Food Microbiology 52 (2015) 106–118

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Understanding the bacterial communities of hard cheese with blowing defect

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ARTICLE INFO

Article history:
Received 17 March 2015
Received in revised form
19 May 2015
Accepted 3 July 2015
Available online 8 July 2015

Keywords:
Grana Padano cheese
Blowing defect
Clostridium
Lactobacillus
Illumina-based sequencing
lysozyme

ABSTRACT

The environment of hard cheese encourages bacterial synergies and competitions along the ripening process, which might lead in defects such as clostridial blowing. In this study, Denaturing Gradient Gel Electrophoresis (DGGE), a quantitative *Clostridium tyrobutyricum* PCR and next-generation Illumina-based sequencing of 16S rRNA gene were applied to study 83 Grana Padano spoiled samples. The aim was to investigate the community of clostridia involved in spoilage, the ecological relationships with the other members of the cheese microbiota, and the effect of lysozyme. Three main genera were dominant in the analysed cheeses, *Lactobacillus, Streptococcus* and *Clostridium*, and the assignment at the species level was of 94.3% of 4,477,326 high quality sequences. *C. tyrobutyricum* and *C. butyricum* were the most prevalent clostridia. Hierarchical clustering based on the abundance of bacterial genera, revealed three main clusters: one characterized by the highest proportion of *Clostridium*, a second where *Lactobacillus* was predominant and the last, dominated by *Streptococcus thermophilus.* Ecological relationships among species were found: cheeses characterized by an high abundance of *S. thermophilus* and *L. rhamnosus* were spoiled by *C. tyrobutyricum* while, when *L. delbrueckii* was the most abundant *Lactobacillus, C. butyricum* was the dominant spoiling species. Lysozyme also shaped the bacterial community, reducing *C. tyrobutyricum* in favour of *C. butyricum*. Moreover, this preservative increased the proportion of *L. delbrueckii* and obligate heterofermentative lactobacilli and lowered *L. helveticus* and non-starter species, such as *L. rhamnosus* and *L. casei.*

1. Introduction

Long ripening times characteristic of hard cheeses production create the favourable conditions for microbial communities able to survive along the dairy process. This could result in beneficial effects or, alternatively, in spoilage defects that damage the final product. Late blowing is one of the most frequent problems still affecting hard cheese production in dairy factories. Spore-forming clostridia are considered the main agents of such damages (Coulon et al., 1991; Guericke, 1993; Ingham et al., 1998; Klijn et al., 1995; Vissers, 2007) thanks to their spores surviving attitude to heat treatment and their added capacity to germinate during cheese ripening. Different clostridial species have been associated to spoiling of hard cheeses, firstly *Clostridium tyrobutyricum* as the main agent (Bergère and Sivelä, 1990; Klijn et al., 1995; Le Bourhis et al., 2007b; Nishihara et al., 2014) followed by *Clostridium sporogenes, Clostridium butyricum, Clostridium beijerinckii,* and less frequently *Clostridium cochlearium, Clostridium perfringens, Clostridium septicum* (Le Bourhis et al., 2007b; Lycken and Borch, 2006; Reindl et al., 2014). All these microorganisms, alone or in association, have been related during time to the blowing problem, but few data are available about their dynamic changes in the cheese shape and their relationships all along the ripening period.

To reduce losses connected to clostridia spoilage, preservatives such as nitrate and lysozyme, which consistently modify the cheese environment in which microorganisms survive and multiply, are added to milk during hard cheese manufacture (Lodi and Stadhouders, 1990; Stadhouders, 1990). The use of nitrate in milk to prevent late blowing defect was also common in hard cheese-making, particularly in Emmental production (Devoyod, 1975; Korenekova et al., 2000), but its employ was banned after the European Food Safety Authority (EFSA) proposed to reduce levels of nitrosamines in food products (EFSA, 2010). In Italy, Grana Padano (GP) hard cheese is produced from raw cow's milk added with natural whey starter cultures and protected from clostridia spoilage

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http://dx.doi.org/10.1016/j.fm.2015.07.004
0740-0020/

by lysozyme addition (www.granapadano.com). Therefore, the use of this preservative may be responsible of different bacterial dynamics in cheese, depending also on dairy conditions and on microbial milk contamination.

The main bacterial populations associated to the cheese alteration process have been previously studied with the use of cultivation-dependent techniques and PCR-based approaches, but on a small number of samples (Cocolin et al., 2004; Garde et al., 2011; Le Bourhis et al., 2007b). The new methodologies based on 16S rDNA amplification together with Next-Generation Sequencing technology (NGS) have the advantage to determine, with a high resolution power, the bacterial communities present in food environments: published examples include cheese (De Filippis et al., 2014; Ercolini et al., 2012; Masoud et al., 2011, 2012), seafood (Kiyohara et al., 2012; Koyanagi et al., 2011; Roh et al., 2010) and dry fermented sausages (Połka et al., 2015). The recent development in Illumina technology, coupled with multiplexing approaches, allows analysing up two 16S rRNA hypervariable regions in thousands sequences per sample, an approach that was shown to gain an almost complete coverage of the bacterial communities of fermented foods, with correct taxonomical assignment at the species level for more than 95% of the analysed reads (Połka et al., 2015). However, these approaches have not yet been applied for the study of microbial communities involved in late blowing spoilage of hard cheese.

The aim of the present work was to assess the microbial communities of spoiled hard cheese using new NGS technologies associated to quantitative and qualitative cultivation-independent techniques. Respectively, a PCR-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) approach to obtain a qualitative characterization of clostridia heterogeneity, a TaqMan qPCR on single *C. tyrobutyricum* species and an NGS approach based on Illumina MiSeq sequencing of the V3–V4 16S rRNA of total bacteria were applied to a total of 83 Grana Padano cheese samples from nine production facilities, with or without the addition of lysozyme and with blowing defects appearance at different ripening times. Information gathered from this study could be useful to assess the effect of lysozyme as a preservative and to measure the effects of ripening time on clostridial population and their relationships with other bacterial species present in the cheese paste.

2. Materials and methods

2.1. Cheese sampling

Eighty-three samples of hard cheese, with anomalous pastry defects and cavities were collected from the Grana Padano cheese production area in Northern Italy. The samples had different ripening times corresponding to the appearance of defect and ranging from 1 to 23 months. In addition, eight hard cheeses without defect were added as negative controls. A total of 56 cheese samples were produced with the addition of lysozyme, while 35 samples were negative to the additive. Collected cheese portions were 50 cm large and 20–30 cm high and were cut directly from the entire cheese shapes; for each cheese, a representative sample was grounded and suddenly frozen at −40 °C in our laboratory until processing. For all analyses reported in the paper, samples were labelled according to this legend: the first two letters indicate the geographical origin (Province), followed by a three numbers code that points the cheese factory, the following number indicates the ripening time in months and "+" or "−" refer to the use or not of lysozyme; an additional letter (a or b) indicates that two samples were collected from the same cheese factory and ripening time.

2.2. Bacterial strains and genomic DNA isolation

Reference type and isolated clostridial and non-clostridial strains used in this study are listed in Table 1. For DNA extraction, 1 ml of culture was collected and centrifuged at 8,000 g for 5 min. Genomic DNA was isolated from the pelleted cells using the Nucleospin Tissue DNA Isolation Kit (Macherey Nagel, Germany) and DNA was verified on a 1% agarose gel containing SYBR®-Safe (Invitrogen Corporation Life technologies).

2.3. DNA extraction from cheese samples

Total bacterial DNA was extracted from 50 g of grounded cheese for each sample; samples were homogenized in 125-μm filter stomacher bags (Biochek, Foster City, Calif.) with 50 ml of sodium-citrate solution. The filtered homogenates were collected and centrifuged at 5000 rpm for 10 min at 4 °C to obtain pellets. The pellets were washed in saline solution and successively processed using the bead-technology based FastDNA® SPIN kit and the FastPrep® Instrument (Qbiogene, Inc., CA) according to the protocols previously described (Bassi et al., 2013a, 2013b). DNA was then finally suspended in 100 μl of DES solution for further applications. All extracted nucleic acids were examined at a 0.8% electrophoresis agarose gel, and quantified using the picogreen method of the Quant-iT™ HS ds-DNA assay kit (Invitrogen, Paisley, UK) in combination with the QuBit™ fluorometer.

2.4. 16S-Based Clostridium cluster-I specific PCR and DGGE analysis

The PCR-DGGE strategy was based on a first amplification of a 235 bp fragment specific for the order of *Clostridiales* in the V3–V4 region of the 16S rRNA gene, using primers DGGECl2 f (5′-GCGGCGTGCCTAATACATGC-3′) and P4 r (5′- ATCTACGCATTTCACCGCTAC-3′). Primers were designed after aligning more than 150 16S rDNA sequences among representatives of the genera *Clostridium, Ruminococcus, Lactobacillus, Bacillus, Streptococcus, Leuconostoc,* and *Enterococcus* that could frequently be present in dairy products in order to avoid cross-reactions. PCR amplifications were performed in a final volume of 25 μl, which included 12.5 μl of 2× MasterMix PCR (Promega), 0.4 μM primers, and 3 μl of genomic DNA. Template DNA was generally diluted 10-fold and 100-fold to minimize PCR inhibitors. Reactions were heated to 95 °C for 5 min and cycled at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. Cycles were repeated 35 times for all samples. Finally, 5 μl of each PCR product was used for visualization on a 2% agarose gel containing SYBR® Safe (Invitrogen Corporation Life technologies). Negative (without DNA) and positive (with DNA from reference strains) controls were included in each amplification run. 24 *Clostridium* strains, and 30 non-clostridial species (*Bacillus, Lactobacillus, Leuconostoc, Listeria, Staphylococcus, Enterococcus, Streptococcus, Ruminococcus*) were selected and used for the sensitivity test of the PCR protocol (Table 1). A nested PCR on the first DNA template was then performed using primers DGGE2Cl f clamped at 5′ with a 33 bp GC sequence (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCC) and P4 r. The reaction was performed in a 25 μl volume, with 12.5 μl of 2× MasterMix PCR (Promega), 0.1 μM of each primer and 1 μl of DNA obtained in the previous PCR reaction. The amplification cycle was the same of the first PCR reaction except for the annealing temperature of 63 °C and the extension performed at 72 °C for 1 min. Denaturing gradient gel electrophoresis (DGGE) was performed using an INGENY phorU-2 (Ingeny International BV, Netherlands) DGGE system. A portion of each PCR template was loaded on a 8% (w/v) acrylamide gel containing a 45–65% (w/v) denaturant gradient of urea and formamide and electrophoresed at 90 V, 60 °C