Systematic and Applied Microbiology 35 (2012) 54–63

Contents lists available at SciVerse ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm

The spatial distribution of bacteria in Grana-cheese during ripening

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ARTICLE INFO

Article history:
Received 8 April 2011
Received in revised form 24 June 2011
Accepted 1 July 2011

Keywords:
Grana cheese
Under rind
Core
Microbial spatial distribution
Dairy LAB characteristics

ABSTRACT

The microbial composition and its spatial distribution of Grana Trentino, a hard Parmesan-like cheese, was determined, from vat milk to cheese. After cutting along the vertical axis of the cheese wheels, three layers were sampled diagonally across the cheese: under the cheese rind, an intermediate section and the cheese core. After two different ripening periods (9 and 18 months), the cheese samples were analysed using traditional culture dependent and culture independent methods. Milk samples were dominated by mesophilic and psychrophilic bacterial counts. Thermophilic bacteria (*Lactobacillus helveticus*) were found in high amounts in cooked whey and natural whey starter cultures. After 9 months of ripening, lactic acid bacteria (LAB) counts were higher than those after 18 months. Furthermore, the LAB numbers in the cheese core was lower than those under the rind or in the intermediate section. The main LAB species isolated from milk (*Lactococcus lactis*, *Pediococcus pentosaceus*, *Streptococcus uberis* and *Lactococcus garvieae*) were not found in the corresponding cheeses. Some differences were observed in the species composition among the three cheese sections. Microbiota under the rind and in the intermediate section was similar and dominated by *Lactobacillus paracasei* and *Lactobacillus rhamnosus*. The core, after 18 months of ripening, was characterized by a total absence of LAB. In each sample, all LAB were genotypically grouped and the different biotypes were subjected to several technological tests indicating that some non-starter LAB (NSLAB) displayed technological features that are favorable for the production of Grana Trentino cheese.

Introduction

Grana Trentino is an Italian hard cooked cheese subjected to a long ripening time (up to 2 years). It is included in the consortium of Grana Padano cheese, but the milk comes from Brown herds bred in the Trentino region (an Alpine area located in the North-Eastern part of Italy). Furthermore, for Grana Trentino, a traditional production is applied with more restrictions than Grana Padano cheese making [7] is followed. It is made out of partially skimmed raw cow's milk and commercial rennet, with the addition of a natural whey starter culture that is daily produced in the dairy factory and contains high amount of thermophilic lactic acid bacteria (LAB). The addition of lysozyme to the vat milk is not allowed.

The Grana-type cheeses are large compared to other cheeses and larger cheese wheel size could affect some diffusion-dependent chemical parameters, e.g. salt concentration. Since salt penetration across the cheese may create different environments [37], the processes of bacterial selection and development may be influenced during cheese ripening across the different sections of the cheese wheel (from the rind to the core). Grana-type cheeses have been investigated for their physical and chemical properties [31], but the microbial composition and distribution have not been determined so far.

During the ripening process the pool of bacterial enzymes available in the curd metabolize milk components such as lactose, protein and fats, and also their degradation products, including lactate, peptides and fatty acids [27]. The bacterial biota involved in cheese ripening is therefore of primary importance in affecting cheese quality. The LAB community found during the manufacturing of Parmigiano Reggiano and Grana Padano is mainly composed of *Lactobacillus helveticus* which is the dominant species in whey starter, followed by *Lactobacillus delbrueckii* subsp. *lactis*, *Lb. delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus* and *Lactobacillus fermentum* [1,15,34,35]. Usually, mesophilic facultatively heterofermentative lactobacilli are the most common NSLAB detected throughout the whole ripening of Parmigiano Reggiano and Grana Padano [5,13,17]. Some studies dealt with the different chemical and biochemical composition in the inner and outer section of hard cheeses like Parmigiano Reggiano during ripening [26,31]. However, no studies have been carried out to date

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doi:10.1016/j.syapm.2011.07.002

on the distribution of LAB microbiota during ripening of Grana cheeses.

Regarding other cheese typologies, Gobbetti et al. [19] studied the microbial distribution in Taleggio, a soft short ripened cheese, by plate counts of the different microbial groups and analysis of enzymatic activities present in cheese extracts. Dolci et al. [6] studied, by a culture-independent approach, the microbial distribution during ripening of Castelmagno cheese, a traditional cheese produced with raw milk, 2–5 months ripened and of smaller size (2–7 kg) than Grana-like cheeses which may reach a final weight of approximately 30–40 kg.

The objectives of this study were: (1) to enumerate the most representative groups of bacteria occurring in milk, natural whey starter and cheese after 9 and 18 months of ripening in three distinct internal zones of Grana Trentino cheese; (2) to identify the different microbial populations using a polyphasic genetic approach; and (3) to characterize, both phenotypically and genetically, LAB and NSLAB isolated from cheese.

Material and methods

Cheese-making

Trials were carried out in a cheese factory that usually produces Grana Trentino cheese. The milk for experimental trials was collected always from the same two farms, immediately cooled to 12 °C after each evening and morning milking, and transported to the cheese factory once a day (in the evening after the second milking) by means of a temperature-controlled road tanker (transport temperature was in accordance to Regulation (EC) No. 853/2004 [33]). The bulk milk was placed into a 1200 L-shallow tank and the creaming was carried out without temperature control. After creaming, skimmed milk was transferred into the typical copper vat and underwent the traditional Grana cheese manufacturing. Vat milk was heated to 22 °C and added with the natural whey starter. Milk coagulation occurred after the addition of calf rennet powder; the curdle was broken in rice-seed size and cooked (temperature raised slowly to 42–44 °C, then quickly to 55–56 °C). The curdle rested in the hot whey for about 1 h and then was removed from vat and cut in two pieces that were moulded for 2 days at about 20 °C in two cheese wheels. The cheeses were then salted by immersion in brine (280–300 g NaCl L^{-1}) at 15–18 °C for 20–25 days. Ripening was carried out at 18 °C and 80% relative humidity. The two cheese wheels were periodically turned upside down during the 18-month ripening.

Sample collection

Vat milk (VM) was sampled after mixing the evening skimmed milk with the morning whole milk in the vat; the natural whey starter culture (WS) was sampled just before its addition to the vat milk and the cooked whey (CW) was sampled after curdle cooking. Sampling was performed during four cheese-making days in June 2007. The two cheeses produced each day from a single vat were considered twin replicates. The first replicate cheese wheel was sampled after 9 months and the second one after 18 months of ripening. The cheese wheels were cut along the vertical axis to obtain two symmetrical halves and three dish-shaped samples were taken from one half. In order to analyse the entire cheese profile, three portions (about 10 g) per sample were collected: UR (under rind), 4 cm from superior cheese dish – 5 cm from cheese side; MS (middle section) 6 cm from superior cheese dish – 15 cm from cheese side; core (Co), 12 cm from superior cheese dish – 25 cm from cheese side.

Bacterial counts and isolation

Vat milk and whey samples were diluted in peptone water (0.1% mycological peptone). First decimal dilution of cheese was obtained by shaking the samples in 90 mL of sterile sodium citrate 2% (w/v) solution by a Laboratory Blender Stomacher 400 (Seward, London, UK) for 2 min at the highest speed. Cell suspensions were plated in duplicate and incubated as follows: total bacterial count (TBC) onto Plate Count Agar added with 1 g L^{-1} skimmed milk (PCA-SkM), incubated aerobically at 30 °C for 24 h; psychrophilic bacteria on PCA-SkM, incubated aerobically for 7 days at 7 °C; coliforms on violet red bile agar (VRBA), incubated anaerobically for 24 h at 37 °C; mesophilic cocci-shaped LAB on M17 agar, incubated at 30 °C aerobically for 48 h; mesophilic rod-shaped LAB on MRS agar acidified to pH 5.5 with 5 mol L^{-1} lactic acid (MRS), incubated at 30 °C anaerobically for 48 h; thermophilic LAB on Whey Agar Medium (WAM) [16], incubated anaerobically for 72 h at 45 °C. All culture media were purchased from Oxoid (Milan, Italy).

Ten colonies from each plate, representing the dominant microbiota of the analysed samples, were randomly picked up for bacterial isolation. Each isolate was purified by subsequent culturing. Pure cultures were kept at −80 °C in glycerol (40% v/v) stocks. Cell morphology was determined by microscopic observation, Gram characterization was performed applying the KOH method [20] and catalase activity was tested after addition of 5% H_2O_2 on the colonies.

Temperature measurement and physico-chemical analysis

Temperature dynamics were registered with the 175-T2 data logger (Testo, Settimo Milanese, Italy). Measurements of pH of milk, whey, and cheese (soon after cheese making, after 8 h and after 9 and 18 months of ripening) samples were carried out by means of a portable pH meter (Knick Portamess 910, Berlin, Germany) connected to the Cheesetrode (Hamilton Co., Reno, NV, USA) electrode.

Water activity (a_w) under the rind and in the core was evaluated by AquaLab® Model Series 3 (Decagon Devices, Inc. Pullman, WA, USA).

Biotypes clustering and identification

All isolates were subjected to randomly amplified polymorphic DNA-PCR (RAPD-PCR). DNAs were extracted from overnight broth cultures. Cells were centrifuged at 10,000 × *g* for 5 min and the pellets were washed twice in 1 mL distilled water. Cell pellets were subjected to lysis by Instagene Matrix (Bio-Rad, Hercules, CA, USA) following the manufacturer's instruction.

RAPD-PCR was carried out with primer PC1 according to Poznanski et al. [32]. PCR products were separated by electrophoresis on 2.5% (w/v) agarose gel (Gibco BRL, Cergy Pontoise, France) and stained with ethidium bromide (0.5 μg L^{-1}). DNA patterns were analysed through the unweighted pair group method and employing arithmetic averages (UPGMA) using the GelCompar II-BioNumerics software (package version 6.0; Applied Maths BVBA, Belgium). Calculation of similarity of the PCR fingerprinting profiles was based on the Pearson product-moment correlation coefficient. Isolates with similarity coefficient higher than 80% were considered to belong to the same biotype, as described by Gatti et al. [16]. The discriminatory power was evaluated by calculating the Simpson's discriminatory index [22].

Genotypic identification of LAB with different RAPD-PCR profiles was carried out by partial 16S rRNA gene sequencing and species-specific PCRs. The 16S rRNA gene sequence analysis was performed using the Lab159f/Uni515r primer pair as described by Heilig et al. [21]. The PCR product (ca. 30 ng) of each strain was purified with Exo-SAP-IT™ kit (USB Co., Cleveland, OH) and sequenced