Short communication: Phenotypic and genetic diversity of wild *Lactococcus lactis* isolated from traditional Pecorino cheeses of Tuscany

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ABSTRACT

Wild *Lactococcus lactis* isolates from traditional Pecorino cheeses in 4 regions of Tuscany were isolated and characterized to evaluate the diversity of autochthonous lactococci. Sixty strains of *Lactococcus* were clustered by the results of carbohydrate utilization and diagnostic enzyme activity. Twenty-one unique strains were then chosen for characterization of salt and temperature tolerance, as well as acidification and proteolytic activity in milk. Genetic analysis of these strains was performed via 16S ribosomal DNA sequencing and multilocus sequence typing (MLST) to elucidate diversity relative to their location of origin. Phylogenetic analysis showed distinct clustering by region within organism subspecies, and phenotypic properties demonstrated concomitant trends. Multilocus sequence typing thus allowed for the regional distinction of isolates separate from those of previous works, supporting the concept that distinctive regional qualities of cheeses are strongly influenced by microbial ecology.

Key words: *Lactococcus lactis*, Pecorino cheese, multilocus sequence typing, microbial ecology

Short Communication

Pecorino is a ewe milk cheese that is very popular throughout Italy and an important export. Traditional Pecorino cheeses are produced using artisanal methods of production, storage, and aging that are consistent with continuous production in a given territory for no less than 25 yr (Decreto Legislativo n.173, 30th April, 1998). Pecorino cheeses are thereby regionally unique and may lose characteristic attributes if produced similarly in other areas. This specificity is partly related to the breed and diet of dairy animals, which determine the physical and chemical properties of the raw milk (Palmquist et al., 1993), and partly to variations in cheese-making practices. Microbiological aspects are

also important, because traditional Pecorino is made by first fermenting raw milk at room temperature to enrich its microbial load, which can vary significantly by location. The resulting products maintain regionally distinct characteristics reflective of these unique autochthonous microbiota.

Lactococcus lactis is the primary constituent of many industrial and artisanal starter cultures used to ferment dairy products (Smit et al., 2005) and is a naturally dominant species within many sheep milk cheeses. The microbiota of ewe milk and its cheeses have been variously quantified before, showing a high prevalence of lactococci, often among similarly high or higher levels of enterococci and lactobacilli (Durlu-Ozkaya et al., 2001; Medina et al., 2001; Feutry et al., 2012). More recent next-generation sequencing approaches that avoid the selectivity pitfalls of culture-dependent isolation and enumeration show that lactococci may actually be heavily dominant in raw ewes' milk cheeses (Alegria et al., 2012; Quigley et al., 2012). The microbial characterization of cheeses, especially those with protected designations of origin, has been of increasing interest as the allure of preserving natural microbiota has grown. Pecorino cheeses from several regions of Italy have been profiled for autochthonous microorganisms (Mannu and Paba, 2002; Randazzo et al., 2006; Aquilanti et al., 2007), and the focus has often been on nonstarter lactic acid bacteria for their role in ripening and flavor development (De Angelis et al., 2001; Randazzo et al., 2008). Yeasts, *Enterobacteriaceae*, and pathogens have also been targeted (Cosentino et al., 2001; Chaves-López et al., 2006; Gardini et al., 2006; Giammanco et al., 2011). However, little is known of lactococcal diversity, distribution, or regional variability in Pecorino cheeses.

Multilocus sequence typing (MLST), the sequence analysis of genes coding for housekeeping and functional proteins, is being used increasingly for the precise characterization of nonpathogenic food bacteria such as *Lactobacillus* spp. (Cai et al., 2007; Diancourt et al., 2007; Tanganurat et al., 2009), *Oenococcus oeni* (de Las Rivas et al., 2004; Bilhère et al., 2009), and *Lactococcus lactis* (Rademaker et al., 2007; Fernández et al., 2011). Multilocus sequence typing is straightforward, unam-

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biguous, scalable, and highly discriminatory compared with most fingerprinting methods. Sequence data are easily reproducible, provide a measure of evolutionary analysis, and are portable between laboratories for comparison, making MLST ideal for measuring ecological diversity. In the present study, wild *Lactococcus* isolates from traditional Pecorino cheeses made in different provinces of Tuscany (Pisa, Pistoia, Siena, and Massa-Carrara) were isolated and select strains were chosen for evaluation via MLST and traditional phenotypic and technological differentiation to help illustrate the regional diversity of *L. lactis* in Tuscan Pecorino cheese.

Pecorino cheeses were sampled from one dairy in each of 4 regions in Tuscany (Table 1). The cheeses were made with fresh raw ewe milk fermented by autochthonous microbes, without any addition of starter culture. Isolation was performed following the procedure described by IDF (1988). Organisms were isolated on M17 agar with 10% lactose (Oxoid, Basingstoke, UK) for each sampling time point: from the milk, curd, and after 2, 7, 14, 21, 28, 35, 42, and 60 d of ripening. Eight isolates from each sampling point were selected and streaked out 3 times on the same agar medium to purify. In total, 320 isolates were obtained from 4 dairies. Of these isolates, 60 were presumptively identified as lactococci based on Gram staining, catalase, oxidase, and hemolytic activity (Bergey and Holt, 1994). Carbohydrate fermentation and enzymatic patterns were determined for these 60 isolates using the API 20 Strep system (API System, bioMérieux, Marcy l'Etoile, France) following the manufacturer's recommendations (Supplementary Table S1; <http://www.journalofdairyscience.org/>). The isolates were grouped via single-linkage cluster analysis performed with distances calculated from API 20 test results using Mesquite v2.75 (Maddison and Maddison, 2011), and representative isolates were selected from each of the major clades, resulting in a total of 21 isolates, including 3 to 7 isolates from each dairy (Figure 1). *Lactococcus lactis* ssp. *lactis* ATCC19435 and *L. lactis* ssp. *cremoris* ATCC 19257 were used as controls for salt and temperature tolerance testing and for genetic characterization. The sources and sampling times of the 21 isolates are outlined in Table 1. Analyses were carried out in triplicate.

The ability of the isolates ($n = 21$) to grow in different salt concentrations (2, 3, 4, 5, 6, and 6.5% wt/vol) at 30°C and at different temperatures (40, 43, and 45°C) was tested in sterile M17 medium and measured for blank-corrected change in optical density at 600 nm (ΔOD_{600}) after 24 h of incubation using a Spectronic 21 spectrophotometer (Figure 2; Bausch and Lomb, Rochester, NY). All isolates grew very well ($\Delta OD_{600} \geq 1.0$) at 40°C and fairly well ($\Delta OD_{600} \geq 0.5$) at 43°C. Almost all grew well in a 4% salt medium as well. Both

temperature and salt tolerance at these levels are classically attributed only to *L. lactis* ssp. *lactis* (Bergey and Holt, 1994). Some isolates even grew at 45°C and in the presence of up to 6% salt.

Acidification and proteolytic activities of selected strains were evaluated as previously described in Turchi et al. (2011). In short, isolates were inoculated (1% vol/vol) into skim milk reconstituted from powder (Oxoid) and incubated at 30°C. Changes in acidity were recorded as decreases in pH from that of uninoculated milk (pH 6.76) after 6 and 24 h. Proteolytic activities were detected spectrophotometrically at 340 nm after 48 h with α -phthalaldehyde. Proteolytic activity of the bacterial isolates was expressed as micrograms of glycine per milliliter using a standard curve of glycine (BDH Chemicals Ltd., Poole, UK). The selected strains varied widely in the acidification and proteolysis of milk (Figure 2). The strains showing a greater decrease in pH at 6 h also generally demonstrated the greatest acidification after 24 h. Proteolytic activities showed trends similar to those of acidification, with the more acidifying isolates generally demonstrating greater proteolysis.

To identify the subspecies of the 21 chosen isolates, total chromosomal DNA was extracted from each isolate by using the DNeasy Tissue kit (Qiagen GmbH, Hilden, Germany) according to the supplier's procedures. Primers 8F and 1391R from Palmer et al. (2006) were used for amplifying bacterial 16S ribosomal

Table 1. Source (region) and ripening time of *Lactococcus* isolates characterized

Strain ID	Region	Ripening time (d)
Lc1	Siena	42
Lc2	Siena	14
Lc3	Siena	14
Lc4	Siena	28
Lc5	Siena	— (curd)
Lc6	Siena	— (milk)
Lc7	Siena	42
Lc8	Pistoia	7
Lc9	Pistoia	7
Lc10	Pistoia	7
Lc11	Pistoia	7
Lc12	Pistoia	14
Lc13	Massa-Carrara	28
Lc14	Massa-Carrara	29
Lc15	Massa-Carrara	30
Lc16	Pisa	7
Lc17	Pisa	7
Lc18	Pisa	7
Lc19	Pisa	7
Lc20	Pisa	2
Lc21	Pisa	2
ATCC 19435	NA ¹	NA
ATCC 19257	NA	NA

¹Not applicable.

Figure 1. Cluster analysis of initial 60 isolates based on API 20 Strep kit (API System, bioMérieux, Marcy l'Etoile, France) results. Polytomies represent identical API results. The 21 labeled isolates were chosen for further genetic and phenotypic characterization. Colors of cluster groups indicate the regions of origin for those isolates. Shades (colors) of cluster groups indicate the regions of origin for those isolates. Each shade of gray (color) is reflected in the regions of a map of Tuscany: light gray (blue) for Pistoia, medium gray (green) for Pisa, dark gray (red) for Siena, and black (purple) for Massa-Carrara. Color version available in the online PDF.

DNA (rDNA) for 30 PCR cycles and the products were sequenced via standard methods. A BLAST search of the NCBI nonredundant GenBank database (<http://blast.ncbi.nlm.nih.gov>) confirmed that all of the strains belonged unequivocally to the genus *Lactococcus*, and subspecies of *L. lactis* were determined with 100% identity. However, isolates Lc5 and Lc6 were identified as *Lactococcus raffinolactis* with 99% identity. The strain

identities of the 11 *lactis* and 10 *cremoris* subspecies isolates are shown in Figure 2.

Multiple protein-coding loci were then evaluated via MLST using the analytical methods employed by Rade-maker et al. (2007). Primers were used for the sequence analysis of 3 housekeeping genes (*atpA*, *pheS*, and *rpoA*) and 3 functional genes (*bcaT*, *pepN*, and *pepX*) selected for their conservation among available lactic acid bacteria genomes and broad distribution across the genome in unlinked locations without multiple copies. The branched-chain aminotransferase BcaT and peptidases PepN and PepX are important for development of aroma precursors and textural qualities in dairy products from metabolism of milk proteins (Yvon et al., 2000; Luoma et al., 2001). The obtained PCR products were purified and sequenced as before. Single forward sequences were trimmed, aligned, and analyzed using MEGA 5 software (Tamura et al., 2011). The *L. raffinolactis* isolates did not generate amplicons for some of the genes and were omitted from MLST analyses.

For each strain, a sequence type (ST) was assigned, derived from the combination of different alleles at the 7 genes. Among the 21 strains, 10 different ST were attributed. No ST were found in more than one dairy, although more than one ST was isolated from half the dairies. The number of polymorphic sites varied considerably among the 7 genes, particularly in *pepX* and *pepN*, which are involved in flavor production, followed by *atpA* and *bcaT* (105, 60, 51, and 50 polymorphic sites respectively). This variation among genes implicated in texture and flavor development highlights the importance of microbiota in cheese terroir.

The complete data set of all 7 gene sequences was then analyzed by the neighbor-joining method (Saitou and Nei, 1987), resulting in a tree reflective of the ST assigned and 2 distinct clusters of similar topology for the *lactis* and *cremoris* genotypes (Figure 2; and for each gene in Supplementary Figure S1; <http://www.journalofdairyscience.org/>). Results of phenotypic characterization tests are reported next to the 2 main clusters, showing several intra-cluster patterns. Trends in salt and temperature tolerance, acidification, and proteolysis clearly paralleled ST.

A major advantage of MLST is the ability to accurately compare strains from different studies provided that the same primers are used. The 21 strains from this study were compared those investigated by Rade-maker et al. (2007) and Fernández et al. (2011). The comparison showed little or no overlap in ST between strains of different origin (Supplementary Figure S2; <http://www.journalofdairyscience.org/>), underscoring the specificity of the strains for a given location and the power of MLST to distinguish strains.

Figure 2. Neighbor-joining cluster analysis of a multilocus sequence typing (MLST) of partial sequences from the *atpA*, *bcaT*, *pepN*, *pepX*, *pheS*, *rpoA*, and 16S rRNA genes of 19 wild *Lactococcus lactis* strains and 2 control strains from the American Type Culture Collection (ATCC, Manassas, VA), compared with their phenotypic characteristics. Bootstrap percentages after 500 simulations are shown at the nodes. The 2 main clusters represent subspeciation, as indicated by boxes next to the tree. Isolates Lc5 and Lc6, strains of *Lactococcus raffinolactis*, are included below for comparison. The results of associated phenotypic tests are represented by shaded cells ranging from light gray (lowest) to black (highest). A white cell with an X indicates that results were not determined. Phenotypic characteristics include growth at 4% and 6% NaCl, growth at 43 and 45°C, and acidification and proteolysis of milk over the indicated incubation periods.

This study has shown examples of methods used for isolation of autochthonous bacteria and characterization of the phylogenetic distribution of such organisms in different cheese-making environments. However, additional elucidation of phenotypic and technological characteristics revealed even greater diversity among strains than a purely genetic approach would allow. The information from these phenotypic and technological tests has shown differentiation beyond that of MLST, differentiating metabolic activities of isolates even with the same ST. Together, these techniques have provided a detailed estimate of regional microbial diversity in an important cheese-making organism and deliver strong evidence that the natural microbiota within Pecorino cheeses vary with their region of origin.

In conclusion, the amount of polymorphism observed via genetic analysis signifies the potency of

MLST as a tool to assess the ecology within microbial populations from different sources, particularly when complemented with relevant phenotypic analyses. Such approaches will likely have the power to discriminate between varieties and localities of cheese by correlating their autochthonous microbial populations, which could be a useful tool to preserve and explore the effect of traditional cheese-making technology. The intraspecies genetic and phenotypic diversity of the strains isolated in this study reinforces the need for a broader understanding of the populations involved in cheese production and their effects on flavor and quality. This diversity supports the conception that distinctive regional qualities of cheeses are strongly influenced by microbial ecology; however, further study will be needed to correlate the roles of these organisms with resulting sensory aspects.

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