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Metabolic gene-targeted monitoring of non-starter lactic acid bacteria during cheese ripening

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ABSTRACT

Long ripened cheeses, such as Grana Padano (GP), a Protected Designation of Origin (PDO) Italian cheese, harbor a viable microbiota mainly composed of non-starter lactic acid bacteria (NSLAB), which contribute to the final characteristics of cheese. The NSLAB species *Lactobacillus rhamnosus, Lb. casei* and *Lb. paracasei* are frequently found in GP, and form a closely related taxonomic group (*Lb. casei* group), making it difficult to distinguish the three species through 16S rRNA sequencing. *SpxB*, a metabolic gene coding for pyruvate oxidase in *Lb. casei* group, was recently used to distinguish the species within this bacterial group, both in pure cultures and in cheese, where it could provide an alternative energy source through the conversion of pyruvate to acetate. The aim of this work was to study the evolution of the metabolically active microbiota during different stages of GP ripening, targeting 16S rRNA to describe the whole microbiota composition, and *spxB* gene to monitor the biodiversity within the *Lb. casei* group. Furthermore, activation of pyruvate oxidase pathway was measured directly in cheese by reverse transcription real time PCR (RT–qPCR). The results showed that *Lb. casei* group dominates throughout the ripening and high-throughput sequencing of *spxB* allowed to identify four clusters inside the *Lb. casei* group. The dynamics of the sequence types forming the clusters were followed during ripening. Pyruvate oxidase pathway was expressed in cheese, showing a decreasing trend over ripening time. This work highlights how the composition of the microbiota in the early manufacturing stages influences the microbial dynamics throughout ripening, and how targeting of a metabolic gene can provide an insight into the activity of strains relevant for dairy products.

1. Introduction

Cheese is a biochemically dynamic product and significant microbial and biochemical changes occur during its ripening. A ripening period of 24 months or longer is common for some Italian and European traditional cheeses. Despite the long ripening time, these cheeses harbor a viable microbiota at the end of ripening, mainly consisting of non-starter lactic acid bacteria (NSLAB). NSLAB can arise from raw milk and dairy environment (Montel et al., 2014), they do not contribute to acid production during cheese-making, but they can strongly influence the biochemistry of cheese maturation, contributing to the final sensorial properties (Beresford et al., 2001; De Filippis et al., 2016; Settanni and Moschetti, 2010).

The evolution of the microbiota during Grana Padano (GP) manufacturing has been extensively studied (Alessandria et al., 2016; De Filippis et al., 2014; Neviani et al., 2013; Santarelli et al., 2013). Among NSLAB, *Lactobacillus rhamnosus*, *Lb. casei* and *Lb. paracasei*, commonly referred to as *Lb. casei* group, are frequently found in ripened cheeses and they usually become the dominant bacterial population during ripening of extra-hard, cooked Italian Protected Designation of Origin (PDO) cheese, such as Grana Padano (GP) (Gatti et al., 2014). The development of NSLAB during ripening has been attributed to their ability to use nutrient sources available in lactose-free ripened cheese, like milk components modified by technological treatments (rennet addition and curd cooking), starter-LAB metabolites and cell lysis products. Recently, the knowledge about *Lb. rhamnosus* growth in cheese has been deepened through proteomics (Bove et al., 2012) and transcriptomic (Lazzi et al., 2014). The growth of this microorganism in a cheese-like medium (Neviani et al., 2009) causes a metabolic shift towards acetate production. In particular, an upregulation of the *spxB* gene, coding for pyruvate oxidase (POX) was observed (Lazzi et al., 2014). POX catalyses the oxidation of piruvate to acetyl-phosphate,

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which is then converted to acetate with the production of ATP from an acetate kinase (ACK). The increased expression of these enzymes suggests a potential role of this pathway in NSLAB growth during cheese ripening.

Furthermore, a recent study has proposed the use of *spxB* coding sequence as a target to distinguish the species belonging to *Lb. casei* group (Savo Sardaro et al., 2016); their identification is often controversial due to their close phylogenetic relationships (Felis and Dellaglio, 2007). Culture-independent approaches based on high-throughput sequencing (HTS) allows an in-depth study of the microbial diversity, potentially revealing also subdominant microbial species (De Filippis et al., 2017; Ercolini, 2013). Nevertheless, 16S rRNA sequencing does not allow the discrimination within *Lb. casei* group, due to the high similarity in 16S sequence. The sequencing-based monitoring of different strains in the microbiota was recently proposed, selecting key genes with significant intra-species heterogeneity such as the *lacZ* and *serB* genes of *Streptococcus thermophilus* (De Filippis et al., 2014; Ricciardi et al., 2016).

In the current study, HTS was used to study the evolution of the metabolically active microbiota during different stages of GP ripening, targeting 16S rRNA to describe the whole microbiota composition, and *spxB* gene to monitor the biodiversity within *Lb. casei* group. Furthermore, activation of POX-ACK pathway was measured directly in cheese by reverse transcription real time PCR (RT–qPCR).

2. Materials and methods

2.1. Sampling

PDO Grana Padano cheese was manufactured with raw cows' milk, using natural whey starter, according to the usual manufacturing technique (EU regulation OJ L 160, 18.6.2011, p. 65–70), at Caseificio San Vitale (Brescia, Italy). Cheese manufacturing was performed in the same day with two different batches of milk, using the same natural whey starter, leading to the production of two cheese-making, hereafter referred to as CM1 (cheese-making 1) and CM2 (cheese-making 2). Cheese was manufactured as reported in the official production guidelines of Grana Padano consortium (http://www.granapadano.it/en/luoghi-produzione-fasi-stagionature-marchi-e-taglio). Briefly, after the coagulation and cooking steps and after “resting time” in the vat, the curd is lifted using wood stoles and linen cloths, then the curd mass is raised from the bottom of the vat and cut into equal parts in order to create two twin wheels. Therefore, for each cheese-making two twin wheels were produced, and ripened on the same shelf of the warehouse. Cheese samples were collected from the twin wheels after 2, 6 and 12 months of ripening, immediately grated, and stored at − 80 °C.

2.2. RNA extraction and cDNA synthesis

Following a strategy employed also in analysis of other cheese varieties, grated cheese samples coming from twin wheels were mixed prior to RNA extraction (Cardinali et al., 2017; Guidone et al., 2016). RNA was extracted in triplicate by modifying a protocol described in a previous work (Monnet et al., 2008). In brief, 150 mg of grated cheese were placed in a 2 ml screw cap tube containing 500 µl of zirconia/glass beads (diameter, 0.1 mm; BioSpec Products, Bartlesville, USA) and immediately added with 1.5 ml of TRIzol (Invitrogen, Milano, Italy). The tubes were processed with a Mini-BeadBeater 8 (BioSpec Products, Bartlesville, USA) by using three 60-s mixing sequences at maximum speed, spaced out with 60-s pauses at room temperature. The homogenized sample was then processed following the TRIzol manufacturer's instruction.

RNA was quantified spectrophotometrically, and the integrity was evaluated by denaturing agarose gel electrophoresis. Triplicate RNA samples were pooled prior to reverse transcription, that was performed with QuantiTect Reverse Transcription Kit (Qiagen, Milan, Italy), including a gDNA contamination removal step, using random hexamer primers and according to the manufacturer instructions.

2.3. 16S rRNA gene amplicon library preparation and sequencing

The microbial diversity was studied by pyrosequencing of the amplified V1–V3 region of the 16S rRNA gene using primers and PCR conditions previously described (Ercolini et al., 2012). 454-adaptors were included in the forward primer followed by a 10 bp sample-specific Multiplex Identifier (MID). PCR products were purified twice by Agencourt AMPure kit (Beckman Coulter, Milano, Italy), quantified using the QuantiFluorTM (Promega, Milano, Italy) and an equimolar pool was obtained prior to further processing. The amplicon pool was used for pyrosequencing on a GS Junior platform (454 Life Sciences, Roche, Italy) by using the GS Junior + emPCR and Sequencing kits (454 Life Sciences, Roche, Italy) according to the manufacturer's instructions.

2.4. SpxB gene amplicon library preparation and sequencing

Recently, Savo Sardaro et al. (2016) have proposed the use of a 151 bp fragment *spxB* gene to identify *Lb. casei* group in cheese matrix, but for pyrosequencing a 684 bp fragment of *spxB* coding region was selected as a target, and primer specificity was assessed as described in the original paper (data not shown). In order to prepare amplicon libraries for *Lb. casei* group *spxB* gene sequencing, the 454 Universal Tailed Amplicon protocol was used with a double PCR step (454 Sequencing System – Guidelines for Amplicon Experimental Design). Only 5 libraries were successfully prepared from the 6 samples. A sequence of 684 bp of the coding region of *spxB* was amplified using the primers Pox_FW 5′-AGACGCAATGATCAAGGTG**Y**T and Pox_RV 5′-GTGATGATCGG**R**ATATGCGTT.The universal primers M13f 5′-TGTAAAACGACGGCCAGT and M13r 5′-CAGGAAACAGCTATGAC were included at 5′ and 3′ ends of the POX primers (Daigle et al., 2011). Each PCR mixture (final volume, 50 µl) contained 100 ng of template cDNA, 0.1 µmol/l of each primer, 0.50 mmol of each deoxynucleoside triphosphate, 2.5 mmol/l $MgCl_2$, 5 µl of 10 × PCR buffer and 2.5 U of native Taq polymerase (Invitrogen, Milano, Italy). The following PCR conditions were used: 95 °C for 3 min, followed by 40 cycles at 94 °C for 45 s, 57 °C for 45 s, 72 °C for 1 min. A final extension was carried out at 72 °C for 5 min. Twenty nanograms of the amplicon were used as template in a second PCR step, where primers M13f and M13r were used, with the addition of 454-adaptors and a 10 bp sample-specific Multiplex Identifier (MID). The PCR mixture was prepared as above described and the PCR conditions were modified as follows: 94 °C for 5 min, 30 cycles at 94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min, and a final extension of 72 °C for 7 min, as previously described (De Filippis et al., 2014). PCR products were purified twice by Agencourt AMPure kit (Beckman Coulter, Milano, Italy) and then quantified using the QuantiFluorTM (Promega, Milano, Italy). An equimolar pool of amplicons was prepared and it was used for pyrosequencing as described above.

2.5. Detection of spxB expression from cheeses

cDNA was also used for qPCR in a Mastercycler ® ep realplex S instrument (Eppendorf). In brief, 1 µl of cDNA template was used in each reaction, using Power SYBR® Green PCR Master Mix (Applied Biosystems, Milano, Italy) and 5 µM of each primer, according to the target to detect, in 15 µl final volume. The primers used were different from the ones used in HTS library preparation: a universal primer pair was used to detect a 130 bp fragment of 16S rRNA gene (Denman and McSweeney, 2006), and *Lb. casei* group specific primers were used to detect *spxB* gene (Savo Sardaro et al., 2016). A third primer pair was designed, targeting a 227 bp fragment of *ackA* gene, and tested for specificity against members of the *Lb. casei* group (data not shown). Details about the primer pairs used for RT qPCR can be found in