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Can the development and autolysis of lactic acid bacteria influence the cheese volatile fraction? The case of Grana Padano

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ABSTRACT

In this study, the relationship between the dynamics of the growth and lysis of lactic acid bacteria in Grana Padano cheese and the formation of the volatile flavor compounds during cheese ripening was investigated. The microbial dynamics of Grana Padano cheeses that were produced in two different dairies were followed during ripening. The total and cultivable lactic microflora, community composition as determined by length heterogeneity-PCR (LH-PCR), and extent of bacterial lysis using an intracellular enzymatic activity assay were compared among cheeses after 2, 6 and 13 months of ripening in two dairies.

The evolution of whole and lysed microbiota was different between the two dairies. In dairy 2, the number of total cells was higher than that in dairy 1 in all samples, and the number of cells that lysed during ripening was lower. In addition, at the beginning of ripening (2 months), the community structure of the cheese from dairy 2 was more complex and was composed of starter lactic acid bacteria (*Lactobacillus helveticus* and *Lactobacillus delbrueckii*) and NSLAB, possibly arising from raw milk, including *Lactobacillus rhamnosus*/*Lactobacillus casei* and *Pediococcus acidilactici*. On the other hand, the cheese from dairy 1 that ripened for 2 months was mainly composed of the SLAB *L. helveticus* and *L. delbrueckii*. An evaluation of the free-DNA fraction through LH-PCR identified those species that had a high degree of lysis. Data on the dynamics of bacterial growth and lysis were evaluated with respect to the volatile profile and the organic acid content of the two cheeses after 13 months of ripening, producing very different results. Cheese from dairy 1 showed a higher content of free fatty acids, particularly those deriving from milk fat lipolysis, benzaldehyde and organic acids, such as pGlu and citric. In contrast, cheese from dairy 2 had a greater amount of ketones, alcohols, hydrocarbons, acetic acid and propionic acid. Based on these results, we can conclude that in the first cheese, the intracellular enzymes that were released from lysis were mainly involved in aroma formation, whereas in the second cheese, the greater complexity of volatile compounds may be associated with its more complex microbial composition caused from SLAB lysis and NSLAB (mainly *L. rhamnosus*/*L. casei*) growth during ripening.

1. Introduction

The microbiota of cheeses that are produced with raw milk and natural starter is very complex, and its composition is crucial for the development of the unique sensory characteristic of each traditional cheese variety. Moreover, if the cheese is long ripened, the microbial population balance changes under the influence of a continuous shift in the environmental conditions and microorganisms interactions; therefore, the characteristics of a cheese also depend on the microflora dynamics (Neviani and Gatti, 2013; Neviani et al., 2013).

The microbiota of ripened cheese are mainly composed of lactic acid bacteria (LAB) and include LAB starter strains (SLAB) and adventitious LAB species, namely non-starter LAB (NSLAB). Both types of bacteria play different roles in cheese-making. SLAB participate in the fermentation process, fermenting lactose to produce high concentrations of lactic acid, while NSLAB do not contribute to acid formation during manufacture but have been implicated in cheese maturation (Beresford et al., 2001).

The microbial communities of Grana Padano (GP), a protected designation of origin (PDO) of Italian extra-hard cheese manufactured with raw milk and natural whey culture, were recently reviewed by Gatti and colleagues (Gatti et al., 2014), highlighting the dynamics of LAB during both cheese making and ripening considering an increase in viable cells and their lysis.

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Indeed, SLAB grow at the beginning of cheese manufacturing, developing mainly during curd acidification. After brining and during ripening, a hostile environment (no residual lactose, pH 5.0–5.3 and 4–6% salt in moisture, moisture decreasing to values of 28 to 30%) is generated, leading to a gradual decline in starter viability. Some of the dying SLAB undergo autolysis, releasing intracellular enzymes mostly in the early steps of ripening (Gatti et al., 2014).

On the other hand, NSLAB are able to grow after cheese brining, surviving the heat and acid stress of the first step of cheese making and developing during maturation. Later, these bacteria begin to autolyse very slowly during the final months of the long maturation, releasing enzymes throughout the entire ripening process (Gatti et al., 2014).

Therefore, biochemical reactions of microbial origin that occur during ripening are the result of the metabolism of viable LAB cells and the activity of enzymes released by the other lysed LAB. In particular, the formation of flavors involves 3 major LAB metabolic pathways: i) metabolism of lactate and citrate, ii) release of free fatty acids and their subsequent metabolism, and iii) proteolysis and the subsequent amino acid catabolism (McSweeney and Sousa, 2000; Yvon and Rejien, 2001). Through bacterial metabolism, sapid volatile and non-volatile compounds are generated, and these molecules in the correct ratios and concentrations identify the cheese flavor (McSweeney and Sousa, 2000; Smit et al., 2005). The volatile fraction of Grana Padano cheese is characterized by the presence of esters, ketones, aldehydes, alcohols, lactones, pyrazines and free fatty acids. The most important compounds in the definition of Grana Padano cheese flavor are ethyl esters, particularly ethyl butanoate and ethyl hexanoate, butanoic and hexanoic acids, and other molecules imparting fruity, green, nutty and coconut notes (Boscaini et al., 2003; Frank et al., 2004; Langford et al., 2012; Moio and Addeo, 1998). The balance between these molecules changes during ripening: Moio and Addeo (1998) observed that during Grana Padano cheese maturation, the number of compounds that were responsible for fruity and green notes decreases, whereas that of volatiles having spicy, nutty and earthy notes increases.

A positive effect in aroma production has been observed in laboratory-scale cheese making when the lysis of the selected strain used as starter was induced by the action of bacteriocin produced by adjunctive *Lactococcus lactis* (Martínez-Cuesta et al., 2001; de Palencia et al., 2004). Amino acid conversion to desirable aroma compounds, such as benzaldehyde and volatile sulphur compounds derived from metionine, has been also observed when autolytic *Lc. lactis* subsp. cremoris was used to produce experimental cheese model Ch-easy (Bourdat-Deschamps et al., 2004). However, to the authors' knowledge, the effect of the lysis of natural starter on aroma cheese compounds has never been investigated. The aim of this study was to investigate the relationship between the dynamics of the growth and lysis of LAB in Grana Padano during cheese ripening and the formation of volatile flavor compounds. To reach this goal, Grana Padano cheeses that were produced in two different dairies were compared after 2, 6 and 13 months of ripening. For all samples, the total and cultivable lactic microflora were counted, and length heterogeneity-PCR (LH-PCR) was carried out to determine the community structure and species diversity. Moreover, the extent of bacterial lysis in cheese was measured using an intracellular enzymatic activity assay and, to better discriminate which LAB species underwent lysis, LH-PCR was carried out on DNA from lysed cells. These data have been discussed considering the volatile profile and the organic acid content of the two cheeses after 13 months of ripening.

2. Material and methods

2.1. Cheese manufacture and sampling

Two dairies (dairy 1 and dairy 2) of the GP production area were considered for this study (GP1 and GP2). Raw milk was treated according to the GP PDO production protocol (Dossier number IT/PDO/0017/0011). As required by GP technical guidelines, milk from a single milking was skimmed by creaming for approximately 8 h at 8–20 °C. Partially skimmed milk was transferred to two twin vats (copper tanks) with a capacity of 1200 l. Skimmed raw milk was supplemented with lysozyme (20 mg/l) as an anticlostridial agent. Natural whey culture (NWC) was used as a starter (2.5–3.2% *v*/v) and was obtained by incubating the whey of the previous day's cheese making at a gradient of temperature from approximately 50 °C to 35–20 °C for 18–24 h. Calf rennet powder was added, and coagulation was performed at 31–33 °C. After coagulation, the curd was cut and then stirred and cooked for 5–15 min at 53–54 °C. After waiting for 40–80 min for curd precipitation, it was extracted from the vat and cut into two twin cheeses that were molded for 48 h. Four twin cheeses were obtained, were salted in saturated brine for 23 days and ripened for 13 months at 18–22 °C and 80–85% relative humidity.

The cheeses were sampled after 2, 6 and 13 months of ripening for the first dairy (GP1) and the second dairy (GP2). Acidified curds (48 h after vat extraction) have also been examined. Cheeses were sampled from each twin wheel and cut into slices. For each of the samples, one of the four cheeses was analyzed as suggested by Gatti et al. (2008a, 2008b). All of the samples were kept at 4 °C after collection and analyzed in a laboratory immediately upon arrival.

2.2. Total and cultivable bacterial counts

The total bacterial count in cheese samples was obtained using the LIVE/DEAD®*Bac*light™ Bacterial Viability kit (Molecular Probes, Oregon, USA) and fluorescence microscopy (Gatti et al., 2006). The grated cheese homogenates in trisodium citrate (15 ml) were centrifuged (10,000 × *g*, 10 min, 4 °C). The obtained pellets were washed twice in 15 ml of 20 g/l trisodium citrate (pH 7.5) (Sigma-Aldrich, St. Louis, USA), resuspended in 15 ml of sterile water and 10-fold diluted. Subsequently, 1 ml of each sample was used for microbial counts according to the manufacturer's instructions. Samples that were stained with LIVE/DEAD® were then filtered onto black polycarbonate filters (0.2-μm pore size) (Millipore Corp., Billerica, MA, USA) and counted as described by Bottari et al. (2010) using an epifluorescence microscope (Nikon 80i, Tokyo, Japan). Three separate counts were performed for each sample. The results were expressed as total cells per gram, obtained by the sum of the viable cells (green) and non-viable (red) cells.

Cultivable LAB counts were determined on de Man, Rogosa and Sharpe (MRS) agar (Oxoid, Basingstoke, United Kingdom). Ten grams of the grated cheese samples were suspended in 90 ml of 20 g/l trisodium citrate (pH 7.5) (Sigma–Aldrich, St. Louis, USA) and homogenized for 2 min in a blender (Seward, London, United Kingdom). Decimal dilutions were made in quarter-strength Ringer solution (Oxoid, Basingstoke, United Kingdom) and plated in triplicate on MRS. The plates were incubated at 30 °C for 2 days under anaerobic conditions.

2.3. Length Heterogeneity PCR (LH-PCR) analysis

To analyze DNA arising from the whole-cell fraction, samples were prepared as reported by Gatti et al. (2008a, 2008b). Ten grams of grated cheese samples were diluted 1:10 in 20 g/l trisodium citrate (pH 7.5) (Sigma-Aldrich, St. Louis, USA) and homogenized in a blender for 3 min; 1 ml of homogenate was centrifuged and washed using 20 g/l trisodium citrate (pH 7.5). Pellets were suspended in 100 μl of pure water and treated with 0.14 U/μl amplification-grade DNase I (Sigma-Aldrich Co., St. Louis, MO) under conditions given by the supplier to digest free DNA arising from lysed cells. DNA extraction was carried out using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Microbial DNA was analyzed by LH-PCR (Applied Biosystems, Foster City, USA).

V1 and V2 16S rRNA gene regions were amplified using primers 63F and 355R as previously described by Lazzi et al. (2004); the 63F primer was 5′ end-labeled with 6-carboxyfluorescein (FAM). The length heterogeneity of the PCR amplicons was detected by capillary