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Microbial changes of natural milk cultures for mozzarella cheese during repeated propagation cycles

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ABSTRACT

Natural milk cultures are undefined starters produced by a selective treatment (milk pasteurization, incubation at high temperature and backslopping), and used for the production of Mozzarella cheese. The objective of this study was to monitor the microbial composition (at or below the species level) and the variability of two model laboratory cultures (LC) and an artisanal culture (NMC) produced at a dairy plant over 13 reproduction cycles using culture independent and dependent methods. PCR-DGGE of V3 region of 16S RNA gene and of a fragment of the *lacSZ* operon of *Streptococcus thermophilus* proved that the cultures were dominated by *S. thermophilus* but other species (*Lactobacillus delbrueckii*, *L. helveticus*, *Lactococcus lactis*, enterococci) were present. These results were partially confirmed by culture dependent analysis. Molecular identification and typing (RAPD-PCR and *lacSZ* PCR-DGGE and partial sequencing of *serB* in *S. thermophilus*) confirmed NMC had the highest diversity, while the two replicate LCs diverged at some early stage. Variability in acid production activity and in aroma production was also observed, and was highest in NMC. *S. thermophilus* bacteriophages were detected by multiplex PCR.

1. Introduction

Artisanal (or natural) cultures are undefined starters reproduced daily by cheese makers using some form of backslopping (Parente, 2006; Parente & Cogan, 2004). Natural whey cultures (NWC) are required for Protected Designation of Origin (PDO) cheeses including Parmigiano Reggiano, Grana Padano, and Mozzarella di Bufala Campana, and are produced by incubating whey at the end of cheese manufacture for 16–24 h, with or without temperature control. Natural milk cultures (NMC) are used for the production of PDO Asiago Pressato, Asiago d'Allevo, Gorgonzola, and traditional specialty guaranteed (TSG) Mozzarella cheese and the techniques used for their production are described below. Details on cheeses requiring the use of natural starter cultures in their standard of identity can be found in the DOOR database (http://ec.europa.eu/agriculture/quality/door/list.html).

Random contamination, selective treatments (high temperatures), intrinsic (pH, lactic acid concentration) and implicit factors (competition, amensalism, bacteriophage) shape the community and population structure and determine the performance of artisanal cultures (Parente, 2006). NWC have been extensively studied (De Filippis, La Storia, Stellato, Gatti, & Ercolini, 2014; Ercolini, Frisso, Mauriello, Salvatore, & Coppola, 2008; Ercolini, Mauriello, Blaiotta, Moschetti, & Coppola, 2004; Gatti, Lazzi, Rossetti, Mucchetti, & Neviani, 2003; Lazzi, Rossetti, Zago, Neviani, & Giraffa, 2004; Rossetti et al., 2008) and are dominated by *Streptococcus thermophilus* and/or by thermophilic lactobacilli (*Lactobacillus helveticus*, *L. delbrueckii* susbp. *lactis*, *L. fermentum*) but mesophilic species (*Lactococcus lactis*) may also be present. A large variety of Operational Taxonomic Units (OTUs) was recently found by Next Generation Sequencing (NGS) of NWC for the production of traditional Italian cheeses, but diversity was relatively low and many samples were dominated by 1–2 species (De Filippis et al., 2014). Estimates on the number of strains present in natural cultures vary with the method used. Using RAPD-PCR and

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phenotypic tests between 3 and 10 biotypes of *L. helveticus* have been found in NWC for Parmigiano Reggiano (Gatti et al., 2003). NGS of a region upstream of *lacSZ* operon of *S. thermophilus* recovered only a limited number of sequence types, and one sequence type was always dominant (De Filippis et al., 2014).

NMC are produced by heat treating (60–63 °C, 10–30 min) raw milk followed by incubation at 37–42 °C to select for thermophilic lactic acid bacteria. Although they are used in the production of several cheeses, less is known on their composition. They are dominated by *S. thermophilus* but other species (*Streptococcus macedonicus*, lactobacilli, enterococci) are frequently present (Carraro et al., 2011; Delgado et al., 2013; Parente, Rota, Ricciardi, & Clementi, 1997).

Variability of performance is expected in undefined starters, but scientific reports are rare (Erkus et al., 2013; Nielsen, 1998). Natural starters are reproduced with limited asepsis, and bacteriophages may act as selective agents. Even if reproduction in the presence of bacteriophages should select for resistant strains (Parente & Cogan, 2004), failures due to the development of virulent bacteriophages have been observed in mesophilic starters (Josephsen, Petersen, Neve, & Nielsen, 1999). Thermophilic lactobacilli in NWC are frequently lysogenic (Zago, Suarez, Reinheimer, Carminati, & Giraffa, 2007) and lytic bacteriophages can be isolated from these cultures (Zago, Comasoni, Neviani, & Carminati, 2005), but reports on *S. thermophilus* bacteriophages occurring in natural starters are sporadic (Bruttin et al., 1997; Zinno, Janzen, Bennedsen, Ercolini, & Mauriello, 2010).

The objective of this study was to obtain knowledge on the composition, diversity and stability of a natural milk culture used for the production of mozzarella cheese. Therefore, model natural starters were propagated in the laboratory for several cycles and composition at the species level or below, activity and occurrence of bacteriophages were compared with that of the corresponding culture propagated in an artisanal cheese making plant.

2. Material and methods

2.1. Sampling

Raw milk batches (fourteen) were obtained daily from a pasta filata cheese making plant, transported to the laboratory at 4 °C and used to produce laboratory milk cultures (LC; cycle 0) using the procedure described in Fig. 1. Two batches (LCA and LCB) were propagated independently, and cross contamination was prevented. Subsequent batches (cycles 1–13) were obtained by backslopping i.e. by inoculating (0.5% v/v) the culture from the previous cycle in raw milk obtained daily from the cheese making plant, followed by heat treatment and incubation as described in Fig. 1. At the end of incubation (on average 16 h) each culture was refrigerated before use for inoculation of the following cycle. For comparison purposes, natural milk cultures were obtained from the cheese making plant providing the raw milk (NMC; cycles 0, 1, 4, 7, 10, 13). The procedure used for propagation of the culture at the cheese plant was identical to that described above, but heat treatment and incubation were carried out in a temperature controlled starter tank (volume 50 L) and control of asepsis during reproduction was less stringent. Microbiological and chemical analyses and samples were performed on refrigerated samples within 1 h, while DNA extraction and headspace analysis were performed on samples stored at −80 °C.

2.2. Physico-chemical and chemical analyses and performance tests

pH of raw milk, model laboratory cultures (LCA and LCB) and natural milk cultures (NMC) was measured using a combination electrode (Hamilton Bonaduz AG, Bonaduz, Switzerland) and a pH-meter (Orion 420A plus, Thermo Fisher Scientific, Rodano, Italy). Titratable acidity was measured using NaOH (0.25 mol/l) with phenolphthalein (1% w/v in ethanol) as an indicator. Acid production activity was assayed by inoculating each sample of NMC and LC (5%, v/v) in pre-warmed (42 °C) reconstituted (11% v/v) Skim Milk (RSM, sterilized at 121 °C for 5 min) and incubating at 42 °C. pH was measured at 0 and 4 h, and pH decrease (ΔpH) was calculated. Three replicates were used.

2.3. Microbial counts

After dilutions in quarter strength Ringer Solution, coliforms and total mesophilic counts were obtained by pour plating in Violet Red Bile Agar (24 h, 35 °C) and Plate Count Agar (for raw and pasteurized milk, 48 h, 30 °C), respectively. Thermophilic streptococci and aciduric lactobacilli were enumerated by spread plating on LM17 (M17 supplemented with 0.5% w/v lactose) and Rogosa (pH 5.4) agar after 48 h at 42 °C and after 5 days at 42 °C, respectively, in anaerobiosis (AnaeroGen bags, Oxoid) respectively.

2.4. Culture independent methods

Denaturing gradient gel electrophoresis (DGGE) was performed for NMC and LCs at cycles 4 and 13. DNA was extracted using Power Food Bacterial DNA Extraction kit (MO BIO Laboratories Inc., Carlsbad, CA, USA). PCR-DGGE of the V3 region of 16S RNA gene was carried out as described by Ercolini et al. (2004) using Q5 Hot Start High-Fidelity Taq (New England Biolabs, Hitchin, UK). Staining was performed using 25 μl of SYBR Gold 10,000X (Invitrogen, Thermo Fisher Scientific, Rodano, Italy) in 250 ml of TAE 1X. Gel images were digitized using a GelDocXR apparatus with an XcitaBlueTM conversion screen, ChemiDocTM XRS filter and Quantity One 1-D analysis software (Bio-Rad Laboratories, Hercules, CA, USA) and converted to .tif images. Ladders including amplified DNA from pure cultures of *Lactobacillus plantarum* subsp. *plantarum* DSM20174, *L. fermentum* DSM20052 (Lfe), *Enterococcus faecium* DSM20477 (Efc), *L. helveticus* DSM20075 (Lhe), *L. delbrueckii* subsp. *lactis* DSM20072 (Lde), *Enterococcus faecalis* DSM20478 (Efl), *Lactococcus lactis* subsp. *lactis* DSM20481 (Lcl), *Streptococcus thermophilus* DSM20617 (Sth), *L. paracasei* subsp. *paracasei* DSM5622 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) were included, and bands which did not match those of the ladders were eluted, re-amplified, checked for purity and sequenced if a single amplification product was obtained.

lacS PCR-DGGE was carried out for *S. thermophilus* strains and for selected milk cultures as described by Ercolini, Fusco, Blaiotta, and Coppola (2005) and staining was performed as described above. DNA from *S. thermophilus* strains belonging to different *lacS* biotypes was used as a ladder.

A multiplex PCR assay for *cos*- and *pac-pac*-type bacteriophages of *S. thermophilus* was performed on filtered (0.45 μ) culture supernatants as described by Quiberoni, Tremblay, Ackermann, Moineau, and Reinheimer (2006).

2.5. Isolation, typing and molecular identification

Colonies were randomly picked from LM17 from cycle 13 and purified on the same medium. DNA was extracted from cell pellets using InstaGene matrix (BioRad Laboratories). RAPD-PCR was carried out using Coc1 primer (Cocconcelli, Porro, Galandini, & Senini, 1995). The collection was deduplicated by removing isolates whose profiles were not significantly different (the average similarity of