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Changes in psychrotrophic microbial populations during milk creaming to produce Grana Trentino cheese

Elena Franciosi [a,*], Giorgia De Sabbata [a], Fausto Gardini [b], Agostino Cavazza [a], Elisa Poznanski [a,1]

[a] IASMA Research and Innovation Centre, Fondazione Edmund Mach, Food Quality and Nutrition Area, Innovative Food Technologies, Via E. Mach 1, 38010 San Michele all'Adige (TN), Italy
[b] Department of Food Science, University of Bologna, Piazza Goidanich 60, 47023 Cesena (FC), Italy

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ABSTRACT

The aim of this study was to study the psychrotrophic microbiota developing during milk creaming of Grana Trentino cheese-making. 138 isolates from raw whole milk, cream and skim milk samples were screened by Randomly amplified polymorphic DNA PCR biotyping and representative strains of each biotype were characterised by partial 16S rRNA gene sequencing and enzymatic activity. *Pseudomonadaceae* were commonly isolated in cream samples while *Streptococcaceae* and *Enterobacteriaceae* in milk samples. *Moraxellaceae* and *Flavobacteriaceae* were found in both cream and milk samples.

More than 80% of psychrotrophic isolates could grow at 37 °C. All *Flavobacteriaceae* and half of *Pseudomonadaceae* biotypes displayed proteolytic activity on milk agar even at low temperatures such as 10 °C. All *Streptococcaceae* and some of *Enterobacteriaceae* displayed acidifying activity and almost all *Acinetobacter* spp. (*Moraxellaceae*) displayed lipolytic activity towards tributyrin.

Even if psychrotrophic bacteria is not the dominant microbial group in raw milk, their total number increases during creaming and becomes one of the most present group together with Lactic Acid Bacteria. Their enzymatic activities may be key players in determining milk quality for cheese making.

1. Introduction

Grana Trentino is a hard-cooked cheese belonging to Grana Padano consortium, that undergoes a ripening period of almost 2 years. It is produced in Trentino region (Alpine area located in the North of Italy) from raw cow's milk (DPR n. 1269, 30 October 1955). The bulk milk derives from two different milkings mixed approximately in 1:1 ratio. The first is carried to dairy factory the evening before cheese-making and is put in large shallow tanks for 9–11 h during which the creaming takes place. After this overnight rest, the partially skim milk lays under the cream in the tank and is manually drained from the cream fat and put in a vat. An about equal amount of whole milk coming from the morning milking is added to the skim milk before cheese-making. Cream is used for butter production. During overnight rest, the milk spontaneous creaming occurs at a temperature kept between 15 and 17 °C to avoid lactic acid bacteria overgrowth. The creaming process is crucial to optimize the fat to casein ratio of milk (Mucchetti and Neviani, 2006), but also has important microbiological consequences because some microorganisms present in raw milk are removed with the fat globules. The recent development of molecular community fingerprinting methods provided a view of the complex microbial ecosystems such as raw milk (Lafarge et al., 2004; Ogier et al., 2004). Raw milk contains bacteria such as lactic acid strains, that can have technological relevance, but also psychrotrophic ones like *Pseudomonas*, *Acinetobacter*, *Enterobacter*, *Flavobacterium*, *Klebsiella*, *Bacillus*, *Clostridium* and *Corynebacterium* (Dogan and Boor, 2003; Munsch-Alatossava and Alatossava, 2006) which produce heat resistant exo-enzymes of considerable spoilage potential (Desmasures et al., 1997; Cousin, 1982). Psychrotrops can grow and spoil the milk or the cream during overnight rest. Nielsen (2002) suggests that proteases and lipases produced by psychrotrophs can be cause of defects in dairy products. As proteases can break casein micelles, the final coagulum can be less compact and more fragile (Manfredini and Massari, 1989). The bacterial psychrotrophic lipases may cause the hydrolysis of triglycerides in the cream fat and off-flavours development decreasing the sensory properties of stored dairy products like butter and cheeses (Craven and Macauley, 1992; Hilton, 2006).

The dynamics of microbial population during hard cheeses maturation and ripening are well known (Gatti et al., 2008; Dolci

* Corresponding author. Tel.: +39 0461 615117.
E-mail address: elena.franciosi@iasma.it (E. Franciosi).
[1] Current address: Free University of Bolzano-Bozen Faculty of Science and Technology, Piazza Università 1, 39100 Bolzano, Italy.

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et al., 2008; Randazzo et al., 2006) but the knowledge of the evolution and characterization of psychrotrophic microbiota is limited to comparison between refrigerated and not refrigerated milk (Lafarge et al., 2004) and works about psychrotrophic populations in cream is still lacking. Studies dealing with the creaming process are mostly focused on the physico-chemical and rheological properties of milk (Ma and Barbano, 2000) or on different creaming conditions (Malacarne et al., 2008). The development of microbial population during spontaneous creaming was studied only *in vitro* (Dellaglio et al., 1969), while the behaviour of inoculated pathogens strains was studied in different creaming conditions (Carminati et al., 2008). The microbial dynamics during creaming in real system was studied by Panari et al. (2007) which considered only the plate counts.

In this study, we monitored the microbiota before and after the spontaneous creaming of milk from raw whole milk collected in the evening to cream and skim milk used to produce Grana Trentino cheese in a dairy located in Non Valley (Trento, Italy). Particular attention was posed on the psychrotrophic microorganisms present in raw milk before and after the rest. In order to understand the composition of psychrotrophic population in milk before and after creaming, a total of 138 colonies were isolated from psychrotrophic microbiota during four different cheese-making processes. The isolates were characterized by genotypic techniques (RAPD-PCR and sequencing); their lipolytic and proteolytic traits and their lecithinase activity was evaluated by means of screening methods on solid media following the screening approach by Hantsis-Zacharov and Halpern (2007). Since growth media and the incubation conditions only select for a fraction of the bacterial populations present, it still remains to be established whether the strains isolated from milk and cream samples are representative of the microbial populations present in them. Despite this bias the use of culture dependent methods (plating and isolates) is a prerequisite to study the single strains composing the community and their phenotypical traits.

2. Materials and methods

2.1. Milk supply, creaming apparatus and sample collection

All the whole milk (WM), skim milk (SM) and cream (C) samples were collected in a cheese factory producing Grana Trentino located in Non valley (Trento, Italy).

The raw cow's whole milk was collected at three farms, equipped with automated milking facilities, and transported to the cheese factory by a single truck. The total raw whole milk was carried to the dairy by a truck supplied with a vacuum pump (AVM12, Acram S.r.l., S. Ambrogio di Valpolicella, Verona, Italy) and milk volumes were recorded by means of magneto inductive measure system. The milk was gravity separated at about 15–17 °C in a flat steel not covered vat (about 2 m × 1.5 m with a depth of 25 cm). After the overnight rest for about 10 h, the skim milk fraction was drained from the bottom of the flat vat and collected in a copper vat that was kept mild stirred till the cheese-making started after the addition of the morning whole milk. The top fraction was the cream and was drained after the skim milk but in a plastic graduated bow to record the volume. All experiments were replicated with milk collected in three consecutive days for four consecutive weeks (12 cheese-making processes). The bulk-tank raw milk and cream were stirred before sampling. In particular the cream was stirred by a mild up-and-down moving to avoid microbial sedimentation in cream layers. After stopping agitation, a litre of milk or cream, measured by a calibrated bottle, were sampled and divided into subsamples by dispensing 2 ml quantities to polypropylene cryovials (Sarstedt, Numbrecht, Germany). Samples for microbiological analysis were stored in liquid nitrogen immediately after collection and kept in a Freezer Fryo 410lt at −80 °C (New Brunswick Scientific Co.; Edison, NJ; USA) until analyzed. Immediately before plating, (usually a week after sampling), the samples were thawed in an agitated water bath at 40 °C. The thawing was never more than 5 min.

Samples for chemical analysis were stored at 4 °C, transferred to the laboratory within an hour and analyzed in 3 h.

2.2. Milk and cream composition analysis

The following analyses were carried out on all milk and cream samples: pH values were measured at the cheese factory using a Portamess® 911 (X) pHmeter (Knick GmbH & Co., Berlin, Germany) connected to a Cheesetrode (Hamilton Co., Reno, NV, USA) electrode after bulk mixing; fat content by infrared analysis (Biggs, 1978) of a bulk sample with a Milko-Scan 134 A/B (Foss Electric, DK-3400 Hillerod, Denmark).

2.3. Enumeration of microorganisms and statistical analysis

The first decimal dilution of cream samples was shacked for 2 min at 260 rpm in a Stomacher Lab Blender 400 BA 7021 (Seward Medical, Worthing, UK) to allow the recovery of bacteria entrapped during the skimming in the fat globule clusters. Samples were diluted in peptone water (0.1 % mycological peptone, Oxoid, Basingstoke, UK) when necessary and plated in duplicate on the following media, all purchased from Oxoid: Plate Count Agar (PCA) incubated at 30 °C for 48 h for aerobic mesophilic bacteria counts, and at 7 °C for 7 days for aerobic psychrotrophic bacteria counts, De Man Rogosa and Sharp (MRS) Agar incubated at 30 °C for 48 h anaerobically for enumeration of lactic acid bacteria (LAB), Pseudomonas Agar Base (PAB) with CFC supplement incubated at 30 °C for 48 h for *Pseudomonadaceae* counts and Violet Red Bile Glucose Agar (VRBGA) incubated at 37 °C for 24 h for *Enterobacteriaceae* counts.

Colonies were randomly picked from psychrotrophic countable PCA plates: one day out of three cheese-making days every week (a total of four out of 12 cheese-making days in four weeks). For each selected day, at least 10 colonies per plate and per sample (Whole Milk, Cream and Skim Milk) were collected for a total of about 35 colonies isolated in each one of the four days. The colonies were streaked onto PCA (incubated at 30 °C for 48 h) and the procedure was repeated four times in order to obtain pure cultures. After purification, the isolates were subcultured in liquid Luria-Bertani (LB) medium (Oxoid). Gram reaction was performed using Gregersen's KOH method (Gregersen, 1978), catalase reactions by transferring fresh colonies from an agar medium to a glass slide and adding 3% H_2O_2, and cytochrome oxidase with Oxidase test (Oxoid). Gram negative, catalase positive and oxidase negative isolates were plated on VRBGA and incubated at 37 °C for 24–48 h to check their belonging to *Enterobacteriaceae* Family; Gram negative, catalase positive and oxidase positive isolates were plated onto Pseudomonas Agar Base with CFC supplement to check their belonging to the *Pseudomonas* spp.

Bacterial isolates were stored at −80 °C in LB medium containing 20% glycerol.

2.4. DNA Extraction and RAPD PCR amplification

Bacterial DNA was extracted from all the isolates after overnight growth in LB Broth at 30 °C. DNA from Gram positive bacteria was extracted using the InstaGene Matrix (Bio-Rad, Milan, Italy) according to the manufacturer's instructions, while from Gram