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ORIGINAL PAPER

Identification, typing and investigation of the dairy characteristics of lactic acid bacteria isolated from "Vastedda della valle del Belìce" cheeses

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Abstract Traditional cheeses made without starter cultures can be characterised by the attribute of instability. The addition of autochthonous starter cultures can ensure stability without compromising the characteristics of the final product. This study aimed to characterise the autochthonous lactic acid bacteria (LAB) population in "Vastedda della valle del Belìce" cheeses, which have a protected designation of origin (PDO) status, in order to develop an ad hoc starter culture to be used in its future production. Winter and spring productions were analysed to ensure isolation of specific LAB that had adapted to perform fermentation at low temperatures. Plate counts revealed total microbial numbers nearing 10^9 CFU.g^{-1}. All of the cheese samples were dominated by coccus-shaped LAB. When enterobacteria were present, their concentrations were at similar levels (3.3–5.6 Log CFU.g^{-1}) in both seasons. All of the colonies that differed in morphological appearance were isolated and differentiated on the basis of phenotypic characteristics and genetic polymorphisms, as analysed by random amplification of polymorphic DNA-polymerase chain reaction. A total of 74 strains were identified and further genotyped by sequencing the 16S rRNA gene, resulting in the identification of 16 LAB species belonging to five genera (*Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Streptococcus*). The species most frequently present were *Streptococcus gallolyticus* subsp. *macedonicus*, *Streptococcus thermophilus*, *Lactococcus lactis* and *Leuconostoc mesenteroides*. The 74 strains were also investigated in vitro for general dairy parameters such as acidification capacity, diacetyl generation and antibacterial activity. Several strains of the most frequently represented species displayed traits relevant to the production of PDO "Vastedda della valle del Belìce".

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1 Introduction

Stretched pasta filata cheeses owe their characteristics to the methods used in their production, which consists of two distinct steps. The first step involves an acidification of the curd, which results in it assuming a plastic consistency. The curd is then heated to a scalding temperature (80–90 °C), allowing it be moulded into the final shape. Following these two steps, the cheese is left to ripen (Salvadori del Prato 1998). Within this group, only a few cheeses are produced from ewes' milk. "Vastedda della valle del Belice" (Vastedda) is a pasta filata cheese from the homonymous valley in Sicily, Italy; it is made from raw ewes' milk without the addition of starter cultures and has been named a protected designation of origin (PDO) cheese by the European Union. Soon after its production, the cheese is sealed under vacuum, refrigerated and sold fresh in local markets (Mucchetti et al. 2008).

From a hygienic perspective, raw milk cheeses deserve greater attention than cheeses made from thermally treated milk, because the final products can become contaminated with pathogenic microorganisms from the raw milk that subsequently survive the cheese-making process (Donnelly 2004). However, the stretching phase, typical of the pasta filata preparation method, strongly contributes to the safety of the resulting cheeses because the high temperatures applied during processing inactivate most microorganisms.

Cheese cannot be made without the activity of certain species of lactic acid bacteria (LAB) (Parente and Cogan 2004). As such, cheese produced from raw milk without the addition of starter cultures relies on the presence of indigenous LAB in the milk and/or those transferred from the processing equipment or the transformation environment. Under these conditions, differences in microbial evolution may produce variability in the final characteristics of the cheese, which cannot easily be controlled by the cheese maker (Franciosi et al. 2008). Species and strain composition may be responsible for not only the inter-factory differences (Antonsson et al. 2003; De Angelis et al. 2001) but also for the differences between cheeses produced at the same factory on different days or in different vats on the same day (Fitzsimons et al. 1999; Williams et al. 2002).

The modern systematic approach to minimising microbial variability and obtaining a cheese with the desired characteristics, consistently over time, is based on the use of starter cultures. However, this technology can compromise the individual characteristics or typicality of the final product. In this context, the only innovation that can be used to attain the typicality of a given cheese is the addition of autochthonous microorganisms. These microorganisms can be indigenous to the milk, or they may derive from the environment or equipment. In some cases, they are highly adapted to the production area and are responsible for the desirable sensory attributes of the cheese (Micari et al. 2007).

Traditionally, Vastedda cheese was produced only during the summer, but due to the increasing demand for this cheese, it is now produced year-round. The particular flavour and typical organoleptic properties of raw milk cheeses are strongly associated with

specific attributes of the raw milk (Beresford et al. 2001), and the generation of the aroma profile relies on metabolites produced by the indigenous microbial populations (Settanni and Moschetti 2010), which may vary across the different seasons.

In order to identify the autochthonous LAB composition of the starter culture required to standardise the year-round production of Vastedda cheese, seasonal samples of this cheese were collected from different dairy factories. In order to select the strains best suited for use in Vastedda cheese production, the LAB populations in the cheese samples were enumerated, isolated and characterised with respect to their acidification capacity, diacetyl production and ability to inhibit the growth of undesirable bacteria.

2 Materials and methods

2.1 Sample collections

Samples of PDO "Vastedda della valle del Belìce" were collected from seven markets supplied by seven dairy factories located in western Sicily (Agrigento, Palermo and Trapani provinces), during the winter (2011–2012) and spring (2012) production periods (Table 1). The Vastedda cheeses were manufactured (Fig. 1) according to regulated production practices that exclude the addition of starter LAB (GUE no. C 42/16 19.2.2010), vacuum-packed and refrigerated. The market samples were collected 11–14 days after production, placed into a portable refrigerator and transferred to the Laboratory of Agricultural Microbiology (Department of Agricultural and Forestry Science, University of Palermo).

2.2 Microbiological analysis

Cheese samples were homogenised with a stomacher (BagMixer® 400, Interscience, Saint Nom, France) for 2 min at the highest speed in a sodium citrate (20 $g.L^{-1}$) cheese/diluent (1:9) solution. Further serial decimal dilutions were prepared using Ringer's solution (Oxoid, Milan, Italy). Cell suspensions were plated and incubated as follows: Total mesophilic count (TMC) bacteria were plated on plate-count agar (PCA) supplemented with 1 $g.L^{-1}$ skimmed milk and incubated aerobically for 72 h at 30 °C; Enterobacteriaceae on double-layered violet-red-bile-glucose agar (VRBGA) and incubated aerobically for 24 h at 37 °C; mesophilic and thermophilic rod-shaped LAB on de Man-Rogosa-Sharpe (MRS) agar, acidified to pH 5.4 with lactic acid (5 $mol.L^{-1}$) and incubated anaerobically for 48 h at 30 and 44 °C, respectively; and mesophilic and thermophilic coccus-shaped LAB on M17 agar and incubated anaerobically for 48 h at 30 and 44 °C, respectively. Anaerobiosis occurred in hermetically sealed jars added with the AnaeroGen AN25 system (Oxoid). All media were purchased from Oxoid. Microbiological counts were performed in triplicate.

Statistical analyses were conducted using STATISTICA software (StatSoft Inc., Tulsa, OK, USA). Due to the winter-produced batch from factory G being unavailable, only the data from cheese factories A–F were analysed. Microbial data were analysed using a generalised linear model that included the effects of the farms (Fa=A to F), the season (Se=W and S) and their interaction (Fa×Se) with one another. Student *t*tests were

Table 1 Samples of Vastedda della valle del Belice cheese analysed

Samples[a]	Days from production	Season	Bacterial count (Log CFU.g^{-1})					
			M17		MRS		PCA	VRBGA
			30 °C	44 °C	30 °C	44 °C	30 °C	37 °C
VC1	14	Winter	7.9±0.4	8.1±0.3	6.6±0.6	7.1±0.5	7.5±0.3	<1
VC2	14	Winter	8.9±0.9	8.9±0.4	8.4±0.2	8.4±0.4	8.6±0.3	3.7±0.2
VC3	14	Winter	9.2±0.7	9.2±0.4	5.7±0.7	8.2±0.1	8.9±0.2	3.4±0.5
VC4	13	Winter	8.1±0.4	8.6±0.6	8.1±0.7	8.0±0.4	7.9±0.3	<1
VC5	11	Winter	7.4±0.8	7.9±0.5	6.7±0.4	6.3±0.3	7.6±0.6	5.1±0.1
VC6	14	Winter	9.0±0.3	9.3±0.4	8.0±0.2	8.1±0.3	8.6±0.6	5.6±0.4
VC1	14	Spring	8.6±0.7	9.0±0.8	8.4±0.3	8.4±0.6	8.1±0.6	<1
VC2	14	Spring	8.8±0.4	8.8±0.6	5.7±0.5	7.0±0.4	8.5±0.3	4.1±0.2
VC3	14	Spring	8.5±0.2	8.4±0.2	7.0±0.7	7.2±0.6	8.2±0.4	3.3±0.8
VC4	12	Spring	8.5±0.1	8.6±0.5	8.3±0.9	8.3±0.8	8.0±0.6	<1
VC5	14	Spring	8.6±0.4	8.5±0.3	8.2±0.5	8.2±0.4	8.2±0.5	5.3±0.5
VC6	13	Spring	8.6±0.3	8.7±0.1	8.4±0.3	8.4±0.3	8.7±0.2	5.6±0.6
VC7	13	Spring	8.4±0.4	8.9±0.0	8.4±0.2	8.4±0.1	8.7±0.2	<1
Statistical significance[b]								
		Factory (F)	***	NS	***	**	***	***
		Season (S)	NS	***	***	*	*	***
		F*S	***	***	***	***	NS	***

Results indicate mean values±SD of three plate counts

NS not significant, *MRS* de Man-Rogosa-Sharpe agar for mesophilic rod LAB, *M17* medium 17 agar for mesophilic coccus LAB, *PCA* plate count agar for total mesophilic count, *VRBGA* violet red bile glucose agar for Enterobacteriaceae, *VC* Vastedda cheese

[a] Origin of samples: VC1, factory A, Salemi (TP); VC2, factory B, Partanna (TP); VC3, factory C, Sambuca di Sicilia (AG); VC4, factory D, S. Margherita Belìce (AG); VC5, factory E, Poggioreale (TP); VC6, factory F, Menfi (AG); VC7, factory G, Contessa Entellina (PA)

[b] Evaluated considering data of Vastedda cheese of the factories A–F

*P<0.05, **P<0.01, ***P<0.001

used to compare the means, and pairwise comparisons were evaluated with a post hoc Tukey's test. A Pvalue <0.05 was deemed significant.

2.3 LAB isolation and phenotypic grouping

Gram-positive (Gregersen KOH method) and catalase-negative bacterial cultures, presumptively considered LAB, were obtained by randomly picking a minimum of four morphologically similar colonies for each of the various colony shapes found on the MRS and M17 agar plated and transferring them to corresponding broth media. Catalase-negative bacteria were identified by their inability to catalyse H_2O_2 (5%) to water. The isolates were purified by successive sub-culturing and stored in broth media containing 20% glycerol (*v/v*) at −80 °C until further analysis.

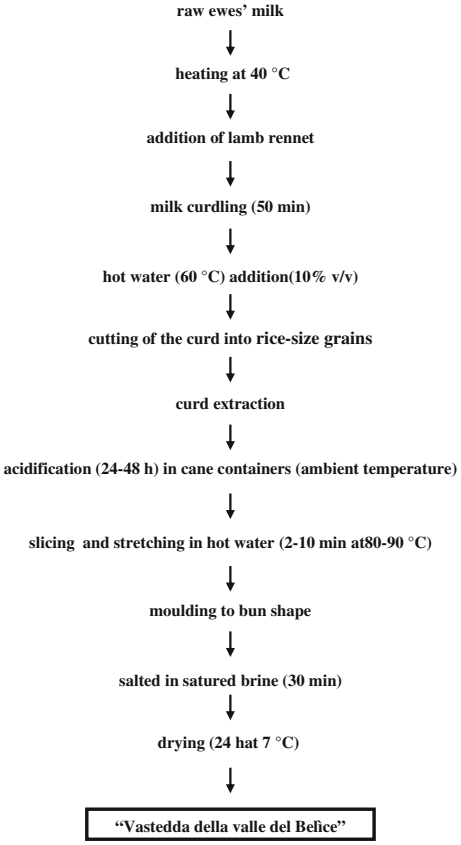

Fig. 1 Flow diagram of Vastedda della valle del Belìce cheese production

The isolates were phenotypically characterised in order to obtain the initial groupings by observing the cell morphology of the LAB isolates using an optical microscope. Subsequently, the presumptive LAB isolates were subjected to further phenotypic assays. Rod- and coccus-shaped LAB isolates were grouped on the basis of their growth characteristics: growth at 15 and 45 °C; resistance at 60 °C for 30 min (Sherman 1937) evaluated as described by Harrigan and McCance (1976); NH_3 production from arginine tested as described by Abd-el-Malek and Gibson (1948)); aesculine hydrolysis determined by the method described by Qadri et al. (1980); acid production from arabinose, ribose, xylose, fructose, galactose, lactose, sucrose and glycerol; and CO_2 production from glucose. The test for CO_2 production was carried out in Durham's tubes with the same growth medium used for isolation, except for the addition of citrate, which can alter gas formation when fermented by certain LAB. The M17 medium had glucose substituted for lactose. Positive results for this test were indicative of hetero-fermentative metabolism. The strains negative for this assay were inoculated into test tubes containing their optimal growth media prepared with a mixture of pentose carbohydrates (xylose, arabinose and ribose, 8 $g.L^{-1}$ each) in place of glucose as described by Settanni et al. (2012)). The strains capable of growth in this medium have a facultative hetero-fermentative metabolism, while the strains unable to grow in the medium have an obligate homo-fermentative metabolism. The

coccus-shaped isolates were further grouped by their ability to grow at pH 9.2 and in the presence of NaCl (6.5 $g.L^{-1}$) to separate enterococci (that can grow in both conditions) from other dairy cocci.

2.4 Genotypic differentiation and identification of LAB

Cells were harvested from cheese isolate cultures grown overnight in broth media at the optimal temperatures, and genomic DNAs were extracted using the Instagene Matrix kit (Bio-Rad, Hercules, CA) as described by the manufacturer. Crude cell extracts were used as templates for the polymerase chain reactions (PCRs).

Strains were differentiated from one another by random amplification of polymorphic DNA (RAPD)-PCR analysis in a 25-μL volume using the single primers M13, AB111 and AB106 as described by Settanni et al. (2012). The PCR products were separated by electrophoresis on 2% (*w/v*) agarose gels (Gibco BRL, Cergy Pontoise, France) and visualised by UV trans-illumination after staining with the SYBR® safe DNA gel stain (Molecular probes, Eugene, OR, USA). The GeneRuler 100 base pair (bp) Plus DNA ladder (M Medical Srl, Milan, Italy) was used as a molecular size marker, and the RAPD patterns were analysed using Gelcompare II software version 6.5 (Applied-Maths, Sint-Marten-Latem, Belgium).

The LAB with different RAPD-PCR profiles were identified genotypically by sequencing 16S rRNA. PCR reactions were performed as described by Weisburg et al. (1991) using the primers rD1 (5′-AAGGAGGTGATCCAGCC-3′) and fD1 (5′-AGAGT TTGATCCTGGCTCAG-3′). The PCR products were visualised, and the amplicons corresponding in size to the molecular weight of the 16S rRNA genes were excised and purified using the QIAquick purification kit (Quiagen S.p.a., Milan, Italy). The resulting DNA was sequenced by Consorzio Regionale di Ricerca BioEvoluzione Sicilia (Santa Margherita del Belice, AG, Italy), using the same primers employed for the PCR amplifications. The sequences were identified by a BLAST search of the GenBank/EMBL/DDBJ database and were deposited under the accession numbers KC545881–KC545901 and KF147882–KF147890.

2.5 Technological screening of the LAB

The different LAB strains were tested for their acidification capacity, diacetyl formation and production of antimicrobial compounds.

The acidifying capacity was assayed at the optimal growth temperature and at 7 °C in 10 mL of full fat, ultra-high temperature (UHT) ewe's milk inoculated with a 1% (*v/v*) cell suspension obtained by growing the cultures overnight in their optimal medium, centrifuging at 5,000×*g* for 5 min, washing and re-suspending in Ringer's solution. To standardise bacterial inocula, the cells were re-suspended in Ringer's solution to an optical density at 600 nm of 1.00, corresponding to approximately 10^9 $CFU.mL^{-1}$, as measured with a 6400 Spectrophotometer (Jenway Ltd., Felsted, Dunmow, UK) and confirmed by plate count with the optimal media. The incubations were at 30 or 44 °C for mesophilic and thermophilic strains, respectively, and at 7 °C for both groups. The pH was measured at 2-h intervals for the first 8 h, and then at 24, 48 and 72 h after inoculation and incubation at 30 and 44 °C. The pH was monitored for 7 days following inoculation and incubation at 7 °C.

Diacetyl production was determined as described by King (1948). Briefly, UHT milk was inoculated with LAB and incubated for 24 h at 30 °C. A 1-mL aliquot of the culture was added to a tube containing 0.5 mL of α-naphthol (1%, *w/v*) and KOH (16%, *w/v*) and incubated at 30 °C for 10 min. Diacetyl generation was indicated by the formation of a red ring at the top of the tube.

The antimicrobial activity of each LAB strain was first detected by the agar-spot deferred method, and the strains displaying positive results were subsequently tested with the well diffusion assay (WDA) (Schillinger and Lücke 1989). Both assays were performed as modified by Corsetti et al. (2008) using *Lactobacillus sakei* LMG2313, *Listeria innocua* 4202 and *Listeria monocytogenes* ATCC 19114 as indicator strains. All tests were carried out in triplicate. The sensitivity of the active supernatants to proteolytic enzymes was tested by digesting them with proteinase K (12.5 $U.mg^{-1}$), protease B (45 $U.mg^{-1}$) and trypsin (10.6 $U.mg^{-1}$) at a final concentration of 1 $mg.mL^{-1}$ in phosphate buffer (pH 7.0). After incubating for 2 h at 37 °C, the remaining activity was measured by a second WDA (Settanni et al. 2005). All enzymes were purchased from Sigma-Aldrich (St. Louis, MO).

3 Results

3.1 Microbiological analyses

The viable cell counts for the Vastedda cheeses are displayed in Table 1. Both the origin of the cheese factory and the season of production significantly affected growth of the mesophilic and thermophilic rod-shaped LAB and Enterobacteriaceae. There were no significant differences between the seasons for the mesophilic coccus-shaped LAB or between the different factories for the thermophilic coccus-shaped LAB. With the exception of the TMC, the interaction between the factories and seasons were significant ($P<0.001$) for all microbial groups.

All of the cheese samples were dominated by coccus-shaped LAB, and there were no statistically significant differences between the population levels of thermophilic and mesophilic bacteria. For the rod-shaped LAB, significant differences in the number of thermophilic and mesophilic bacteria present in the VC3 sample produced in the winter and the VC2 sample produced in the spring were observed. In general, the cell counts for the thermophilic groups were higher than those for the mesophilic LAB. Enterobacteria were present in the VC2, VC3, VC5 and VC6 samples from the winter and spring productions, with the bacterial concentrations in the VC5 and VC6 samples being the highest in both seasons.

3.2 Isolation and grouping of LAB

Based on their appearance, about four colonies with similar attributes (colour, edge, surface and elevation) were isolated from each thermophilic and mesophilic LAB culture for each of the morphologies identified on the agar plates. Colonies were picked from the plates inoculated with the most diluted sample. A total of 1,044 colonies were collected from 39 cheese samples. All of the cultures were inspected microscopically and classified as cocci (928) or rods (116). Gram-positive and

catalase-negative cultures were considered presumptive LAB and produced 894 of the cocci and all 116 of the rod cultures requiring further examination.

Based on several phenotypic features of the cultures and combinations of these features, the 1,010 LAB cultures were separated into 29 groups (Table 2), 3 for rods and 26 for cocci. The largest groups, composed of more than 100 isolates, were groups 22 and 27. In contrast, groups 13, 14 and 18 had only 2 or 3 isolates. Groups 1 and 2 were determined to have a homo-fermentative metabolism and were further characterised by their growth or lack thereof in the presence of ribose. Group 1 was found to have an obligate homo-fermentative metabolism that was characterised by the ability to grow at 45 °C but not at 15 °C, resulting in the classification of the group as thermophilic lactobacilli. Group 2 was determined to be composed of facultative homo-fermentative mesophilic lactobacilli that grew at 15 °C but not at 45 °C.

3.3 Differentiation and identification of LAB

Following the methodology described by De Angelis et al. (2001), approximately 30% of the isolates from each phenotypic group, for a total of 336 isolates, were selected from the different VC samples and subjected to RAPD analysis. The reproducibility of the RAPD-PCR fingerprints was assessed by comparing the PCR products obtained with the primers M13, AB106 and AB111 using DNA extracted from three separate cultures of two strains from each phenotypic group. The lowest similarity found was 92%, indicating the results were highly reproducible using this technique under the applied conditions (data not shown). The genotypic differentiation by RAPD-PCR analysis distinguished 74 strains (Fig. 2). Although the strains belonging to the phenotypic groups 15 and 27 had very similar phenotypic profiles, they clustered quite far apart from one another after the RAPD-PCR analysis.

The 74 strains were identified by sequencing of the 16S rRNA gene (Tables 3 and 4). The sequence lengths ranged from 1,454–1,509 bp and covered most of the 16S rRNA genes occurring in LAB, which in some species is longer than 1,600 bp. The BLAST searches produced percentages of identity with sequences available in the NCBI database of at least 97%, which is the minimum level of similarity required between 16S rRNA genes from two strains to be considered as belonging to the same species (Stackebrandt and Goebel 1994), although Stackebrandt and Ebers (2006) have proposed that "a 16S rRNA gene sequence similarity range above 98.7–99% should be mandatory for testing the genomic uniqueness of a novel isolate". The unequivocal identification of the strains belonging to the *Enterococcus* genus was obtained by applying the multiplex PCR assay on the *sodA* gene as described by Jackson et al. (2004). All of the strains (31 mesophilic and 43 thermophilic) were confirmed to belong to 16 species included in the LAB group. The species with the highest number of strains were *Leuconostoc mesenteroides* (*n*=17), *Streptococcus thermophilus* (*n*=15), *Lactococcus lactis* (*n*=7) and *Streptococcus gallolyticus* subsp. *macedonicus* (*n*=13). One strain (PON203) could not be classified as any of the described species because it shared only 96% identity with *L. lactis* subsp. *lactis* S49-2 (Acc. No. HM058666).

Table 2 Phenotypic grouping of the LAB isolated from Vastedda della valle del Belice cheeses

Characters	Clusters														
	1 (*n*=72)	2 (*n*=10)	3 (*n*=34)	4 (*n*=14)	5 (*n*=20)	6 (*n*=18)	7 (*n*=67)	8 (*n*=47)	9 (*n*=55)	10 (*n*=17)	11 (*n*=51)	12 (*n*=11)	13 (*n*=2)	14 (n=3)	15 (n=68)
Morphology	R	R	R	C	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	sc	sc	sc
Growth:															
15 °C	−	+	−	+	+	+	+	+	+	+	+	+	+	+	+
45 °C	+	−	+	−	−	−	−	−	−	−	−	+	+	+	−
pH 9.2	nd	nd	nd	−	−	−	−	−	−	−	−	−	−	−	+
6.5% NaCl	nd	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	−	−	+	+	+	+	+	+	+	+	+	+	+	+	−

Table 2 (continued)

Characters	Clusters													
	16 (n=10)	17 (n=21)	18 (n=2)	19 (n=14)	20 (n=72)	21 (n=14)	22 (n=127)	23 (n=16)	24 (n=57)	25 (n=17)	26 (n=28)	27 (n=115)	28 (n=11)	29 (n=17)
Morphology	C	C	C	C	C	C	C	C	C	C	C	C	C	C
Cell disposition	sc	sc	sc	lc	lc	lc	lc	lc	lc	lc	lc	lc	lc	lc
Growth:														
15 °C	+	+	+	−	−	−	−	−	−	−	−	−	−	−
45 °C	−	+	+	+	+	+	+	+	+	+	+	+	+	+
pH 9.2	+	+	+	−	−	−	−	−	−	−	−	−	−	−
6.5% NaCl	−	+	+	−	−	−	−	−	−	−	−	−	−	−
Resistance to 60 °C	−	−	−	−	−	−	−	−	−	+	+	+	+	+
Hydrolysis of														
Arginine	+	+	+	−	−	−	−	−	−	−	−	−	−	−
Aesculin	+	+	+	+	+	+	−	−	−	−	+	−	−	−
Acid production from														
Arabinose	−	+	+	−	−	−	−	−	−	−	−	−	−	−
Ribose	+	+	+	+	+	−	+	−	−	+	+	+	−	+
Xylose	−	+	+	−	−	−	−	−	−	−	−	−	−	−
Fructose	+	+	+	−	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	−	+	+	+	+	+	+	−	+	+	+	+
Glycerol	+	+	+	+	+	+	+	−	+	−	+	+	+	−
CO_2 from glucose	−	−	−	−	−	−	−	−	−	−	−	−	−	−

R rod, *C* coccus, *sc* short-chain, *lc* long-chain, *nd* not determined

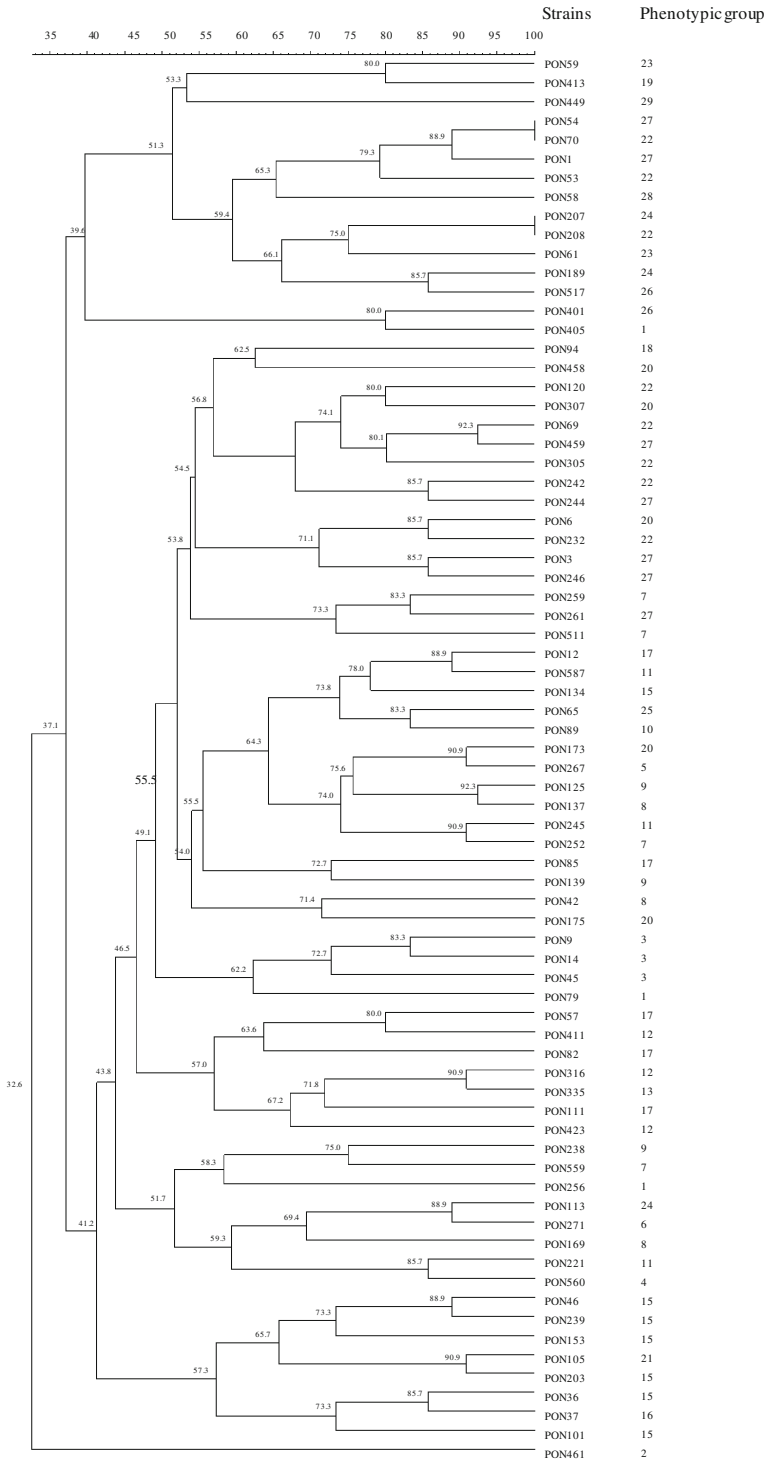

Fig. 2 Dendogram obtained from combined RAPD-PCR patterns of the LAB strains isolated from Vastedda della valle del Belice cheeses. Scale *bar* indicate the percentage of similarity

Table 3 Identification and inhibitory activity of the mesophilic LAB isolated from Vastedda della valle del Belìce cheeses

Phenotypic group	Species	Strain	Cheese sample	Season of first isolation	Persistence[a]	Acc. no.	BLAST homology (%)	Sequence length (bp)	Diacetyl production	Bacteriocin-like inhibitory activity[b]		
										Indicator strains[c]		
										19114	4202	2313
2	*L. curvatus*	PON461	VC4	Spring	n.e.	KC545936	99	1,508	−	−	−	−
4	*L. mesenteroides*	PON560	VC5	Spring	n.e.	KC545940	99	1,488	−	−	−	−
5	*L. mesenteroides*	PON267	VC1	Winter	X	KC545923	99	1,486	−	−	−	−
6	*L. mesenteroides*	PON271	VC1	Winter	n.f.	KC545924	99	1,483	−	−	−	−
7	*L. mesenteroides*	PON252	VC1	Winter	n.f.	KC545920	99	1,485	+	−	−	−
7	*L. mesenteroides*	PON259	VC1	Winter	n.f.	KC545921	100	1,498	+	−	−	1.10±0.12
7	*L. mesenteroides*	PON511	VC6	Spring	n.e.	KC545937	99	1,487	−	−	−	−
7	*L. mesenteroides*	PON559	VC5	Spring	n.e.	KC545939	99	1,492	−	−	−	−
8	*L. mesenteroides*	PON42	VC2	Winter	n.f.	KC545888	99	1,483	−	−	−	−
8	*L. mesenteroides*	PON137	VC5	Winter	n.f.	KC545907	99	1,478	−	−	−	−
8	*L. mesenteroides*	PON169	VC6	Winter	n.f.	KC545910	99	1,487	+	1.10±0.12	−	1.40±0.10
9	*L. mesenteroides*	PON139	VC5	Winter	n.f.	KC545908	99	1,484	−	−	−	−
9	*L. mesenteroides*	PON125	VC5	Winter	n.f.	KC545905	99	1,479	+	−	−	−
9	*L. mesenteroides*	PON238	VC2	Winter	n.f.	KC545943	99	1,492	−	−	−	−
10	*L. mesenteroides*	PON89	VC5	Winter	X	KC545899	99	1,498	+	1.60±0.17	1.17±0.06	−
11	*L. mesenteroides*	PON221	VC2	Winter	n.f.	KC545914	99	1,488	+	−	−	1.20±0.00
11	*L. mesenteroides*	PON245	VC1	Winter	n.f.	KC545881	99	1,488	+	−	−	−
11	*L. mesenteroides*	PON587	VC5	Spring	n.e.	KC545941	99	1,494	+	−	−	−
12	*Leuconostoc lactis*	PON316	VC3	Spring	n.e.	KC545927	100	1,474	−	1.60±0.17	1.30±0.06	1.10±0.06
12	*Leuconostoc lactis*	PON411	VC1	Spring	n.e.	KC545931	99	1,503	−	1.40±0.17	1.30±0.15	1.10 ±0.06

Table 3 (continued)

Phenotypic group	Species	Strain	Cheese sample	Season of first isolation	Persistence[a]	Acc. no.	BLAST homology (%)	Sequence length (bp)	Diacetyl production	Bacteriocin-like inhibitory activity[b]		
										Indicator strains[c]		
										19114	4202	2313
12	*Leuconostoc lactis*	PON423	VC1	Spring	n.e.	KC545933	99	1,499	−	1.10±0.12	1.30±0.12	−
13	*Leuconostoc lactis*	PON335	VC3	Spring	n.e.	KC545928	98	1,487	−	1.00±0.06	1.20 ±	1.30±0.12
14	*Leuconostoc lactis*	PON57	VC3	Winter	n.f.	KC545892	99	1,486	+	1.30±0.12	1.10±0.12	−
15	*L. lactis*	PON101	VC6	Winter	X	KC545901	100	1,471	−	−	−	−
15	*L. lactis*	PON134	VC5	Winter	n.f.	KC545906	99	1,484	+	−	−	−
15	*L. lactis*	PON153	VC6	Winter	n.f.	KC545909	99	1,480	+	−	−	1.30±0.10
15	*Lactococcus* spp.	PON203	VC3	Winter	X	KC545912	96	1,501	−	−	−	−
15	*L. lactis*	PON239	VC2	Winter	n.f.	KC545916	99	1,469	−	−	−	−
15	*L. lactis*	PON36	VC2	Winter	X	KC545886	99	1,484	+	1.10±0.15	−	1.20±0.12
15	*L. lactis*	PON46	VC2	Winter	n.f.	KC545890	99	1,483	−	−	−	−
16	*L. lactis*	PON37	VC2	Winter	n.f.	KC545887	99	1,487	+	−	−	−

n.e. not evaluated, *n.f.* not found in spring cheese

Symbols: *X* found also in spring cheeses; *plus sign* positive for diacetyl production; *minus sign* negative for diacetyl production or, in case of antibacterial tests, no inhibition found

[a] Evaluated only for the strains isolated from winter cheese productions

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

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

Table 4 Identification and inhibitory activity of the thermophilic LAB isolated from Vastedda della valle del Belìce cheeses

Phenotypic group	Species	Strain	Cheese sample	Season of first isolation	Persistence[a]	Acc. no.	BLAST homology (%)	Sequence length (bp)	Diacetyl production	Bacteriocin-like inhibitory activity[b]		
										Indicator strains[c]		
										19114	4202	2313
1	*L. delbrueckii*	PON79	VC4	Winter	X	KC545897	100	1,500	−	−	−	−
1	*L. delbrueckii*	PON256	VC1	Winter	n.f.	KC545944	99	1,456	−	−	−	1.00±0.12
1	*L. delbrueckii*	PON405	VC1	Spring	n.e.	KC545930	99	1,507	−	−	−	−
3	*L. fermentum*	PON9	VC1	Winter	n.f.	KC545884	99	1,509	−	1.30±0.06	1.20±0.00	1.30±0.15
3	*L. fermentum*	PON14	VC1	Winter	n.f.	KC545885	99	1,505	−	1.30±0.12	1.30±0.12	1.40±0.10
3	*L. fermentum*	PON45	VC2	Winter	n.f.	KC545889	99	1,508	−	−	1.10±0.15	−
17	*E. durans*	PON12	VC12	Winter	n.f.	KF147885	99	1,500	−	−	−	−
17	*E. gallinarum*	PON82	VC5	Winter	n.f.	KF147889	99	1,495	−	−	−	−
17	*E. faecalis*	PON85	VC5	Winter	n.f.	KC545898	99	1,494	+	−	−	−
17	*E. faecium*	PON111	VC6	Winter	n.f.	KF147883	99	1,498	+	1.10±0.12	1.00±0.06	1.20±0.00
18	*E. faecium*	PON94	VC5	Winter	n.f.	KC545900	99	1,497	+	1.00±0.12	1.00±0.06	1.00±0.12
19	*S. thermophilus*	PON413	VC1	Spring	n.e.	KC545932	99	1,472	−	−	−	−
20	*S. thermophilus*	PON6	VC1	Winter	n.f.	KC545883	100	1,481	+	−	−	1.00±0.06
20	*S. thermophilus*	PON307	VC3	Spring	n.e.	KC545926	100	1,473	+	−	−	−
20	*S. thermophilus*	PON458	VC4	Spring	n.e.	KC545934	100	1,508	−	−	1.10±0.06	−
20	*S. gallolyticus* subsp. *macedonicus*	PON173	VC6	Winter	n.f.	KF147882	99	1,481	−	−	−	−
20	*S. lutetiensis*	PON175	VC5	Winter	n.f.	KF147890	99	1,480	+	−	−	−
21	*S. lutetiensis*	PON105	VC6	Winter	n.f.	KC545902	99	1,483	+	−	−	−
22		PON53	VC3	Winter	n.f.	KC545891	99	1,483	−	−	−	−

Table 4 (continued)

Phenotypic group	Species	Strain	Cheese sample	Season of first isolation	Persistence[a]	Acc. no.	BLAST homology (%)	Sequence length (bp)	Diacetyl production	Bacteriocin-like inhibitory activity[b]		
										Indicator strains[c]		
										19114	4202	2313
	S. gallolyticus subsp. *macedonicus*											
22	*S. thermophilus*	PON69	VC5	Winter	X	KC545895	99	1,484	+	−	−	−
22	*S. gallolyticus* subsp. *macedonicus*	PON70	VC4	Winter	n.f.	KC545896	99	1,487	−	−	−	−
22	*S. thermophilus*	PON120	VC5	Winter	n.f.	KC545904	99	1,490	−	−	−	−
22	*S. gallolyticus* subsp. *macedonicus*	PON208	VC3	Winter	n.f.	KC545913	99	1,479	−	−	−	−
22	*S. bovis*	PON232	VC2	Winter	n.f.	KC545915	98	1,486	−	1.40±0.15	1.10±0.06	1.10±0.06
22	*S. thermophilus*	PON242	VC1	Winter	n.f.	KC545917	99	1,480	+	−	−	−
22	*S. thermophilus*	PON305	VC3	Spring	n.e.	KC545925	99	1,478	−	−	−	−
23	*S. gallolyticus* subsp. *macedonicus*	PON59	VC3	Winter	n.f.	KC545894	99	1,484	+	1.50±0.17	1.00±0.12	1.00±0.06
23	*S. gallolyticus* subsp. *macedonicus*	PON61	VC3	Winter	n.f.	KC545942	99	1,488	−	1.10±0.12	−	−
24	*S. thermophilus*	PON113	VC5	Winter	n.f.	KC545903	99	1,480	+	−	−	−
24	*S. gallolyticus* subsp. *macedonicus*	PON189	VC3	Winter	n.f.	KC545911	99	1,466	−	−	−	1.20±0.00
24	*S. gallolyticus* subsp. *macedonicus*	PON207	VC3	Winter	n.f.	KF147886	99	1,482	−	−	−	−
25	*S. gallolyticus* subsp. *macedonicus*	PON65	VC3	Winter	n.f.	KF147884	99	1,483	+	−	−	−

Table 4 (continued)

Phenotypic group	Species	Strain	Cheese sample	Season of first isolation	Persistence[a]	Acc. no.	BLAST homology (%)	Sequence length (bp)	Diacetyl production	Bacteriocin-like inhibitory activity[b]		
										Indicator strains[c]		
										19114	4202	2313
26	*S. thermophilus*	PON401	VC1	Spring	n.e.	KC545929	99	1,480	−	−	−	−
26	*S. infantariusus*	PON517	VC6	Spring	n.e.	KC545938	99	1,486	−	−	−	−
27	*S. gallolyticus* subsp. *macedonicus*	PON1	VC1	Winter	n.f.	KC545945	98	1,496	+	−	−	−
27	*S. thermophilus*	PON3	VC1	Winter	n.f.	KC545882	100	1,494	−	−	−	−
27	*S. gallolyticus* subsp. *macedonicus*	PON54	VC3	Winter	n.f.	KF147887	99	1,489	−	−	−	−
27	*S. thermophilus*	PON244	VC1	Winter	n.f.	KC545918	100	1,454	+	1.30±0.12	1.10±0.12	−
27	*S. thermophilus*	PON246	VC1	Winter	n.f.	KC545919	99	1,482	+	−	−	−
27	*S. thermophilus*	PON261	VC1	Winter	n.f.	KC545922	99	1,482	+	−	−	−
27	*S. termophilus*	PON459	VC4	Spring	n.e.	KC545935	100	1,479	−	−	−	−
28	*S. gallolyticus* subsp. *macedonicus*	PON58	VC3	Winter	n.f.	KC545893	99	1,480	+	−	−	−
29	*S. gallolyticus* subsp. *macedonicus*	PON449	VC4	Spring	n.f.	KF147888	99	1,478	+	−	−	−

L Lactobacillus, *E Enterococcus*, *S Streptococcus*, *n.e.* not evaluated, *n.f.* not found in spring cheese

Symbols: *X* found also in spring cheeses; *plus sign* positive for diacetyl production; *minus sign* negative for diacetyl production or, in case of antibacterial tests, no inhibition found

[a] Evaluated only for the strains isolated from winter cheese productions

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

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

3.4 Evaluation of the general dairy characteristics of the LAB

The results of the acidification capacity assay done at the optimal growth temperature for each of the 74 LAB strains are reported in Tables 5 and 6. Milk cultures inoculated with *S. thermophilus* PON244 and PON305 (Table 5), *Lactococcus* spp. PON203 and *Leuconostoc lactis* PON335 (Table 6) displayed the greatest decrease in pH as these strains after 24 h. The results from acidifying the milk during refrigeration (results not shown) were as follows: streptococci and lactobacilli reduced the milk pH to 6.51-6.68 after 7 d, with the exception of *Streptococcus lutetiensis* PON105, which decreased the pH to 5.18; enterococci slightly reduced the milk pH, reaching 6.17 for *E. gallinarum* PON82 by day 7; all of the leuconostocs and lactococci reduced the milk pH, but the majority did not produce decreases below 5.50. Interestingly, three *L. lactis* (PON101, PON134 and PON153) and four *L. mesenteroides* strains (PON125, PON137, PON139 and PON271) acidified the milk to a pH of 4.52–4.65 after 7 days.

Diacetyl production (Tables 3 and 4) was found to be strain-dependent with 13 mesophilic isolates and 18 thermophilic isolates showing a positive result. The majority of the LAB strains with this characteristic were among the *L. mesenteroides* and *S. thermophilus* species.

Out of the LAB isolates, 23 exhibited antibacterial activities (Tables 3 and 4), inhibiting at least one of the indicator strains. All *Lactobacillus fermentum* and *Leuconostoc lactis* strains were antibacterial compound producers and the highest activity, as measured by the inhibition area, was observed for *Leuconostoc lactis* PON316. Treating the culture supernatants with proteolytic enzymes eliminated all inhibitory activity, confirming that the toxins were proteinaceous, which is a general characteristic of bacteriocins (Jack et al. 1995). Because we did not characterise the amino acid and nucleotide sequences for these substances in this study, we will be referring to them as bacteriocin-like inhibitory substances (BLIS) (Corsetti et al. 2008). With the exception of the BLIS produced by the PON6, PON153, PON189, PON221, PON256 and PON259 isolates, all of the other BLIS were active against *Listeria* spp.

4 Discussion

Starter LAB cultures are added in cheese production to determine a rapid increase in the lactic acid concentration during curd acidification or fermentation (Settanni and Moschetti 2010). One of the most promising strategies to control undesirable indigenous microbial populations is to employ well-characterised LAB. However, to avoid a loss of typicality, the addition of autochthonous microorganisms is strongly suggested (Franciosi et al. 2008).

Vastedda della valle del Belìce PDO is a particular type of pasta-filata cheese produced from raw milk following the same process used for the long-term ripening of other pasta-filata cheeses, which includes acidification of the curd between 24 and 48 h (GUE no. C 42/16 19.2.2010) but is then consumed soon after production. Since Vastedda cheese is intended for fresh consumption and is kept under refrigeration, a real ripening of this cheese does not occur. Thus, although starter LAB are generally

Table 5 Values of pH registered for the mesophilic LAB isolated during different productions of Vastedda della valle del Belice cheeses

Species	Strain	Time (h)							
		0	2	4	6	8	24	48	72
L. curvatus	PON 461	6.57	6.57	6.36	6.36	6.30	4.74	3.90	3.69
L. mesenteroides	PON 560	6.65	6.65	6.56	6.31	6.12	4.32	4.24	4.21
L. mesenteroides	PON 267	6.65	6.65	6.35	6.23	6.20	4.42	4.17	4.16
L. mesenteroides	PON 271	6.65	6.65	6.62	6.54	6.28	4.09	3.74	3.71
L. mesenteroides	PON 252	6.65	6.65	6.35	6.34	6.11	4.36	4.12	4.10
L. mesenteroides	PON 259	6.67	6.67	6.36	6.21	5.45	4.25	4.15	4.15
L. mesenteroides	PON 511	6.65	6.65	6.57	6.28	5.68	4.30	4.28	4.27
L. mesenteroides	PON 559	6.64	6.64	6.54	6.21	5.54	4.32	4.28	4.26
L. mesenteroides	PON 42	6.66	6.66	6.49	6.41	6.23	4.33	4.09	4.07
L. mesenteroides	PON 137	6.67	6.67	6.47	6.27	6.16	4.65	4.16	4.12
L. mesenteroides	PON 169	6.68	6.68	6.48	6.39	6.38	4.51	4.19	4.19
L. mesenteroides	PON 139	6.66	6.66	6.32	6.07	5.87	5.12	4.45	4.33
L. mesenteroides	PON 125	6.59	6.59	6.47	6.36	6.18	4.82	4.14	3.86
L. mesenteroides	PON 238	6.65	6.65	6.59	6.37	5.88	4.38	4.38	4.38
L. mesenteroides	PON 89	6.67	6.67	6.52	6.39	6.38	4.78	4.15	4.18
L. mesenteroides	PON 221	6.67	6.67	6.60	6.41	6.28	4.47	4.31	4.30
L. mesenteroides	PON 245	6.61	6.61	6.55	6.36	5.89	4.46	4.18	4.03
L. mesenteroides	PON587	6.66	6.66	6.42	6.19	6.06	4.85	4.33	4.29
Leuconostoc lactis	PON316	6.65	6.65	6.29	6.15	6.01	4.54	4.31	4.27
Leuconostoc lactis	PON411	6.67	6.67	6.27	6.16	6.12	5.04	4.41	4.33
Leuconostoc lactis	PON423	6.64	6.64	6.33	6.15	6.09	5.11	4.42	4.32
Leuconostoc lactis	PON335	6.65	6.65	6.53	5.99	5.20	4.35	4.32	4.30
Leuconostoc lactis	PON57	6.67	6.67	6.43	6.30	6.15	4.99	4.27	4.24
L. lactis	PON 101	6.57	6.57	6.21	6.21	6.06	4.51	4.14	3.93
L. lactis	PON 134	6.62	6.62	6.43	6.24	5.95	4.93	3.96	3.76
L. lactis	PON 153	6.66	6.66	6.20	5.89	5.52	4.26	4.12	4.12
Lactococcus spp.	PON 203	6.66	6.66	6.11	5.32	4.66	4.20	4.20	4.18
L. lactis	PON 239	6.66	6.66	6.59	6.25	5.45	4.35	4.35	4.33
L. lactis	PON36	6.66	6.66	6.19	5.90	5.45	4.27	4.25	4.18
L. lactis	PON 46	6.66	6.66	6.63	6.51	6.43	4.45	4.36	4.29
L. lactis	PON 37	6.63	6.63	6.06	5.83	5.71	4.99	4.39	4.09

selected only for their acidification capacity (Settanni and Moschetti 2010), for Vastedda cheese production, the LAB should also produce the aroma necessary to maintain the typicality of this cheese within a short period of time.

With the aim of developing an ad hoc starter culture for the year-round production of Vastedda cheese that retains its typical characteristics, different PDO Vastedda della valle del Belice cheeses produced in the winter and spring were used as sources of autochthonous LAB. Starter LAB cultures employed in pasta filata cheese

Table 6 Values of pH registered for the thermophilic LAB isolated during different productions of Vastedda della valle del Belice cheeses

Species	Strain	Time (h)							
		0 h	2 h	4 h	6 h	8 h	24 h	48 h	72 h
L. delbrueckii	PON79	6.67	6.67	6.59	6.47	6.37	4.63	4.30	3.90
L. delbrueckii	PON256	6.65	6.65	6.28	6.18	5.92	4.29	3.91	3.77
L. delbrueckii	PON405	6.63	6.63	6.57	6.53	6.27	4.14	3.72	3.61
L. fermentum	PON9	6.64	6.64	6.40	6.20	6.10	4.41	4.18	4.03
L. fermentum	PON14	6.68	6.68	6.51	6.48	6.45	4.97	4.17	4.17
L. fermentum	PON45	6.63	6.63	6.26	6.24	6.21	4.86	4.22	4.00
E. durans	PON12	6.66	6.61	6.29	6.18	6.03	5.45	4.21	4.11
E. gallinarum	PON82	6.67	6.66	6.44	6.42	6.36	5.66	4.24	4.19
E. faecalis	PON85	6.60	6.60	6.42	6.41	6.29	4.43	4.15	4.10
E. faecium	PON111	6.61	6.59	6.59	6.55	6.22	4.51	4.09	3.99
E. faecium	PON94	6.59	6.59	6.50	6.38	6.21	4.61	4.12	3.93
S. thermophilus	PON413	6.62	6.62	6.13	5.95	5.88	5.04	4.46	4.35
S. thermophilus	PON6	6.66	6.66	6.41	6.03	6.02	4.63	4.16	3.90
S. thermophilus	PON307	6.65	6.65	6.10	5.81	5.36	4.29	4.18	4.18
S. thermophilus	PON458	6.59	6.59	6.56	6.54	6.22	4.43	4.09	3.82
S. gallolyticus subsp. *macedonicus*	PON173	6.66	6.66	6.30	6.29	6.13	4.99	4.18	4.15
S. lutetiensis	PON175	6.62	6.59	6.51	6.38	6.18	4.69	4.15	3.99
S. lutetiensis	PON105	6.66	6.66	6.39	6.29	6.26	5.45	4.21	4.19
S. gallolyticus subsp. *macedonicus*	PON53	6.65	6.65	6.25	6.08	5.90	5.00	3.94	3.71
S. thermophilus	PON69	6.64	6.64	6.30	6.18	6.02	4.64	4.11	3.92
S. gallolyticus subsp. *macedonicus*	PON70	6.55	6.55	6.32	6.28	6.04	5.13	4.14	3.84
S. thermophilus	PON120	6.58	6.58	6.60	6.53	6.30	4.64	4.16	3.89
S. gallolyticus subsp. *macedonicus*	PON208	6.65	6.65	6.30	6.29	6.15	4.82	4.18	3.90
S. bovis	PON232	6.64	6.64	6.19	6.03	5.64	4.70	4.28	4.00
S. thermophilus	PON242	6.65	6.65	6.08	5.48	4.78	4.20	4.14	4.14
S. thermophilus	PON305	6.64	6.64	5.94	5.26	4.68	4.23	4.21	4.21
S. gallolyticus subsp. *macedonicus*	PON59	6.69	6.69	6.51	6.39	6.29	5.15	4.17	4.15
S. gallolyticus subsp. *macedonicus*	PON61	6.65	6.65	6.50	6.34	6.16	4.70	4.23	4.03
S. thermophilus	PON113	6.69	6.69	6.62	6.58	6.50	4.38	4.10	4.10
S. gallolyticus subsp. *macedonicus*	PON189	6.66	6.66	6.39	6.31	6.16	4.81	4.05	3.81
S. gallolyticus subsp. *macedonicus*	PON207	6.66	6.64	6.59	6.50	6.21	4.11	3.85	3.74
S. gallolyticus subsp. *macedonicus*	PON65	6.69	6.69	6.59	6.44	6.28	5.98	4.22	4.11
S. thermophilus	PON401	6.65	6.65	6.35	6.14	5.86	4.35	3.85	3.71
S. infantariusus	PON517	6.65	6.65	6.28	6.24	6.23	5.06	3.79	3.63
S. gallolyticus subsp. *macedonicus*	PON1	6.69	6.69	6.36	6.24	6.16	4.45	4.20	4.20
S. thermophilus	PON3	6.69	6.69	6.55	6.50	6.48	6.03	4.62	4.21
S. gallolyticus subsp. *macedonicus*	PON54	6.68	6.66	6.55	6.49	6.17	4.33	3.99	3.91
S. thermophilus	PON244	6.65	6.65	5.92	5.21	4.62	4.18	4.16	4.16
S. thermophilus	PON246	6.65	6.65	6.31	6.27	6.02	5.12	3.91	3.79

Table 6 (continued)

Species	Strain	Time (h)							
		0 h	2 h	4 h	6 h	8 h	24 h	48 h	72 h
S. thermophilus	PON261	6.66	6.66	6.61	6.51	5.68	4.26	4.02	3.81
S. thermophilus	PON459	6.66	6.66	6.64	6.64	6.46	4.40	4.01	3.81
S. gallolyticus subsp. *macedonicus*	PON58	6.69	6.69	6.52	6.41	6.29	4.91	4.15	4.12
S. gallolyticus subsp. *macedonicus*	PON449	6.67	6.65	6.58	6.41	6.16	4.79	4.33	4.13

production are commonly thermophilic. However, because we did not believe that LAB isolated from cheeses produced during the summer would be capable of meeting our goal of developing a starter culture that could acidify the curd at low winter temperatures (below 15 °C), the summer productions of Vastedda cheese were not included in this study.

All of the cheese samples were dominated by coccus-shaped LAB, with the highest levels approaching 9.3 Log CFU.g^{-1}. The TMC and LAB concentrations detected in this study were, on average, 1 Log cycle higher than those reported by Mucchetti et al. (2008), who studied the influence of cheese-making technologies on the microbial populations of Vastedda cheese. This may be due to the difference in time between the productions and analyses of the cheeses used in the two studies. Members of the Enterobacteriaceae family were not always found, but their level was estimated to be up to 10^5 CFU.g^{-1}. Interestingly, when they were present, the concentration of Enterobacteriaceae did not greatly vary between the winter and spring productions, suggesting that their presence may have been due to insufficient hygienic conditions during milking and/or cleaning of the cheese factories.

Colonies of LAB were isolated from the cheese samples and purified in order to be characterised, differentiated and identified. The isolates were subjected to several tests generally employed to perform the phenotypic grouping of LAB based on their cell morphology, physiological traits and biochemical characteristics (Settanni et al. 2012). With this approach, 29 LAB groups were identified. A representative percentage of the isolates was examined by RAPD-PCR, a technique commonly applied, alone or in combination with other methodologies, to discriminate LAB strains associated with food matrices. In this study, 74 different strains were found, demonstrating that high levels of LAB biodiversity exist in Vastedda cheeses.

Seventy-three strains were identified at the species level, and all of them belonged to the LAB group. One isolate, PON203, remained unidentified, but it is probably a new *Lactococcus* species, given its 96% similarity to *L. lactis* subsp. *lactis*. The species *S. thermophilus* has been found to dominate bacterial populations during the manufacturing of other Italian stretched cheeses lacking commercial starters such as Provolone del Monaco (Aponte et al. 2008), Scamorza Altamurana (Baruzzi et al. 2002) and Caciocavallo Palermitano (Settanni et al. 2012). This species has been detected in the LAB employed as thermophilic starter cultures, and it is generally present in the natural whey starter culture used for cooked cheese and pasta-filata cheese production (Parente et al. 1998; Settanni and Moschetti 2010). Thermophilic

streptococci have also been found in high numbers during the fermentation of the Greek cheese Kasseri (Tsakalidou et al. 1994, 1998; Moatsou et al. 2001), but we have found no reports of the *S. thermophilus* species in raw ewes' milk pasta-filata cheeses in the literature.

Among the species identified with a large number of strains, *L. lactis* and *L. mesenteroides* were dominant in the Vastedda cheeses. These species are also the main components of the mesophilic milk starter cultures used for dairy fermentations (Settanni and Moschetti 2010). This phenomenon is due to their thermal resistance during scalding of the acidified curds. Several strains within these two species were able to develop colonies after treatment at 60 °C for 30 min. Exposure of the strains to high temperatures during curd stretching (80–90 °C) occurs for a short period, which may explain the presence of other leuconostocs and lactococci after 14 days of ripening.

Strains in another large group belonged to the species *S. gallolyticus* subsp. *macedonicus*, previously classified as *Strepotococcus macedonicus*, but reclassified by Schlegel et al. (2003). This species is being considered to develop adjunct cultures for use in cheese making (Settanni et al. 2011). Three strains were identified as *Lactobacillus delbrueckii*, which is present in other thermophilic starter cultures (Parente and Cogan 2004), while the other species with dairy relevance (*E. faecium*, *Enterococcus faecalis*, *Lactobacillus curvatus* and *L. fermentum*) are members of the non-starter LAB community (Chamba and Irlinger 2004).

Other *Streptococcus* spp. were identified in the samples of Vastedda cheese analysed, in addition to *S. thermophilus* and *S. gallolyticus* subsp. *macedonicus*. *S. bovis* is associated with dairy environments (Jans et al. 2012) and is considered a pathogenic bacterium (Vaska and Faoagali 2009). *Streptococcus infantarius* has been found in raw ewes' milk cheese (Todaro et al. 2011), and *S. lutetiensis* was reclassified from *S. infantarius* subsp. *coli* (Poyart et al. 2002). All of these species belong to the group D streptococci and are undesirable in cheese.

Some strains (5%) were found to persist during the two consecutive seasons of production (Table 3), highlighting their strong adaptation to the environment and to the transformation methods. This phenomenon is unsurprising in traditional cheese-making processes, especially in those carried out using wooden equipment, which allows the formation of microbial biofilms. Several wooden vats have been found to host LAB (Lortal et al. 2009; Didienne et al. 2012), and some strains have persisted over time (Settanni et al. 2012). As Vastedda cheese production is carried out using wooden vats, they may provide a reservoir of LAB to inoculate the milk, resulting in the LAB found in the final products.

With a view to selecting autochthonous LAB species to standardise the production of Vastedda cheese, all of the LAB strains were evaluated based on characteristics that facilitate cheese production. The acidification capacity is of paramount importance, and an optimal starter LAB culture contains isolates that can highly acidify milk within a short period of time (Franciosi et al. 2009). The presence of diacetyl, which is a flavour compound generated as an end product of citrate metabolism by some LAB, was also evaluated and found to be produced by several strains. Furthermore, all of the strains were tested for antibacterial compound production, and a consistent percentage of the LAB isolated from Vastedda della valle del Belìce PDO were BLIS producers, a positive attribute that ensures a competitive advantage for the starter strains. Since Vastedda cheese is refrigerated soon after production, the presence of LAB able to ferment at low

temperatures should ensure the development of useful characteristics. Acidification at low temperatures was mediated by some strains of *L. lactis* and *L. mesenteroides*, which, with the exception of *L. mesenteroides* PON271, were all resistant to high temperatures.

5 Conclusion

Vastedda della valle del Belìce PDO cheeses produced in the winter and spring were characterised by high levels of thermophilic and mesophilic LAB. All of the major groups of strains belonged to species commonly employed as starter cultures in different cheese productions. Some strains belonging to the thermophilic species *L. delbrueckii* and *S. thermophiles* and the mesophilic species *L. lactis* and *L. mesenteroides* displayed characteristics favourable to dairy production, suggesting that they may be useful in the Vastedda cheese-making process. Thus, the strains *L. delbrueckii* PON79, PON256 and PON405; *L. lactis* PON36, PON153 and PON203; *L. mesenteroides* PON169, PON259 and PON559; and *S. thermophilus* PON244, PON120 and PON242 will be evaluated in situ for their potential to act as starter cultures for the continuous four-season production of Vastedda cheese.

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