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Short communication

Microbial evolution of traditional mountain cheese and characterization of early fermentation cocci for selection of autochtonous dairy starter strains

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ABSTRACT

The microbial population of Traditional Mountain (TM) cheese was investigated and characterized for the selection of cocci suitable for developing new starter cultures. Samples of milk, curd and cheese at different ripening times were enumerated in selective culture media and 640 colonies were isolated from curd and cheese after 24 h of ripening. The Lactic Acid Bacteria (LAB) isolated from M17 were clustered into 231 biotypes by RAPD-PCR analysis and identified as *Lactococcus lactis*, *Streptococcus thermophilus* and *Enterococcus faecalis*. Forty percent of enterococci showed the *in vitro* ability to inhibit raw milk resident coliforms, but they were excluded as possible starters due to the presence of associated risk factors. All lactococci and streptococci were tested for their technological properties; 4 *Lc. lactis subsp. lactis* and 2 *Sc. thermophilus* which were fast acidifiers and did not produce unpleasant flavours were subjected to the freeze-drying stability test. *Lc. lactis subsp. lactis* biotype 68 and *Sc. thermophilus* biotype 93 showed the best technological properties and may be appropriate for cheese production. This work gave evidence of the high biodiversity of TM-cheese autochthonous biotypes which could be used as starter cultures for the improvement of TM-cheese technology.

1. Introduction

Trentino alpine area (North Eastern Italy) has an ancient tradition of dairy products. TM-cheese is a raw cow's milk cheese produced in small farms, called *Malga*, which are located between 1400 and 2000 m above sea level. Malga farms are open only during the summer season and are set up to house cows for grazing and to manufacture cheese following a traditional procedure that is not standardized and differs depending on the area of production. TM-cheese-makers do not use any commercial starter culture, which is known to standardize the organoleptic properties of the final product (Yann and Pauline, 2014); therefore, the microbial fermentation is totally spontaneous and carried out by the indigenous microbiota, deriving from the raw milk and the Malga-farm environment. The traditional methods used for the production and ripening of raw milk cheeses are still followed to keep the richness and variability of the microbial population in cheese, generating different characteristics in ripened traditional cheeses (Montel et al., 2014). When no starter cultures are added, the autochtonous microbiota contributes to both the fermentation and ripening processes, either directly via its metabolic activity, or indirectly via the release of enzymes into the cheese matrix after autolysis (Pereira et al., 2010). For these reasons, traditional and spontaneously fermented cheeses, like TM-cheese, could be considered as possible sources of microbial diversity and wild LAB harbouring good properties for dairy production.

By contrast, starter and/or adjunct cultures as *Streptococcus thermophilus* and *Lactococcus lactis* (Axelsson, 2004; Iyer et al., 2010) are added to the milk in order to obtain cheeses with desired and predictable properties and the cheese-making process is carried out following standardized procedures (Beresford et al., 2001; Høier et al., 2010).

To our knowledge, few studies focused on the technology and microbial population of mountain cheeses (Bonizzi et al., 2009; Franciosi et al., 2008, 2009, 2015; Giannino et al., 2009; Poznanski et al., 2004), and only one on spontaneously fermented TM-cheese (Carafa et al., 2015).

The objectives of this study were i) to follow the microbial

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evolution, ii) to characterize the autochtonous cocci strains involved in the early fermentation process of spontaneous fermented TM-cheese, iii) to identify wild strains to be used in mixed starter cultures with non-starter lactobacilli previously isolated (Carafa et al., 2015) for TM-cheese and other dairy productions.

The isolated LAB were genetically characterized and tested for their ability to inhibit the growth of raw milk-resident coliforms. Lactococci and streptococci were screened for their ability to grow at non-optimal temperatures, proteolytic, acidifying and autolytic activities, and production of acetoin and desired flavours notes.

2. Materials and methods

2.1. Sampling and microbiological counts

The TM-cheese making process was followed in eight different Malga-farms located in the Alpine area of Trentino. For each farm, 3 cheese-making days were followed, and samples of raw milk, curd and cheese at different stages of ripening (24 h, 1 month and 7 months), were collected for each day of cheese-production, for a total of 120 samples. The milk samples were collected from the vat and the curd samples were collected after the extraction. The pH of cheese 24 h, 1 month and 7 months following production was measured using the pH meter PT1000 (Knick, Berlin, Germany) equipped with a Hamilton electrode (Hamilton Bonaduz, Bonaduz, Switzerland).

All milk and cheese samples have been analysed within 24 h for microbiological counts. Cheese samples were homogenized as reported previously by Carafa et al. (2015). Cell suspensions and samples of milk were serially diluted, plated in triplicate and incubated as follows: the total bacterial (TB) were counted onto Plate Count Agar supplemented with 10 g/L of skimmed milk (PCA-SM) and incubated aerobically at 30 °C for 24 h; enterococci onto Kanamycin Aesculin Azide (KAA) agar and incubated aerobically for 24 h at 37 °C, mesophilic cocci-shaped lactic acid bacteria (LAB) onto M17 agar, incubated at 30 °C aerobically for 48 h; thermophilic cocci-shaped LAB onto M17 agar, incubated at 45 °C anaerobically for 72 h and mesophilic rod-shaped LAB onto MRS agar acidified to pH 5.5 with 5 mol/L lactic acid, incubated at 30 °C anaerobically for 48 h. All culture media were purchased from Oxoid (Milan, Italy).

2.2. Pathogen and spoilage bacteria detection

The presence of *Listeria* was detected inoculating the sample in Listeria Enrichment Broth (LEB, Merck, Darmstadt, Germany). After 48 h of incubation at 30 °C, the inoculated broths were streaked on PALCAM Listeria selective agar (Merck) and incubated at 35 °C in anaerobic conditions for up to 48 h. Presumptive *Listeria monocytogenes* strains grow as grey–green coloured colonies with a black area.

Coliforms were counted using violet red bile agar (VRBA, Oxoid). Purple–pink colonies were detected after 24 h of incubation at 37 °C, anaerobically. The detection of *Staphylococcus aureus* was carried out by plating on *Staphylococcus* agar No. 110 (Laboratorios Conda S.A., Madrid, Spain). The presence of *Clostridium* spores in milk and 24h-cheese samples was estimated by using the most probable number (MPN) technique with a 3×3 scheme, as follows: after pasteurization (85 °C for 15 min), the first three decimal dilutions were inoculated in Reinforced Clostridial Medium (RCM) supplemented with 1.4% (v/v) Na-lactate (Merck); the tubes were sealed with sterile melted paraffin:vaseline (1:6) and observed for gas production after incubation at 37 °C for 7 days.

2.3. Bacteria isolation and genotypic characterization

Ten or more colonies were randomly picked up from countable M17 agar plates of curd and cheese after 24 h of ripening for the subsequent starter selection. The selection of putative LAB and their purification and store was done as previously described by Carafa et al. (2015).

The biotype clustering and species identification of the putative LAB isolates was carried out by using Randomly Amplified Polymorphic DNA-PCR (RAPD-PCR) and partial 16S rRNA gene sequencing following the approach described by Carafa et al. (2015). The similarity value for biotypes clustering was set at 85% after testing the RAPD-PCR repeatability as determined by Foschino et al. in a previous work (2008).

Lc. lactis subspp. lactis and *cremoris, Sc. thermophilus* and *Enterococcus faecium* species were also confirmed by means of species-specific PCR according with Corroler et al. (1998), Lick et al. (1996) and Cheng et al. (1997). All amplifications were performed with a T100™ ThermalCycler (Bio-Rad Laboratories).

2.4. Screening for selection of dairy starter strains

One isolate representative of each biotype was analysed for the detection of prophage, adapting the method of Cochran and Paul (1998) as reported by Carafa et al. (2015).

All biotypes were screened for their ability to inhibit the growth of milk resident coliforms using the Well Diffusion Assay described by Corsetti et al. (2004), with some modifications. One mL of raw cow's milk containing 5.7 Log CFU/mL of coliforms was plated under a bilayer of VRBA with 4-methylumbelliferyl-β-D-glucuronide (MUG) by Oxoid. Thereafter, wells of 10 mm in diameter were generated into the bilayer and 200 μL of cell-free supernatants were inoculated into each well. In order to eliminate the inhibitory effect of lactic acid, supernatants were previously adjusted to pH 7 ± 0.2 with KOH and filtered through a 0.22 μm pore size filter (Minisart®, Sartorius Lab Holding GmbH, Goettingen, Germany). Plates were refrigerated at 4 °C for 4 h to allow the radial diffusion of the compounds contained in the supernatant and finally incubated for 24 h at 37 °C. The inhibitory effect of strains on the growth of coliforms was shown by the presence of a clear halo around each well. A solution of chloramphenicol (1 mg/mL) was used as positive control.

More technological analyses were performed in triplicate on phage-free lactococci and streptococci. The growth ability at different temperatures (30 and 45 °C) was detected as reported previously by Carafa et al. (2015).

The exocellular proteolytic activity was evaluated following the method reported by Franciosi et al. (2009).

The acidifying ability of cell suspensions (1%, v/v) was evaluated in 10 mL of sterile UHT milk (Latte Trento, Trento, Italy) following the method reported by Morandi and Brasca (2012); pH measurements were carried out using a pH meter PT1000 (Knick, Berlin, Germany) equipped with a Hamilton electrode (Hamilton Bonaduz, Bonaduz, Switzerland), every 15 min for 24 h.

The olfactory flavour notes developed by lactococci and streptococci were analysed following a sniffing test as described by Sánchez et al. (2000), with some modifications. Revitalized cultures were inoculated (1%, v/v) in 15 mL of microfiltered whole milk (Latte Trento, Trento, Italy) for 48 h at optimal conditions. Each sample was evaluated by a panel of eight untrained assessors. Odour attributes (lactic-acid, yoghurt, butter, sulphur, spicy, fruity and fermented herbs) were evaluated and rated in null (0), light (1), medium (2) and strong (3). A statistical interpretation of the results was done by using the principal component analysis (PCA) on the correlation matrix of STATISTICA (data analysis software system),