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Population dynamics of lactobacilli in Grana cheese

Miriam ZAGO, Maria Emanuela FORNASARI, Lia ROSSETTI, Barbara BONVINI, Laura SCANO, Domenico CARMINATI, Giorgio GIRAFFA*

C.R.A. - Istituto Sperimentale Lattiero Caseario, Via A. Lombardo 11, 26900 Lodi, Italy

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Abstract - A survey on the presence, microbial diversity, and population dynamics of lactobacilli in Grana cheese is presented. Evolution of thermophilic rod lactic acid bacteria within the first two days from cheese making and during ripening was different according to different bacterial groups, which were selectively enumerated and identified by molecular methods. Species-specific microbial counts indicated prevalence of *Lactobacillus helveticus* in both the whey starter and the cheese at moulding, and of *Lactobacillus delbrueckii* subsp. *lactis* in cheese after two months of ripening. In more advanced ripening, a decrease of total thermophilic lactobacilli and an increase of mesophilic lactobacilli (mostly belonging to *Lactobacillus casei/paracasei* and *Lactobacillus rhamnosus)* was observed. PCR fingerprinting of lactobacilli, which was performed by PCR-fingerprinting, indicated a marked microbial heterogeneity within the *Lactobacillus* spp. populations, which enabled strain (or group)-specific fingerprints to be observed.

Key words: lactic acid bacteria, *Lactobacillus*, microbial diversity, molecular identification, Grana cheese, microbial counts.

INTRODUCTION

In the microbiology of Grana and Parmigiano Reggiano cheeses, the most widespread Italian hard-cheese varieties (Ottogalli, 2000), the lactic acid bacteria (LAB) microflora deriving from both raw milk and cheese-whey starter plays an important role in the achievement of the typical and appreciated sensory characteristics of the cheese.

The whey starter represents a complex, mixed-strain culture, due to different ecological, biological, technological, and biochemical phenomena that take place and act concomitantly. The role of these cultures is essentially to allow an acidification by lactic fermentation, which favours the curd whey drainage by the increase of the acidity of cheese vat milk. It is well established that whey starters for hard cooked cheeses are dominated by a LAB microbiota belonging to *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. d. bulgaricus)*, *Lactobacillus delbrueckii* subsp. *lactis* (*L. d. lactis)*, *Lactobacillus fermentum*, and *Streptococcus thermophilus* (Giraffa *et al*., 1997; Parente and Cogan, 2004). It has also been suggested a contribution of this microflora on the ripening process, but further studies are still needed on this subject. Moreover, a heterogeneous non-starter LAB (NSLAB) microflora predominates in mature Grana Padano and Parmigiano Reggiano cheeses. The composition of this community may vary but *Lactobacillus casei*-group, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, and *Pediococcus* spp. constitute the major part (Gobbetti, 2004). Such micro-organisms are believed to be involved in a number of biological modifications, enabling milk transformation and participating, together with the biochemical activities of the starter, in the ripening process (Giraffa *et al*., 1997; Coppola *et al*., 1997, 2000; Gobbetti 2004).

The present article is aimed at reporting main progress as well as recent unpublished results on the presence and role of lactobacilli in Grana cheese. Particular attention is paid on possibilities offered by molecular biology techniques in the identification and characterisation of isolated strains.

MATERIALS AND METHODS

Strains, media, and cultivation conditions. The lactobacilli studied included: isolates from whey starter cultures for Grana cheese; isolates from Grana cheese curd after moulding and during ripening; the type strains *L. helveticus* ATCC 15009^T, *L. d. bulgaricus* ATCC 11842^T, and *L. d. lactis* ATCC 12315^T. The strains were maintained as frozen stocks at -80 °C in the presence of 15% of glycerol as a cryoprotective agent. Unless otherwise specified, strains were routinely reactivated overnight at 37 °C in MRS broth medium (Biokar, Beauvais, France).

Microbiological analysis of whey starter and Grana Padano cheese. In the cheese plant of C.R.A. - Istituto Sperimentale Lattiero Caseario (Lodi, Italy), Grana cheese was manufactured from partially skimmed raw milk according to well known technology (Gobbetti, 2004). Lactobacilli in the cheese form were counted at moulding, at 6 and 48

* Corresponding author. Phone: +39 037145011; Fax: +39 037135579; E-mail: ggiraffa@ilclodi.it

h after moulding, and after 2, 4, and 8 months ripening. The counts of lactobacilli in whey starter cultures and cheese were performed using MRS agar (pH 6.5 ± 0.2) as the culture medium. Mesophilic and thermophilic LAB were counted after incubation of MRS agar plates at 30 and 44 °C for 48 to 72 h under anaerobic conditions.

The specific detection of *L. helveticus*, *L. d. bulgaricus*, and *L. d. lactis* was performed by colony hybridization. Bacterial colonies were grown on Hybond N^+ membranes (Amersham) layered on MRS solid medium under the conditions described above. After the colonies had grown, membrane layered MRS master plates were replicated onto fresh MRS agar plates and, after a brief incubation on fresh MRS agar plates containing 100 µg/ml of ampicillin, colonies remained on the membranes were treated for bacterial lysis. The membranes were placed on filter paper (Whatman 3) soaked in 10% sodium dodecyl sulphate (SDS) and incubated at room temperature for 3 min. The membranes were then placed on a mixture containing Tris-HCl 50 mmol/l pH 7.5, glucose 50 mmol/l and 20 mg/ml lysozyme and incubated at 37 °C for 45 min. Cell lysis was then achieved by incubation on NaCl 1.5 mol/l, NaOH 0.5 mol/l for 15 min. The membranes were neutralised with two washes with NaCl 1.5 mol/l, Tris-HCl 0.5 mol/l, pH 7.5 for 5 min and then washed with 30 mmol/l Na_3 citrate, 300 mmol/l NaCl (SSC), pH 7.0. DNA was denatured and bound by placing the membranes in 0.4 mol/l NaOH for 20 min or by baking at 80 °C for 1-2 h. The filter was then ready for DNA hybridization, which was carried out using the enhanced chemiluminescence (ECL)-direct nucleic acid labeling and detection systems (Amersham), according to supplier's instructions. Hybridization was performed under stringent conditions (6 M urea, 42 °C) with DNA probes that were specific for the identification of *L. helveticus* (de los Reyes-Gavilàn *et al*., 1992), *Lactobacillus delbrueckii* (Delley *et al*., 1990), and *L. d. lactis* (Giraffa and Mora, 1999). Hybridization between the probes and DNA extracted from the three type strains were used as positive control signals.

To confirm the reliability of colony hybridizations, randomly isolated lactobacilli from MRS agar plates grown at 30 and 44 °C were identified by molecular (i.e. species-specific PCR and PCR-ARDRA) methods. About 500 colonies were randomly isolated from MRS agar plates and grown in MRS broth (pH 7.4 ± 0.1). After microscopic examination, 480 rod-shaped isolates (of which 280 were thermophilic and 200 mesophilic) were further identified.

Identification of isolated lactobacilli.

Amplification and restriction analysis of ribosomal DNA (PCR-ARDRA). The polymerase chain reaction (PCR) was used to amplify the 16S rRNA gene from different thermophilic lactobacilli to confirm their taxonomic position after colony hybridization. PCR fragments of approx. 1600 bp corresponding to the 16S rRNA genes were amplified from total DNA by using the primers described by Rodtong and Tannock (1993). Lactobacilli were lysed and their total DNA was extracted according to a protocol previously described (Giraffa *et al*., 1998a). 16S rRNA genes of the different strains were amplified in a volume of 100 µL, containing 50 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 200 µM of each dNTP, 0.4 µM of each primer (CELBIO, Milan, Italy), 100 ng of genomic DNA, and 2.5 U of AmpliTaq Gold (Applied Biosystems, Monza, Italy). DNA amplifications were performed in a Perkin Elmer (mod. 2400) thermal cycler. With AmpliTaq Gold, a 10-min pre-incubation at 94 °C was suggested by manufacturer to initiate activation of the enzyme and as initial DNA denaturation. Initial denaturation was followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 30 s, and extension at 72 °C for 2.5 min, and a final chain elongation step at 72 °C for 10 min. Before endonuclease digestion, PCR products were visualised by agarose (1.2% w/v) gel electrophoresis and purified with Geneclean III spin kit (Bio 101, La Jolla, CA, USA). DNA quantification and purity were obtained by spectrophotometric determination or by ethidium bromide fluorescence as described by Sambrook *et al.* (1989). Restriction was carried out during 2 h at 37 °C in 100 µL volumes of incubation buffer (Life Technologies) containing 8-12 U of the restriction enzyme *Eco*RI and 250 ng of purified PCR product.

Species-specific PCR. Mesophilic lactobacilli were identified by species-specific PCR according to previously described primers and amplification conditions (Rossetti and Giraffa, 2005).

PCR fingerprinting. The total DNA of the isolates was used as template for PCR fingerprinting. The M13 minisatellite core sequence 5'-GAGGGTGGCGGTTCT-3' (Huey and Hall, 1989), which has successfully been used to obtain strain-specific patterns of lactobacilli (Andrighetto *et al*., 1998), was used as PCR primer. Amplification conditions consisted of 40 cycles of 1 min at 94 °C, 20 s at 45 °C, and 120 s at 72 °C; plus one additional cycle with a final 10-min chain elongation at 72 °C. PCR profiles were visualised after electrophoresis in 1.8% agarose (Sigma Italia) gels and staining with ethidium bromide. The photographs of the gels were scanned with a laser densitometer and the image was analysed with the pattern analysis software package GelCompar Version 4.0 (Applied Maths, Kortrjik, Belgium). Calculation of similarity of the band profiles was based on the Pearson correlation coefficient *r*. Clustering was accomplished by using the unweighted pair group method with arithmetic average (UPGMA).

RESULTS AND DISCUSSION

The level of total thermophilic lactobacilli in the whey starter before vat inoculation was about 10^8 CFU/ml; after starter addition into the vat, microbial count was about 10^6 CFU/ml cheese milk. The evolution of thermophilic lactobacilli within the first two days from cheese making and during ripening was different according to different bacterial species, which were selectively enumerated by colony hybridization. *Lactobacillus helveticus* dominated in the whey starter (about 5×10^8 cells/ml) and in the curd (about 2×10^8 cells/g); then it dropped to approx. 10^7 cells/g in the cheese at moulding, and to 2×10^4 cells/g after two months of ripening. On the other hand, *L. delbrueckii* was not detectable (< 10^6 cells/ml or /g) in the whey starter and cheese curd but it progressively increased to about 10^6 cells/g in cheese after two months of ripening. Sequential hybridizations with different DNA probes specific for *L. delbrueckii* and *L. d. lactis* indicated that approx the 99% of the *L. delbrueckii* population belonged to *L. d. lactis* (data not shown).

Further molecular, PCR-ARDRA-based identification of strains from 280 colonies randomly isolated from MRS agar plates at 44 °C confirmed the reliability of the DNA probes used in our colony hybridization experiments. PCR-ARDRA indicated that the most part (i.e. more than 90%) of isolates were identified as *L. helveticus* and *L. delbrueckii* and confirmed also that, within the *L. delbrueckii* strains, the most part belonged to *L. d. lactis*. Indeed, the 16S rRNA genes amplified by PCR from all the *L. helveticus* and *L. delbrueckii* isolates showed an already reported (Andrighetto *et al*., 1998; Giraffa *et al*., 1998a) clearly resolvable, RFLP between the two species after digestion with *Eco*RI (Fig. 1).

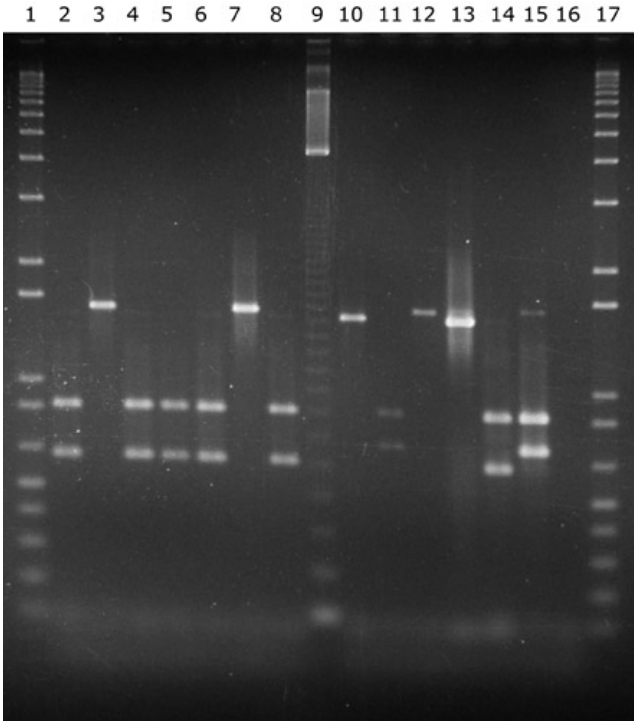

FIG. 1 - ARDRA analysis of thermophilic lactobacilli. Lanes 2, 4, 5, 6, and 8: 16S rDNA of *Lactobacillus helveticus* cut with *Eco*RI; lanes 10: *Lactobacillus delbrueckii* subsp. *lactis* cut with *Eco*RI; lane 11: *Lactobacillus delbrueckii* subsp. *bulgaricus* cut with *Eco*RI; lanes 13, 14 and 15: 16S rDNA of the type strains (i.e. ATCC 15009, ATCC 12315 and ATCC 11842, respectively) of the three species cut with the enzyme; lanes 3, 7, and 12: uncut 16S rDNA of the type strains. Lanes 1, 9, and 17: Molecular size marker 1-kb plus (lanes 1 and 17) and 100 bp ladder (lane 9) (Invitrogen Italia, Milano, Italy).

In more advanced stages of ripening, i.e., from 2-4 to 8 months ripening, thermophilic lactobacilli progressively decreased whereas the mesophilic *Lactobacillus* spp. population, i.e., that counted in MRS agar plates at 30 °C, started to increase reaching levels up to 10^6-10^7 CFU/g. Species-specific PCR identification of about 200 strains randomly isolated from MRS agar plates at 30 °C showed that more than the 95% of the mesophilic population of lactobacilli belonged to *L. casei/L. paracasei* and *L. rhamnosus*, whereas *L. plantarum* was less frequently recovered.

PCR fingerprinting of isolates, which was performed using the M13 minisatellite sequence, allowed us to observe a certain microbial heterogeneity within the *Lactobacillus* spp. populations, which enabled strain (or group)-specific fingerprints to be obtained (Fig. 2 - A, B, C: *L. helveticus*, *L. d. lactis*, and *L. casei/L. paracasei* isolates, respectively, shown as example). Overall 28, 15, 30, and

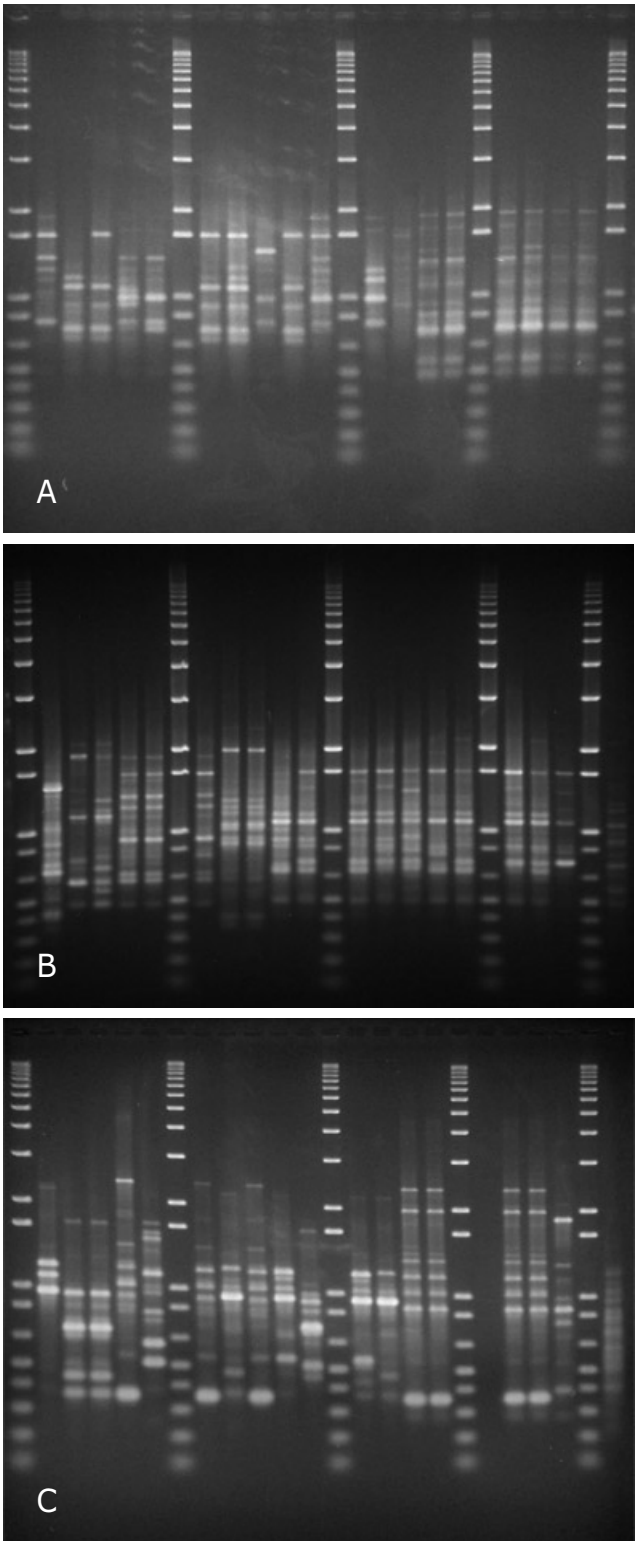

FIG. 2 - PCR fingerprinting of some of the lactobacilli isolated from Grana cheese. A: *Lactobacillus helveticus*; B: *Lactobacillus delbrueckii* subsp. *lactis*; C: *Lactobacillus casei*. Lanes 1, 7, 13, 18, and 23: Molecular size marker 1-kb plus (Invitrogen Italia).

10 genotypically different biotypes were recovered from *L. helveticus*, *L. d. lactis*, *L. casei/L. paracasei*, and *L. rhamnosus*, respectively (data not shown).

In the present study, microbial variations of lactobacilli population during Grana Padano cheese manufacture were scored. Thermophilic lactobacilli dominate during the early stages of ripening and, after their rapid autolysis, they are substituted by mesophilic lactobacilli, thus confirming previous findings on Grana and Parmigiano cheeses (Bosi *et al*., 1993; Neviani and Carini, 1994; Coppola *et al*., 1997, 2000; Ranalli *et al*., 2000). It is well established that thermophilic lactobacilli largely prevail during manufacturing, undergo to species and strain variations, and are then replaced during ripening from non-starter LAB which are originally present in raw milk (Bottazzi, 1993; Neviani and Carini, 1994; Gobbetti, 2004). This suggests that both mesophilic and thermophilic LAB and, after microbial autolysis, their released enzymes may play an important role for the ripening of Grana cheese.

The identification of many isolates from Grana cheeses indicated that *L. helveticus* is the dominant species in the natural whey starter and during the early stages of cheese making whereas *L. d. lactis* predominated later, after two months of ripening. This confirmed previous findings indicating the two above species as the majority of thermophilic lactic microflora recovered from Grana cheese during the first stages of ripening (Bottazzi, 1993; Neviani and Carini, 1994; Parente and Cogan, 2004). The different species of thermophilic lactobacilli are selected since the extraction of the curd from the kettle by the temperature gradient between the external and the internal cheese zones (Giraffa *et al*., 1998b).

Amongst the *L. delbrueckii* isolates, the prevalence of *L. d. lactis* in both the experimental and the commercial Grana cheeses is not surprising and evidences a difficulty to isolate the subspecies *bulgaricus* from this cheese (Andrighetto *et al*., 1998). Also studying Parmigiano Reggiano cheese, Cocconcelli *et al*. (1997) reported that *L. helveticus* and *L. d. lactis* are the dominating species in natural whey culture *Lactobacillus* population. *L. d. lactis* seems, therefore, a bacterium well adapted to the cheese environment.

PCR fingerprinting has been used successfully for species differentiation of dairy lactobacilli (for a review, see Giraffa and Neviani, 2000). When PCR fingerprinting was applied for differentiating between dominant *L. helveticus* and *L. d. lactis* populations, this method enabled us to observe strain (or group)-specific molecular markers, which could be applied to ecological studies, e.g. to study population dynamics in mixed strain fermentation. In a previous study (Giraffa and Neviani, 1999), separate groups of *L. helveticus* strains, differently distributed between the whey starter and the cheese, were discriminated. In particular, two clearly distinguished biotypes dominated the *L. helveticus* populations of the whey starter and cheese curd. Phenotypic and genotypic heterogeneity of *L. helveticus* strains isolated from Grana and Provolone cheeses enabled a strain subdivision according to the different dairy niches used as sources of strains to be also obtained (Gatti *et al*., 1999; Giraffa *et al*., 2000). It can be concluded, therefore that the community of dairy thermophilic lactobacilli is composed of more than 30 biotypes which are peculiar of different microbial ecosystems.

Microbial agents responsible for the development of the characteristic Grana cheese flavour derive also from raw milk. Most raw milk micro-organisms associated wit hard cheese varieties are mesophilic, NSLAB mainly belonging to the *Lactobacillus* casei-plantarum group (Cogan *et al*., 1997; Coppola *et al*., 1997, 2000; Corroler *et al*., 1998; Dellaglio *et al*., 1998; Bouton *et al*., 2002; Casey *et al*., 2006). In the present investigation, the mesophilic lactobacilli were recovered at high numbers at advanced stages of Grana cheese ripening and, after further identification, they mostly belonged to *L. casei/L. paracasei* and *L. rhamnosus*. The PCR applied to identify *L. casei* does not distinguish this organism from *L. paracasei* (Drake *et al*., 1996) However, according to recent taxonomic studies, *L. casei* is indistinguishable from many strains of *L. paracasei* and, therefore, this latter should be rejected (Felis *et al*., 2001; Chavagnat *et al*., 2002). The presence of these two NSLAB agrees with findings from Coppola *et al*. (1997, 2000), which isolated *L. paracasei* subsp. *paracasei* and *L. rhamnosus* from Parmigiano Reggiano up to 24 months of ripening. The NSLAB, which are able to survive longer into the cheese, are considered important microflora for the development of cheese flavour because of their proteolytic and peptidolytic activities (Bottazzi, 1993; Bosi *et al*., 1993; Dellaglio *et al*., 1998; Gobbetti, 2004). PCR fingerprinting of mesophilic lactobacilli evidenced a high strain heterogeneity, which was partly expected because of the widespread sources of origin of this microflora (raw milk, dairy environment, cheese production environment).

Grana cheese represents a complex microbiological ecosystem which evolved in parallel with changes in milk quality and cheese technology. Both mesophilic and thermophilic LAB may play an important role for the ripening of Grana cheese. The differences evidenced in bacterial species- and strain-composition between whey-starter and curd/cheese agree with the different biochemical and biophysical characteristics of the two ecosystems.

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