International Journal of Food Microbiology 124 (2008) 164-170

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Microbial diversity in Natural Whey Cultures used for the production of Caciocavallo Silano PDO cheese

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ARTICLE INFO

Article history:
Received 22 February 2008
Received in revised form 18 March 2008
Accepted 18 March 2008

Keywords:
Caciocavallo Silano PDO cheese
Natural Whey Cultures
DGGE
DHPLC
Geographical origin

ABSTRACT

The microbial diversity of sixty-three Natural Whey Cultures (NWCs) for the manufacture of Caciocavallo Silano cheese PDO was studied. The NWCs were collected from different dairies covering the whole territory of PDO production including five different regions of southern Italy. The microbial species diversity was determined by direct DNA extraction from NWCs and Polymerase Chain Reaction (PCR) amplification of variable regions of the 16S rRNA gene followed by denaturing gradient gel electrophoresis (DGGE) and denaturing high performance liquid chromatography (DHPLC). DGGE and DHPLC fingerprinting yielded the same results in terms of number of bands/peaks and specific migration/retention time of the amplicons. The DHPLC technique was used in this study for the first time to assess a food-related mixed microbial community by a culture-independent approach and proved to be at least as effective as DGGE in profiling species diversity in NWCs. Cluster analysis of DGGE and DHPLC data revealed that the species-related groups of similarity were not dependent on the geographical origin of the NWCs. The presence of three groups of 10–14 NWCs at 100% of species similarity indicated that some species associations are very commonly occurring in the NWCs for Caciocavallo Silano cheese PDO. A RAPD-PCR analysis of the NWCs was also performed for the members of the above groups and it showed that, though characterized by the same species diversity, most of the identical NWCs included different biotypes. The sequences of DGGE bands and DHPLC peaks revealed the occurrence of mainly thermophilic lactic acid bacteria such as *Lactobacillus delbrueckii*, *L. helveticus* and *Streptococcus thermophilus* even though the mesophilic *Lactococcus lactis* also occurred in some NWCs. In conclusion, the results of this study indicate that the microbial diversity of NWCs used for the Caciocavallo Silano PDO cheese is not high, it is not dependent on the geographical origin and the same microbial species occur within the territory examined. The microbiota of fermented PDO products and its possible link with territory should be studied case by case in order to have useful evidences for the assessment of product quality and authenticity.

1. Introduction

Caciocavallo Silano is a semi-hard "pasta-filata" cheese that is granted a Protected Designation of Origin (PDO) label (European Regulation 1236/96) and is produced in five different regions of southern Italy, namely Campania, Basilicata, Calabria, Puglia and Molise. It is produced from raw or mildly thermally treated (58 °C for 30 s) whole cow's milk with initial addition of rennet at 36–38 °C. The fermentation is carried out for 4–10 h by addition of Natural Whey Cultures (NWCs) arising from the previous manufacture according to the traditional back-slopping procedure. The fermentation is stopped empirically when the curd is ready to be stretched in hot water. After stretching, the cheese is flask-like shaped in 1–2.5 kg sizes, cooled in water, salted in brine for 6 h, hanged, air dried and ripened for 1–3 months. Different types of Caciocavallo can be produced with different technologies varying in raw material, starter culture, curd and cheese ripening conditions and leading to products characterized by different traits. However, Caciocavallo Silano cheese producers are obliged to follow the regulations for production of the PDO designation (DPCM 196/93, Rome, Italy) that include ruling of milk origin and treatment, stretching procedure, timing, etc. Several studies have focused on the microbiology and/or biochemistry of intermediates of production and final products describing also caciocavallo cheeses different from Silano (Corsetti et al., 2001; Gobbetti et al., 2002; Coppola et al., 2003; Piraino et al., 2005). The final products were found to be characterized mainly by mesophilic non-starter lactic acid bacteria

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doi:10.1016/j.ijfoodmicro.2008.03.007

(LAB) such as *Lactobacillus plantarum*, *L. paracasei*, *L. fermentum* (Corsetti et al., 2001; Coppola et al., 2003; Piraino et al., 2005). However, no data are yet available on the microbial composition of the NWCs used for the manufacture of Caciocavallo Silano PDO cheese, although the use of the natural starter is a common feature for this production (Gobbetti et al., 2002).

In the traditional cheese making procedures the NWCs are natural microbial cultures occurring in the whey drained after curd ripening. Part of this whey is stored and employed as starter in the manufacture of the following day. In previous studies these type of starters used for Mozzarella cheese manufacture have been characterized using both traditional and molecular procedures (Coppola et al., 1988, 1990; Ercolini et al., 2001, 2004, 2005) and defined as complex consortia of microorganisms of great importance for the quality of a traditional product. The attention of the scientific community towards traditional and labelled fermented foods has grown in the last decade and several works reported on the use of advanced molecular tools for the assessment of microbial diversity of traditional cheeses (Randazzo et al., 2002; Ercolini et al., 2003, 2004; Giraffa, 2004; Duthoit et al., 2005; Callon et al., 2006; Delbes et al., 2007; Aponte et al., 2008; Coppola et al., 2008).

The aim of this work was to investigate the microbial diversity of 63 NWCs used for the manufacture of Caciocavallo Silano cheese PDO and arising from dairies covering the whole territory of production according to the PDO regulation. This aim was pursued by using a culture-independent PCR based approach for the typing of NWCs both at species and beyond-species level. Moreover, a novel PCR–DHPLC approach coupled with direct sequencing is employed and compared to the more traditional PCR–DGGE technique to assess the microbiota at species level.

2. Materials and methods

2.1. NWC samples

The 63 NWCs used in this study were collected in 63 dairies located in 5 different regions of Southern Italy belonging to the area of production of Caciocavallo Silano PDO cheese (Regions: A, Campania; B, Basilicata; C, Calabria; D, Puglia; E, Molise). All the factories providing samples were part of an organized consortium for the preservation of the certified typical product "Caciocavallo Silano cheese PDO". The NWCs arose from the Caciocavallo cheese manufacture of the previous day and were ready to be used as starter for the current day processing. The NWCs had a load of lactic acid bacteria counted on MRS and M17 at 37 °C ranging between 5×10^6 and 5×10^8 CFU ml^{-1}. After the collection, the samples were cooled at 4 °C and, having been transferred to the laboratory, were frozen at −20 °C.

2.2. DNA extraction

Each sample of natural starter was also subjected to DNA extraction as previously described (Mauriello et al., 2003). The protocol described by the Wizard DNA purification kit (Promega, Madison, Wiscon) was applied as follows: 1 ml of NWC sample was centrifuged at 17,000 ×*g* for 5 min at 4 °C and the resulting pellet was resuspended in 100 μl of TE buffer (100 mM TRIS, 10 mM EDTA); then 160 μl of 0.5 M EDTA/Nuclei Lysis Solution in 1/4.16 ratio, 5 μl of RNAse (10 mg/ml^{-1}, Sigma) and 20 μl of pronase E (20 mg/ml^{-1}, Sigma) were added and the mixture was incubated for 60 min at 37 °C. After incubation, 1 vol. of ammonium acetate 5 M was added to the sample that was then centrifuged at 17,000 ×*g* for 5 min at 4 °C. The supernatant was precipitated with 0.7 vol. of isopropanol and centrifuged at 29,000 ×*g* for 5 min. Finally, the pellet was dried and resuspended in 50 μl of DNA Rehydration Solution by incubation at 55 °C for 45 min.

2.3. PCR amplification

Primers spanning the 200 bp V3 region of the 16S ribosomal DNA of *E. coli* were used in PCR amplification as previously described (Ercolini et al., 2003). Moreover, the V6–V8 region was also amplified by using primers U968 and L1401 (Zoetendal et al., 1998), giving PCR products of about 450 bp. A GC-clamp was added to the forward primers, according to Muyzer et al. (1993). Amplifications were performed in a programmable heating incubator (MJ Research Inc., Watertown, MA, USA). Each mixture (final volume, 50 μl) contained 20 ng of template DNA, each primer at a concentration of 0.2 μM, each deoxynucleoside triphosphate at a concentration of 0.25 mM, 2.5 mM $MgCl_2$, 2.5 μl of 10× PCR buffer and 2.5 U of *Taq* polymerase (Invitrogen, Milano, Italy). For both PCR amplifications, template DNA was denatured for 5 min at 94 °C and a touchdown PCR was performed according to Muyzer et al. (1993). The initial annealing temperature was 66 °C and this was decreased 1 °C every cycle for 10 cycles, finally 20 cycles were performed at 56 °C. The extension for each cycle was carried out at 72 °C for 3 min while the final extension was at 72 °C for 10 min. Aliquots (2 μl) of PCR products were routinely checked on 1.5% agarose gels.

2.4. DGGE analysis

PCR products were analyzed by DGGE by using a Bio-Rad Dcode apparatus. V6–V8 and V3 PCR amplicons were applied to 7% and 8% (wt vol^{-1}) polyacrylamide gels, respectively, in 1X TAE buffer. Parallel electrophoresis experiments were performed at 60 °C by using gels containing a 25 to 50% urea–formamide denaturing gradient (100% corresponded to 7 M urea and 40% (wt vol^{-1}) formamide). The gels were run for 10 min at 50 V, followed by 4 h at 200 V. They were then stained with ethidium bromide for 3 min, rinsed for 15 min in distilled water and observed using a Gel Doc apparatus (Bio-Rad). Bands were automatically detected by using Quantity one acquisition software (Bio-Rad).

2.5. Sequencing of DGGE fragments

DGGE bands to be sequenced were purified in water according to Ampe et al. (1999). One μl of the eluted DNA of each DGGE band was re-amplified by using the primers and the conditions described above. PCR products that gave a single band co-migrating with the original band were then purified by QIaex PCR purification kit (Qiagen, Milano, Italy) according to the manufacturer's instructions and sequenced. Sequencing was performed by Deoxy terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems) using the reverse primers used for the amplification of V3 (Muyzer et al., 1993) and V6–V8 (Zoetendal et al., 1998) regions of the 16S rRNA gene. To determine the closest known relatives of the partial 16S rRNA gene sequences obtained, searches were performed in public data libraries (GenBank) with the Blast search program.

2.6. DHPLC analysis

PCR fragments arising from the amplification of the variable V3 region of the 16S rRNA gene of NWCs were separated by DHPLC analysis (Wave system 3500, Transgenomic, Omaha, NE). The PCR product was injected on a preheated C18 reversed-phase column (DNASep, Transgenomics, Omaha, NE) and was eluted by a linear acetonitrile gradient formed by mixing buffer A (0.1 mmol l^{-1} triethylamine acetate TEAA, pH7.0) and buffer B (0.1 mmol l^{-1} TEAA, pH7.0 with 250 ml l^{-1}acetonitrile). The increase of buffer B was 0.4% per min at a flow rate of 0.9 ml min^{-1}. The initial concentration of buffer B was adjusted to 45%, to obtain retention times between 13 and 20 min, the ended concentration of buffer B was 58%, after 31 min of chromatographic run. The oven temperature for optimal separation was set to 65 °C. Minimum absorbance of 1 mV was considered as required for a peak to be considered as such.