lortal et al., 2009). In conclusion, although the duration of this study was not sufficient to exclude the possibility that changes in the surface roughness and/or biofilm composition evolution compromise the safety of the wooden vat system, the repeated daily treatment at 80°C with deproteinized whey represents a hurdle to the survival of pathogenic bacteria, as confirmed by several works performed on aged wooden vats. The levels of the different LAB groups on a given wood type were almost comparable. However, the wood type influenced the levels of LAB; high levels were registered on the surfaces of cedar, ash, walnut, and poplar vats. Within this bacterial group, enterococci were only detected on cedar and cherry woods. The LAB strains isolated from the eight biofilms analyzed were identified as species commonly associated with the traditional dairy products. The technological characterization of the LAB found at high numbers on the surfaces of the wooden vats of this study showed interesting dairy properties. These observations strengthen the importance of using the wooden vats for traditional cheese production and provide evidence to valorize Sicilian forest wood resources via the production of traditional dairy equipment. For this purpose, further studies are being prepared to better investigate the influence of the different wooden vats on the characteristics of the final cheeses.

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CHAPTER IV

Influence of early vat bacterial biofilms developed on different wood types on the final characteristics of Vastedda della valle del Belìce cheese

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Influence of early vat bacterial biofilms developed on different wood types on the final characteristics of Vastedda della valle del Belìce cheese

ABSTRACT

This study aimed to describe the influence of early vat bacterial biofilms developed on different wood types (*Castanea sativa* Miller, *Cedrus libani*, A. Rich., *Prunus avium* L., *Fraxinus ornus* L., *Juglans regia* L., *Pinus nigra* J.F. Arnold and *Populus nigra* L.) on microbiological, chemical, physical and sensory characteristics of PDO Vastedda della valle del Belìce cheese. To this purpose, experimental cheeses after 15 days of refrigerated storage were examined. Lactic acid bacteria (LAB) populations dominated the microbial community of all samples. The species of LAB more frequently identified were *Lactococcus lactis* among starter LAB and *Lactobacillus paracasei*, *Lactobacillus rhamnosus* and *Lactobacillus fermentum* among non starter LAB. Generally, the use of Sicilian tree species did not negatively affect the chemico-physical composition of VdB cheeses. Some cheeses reveled a slight decrease of lightness. Among chemical parameters, significant variations were registered with regards to a_w, oxidation state, polyphenols and volatile organic compounds (VOCs). The differences detected among the VOCs emitted from cheeses were not perceived by the panelists who recognized all cheeses from the different trails as similar. This study showed the suitability of the different Sicilian tree species used for the production of the wooden vats employed in cheese making for traditional dairy productions. Further investigations on the total bacterial composition are under processing by next generation sequencing analysis.

4.1. INTRODUCTION

In the last decades, the consumers are demanding more and more foods with no or reduced chemical preservatives and able to exert health benefits (Leite et al., 2006). In this context the request for traditional dairy products has increased (Gaglio et al., 2014b) determining a re-discovery of typical cheeses (Settanni et al., 2012). The most important traditional Sicilian cheeses enjoying a PDO status are Ragusano, Pecorino Siciliano, Piacentinu Ennese and Vastedda della valle del Belìce.

The traditional production protocols for these cheeses are based on the use of raw milk of the indigenous breeds, artisan animal rennet and wooden equipment (Scatassa et al., 2015). Among the microbial sources, the wooden vats used for centuries to collect and transform milk by farmers and cheesemakers represent the main reservoir of desirable dairy LAB (Cruciata et al., 2018; Di Grigoli et al., 2015; Scatassa et al., 2015). In order to better investigate the microbial ecology of these microbial systems, several works were carried out by Italian and French groups to study the bacterial biofilms associated to the wooden vats used in cheese making (Licitra et al., 2007; Lortal et al., 2009; Didienne et al., 2012; Scatassa et al., 2015; Gaglio et al., 2016).

Microbiological investigations showed the persistence and the dominance of certain LAB species, in particular known starter LAB (SLAB) such as *Lactobacillus helveticus*, *Lactococcus lactis* and *Leuconostoc mesenteroides* responsible of the curd acidification and several non starter LAB (NSLAB) such as *Lactobacillus plantarum* and *Lactobacillus casei* playing defining roles during ripening (Settanni and Moschetti, 2010). The specific investigation of pathogenic bacteria never revealed their presence on these wooden equipment, probably due to the ability of biofilmogenic LAB to produce antimicrobial compounds such as bacteriocin together to the inhibitory action exerted by the organic acids produced during fermentation (Lortal et al., 2009).

Industrial cheese productions are obtained using pasteurized milk transformed in stainless steel equipment. Since the vat used for milk clotting does not host LAB these productions necessitate the inoculation of commercial starter cultures for the acidification of curd (Di Grigoli et al., 2015). This may influence the features of the final cheeses, since LAB biodiverdity associated to raw milk and wooden equipment is considered a key factor for the organoleptic features of artisanal cheeses (Scatassa et al., 2015; Gaglio et al., 2016). A study conducted by Settanni et al. (2012) on the microbiological characterization of both traditional and standard technologies applied to obtain Caciocavallo Palermitano cheeses showed that, following the traditional protocol of production, a clear dominance of the *Streptococcus thermophilus* strains and other species of vat origin identified as members of the NSLAB population ensure cheese typicality.

A recent study conducted in order to valorize the Sicilian forestry resources showed the ability of LAB to adhere and survive on several wood typologies including those not traditionally employed in cheese making, indicating the suitability of local woods in traditional dairy processes (Cruciata et al., 2018). Following the previous study, this work was carried out to evaluate the influence of early vat bacterial biofilms developed on different wood types on the final characteristics of Vastedda della valle del Belìce cheese, in order to legitimate the use of local tree species in cheese production.

4.2. MATERIALS AND METHODS

4.2.1. Cheese production and sample collection

Seven experimental wooden vats (15 L) made from tree species grown in Sicily (*Castanea sativa* Miller, *Cedrus libani*, A. Rich., *Prunus avium* L., *Fraxinus ornus* L., *Juglans regia* L., *Pinus nigra* J.F. Arnold and *Populus nigra* L.) and a control vat made of Calabrian chestnut (*Castanea sativa* Miller) were used for PDO Vastedda della valle del Belìce cheese making, after biofilm formation as reported by Cruciata et al. (2018). VdB cheese production was performed according to the EU Regulation n. 971 (OJC no. C 42/16 19.2.2010)

Two cheese making trials were carried out at an artisanal dairy farm ("Ovini e Natura" Società Agricola di firpo F & C s.a.s., Santa Margherita Belìce, Italy) belonging to consortium for the production of PDO VdB cheese. Experimental cheeses were obtained from 12 liter of raw ewes' milk. Cheese productions were carried out in duplicate at 7 d interval.

Samples of bulk milk (BM), acidified curd (AC) before stretching, cheese just after stretching (V_0) and cheese after 15 d of refrigerated storage at 7°C (V_{15}) were collected for each cheese making.

4.2.2. Microbiological analysis and isolation LAB

The main pro-technological microbial groups associated with food production and those investigated for quality, hygiene, and safety aspects were analysed in all dairy samples collected during VdB cheese production. Bulk milks were subjected to decimal serial dilutions in Ringer's solution (Oxoid), while 15 g of each solid sample (AC, V_0 and V_{15}) were first subjected to homogenization in a stomacher (BagMixer® 400, Interscience, Saint Nom, France) for 2 min at the highest speed in sodium citrate (2% w/v) solution and

then serially diluted in Ringer's solution. Microbial suspensions were plated and incubated as follows: total mesophilic microorganisms (TMM) on plate count agar (PCA) with 1 g L^{-1} added skimmed milk (SkM), incubated aerobically at 30°C for 72 h; total psychrotrophic counts (TPC) on PCA-SkM, incubated aerobically at 7°C for 7 d; mesophilic and thermophilic rod LAB on MRS agar, acidified at pH 5.4 with lactic acid (5 mol L^{-1}), incubated anaerobically for 48 h at 30 and 44°C, respectively; and mesophilic and thermophilic coccus LAB on M17 agar, incubated anaerobically for 48 h at 30 and 44°C, respectively; enterococci on kanamycin azide aesculin agar (KAA) incubated aerobically for 24 h at 37°C; members of the *Enterobacteriaceae* family on Violet Red Bile Glucose Agar (VRBGA) incubated aerobically for 24 h at 37°C; coagulase-positive staphylococci (CPS), *Salmonella* spp., *Listeria monocytogenes*, and *Escherichia coli* were analyzed as reported by Cruciata et al. (2018). Microbiological counts were carried out in duplicate for all samples. Anaerobic conditions were obtained with the Anaeroben AN25 (Oxoid) system in hermetically sealed jars. All media and supplements were purchased from Oxoid.

LAB from refrigerated cheeses (W1 V_{15} – W8 V_{15}) were isolated, about five colonies per morphology, from the highest dilutions of cell suspensions plated on MRS and M17 media. All different morphologies were considered in order to evaluate the total LAB diversity. The isolates were streaked by successive subculturing, and their purity was verified under an optical microscope. All Gram-positive (Gregersen KOH method) and catalase-negative bacterial cultures were considered presumptive LAB and were stored in glycerol stocks at -80°C for further investigation.

4.2.3. Phenotyping grouping, strain differentiation and identification of LAB

Phenotypic characterization was carried out in order to obtain an initial grouping of the isolates. After microscopic inspection, rods and cocci were subgrouped on the basis of

growth at 15 and 45°C, hydrolysis of arginine and esculin, acid production from several sugars as arabinose, ribose, xylose, fructose, galactose, lactose, sucrose, and glycerol, and CO_2 production from glucose tested with Durham's tubes. In order to separate enterococci from other dairy cocci were also grouped according to their capacity of growth at pH 9.2 and in the presence of 6.5 g/L NaCl (Gaglio et al., 2014a).

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

LAB strains were differentiated from one another by random amplification of polymorphic DNA (RAPD)-PCR using the single primers M13, AB111 and AB106 as reported by Settanni et al. (2012). PCR products and the GeneRuler 100 bp Plus DNA ladder (M Medical Srl, Milan, Italy) were separated by electrophoresis on 1.5% (w/v) agarose gel (Gibco BRL, Cergy Pontoise, France) and visualized by UV transillumination after staining with the SYBR® safe DNA gel stain (Molecular Probes, Eugene, OR). The comparison of RAPD patterns was performed with GelCompar II software, version 6.5 (Applied Maths, Sint-Marten-Latem, Belgium), and the isolates with different RAPD profiles were considered as different strains.

The genotypic identification of the LAB with different RAPD patterns was carried out by amplification and successively sequencing of the 16S rRNA gene. PCRs were performed as described by Weisburg et al. (1991) while DNA sequencing reactions were performed at Istituto Zooprofilattico Sperimentale della Sicilia "A. Mirri" (IZS, Palermo, Italy) as described by Cruciata et al., (2018). The identities of the sequences were determined by a blast search against the NBCI nonredundant sequence database and by comparison with the sequences of the sole type strains within the EZTaxon database (http://eztaxon-e.ezbiocloud.net/taxonomy) (Chun et al., 2007).

4.2.4. Chemical- physical composition

Cheese samples were analyzed for dry matter (DM), fat, protein (TN×6.38) and ash content according to IDF standards 4A (IDF 1982), 5B (IDF 1986), 25 (IDF 1964a) and 27 (IDF 1964b), respectively. Salt content was determined by Volhard method (AOAC, 2000). Measurements of pH were performed electrometrically by the pH-meter DocuMeter Sartorius (Data Weighing Systems, Inc., Elk Grove, IL, USA). Water activity (a_w) was determined according to the ISO 21807 (2004a) using the HygroPalm water activity indicator (Rotronic, Bassersdrof, Germany).

Measurements of peroxide value on VdB cheeses were determined accordingly to IDF standard method 74A:1991 (IDF, 1991), peroxide value of cheese lipid was recorded as milli-equivalents of oxygen per kilogram of cheese lipid.

4.2.5. Surface colour

VdB cheese color was analyzed on the top surface by a Minolta tristimulus Chromometer CR-300 (Minolta, Osaka, Japan) using CIELAB L*a*b* values (Hunter, 1975). The measure of lightness (L* values, range 0–100) represents black to white, the redness measurement (a* values) describes green to red, and the yellowness measurement (b* values) represents blue to yellow. Beside these attributes, a* and b* values were also used to determines the parameters hue angle and chroma: hue angle (a*/b*) gives the predominant wavelength composing the color; chroma or saturation [$\sqrt{(a^2 + b^2)}$] accounts for the vividness or the color purity. The chromometer was standardized using a white standard plate. The results reported are averages of five measurements on the same cheese slice.

4.2.6. Analysis of fatty acids, oxidation state and polyphenols

Fatty acids (FA) were determined in lyophilized cheese samples (100 mg) which were directly methylated with 2 mL of 0.5 M $NaOCH_3$ at 50°C for 15 min, followed by 1 mL of 5% HCl in methanol at 50°C for 15 min (Lee & Tweed, 2008). Fatty acid methyl esters (FAME) were recovered in hexane (1.5 ml). One microliter of each sample was injected by auto-sampler into an HP 6890 gas chromatography system equipped with a flame-ionization detector (Agilent Technologies Inc., Santa Clara, CA).

Fatty acid methyl esters from all samples were separated using a 100-m length, 0.25-mm i.d., 0.25-μm capillary column (cp-sil 88; Chrompack, Middelburg, the Netherlands). The injector temperature was kept at 255°C and the detector temperature was kept at 250°C, with an H_2 flow of 40 ml/min, air flow of 400 mL/min, and a constant He flow of 45 ml/min. The initial oven temperature was held at 70°C for 1 min, increased at 5°C/min to 100°C, held for 2 min, increased at 10°C/min to 175°C, held for 40 min, and then finally increased at 5°C/min to a final temperature of 225°C and held for 45 min. Helium, with a head pressure of 158.6 kPa and a flow rate of 0.7 ml/min (linear velocity of 14 cm/s), was used as the carrier gas. Fatty acid methyl ester hexane mix solution (Nu-Chek Prep Inc., Elysian, MN, USA) was used to identify each FA. The identification of the conjugated linoleic acid (CLA) isomers was performed using a commercial mixture of cis- and trans-9,11- and 10,12-ocdecadienoic acid methyl esters (Sigma-Aldrich, Milano, Italy) and published isomeric profile (Kramer et al., 2004; Luna, de la Fuente and Juárez, 2005).

The oxidation status of cheese fat was assessed on freeze-dried samples by determination of peroxide value (POV, mEq O_2/kg fat), as index of primary lipid oxidation (74A; IDF 1991). In addition, thiobarbituric acid-reactive substances (TBARs), expressed as μg malonylaldehyde (MDA)/kg DM), used as a measure of the secondary lipid oxidation products, was determined according to the method proposed by Tarladgis et al. (1960) and

modified by Mele et al. (2011). Cheese extracts were prepared, according to the method of Rashidinejad et al. (2013) with slight modifications, to measure cheese antioxidant status by the determination of total phenolic compound content, measured using the Folin–Ciocalteau colorimetric method, as described by López-Andrés et al. (2014).

4.2.7. Analysis of cheese volatile organic compounds

Volatile organic compounds (VOC) emitted from VdB cheeses were determined using the headspace solid-phase microextraction (SPME) method coupled with gas chromatography with mass spectrometric detection. The SUPELCO SPME (Bellefonte, PA) fiber holder and fiber used were coated with divinylbenzene/polydimethylsiloxane (DV/PDMS), 65 mm. Cheese samples were kept at -20°C until analysis. Before analysis, each sample (10 g) was grated, transferred into a 35 mL vial, added with 10 mL of H2O, 200 μL of internal standard solution (1-Heptanol, 35 mg/L in 20% ethanol aqueous solution) and 1 g of NaCl, the latter added to increase extraction rate of VOCs. Extraction temperature of head-space and time were 60°C and 30 min, respectively. The samples were gently vortexed during extraction using a magnetic stirrer. Fiber exposition was prolonged for 30 min at 60°C (Gaglio et al., 2014). Thermal desorption was performed in the injector at 250°C for 2 min into a Finnegan Trace MS for GC/MS (Agilent 6890 Series GC system, Agilent 5973 Net Work Mass Selective Detector; Milan, Italy) equipped with a DB-WAX capillary column (Agilent Technologies; 30 m. 0.250 mm i.d. film thickness 0.25 μm, part no 122–7032). The GC-MS system and chromatographic conditions described by Sannino et al. (2013) were used for analysis. Mass spectra were recorded by electronic impact at 70 eV using the ion source temperature of 200°C. All compounds of m/z 33–495 atomic mass units (amu) were detected with this scan mode. Individual peaks were identified by comparing their retention indices to those of control samples and by comparing their mass spectra with those within the NIST/EPA/NIH Mass Spectral Library database (Version 2.0d. build

2005). Volatile compounds were expressed as µg/kg. All solvents and reagents were purchased from WWR International (Milan, Italy). All analyses were performed in triplicate.

4.2.8. Sensory analysis

After 15 days of refrigerate storage (7°C) under vacuum, VdB cheeses were evaluated for their sensory characteristics through a panel test carried out following the ISO (2003a) indications. The analysis included a total of 16 cheeses, 8 for each cheese making trial. The effect of wood type on the sensory characteristics of the cheeses was evaluated by 12 trained judges (six men and six woman; 22 and 53 years old). Experimental cheese samples before tasting were cut into pieces (3 x 3 x 3 cm) and acclimated for 1 hour at ambient temperature (about 20°C) before being administered to the judges. The judges evaluated several parameters regarding the aspect (color and uniformity of structure), the smell (strength of odor, milk, butter and unpleasant smell), the taste (salty, sweet, acid, spicy and bitter taste), the consistency (soft/hard, solubility and grittiness following mastication) and the overall acceptability of the product for each sample.

4.2.9. V3-V4 amplification and sequencing strategy

The 16S rRNA gene consists of nine hypervariable regions flanked by regions of more conserved sequence. To maximize the effective length of the MiSeq's 300PE sequencing reads, a region of approximately 469 bp encompassing the V3 and V4 hypervariable regions of the 16S rRNA gene was targeted for sequencing. This region provides ample information for taxonomic classification of microbial communities.

Genomic DNA was extracted from cheese samples using QIAamp DNA Mini Kit and diluted to 5 ng/µl in 10 mM Tris pH 8.5 as requested by Illumina protocol 16S Metagenomic Sequencing Library Preparation, 15044223 Rev. B. Briefly, to amplify and

sequence the V3-V4 hypervariable region of the 16S rRNA gene, primers were designed that contained overhang adapter sequences that must be appended to the primer pair sequences for compatibility with Illumina (Illumina, San Diego, CA, USA) index and sequencing adapters. Amplification of fragment was obtained following PCR condition suggested by the above mentioned Illumina protocol and the expected fragment's size was ~550 bp. In the following step, adapters and dual-index barcodes were added to the amplicon target obtaining a fragment of ~630 bp. After, PCR Clean-Up step, the obtained libraries (~630 bp in lenght) were quantified with Agilent Bioanalyzer 2100 and QuBit 2.0 Fluorometer (Invitrogen), then normalized to 4nM and, finally, pooled. PhiX Control library (v3) (Illumina) was combined with the amplicon library (expected at 5%). The libraries were sequenced with MiSeq Reagent Kit v3, 600 Cycles sequencing kit (MS-102-3003) on MiSeq System (Illumina).

4.2.10. Statistical analysis

Microbiological and volatile organic compounds data were subjected to one-way analyses of variance (ANOVAs). Pair comparisons of treatment means were achieved by using Tukey's procedure at a *P* value of <0.05.

Chemical and physical parameters were analyzed with repeated-measures linear analyses of variance (GLM procedure, SAS 9.1.2 software), which included the fixed effect Wood type. Comparisons among least-square-means was performed by *t* test; differences were considered significant at $P < 0.05$.

4.3. RESULTS

4.3.1. Microbiological analysis

The levels of the different microbial groups investigated in this study are reported in Table 1. The results for *Salmonella* spp. and *L. monocytogenes* are not reported because these pathogens were not detected in any sample analyzed. The levels of TMM and TCP of BM were 6.99 and 4.57 log CFU/ml, respectively. During the different steps of cheese making, TMM were higher than TPC. The levels of TMM in AC were in the range 7.22 - 7.89 log CFU/g and decreased slightly in the cheeses at time zero (V_0) and after 15 days (V_{15}). TCP levels of all samples (AC, V_0 and V_{15}) were lower than TMM (1.97 - 4.20 log CFU/ml for cheese after 15 d). Members of the *Enterobacteriaceae* family in bulk were at 4.52 log CFU/ml and increased in AC until 6.83 log CFU/g; lower levels were detected in cheeses in the range 1.42 - 2.96 log CFU/g. A decreasing trend was observed CPS and *E. coli* which were undetectable in some cheeses after 15 d of refrigerate storage.

The levels of mesophilic and thermophilic rod LAB in raw milk were comparable at about 6 log CFU/ml, while mesophilic and thermophilic cocci LAB showed about 2 log cycles lower. LAB populations dominated all acidified curds and reached values between 8 - 9 log CFU/g. These levels remained almost constant in V_0 and V_{15} cheeses. Enterococci were registered at approximately 5 log CFU/ml in raw milk and increased of about 1 log cycle during cheese production.

Table 1. Microbial evolution from curd acidification to experimental Vastedda della valle del Belìce

Samples	Bacterial counts									
	Mesophilic coccus LAB	Thermophilic coccus LAB	Mesophilic rod LAB	Thermophilic rod LAB	TMM	TPC	*Enterobacteriaceae*	Enterococci	CPS	*E. coli*
BM	5.52 ± 0.04	3.84 ± 0.08	6.10 ± 0.79	6.16 ± 0.06	6.99 ± 1.15	4.57 ± 0.00	4.52 ± 0.58	4.68 ± 0.71	3.25 ± 0.24	3.18 ± 1.27
W1 AC	9.06 ± 0.27 A	8.33 ± 0.63 A	8.85 ± 0.42 A	7.35 ± 0.21 A	7.76 ± 0.31 A	4.59 ± 0.26 A	5.98 ± 0.09 B	6.07 ± 0.20 A	4.92 ± 0.13 AB	5.70 ± 0.16 A
W2 AC	7.89 ± 0.26 B	8.28 ± 0.69 A	8.97 ± 0.25 A	7.96 ± 0.27 A	7.65 ± 0.30 AB	3.62 ± 0.31 A	5.94 ± 0.14 B	5.86 ± 0.19 A	5.20 ± 0.18 A	5.65 ± 0.31 A
W3 AC	8.98 ± 0.35 A	8.42 ± 0.66 A	8.79 ± 0.40 A	7.85 ± 0.08 A	7.44 ± 0.14 AB	4.06 ± 0.08 A	6.83 ± 0.14 A	6.51 ± 0.30 A	5.03 ± 0.12 AB	5.71 ± 0.26 A
W4 AC	9.16 ± 0.23 A	8.31 ± 0.54 A	7.66 ± 0.51 A	8.30 ± 0.43 A	7.67 ± 0.07 AB	3.46 ± 0.48 A	5.98 ± 0.14 B	5.97 ± 0.36 A	4.61 ± 0.19 AB	5.78 ± 0.17 A
W5 AC	8.98 ± 0.24 A	8.33 ± 0.65 A	8.43 ± 0.59 A	8.41 ± 0.42 A	7.22 ± 0.05 B	3.53 ± 0.24 A	6.15 ± 0.08 B	6.72 ± 0.28 A	4.09 ± 0.17 B	5.78 ± 0.23 A
W6 AC	8.86 ± 0.20 AB	8.18 ± 0.85 A	8.76 ± 0.40 A	8.09 ± 0.51 A	7.89 ± 0.03 A	3.86 ± 1.06 A	6.15 ± 0.11 B	6.31 ± 0.65 A	4.86 ± 0.76 AB	4.70 ± 0.16 B
W7 AC	9.01 ± 0.71 A	8.16 ± 1.16 A	8.85 ± 0.72 A	8.13 ± 0.55 A	7.87 ± 0.18 A	3.80 ± 1.26 A	6.10 ± 0.02 B	6.24 ± 0.49 A	4.44 ± 0.38 AB	4.81 ± 0.20 AB
W8 AC	8.84 ± 0.23 AB	7.56 ± 1.75 A	8.81 ± 0.39 A	8.55 ± 0.08 A	7.86 ± 0.12 A	4.65 ± 0.18 A	6.24 ± 0.29 AB	6.28 ± 0.24 A	4.66 ± 0.28 AB	4.51 ± 0.05 B
	*	ns	ns	ns	**	ns	***	ns	*	***
W1 V_0	8.67 ± 0.83 A	8.43 ± 0.49 A	8.25 ± 1.26 AB	7.28 ± 0.11 A	7.06 ± 0.77 A	2.45 ± 0.63 B	2.38 ± 0.43 A	5.49 ± 0.30 A	1.31 ± 0.85 A	2.07 ± 0.67 AB
W2 V_0	8.65 ± 0.49 A	8.56 ± 0.39 A	7.74 ± 0.21 AB	8.13 ± 0.74 A	6.66 ± 0.33 A	2.11 ± 0.05 B	2.92 ± 0.40 A	5.61 ± 0.30 A	1.98 ± 0.39 A	2.15 ± 1.20 AB
W3 V_0	8.57 ± 0.38 A	8.49 ± 0.37 A	7.78 ± 0.31 AB	8.23 ± 0.65 A	6.73 ± 0.09 A	2.32 ± 0.66 B	2.42 ± 0.44 A	5.69 ± 0.03 A	1.30 ± 0.84 A	2.08 ± 0.34 AB
W4 V_0	8.71 ± 0.26 A	8.57 ± 0.44 A	6.77 ± 0.06 B	8.33 ± 0.50 A	7.04 ± 0.51 A	2.28 ± 0.11 B	3.00 ± 0.05 A	6.24 ± 0.51 A	2.49 ± 0.37 A	2.87 ± 0.04 A
W5 V_0	8.60 ± 0.33 A	8.55 ± 0.53 A	7.81 ± 0.16 A	8.23 ± 0.63 A	7.15 ± 0.11 A	2.89 ± 0.16 AB	2.61 ± 0.23 A	6.15 ± 0.49 A	2.06 ± 0.31 A	2.39 ± 0.20 AB
W6 V_0	8.64 ± 0.40 A	8.61 ± 0.41 A	7.97 ± 0.05 A	8.26 ± 0.63 A	6.82 ± 0.16 A	3.53 ± 0.58 A	1.79 ± 1.12 A	5.73 ± 0.17 A	2.85 ± 0.91 A	1.06 ± 0.49 B
W7 V_0	8.21 ± 1.03 A	8.59 ± 0.47 A	7.89 ± 0.08 A	8.34 ± 0.99 A	7.38 ± 0.65 A	3.03 ± 0.41 AB	1.84 ± 1.18 A	5.71 ± 0.29 A	2.09 ± 0.12 A	1.24 ± 0.75 AB
W8 V_0	8.70 ± 0.48 A	8.67 ± 0.46 A	7.64 ± 0.21 AB	8.32 ± 0.66 A	6.78 ± 0.13 A	3.28 ± 0.47 AB	2.71 ± 0.20 A	5.35 ± 0.14 A	2.67 ± 0.09 A	2.36 ± 0.35 AB
	ns	ns	**	ns	ns	*	ns	ns	ns	*
W1 V_{15}	8.60 ± 0.56 A	8.63 ± 0.20 A	7.66 ± 0.39 B	8.22 ± 0.57 A	6.82 ± 0.35 A	2.64 ± 0.48 A	1.42 ± 0.60 A	5.66 ± 0.01 A	<1 A	<1 B
W2 V_{15}	8.86 ± 0.19 A	8.88 ± 0.29 A	8.23 ± 0.60 AB	8.52 ± 0.74 A	6.66 ± 0.48 A	2.49 ± 0.34 A	1.62 ± 0.87 A	5.63 ± 0.22 A	1.23 ± 0.74 A	1.04 ± 0.47 A
W3 V_{15}	8.98 ± 0.03 A	8.91 ± 0.12 A	8.94 ± 0.54 A	8.58 ± 0.57 A	7.15 ± 0.00 A	1.97 ± 1.37 A	2.62 ± 0.54 A	6.04 ± 0.33 A	1.00 ± 0.41 A	2.56 ± 0.54 A
W4V_{15}	9.00 ± 0.20 A	8.85 ± 0.37 A	8.70 ± 0.60 AB	8.63 ± 0.53 A	7.24 ± 0.29 A	3.24 ± 0.87 A	2.96 ± 0.12 A	6.14 ± 0.87 A	1.28 ± 0.81 A	2.72 ± 0.35 AB
W5 V_{15}	8.88 ± 0.53 A	8.77 ± 0.29 A	8.20 ± 0.39 AB	8.55 ± 0.57 A	6.54 ± 0.33 A	3.47 ± 0.86 A	2.34 ± 0.19 A	6.27 ± 0.76 A	<1 A	1.07 ± 0.52 AB
W6 V_{15}	8.81 ± 0.56 A	8.81 ± 0.25 A	8.42 ± 0.39 AB	8.49 ± 0.27 A	6.83 ± 0.20 A	4.20 ± 1.49 A	1.72 ± 1.02 A	5.27 ± 0.10 A	1.17 ± 0.66	1.07 ± 0.52 AB
W7 V_{15}	9.23 ± 0.02 A	8.81 ± 0.05 A	8.07 ± 0.19 AB	8.61 ± 0.13 A	7.00 ± 0.11 A	3.30 ± 0.44 A	1.75 ± 1.06 A	5.32 ± 0.34 A	<1 A	1.12 ± 0.58 AB
W8 V_{15}	8.81 ± 0.25 A	8.82 ± 0.36 A	8.25 ± 0.35 AB	8.30 ± 0.93 A	6.79 ± 0.06 A	3.83 ± 1.18 A	1.64 ± 0.90 A	5.35 ± 0.20 A	<1 A	<1 B
	ns	ns	*	ns	ns	ns	ns	ns	ns	**

Abbreviation: TMM, total mesophilic microorganisms; TPC, total psychrotrophic count; CPS, coagulase-positive staphylococci; BM, Bulk milk; W1 AC, acid curd produced in Calabrian Chestnut wooden vat; W1 V_0, Vastedda cheese produced in Calabrian Chestnut wooden vat; W1 V_{15}, Vastedda cheese produced in Calabrian Chestnut wooden vat after 15 days of refrigerate storage; W2 AC, acid curd produced in Sicilian Chestnut wooden vat; W2 V_0, Vastedda cheese produced in Sicilian Chestnut wooden vat; W2 V_{15}, Vastedda cheese produced in Sicilian Chestnut wooden vat after 15 days of refrigerate storage; W3 AC, acid curd produced in Cedar wooden vat; W3 V_0, Vastedda cheese produced in Cedar wooden vat; W3 V_{15}, Vastedda cheese produced in Cedar wooden vat after 15 days of refrigerate storage; W4 AC, acid curd produced in Cherry wooden vat; W4 V_0, Vastedda cheese produced in Cherry wooden vat; W4 V_{15}, Vastedda cheese produced in Cherry wooden vat after 15 days of refrigerate storage; W5 AC, acid curd produced in Ash wooden vat; W5 V_0, Vastedda cheese produced in Ash wooden vat; W5 V_{15}, Vastedda cheese produced in Ash wooden vat after 15 days of refrigerate storage, W6 AC, acid curd produced in Walnut wooden vat; W6 V_0, Vastedda cheese produced in Walnut wooden vat; W6 V_{15}, Vastedda cheese produced in Walnut wooden vat after 15 days of refrigerate storage; W7 AC, acid curd produced in Balck pine wooden vat; W7 V_0, Vastedda cheese produced in Black pine wooden vat; W7 V_{15}, Vastedda cheese produced in Black pine wooden vat after 15 days of refrigerate storage; W8 AC, acid curd produced in Poplar wooden vat; W8 V_0, Vastedda cheese produced in oplar wooden vat; W8 V_{15}, Vastedda cheese produced in Poplar wooden vat after 15 days of refrigerate storage.Units are log CFU/ml for liquid sample and log CFU/gr for solid sample. Results indicate mean values ± standard deviation (SD) of four plate counts (carried out in duplicate for two independent productions). * P<0.05; ** P<0.01; *** P<0.001

4.3.2. Composition of cheese LAB

A total of 465 presumptive LAB colonies were isolated from VdB cheeses obtained through vats made with different wood types. All cultures, after purification and microscopic analysis, were separated into 298 cocci and 167 rods. Gram and catalase tests indicated that 271 cocci and 153 rods could be considered presumptive LAB cultures. From the combination of the different phenotypic features the cultures were separated into 4 groups (Table 2).

Table 2. Phenotypic grouping of the LAB isolated from Vastedda della valle del Belìce.

Characters	Clusters			
	1 (n=103)	2 (n=98)	3 (n=84)	4 (n=139)
Morphology	R	R	R	C
Cell disposition	sc	sc	sc	sc
Growth:				
15°C	-	+	+	+
45°C	+	+	+	-
pH 9.6	n.d.	n.d.	n.d.	+
6.5% NaCl	n.d.	n.d.	n.d.	-
Resistance to 60°C	+	+	+	+
Hydrolysis of:				
arginine	+	+	+	+
aesculin	+	+	+	+
Acid production from:				
arabinose	+	+	-	-
ribose	+	+	+	+
xylose	+	+	-	-
fructose	+	+	+	+
galactose	+	+	+	+
lactose	+	+	+	+
sucrose	+	+	+	+
glycerol	+	+	+	+
CO_2 from glucose	+	-	-	-

About 30% of the total cultures collected, representative for the different VdB cheeses analysed, were subjected to RAPD analysis. The genotyping differentiation performed so far (this analysis is still in progress) revealed the presence of 12 distinct strains (data not shown). The sequencing of the 16S rRNA gene and the sequence comparison within two

distinct databases (BLAST and Ez-Taxon) identified 4 main species: *L. lactis, Lactobacillus fermentum, Lactobacillus paracasei* and *Lactobacillus rhamnosus* (Table 3).

Table 3. Identification of LAB from cheeses

Strains	Species	Sample	Similarity		bp
			Blast	Ez-taxon	
TV605	*Lactococcus lactis*	W8 V_{15}	99% MF628990.1	99.77 JCM 5805(T)	1302
TV538	*Lactococcus lactis*	W4 V_{15}	99% MG551180.1	99.92%NBRC00931(T)	1270
TV534	*Lactobacillus fermentum*	W4 V_{15}	99% CP016803.1	100% CECT 562(T)	1245
TV520	*Lactobacillus fermentum*	W3 V_{15}	98% MG551079.1	99.34% CECT 562(T)	1213
TV527	*Lactobacillus paracasei*	W3 V_{15}	99% AY773951.1	99.69% JCM 1171(T)	1356
TV593	*Lactobacillus paracasei*	W7 V_{15}	99% CP016355.1	100% ATCC 25302	1432
TV607	*Lactobacillus paracasei*	W8 V_{15}	99% MG551251.1	100.00 JCM 1171(T)	1425
TV518	*Lactobacillus paracasei*	W3 V_{15}	99% MG551251.1	100% JCM 1171(T)	1330
TV529	*Lactobacillus paracasei*	W3 V_{15}	99% MG551251.1	99.92% JCM 1171(T)	1331
TV543	*Lactobacillus paracasei*	W4 V_{15}	99% KU315089.1	99.30% JCM 1171(T)	1301
TV496	*Lactobacillus paracasei*	W1 V_{15}	99% CP017261.1	100% JCM 1171(T)	1396
TV535	*Lactobacillus rhamnosus*	W4 V_{15}	100% MG437361.1	100% JCM 1136(T)	1250

4.3.3. Chemico-physical parameters, surface colour of cheese sample

The chemico-physical parameters of the experimental VdB after 15 days of refrigerated storage are reported in Table 4. The different wooden vats did not determine significant variations of the main chemico-physical parameters except for a_w. VdB cheeses produced in Calabrian chestnut and walnut vat (W1 V_{15}; W6 V_{15}) were characterized by the lowest a_w value (0.97).

Color parameters were reported in Table 5. Lightness (L*) and hue angle of cheeses were influenced by the wood type. The cheeses obtained with Sicilian chestnut, walnut and black pine (W2 V_{15}; W6 V_{15}, W7 V_{15}) decrease L* causing a slight browning, while ash (W5 V_{15}) increased L*. Hue angle of W1 V_{15} and W2 V_{15} cheeses was lower than other samples. W1 V_{15} and W2 V_{15} cheeses were characterized by a less intense yellow. The parameter a* (red–green), b* (yellow–blue) and chroma values did not show significant changes between cheeses obtained with different woods.

Table 4. Chemical-physical cheese parameters in experimental Vastedda della valle del Belìce

Cheese samples	pH	Dry matter[a]	Fat[a]	Protein[a]	N soluble[a]	Ash[a]	a_w	Salt[a]
W1 V_{15}	5.44	54.47	44.71	45.30	0.63	5.03	0.97	0.82
W2 V_{15}	5.43	54.75	45.28	45.04	0.59	4.95	0.99	0.76
W3 V_{15}	5.43	55.83	47.35	44.47	0.65	4.99	0.99	0.79
W4 V_{15}	5.37	55.84	43.73	45.43	0.61	5.07	0.99	0.73
W5 V_{15}	5.40	55.51	45.18	44.05	0.62	4.86	0.99	0.53
W6 V_{15}	5.44	56.33	46.33	45.17	0.57	5.11	0.97	0.79
W7 V_{15}	5.45	55.69	45.76	44.40	0.60	4.98	0.98	0.76
W8 V_{15}	5,45	55,71	45,81	45,04	0,60	5,00	0,99	0,79
SEM	0.03	0.63	0.92	0.90	0.03	0.05	0.006	0.13
Wooden vat	ns	ns	ns	ns	ns	ns	*	ns
Cheese making	ns	ns	*	ns	***	**	*	*

Abbreviation: a_w, water activity; W1 V_{15}, Vastedda cheese produced in Calabrian Chestnut wooden vat after 15 days of refrigerate storage; W2 V_{15}, Vastedda cheese produced in Sicilian Chestnut wooden vat after 15 days of refrigerate storage; W3 V_{15}, Vastedda cheese produced in Cedar wooden vat after 15 days of refrigerate storage; W4 V_{15}, Vastedda cheese produced in Cherry wooden vat after 15 days of refrigerate storage; W5 V_{15}, Vastedda cheese produced in Ash wooden vat after 15 days of refrigerate storage, W6 V_{15}, Vastedda cheese produced in Walnut wooden vat after 15 days of refrigerate storage; W7 V_{15}, Vastedda cheese produced in Black pine wooden vat after 15 days of refrigerate storage; W8 V_{15}, Vastedda cheese produced in Poplar wooden vat after 15 days of refrigerate storage.

[a] Units are %.

SEM, standard error of means; ns = not significant.

* P<0.05; ** P<0.01; *** P<0.001

Table 5. Influence of wooden vat on the colorimetric characteristic of experimental Vastedda della valle del Belìce

Cheese samples	L	a*	b*	Croma[1]	Hue angle[2]
W1 V_{15}	83.46	3.96	14.40	14.94	0.27
W2 V_{15}	82.10	3.97	15.02	15.53	0.26
W3 V_{15}	83.61	4.04	14.01	14.58	0.29
W4 V_{15}	83.17	4.37	14.95	15.58	0.29
W5 V_{15}	84.47	4.03	13.80	14.38	0.29
W6 V_{15}	82.10	4.34	14.54	15.18	0.30
W7 V_{15}	82.65	3.88	13.23	13.78	0.29
W8 V_{15}	83.38	4.26	13.96	14.60	0.30
SEM	0.53	0.15	0.50	0.52	0.005
Wooden vat	*	ns	ns	ns	***
Observation	**	*	ns	ns	***
Cheese making	*	***	***	***	*

Abbreviation: W1 V_{15}, Vastedda cheese produced in Calabrian Chestnut wooden vat after 15 days of refrigerate storage; W2 V_{15}, Vastedda cheese produced in Sicilian Chestnut wooden vat after 15 days of refrigerate storage; W3 V_{15}, Vastedda cheese produced in Cedar wooden vat after 15 days of refrigerate storage; W4 V_{15}, Vastedda cheese produced in Cherry wooden vat after 15 days of refrigerate storage; W5 V_{15}, Vastedda cheese produced in Ash wooden vat after 15 days of refrigerate storage, W6 V_{15}, Vastedda cheese produced in Walnut wooden vat after 15 days of refrigerate storage; W7 V_{15}, Vastedda cheese produced in Black pine wooden vat after 15 days of refrigerate storage; W8 V_{15}, Vastedda cheese produced in Poplar wooden vat after 15 days of refrigerate storage.

[1]Croma=$\sqrt{(a^2+b^2)}$; [2]Hue angle=a/b.

SEM, standard error of means; ns = not significant.

* P<0.05; ** P<0.01; *** P<0.001.

4.3.4. Fatty acid composition, oxidation state of cheese and polyphenol level

The effect of the wood type on cheese fatty acid composition is reported in Table 6. Several fatty acids did not result significantly influenced by wooden vats. Only C17:0 *anteiso* and BCFA showed significant variations.

Table 6. Cheese fatty acid (mg/g)

Fatty acid	W1 V15	W2 V15	W3 V15	W4 V15	W5 V15	W6 V15	W7 V15	W8 V15	SEM[1]	P-value
C4:0	25.6	26.7	27.0	29.6	26.2	27.5	26.4	27.0	1.2	ns
C6:0	21.7	22.5	22.8	25.3	22.2	23.3	22.4	22.6	1.1	ns
C8:0	19.6	20.3	20.5	22.8	20.2	21.0	20.3	20.3	0.9	ns
C9:0	0.2	0.2	0.2	0.3	0.4	0.2	0.2	0.2	0.0	ns
C10:0	52.6	54.3	54.2	60.3	53.9	55.3	54.0	53.9	2.2	ns
C11:0	2.5	2.6	2.6	2.9	2.6	2.6	2.6	2.6	0.1	ns
C12:0	31.9	32.7	32.2	35.4	32.3	32.7	32.4	32.5	1.1	ns
C13:0	1.4	1.4	1.4	1.5	1.4	1.4	1.4	1.4	0.0	ns
C14:0 *iso*	1.0	1.1	1.1	1.1	1.1	1.1	1.0	1.1	0.0	ns
C14:0	96.2	97.5	94.3	103.0	96.0	96.0	96.2	96.4	2.7	ns
C15:0 *iso*	2.4	2.4	2.4	2.5	2.4	2.3	2.4	2.4	0.1	ns
C15:0 *anteiso*	4.4	4.5	4.4	4.7	4.4	4.4	4.5	4.6	0.1	ns
C14:1 *c9*	1.8	1.8	1.7	1.8	1.8	1.8	1.7	1.8	0.1	ns
C15:0	12.6	12.6	12.2	13.2	12.5	12.4	12.5	12.6	0.3	ns
C16:0 *iso*	3.2	3.2	3.1	3.3	3.2	3.1	3.1	3.1	0.1	ns
C16:0	212.9	213.4	204.3	221.1	210.8	206.7	211.3	211.0	5.3	ns
C17:0 *iso*	6.5	5.6	5.7	6.7	6.0	5.5	6.5	6.3	0.3	ns
C17:0 *anteiso*	3.8[a]	3.8[a]	3.6[a]	3.5[a]	3.7[a]	2.9[b]	3.7[a]	3.7[a]	0.2	*
C16:1 *c9*	12.7	12.8	12.4	13.3	12.6	12.1	12.4	12.7	0.3	ns
C17:0	8.8	8.8	8.5	9.1	8.8	8.5	8.7	8.7	0.2	ns
C18:0	93.7	93.3	89.4	96.2	93.0	90.2	92.7	92.2	2.1	ns
C18:1 *t11* , VA[2]	31.8	31.9	30.3	33.1	31.5	30.6	31.4	31.6	0.9	ns
C18:1 *c9*	161.4	161.1	155.2	165.1	161.0	156.3	160.6	154.2	4.2	ns
C18:2 n-6 *c9 c12* LA[3]	23.4	23.4	22.4	24.3	23.4	22.6	23.5	23.2	0.6	ns
C18:3 n-3 ALA[4]	17.7	17.6	16.8	18.5	17.4	16.9	17.6	17.3	0.5	ns
CLA[5] C18:2 *c9 t11*, RA[6]	12.2	12.1	11.7	12.7	12.2	11.8	12.0	11.9	0.3	ns
CLA isomers	4.1	3.4	2.9	3.1	3.5	3.1	3.6	3.9	0.3	ns
C20:5 n-3, EPA[7]	1.0	1.1	1.0	1.2	1.1	1.0	1.0	1.0	0.1	ns
C22:5 n-3, DPA[8]	2.2	2.2	2.1	2.3	2.2	2.2	2.2	2.1	0.1	ns
Saturated FA	611.4	617.8	600.1	653.3	611.7	606.8	613.6	612.6	16.2	ns
Monounsaturated FA	258.6	260.7	248.9	267.2	258.5	250.2	258.3	257.2	6.4	ns
Polyunsaturated FA	83.6	84.9	79.8	87.5	85.1	81.9	86.7	84.4	2.7	ns
Unsaturated FA	342.2	345.6	328.7	354.7	343.6	332.1	345.0	341.6	9.0	ns
Total FA	954.7	963.4	928.8	999.8	955.3	938.9	958.6	954.2	24.4	ns
Unsaturated/Saturated	0.56	0.56	0.55	0.55	0.56	0.55	0.56	0.56	0.0	ns
Σ omega-6	45.7	47.7	44.6	49.0	47.9	46.2	49.5	47.2	1.8	ns
Σ omega-3	21.7	21.7	20.6	22.8	21.6	20.8	21.6	21.3	0.6	ns
Omega-6/omega-3	2.2	2.3	2.2	2.2	2.3	2.3	2.4	2.3	0.1	ns
BCFA[9]	21.8[a]	21.1[a]	20.7[a]	22.3[a]	21.3[a]	19.8[b]	21.9[a]	21.1[a]	0.4	*

W1 V_{15}, Vastedda cheese produced in Calabrian Chestnut wooden vat after 15 days of refrigerate storage; W2 V_{15}, Vastedda cheese produced in Sicilian Chestnut wooden vat after 15 days of refrigerate storage; W3 V_{15}, Vastedda cheese produced in Cedar wooden vat after 15 days of refrigerate storage; W4 V_{15}, Vastedda cheese produced in Cherry wooden vat after 15 days of refrigerate storage; W5 V_{15}, Vastedda cheese produced in Ash wooden vat after 15 days of refrigerate storage, W6 V_{15}, Vastedda cheese produced in Walnut wooden vat after 15 days of refrigerate storage; W7 V_{15}, Vastedda cheese produced in Black pine wooden vat after 15 days of refrigerate storage; W8 V_{15}, Vastedda cheese produced in Poplar wooden vat after 15 days of refrigerate storage.
a, b, means within a row with different superscripts differ ($P \leq 0.05$), [1]standard error of mean; [2]vaccenic acid; [3]linoleic acid; [4]α-linolenic acid; [5]conjugated linoleic acid; [6]rumenic acid; [7]eicosapentaenoic acid; [8]docosapentaenoic acid; [9]branched chain fatty acids.

Table 7 reports the oxidation state and polyphenols levels of experimental VdB cheeses. The oxidation state of the cheeses was influenced significantly by wood type. In particular, W8 V_{15} cheese showed a lower primary oxidation (1.68 mq/kg) than control cheese (3.56

mq/kg). Moreover, W8 V_{15} sample showed the highest levels of polyphenols (almost 700 mg GAE/100 g). In addition, polyphenols level were significantly influenced by the wooden vats.

Table 7. Influence of wooden vat on the oxidation state of experimental Vastedda della valle del Belìce

Cheese samples	Peroxidase (mq/kg)	TBARs (μg MDA/100 g fat)	Polyphenols (mg GAE/100 g)
W1 V_{15}	3.56	4.4	411
W2 V_{15}	2.86	4.5	366
W3 V_{15}	2.55	4.8	436
W4 V_{15}	2.94	5.5	341
W5 V_{15}	2.58	5.3	492
W6 V_{15}	2.72	4.7	393
W7 V_{15}	3.12	4.8	498
W8 V_{15}	1.68	4.2	692
SEM	0.28	0.2	110
Wooden vat	***	**	*
Cheese making	***	***	***

Abbreviation: TBARs, Thiobarbituric Acid Reactive Substances Test; W1 V_{15}, Vastedda cheese produced in Calabrian Chestnut wooden vat after 15 days of refrigerate storage; W2 V_{15}, Vastedda cheese produced in Sicilian Chestnut wooden vat after 15 days of refrigerate storage; W3 V_{15}, Vastedda cheese produced in Cedar wooden vat after 15 days of refrigerate storage; W4 V_{15}, Vastedda cheese produced in Cherry wooden vat after 15 days of refrigerate storage; W5 V_{15}, Vastedda cheese produced in Ash wooden vat after 15 days of refrigerate storage, W6 V_{15}, Vastedda cheese produced in Walnut wooden vat after 15 days of refrigerate storage; W7 V_{15}, Vastedda cheese produced in Black pine wooden vat after 15 days of refrigerate storage; W8 V_{15}, Vastedda cheese produced in Poplar wooden vat after 15 days of refrigerate storage.
SEM, standard error of means.
P<0.05; ** P<0.01; *** P<0.001.

4.3.5. Volatile organic compound composition

The volatile organic compounds emitted from VdB are reported in table 8. Twenty-five volatile compounds were identified in the headspace of the cheeses: 9 acids, 6 alcohols, 4 esters, 3 ketones, 2 aldehydes and 1 aromatic hydrocarbons. Some differences were revealed among the VOCs of the different samples. In particular, the samples W4 V_{15}, W7 V_{15} and W8 V_{15} showed the highest concentration of volatile compounds, while sample W2 V_{15} the lowest. In particular, acids (from C4 to C16) were registered at higher concentrations in the samples W8 V_{15}, W7 V_{15} and W4 V_{15}. Hexanoic, octanoic and decanoic acids were the compounds highly concentrated in all samples (on average 1691, 1506 and 928 μg/kg, respectively). Alcohols were present at higher concentration in W4,

followed by the samples W7 V_{15} and W8 V_{15}. High level of isoamyl alcohol were registered in all samples except W2 V_{15}. Aldehydes were detected at higher concentrations in W7 V_{15}, W8 V_{15} and W4 V_{15}, although acetoin was mostly concentrated in W1 V_{15} (195 μg/kg). Ketons were more present in W3 V_{15}, W4 V_{15} e W5

4.3.6. Sensory analysis

Figure 1 reports the graphic representation of the sensory characteristics evaluated on the cheeses made with wooden vats of different origin. The judges did not score differently the sensory attributes of the cheeses, indicating that the different wood types did not affect the final characteristics of VdB cheese.

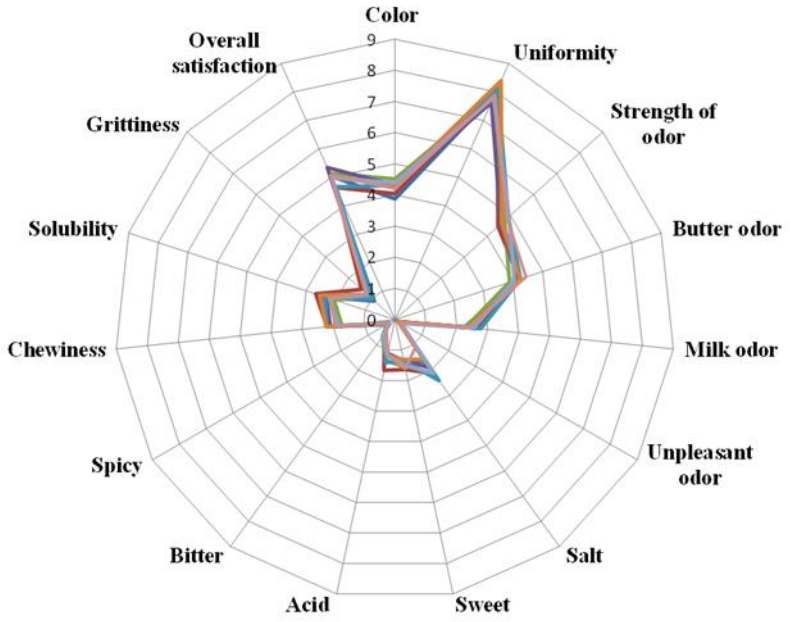

Fig. 1. Immage of sensory evaluation of Vastedda della valle del Belìce.

Table 8. Analysis of volatile organic compounds emitted from experimental Vastedda della valle del Belìce

	Chimical compounds	W1 V_{15}	W2 V_{15}	W3 V_{15}	W4 V_{15}	W5 V_{15}	W6 V_{15}	W7 V_{15}	W8 V_{15}	P-value
Acids	Acetic acid	194.40 ± 3.14 C	160.39 ± 4.76 D	166.23 ± 3.63 D	305.81 ± 9.50 B	292.20 ± 5.58 B	144.10 ± 3.51 D	405.48 ± 13.52 A	410.87 ± 9.83 A	***
	Butyric acid	551.63 ± 12.34 A	230.89 ± 5.21 D	260.07 ± 9.56 D	373.78 ± 8.84 C	355.50 ± 8.74 C	193.06 ± 9.20 E	500.48 ± 13.69 B	517.75 ± 11.67 B	***
	Hexanoic acid	1835.49 ± 66.31 B	1073.49 ± 44.05 D	1455.52 ± 45.66 C	2143.78 ± 84.13 A	1995.38 ± 78.31 AB	1175.46 ± 46.23 D	1864.05 ± 41.69 B	1988.47 ± 51.68 AB	***
	Heptanoic acid	12.51 ± 0.26 C	9.23 ± 0.35 D	5.96 ± 0.21 E	21.56 ± 0.47 B	19.79 ± 0.89 B	13.19 ± 0.67 C	23.46 ± 0.64 A	23.78 ± 0.57 A	***
	Octanoic acid	988.55 ± 22.29 D	941.42 ± 35.25 D	1288.61 ± 52.88 C	2325.12 ± 57.19 A	1714.02 ± 64.19 B	1266.94 ± 63.29 C	1722.61 ± 28.17 B	1799.40 ± 62.24 B	***
	Nonanoic acid	4.64 ± 0.11 DE	6.66 ± 0.25 D	8.14 ± 0.14 D	14.20 ± 0.53 C	0.00 ± 0.00 E	18.09 ± 0.94 C	68.73 ± 1.43 B	186.54 ± 4.33 A	***
	Decanoic acid	386.53 ± 8.99 F	522.09 ± 12.92 E	835.26 ± 20.54 D	1344.89 ± 40.94 A	914.85 ± 21.88 CD	949.24 ± 49.50 C	1282.91 ± 23.78 AB	1191.68 ± 24.77 B	***
	Undecanoic acid	39.02 ± 0.76 G	51.09 ± 2.29 G	106.66 ± 3.51 E	130.98 ± 3.13 D	76.05 ± 2.84 F	143.35 ± 6.55 C	265.66 ± 4.41 A	169.00 ± 2.80 B	***
	Hexadecanoic acid	14.64 ± 0.38 F	18.75 ± 0.56 F	125.05 ± 3.16 D	56.26 ± 2.14 E	41.44 ± 1.05 E	182.09 ± 7.38 C	388.25 ± 11.55 B	644.57 ± 10.76 A	***
alcohols	2.3-Butanediol	250.07 ± 4.19 D	151.41 ± 5.57 F	163.32 ± 2.85 F	323.43 ± 7.91 B	183.73 ± 4.41 E	70.32 ± 2.48 G	380.62 ± 8.59 A	293.07 ± 5.02 C	***
	Isoamyl alcohol	433.53 ± 7.09 D	141.91 ± 4.58 E	624.52 ± 9.35 C	964.48 ± 37.85 A	758.35 ± 29.76 B	509.69 ± 11.35 D	751.27 ± 22.48 B	450.19 ± 11.32 D	***
	1-Pentanol	8.98 ± 0.31 D	7.49 ± 0.30 D	22.92 ± 0.97 B	22.33 ± 0.56 B	27.45 ± 0.64 A	22.62 ± 1.01 B	13.15 ± 0.31 C	28.94 ± 0.78 A	***
	1-Hexanol	5.87 ± 0.14 G	11.92 ± 0.37 F	32.43 ± 0.77 C	77.67 ± 1.16 A	28.52 ± 0.69 D	16.96 ± 0.40 E	34.79 ± 0.95 C	39.09 ± 0.91 B	***
	2-Heptanol	11.06 ±0.25 G	0.00 ± 0.00 H	73.51 ± 2.73 D	140.25 ± 3.45 B	85.74 ± 3.07 C	63.07 ± 1.41 E	150.94 ± 5.55 A	40.60 ± 0.97 F	***
	2-Phenylethanol	104.69 ± 1.71 E	98.39 ±2.20 E	159.14 ± 5.00 D	254.63 ± 6.02 B	160.09 ± 4.05 D	115.53 ± 5.02 E	203.3 ± 4.23 C	276.72 ± 10.00 A	***
Aldehydes	Acetoin	194.88 ± 6.11 A	11.49 ± 0.32 D	13.19 ± 0.22 D	0.00 ± 0.00 E	14.80 ± 0.67 D	10.96 ± 0.34 D	144.90 ± 3.35 B	86.49 ± 1.73 C	***
	Benzaldehyde	23.75 ± 0.73 E	29.52 ± 1.06 E	45.79 ± 1.08 C	50.15 ± 1.10 C	78.33 ± 2.45 B	36.68 ± 1.92 D	100.38 ± 1.64 A	81.75 ± 1.99 B	***
Aromatic	p-Cymene	22.70 ±0.68 F	0.00 ± 0.00 G	43.04 ± 1.58 C	51.24 ± 1.26 B	76.31 ± 2.99 A	43.41 ± 1.61 C	35.59 ± 0.99 D	30.12 ± 0.47 E	***
Esters	Ethyl octanoato	8.57 ± 0.21 E	7.68 ± 0.17 E	29.27 ± 0.72 C	35.48 ± 1.04 B	28.41 ± 0.67 C	17.77 ± 0.53 D	88.08 ± 2.75 A	18.73 ± 0.51 D	***
	2-Propylfuran	6.66 ± 0.15 D	38.53 ± 1.16 A	20.32 ± 0.62 C	22.50 ± 0.68 BC	20.70 ± 0.79 C	24.74 ± 1.04 B	38.52 ± 0.87 A	21.30 ± 0.68 C	***
	Ethyl decanoate	10.36 ± 0.34 F	14.19 ± 0.43 F	58.64 ± 1.08 E	135.74 ± 5.05 C	101.21 ± 2.35 D	69.57 ± 3.26 E	276.77 ± 6.45	237.63 ± 5.09 B	***
	Estragol	44.63 ± 1.01 D	45.92 ± 1.10 D	109.45 ± 3.21 AB	117.04 ± 4.35 A	64.94 ± 2.43 C	104.14 ± 5.20 B	112.18 ± 1.75 AB	111.79 ± 2.30 AB	***
Ketones	3.5-Octadien-2-one	15.63 ± 0.53 E	10.67 ± 0.32 F	36.12 ± 0.85 C	46.05 ± 1.10 B	59.45 ± 1.74 A	31.33 ± 1.45 D	39.01 ± 0.62 C	62.27 ± 0.97 A	***
	2-nonanone	11.36 ± 0.19 E	13.17 ± 0.54 E	137.80 ± 5.41 A	50.63 ± 1.24 BC	44.01 ± 1.86 C	16.50 ± 0.50 E	57.51 ± 1.56 B	27.80 ± 0.87 D	***
	2-Heptanone	9.28 ± 0.21 F	12.20 ± 0.45 F	39.88 ± 1.20 D	64.07 ± 2.08 B	58.58 ± 1.70 C	75.65 ± 2.16	27.17 ± 0.43 E	27.60 ± 0.57 E	***

Abbreviation: W1 V_{15}, Vastedda cheese produced in Calabrian Chestnut wooden vat after 15 days of refrigerate storage; W2 V_{15}, Vastedda cheese produced in Sicilian Chestnut wooden vat after 15 days of refrigerate storage; W3 V_{15}, Vastedda cheese produced in Cedar wooden vat after 15 days of refrigerate storage; W4 V_{15}, Vastedda cheese produced in Cherry wooden vat after 15 days of refrigerate storage; W5 V_{15}, Vastedda cheese produced in Ash wooden vat after 15 days of refrigerate storage, W6 V_{15}, Vastedda cheese produced in Walnut wooden vat after 15 days of refrigerate storage; W7 V_{15}, Vastedda cheese produced in Black pine wooden vat after 15 days of refrigerate storage; W8 V_{15}, Vastedda cheese produced in Poplar wooden vat after 15 days of refrigerate storage. Results indicate mean values of three measurements and are expressed (in μg/kg).

4.4. DISCUSSION

The final characteristics of traditional Sicilian cheeses made according to PDO disciplinaries (OJC no. C 42/16 19.2.2010) depend on several factors, including raw materials (milk and artisan rennet) (Franciosi et al., 2008; Cruciata et al., 2014), wooden equipment (Lortal et al., 2009; Licitra et., 2007; Scatassa et al., 2015; Di Grigoli et al., 2015), dairy environments and dairy technologies (Settanni and Moschetti, 2014). In recent years, several studies have highlighted the scientific value of the microbial biofilm on the acidification of the curd and ripening of traditional cheeses (Licitra et al., 2007; Lortal et al., 2009; Didienne et al., 2012; Scatassa et al., 2015; Di grigoli et al., 2015; Gaglio et al., 2016). Furthermore, Aviat et al. (2016) stated that the wood in contact with foods has not been found responsible for any foodborne outbreak. Thus, the conditions of production of typical Sicilian cheeses do not compromise the safety of the production chain.

In this study, 7 wooden vats made with Sicilian tree species and were used in comparison to one control vat made of Calabrian chestnut to produce PDO VdB cheeses in order to evaluate the influence of the different woods on the final products kept refrigerated for 15 d. No sample contained pathogenic *Salmonella* spp. and *L. monocytogenes*, while *E. coli* and CPS, initially present in milk, decreased their levels from stretching. The decrease registered could be a consequence of the high temperature of stretching and directly related to the dominance of the pro-technological bacteria (Mariani et al., 2011). The evolution of LAB populations from acidification of the curds until the end of refrigerated storage were comparable for all trials, including control production. These microorganisms dominated during all phases of the production process and the highest levels were registered for coccus LAB (9.23 UFC/g). These findings were superimposable to the results reported by Gaglio et al. (2016). Enterococci followed the same trend registered for other LAB groups,

but their levels were lower, about 3 Log cycles. Similar data were previously reported by Mucchetti et al. (2008). Despite being indicators of low hygienic quality (Giraffa et al., 2003), the presence of enterococci is directly linked with cheese typicality (Folquié Moreno et al., 2006).

LAB from final cheeses were isolated and purified in order to be investigated at strain and species level. Four main phenotypic groups were detected and examined by RAPD-PCR. With this method allowed the recognition of 12 strains. The species more frequently identified were *L. lactis* among SLAB and *L. paracasei, L. rhamnosus* and *L. fermentum* among NSLAB. In terms of cell densities, the dominant species were *L. lactis* followed by *L. paracasaei*. The direct comparison of the polymorphic profiles of the strains isolated from the final cheeses to those of wooden vat surface origin carried out by Cruciata et al. (2018) showed that *L. lactis* and *L. fermentum* strains found in cheese derived from the wooden vats. Among these strains, *L. lactis* were characterized by a very fast acidification and a rapid autolysis, whereas *L. fermentum* showed antimicrobial activity, a parameter technologically relevant in cheese manufacture because it contributes to the inhibition of pathogenic bacteria (Cruciata et al., 2018).

Several chemico-physical parameters (fat, protein, dry matter and ash content, as well as pH) were within the range previously reported for VdB cheeses (Todaro et al., 2017). Water activity is the most important factor that affect cheese stability (Di Marzo et al., 2006). The effect of wooden vat significantly influenced a_w and the lowest values were recorded for VdB cheeses processed with Calabrian chestnut, black pine and poplar wooden vats. This phenomenon is considered positive, because an increase of a_w values during storage promotes the development of undesirable microbial groups (Robertson et al., 1993).

Between colour parameters, the effect of wooden vat significantly influenced only lightness (L*) and hue angle. Lightness decreased for cheeses obtained with Sicilian chestnut, walnut and Black pine causing a slight browning of the VdB. This fact is probably due of increased melanoidin concentrations responsible for brown pigmentation (Fox et al., 2000). Melanoidin formation is a non enzymatic browning reaction that occurs in cheese and dairy products when galactose produced from lactose hydrolysis reacts with AA produced from proteolytic breakdown (Corzo et al., 2000). Another possible explanation for this color difference could be due to increased protein hydration which reflects a decrease in the number of free moisture droplets, and thereby a reduced degree of light scattering (Sánchez-Macías et al. 2010).

Analysis of cheese fatty acids revealed higher level as previously reported by Todaro et al. (2014; 2017), maybe due to different on fatty acid composition of the ruminants' milk that depend by changes in animal feeding (Lestingi and De Palo, 2006).

VdB produced with poplar vat showed a low oxidation, probably due to the effect of natural antioxidant transferred from the wood to the milk; in fact, some works carried out on the extract of poplar buds reported high concentrations of polyphenols, including phenolic acid and some flavonoids, as well as the moderate antioxidant capacity of the extract (Dudonnè et al., 2011).

Cheese flavour is derived from a wide range of compounds resulting from the hydrolysis or metabolism of carbohydrates, proteins and fats, along with compounds added during processing or directly from the milk (Fox et al., 2000)

Milk fat is relevant for cheese flavor because it undergoes various reactions such as hydrolysis, oxidation, and esterification and produces free fatty acid, lactones, esters, and ketones that contribute to the overall flavor of cheese (McSweeney and Sousa, 2000; Alewijn et al.,2005). The main components of the volatile fraction of VdB cheeses

analyzed in this study were free fatty acids mainly represented by hexanoic, octanoid and butyric acid. Similar free fatty acid profile were observed for the Provola dei Nebrodi, another traditional stretched Sicilian cheese (Ziino et al., 2005). These compounds derive mainly from the action of the lamb rennet used for curdling that is responsible for the high amount of short-chain free fatty acids (Virto et al., 2003). Different results were reported for other cheese varieties. Rychlik and Bosset (2001) reported acetic, butanoic and propanoic as the major acids in Swiss Gruyère and Ur-Rehman et al. (2000) reported decanoic, octanoic and hexanoic acids as the major free fatty acids in Cheddar cheese. This class of compound were more represented in aged cheeses because they increase during ripening time (Fernandez et al., 2004). Furthermore, differences in free fatty acid composition were documented between whole- and reduced-fat cheeses (Tungjaroenchai et al., 2004). Free fatty acids can also react with alcohol groups to form esters which are volatile and odour-active and are important compounds influencing the final flavour of many cheeses (Fox et al., 2000) and provide fruity flavors to dairy products (Urbach, 1997). Esters profile in VdB samples resulted similar to that reported Verzera et al. (2010) who analyzed several Vastedda cheeses. Different results were reported for Canestrato Pugliese and Pecorino Romano (Di Cagno et al., 2003) characterized by a lower content in total esters and different chemical profiles.

In general, a number of key aromatic compounds are derived from the metabolism of carbohydrates (lactose and citrate) by LAB resulting in acetate, 2,3-butanediol, acetaldehyde, acetoin (3-hydroxy 2-butanone), ethanol, propionate and lactate (Fox et al., 2000). Among these acetoin and 2,3-butanediol were detected in all VdB cheeses.

The significant differences in the compounds emitted by the cheeses were not confirmed by sensory analyses. Sensory evaluation showed that the different woods used for the manufacture of VdB enable the production of cheese with similar sensory characteristics.

Furthermore, the resulting cheeses were comparable to those made in control Calabrian chestnut.

In conclusion, this study demonstrated the persistence and dominance of LAB of wooden vat origin in all cheeses at 15 days of refrigerated storage and the general absence of undesired pathogenic bacteria. The use of Sicilian tree species did not negatively affect the chemical composition of VdB cheeses. A slight browning of cheeses produced in chestnut, walnut and black pine wooden vats was observed. The cheeses produced with poplar vat showed the lowest oxidation. The differences in VOCs detected in the cheeses from the different trails were not perceived by the panelists who recognized all cheeses as similar. This study showed the suitability of the different Sicilian tree species in traditional dairy productions.

Furthermore, V3-V4 amplification and sequencing are under process in order to better investigate the influence of the different wooden vats on the characteristics of the final cheeses.

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CONCLUSIONS

The extended year round production of VdB cheese generated marked differences among the production seasons. Furthermore, since the production protocol does not include the addition of starter cultures, the final quality is quite unpredictable. This because LAB populations might be greatly affected by the different temperatures registered in Sicily during the different seasons, going from below 15°C in winter until above 35°C during summer.

The modern systematic approach to minimize the microbial variability among productions and obtain cheeses with stable desired characteristics is basically based on the use of selected starter cultures. In order to develop starters for traditional cheeses, only autochthonous LAB can be considered to maintain their typicality. In particular, the strains suitable for VdB production have to be characterized by heat resistance during stretching and the ability to carry out the acidification at high temperatures during summer as well as at low temperatures during winter. In order to approach this issue, our research group characterized the indigenous LAB populations isolated from VdB cheeses produced in different seasons and selected three *Lc. lactis* subsp. *cremoris* (PON36, PON153 and PON203) with interesting dairy properties. These strains showed high performances in standard conditions, obtained in an experimental cheese making plant including stainless steel equipment.

In this PhD project, a strategy to transfer the selected lactococci in the traditional production system through the development of biofilms onto the surfaces of virgin (new) wooden vats was developed. The system was tested in controlled and uncontrolled conditions. The controlled conditions were realized at pilot plant level with milk from a single farm characterized by low levels of microbial contamination in an experimental dairy plant, while the uncontrolled conditions were those of an industrial plant that

transforms bulk milk from several farms. In each condition, two virgin vats were activated, one (control vat) following the traditional protocol applying deproteinized whey from the previous day cheese making and the other one (experimental vat) with a whey fermented by the selected strains. The selected lactococci were able to develop the biofilm necessary for milk inoculation more rapidly than whey of the control vats. Furthermore, the genotypic monitoring of these strains showed that they dominated cheese LAB populations during storage. The productions performed in winter and in summer seasons indicated that the biofilm developed *ad hoc* with the autochthonous selected lactococci determined the microbial and sensory stabilization of VdB during the year round production.

If on the one hand the uncontrolled evolution of LAB may determine marked differences among cheese productions and unpredictable final characteristics, on the other hand, the application of commercial starter cultures might determine a flattening of cheese taste with the consequence that cannot be clearly distinguishable by production technology and/or geographical origin. Therefore, the selection and application of autochthonous starter strains is mandatory to drive a fermentation process of typical cheeses. The microbial activation of the virgin wooden vats with selected *L. lactis* subsp *cremoris* strains, through addition of natural whey starter culture, reduced dominant LAB biodiversity and generated cheeses with sensory attributes comparable to those of commercial cheese. This strategy determined a positive innovation respectful of the traditional process and the PDO disciplinary. Hence, this strategy will be provided to the consortium for the production of PDO VdB cheese in order to stabilize the microbial and sensorial attributes of cheese throughout the year.

This PhD project pursued also the objective to valorize the local forestry resources in order to keep the entire cheese making process regional. Nowadays, the wood used for

the production of traditional dairy equipment used for the Sicilian cheese productions is imported from other regions. The ancient tradition of the use of wood in Sicily is disappearing with the direct consequence that the cultivable areas with the local tree species have decrease notably in the last years. With this in mind, our research aimed also at the evaluation of the suitability of local woods for the production of equipment to be used in cheese making. With this perspective, the indigenous milk LAB were also investigated for the presence of starter and non starter cultures able to develop stable biofilm necessary for curd fermentation and cheese ripening, respectively.

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

Furthermore, the study of the influence of early vat bacterial biofilms developed on different wood types on the final characteristics of VdB cheese legitimate the use of local

tree species in cheese production. In fact, it was demonstrated the persistence and dominance of LAB of wooden vat origin in all cheeses at 15 d of refrigerated storage and the general absence of undesired pathogenic bacteria. The use of Sicilian tree species did not negatively affect the chemical composition of VdB cheeses. The differences in VOCs detected in the cheeses from the different trails were not perceived by the panelists who recognized all cheeses as similar. Thus, this project provided evidences on the suitability of the different Sicilian tree species in traditional dairy productions.

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