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The influence of the wooden equipment employed for cheese manufacture on the characteristics of a traditional stretched cheese during ripening

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ABSTRACT

The influence of the wooden equipment used for the traditional cheese manufacturing from raw milk was evaluated on the variations of chemico-physical characteristics and microbial populations during the ripening of Caciocavallo Palermitano cheese. Milk from two farms (A, extensive; B, intensive) was processed in traditional and standard conditions. Chemical and physical traits of cheeses were affected by the farming system and the cheese making technology, and changed during ripening. Content in NaCl and N soluble was lower, and paste consistency higher in cheese from the extensive farm and traditional technology, whereas ripening increased the N soluble and the paste yellow and consistency. The ripening time decreased the number of all lactic acid bacteria (LAB) groups, except enterococci detected at approximately constant levels (10^4 and 10^5 cfu g^{-1} for standard and traditional cheeses, respectively), till 120 d of ripening. In all productions, at each ripening time, the levels detected for enterococci were lower than those for the other LAB groups. The canonical discriminant analysis of chemical, physical and microbiological data was able to separate cheeses from different productions and ripening time. The dominant LAB were isolated, phenotypically characterised and grouped, genetically differentiated at strain level and identified. Ten species of LAB were found and the strains detected at the highest levels were *Pediococcus acidilactici* and *Lactobacillus casei*. Ten strains, mainly belonging to *Lactobacillus rhamnosus* and *Lactobacillus fermentum* showed an antibacterial activity. The comparison of the polymorphic profiles of the LAB strains isolated from the wooden vat with those of the strains collected during maturation, showed the persistence of three enterococci in traditional cheeses, with *Enterococcus faecalis* found at dominant levels over the *Enterococcus* population till 120 d; the absence of these strains in the standard productions evidenced the contribution of vat LAB during Caciocavallo Palermitano cheese ripening.

1. Introduction

Many traditional cheeses are manufactured in small size farms with raw milk from animals of indigenous breeds that are fed mainly on natural pasture. This is the case of Caciocavallo Palermitano cheese, a "pasta-filata" product, manufactured within the Palermo province (Sicily, Italy) mainly with milk from the autochthonous breed cows (Cinisara and Modicana) processed raw. The traditional cheese making is carried out employing the wooden dairy equipment without the addition of lactic acid bacteria (LAB) (Bonanno et al., 2004).

Recently, some variations in the traditional production system of Caciocavallo Palermitano cheese have been registered for some dairy factories, especially those characterised by high volumes of milk. Since cheese cannot be made without the action of certain species of LAB (Parente and Cogan, 2004), any innovation based on the thermal treatment of milk may compromise the characteristic features that contribute to the definition of cheese typicality. In general, cheese production comprises two different microbiological steps in which different LAB are involved: starter LAB (SLAB) during

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manufacturing, and non starter LAB (NSLAB) during ripening (Settanni and Moschetti, 2010).

The microbiota of a typical cheese is defined for the final characteristics of the resulting product and it often reflects the environment and the system of production (Micari et al., 2007). The typical flavour of a given cheese depends also on the microbial activity during ripening, especially due to the enzymatic degradation of milk lactose, fat and protein, producing volatile organic compounds with aromatic properties (Urbach, 1997).

Several cheeses are niche products that are linked to the production area not only for the traditions that are handed down over time, but also and, above all, for the presence of microbial species and strains colonizing the environment of transformation and the equipment employed during processing (Settanni and Moschetti, 2014). This phenomenon is particularly evident when the equipments used for transformation are made with material (e.g. wood) that can help the formation of microbial biofilms (Lortal et al., 2009; Didienne et al., 2012) strongly contributing to the typicality definition.

Currently, some Caciocavallo Palermitano producers follow a standard scheme: milk is pasteurised, the equipment is in stainless steel and commercial SLAB are added into the milk before coagulation. This actual trend involves the simultaneous presence of "traditional" and "standard" products, both designed as "Caciocavallo Palermitano" on the market, that indeed differ substantially (Settanni et al., 2010). A study conducted on the microbiological characterization of both traditional and standard technologies applied to obtain this cheese revealed that, following the traditional protocol, a clear dominance of the *Streptococcus thermophilus* strains of wooden vat origin emerged during the entire cheese manufacture till stretched curd moulding, highlighting the influence of the traditional equipment during the first stages of the production process (Settanni et al., 2012). However, other species of vat origin were identified as members of the NSLAB population, some of which are reported to be linked to the cheese typicality.

With this in mind, the traditional cheeses have been followed from the manufacturing stage to ripening, then sampled at different times. The specific objectives of this work were to: enumerate and isolate LAB from traditional and standard productions after 30, 60 and 120 days of ripening; characterize, differentiate and identify all dominant LAB; compare the polymorphic profiles of the strains isolated during ripening with those previously isolated from the wooden vat before milk was processed into cheese; evaluate the chemical and physical changes of the cheeses during ripening.

2. Materials and methods

2.1. Cheese production and sample collection

The experimental cheeses object of this study were previously produced (Settanni et al., 2012; Bonanno et al., 2013) using the bulk milk from two farms (A and B) located within the Palermo province (Sicily, Italy). In the farm A, cows of autochthonous breed (Cinisara) were fed mainly at natural pasture, whereas in the farm B specialized dairy cows of Brown breed received a diet based on hay and concentrate.

Four productions, two traditional (TA and TB) carried out following the local cheese making protocol with the wooden equipment, and two standard (SA and SB) carried out in a stainless steel vat added with a commercial SLAB culture (LYOBAC-D T, Alce International s.r.l., Quistello, Italy), were performed in a dairy factory close to the farms. The main wooden equipment for Caciocavallo Palermitano cheese production (Tornambé et al., 2009) consisted of a vat (*tina*) for milk coagulation, a stick (*rotula*) for curd breaking, a bowl (*cisca*) for curd pressing, a cane plan (*cannara*) for residual whey loss by pressing, a horizontal stick (*appizzatuma*) for curd acidification, a truncated conical vat (*piddiaturi*) for curd stretching through a stick (*maciliatuma*) and a form (*tavuleri*) for moulding. Each cheese production was performed in triplicate in three consecutive weeks for a total of three cheese making trials. Cheeses from all productions were sampled at 30, 60 and 120 days during ripening from the same form by covering the cut surface with paraffin. On the whole, 36 samples [three replicates for each of the four productions (TA, TB, SA and SB) at the three ageing times (30, 60 and 120 d)] were collected. For microbiological analysis, cheese samples were transferred into sterile Stomacher bags, kept into a portable cooler during transport and, once in laboratory, immediately analysed. Successively, the samples were stored frozen (−20 °C) until other analysis.

2.2. Chemico-physical analyses

Each cheese sample was analysed for dry matter (DM), fat, protein ($N \times 6.38$), and ash content according to International Dairy Federation (IDF) standards [4A:1982 (IDF, 1982), 5B:1986 (IDF, 1986), 25:1964 (IDF, 1964a), and 27:1964 (IDF, 1964b), respectively]. Soluble nitrogen (N) was determined on an aqueous filtrate using the Kjeldahl method (MAF, 1986), and NaCl according to the IDF procedure (17A:1972; IDF, 1972).

Colour was measured by Minolta Chroma Meter (CR-300; Minolta, Osaka, Japan) using illuminant C, and expressed as lightness (L*), redness (a*), and yellowness (b*), according to the (CIE) L*a*b* system. The maximum resistance to compression (compressive stress, N mm^{-2}) was measured with an Instron 5564 tester (Instron, Trezzano sul Naviglio, Milano, Italy).

2.3. Microbiological analyses

Twenty-five grams of each cheese sample were suspended in 225 mL sodium citrate (2% w/v) solution and homogenised for 2 min at high speed with a stomacher (BagMixer® 400, Interscience, Saint Nom, France). Further serial decimal dilutions were performed in Ringer's solution (Sigma–Aldrich, Milan, Italy). Total mesophilic counts (TMC), total psychrotrophic counts (TPC), coliforms, enterococci, pseudomonads, mesophilic and thermophilic rod LAB, mesophilic and thermophilic cocci LAB, and yeasts were cultivated and incubated as reported by Settanni et al. (2012). Microbiological counts were performed in duplicate.

2.4. Isolation of LAB and phenotypic grouping

After growth, at least four colonies for each different morphology of presumptive LAB, including enterococci, were picked up from count plates and transferred to the corresponding broth media. The isolates from kanamycin aesculin azide (KAA) were cultivated in M17 broth, while the cultures from whey-based agar medium (WBAM) were inoculated into de Man-Rogosa-Sharpe (MRS) broth medium. The isolates were purified by successive sub-culturing, checked microscopically for purity and cell morphology and those Gram-positive (Gregersen KOH method) and catalase negative [determined by transferring fresh colonies from a Petri dish to a glass slide and adding 5% (w/v) H_2O_2] were stored in glycerol at −80 °C.

Phenotypic characterization was carried out as reported by Gaglio et al. (2014) based on growth at 15 and 45 °C, resistance at 60 °C for 30 min, NH_3 production from arginine, aesculine hydrolysis, acid production from arabinose, ribose, xylose, fructose, galactose, lactose, sucrose and glycerol, and CO_2 production from glucose. For coccus isolates, the sub-grouping also included the

evaluation of growth at pH 9.6 and in presence of NaCl 6.5% (w/v) since, unlike other dairy cocci, enterococci can grow in both conditions.

2.5. Genotypic differentiation and identification of LAB

DNA from LAB cultures was extracted by cell lysis using the Instagene Matrix kit (Bio-Rad, Hercules, CA) as described by the manufacturer. Crude cell extracts were then used as templates for PCR.

Strain differentiation was performed by random amplification of polymorphic DNA-PCR (RAPD-PCR) following the scheme reported by Settanni et al. (2012) by means of T1 Thermocycler (Biometra, Göttingen, Germany) to generate amplicons and the pattern analysis software package Gelcompare II Version 6.5 (Applied Maths, Sin-Martens-Latem, Belgium) to analyse their profiles.

Genotypic identification of the LAB characterised by different RAPD-PCR patterns was carried out by 16S rRNA gene sequencing (Weisburg et al., 1991). DNA fragments of about 1600 bp were purified by the QIAquick purification kit (Quiagen S.p.a., Milan, Italy) and sequenced at PRIMM (Milan, Italy). The sequences were compared by a BLAST search in GenBank/EMBL/DDBJ database. Furthermore, the multiplex PCR assay based on *sodA* gene reported by Jackson et al. (2004) was applied to confirm species identity of enterococci.

2.6. Antibacterial substances produced by LAB

The antibacterial activity of each LAB was evaluated against three strains (*Lactobacillus sakei* LMG2313, *Listeria innocua* 4202, and *Listeria monocytogenes* ATCC 19114) highly sensitive to bacteriocins (Hartnett et al., 2002; Corsetti et al., 2008). The inhibitory activities were first tested through the agar-spot deferred method, and the strains displaying antimicrobial properties were further subjected to the well diffusion assay (WDA) as reported by Corsetti et al. (2008). All tests were carried out in triplicate. The proteinaceous nature of the active compounds was tested against proteolytic enzymes as described by Settanni et al. (2005). All enzymes were purchased from Sigma–Aldrich (St. Louis, MO).

2.7. Statistical analysis

The GLM and CANDISC procedures of the SAS software package version 9.2 (SAS, 2010) were used for the statistical analysis. Chemico-physical and microbiological data were analysed by GLM procedure including the effects of farm (F = A, B), cheese technology (TC = *T*, traditional; S, standard), ripening time (R = 30, 60, 120 d), and their interaction F*TC*R. The Student "t" test was used for means comparisons at $P \leq 0.05$ significance level. A multivariate statistical approach were performed by a canonical discriminant analysis according to the CANDISC procedure, in order to ascertain the ability of chemical and physical parameters and microbiological counts in discriminating cheeses from different productions and during ripening.

3. Results

3.1. Chemico-physical analyses

Yield, chemical composition, and colour parameters (L*, a* and b*) of cheeses were affected by the farm (Table 1). On the whole, cheeses produced in the extensive farm A showed higher yield and protein percentage, lower fat, NaCl and soluble N contents, and a

Table 1
Chemico-physical characteristics of cheese samples collected through Caciocavallo Palermitano cheese ripening (means ± SD).

Production	TA			TB			SA			SB			Statistical significance[f]			
	30 d	60 d	120 d	30 d	60 d	120 d	30 d	60 d	120 d	30 d	60 d	120 d	Farm (F)	Technology (TC)	Ripening (R)	F*TC*R
Cheese yield, %	8.79 ± 0.55	8.63 ± 0.51	8.8 ± 0.50	7.78 ± 0.37	7.52 ± 0.33	7.26 ± 0.35	9.33 ± 0.65	9.15 ± 0.61	8.85 ± 0.58	8.07 ± 0.14	7.85 ± 0.12	7.56 ± 0.13	***	*	*	NS
DM, %	61.72 ± 1.00	60.58 ± 1.10	62.68 ± 0.58	65.15 ± 0.68	64.64 ± 1.60	68.91 ± 1.01	59.37 ± 2.22	56.72 ± 1.98	60.22 ± 2.10	61.39 ± 2.76	57.94 ± 0.75	62.21 ± 1.96	***	***	***	NS
Protein, % DM	48.62 ± 0.39	49.00 ± 0.33	49.34 ± 0.41	46.40 ± 1.87	46.77 ± 2.71	47.25 ± 1.60	48.76 ± 1.82	49.47 ± 2.12	48.79 ± 1.85	48.53 ± 1.63	49.00 ± 1.33	49.02 ± 1.37	*	+	NS	NS
Fat, % DM	39.14 ± 0.75	39.94 ± 0.51	39.90 ± 0.51	41.11 ± 2.65	41.77 ± 2.63	41.29 ± 1.94	37.39 ± 2.18	38.17 ± 1.92	37.68 ± 2.07	39.04 ± 1.40	38.60 ± 1.36	38.82 ± 1.65	*	***	NS	NS
Ash, % DM	8.32 ± 0.21	7.24 ± 0.33	7.72 ± 0.65	9.20 ± 0.66	7.93 ± 0.49	8.62 ± 1.00	10.03 ± 1.35	8.48 ± 1.00	10.08 ± 1.96	9.78 ± 0.48	8.61 ± 0.51	9.21 ± 0.24	NS	***	**	NS
NaCl, g/100 g	2.39 ± 0.14	1.62 ± 0.21	2.01 ± 0.35	3.48 ± 0.20	2.55 ± 0.05	3.28 ± 0.61	3.58 ± 0.75	2.40 ± 0.77	3.41 ± 1.11	3.64 ± 0.55	2.69 ± 0.34	3.33 ± 0.25	**	**	***	NS
Soluble N, % DM	0.58 ± 0.24	0.87 ± 0.15	1.01 ± 0.03	0.67 ± 0.34	0.97 ± 0.13	1.08 ± 0.12	1.05 ± 0.36	1.18 ± 0.23	1.62 ± 0.15	1.21 ± 0.32	1.70 ± 0.21	1.74 ± 0.41	*	***	***	NS
SN/TN[a]	7.67 ± 3.22	11.37 ± 1.91	13.11 ± 0.47	9.14 ± 4.25	13.17 ± 1.00	14.60 ± 1.07	13.81 ± 4.78	15.31 ± 3.64	21.20 ± 2.62	15.99 ± 4.63	22.24 ± 3.10	22.76 ± 5.93	*	***	***	NS
L* [b]	83.60 ± 0.66^{abc}	83.10 ± 0.66^{abcd}	80.28 ± 0.86^{def}	85.57 ± 1.71^{a}	84.67 ± 0.77^{ab}	80.90 ± 1.12^{cdef}	81.76 ± 3.19^{bcde}	81.39 ± 2.56^{bcde}	78.07 ± 2.44^{f}	79.02 ± 1.73^{ef}	77.94 ± 1.91^{f}	72.28 ± 3.30^{g}	+	***	***	*
A* [c]	−4.10 ± 0.10	−4.30 ± 0.27	−5.04 ± 0.09	−4.27 ± 0.23	−4.06 ± 0.03	−4.13 ± 0.12	4.54 ± 0.51	−4.47 ± 0.05	−5.32 ± 0.23	−5.29 ± 0.39	−5.02 ± 0.32	−6.04 ± 0.49	**	***	***	+
B* [d]	26.79 ± 0.55^{ef}	28.09 ± 1.30^{de}	29.04 ± 1.11^{cd}	29.81 ± 0.59^{bc}	30.81 ± 1.12^{ab}	32.16 ± 0.86^{a}	25.27 ± 0.92^{f}	25.32 ± 1.24^{f}	25.19 ± 1.16^{f}	29.53 ± 0.17^{bcd}	29.50 ± 0.59^{bcd}	28.25 ± 1.46^{cde}	***	***	NS	*
CS[e], N/mm^2	0.25 ± 0.03	0.24 ± 0.05	0.41 ± 0.13	0.27 ± 0.12	0.31 ± 0.05	0.36 ± 0.19	0.15 ± 0.05	0.16 ± 0.02	0.23 ± 0.02	0.13 ± 0.03	0.11 ± 0.01	0.23 ± 0.18	NS	***	*	NS

Means within a row with different superscripts (a, b, c, d, e, f, g) differ ($P \leq 0.05$).
[a] SN/TN = soluble N/total N.
[b] L* = lightness.
[c] a* = redness.
[d] b* = yellowness.
[e] CS = compressive stress.
[f] *P* value: ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; +, $P \leq 0.10$; NS, not significant.

less intense yellow colour, as indicated by the lower b* values, than farm B.

Cheese making technology significantly influenced all chemico-physical parameters. Compared with cheeses from standard productions, those produced with the traditional technology had higher DM and, consequently, lower cheese yield. Moreover, traditional cheeses showed higher fat content and values of L*, a* and b* colour indexes, and were more resistant to compression, indicating a more compact cheese paste than S cheeses; in addition, their content of NaCl and soluble N was lower.

A significant trend due to ripening time was observed for most of the cheese parameters. A marked increase in soluble N and compressive stress test was detected during ripening, whereas L* and b* decreased and increased, respectively, only in traditional cheese, explaining the significant F*TC*R interactions. However, between 30 and 60 d of ageing, a reduction in both DM and NaCl content was registered.

3.2. Microbial evolutions during ripening

The viable counts of the 10 microbial groups investigated in this study are reported in Table 2. Coagulase positive staphylococci and clostridia were not investigated since they were not detected in the stretched curd processed into cheese (Settanni et al., 2012). The effects of farm, cheese making conditions and ripening time affected significantly the development of total psychrotrophic microorganisms and, consequently, pseudomonads. In general, except for enterococci, the most evident effect on the growth of the several microbial groups analysed was showed by the ripening time ($P < 0.001$). The interactions of the three effects considered resulted significant only for psychrotrophic microorganisms and coliforms.

A general decreasing trend was observed for TMC, coliforms, yeasts and all LAB groups, during ripening. Enterococci within each production did not statistically ($P > 0.05$) vary at the different times of analysis, but their levels estimated in the cheeses from traditional productions were, on average, about 1 Log cycle higher than those from the corresponding standard productions. However, the group of enterococci was counted at levels lower than those detected for the other LAB groups in all productions for each collection time. The highest levels were observed for mesophilic rod LAB at 30 d of ripening, while the lowest levels were registered for thermophilic coccus LAB at 120 d.

3.3. Canonical discriminant analysis

The canonical discriminant analysis, performed simultaneously on chemical, physical and microbiological data, was able to distinguish clearly the Caciocavallo Palermitano cheeses manufactured according the different productions and during ripening.

The plot generated by the canonical discriminant analysis (Fig. 1) showed a wider separation, due to the canonical variable 1 (*y*-axis), among cheeses produced with different technologies (traditional and standard). In addition, a discriminant effect of the farm, also due mainly to the canonical variable 1, emerged within both cheese technologies. Whereas the separation effect of ripening, linked to the canonical variable 2 (*x*-axis), was quite evident, even though weaker for traditional cheeses of the farm A.

Table 3 shows the correlation coefficients for the parameters considered with the canonical variables. The variables 1, which contributed to separate the cheeses on the basis of technology and, to a lesser extent, of farm, explained the 54% of variance and was mainly correlated to chemical and physical parameters, especially the yellow index b* (0.76), even though the highest coefficient was recorded with the enterococci (0.81). The canonical variable 2, responsible for the separation among cheeses due to the ripening

Table 2
Microbial load (log cfu g^{-1}) of cheese samples collected through Caciocavallo Palermitano cheese ripening (means ± SD).

Production	TA			TB			SA			SB			Statistical significance			
	30 d	60 d	120 d	30 d	60 d	120 d	30 d	60 d	120 d	30 d	60 d	120 d	Farm (F)	Technology (TC)	Ripening (R)	F*TC*R
PCA-SkM 7 °C	3.20 ± 0.26^{ab}	2.67 ± 0.15^{c}	2.13 ± 0.11^{d}	3.47 ± 0.30^{a}	3.20 ± 0.10^{ab}	2.97 ± 0.21^{bc}	2.20 ± 0.40^{d}	1.67 ± 0.35^{e}	1.77 ± 0.30^{de}	3.07 ± 0.40^{abc}	3.10 ± 0.20^{abc}	3.17 ± 0.15^{ab}	***	***	***	**
PCA-SkM 30 °C	7.73 ± 0.40	7.13 ± 0.30	6.40 ± 0.56	7.77 ± 0.25	7.43 ± 0.21	6.57 ± 0.40	7.63 ± 0.40	7.53 ± 0.50	6.67 ± 0.68	8.20 ± 0.36	7.17 ± 0.40	7.20 ± 0.40	NS	NS	***	NS
VRBA	4.10 ± 0.40^{ab}	3.80 ± 0.26^{abc}	2.87 ± 0.35^{de}	3.93 ± 0.51^{ab}	3.13 ± 0.50^{cd}	2.67 ± 0.42^{de}	3.20 ± 0.26^{cd}	2.67 ± 0.25^{de}	2.40 ± 0.40^{e}	4.33 ± 0.61^{a}	3.60 ± 0.10^{bc}	2.60 ± 0.60^{de}	NS	NS	***	*
KAA	5.40 ± 0.36	5.63 ± 0.25	5.57 ± 0.38	5.63 ± 0.23	5.73 ± 0.50	5.90 ± 0.20	4.43 ± 0.45	4.83 ± 0.35	4.33 ± 0.35	4.47 ± 0.50	4.93 ± 0.45	4.83 ± 0.06	NS	***	NS	NS
PAB	2.63 ± 0.15	2.30 ± 0.26	2.33 ± 0.21	3.57 ± 0.21	3.53 ± 0.31	2.90 ± 0.20	2.27 ± 0.30	2.30 ± 0.30	1.90 ± 0.10	3.00 ± 0.50	2.83 ± 0.35	2.87 ± 0.15	***	***	*	NS
MRS	8.07 ± 0.30	7.43 ± 0.40	6.80 ± 0.35	8.20 ± 0.36	7.63 ± 0.32	6.47 ± 0.55	8.10 ± 0.66	7.00 ± 0.10	6.07 ± 0.29	7.93 ± 0.31	7.23 ± 0.25	6.97 ± 0.45	NS	NS	***	NS
M17 30 °C	7.17 ± 0.21	6.93 ± 0.11	6.83 ± 0.11	7.13 ± 0.23	6.53 ± 0.32	5.87 ± 0.42	7.67 ± 0.23	6.97 ± 0.65	6.30 ± 0.50	7.77 ± 0.70	7.23 ± 0.75	6.77 ± 0.45	NS	*	***	NS
WBAM	6.97 ± 0.06	6.53 ± 0.45	5.80 ± 0.30	7.13 ± 0.65	6.83 ± 0.50	6.10 ± 0.72	7.23 ± 0.23	6.80 ± 0.30	6.13 ± 0.25	7.53 ± 0.55	6.60 ± 0.40	6.27 ± 0.25	NS	NS	***	NS
M17 44 °C	3.20 ± 0.66	2.67 ± 0.70	2.13 ± 0.35	3.47 ± 0.65	3.20 ± 0.17	2.97 ± 0.23	2.20 ± 0.51	1.67 ± 0.22	1.77 ± 0.06	3.07 ± 0.46	3.10 ± 0.46	3.17 ± 0.30	NS	*	***	NS
DRBC	7.73 ± 0.50	7.13 ± 0.25	6.40 ± 0.36	7.77 ± 0.31	7.43 ± 0.15	6.57 ± 0.58	7.63 ± 0.20	7.53 ± 0.06	6.67 ± 0.50	8.20 ± 0.55	7.17 ± 0.45	7.20 ± 0.20	*	NS	***	NS

Results indicate mean values ± S.D. of six plate counts (carried out in duplicate for three independent productions).
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; VRBA, violet red bile agar for coliforms; KAA, kanamycin aesculin azide agar for enterococci; PAB, *Pseudomonas* agar base for pseudomonads; MRS, de Man-Rogosa-Sharpe agar for mesophilic rod LAB; 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; WBAM, whey-based agar medium for thermophilic rod LAB; DRBC, dichloran rose bengal chloramphenicol agar for yeasts.
P value: ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; NS, not significant.
Means within a row with different superscripts (a, b, c, d, e) differ ($P \leq 0.05$).

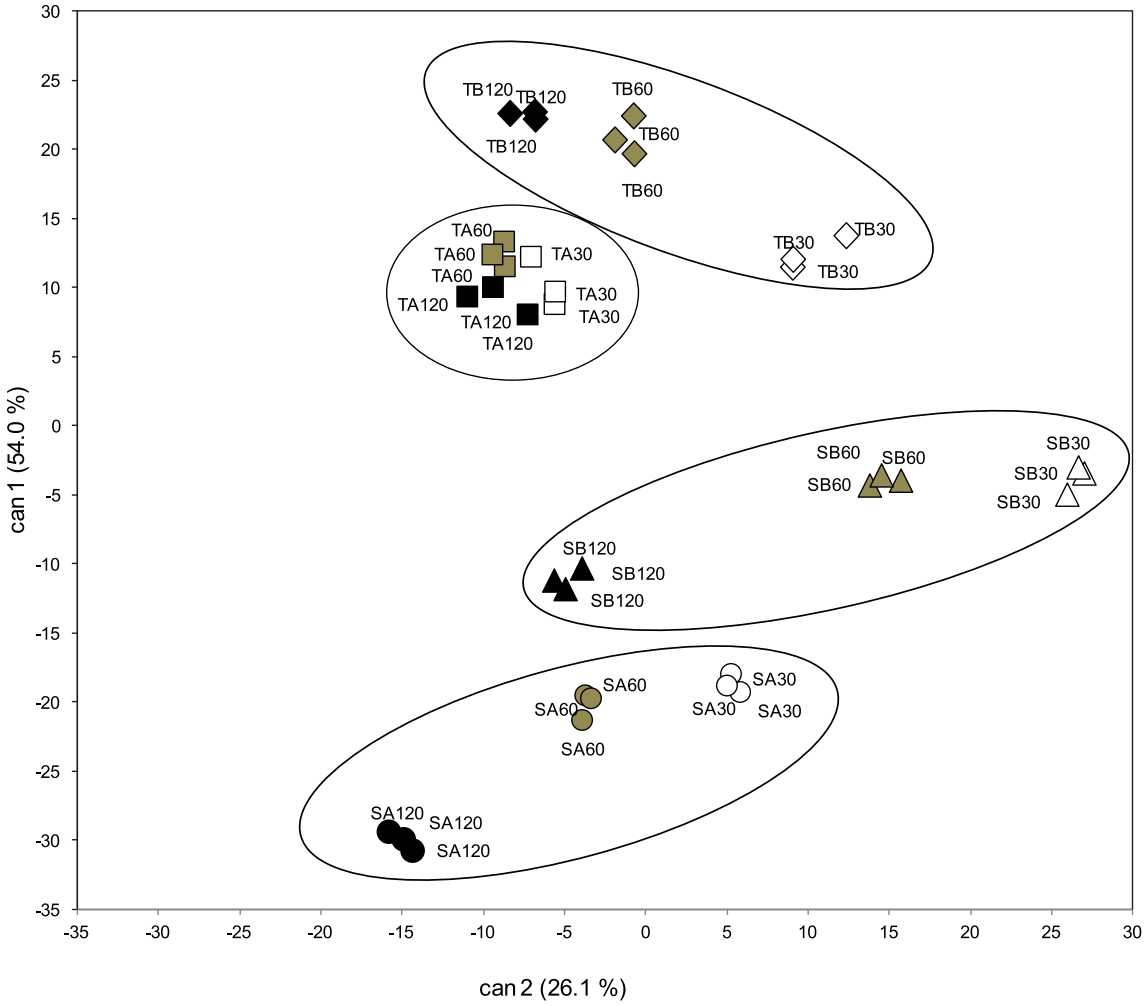

Fig. 1. Plot from canonical discriminant analysis in which Caciocavallo Palermitano cheeses from different productions are distributed in function of canonical variables 1 and 2 based on chemico-physical parameters and microbiological counts.

time, explained the 26% of the variance, and was mainly and positively correlated to all microbiological groups, with the exception of enterococci, with which it showed a lower and negative correlation.

3.4. Isolation and grouping of LAB

On the basis of appearance, about four colonies showing similar morphological characteristics were isolated from each medium used for LAB counts, at the highest dilutions of samples, in order to detect the dominant strains. A total of 882 colonies were collected from 36 cheese samples. All cultures were subjected to microscopic inspection and separated in 683 cocci and 199 rods. After Gram characterisation and catalase test, 612 cocci and 191 rods were still considered presumptive LAB cultures, as being Gram-positive and catalase-negative.

Based on several phenotypic features of the cultures and combinations of these features, the 803 LAB cultures were separated into 13 groups (Table 4), 7 for cocci and 6 for rods. The most numerous groups were group I and III, including 195 and 169 isolates, respectively. However, the unequivocal determination of the fermentative metabolism of LAB included between the groups IX to XIII needed the evaluation of their growth in presence of pentose sugars, that evidenced an obligate homofermentative metabolism for the isolates of group IX, and showed a facultative heterofermentative metabolism for the isolates of the groups X to XIII.

3.5. Differentiation and identification of LAB

Two hundred and forty-one isolates (about 30% of the total cultures collected) were selected from each phenotypic group (PG) as being representative of the different productions and ripening times and subjected to the RAPD analysis. The genotypic differentiation distinguished 30 strains as shown by the resulting dendrogram (Fig. 2).

All 30 strains were identified by 16S rRNA gene sequencing. The BLAST search evidenced a percentage of identity with sequences available in the NCBI database of at least 97%, which is considered the minimum level of similarity for 16S rRNA genes of two strains belonging to the same species (Stackebrandt and Goebel, 1994). This method allowed the identification of all strains at species level (Table 5) and all of them were confirmed to belong to the group of LAB. The species with the highest number of strains were *Pediococcus acidilactici* and *Lactobacillus casei*.

Table 3
Canonical discriminant analysis: correlation coefficients for chemico-physical parameters and microbiological counts with the canonical variables 1 and 2 in the canonical discriminant analysis of Caciocavallo Palermitano cheese from different productions during ripening.

	Canonical variable 1	Canonical variable 2
Variance %	53.99	26.08
Cheese yield	−0.5270	−0.1802
DM	0.6769	−0.1227
Protein	−0.3586	−0.1064
Fat	0.6276	−0.0212
Ash	−0.5092	0.2755
NaCl	−0.2954	0.3592
Soluble N	−0.5313	0.0402
SN/TN[a]	−0.5074	0.0461
L* [b]	0.4781	0.0126
A* [c]	−0.3785	0.0224
B* [d]	0.7579	0.2528
CS[e], N mm^{-2}	0.4720	−0.4366
PCA-SkM 7 °C	0.6086	0.4207
PCA-SkM 30 °C	−0.0610	0.5863
VRBA	0.3176	0.5753
KAA	0.8116	−0.2498
PAB	0.5656	0.4600
MRS	0.2357	0.5483
M17 30 °C	−0.2118	0.5854
WBAM	−0.0485	0.6064
M17 44 °C	0.3927	0.4565
DRBC	−0.0355	0.6468

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; VRBA, violet red bile agar for coliforms; KAA, kanamycin aesculin azide agar for enterococci; PAB, *Pseudomonas* agar base for pseudomonads; MRS, de Man-Rogosa-Sharpe agar for mesophilic rod LAB; 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; WBAM, whey-based agar medium for thermophilic rod LAB; DRBC, dichloran rose bengal chloramphenicol agar for yeasts.
[a] SN/TN = soluble N/total N.
[b] L* = lightness.
[c] a* = redness.
[d] b* = yellowness.
[e] CS = compressive stress.

3.6. Comparison of RAPD profiles of vat and cheese LAB

In order to evaluate the persistence of the LAB of wooden vat origin during the ripening of Caciocavallo Palermitano cheese, the RAPD profiles of the strains, isolated from the vat before milk was processed into cheese, were compared to those of the LAB strains collected during cheese maturation. The direct comparison of the RAPD profiles (Fig. 3) was able to evidence the persistence of three enterococci identified as *Enterococcus faecalis* FMA721, *Enterococcus gallinarum* FMA288 and *Enterococcus casseliflavus* FMA108 at the time of isolation from the wooden vat and identified, in this work, from ripened cheeses as *E. faecalis* FMAC219, *E. gallinarum* FMAC104 and *E. casseliflavus* FMAC98, respectively. All three strains were not found, at least at dominant levels, in the standard productions and were isolated from both traditional (A and B) productions. In particular, *E. casseliflavus* FMAC98 was isolated from TA and TB no longer than 30 d, *E. gallinarum* FMAC104 was isolated at 30 d from TA and TB, but at 60 d only from TB, while *E. faecalis* FMAC219 persisted till 120 d in both productions.

3.7. Inhibitory activity of LAB

In order to evaluate the competitive advantages of the strains isolated during ripening of Caciocavallo Palermitano cheeses, the strains were tested for antibacterial compound production against three indicator strains with high sensitivity to bacteriocins. Ten strains, all lactobacilli, showed an antibacterial activity at least against one of the indicator strains, with *Lactobacillus rhamnosus* FMAC62 and *Lactobacillus fermentum* FMAC1 showing the highest inhibition both in terms of number of indicator strains and width of the inhibition areas (Table 5). All active compounds were inactivated by proteolytic enzymes (data not shown), proving their protein nature. Since the active substances were not characterised for amino acid and/or gene sequences, they shall be referred to as bacteriocin-like inhibitory substances (BLIS) (Corsetti et al., 2008).

4. Discussion

In the last years, although the innovation in food technologies allowed the production of several safe foods with extended shelf-life, a re-discovery of traditional products is being registered (Settanni and Moschetti, 2014). Regarding the traditional food processes, especially those applied in cheese manufacture, they are not immutable in principle (Cavazza et al., 2011). Several factors may change over time: hygiene of breeding, milk production, transformation environment conditions, manufacturer's origin and experience and government regulations. As a matter of fact, all these changes may affect the concept of food typicality which is expression of the characteristics of a territory, its history and tradition (Iannarilli, 2002).

This work was carried out within a research project aimed to determine the influence of the traditional equipment on the quality of Caciocavallo Palermitano cheese during ripening.

Chemical and physical traits of cheeses resulted highly influenced by the farming system and the cheese making technology, as also emerged by the canonical discriminant analysis, confirming the contribution of the environment of milk production and cheese manufacture in characterizing cheese quality. Under extensive farming system (farm A), the higher cheese yield was linked to the higher casein content that characterized the low milk produced by the rustic autochthonous Sicilian cows, whereas the lower cheese fat was probably connected to the milking system of the autochthonous cows, according to which the last and most fat rich-milk is destined for the calf (Alabiso et al., 2000). Moreover, in cheeses from farm A, the lower NaCl may depend on the higher moisture which diluted the salt, the lower N soluble content may reflect the lower milk urea (Martin et al., 1997), and the lower yellow index (b*) was probably due to a lower level of carotenoids in comparison with the cheese from intensive farm B where the cows consumed a maize-based concentrate. The strong pressure action exerted on the cheese paste to eliminate the residual whey during the traditional cheese making technology (Tornambé et al., 2009) may be responsible for the higher DM, the reduced cheese yield, and the more compact cheese paste in comparison with the standard technology. The lower NaCl content of traditional cheeses could be imputable to either lower moisture or harder paste consistency, both contributing to reduce salt absorption. The indigenous lactic microorganisms active in the traditional cheese making (Settanni et al., 2012) could be responsible for the higher colour indexes, indicating a more intense cheese colour (Buffa et al., 2001), and also for the less pronounced proteolytic activity, resulting in the lower levels of N soluble, in comparison with the selected LAB used in the standard productions. During ripening, as expected, both traditional and standard cheeses showed an increasing trend for soluble N, derived from microbial proteolysis, and paste consistency, whereas the changes in the colour indexes (L* increase and a* decrease) interested only the traditional cheeses, due to the higher ability of the native microbiota to confer a more intense yellow colour during ripening than the microorganisms of the commercial starter (Buffa et al., 2001). The reduction in both DM and NaCl content between 30 and 60 d of ageing, observed in all cheeses, was

Table 4
Phenotypic grouping of LAB isolates collected through Caciocavallo Palermitano cheese ripening.

Characters	Clusters												
	I (n = 195)	II (n = 40)	III (n = 169)	IV (n = 81)	V (n = 56)	VI (n = 41)	VII (n = 30)	VIII (n = 48)	IX (n = 71)	X (n = 28)	XI (n = 12)	XII (n = 9)	XIII (n = 23)
Morphology[a]	C	C	C	C	C	C	C	R	R	R	R	R	R
Cell disposition[b]	sc	sc	tr	tr	tr	tr	tr	sc	sc	sc	sc	sc	sc
Growth:													
15 °C	+	+	+	+	+	+	+	−	−	+	+	+	+
45 °C	+	+	+	+	+	+	+	+	+	−	−	−	+
pH 9.2	+	+	+	+	+	+	+	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6.5% NaCl	+	+	+	+	+	+	+	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Resistance to 60 °C	+	+	+	−	+	+	+	+	−	+	+	−	+
Hydrolysis of:													
Arginine	+	−	−	−	−	+	+	−	−	−	+	−	−
Aesculin	+	−	−	−	+	−	+	−	−	−	+	−	+
Acid production from:													
Arabinose	+	+	+	+	+	+	+	+	−	+	+	+	+
Ribose	+	+	+	+	+	+	+	+	−	+	+	+	+
Xylose	+	+	+	+	+	+	+	+	−	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+	+	−	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+	+	+	+	+	+	+	+
CO_2 from glucose	−	−	−	−	−	−	−	+	−	−	−	−	−
Growth in presence of pentose carbohydrates	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	−	+	+	+	+

n.d., not determined.
[a] R, rod; C, coccus.
[b] sc, short chain; tr, tetrads.

opposite to that expected; this particular trend could be explained by the sampling method using the paraffin covering that, reducing the exposure of the central slice of cheese to the air, may have slowed down dehydration and NaCl penetration into the 60-d aged cheese samples (Bonanno et al., 2013).

The evaluation of the microbiological characteristics of traditional and standard productions, conducted in a previous study (Settanni et al., 2012), revealed that, following the traditional protocol, a clear dominance of the *S. thermophilus* strains, typical thermophilic SLAB, of wooden vat origin was registered during the entire cheese manufacture till stretched curd moulding. In that study, other species found in the wooden vat were identified and recognized as common members of the NSLAB population. Thus, the main hypothesis of the present experimentation was the persistence of some LAB forming biofilms on the wooden vat surface during the ripening of cheese manufactured traditionally. This because the persistence of vat LAB in cheese, over time, undoubtedly shows a clear active role of these bacteria during ripening. The traditional cheeses followed at the manufacturing stage, together with those carried out in standard conditions, were then subjected to ripening and the data retrieved from the samples collected at 30, 60 and 120 days of ripening are showed in this paper.

In general, the ripening time significantly affected the development of all LAB groups, except enterococci, as clearly confirmed by the canonical discriminant analysis where all the microbiological groups contributed to separate cheeses at different ripening time, except enterococci. In fact, this last group was detected at levels lower than those detected for the other LAB groups in all productions at each collection time and, interestingly, their levels registered in the traditional cheeses were almost one order of magnitude higher than those observed for the corresponding standard cheeses. The other LAB groups showed a decreasing trend in concentration over time. The highest levels were observed for mesophilic rod LAB at 30 d of ripening (in the range 10^7–10^8 cfu g^{-1}) while the lowest levels were registered for thermophilic coccus LAB at 120 d (in the range 10^5–10^6 cfu g^{-1}). These levels are in the same range of those reported for other Caciocavallo cheeses produced in southern Italy such as Caciocavallo Pugliese analysed at 60 d that showed ca. 10^8 cfu g^{-1} for mesophilic rod LAB and ca. 10^6 cfu g^{-1} for mesophilic coccus LAB, while thermophilic LAB were not higher than 10^6 cfu g^{-1} (Gobbetti et al., 2002) and different Caciocavallo produced in Calabria, Campania and Basilicata regions, collected from retail markets, for which the maximum levels were in the range 8.8–8.9 Log cfu g^{-1} on MRS (Piraino et al., 2005).

The different dominating LAB colonies were isolated from the various plate counts of the cheese samples and 803 isolates were first subjected to several phenotypic tests from which thirteen groups were obtained. Two-hundred and forty-one isolates representative of the different ripening times of the traditional and standard cheeses were differentiated by RAPD-PCR in 30 strains, evidencing a limited LAB biodiversity in the experimental cheeses. All 30 strains were identified by 16S rRNA gene sequencing as *P. acidilactici*, *Pediococcus pentosaceus*, *E. casseliflavus*, *E. gallinarum*, *E. faecalis*, *L. rhamnosus*, *L. casei*, *Lactobacillus delbrueckii*, *L. fermentum* and *Lactobacillus paracasei*.

Except *E. casseliflavus*, *E. gallinarum*, and *L. delbrueckii*, most of the species identified are commonly reported to be part of the NSLAB population in several cheeses (Settanni and Moschetti, 2010). Furthermore, the species *L. fermentum*, *L. paracasei*, *L. rhamnosus*, *L. casei*, *L. delbrueckii*, and *E. faecalis* were found in other Caciocavallo type cheeses produced in South Italy (Gobbetti et al., 2002; Piraino et al., 2005; Morea et al., 2007). In our work, enterococci were detected at approximately constant levels (10^5 cfu g^{-1} for traditional and 10^4 cfu g^{-1} for standard cheeses), till 120 d of ripening. Similar levels of enterococci have been reported for other Italian raw cows' milk cheeses (Franciosi et al., 2009).

In order to better investigate the low biodiversity of LAB isolated during ripening, all LAB strains were tested for antibacterial compound production, since this character may confer advantages from

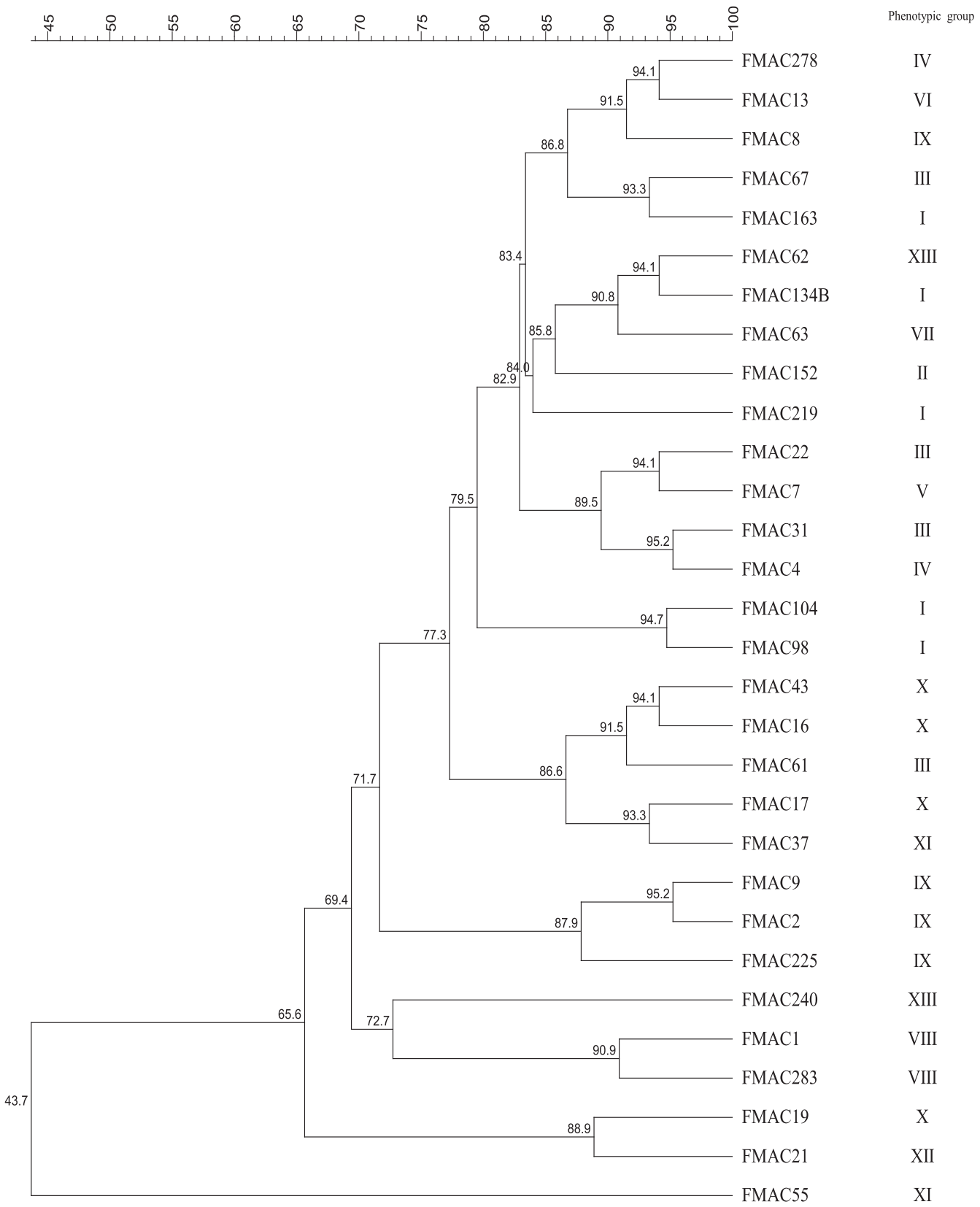

Fig. 2. Dendrogram obtained from RAPD-PCR patterns of LAB strains from traditional and standard Caciocavallo Palermitano cheese productions.

Table 5
Identification of LAB strains through Caciocavallo Palermitano cheese production.

Strains	Phenotypic group	Cheese samples	Ripening time	Species	Ac. No.	Bacteriocin-like inhibitory activity[a]		
						Indicator strains[b]		
						19114	4202	2313
FMAC98	I	TB	30 d	*E. casseliflavus*	KF060255	–	–	–
FMAC104	I	TB	60 d	*E. gallinarum*	KF060264	–	–	–
FMAC134B	I	SB	30 d	*E. durans*	KF029506	–	–	–
FMAC163	I	TA	120 d	*E. casseliflavus*	KF060266	–	–	–
FMAC219	I	SB	120 d	*E. faecalis*	KF060261	–	–	–
FMAC152	II	TA	30 d	*E. casseliflavus*	KF060267	–	–	–
FMAC22	III	TB	30 d	*P. acidilactici*	KF060269	–	–	–
FMAC31	III	TB	120 d	*P. acidilactici*	KF060262	–	–	–
FMAC61	III	SB	60 d	*P. pentosaceus*	KF060257	–	–	–
FMAC67	III	SB	120 d	*P. pentosaceus*	KF029505	–	–	–
FMAC4	IV	TA	30 d	*P. acidilactici*	KF060271	–	–	–
FMAC278	IV	SB	30 d	*P. acidilactici*	KF060253	–	–	–
FMAC7	V	TA	60 d	*P. acidilactici*	KF060254	–	–	–
FMAC13	VI	TA	120 d	*P. acidilactici*	KF060270	–	–	–
FMAC63	VII	SB	60 d	*P. pentosaceus*	KF060272	–	–	–
FMAC1	VIII	TA	30 d	*L. fermentum*	KF060268	1.8 ± 0.06	1.9 ± 0.10	2.0 ± 0.00
FMAC283	VIII	SB	60 d	*L. fermentum*	KF060259	–	1.4 ± 0.17	1.2 ± 0.10
FMAC2	IX	TA	30 d	*L. delbrueckii*	KF029498	1.3 ± 0.15	1.5 ± 0.10	1.8 ± 0.06
FMAC8	IX	TA	60 d	*L. delbrueckii*	KF060252	–	–	–
FMAC9	IX	TA	60 d	*L. delbrueckii*	KF060256	–	1.4 ± 0.10	–
FMAC225	IX	TA	30 d	*L. delbrueckii*	KF060263	–	–	–
FMAC16	X	TA	120 d	*L. casei*	KF029499	–	–	–
FMAC17	X	TA	120 d	*L. casei*	KF029500	–	–	–
FMAC19	X	TB	30 d	*L. casei*	KF029501	–	1.6 ± 0.17	–
FMAC43	X	SA	60 d	*L. casei*	KF029503	–	–	–
FMAC37	XI	SA	30 d	*L. casei*	KF029502	1.2 ± 0.06	1.1 ± 0.12	1.2 ± 0.10
FMAC55	XI	SB	30 d	*L. casei*	KF060258	1.4 ± 0.00	1.7 ± 0.06	1.8 ± 0.00
FMAC21	XII	TB	30 d	*L. paracasei*	KF060265	1.4 ± 0.12	1.5 ± 0.10	1.7 ± 0.17
FMAC62	XIII	SB	60 d	*L. rhamnosus*	KF029504	1.9 ± 0.00	2.1 ± 0.00	2.1 ± 0.06
FMAC240	XIII	TA	120 d	*L. rhamnosus*	KF060260	1.3 ± 0.06	1.5 ± 0.12	1.2 ± 0.15

Abbreviations: *E.*, *Enterococcus*; *P.*, *Pediococcus*; *L.*, *Lactobacillus*.
Symbols: plus sign positive for diacetyl production; minus sign negative for diacetyl production or, in case of antibacterial tests, no inhibition found.
[a] Width of the inhibition zone (mm). Results indicate mean ± S.D. of three independent experiments.
[b] Bacterial species: *Listeria monocytogenes* ATCC 19114; *Listeria innocua* 4202; *Lactobacillus sakei* 2313.

competitiveness with other strains. Ten lactobacilli showed the capacity of producing BLIS and this allows to improve the safety, control the fermentation microbiota, speed maturation and increase the shelf life of the final cheeses (Deegan et al., 2006; Garde et al., 2007).

In order to evaluate the influence of the LAB forming biofilms on the wooden vat during the ripening of Caciocavallo Palermitano cheese, the strains isolated from the vat before milk addition were compared (by RAPD profiles) to those collected during cheese maturation. Three strains belonging to the species *E. faecalis*, *E. casseliflavus* and *E. gallinarum* were found in the vat and also during ripening, even though only *E. faecalis* was detected till the end of the ripening period at levels of 10^5 cfu g^{-1} that were dominant within the enterococcal population. These strains were not found in the standard productions, evidencing the defining role of the wooden vat in enriching the milk LAB diversity at the time of cheese making.

The presence of the enterococci in cheese is usually attributed to faecal contamination; but some authors assessed that the presence of these bacteria in the food matrices is not always due to the direct contact with contaminated material (Mundt, 1986; Birollo et al., 2001). Although the enterococci do not represent the major humans pathogenic, they are recognized as responsible of numerous nosocomial infections (Coque et al., 1996). However, several authors suggest that the presence of some strains of *Enterococcus*, established their harmlessness, is desirable,

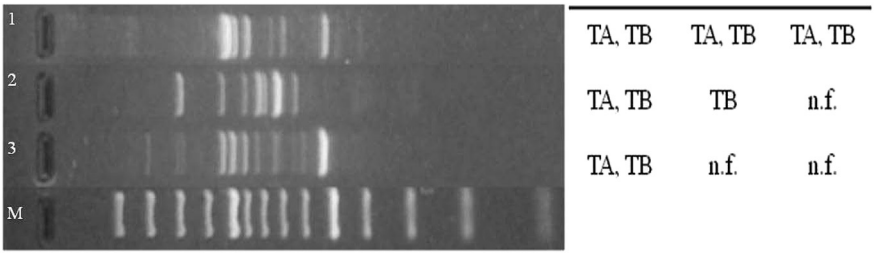

Fig. 3. Persistence of LAB [carried out by RAPD (with primer M13) profile comparison] of wooden vat origin during the ripening of traditional Caciocavallo Palermitano cheeses. Lines M, 1-kb DNA molecular size markers (Invitrogen). Lines: 1, *E. faecalis* FMA721; 2, *E. gallinarum* FMA288; 3, *E. casseliflavus*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

especially in long ripened traditional cheeses, as their contribution in developing the aroma is believed to be fundamental. Moreover, numerous strains of this group, originating from raw milk, are tightly linked to typicality of the final cheese (Foulquié Moreno et al., 2006). The presence of enterococci at dominant levels during ripening has been reported for cheeses produced in the Mediterranean basin, as well as for other Sicilian cheeses (Randazzo et al., 2008). In this work, within the enterococci isolated, only *E. faecalis* is generally associated with cheese (Settanni and Moschetti, 2010). The influence of the enterococci bacteria on the sensory properties of cheese seems to be due to specific biochemical traits such as proteolytic and lipolytic activities, citrate utilization, and production of several aromatic volatile compounds (Oliszewski et al., 2013). Due to their positive contribution to cheese flavour and their role in acceleration of the ripening process (Gardiner et al., 1999), enterococci are being proposed as adjunct cultures (De Vuyst et al., 2011; Oliszewski et al., 2013).

The other NSLAB species had not been previously isolated from the wooden vat. This finding cannot exclude their presence in the wooden vat at subdominant (undetectable) levels or in a dormant/viable but not cultivable (VBNC) state. However, since standard cheese making was carried out in a stainless steel vat, the strain comparison between LAB collected from both traditional and standard productions, clarified the doubt on the origin of the strains. From this comparison, only for *L. delbrueckii* the milk or rennet origin has to be excluded, since it was only found in the traditional production (both A and B), highlighting the higher LAB biodiversity of the traditional cheese productions compared to the standard ones.

5. Conclusions

In this work, the Caciocavallo Palermitano cheeses manufactured with traditional or standard technologies, performed using bulk milk derived from two farming systems, were analysed at different times in order to investigate the changes in chemical, physical and microbiological characteristics induced by the production system and during ripening.

With regards to the objectives, several conclusions may be drafted. The contribution of the farming systems and cheese technology in changing chemical and physical traits was mainly evidenced by the lower contents in NaCl and soluble N, and the higher paste consistency recorded in cheeses from extensive farm and traditional technology, whereas during ripening the soluble N content and the paste yellow and consistency increased. LAB levels of the experimental cheeses were in the same range of those reported for other Caciocavallo cheeses produced in southern Italy. The ripening time affected the development of several LAB groups except enterococci, whose concentration was constant during ripening. The majority of the 10 LAB species identified are commonly reported to be part of the NSLAB population in several cheeses, including Italian Caciocavallo cheeses. The persistence of LAB from the wooden vat during ripening was evaluated by direct comparison of the polymorphic profiles and three strains belonging to the species *E. faecalis*, *E. casseliflavus* and *E. gallinarum* were found to be present during cheese maturation only in the traditional productions. In particular, *E. faecalis* FMA288 was found to dominate the enterococcal population at the end of the ripening period, evidencing the defining role of the wooden vat in the modification of LAB composition during Caciocavallo Palermitano cheese ripening. Other studies are being prepared in order to evaluate the individual contribution of each persistent strain to the aromatic and safety aspects of the final cheeses.

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