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Microbial dynamics of Castelmagno PDO, a traditional Italian cheese, with a focus on lactic acid bacteria ecology

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Abstract

The dynamics of dominant microflora throughout the manufacture and ripening processes were evaluated in three batches of traditional Castelmagno PDO cheese.

Milk, curd and cheese samples, at different stages during cheesemaking, were collected and subjected to culture-dependent and -independent analysis. Traditional plating and genetic identification of lactic acid bacteria (LAB) isolates, and PCR-DGGE analysis of V1 region of 16S rRNA gene were carried out. The collected samples were also monitored by HPLC for the presence of organic acids, sugars and ketones.

LAB resulted to be the prevailing microflora in all production stages although enterococci, coagulase-negative cocci and yeasts also showed considerable viable counts probably related to the presence, in the dairy samples analysed, of free short-chain fatty acids detected by HPLC. *Lactococcus lactis* subsp. *lactis* was the species most frequently isolated during Castelmagno PDO manufacture, while *Lactobacillus plantarum* and *Lactobacillus paracasei* were isolated with the highest frequencies from ripened Castelmagno PDO cheese samples. Occasionally strains of *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus coryniformis* subsp. *torquens* and *Lactobacillus casei* were isolated. The results obtained on Castelmagno PDO microflora underlines a partial correspondence between culture-dependent method and DGGE analysis. Thus, in this study, it is highlighted once more the importance to combine molecular culture-independent approaches with classical microbiological methods for the study of complex environmental communities occurring in food matrices.

Keywords: Castelmagno PDO; Microbial dynamics; Lactic acid bacteria; PCR-DGGE

1. Introduction

Italian dairy tradition has been recognized from European Community with the attribution of the "Protected Designation of Origin" to 31 traditional cheeses (E.U., 2006). One of the most famous and known is the Castelmagno PDO cheese, whose technology has been handed down since the twelfth century and now recorded in the current national legislation (Official Gazette, No. 173, July 2006).

Castelmagno PDO is a hard and pressed cheese produced in Piedmont, in the Grana Valley (Italian Maritime Alps, northwest Italy). The shape is cylindrical with flat surfaces and the weight is of about 5–7 kg, the rind is wrinkled and reddish-grey. The texture of aged cheeses is compact but friable, the flavour salty and moderate piquant. The surface of the cheese is usually colonised by *Penicillium* spp. coming from the environment but in some cases it can be found also inside the loaf. In that case Castelmagno PDO is considered a blue cheese. Though Castelmagno is commonly classified as blue cheese, in recent years the practice of the "drilling" of the core (Official Gazette, No. 173, July 2006), to obtain mould growth, is falling into disuse and most of the commercialized Castelmagno cheese does not present inner moulds.

In Castelmagno PDO cheesemaking, raw cow milk is partially skimmed and coagulated by addition of liquid or powdered calf rennet. Rarely, a mixture of cows', ewes' and goats' milks is used.

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Traditional technology does not allow the use of starter cultures and acidification is due to indigenous lactic acid bacteria (LAB). After cutting the coagulum and removing most of the whey, the curd is traditionally harvested in cloth bags which are hung, or left on a sloping surface for at least 18 h, allowing the elimination of further whey. Then, the curd is roughly cut, and covered for 3–6 days with acidified whey obtained from previous cheesemaking. Finally the curd is crumbled, salted and strongly pressed in moulds. The cheese is ripened in natural caves at 10–12 °C for at least 60 days, frequently for 90 days; for peculiar production the ripening time reaches or exceeds 180 days.

Castelmagno PDO is an artisanal cheese made by farmers on a small scale using traditional practices. In the last years, the growth of industrial dairy plants producing a more uniform and standardized product, led to a growing concern for the loss of typical organoleptic features of Castelmagno PDO strictly related with the indigenous microbial population present in raw milk and selected by cheesemaking environment.

The first step towards protecting the microbial biodiversity in Castelmagno PDO cheese is the knowledge of microflora evolution in the cheese during traditional manufacturing and ripening processes. Thus the aim of this work was to acquire information about different microbial groups involved in the fermentation and ripening. In particular the objective was to follow the contribution of LAB to the achievement of the final fermented product.

The approach selected in this study was the use of both culture-dependent and -independent methods. LAB, isolated by conventional cultivation, were identified by molecular methods using a combination of PCR 16S–23S rRNA gene spacer analysis (RSA), species-specific primers and 16S rRNA gene sequencing (Nour et al., 1995; Moschetti et al., 1997; Moschetti et al., 1998; Coppola et al., 2001; Fortina et al., 2003).

In parallel, PCR-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) was carried out to understand the composition and dynamics of dominant microflora directly in food matrix (Ercolini, 2004). PCR-DGGE is a culture-independent technique based on separation and comparison, in denaturing gradient gel, of relatively conserved regions in the genome. This approach allows separation of DNA molecules that differ by single bases and hence represents a powerful tool in following microbial species arising during manufacture and ripening of fermented products (Cocolin et al., 2001; Randazzo et al., 2002; Ercolini et al., 2003; Cocolin et al., 2004; Belen Floréz and Mayo, 2006; Randazzo et al., 2006). Moreover, HPLC analysis to quantify organic acids, sugars and ketones was carried out on Castelmagno PDO during manufacture and ripening.

2. Materials and methods

2.1. Dairy samples

Milk, curd and cheese samples were collected from three subsequent manufacturing batches in one small size traditonal dairy plant in the Grana Valley (Italian Maritime Alps, northwest Italy) during the summer alpine pasture. For each batch it was sampled milk in the coagulation tank (M), curd after cutting (Cu), curd after 24 h (Cu24), curd after 3 days rest in whey (Cu3W), cheese after 3 days salting (Ch3S), cheese after 30 days ripening (Ch30), cheese after 60 days ripening (Ch60), and cheese after 90 days ripening (Ch90). All samples were transported to the laboratory under refrigerated conditions not later than 3 h from the collection, and subjected to microbiological, molecular and chemical analysis.

2.2. pH measurements

Potentiometric pH measurements were obtained with the pin electrode of a pH meter (Portamess 913, Knick, Berlin, Germany) that was inserted directly into the inner part of the samples. Three independent measurements were carried out for each sample. Means and standard deviations were calculated.

2.3. Enumeration and isolation of microorganisms

Twenty millilitres of milk and 20 g of curd and cheese samples were homogenized in sterile Ringer solution (Oxoid, Basingstoke, UK) with a Stomacher machine, serially diluted in Ringer solution and plated on specific media for viable counts. The following analyses were performed: mesophilic aerobic bacteria on Plate Count Agar (Oxoid) at 30 °C for 48 h, mesophilic and thermophilic cocci on M17 agar (Fluka, Buchs SG, Switzerland), incubated aerobically, respectively at 30 °C and 37 °C for 48 h, mesophilic and thermophilic lactobacilli on MRS agar (Oxoid), incubated anaerobically, respectively at 30 °C and 37 °C for 48 h, enterococci on Kanamycin Aesculin Azide Agar (Fluka) at 37 °C for 48 h, coagulase-negative cocci (CNC) on Mannitol Salt Agar (Oxoid) at 30 °C for 48 h, coliforms on Violet Red Bile Lactose Agar (Fluka) at 37 °C for 24 h, yeast and moulds on Malt Agar (Oxoid) supplemented with tetracycline (1 μg/ml, Fluka) at 25 °C for 96 h. After counting, a two way ANOVA was performed by Statistica 7.1 (StatSoft Inc., Tulsa, Oklahoma, USA) to compare the microbial loads of the three batches at each sampling points. Then means and standard deviations of count values were calculated.

From M17 agar and MRS agar plates, 12 randomly selected colonies of LAB were isolated for each collection step. They were purified by two subsequent subcultures on M17 and MRS agar and finally stored at −20 °C in M17 and MRS broth containing 30% glycerol, before being subjected to molecular analysis.

2.4. DNA extraction from pure cultures

Genomic DNA from LAB isolates was extracted from 100 μl of an overnight culture diluted with 300 μl TE 1X buffer (10 mM Tris–HCl, 1 mM Na_2EDTA, pH 8.0) as described by Mora et al. (2000).

2.5. Identification of LAB isolates

Identification of LAB isolates was carried out by combining PCR 16S–23S rRNA gene spacer analysis (RSA), species-

specific primers and 16S rRNA gene sequencing. The RSA was carried out with primers G1 and L1 (Table 1). This analysis allowed to cluster the isolates on the basis of polymorphism of 16S–23S rRNA gene spacer region, and to obtain an indication of the hypothetical genus they could belong to (Coppola et al., 2001, Fortina et al., 2003). According to RSA results, species-specific PCRs were planned in order to determine the taxonomic positions of the isolates at species level. The primers used and their corresponding references are reported in Table 1. Isolates for which it was not possible to establish a well defined RSA profile were submitted to partial 16S rRNA gene amplification with primers p8FPL and p806R (Table 1). The amplified fragments were sent for the sequencing to MWG Biotech (Ebersberg, Germany) and the sequences obtained were aligned with those in GenBank with Blast program (Altschul et al., 1997) to determine the closest known relatives of isolates.

2.6. DNA extraction from dairy samples

At each sampling point, 20 g samples, in triplicate, were homogenized in a stomacher bag with 100 ml of Ringer solution for 1 min. Big debris was allowed to deposit for 3 min and 2 ml of supernatant was collected and centrifuged at 14,000 rpm for 5 min to pellet the cells. After supernatant discarding, pellet was resuspended in 150 μl of proteinase K buffer (50 mM Tris–HCl, 10 mM EDTA pH 7.5, 0.5% [wt/vol] sodium dodecyl sulfate) and 25 μl of proteinase K (25 mg/ml Sigma, Milan, Italy) was added, and submitted to heat treatment at 50 °C for 1 h. At this point, the suspension was transferred to tubes containing 0.3 g of glass beads (Sigma). One hundred-fifty microliters of 2× breaking buffer (4% [vol/vol] Triton X-100, 2% [wt/vol] sodium dodecyl sulfate, 200 mM NaCl, 20 mM Tris [pH 8], 2 mM EDTA [pH 8]) and 300 μl of phenol–chloroform–isoamyl alcohol (25:24:1, pH 6.7, Sigma) were added in the tubes and mixed with a Vortex mixer for approximately 5 min. After the treatment, 300 μl of TE (10 mM Tris, 1 mM EDTA) were added in the tubes and a centrifugation at 14,000 rpm for 5 min was performed. The DNA in the aqueous phase was precipitated with 800 μl ice-cold absolute ethanol and it was collected by centrifugation at 14,000 rpm for 10 min, washed briefly in 70% ethanol and resuspended in 50 μl of TE buffer (Cocolin et al., 2004).

2.7. PCR amplification

To investigate the dominant bacterial species, the variable V_1 region of 16S rRNA gene was amplified with primers P_1V_1GC (5′-GCG GGC CGC GCG ACC GCC GGG ACG CGC GAG CCG GCG GCG GGC GGC GTG CCT AAT ACA TGC-3′) (the underlined sequence represents the GC clamp as described by Sheffield et al., 1989) and P_2V_1 (5′-TTC CCC ACG CGT TAC TCA CC-3′) (Cocolin et al., 2001). PCR was performed in a final volume of 25 μl containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 1.25 U of Taq polymerase (Eppendorf, Hamburg, Germany), and 0.2 μM concentration of each primer. One microliter of template DNA was added to the mixture. A touchdown PCR was performed in a MyCyler (BioRad, Hercules, CA, USA). The initial annealing temperature was 60 °C and it was decreased to 52 °C in intervals of 2 °C every two cycles; finally 20 cycles were performed at 50 °C. A denaturation of 95 °C was used, while the extension for each cycle was carried out at 72 °C for 1.5 min and the final extension was at 72 °C for 5 min (Cocolin et al., 2004). PCR products were analysed by electrophoresis in 2% Tris-acetate-EDTA agarose gels.

2.8. DGGE analysis

The Dcode universal mutation detection system (BioRad) was used for DGGE analysis. PCR products obtained with

Table 1
Nucleotidic sequence, target gene and reference of primers used for LAB isolates identification

	Primers (5′ to 3′)	Target gene	Reference
G1	GAAGTCGTAACAAGG	Bacterial 16S–23S rRNA gene spacer region	Jensen et al. (1993)
L1	CAAGGCATCCACCGT		
p8FPL	AGAGTTTGATCCTGGCTCAG	Bacterial 16S rRNA region	Teymoortash et al. (2002)
p806R	CTACGGCTACCTTGTTACGA		
EM1A	TTGAGGCAGACCAGATTGACG	Nucleotide sequence conserved in *E. faecium*	Cheng et al. (1997)
EM1B	TATGACAGCGCTCCGATTCC		
E1	ATCAAGTACAGTTAGTCTT	Ddl gene in *E. faecalis*	Dutka-Malen et al. (1995)
E2	ACGATTCAAAGCTAACTG		
St1	CACTATGCTCAGAATACA	Lac Z gene in *S. thermophilus*	Lick et al. (1996)
St2	CGAACAGCATTGATGTTA		
His1	CTTCGTTATGATTTTACA	Histidine biosynthesis operon in *L. lactis*	Corroler et al. (1999)
His2	CAATATCAACAATTCCAT		
pLG-1	CATAACAATGAGAATCGC	16S rRNA gene in *L. garvieae*	Zlotkin et al. (1998)
pLG-2	GCACCCTCGCGGGTTG		
para	CATAACAATGAGAATCGC	16S rRNA gene in *L. paracasei*	Ward and Timmins (1999)
Y2	GCACCCTCGCGGGTTG		
16	CATAACAATGAGAATCGC	16S–23S rRNA gene spacer region in *L. plantarum*	Berthier and Ehrlich (1998)
Lpl	GCACCCTCGCGGGTTG		

Table 2
Mean log microbial counts of main microflora during manufacture and ripening of Castelmagno PDO cheese [a] and pH values

Sample from manufacture and ripening [b]	pH		Microbial log counts (expressed as mean of cfu mL^{-1} for milk and cfu g^{-1} for curd and cheese) and standard deviations (SD)																	
			PCA		M17 30 °C		M17 37 °C		MRS 30 °C		MRS 37 °C		KAA		MSA		MALT AGAR		VRBA	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
M	6.70	0.01	5.3	1.2	5.6	1.0	5.4	1.1	5.3	1.1	5.4	1.1	4.7	0.7	4.4	0.4	4.7	0.4	4.3	0.5
Cu	6.66	0.03	6.6	0.6	6.1	1.1	5.9	1.0	6.6	0.4	6.6	0.6	5.8	1.0	5.4	0.5	6.1	0.5	5.6	0.3
Cu24	5.16	0.18	8.8	0.6	8.9	0.8	8.6	0.9	8.2	1.0	7.5	0.4	7.2	0.7	6.9	0.5	8.5	0.8	6.6	0.6
Cu3W	4.78	0.15	9.9	0.2	9.8	0.4	9.4	0.5	9.6	0.7	9.6	0.6	7.7	0.5	6.9	0.3	8.6	0.5	5.5	0.4
Ch3S	4.73	0.06	9.3	0.3	9.1	0.3	9.0	0.4	9.1	0.5	8.4	1.3	7.3	0.2	5.9	0.3	8.2	0.9	4.6	1.2
Ch30	5.30	0.09	8.2	0.5	9.4	1.1	8.5	0.7	7.7	0.8	8.2	0.2	7.0	1.2	7.7	1.4	6.3	0.5	2.4	0.4
Ch60	5.10	0.11	6.8	0.2	6.8	0.4	6.9	0.2	6.4	0.1	6.8	0.1	6.1	0.2	4.8	0.4	4.5	0.7	1.4	0.4
Ch90	5.00	0.08	6.7	0.1	6.6	0.1	6.6	0.4	6.7	0.1	6.6	0.1	5.9	0.4	4.6	0.3	3.5	0.4	<10	

[a] Mean values of samples from three subsequent manufacturing batches.
[b] M: milk; Cu: curd after cutting; Cu24: curd after 24 h; Cu3W: curd after 3 days rest in whey; Ch3S: cheese after 3 days salting; Ch30: cheese after 30 days ripening; Ch60: cheese after 60 days ripening; Ch90: cheese after 90 days ripening.

primers P_1V_1GC and P_2V_1 were applied to an 8% (wt/vol) polyacrylamide gel (acylamide-bis acrylamide, 37.5:1) with a denaturant gradient from 30 to 60%, in a 1X TAE buffer (2 M Tris base, 1 M glacial acetic acid, 50 M EDTA [pH 8]). Gels were subjected to a constant voltage of 120 V for 4 h at 60 °C. DNA bands were stained in 1× TAE containing 1× SYBR Green I (Sigma), and analysed under UV by using UVIpro Platinum 1.1 Gel Software (Eppendorf).

2.9. Cloning and sequencing of DGGE fragments

Selected DGGE bands were extracted from the gels, transferred into 50 μl sterile water and incubated overnight at 4 °C. Two microliters of the eluted DNA was reamplified by using the conditions described above and checked by DGGE. PCR products that gave a single band comigrating with the control were then amplified with the primer without GC clamp, purified by Perfectprep Gel Clean up (Eppendorf) and cloned using the pGEM^T-plasmid vector system (Promega, Madison, Wis., USA). Selected transformants were heat lysated (treatment for 10 min at 100 °C), V_1 16S rRNA gene regions were amplified with primers P_1V_1GC and P_2V_1 and, after DGGE analysis, compared to the original band cut from the sample. The clones that produced a single DGGE amplicon comigrating with the control were sent for plasmid insert sequencing to MWG Biotech.

Table 3
Means and standard deviations (SD) of chemical compound concentrations determined by HPLC

Chemical compounds	Samples from manufacture and ripening [a]															
	M		Cu		Cu24		Cu3W		Ch3S		Ch30		Ch60		Ch90	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Acetic acid	0.025	0.021	0.002	0.003	0.332	0.086	1.127	0.013	1.097	0.037	0.835	0.173	0.877	0.302	0.697	0.310
Butyric acid	nd	–	nd	–	nd	–	0.005	0.005	0.002	0.003	0.043	0.055	0.010	0.010	0.112	0.028
Citric acid	1.727	0.194	1.360	0.162	1.910	0.467	0.575	0.160	0.288	0.096	0.065	0.039	0.032	0.008	0.032	0.008
Formic acid	0.225	0.030	0.226	0.103	0.238	0.094	0.214	0.168	0.216	0.051	0.340	0.064	0.198	0.101	0.064	0.090
Hippuric acid	0.022	0.009	0.024	0.022	nd	–	nd	–	Nd	–	nd	–	nd	–	nd	–
Iso-butyric acid	nd	–	nd	–	nd	–	nd	–	Nd	–	nd	–	nd	–	nd	–
Iso-valeric acid	nd	–	nd	–	nd	–	0.055	0.095	Nd	–	0.015	0.015	0.103	0.084	0.170	0.202
Lactic acid	0.077	0.011	0.122	0.055	17.387	2.423	28.580	1.610	26.480	1.245	19.755	1.531	14.957	1.006	8.090	0.438
n-Valeric acid	nd	–	nd	–	nd	–	nd	–	Nd	–	nd	–	nd	–	0.003	0.006
Orotic acid	0.025	0.007	0.033	0.015	0.012	0.003	0.008	0.003	0.008	0.003	0.022	0.020	0.008	0.006	0.038	0.025
Oxalic acid	0.390	0.042	0.353	0.093	0.980	0.076	0.318	0.252	0.035	0.017	0.057	0.089	0.028	0.036	0.243	0.235
Propionic acid	nd	–	nd	–	0.081	0.033	0.081	0.004	0.133	0.013	0.721	0.305	1.053	0.155	1.449	0.644
Pyruvic acid	nd	–	0.005	0.000	0.092	0.038	0.210	0.109	0.303	0.165	0.203	0.061	0.120	0.080	0.038	0.003
Uric acid	0.013	0.003	0.023	0.003	0.010	0.000	0.003	0.006	0.005	0.005	0.005	0.005	0.025	0.020	0.060	0.025
Galactose	0.058	0.022	0.095	0.007	0.173	0.169	0.245	0.024	0.034	0.033	nd	–	nd	–	nd	–
Glucose	0.005	0.007	0.007	0.008	nd	–	0.068	0.037	nd	–	0.012	0.020	0.010	0.017	0.007	0.012
Lactose	49.125	1.520	35.395	3.614	7.677	1.570	1.367	1.139	0.273	0.127	nd	–	nd	–	nd	–
Acetoin	nd	–	nd	–	0.003	0.003	0.065	0.054	0.030	0.030	0.015	0.026	0.022	0.023	0.072	0.033
Diacetyl	nd	–	nd	–	0.075	0.130	0.472	0.143	0.482	0.238	0.603	0.391	0.463	0.215	0.783	0.122

Reported values were expressed as g kg^{-1} of milk, curd and cheese (nd—not determined).
[a] M: milk; Cu: curd after cutting; Cu24: curd after 24 h; Cu3W: curd after 3 days rest in whey; Ch3S: cheese after 3 days salting; Ch30: cheese after 30 days ripening; Ch60: cheese after 60 days ripening; Ch90: cheese after 90 days ripening.

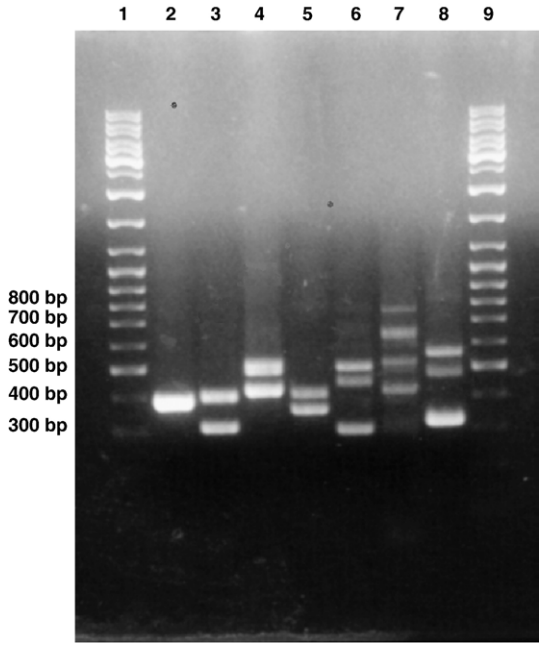

Fig. 1. RSA profiles obtained from the LAB isolated from Castelmagno PDO cheese during manufacturing and ripening. Lanes 1 and 9: 1 Kb DNA Ladder (MBI); Lanes 2 to 8: amplification profiles obtained by RSA analysis.

2.10. Sequence analysis

To determine the closest known relatives of the clones, partial 16S rRNA gene sequences were aligned with those in GenBank with Blast program (Altschul et al., 1997).

2.11. HPLC analysis

Milk, curd and cheese samples were subjected to determination of organic acids (citric, orotic, pyruvic, lactic, oxalic, uric, hippuric, formic, acetic, propionic, butyric, isobutyric, valeric and iso-valeric), sugars (lactose, glucose and galactose), and ketones (diacetyl and acetoin) by high performance liquid chromatography according to the method described by Zeppa et al. (2001). Each sample was analysed in triplicates.

3. Results

3.1. Dynamic of microbial counts throughout manufacturing and ripening

The two way ANOVA did not highlight significant differences among the microbial loads of the three batches at each sampling point. The mean of microbial counts during cheese-making and ripening of Castelmagno PDO cheese, and the pH values of milk, curd and cheese samples, are summarized in Table 2.

The main microflora present in milk, curd and cheese samples from three subsequent manufacturing batches is shown. The total aerobic bacteria count was 7.4×10^5 cfu/mL in raw milk and reached the highest value 7.9×10^9 cfu/g in the curd after 3 days rest in whey.

LAB present in raw milk (10^5 to 10^6 cfu/mL) increased to the highest value 7.6×10^9 cfu/g within 5 days of manufacture immediately before salting, when lactic acid production was also completed (Table 3). LAB kept values ranging from 10^8 to 10^9 cfu/g until 30 days of ripening, then they started to strongly decrease reaching the lowest load of 10^6 cfu/g after 90 days of ripening. Strong differences between mesophilic and thermophilic LAB microflora counts were not revealed as between presumptive lactococci and lactobacilli isolated from M17 and MRS agar plates, respectively. Enterococci showed a development trend comparable to lactococci and lactobacilli even if characterized by lower microbial counts.

The CNC were present with 2.8×10^4 cfu/mL in raw milk and increased during manufacture to values not higher than 10^7 cfu/g. The salting process initially decreased the speed of development of CNC; they reached the maximum count value of 5.9×10^8 cfu/g after 30 days ripening, when LAB, still strongly present, started to decrease and consequently the pH value slightly increased. The increase of the pH to 5.3 can be also explained by the proteolytic activity of microorganisms involved in the fermentation. Yeasts reached the highest level in curd samples, then they started to decrease; we did not detect any mould growth on Malt Agar plates. Coliforms rapidly reached values of 8.8×10^6 cfu/g in the curd after 24 h. Their

Table 4
Number of isolates of LAB species found in samples[a] from manufacture and ripening of the three batches (I, II, III)

Species	M			Cu			Cu24			Cu3W			Ch3S			Ch30			Ch60			Ch90		
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
L. lactis lactis	4	5	4	3	3	2	9	11	13	5	5	6	6	6	11									
L. lactis cremoris				1	2	1	1	1	1	1		1	1											
L. paracasei													2	1	1	8	6	8		2	4	1	2	2
L. plantarum															1	2	1	1	6	5	6	5	2	4
L. coryniformis torquens																							4	
L. delbrueckii lactis												4												
L. casei																								1
Enterococcus sp.						1	1							1	1	4	9	3	5	1	1	2	4	5
E. faecium				2	1	1	1	1								1	1	3	2	2		2	3	3
E. faecalis	1	3	1		1	1											2	1	8	3				
E. durans																						1		

[a] M: milk; Cu: curd after cutting; Cu24: curd after 24 h; Cu3W: curd after 3 days rest in whey; Ch3S: cheese after 3 days salting; Ch30: cheese after 30 days ripening; Ch60: cheese after 60 days ripening; Ch90: cheese after 90 days ripening.

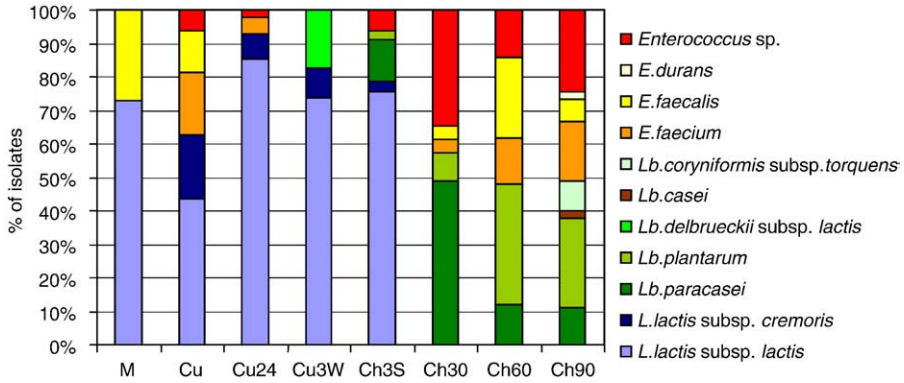

Fig. 2. Frequency of isolation of LAB species throughout 3 productions of Castelmagno PDO. M, milk; Cu, curd after cutting; Cu 24, curd after 24 h; Cu3W, curd after 3 days rest in whey; Ch3S, cheese after 3 days salting; Ch30, cheese after 30 days ripening; Ch60, cheese after 60 days ripening; Ch90, cheese after 90 days ripening.

following decrease can be correlated with the pH drop and the salting process. At 60 days of ripening they were <10 cfu/g.

3.2. Molecular identification of LAB isolates

A total of 274 presumptive LAB isolates were randomly selected from agar plates and subjected to molecular identification. A first clustering identification step was reached by RSA obtaining seven different profiles (Fig. 1). Cluster I was composed of 101 isolates and its profile was characterized by a unique band migrating approximately 390 bp (Fig. 1, lane 2). In cluster II, 51 isolates were grouped, characterized by two bands migrating at 300 and 400 bp (Fig. 1, lane 3). For other 32 isolates two amplification bands at 400 and 500 bp were obtained (cluster III) (Fig. 1, lane 4). Only one isolate showed a RSA profile characterized by two bands migrating at 350 and 400 bp (cluster IV) (Fig. 1, lane 5). Clusters V, VI and VII, including 39, 25 and 10 isolates, respectively, exhibited a three or four bands profile (Fig. 1, lanes 6, 7, 8).

Species-specific primers were necessary to finally establish the taxonomic positions of isolates. Within cluster I the isolates were discriminated between the two major subspecies of *Lactococcus lactis*, *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*. Clusters II, III and IV included enterococcal isolates belonging respectively to the species *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus durans*. The remaining 39 isolates from clusters II and III were classified as belonging to *Enterococcus* genus. The isolates within group V were identified as *Lactobacillus paracasei*, while isolates within both clusters VI and VII resulted positive for *Lactobacillus plantarum* specific primers.

For 15 isolates it was not possible to establish a well defined RSA profiles thus they were submitted to 16S rRNA gene sequencing. Four isolates resulted to belong to the species *Lactobacillus delbrueckii* subsp. *lactis*, 4 to *Lactobacillus coryniformis* subsp. *torquens* and 1 to *Lactobacillus casei*. The remaining 6 isolates were classified as species belonging to *Staphylococcus* genus.

In Table 4 the distribution, among batches and sampling times, of the number of isolates of LAB species is reported.

L. lactis subsp. *lactis* was dominant in the milk, in the curd samples and in the cheese after 3 days of salting, and its frequency ranged from 43.8% to 85.4% among LAB isolates (Fig. 2). From the same samples *L. lactis* subsp. *cremoris* was also isolated even if the percentages were not higher than 18.8%. Thus lactic fermentation process involved mainly lactococcal species that subsequently decreased during the ripening when populations of *Lactobacillus* dominated. *L. plantarum* and *L. paracasei* were the most frequently species isolated in aged cheese samples with frequencies ranging from 3.0% to 36.0% and from 11.1% to 49.0% respectively. *L. delbrueckii* subsp. *lactis*, *L. coryniformis* subsp. *torquens*

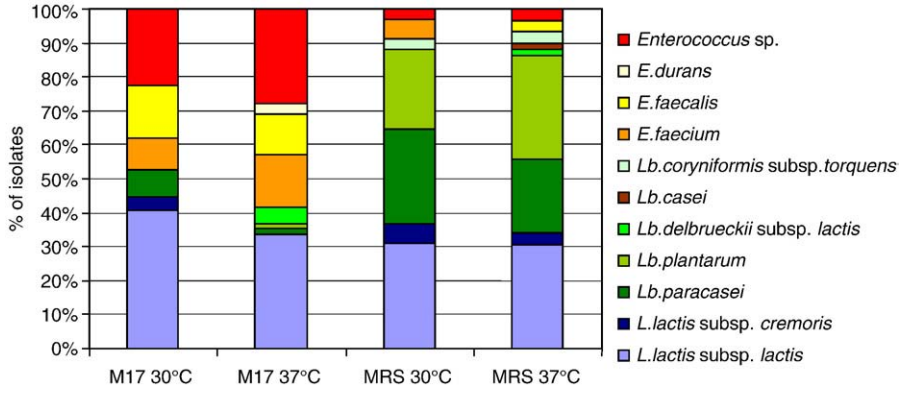

Fig. 3. Prevalence of LAB species among isolates randomly selected on M17 agar and MRS agar plates incubated at both 30 and 37 °C.

and *L. casei* were occasionally isolated, as reported above, with low percentage (Fig. 2).

E. faecium and *E. faecalis* prevailed among enterococci. Together with other *Enterococcus* sp. isolates, these species were strongly present in milk and curd samples; their presence decreased during later manufacturing stages while again they showed an important presence during ripening. One *E. durans* isolate was found in a 90 days ripened cheese sample.

In Fig. 3 the frequency of isolation of each LAB species in correlation to the selective isolation LAB media is reported. M17 and MRS agar showed low selectivity towards lactococcal isolates obtained from both media with comparable percentages. On the contrary enterococci grew preferably on M17 agar plates while MRS agar was quite discriminating for lactobacilli. The incubation temperature used in the study did not allow to obtain a growth differentiation among LAB species isolated belonging to mesophilic microflora; however it is noteworthy that the only thermophilic species isolated, *L. delbrueckii* subsp. *lactis*, grew on agar plates incubated at 37 °C.

3.3. PCR-DGGE monitoring of microbial dynamics in Castelmagno PDO cheese

Total microbial DNA from all three batches was extracted from samples ranging from raw milk to 90 days ripened cheese and analysed by PCR-DGGE. Fig. 4 shows DGGE profiles of the PCR amplicons belonging to V1 regions of bacterial 16S rRNA gene of one batch analysed and chosen as representative; important differences in fingerprints were not observed among the three batches

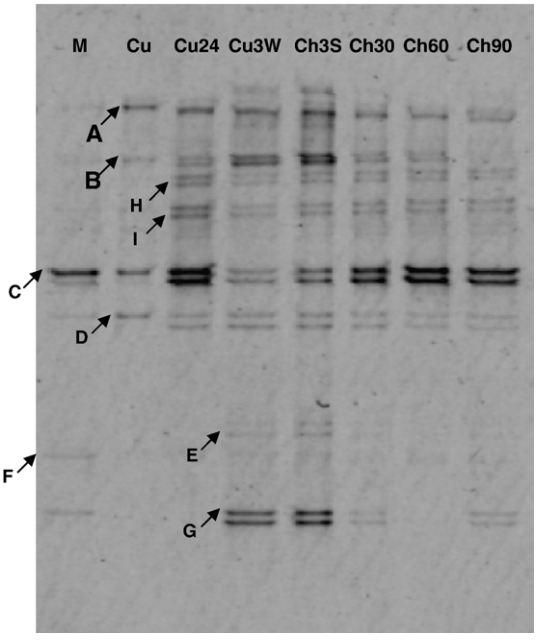

Fig. 4. DGGE profiles of PCR products of the bacterial V1 region of the 16S rDNA of samples taken during artisanal Castelmagno PDO cheese manufacturing and ripening. Lanes: M, milk; Cu, curd after cutting; Cu 24, curd after 24 h; Cu3W, curd after 3 days rest in whey; Ch3S, cheese after 3 days salting; Ch30, cheese after 30 days ripening; Ch60, cheese after 60 days ripening; Ch90, cheese after 90 days ripening. Bands A to G were identified and the results obtained are reported in Table 5.

Table 5
Sequence information for dominant fragments in DGGE profiles obtained by analysing the total microbial community in Castelmagno PDO manufacturing and ripening

Band	Closest sequence relative	% Identity	GenBank accession no.
A	*Lactobacillus plantarum*	100%	EF185922
B	*Streptococcus agalactiae*	100%	DQ232516
C	*Lactococcus lactis* subsp. *lactis*	100%	EF114309
D	*L. lactis* subsp. *cremoris*	100%	CP000428
E	*Lactobacillus* sp.	97%	AB262680
F	*Macrococcus caseolyticus*	98%	EF032686
G	*Lactobacillus kefiranofaciens*	98%	AJ575262

and the variability in microbial composition, assessed by number of bands and migration position in DGGE gels, was low among milk, curd and cheese samples analysed (data not shown).

Generally no dramatic changes were noticed in terms of appearance and disappearance of bands in DGGE profiles of collected samples.

In order to eliminate band duplicates probably due to unfinished extensions of amplicons from primer with GC clamp, final extension time was elongated to 30 min, as advised by Janse et al. (2004), but without improvement.

After excision from DGGE gels, bands A to G were re-amplified with primers P_1V_1 and P_2V_1 and provided suitable amplicons for identification. Unfortunately, purification and reamplification of bands H and I did not prove successful.

After cloning, bands A to G were sent to MWG Biotech for sequencing and the results obtained after alignment in GenBank are shown in Table 5. Band A originated from *L. plantarum* and band G from *Lactobacillus kefiranofaciens*; clones corresponding to band E derived from species belonging to the *Lactobacillus* genus. *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* were referred respectively to bands C and D. Band B belonged to *Streptococcus agalactiae* and band F to *Macrococcus caseolyticus*.

Bands C and D were both detected in all collected samples. Band A was present in every sample however in milk it was almost imperceptible. Band B was detected from milk to 60 day ripened cheese. Band G was strongly detected in the curd after rest in whey and after 3 days salting. Band E weakly appeared in curd samples after rest in whey and at the beginning of ripening while band F was weakly present in milk, curd and cheese samples.

3.4. HPLC analysis

The composition in organic acids, sugars and ketones of the dairy samples analysed is reported in Table 3. A low variability was observed for all examined compounds in samples from the three subsequently batches. Lactic acid metabolism, and lactose consumption were strongly related to LAB growth trend, as well as consumption of glucose and galactose metabolized by LAB.

4. Discussion

The cheese microflora play a critical and pivotal role in the development of the unique characteristics of each cheese variety.

High densities of indigenous microorganisms selected by the cheesemaking environment are present throughout manufacture and ripening of artisanal cheeses and play a significant role in the maturation process.

The main objective of cheese microbiology is to develop a clear view of microflora evolution throughout cheese manufacture and ripening. It is important that the whole microflora is monitored and that individual components are accurately identified and characterised. We attempted to reach this objective acquiring information about the different microbial groups involved in Castelmagno PDO production.

Microbiological counts obtained from Castelmagno PDO samples highlighted a significant presence of all microbial groups targeted with values comparable to those reported for other italian ripened cheeses (Randazzo et al., 2002; Randazzo et al., 2006). According to other authors (Ampe et al., 1999; Randazzo et al., 2002; Ercolini et al., 2003) it is noteworthy that some of the used media were not very selective; in fact staphylococci isolates were found on M17 and MRS agar plates.

LAB resulted to be the prevailing microflora in all production stages; they reached the highest levels in the curd after 3 days rest in whey, a typical stage in the manufacture of this cheese. They started to strongly decrease in aged cheeses as a result of autolysis and microbial competition events (Fox et al., 2004).

The incubation temperature used in the study did not allow to differentiate mesophilic and thermophilic LAB species, except for the thermophilic *L. delbrueckii* subsp. *lactis*, which grew exclusively at 37 °C. Other authors used this incubation temperature (Fortina et al., 2003; Torres-Llanez et al., 2006) to discriminate mesophilic and thermophilic microflora in dairy environments; however we should consider the possibility to broaden the temperature range.

Enterococci, CNC and yeasts also showed considerable viable counts and they may be responsible for the moderate lipolytic activity detected by HPLC. In fact the combined lipolytic activity of these microorganisms could be the reason for the presence, in the dairy samples analysed, of free short-chain fatty acids as formic, acetic, propionic, butyric, valeric and isovaleric, during cheese manufacturing and especially ripening. The presence of these compounds in dairy environments has been related from many authors to the intensity of the lipolytic activities of secondary microflora and enterococci (Fox et al., 2004; Mucchetti and Neviani, 2006).

Diacetyl and acetoin production in ripened cheese samples could be correlated to enterococci activity. Citrate metabolism from enterococci has been widely described (Coppola et al., 1988; Parente et al., 1989; Litopoulou-Tzanetaki et al., 1993; Centeno et al., 1996).

As expected coliforms progressively decreased in number as a consequence of pH drop during ripening process and they were not detected (detection limit 10 cfu/g) at the end of the study.

L. lactis subsp. *lactis* was the species most frequently isolated during Castelmagno PDO manufacture, and thus responsible for acid production and pH drop. Lactic acid production carried out by lactococcal isolates was followed by its slowly consumption in ripening stages. This reduction could be explained, in Castelmagno PDO samples analysed, with the appearance of lactobacilli, in particular *L. plantarum*, characterized by oxidation activity (Mucchetti and Neviani, 2006).

Although lactococcal isolates are strongly inhibited from the salt, high percentages of *L. lactis* subsp. *lactis* were isolated from Castelmagno PDO cheese samples after 3 days salting. High occurrence of wild *L. lactis* subsp. *lactis* as predominant species in cheesemaking was detected in many European artisanal dairy products (Cogan et al., 1997).

Considerable amount of other LAB is commonly found during cheese ripening (Beresford et al., 2001), which promotes in particular the development of heterofermentative lactobacilli belonging to the so called "secondary culture" strongly contributing to flavour development.

L. plantarum and *L. paracasei* species were isolated from ripened Castelmagno PDO cheese samples with the highest frequencies. These species, generally absent in the milk, occur in dairy ecosystems and dominate the bacterial flora of many ripened semihard cheeses (Beresford et al., 2001). Occasionally the species *L. delbrueckii* subsp. *lactis*, *L. coryniformis* subsp. *torquens* and *L. casei*, characteristics of dairy environment (Bélen Flòrez and Mayo, 2006; Coppola et al., 2003; Fortina et al., 2003; Randazzo et al., 2002), were isolated from the 3 Castelmagno PDO batches.

The energy source used by lactobacilli for growth has not yet been clearly defined, since at the time of most active growth of lactobacilli, lactose has been exhausted (Beresford et al., 2001). In Castelmagno PDO cheese citric and lactic acids could be hypothesized as potential energy source (Cogan et al., 1997; Mucchetti and Neviani, 2006).

E. faecalis and *E. faecium* species were isolated with high percentage from Castelmagno PDO milk and curd samples as well as aged cheese. Although their presence in manufacturing samples is mainly associated to low hygienic conditions during milking and storing process (Garcia Fontan et al., 2001), their occurrence in ripened cheese could be related to an intense lipolytic activity (Mucchetti and Neviani, 2006). Enterococcal presence is widely reported in artisanal dairy products (Cogan et al., 1997).

The development of culture-independent methods for microbial analysis has revolutionised microbial ecology and its application to cheese microbiology is leading to major new insights into this complex microbial ecosystem. The results obtained on Castelmagno PDO microflora underlines a partial correspondence between culture-dependent and culture-independent methods. DGGE revealed a predominance of *L. lactis* subsp. *lactis* throughout Castelmagno PDO cheesemaking and ripening. This species was isolated with high frequency on agar plates from milk and curd samples but then it was not found among isolates from ripened cheeses. The same divergence was noticed for *L. lactis* subsp. *cremoris*, detected by both methods with lower incidence compared to *L. lactis* subsp. *lactis*. *L. plantarum* was detected in all samples by DGGE while it was isolated on agar plates exclusively from aged cheese samples. *L. paracasei*, found in cheese samples with moderate incidence, was not highlighted by DGGE. *L. kefiranofaciens* was detected in DGGE gels in the last stages of manufacture while this species was not isolated by culturing on selective media. *Lactobacillus* species occasionally isolated by culture-dependent method from cheesemaking and ripening were *L. delbrueckii* subsp. *lactis*, *L. casei* and *L. coryniformis* subsp.

torquens. *M. caseolyticus* was weakly detected by DGGE in milk, curd and cheese samples; it forms part of the normal microbiota of cattle and other animals which explains its presence in the milk. As also reported for other dairy products (Randazzo et al., 2002) its presence was not revealed by culture-dependent methods. *S. agalactiae* was observed in DGGE gels in Castelmagno PDO samples, while was not detected on agar plates. This species is found in the milk as mastitis pathogen (Oliver et al., 2004).

Culturing on selective media showed an high incidence of enterococcal isolates, especially *E. faecium* and *E. faecalis*, which were not detected by DGGE.

Divergences in bacterial species detection between culture-dependent and culture-independent methods could be due to different reasons; for example the permanence in cheese matrix of bacterial DNA coming from cellular autolysis, or the high selectivity of some media towards specific microorganisms which find optimal conditions for their growth. Thus the present research highlights the importance to combine molecular culture-independent approaches with classical microbiological methods for the study of complex environmental communities from food matrix.

The results obtained represent the first approach to the understanding of microbial dynamics in Castelmagno PDO, a traditional Italian cheese characterized by typical cheesemaking without the addition of any starter culture. The research was focused on summer alpine production from which the traditional salty and moderate piquant aged Castelmagno PDO cheese comes from. Further studies on winter valley floor production will be considered to give a more wide-ranging knowledge of the microbiological properties of Castelmagno PDO cheese.

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