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

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 (*L. helveticus* and *L. delbrueckii*) and NSLAB, possibly arising from raw milk, including *L. rhamnosus/L. 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.

Keywords

lactic acid bacteria; autolysis; Grana Padano; cheese ripening; aroma;

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 et al., 2013a, 2013b).

The microbiota of ripened cheese are mainly composed of lactic acid bacteria (LAB) and include LAB starters 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.

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).

79 Therefore, biochemical reactions of microbial origin that occur during ripening are the result of the
80 metabolism of viable LAB cells and the activity of enzymes released by the other lysed LAB. In
81 particular, the formation of flavors involves 3 major LAB metabolic pathways: i) metabolism of
82 lactate and citrate, ii) release of free fatty acids and their subsequent metabolism, and iii) proteolysis
83 and the subsequent amino acid catabolism (McSweeney and Sousa, 2000; Yvon and Rijnen, 2001).
84 Through bacterial metabolism, sapid volatile and non-volatile compounds are generated, and these
85 molecules in the correct ratios and concentrations identify the cheese flavor (McSweeney and Sousa,
86 2000; Smit 2005). The volatile fraction of Grana Padano cheese is characterized by the presence of
87 esters, ketones, aldehydes, alcohols, lactones, pyrazines and free fatty acids. The most important
88 compounds in the definition of Grana Padano cheese flavor are ethyl esters, particularly ethyl
89 butanoate and ethyl hexanoate, butanoic and hexanoic acids, and other molecules imparting fruity,
90 green, nutty and coconut notes (Boscaini et al., 2003; Frank et al., 2004; Langford et al., 2012; Moio
91 and Addeo, 1998). The balance between these molecules changes during ripening: Moio and Addeo
92 (1998) observed that during Grana Padano cheese maturation, the number of compounds that were
93 responsible for fruity and green notes decreases, whereas that of volatiles having spicy, nutty and
94 earthy notes increases.
95 A positive effect in aroma production has been observed in laboratory-scale cheese making when the
96 lysis of the selected strain used as starter was induced by the action of bacteriocin produced by
97 adjunctive *Lactococcus lactis* (Martínez-Cuesta et al 2001, de Palencia et al. 2004). Amino acid
98 conversion to desirable aroma compounds, such as benzaldehyde and volatile sulphur compounds
99 derived from metionine, has been also observed when autolytic Lactococcus lactis subsp. cremoris
100 was used to produce experimental cheese model Ch-easy (Bourdat-Deschamps et al. 2004). However,
101 to the authors' knowledge, the effect of the lysis of natural starter on aroma cheese compounds has
102 never been investigated. The aim of this study was to investigate the relationship between the
103 dynamics of the growth and lysis of LAB in Grana Padano during cheese ripening and the formation
104 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 liters. 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. (2008). 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 (10000 × *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.

2.4 Length Heterogeneity PCR (LH-PCR) analysis

To analyze DNA arising from the whole-cell fraction, samples were prepared as reported by Gatti et al. (2008). 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 electrophoresis (ABI Prism 310, Applied Biosystems, Foster City, USA). The PCR and capillary electrophoresis conditions were as described by Bottari et al. (2010). The fragment sizes (base pairs) were determined using GeneMapper software version 4.0 (Applied Biosystems, Foster City, USA) and the local Southern method to generate a sizing curve from the fragment migration of the internal size standard (GS500 LIZ®; Applied Biosystems Foster City, USA), and the minimum noise threshold was set at 150 fluorescence units. The peaks, corresponding to amplicons of specific length on the electropherogram profiles, represent fragments of different sizes, and the areas under the peaks depend on the number of fragments (Lazzi et al. 2004). Each peak, corresponding to amplicons of specific length on the electropherogram profiles, was attributed to bacterial species according to published databases (Lazzi et al., 2004; Gatti et al., 2008), and the areas under the recognized peaks were used in this study to measure the number of the recognized species in the samples. The total area under all of the peaks (sum of attributed and unattributed peaks) of the LH-PCR electropherograms was used in this work to measure the total amount of DNA arising from intact cells.

180 **2.5 Cell lysis evaluation in cheese**

181 The extent of bacterial lysis occurring in all of the cheese samples was determined by i) the activity
182 of aminopeptidase located intracellularly and ii) LH-PCR analysis of the lysed cell fraction.
183 For the first analysis, sample extracts were prepared according to De Dea Lindner et al. (2008). A
184 dialysis step was included to eliminate any low-molecular-weight substances, such as salts, which
185 could interfere with the successive reactions. Then, 50 µL of the sample suspension obtained after 24
186 h of dialysis in cellulose tubing (Spectra/por, Spectrum Laboratories Inc., USA) with a cutoff of 3000
187 g/mol was centrifuged (10000 × *g*, 10 min, 4 °C) and filtered through a cellulose acetate membrane
188 with 0.22-µm pore size (Sartorius, Italy). The filtrate (50 µl) was added to 125 µl of 1.312 mmol/L
189 Leucine β-naphthylamide (βNA) derivate solution (Bachem Feinchemikalien AG, Switzerland), 125
190 µl of phosphate buffer 0.05 mol/l pH 7.0, and 200 µl of bidistilled water and then incubated at 40 °C
191 for 3 h. The reaction was stopped by the addition of 250 µl of 2.0 mol/L HCl. The degree of hydrolysis
192 was determined by measuring the colored product of an azocopulation reaction by reading
193 spectrophotometrically at 580 nm (A580 nm) according to Boquien et al. (1989). Each assay was
194 carried out in triplicate, and the average values were calculated.
195 Sample preparation for the LH-PCR analysis of the lysed cell fraction was carried out as described
196 by Gatti et al. (2008). Briefly, grated cheese samples were diluted 1:10 in 20 g/L trisodium citrate
197 (pH 7.5) (Sigma-Aldrich, St. Louis, USA) and homogenized in a blender for 3 min; 1 ml of sample
198 was filtered through a 0.22-µm filter (Whatman GmbH, Dassel, Germany) to obtain a cell-free
199 fraction. DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany)
200 according to the manufacturer's instructions. The attribution and quantification of DNA arising from
201 lysed cells were performed as previously described for DNA that was extracted from intact cells.
202

203 **2.6 Volatile fraction composition**

204 The determination was carried out on 13-month-old Grana Padano cheese samples. A
205 divinylbenzene/carboxen/polydimethylsiloxane, 50/30 µm, 2-cm-long fiber (Supelco, Bellefonte,

206 PA) was used to collect volatile fractions by SPME. Five grams of grated cheese was weighed in a
207 20-ml crimp-top vial and sealed with an aluminum cap provided with a pierceable septum (23 × 75
208 mm, Varian, Palo Alto, CA). The sample was allowed to equilibrate to 45 °C in a thermostatic bath
209 for 5 min without agitation, and the fiber was exposed to the headspace for 30 min. The gas
210 chromatographic analysis of the volatile compounds that adsorbed onto the SPME fiber was carried
211 out with a CP-WAX 52CB capillary column (Varian; 60 m long, 0.32 mm i.d., 0.5 μm film thickness).
212 An Agilent (Palo Alto, CA) 7890A gas chromatograph that was coupled with an Agilent 5975C mass
213 spectrometer was used. During the injection phase, a 3-min splitless mode was applied, and the
214 injector temperature was held at 250 °C. The oven temperature was held at 40 °C for 8 min,
215 programmed to 220 °C at a rate of 4°C/min, and held at 220 °C for 20 min. Helium was used as carrier
216 gas at a flow rate of 1.5 ml/min. The MS temperatures that were used were as follows: interface 220
217 °C, source 200 °C, and quadrupole 150 °C; acquisition was performed in electron impact (EI) mode
218 (70 eV) by 1.6 scans per second, and the mass range was *m/z* 35 to 270. The identification of volatile
219 compounds was performed with the following criteria: comparison with the mass spectra of the
220 W8N08 library (John Wiley and Sons, Inc., New York, NY), injection of authentic standards analyzed
221 under the same GC-MS conditions, and calculation of retention indices (RI) followed by comparison
222 with those obtained from both authentic standards and literature. Analyses were repeated three times.
223 Values were expressed as area units/1,000,000. Statistical analysis was performed using the XLSTAT
224 7.5 package (Addinsoft, France).
225
226 **2.7 Organic acid determination**
227 Pyroglutamic (pGlu) and citric acids were determined on 13-month-old Grana Padano cheese by
228 HPLC as described by Bouzas et al. (1991). Twenty-five milliliters of 0.009 N H_2SO_4 were added to
229 5 g of grated cheese and mixed with a magnetic stirrer for 30 min. The mixture was centrifuged at
230 5000 × *g* for 10 min. The supernatant was filtered through a 0.45-μm cellulose acetate membrane
231 (Bio-Rad Laboratories, Richmond, CA). The HPLC analysis was carried out isocratically at 0.6

232 ml/min and 65 °C using a 300 × 7.8 mm i.d. cation exchange column (Aminex HPX-87H) with a
233 Cation H^+ Microguard cartridge (Bio-Rad Laboratories, Richmond, CA). Analyses were repeated
234 three times. Statistical analysis was performed using the XLSTAT 7.5 package (Addinsoft, France).

235 **3. Results and discussion**

236 **3.1 Evolution of microbial growth and lysis**

237 Microbial dynamics, in terms of total counts, and community composition were followed during the
238 ripening of the GP cheeses produced in two different dairies. The quantitative and qualitative
239 microbial composition of the 2-month-aged sample and its evolution until 13 months of ripening in
240 GP1 and GP2 were different. At the beginning of ripening (2-month cheeses), the number of total
241 cells, as evaluated directly by fluorescence microscopy, in GP2 was comparable with that counted in
242 GP1 and it was higher during ripening (dark bars in Fig. 1). In particular, the decrease in total cells
243 during ripening was significantly greater in GP1 than in GP2 at 13 months, as the GP2 total cell
244 number was 7.27 log cell/g compared to 6.78 log cell/g of GP1 ($p<0.05$). This result means that the
245 number of cells that were lysed during ripening was greater in GP1 than in GP2 and that the
246 cytoplasmic enzymes in cheese GP1 were released earlier than in cheese GP2. Consequently, the
247 more enzymes in GP1 had more time to act in the cheese.

248 Santarelli et al. (2013) recently highlighted that in GP cheese, the 2-month time point seemed to be a
249 crucial moment for GP microbial evolution. With a complex and complete sampling, Santarelli and
250 colleagues demonstrated that during this early period of ripening, evenness and richness were
251 different, with the highest bacterial growth and diversity mainly regarding lysed microbial cells
252 (Santarelli et al., 2013). This microbial aspect is very relevant because it can affect the aromatic
253 features of the cheese. The formation of aroma compounds relies on the concerted action of the
254 enzyme activities that are secreted in food matrix after cell lysis and the metabolic pathway that is
255 present in intact and metabolically active microbial cells (Smid and Kleerebezem, 2014). Indeed, the

conversion of precursor molecules in aroma compounds can occur inside or outside the cell. The latter is the case of activity of cytoplasmatic enzymes that are released by cell lysis. This phenomenon allows an easier contact of the enzyme with the precursor that is accessible in the cheese, advancing cheese ripening. On the other hand, cell lysis leads to the release of substrates that are available for the subsequent catabolism by the remaining intact cells in the cheese (Smid and Kleerebezem, 2014). To characterize the whole microflora that evolved during GP ripening, LH-PCR on DNA were extracted from intact cells. In the whole-cell fraction that was recovered from 2-month GP, the number of cells and the species composition were different between the two cheese-making processes (Fig. 2). The LH-PCR patterns of 2-month GP1 cheese were composed mainly of peaks that were putatively assigned to SLAB, *L. helveticus* and *L. delbrueckii*. On the other hand, the LH-PCR patterns of 2-month GP2 were more complex and putatively attributed to SLAB (*L. helveticus* and *L. delbrueckii*) and NSLAB (*L. rhamnosus/L. casei* and *Pediococcus acidilactici*). In both samples, a low number of peaks unattributed to the database set were detected (Gatti et al., 2008; Lazzi et al., 2004). Cell lysis has been estimated in three different ways: i) decrease of total cells as directly counted by fluorescence microscopy, ii) intracellular aminopeptidase activity measuring optical density as modified by the hydrolysis of H-Leu-BNA derivate, and iii) evaluation of the LH-PCR profiles of the free-DNA fraction. This last method, described by Gatti et al. (2008) and previously adopted by Santarelli et al. (2013), allowed us to determine whether SLAB and NSLAB underwent lysis during the two cheese-making processes.

During GP1 cheese making, a great cell lysis occurred in the cheese already after two months. Respect to 48 hours of acidified curds (similar for the two GPs), a larger decrease in the cell number was observed in 2-month GP1, associated with a more than two-fold-higher H-Leu BNA activity compared to GP2 (Fig. 1). The LH-PCR profile of lysed cells for the two 2-month GPs demonstrated that the greater lysis of GP1 was mainly attributable to the SLAB species *L. helveticus* and *L. delbrueckii* (Fig. 2). The microbial composition of the 6-month GP was different. The total cell number in GP2 remained higher than that in GP1. In GP1, SLAB continued to be the dominant

microbiota, and new NSLAB species, such as *Pediococcus acidilactici,* increased in proportion. The microbiota of GP2 mainly constituted NSLAB species, such as *L. rhamnosus/L. casei*, while peaks of SLAB species, *L. helveticus* and *L. delbrueckii,* were smaller. Microbial lysis as estimated in this time of ripening (6-month) can be considered comparable in both cheese-making processes, mainly regarding H-Leu βNA activity, which peaked in this phase. The LH-PCR profiles of whole cells in the GPs after 13 months of ripening showed a decrease in the peaks area in both samples, highlighting the decreased amount of DNA that was extracted from whole cells due to the decrease in the number of intact cells (Fig. 2). This decrease was already revealed by direct microscopic counts. The most abundant species in GP1 were *P. acidilactici* and *L. delbrueckii* and in GP2 were *L. rhamnosus/L. casei* and *L. helveticus*. The microbial lysis of the two 13-months cheeses was very different. Despite the similarity between the H-Leu BNA activity values, the number of total cells in GP1 was lower than that in GP2, and the species of lysed cells were different. Comparing the total of number cells (log 6.78 cells per g GP1 and log 7.27 cells in GP2) compared to the number of cultivable in MRS (log 5.10 cfu per g in GP1 and log 5.02 cfu per g in GP2), it is possible that viable but not cultivable (VNC) cells were present in both samples (Fig. 1). However, GP2 showed a greater number of VNC microorganisms than did GP1. Based on these results, we can conclude that the evolution of whole and lysed microbiota in the two cheeses during ripening was very different. GP1 was characterized mainly by SLAB, which underwent anticipated lysis after brining, while GP2 was characterized by a more complex microbial composition, not always cultivable, where *L. rhamnosus*/*L. casei*, possibly arising from raw milk, was always dominant. This NSLAB microbiota, by adapting more easily to environmental stress, persisted, thus maintaining cellular integrity for up to 13 months. We can also underlying how only using LH-PCR profiles of the free-DNA fraction, it was possible to determine whether SLAB and NSLAB underwent lysis during the two cheese-making processes. The other two methods that were used to evaluate cell lyses were not able to evidence this difference. The limitations of the culture-dependent method, such as LAB count in MRS, are well known, mainly considering the inability to estimate viable but not cultivable (VBNC), stressed and/or injured cells (Cocolin et

al. 2013). The limit of intracellular aminopeptidase activity determination, as assessed using Leucine β-naphthylamide to reproduce the specificity of aminopeptidase N (Gatti et al. 2008), was due to the strain specificity of the LAB proteolytic system. Two long-ripened cheese samples, with different lysis, can have the same value of aminopeptidase activity due to the presence of species or strains that are characterized by different peptidase systems (Christensen et al. 1999).

3.2 Aromatic profile and organic acids content in cheeses

The composition of the volatile fraction of 13-month cheeses from GP1 and GP2 is reported in table 1. Most of the molecules detected have already been found in the volatile fraction of Grana Padano and Parmigiano Reggiano (Barbieri et al, 1994; Frank et al., 2004; Langford et al., 2012; Moio et al., 1998; Qian and Reineccius, 2002). The aromatic profile was mainly composed of low-molecular-weight fatty acids (FA) (48-56%); ketones (12-18%); ethyl esters of short- and medium-chain fatty acids (approximately 10%); primary, secondary and branched alcohols (7-8%); hydrocarbons (11-15%); aldehydes (0.6-1.7%); and pyrazines, lactones and other compounds in smaller amounts (Table 1). GP1 cheese showed a higher content, compared to GP2 cheese, of free fatty acids, particularly those deriving from milk fat lipolysis (C4-C10), benzaldehyde and organic acids, such as pGlu (GP1=465.6 ± 32.7 mg/100 g per sample; GP2=399.0 ± 36.0 mg/100 g per sample) and citric (GP1= 97.0 ± 5.0 mg/100 g per sample; GP2= 66.3 ± 5.2 mg/100 g per sample). In contrast, GP2 had a higher amount of ketones, alcohols, hydrocarbons, acetic and propionic acid.

The higher and earlier LAB lysis observed GP1 could have determined the release and action of intracellular enzymes in the cheese starting from brining. It is worth noting that most esterases of LAB, which are responsible for the hydrolysis of fatty acids up to 10 carbon atoms from triglycerides, seem to be located intracellularly (Chich et al., 1997; El-Soda et al., 1986). Similarly, the production of benzaldehyde arises from the conversion of phenylalanine, depending on the activity of

cytoplasmic enzymes (Smid and Kleerebezem, 2014). In addition, the higher amount of pGlu in 13-month GP1 can arise from intracellular enzymes released after lysis. Indeed, pGlu derives from glutamine by the cyclase activity of lactic acid bacteria, mainly *L. helveticus*, and accumulates in greater amounts during the long ripening of Grana Padano, when *L. helveticus* lyses gradually occur (Gatti et al. 2014; Mucchetti et al., 2000). The greater complexity of volatile compounds in GP2 cheese may be associated with its more complex microbial composition caused not only by SLAB lysis but also by NSLAB (mainly *L. rhamnosus*/*L. casei*) growth during ripening. Acetoin and ketones, common constituents of cheese aroma (Curioni and Bosset, 2002), are produced by the metabolic pathways of citrate and by the β-oxidation and decarboxylation of free fatty acids, respectively (Marilley and Casey, 2004; McSweeney and Sousa, 2000). Accordingly, a lower content of citrate was found in 13-month GP2. Furthermore, Sgarbi and colleagues (2013) observed that the number of ketones that were produced in medium-mimic cheese ripening generally increased when *L. casei* and *L. rhamnosus* grew. In this study, *L. rhamnosus*/*L. casei* was always dominant during the ripening of GP2. For the same reason, we found higher amounts of acetone. Indeed, this volatile compound is produced by acetyl-CoA metabolic pathways in *L. casei* (Budinich et al., 2011) and *L. rhamnosus* (Ramzan et al., 2010). Secondary alcohols are formed by the reduction of the corresponding aldehydes and methyl ketones, and their production has been associated with *L. casei* and *L. rhamnosus* growth in medium-mimic cheese ripening (Sgarbi et al., 2013). However, based on their weak aroma intensities, these compounds are considered unimportant to Parmesan aroma (Qian et al., 2002). The greater amount of acetic acid in the cheese in which *L. rhamnosus*/*L. casei* was always dominant during ripening confirmed their mechanism of adaptation to the cheese environment, leading to the production of this organic acid coupled with ATP generation (Bove et al., 2012; Lazzi et al., 2014). Propionic acid production can be associated with the development of propionic bacteria, which were not revealed in this study but are commonly found in long-ripened cheeses (Alyson et al. 2014). Several hydrocarbons, having both straight and branched chains, were

found, can derive from the autoxidation of unsaturated fatty acids (Grosch, 1982) and cannot be correlated with microbiological results.

4. Conclusion

To conclude, we will quote a sentence of an elegant review of Smid and Kleerebezem (2014): "The formation of aroma compounds in food fermentation processes [long ripened cheeses in the present study] relies on the concerted action of two different microbial processes: the activities of (a) various enzymes that are mostly secreted in the food matrix by the fermenting microbes [by SLAB lysis in the present study] and (b) complete metabolic pathways present in intact and metabolically active microbial cells [NSLAB in the present study]" (adapted from Smid et al., 2014). When one of the two processes becomes more important, we are able to notice its effect. The dynamics of the growth and lysis of LAB during cheese ripening and volatile flavor compounds at the end of ripening of two GP cheeses aged 13 months were different. One cheese was mainly characterized by an anticipated SLAB cells lysis after brining, while the other was characterized by a more complex microbial composition, not always cultivable, where NSLAB was always dominant. Intracellular enzymes that were released from lysis in the first cheese may be one of the main microbiological agents responsible for aroma formation, while the metabolic pathway of NSLAB growing under hostile conditions leads to metabolic products of microbial origin involved in volatile flavor characterization of the second GP.

The anticipated SLAB cells lysis observed for one GP and the greater development of NSLAB for the other could be due to the different physiological characteristics of different biotypes of the same species. More autolytic *L. helveticus* strains could have been present in the natural whey starter that was used for GP1, and less nutritionally demanding *L. rhamnosus*/*L. casei* strains could have been present in the milk of GP2.

380 The results that were obtained in this work not only have confirmed what Santarelli et al. (2013) state,
381 which is that 2 months of ripening is crucial for GP microbial characterization, but also suggest how
382 the development of NSLAB and the lysis of SLAB, which take place between the acidification of the
383 curd and the cheese after brining, can differently influence the aromatic definition of ripened cheese.

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521 Table 1. Volatile compounds of 13-month-old Grana Padano cheeses (data are expressed as area
522 values/1.000.000)

Compound[1]	RI[2]	identification method[3]		GP1		GP2	
				mean	SD	mean	SD
Ketones							
acetone	817	MS/PI	*[4]	17.0	3.38	32.5	3,80
2-butanone	905	MS/PI		6.0	0.59	5.8	0.42
2-pentanone	983	MS/PI	*	75.7	6.25	155.2	14.41
2-hexanone	1084	PI		1.3	0.26	2.2	0.56
2-heptanone	1190	MS/PI		66.2	4.38	76.0	9.07
2-nonanone	1397	MS/PI	*	13.6	0.62	20.6	2.21
2-undecanone	1609	MS/PI		2.7	0.45	2.5	0.60
Σ of ketones				182.4		294.8	
Esters							
ethyl acetate	891	MS/PI		11.6	1.74	15.7	2.25
ethyl butanoate	1043	MS/PI		61.0	6.72	56.5	5.63
ethyl hexanoate	1242	MS/PI		67.6	9.95	67.7	9,26
ethyl octanoate	1442	MS/PI		9.5	1.16	9.0	1.05
ethyl decanoate	1646	MS/PI		2.3	0.34	1.8	0.51
Σ of esters				152.0		150.8	
Alcohols							
ethanol	937	MS/PI	*	45.6	4,89	68.8	5.75
2-pentanol	1121	PI	*	25.9	2,41	15.7	2.22
1-butanol	1147	PI	*	7.4	1,03	10.9	1.15
3-methyl-1-butanol	1211	MS/PI	*	5.1	0,60	7.4	0.47
3-methyl-3-buten-1-ol	1255	PI	*	4.2	0,33	9.2	2.27
2-heptanol	1322	PI	*	7.3	0,99	4.8	0.62
3-methyl-2-buten-1-ol	1326		*	1.1	0,11	3.2	0.39
1-hexanol	1356	PI	*	2.5	0,16	5.5	0.83
Σ of alcohols				99.2		125.4	
Aldehydes							
3-methylbutanal	921	MS/PI	*	3.7	0,23	6.2	0.45
2-butenal	1046	PI	*	16.2	1,70	1.8	0.20
hexanal	1086	MS/PI		1.8	0,08	1.4	0.28
benzaldehyde	1544	PI		2.9	0,20	-	0.00
Σ of aldehydes				24.6		9.3	
Acids							
acetic	1460	MS/PI	*	218.6	16,54	340.8	25.98
propaonoic	1552	MS/PI	*	2.5	0,55	6.0	1.13
butanoic	1639	MS/PI	*	241.0	11,60	192.7	18.24
isopentanoic	1681	MS/PI	*	48.9	3,59	39.6	2.85
pentanoic	1748	MS/PI		3.1	0,42	2.6	0.45
hexanoic	1855	MS/PI	*	227.2	9,90	150.6	13.22
heptanoic	1964	MS/PI		2.7	0,67	1.7	0.24
octanoic	2071	MS/PI	*	51.9	3,88	33.3	3.93
nonanoic	2177	MS/PI	*	4.3	0,84	1.3	0.33
decanoic	2283	MS/PI	*	20.3	2,34	12.9	2.43
Σ of acids				820.6		781.4	
Pyrazines							
2,6-dimethylpyrazine	1336	PI		7.1	0,87	5.3	1.36
3-ethyl- 2,5-dimethylpyrazine	1454	PI		2.9	0,70	2.6	0.26

Σ of pyrazines				10.0		7.9	
Lactones							
γ-hexalactone	1727	PI	*	1.8	0,40	0.6	0.06
δ-decalactone	1997	PI		0.3	0,11	0.4	0.08
lactone n.i.	2226			1.7	0,24	1.4	0.33
Σ of lactones				3.8		2.3	
Hydrocarbons							
pentane	500		*	1.7	0,17	2.7	0.37
2,2-dimethylbutane	517		*	1.1	0,18	2.5	0.73
2-methylpentane	554		*	0.7	0,24	1.3	0.20
hexane	600		*	7.0	0,85	1.0	0.09
1-pentene	628		*	8.0	1,10	20.2	3.35
2-methyl-1-pentene	640			4.5	1,02	4.5	1.24
heptane	700		*	-	0,00	1.9	0.45
4-methylheptane	754			19.9	1,87	16.6	1.21
octane	800			5.0	1,30	4.6	0.45
2,4-dimethyl-1-heptene	876			26.0	5,09	22.4	4.17
2,2,5,5-tetramethylhexane	926		*	17.9	2,72	32.5	5.73
2,2,4,6,6-pentamethylheptane	944		*	25.9	1,38	44.3	11.22
butyl cyclohexane	1078		*	7.6	0,52	14.8	3.56
hexyl cyclohexane	1293			2.0	0,49	3.3	1.28
Σ hydrocarbons n.i.				41.9		65.0	5.39
Σ of all hydrocarbons				169.1		237.7	
Other compounds							
acetoin	1295	PI	*	1.2	0.11	2.3	0,46
limonene	1206	MS/PI		-	0,00	0.9	0.08
dimethylsulphone	1926		*	0.8	0,09	1.4	0.23

[1] Identification by comparison with mass spectra of Wiley Library

[2] Retention index measured in cheese samples

[3] PI = Retention indexes published (Moio and Addeo, 1998; Qian and Reineccius, 2002; Frank et al., 2004; Acree and Arn, 2004; Bianchi et al., 2007); MS = mass spectra of authentic compounds

[4] Significant difference: *, $p < 0.05$

Figures legends

Figure 1. Total (dark) and cultivable (grey) bacterial counts of cheese samples 48 h after vat extraction and at 2, 6, and 13 months of ripening from GP1 and GP2. Total counts, as determined by fluorescence microscopy, are expressed as log cell/g. Cultivable counts in MRS are expressed as log cfu/g. In the box is reported the aminopeptidase activity expressed as $A_{580\ nm,}$ as indices for cell lysis. The reported data are the mean of triplicate experiments ± standard deviation.

Figure 2. The chart shows the evolution of whole and lysed species in cheese samples at 2, 6, and 13 months of ripening from GP1 and GP2. The data were calculated for electropherograms of each sample as obtained from LH-PCR. Each peak was putatively attributed to bacterial species according to published databases (Lazzi et al., 2004; Gatti et al., 2008), and the areas under the recognized peaks were used to measure the amount of the recognized species in the samples prepared differently as described in the material and methods. The letters identify the species presented as peaks in each electropherogram: a) *L. helveticus*; b) *L. delbrueckii* subsp. *lactis*/*bulgaricus*; c) *L. rhamnosus* or *L. casei/paracasei*; d) *S. thermophilus* or *L. lactis* subsp. *lactis* or *S. uberis*; e) *P. acidilactici*; f) *L. fermentum*; and g) other species.