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A large factory-scale application of selected autochthonous lactic acid bacteria for PDO Pecorino Siciliano cheese production

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ABSTRACT

The main hypothesis of this study was that the autochthonous lactic acid bacteria (LAB) selected for their dairy traits are able to stabilize the production of PDO (Protected Denomination of Origin) Pecorino Siciliano cheese, preserving its typicality. The experimental plan included the application of a multi-strain lactic acid bacteria (LAB) culture, composed of starter (*Lactococcus lactis* subsp. *lactis* CAG4 and CAG37) and non starter (*Enterococcus faecalis* PSL71, *Lactococcus garviae* PSL67 and *Streptococcus macedonicus* PSL72) strains, during the traditional production of cheese at large scale level in six factories located in different areas of Sicily. The cheese making processes were followed from milk to ripened cheeses and the effects of the added LAB were evaluated on the microbiological, chemico-physical and sensorial characteristics of the final products. Results highlighted a high variability for all investigated parameters and the dominance of LAB cocci in bulk milk samples. The experimental curds showed a faster pH drop than control curds and the levels of LAB estimated in 5-month ripened experimental cheeses (7.59 and 7.27 Log CFU/g for rods and cocci, respectively) were higher than those of control cheeses (7.02 and 6.61 Log CFU/g for rods and cocci, respectively). The comparison of the bacterial isolates by randomly amplified polymorphic DNA (RAPD)-PCR evidenced the dominance of the added starter lactococci over native milk and vat LAB, while the added non starter LAB were found at almost the same levels of the indigenous strains. The sensory evaluation showed that the mixed LAB culture did not influence the majority of the sensory attributes of the cheeses and that each factory produced cheeses with unique characteristics. Finally, the multivariate statistical analysis based on all parameters evaluated on the ripened cheeses showed the dissimilarities and the relationships among cheeses. Thus, the main hypothesis of the work was accepted since the quality parameters of the final cheeses were stabilized, but all cheeses maintained their local typicality.

1. Introduction

Pecorino Siciliano is an Italian cheese enjoying the Protected Designation of Origin (PDO) status produced in throughout the Sicily region (25,711 km^2) in South Italy. Pecorino Siciliano is a semi-hard cheese manufactured following traditional techniques from raw ewe's milk without any addition of bacterial starters, according to the protocol of production (GURI n. 295/1955). In these conditions, cheese production relies on lactic acid bacteria (LAB) present in milk, on those transferred by the equipments and from the dairy environments (Settanni and Moschetti, 2014) that together with pH, salt content, ripening conditions and chemical changes occurring during ripening contribute to the microbiological stability of the final product (Johnson et al., 1990). Raw milk is generally employed to produce extra-hard cheeses that are ripened for a long period, until 24 months or even longer (Gobbetti, 2004). Aged, ripened cheeses retain their sensorial traits for long time thanks their low pH, low water activity, and low redox potential (Ledenbach and Marshall, 2010). Despite the stressing chemico-physical parameters that characterize ripened cheese, Todaro

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et al. (2011) found undesired potentially pathogenic microorganisms in ripened PDO Pecorino Siciliano cheese samples.

Pecorino cheese production is widespread in South and Central Italy, but several regional differences are found. The raw materials are directly responsible for the characteristics of this cheese typology (Suzzi et al., 2015; Tofalo et al., 2015), while the indigenous milk microbiota might be defining for the final safety of the ripened products (Schirone et al., 2011, 2013; Guarcello et al., 2015). The differences in the fermenting LAB of the several Pecorino cheeses depend strongly on the technology of transformation and on the use of a natural whey starter culture (Di Cagno et al., 2003).

In cheese making production, starter cultures are generally added in order to rapidly dominate over the native microbiota, but the indiscriminate use of starter strains might determine a flattening of the taste in the final products, with the risk that the obtained cheeses may no longer be distinguishable by production technology and/or geographical origin. The application of autochthonous microorganisms, that are adapted to the production area (environment), the local raw materials (substrates) and the traditional protocol (technology), provide a cheese with the typical characteristics that cannot be reproduced elsewhere (Settanni and Moschetti, 2014).

Recently, same studies have been published on selection of LAB strains to be used as starter for Pecorino Siciliano cheese production (Randazzo et al., 2006, 2007; Franciosi et al., 2009), valuating their effects on volatile compounds (Randazzo et al., 2008) and on microbiological characteristics (Settanni et al., 2013). In particular, in the latter work Settanni et al. (2013) evaluated the effect of different starter lactic acid bacteria (SLAB), alone or in combination with non SLAB (NSLAB), on the microbiological quality and sensory traits of 5-month ripened cheeses.

The aim of the present work was to evaluate the suitability of a multiple strain SLAB/NSLAB culture on the final characteristics of PDO Pecorino Siciliano cheese produced in several areas of Sicily different for pedoclimatic conditions, sheep breed, pasture and dairy farmer.

2. Materials and methods

2.1. Microorganisms and characteristics of milk

The mixed LAB culture used in this study was composed of two SLAB strains (*Lactococcus lactis* subsp. *lactis* CAG4 and CAG37) and three NSLAB strains (*Lactococcus garvieae* PSL67, *Enterococcus faecalis* PSL71 and *Streptococcus macedonicus* PSL72), isolated from PDO Pecorino Siciliano cheeses (Todaro et al., 2011), previously selected for their technological performances and already applied at factory-scale level in a restricted area of western Sicily (Agrigento province) (Settanni et al., 2013).

LAB cultures were grown in M17 broth (Oxoid, Basingstoke, UK) for 18 h and centrifuged at 5000 $\times g$ for 5 min. The cells were washed twice in Ringer's solution (Sigma-Aldrich, Milan, Italy) and re-suspended in the same solution till reaching an optical density (OD) of ca. 1.00, determined by means of a 6400 Spectrophotometer (Jenway Ltd., Felsted Dunmow, UK) at 600 nm wavelength, which approximately corresponds to a concentration of 10^9 CFU/mL, as verified by plate counting.

Bulk raw ewes' milk samples were analysed for somatic cell count (SCC), fat, protein, casein, and lactose using the infrared method (Combi-foss 6000, Foss Electric, Hillerod, Denmark). Urea content was determined by enzymatic method using the difference in pH (CL-10 Plus, Eurochem, Roma, Italy), pH values using a HI 9025 pH-meter (Hanna Instruments, Ann Arbor, MI, USA).

2.2. Cheese production and sampling points

Cheese productions were performed in six dairy factories (Table 1) producing PDO Pecorino Siciliano cheese daily, located in different production areas throughout the Sicily region and gathered into a consortium for the protection of this traditional PDO cheese production. The sampling points, the number of samples analysed and the analysis performed are reported in Table 2.

Bulk milk (100 L) used for the production of experimental cheeses (EC) in each factory was collected from a higher volume of milk left, under manual agitation for approximately 15 min, in a wooden vat and transferred into two plastic vats (50 L each) representing two different trials. One vat was inoculated with the mixed LAB culture (500 mL) to reach, in the final volume of milk, the concentration of 10^7 and 10^3 CFU/mL for SLAB and NSLAB, respectively, and used to produce the experimental cheese (EC). The second vat was added with 500 mL of Ringer's solution without bacteria to obtain the control cheese (CC). Both bulks were then subjected to the traditional cheese making provided by the production protocol of PDO Pecorino Siciliano cheese (GURI, 1955) and ripened for 5 months as follows: 2 months in a storage chamber at 16 °C and 85% of relative humidity (RH) and then 3 months into a natural cave at approximately 16 °C and 90% of RH. The cheese trials were carried out in duplicate in two consecutive weeks in April 2014.

All production processes were entirely monitored; samples were collected from bulk milk at delivery (BM1), bulk milk after resting in wooden vat (BM2), bulk milk with inoculum (BM3), curds just after curdling, acidified curds and ripened cheeses. The wooden vat surfaces (400 cm^2) were sampled, just before cheese production took place, as reported by Didienne et al. (2012) using UV-treated paper squares positioned halfway up the side and on the bottom. Curds were collected after whey discharge, just before reversal in the rattan baskets where they assumed the final shape. Furthermore, in order to follow the curd acidification, two samples of curd were collected for each production and kept at ambient temperature for 7 days. One curd sample was subjected to the monitoring of pH, performed by the portable pH meter (waterproof pHTestr 30, Eutech Instruments, Nijkerk, The Nederlands) at 2-h intervals for the first 8 h and then after 1, 2, 3 and 7 days from milk curdling. The second sample of curd was used for microbiological analysis. Cheeses were sampled after five months of ripening.

2.3. Microbiological analyses

The bulk milk samples, (10 mL), curds (10 g) and wooden vat surfaces (1 mL of the cell suspension obtained after homogenisation of the gauze) collected during cheese making, and the cheese samples (25 g), after 5 months of ripening, were subjected to serial decimal dilution in Ringer's (Sigma-Aldrich, Milan, Italy) solution. Curds and cheeses were homogenised in sodium citrate solution (2% w/v) by a stomacher (BagMixer® 400, Interscience, Saint Nom, France) for 2 min at the highest speed. This step is necessary because sodium citrate is a calcium-sequestering agent that determines the disruption of casein micelles (Corredig et al., 2003) allowing the accessibility to the microorganisms associated to fat globules (Griffiths, 2000). Microbial suspensions were plated and incubated as follows: total psychrotrophic counts (TPC) on Plate Count Agar plus Skimmed Milk, incubated aerobically at 7 °C for 7 days; *Enterobacteriaceae* counts on Violet Red Bile Glucose Agar, aerobically incubated at 37 °C for 24–48 h; enterococci on Kanamycin Aesculin Azide aerobically incubated at 37 °C for 24–48 h; coagulase-positive staphylococci (CPS), on Baird Parker supplemented with Rabbit Plasma Fibrinogen (RPF) supplement and