Microbial diversity, dynamics and activity throughout manufacturing and ripening of Castelmagno PDO cheese

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Microbial diversity, dynamics and activity throughout manufacturing and ripening of Castelmagno PDO cheese (Running title: Microbiology of Castelmagno PDO cheese)

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ABSTRACT

The diversity, dynamics and activity of Castelmagno PDO cheese microbiota were studied in three batches produced in a floor valley farm, in the Grana Valley (northwest Italy), during the wintertime. Samples of milk, curd and cheese (core and subsurface) at different ripening time were submitted to both culture-dependent and –independent analysis. In particular, DNA and RNA directly extracted from the matrices were studied by PCR-*Denaturing gradient gel electrophoresis* (DGGE) and *reverse transcription* (RT)-PCR-DGGE. Culture-dependent methods highlighted the initial dominance of a thermophilic streptococcal population with the species *Streptococcus thermophilus* and *Streptococcus agalactiae*. Then, mesophilic lactococci occurred among isolates during manufacturing, with *Lactococcus lactis* which was also well represented in the first month of Castelmagno PDO maturing. At this point and throughout the ripening, lactobacilli prevailed in cheese samples, represented from *Lactobacillus plantarum* and *Lactobacillus casei*. Culture-independent analysis underlined the undoubted role of *L. lactis*, actively involved in both Castelmagno PDO manufacturing and ripening. Despite *Lactobacillus helveticus* was never isolated on selective media, a DGGE band referred to this microorganism was detected, at RNA level, in samples from ripened cheeses. On the other hand, *Lb. plantarum* was widely isolated from the plates, among lactobacilli, but never detected by direct analysis. Due to the importance of microbiota in the sensory richness and properties of traditional cheeses, new information have been added, in this work, on microbial diversity of Castelmagno PDO cheese.

Keywords: microbial ecology; PCR-DGGE; RT-PCR-DGGE; lactic acid bacteria; Castelmagno PDO cheese.

INTRODUCTION

The complex technology of Castelmagno cheese, bearing a protected designation of origin (PDO), favours the growth of an active microbiota which, together with milk and rennet characteristics, imparts unique properties to this product. In figure 1, the main steps in the technology of this cheese are reported. Briefly, raw milk from cows bred and fed in Grana Valley (northwest Italy), is collected and added of calf rennet. The traditional technique does not provide for the addition of natural or selected starter cultures and, in this sense, Castelmagno PDO is designed as an "artisanal" cheese (Randazzo et al., 2009). Then the coagulum is cut and pulled out from the whey which is further removed by hunging the collected curd in cloth bags or by leaving it on a slanted top for at least 18 hours. At this point, the curd is dip in tanks filled with whey coming from previous cheesemaking, for 3-6 days. This is a critical step in the technology of Castelmagno PDO, imparting, together with the subsequent crumbling, the typical texture, compact and friable at the same time, to the product. After whey treatment, the curd is submitted to crumbling and salting. Finally, it is pressed in moulds and ripened in natural caves at 10-12 °C, for ripening time ranging from 60 to 90 days, or up to 150 and 180 days for peculiar productions.

The knowledge of the microbial communities present in raw milk, and the possibility to follow them in cheesemaking and ripening processes, would promote better understanding of how cheese characteristics vary with respect to microbial growth and activity (Beresford et al., 2001). Nowadays, it is well known the need to combine culture-independent approaches and traditional microbiological methods to describe complex microbial ecosystems as cheese matrices (Alegria et al., 2009; Bonetta et al., 2008; Casalta et al., 2009; Dolci et al., 2008b; Ercolini et al., 2003; Randazzo et al., 2006). In order to overcome the limitations arising from culture-dependent techniques (Hugenholtz et al., 1998), *Denaturing gradient gel electrophoresis* (DGGE), based on

the species-specific sequence separation of amplified regions of the 16S rRNA gene, has been already applied in the study of the microbiota in several dairy products (Alegrìa et al., 2009; Flòrez and Mayo, 2006; Randazzo et al., 2006; Rantsiou et al., 2008).

Due to the importance of microbiota in the sensory richness and variety of traditional cheeses, in this work, new information have been added on floor valley Castelmagno PDO cheese manufactured in the wintertime, with remark for lactic acid bacteria (LAB). This in addition and compared with a previous study on summer alpine pasture productions of the same cheese (Dolci et al., 2008a). Both culture-dependent and –independent methods, on cheese core and subsurface layer, have been applied and, in particular, in order to reveal metabolically active populations, RT-PCR-DGGE has been also performed (Randazzo et al., 2002; Rantsiou et al., 2008).

MATERIALS and METHODS

Sampling

Three batches from a floor valley farm in the Grana Valley (northwest Italy) were studied during the wintertime. There were three-day intervals between the batches and different production steps were analysed (Figure 1): raw milk (M), curd after cutting (Cu), curd after 24h from the production (Cu24), curd after 3 days of rest in whey (Cu3W), cheese after 3 days salting (Ch3S), cheese after 30 days ripening (Ch30), cheese after 60 days ripening (Ch60), cheese after 90 days ripening (Ch90), cheese after 150 days ripening (Ch150). Each sample was subjected to microbiological analysis not more than 2 hours after collecting.

Microbial counts and collection of isolates

Twenty milliliter of milk and 20 g of curd and cheese samples were 1:5 homogenized in Ringer ¼ strength solution (Oxoid, Milan, Italy). Two layers were cut up and analysed for each cheese sample: a 4-cm-thick section from the core of the product and a 3-cm-thick section from the subsurface, precisely 2 cm under the rind. Milk, curd and cheese samples were subjected to the detection of the following microbial groups: aerobic mesophilic bacteria on Gelatin Peptone Agar

(GEL) (Oxoid) at 30 °C for 48 h; lactococci on M17 agar (Fluka, Buchs SG, Switzerland) at 37 °C for 48 h; lactobacilli on MRS agar (Oxoid), incubated anaerobically in jar, at 37 °C for 48 h; enterococci on Kanamycin Aesculin Azide Agar (KAA) (Fluka) at 37 °C for 48 h; coagulase-negative cocci (CNC) on Mannitol Salt Agar (MSA) (Oxoid) at 30 °C for 48 h; *Staphylococcus aureus* on Baird Parker medium (Oxoid) with added egg yolk tellurite emulsion at 37 ºC for 48 h; coliforms on Violet Red Bile Lactose Agar (VRBA) (Oxoid) at 37 °C for 24 h; yeasts and moulds on Malt Agar (Oxoid) supplemented with tetracycline (1 μg/ml) (AMT) at 25 °C for 96 h; *Listeria* spp. on chromogenic media PALCAM (Oxoid), upon sample enrichment in Fraser broth (Oxoid).

Between five to fifteen randomly selected colonies of presumptive lactococci and lactobacilli were isolated for each collection step, respectively, from M17 and MRS agar plates. Colonies were restreaked for purification and stored at -80 °C in M17 and MRS broth, mixed with 30% glycerol, prior to molecular analysis.

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 point. Then means and standard deviations of count values were calculated. Moreover, significant differences among means were further analysed singularly for each specific medium and time of ripening, between core and subsurface layers, using the Duncan test.

The pH measurements were determined by placing a penetration electrode (Portamess 913, Knick, Berlin, Germany) in contact with milk, curd and cheese mass. All analysis were performed in triplicates.

Molecular identification of lactic acid bacteria (LAB) isolates

Cell pellets of LAB isolates were subjected to DNA extraction as previously reported (Cocolin et al., 2001). The molecular identification of the isolates was performed by DGGE analysis and sequencing of partial 16S rRNA according to the methods described by Cocolin et al. (2001) and Rantsiou et al. (2008).

Analysis of dairy samples by culture-independent methods

To detect the main bacterial species involved at each sampling point, DNA and RNA were directly extracted from the matrices and subjected to PCR and RT-PCR, respectively, with universal primers annealing the V_3 region of 16S rRNA gene, followed by DGGE.

Nucleic acid extraction

Sample preparation and nucleic acid extraction were performed according to the protocol reported by Rantsiou et al. (2008). The presence of residual DNA, in RNA samples, was checked by PCR (Cocolin et al., 2001).

PCR-DGGE and RT-PCR-DGGE

PCR and RT-PCR were performed with primers 338f and 518r as described by Alessandria et al. (in press). The amplicons obtained were analysed by DGGE according to the protocol described by Dolci et al. (2008b). DNA and cDNA bands were sent for sequencing to Eurofins MWG Operon (Dolci et al., 2008b).

RESULTS

Microbial counts and pH

The microbial counts, reported in Table 1, are expressed as means and standard deviations of the values of the three batches analysed. According to the two way ANOVA analysis, there was no significant differences between the batches.

Due to the shape of Castelmagno PDO rounds, reaching to 20 cm height, the microbial dynamics were followed both in the core of the cheese and in the subsurface layer. Actually, the trends were very similar and the main microbial groups developed mostly with comparable counts according to Duncan test.

Presumptive lactococci and lactobacilli varied, in the milk, from 6.5 and 6.8 Log cfu/g, respectively, to 8.7 $\log_{10}$ Log in the curd after 3 days rest in whey. They were still found with values of 8.1 Log cfu/g in the cheese 3 days after the salting treatment, and then they decreased to 7.0 Log cfu/g and to about 6.0 Log cfu/g in the cheese after 30 and 150 days of ripening, respectively. Enterococci

developed with a similar trend to lactococci and lactobacilli but with lower count values reaching, in the product after 3 days of salting treatment, the maximum of 6.5 and 6.2 Log cfu/g in the cheese core and subsurface, respectively. CNC were present, in raw milk, with values of 4.4 Log cfu/g and reached 5.0 Log cfu/g in the cheese after 3 days of salting treatment. At this point, at subsurface level, CNC population showed a light increase, during ripening, not exceeding 5.6 $\log_{10}$ cfu/g. Differently, in the core, CNC established on values ranging from 3.7 Log cfu/g to 4.6 Log cfu/g. Yeasts reached the highest values of 4.9 Log cfu/g and 5.5 Log cfu/g, in the core and in the layer under the rind, respectively, in cheese samples 30 days ripened, and, then, markedly declined to about 2.0 Log cfu/g in cheeses ripened for 150 days. No moulds were detected on AMT plates. Coliforms, found with values of 5.4 Log cfu/g in the curd after 24 hours from the production, dropped to 3.9 Log cfu/g in the curd after 3 days of rest in whey, in line with the pH drop,. *Staphylococcus aureus* was < 5 cfu/mL or cfu/g in all the samples analysed while *Listeria* spp. were never detected.

LAB isolate identification and distribution throughout Castelmagno PDO manufacturing and ripening

According to LAB identification, in table 2, the overall distribution of the main recurring LAB species, in the manufacturing and ripening process, has been shown. For each sampling point, the sum of the isolates obtained from the three different productions and belonging to different LAB species, has been reported.

During the manufacture process, only coccoid isolates were obtained and identified, with the exception of *Lactobacillus plantarum*, found in curd samples after 3 days of rest in whey. *Streptococcus thermophilus* and *Streptococcus agalactiae* dominated milk and curd samples after the cutting, while in the curds sampled after 24 h from the beginning of the manufacture and after whey treatment, *Lactococcus lactis* subsp. *lactis* isolates were also found. *L. lactis* subsp. *lactis* population increased at the beginning of the ripening, with, in addition, the modest occurrence of *Lactococcus lactis* subsp. *cremoris*. *Lb. plantarum* isolates were strongly present throughout the

ripening. They prevail among LAB population in cheese samples at 30, 90 and 150 days, while, at 60 days of maturation, *Lactobacillus casei* was isolated with the highest percentage among LAB population, exclusively composed, at this stage, of lactobacilli. *Lactobacillus coryniformis* subsp. *torquens* and *Lactobacillus acidipiscis* were also found during the ripening process even if with lower frequencies (Table 2). Occasionally, isolates belonging to the following species were found in matured samples: *Lactobacillus crustorum*, *Lactobacillus kefiri*, *Lactobacillus parabuchneri*, *Enterococcus faecalis*, *Enterococcus malodoratus*, *Corynebacterium aurimucosum*, *Pediococcus siamensis*, *Micrococcus luteus* and *Pseudomonas* sp. Totally, 386 isolates were identified as belonging to the main LAB microbiota. Twenty two more isolates were occasionally found in the samples analysed, referred to the LAB and no-LAB species reported above. No marked differences have been detected in the distribution of the bacterial species between the core of the cheese and the subsurface, though, among environmental occasional microbiota, *M. luteus*, *Pseudomonas* sp. and *C. aurimucosum* were isolated exclusively from the subsurface.

Bacterial species involved in Castelmagno PDO manufacturing and ripening detected by culture-independent methods

Direct analysis of bacterial DNA from the matrices highlighted the persistence, throughout manufacturing (M) (referred to samples from milk to the curd after 3 days of rest in whey) and ripening (R) (referred to samples of cheese from 3 to 150 days of ripening) (Figure 2a), of the species *L. lactis* (band b) and *S. agalactiae* (band a). Moreover, a band referred to *L. lactis* subsp. *cremoris* (band c) was detected in all cheese samples, since 3 days of maturing. RNA analysis (Figure 2b) showed the viability of *S. agalactiae* (band d) and *L. lactis* subsp. *cremoris* (band c) in milk, curd and cheese samples. *Lactobacillus helveticus* (band e) was detected, during the maturing, in cheese samples at 3, 60 and 150 ripening days. At 60 days, a band referred to the genere *Sphingomonas* sp. (band f) occurred on DGGE profiles. Direct analysis of the matrices did not highlight differences in the bacterial ecology of core and subsurface. The sequence information relating to the bands excised from the gels are reported in Table 3.

DISCUSSION

The cell numbers of the main microbial groups detected in Castelmagno PDO samples, were generally within the limits observed by other authors (Alegrìa et al., 2009; Gobbetti et al., 1997; Ercolini et al., 2003; Randazzo et al., 2006); however it is worth noting the lower amounts of LAB population, especially if compared to data from summer alpine pasture productions of Castelmagno PDO cheese (Dolci et al., 2008a). A season influence on microbial population level could be hypothesized, as also noticed, from other authors, in different studies on dairy matrices (Bonetta et al., 2008; Gaya et al., 2003).

In milk and fresh curds, *S. thermophilus* and *S. agalactiae* predominated the bacterial population. Their prevalence was quite unexpected considering the thermic treatment of the milk not exceeding 33 °C. *L. lactis* subsp. *lactis* occurred among isolates during manufacturing and it was well represented in the first month of Castelmagno PDO maturing. Mesophilic *Lb. plantarum* and *Lb. casei* prevailed throughout the Castelmagno PDO maturing, suggesting their central role, as so-defined "secondary culture", in the definition of cheese flavour characteristics (Berta et al., 2009; Madrau et al., 2006; Van-Horde et al., 2008). *Lb. coryniformis* subsp. *torquens* isolates were also found, even if with more modest numbers. Their presence have been already detected in a previous work on Castelmagno PDO cheese (Dolci et al., 2008a), produced during the summer alpine pasture. Thus, an involvement of this species in the definition of the typical aromatic characteristics of this traditional cheese could, eventually, be suggested.

The direct analysis of both bacterial DNA and RNA highlighted the persistence of active cells of *S. agalactiae* not only in milk and curd samples, where it usually is recovered (Braun and Preuss, 2007; Fortina et al., 2003), but also in cheese throughout the whole ripening period. Lactococci have been also detected by PCR-DGGE and RT-PCR-DGGE. A DGGE band, amplified from DNA pool, and referred to *Lactococcus lactis*, was detected during both manufacturing and maturing. In ripened samples, an upper band appeared in DGGE profiles and the closest relatives was found *L.*

lactis subsp. *cremoris*. This subspecies also appeared, as persistent band, in DGGE profiles obtained from RNA analysis. It is known that V3 region of the rRNA 16S gene has a low discriminative power (Ogier et al., 2002) in relation to the subspecies *lactis* and *cremoris* belonging to the species *L. lactis*, and the results obtained from the sequencing of DGGE bands should be cautiously considered. Nevertheless, it is undoubted that the species *L. lactis* has a main role in Castelmagno PDO production, showing an active involvement during both manufacturing and ripening. In fact, culture-independent methods indicated that *L. lactis* dominated until the end of ripening even if, according to traditional approach, lactococci tended to fall during maturing. The role of *L. lactis* in the fermentative transformation of raw milk has been reported from several authors; on the contrary, its participation to the ripening and its possible contribution to the characteristics of the final product has been rarely considered. However, recently, some authors hypothesized an active presence of *L. lactis* throughout cheese ripening (Florez et al., 2006; Rantsiou et al., 2008) and perhaps this possibility should be better considered and analysed. In contrast, lactobacilli, widely isolated on selective media, was poorly depicted on DGGE gels. At RNA level, a band referred to *Lb. helveticus* was detected in samples from ripened cheeses and the role of this microorganism in flavour development, as it has been already reported for several cheese (Coppola et al., 2005; Ercolini et al., 2008; Fox et al., 2004; Gala et al., 2008) should be taken into account for Castelmagno PDO. Noteworthy, *Lb. helveticus* was never isolated on selective media, due probably to the incubation temperature used in this study which could have favoured mesophilic lactobacilli. On the contrary, other lactobacilli found on the media, as Lb. plantarum, were not detected in DGGE assay, probably due to their low abundance compared with other species. PCR primer annealing should be affected by the relative abundance of DNA molecules from the different species. Finally, *Sphingomonas* sp. was detected, during the ripening, at RNA level. *Sphingomonas* is a bacterium isolated typically from the soil; however, its capability to use lactose as carbon source has been reported (Fialho et al., 1999; Silva et al., 2004) and could justified its adaptation to a dairy environment. The identification, at the species level, could not be

achieved, probably due to the low variability of the V_3 region of 16S rRNA gene, among the genere *Sphingomonas*.

The overview, arising from the present work, of the microbiota acting in manufacturing and ripening of winter floor valley Castelmagno PDO cheese, partially matches with the results obtained in a previous research (Dolci et al., 2008a) on the microbiota of summer alpine pasture production of the same cheese, where *L. lactis* and *Lb. plantarum* represented the main microbiota. DGGE analysis highlighted a major biodiversity in summer productions, compared to the winter batches, probably due to a major microbial richness of milk from alpine pasture. Once again, these kind of studies, besides adding new information on traditional products, provides evidence that natural environments are rich in biodiversity and can be considered as proper sources of new strains.

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Table 1. Count values of the main microflora (mean ± standard deviation of samples from three subsequent batches) at various stages of production of Castelmagno PDO cheese: milk (M); curd after cutting (Cu); curd after 24 hours (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); cheese after 90 days ripening (Ch90); cheese after 150 days ripening (Ch150).

Sample from manufacture and ripening		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)													
				GEL		M17		MRS		KAA		MSA		AMT		VRBA	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
M		6.59	0.01	6.3	0.3	6.5	0.8	6.8	0.7	5.0	1.1	4.4	0.5	4.8	0.8	3.0	0.4
Cu		6.57	0.01	6.1	0.6	7.2	0.8	7.3	0.3	4.8	0.8	4.6	0.6	4.8	0.6	3.1	0.3
Cu24		5.06	0.02	6.9	0.4	8.8	0.2	8.7	0.2	5.6	1.0	5.0	0.5	4.8	0.4	5.4	0.3
Cu3W		4.71	0.07	4.9	0.2	8.7	0.2	8.7	0.3	5.9	0.6	4.4	0.5	4.9	1.0	3.9	1.1
Ch3S	core	4.72	0.07	6.4^{a}	0.2	8.1	0.5	8.1	0.2	6.5	1.0	5.0	0.5	4.8	0.4	3.8	1.7
	subsurface	4.75	0.05	7.6^{b}	0.4	8.0	0.5	8.1	0.7	6.2	1.2	5.0	0.4	4.7	0.6	3.8	1.7
Ch30	core	4.66	0.01	6.4	0.3	6.8	0.4	6.9	0.6	5.7	0.2	4.0^{a}	1.3	4.9	0.9	2.2	0.6
	subsurface	4.76	0.10	6.3	0.5	7.0	0.4	7.1	0.3	5.8	0.2	5.3^{b}	0.3	5.5	0.4	2.6	1.1
Ch60	core	4.73	0.06	5.3	0.7	7.2	0.8	7.2	0.8	5.3	0.4	4.6	0.5	3.4^{a}	1.0	1.1	0.4
	subsurface	4.87	0.13	5.9	0.6	6.8	0.7	6.9	0.6	5.2	0.6	4.8	0.5	5.2^{b}	1.4	1.2	0.5
Ch90	core	4.92	0.03	5.2	0.6	6.7	0.4	6.6	0.5	4.6	0.5	3.7^{a}	0.7	3.8	1.2	1.4	0.6
	subsurface	5.12	0.12	5.0	0.2	6.7	0.4	6.8	0.3	4.6	0.4	4.8^{b}	0.6	3.6	0.9	1.2	0.6
Ch150	core	4.89	0.11	4.3	0.2	5.8	1.0	6.1	0.4	4.6	0.9	4.3^{a}	0.6	2.6	0.7	<5	-
	subsurface	5.09	0.10	5.4	0.6	5.6	0.9	6.7	0.2	4.6	0.2	5.6^{b}	0.7	2.2	0.5	<5	-

[ab]Mean values significantly different ($P < 0.05$) according to Duncan test are marked with a different superscript. The test was carried out considering differences, between core and subsurface, individually for each specific medium and time of ripening.

Table 2. Overall distribution of the main recurring LAB species isolates throughout Castelmagno PDO cheese manufacturing[a] and ripening[b] of the three batches analysed.

Species		M	Cu	Cu24	Cu3W	Ch3S	Ch30	Ch60	Ch90	Ch150
Lactococcus lactis subsp. *lactis*	core			5	4	11	4			
	subsurface					12	3			
Lactococcus lactis subsp. *cremoris*	core				4	2	1			
	subsurface					1				
Lactobacillus plantarum	core				2	10	7	7	20	22
	subsurface					9	8	11	27	25
Lactobacillus casei	core						3	27	15	15
	subsurface						1	23	15	15
Lactobacillus coryniformis subsp. *torquens*	core						3	4	8	6
	subsurface						2	1	4	2
Lactobacillus acidipiscis	core									3
	subsurface							3	1	1
Streptococcus thermophilus	core	6	8	4	4					
	subsurface									
Streptococcus agalactiae	core	6	8	3						
	subsurface									

[ab] M: milk; Cu: curd after cutting; Cu24: curd after 24 hours; 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; Ch150: cheese after 150 days ripening.

Table 3. Sequence information for bands detected on DGGE gels through direct analysis of Castelmagno PDO cheese samples.

Band*	Closest sequence relative	% Identity	GenBank accession no.
a	*Streptococcus agalactiae*	98%	EU075069
b	*Lactococcus lactis*	99%	EF204360
c	*Lactococcus lactis* subsp. *cremoris*	98%	AM406671
d	*Streptococcus agalactiae*	98%	CP000114
e	*Lactobacillus helveticus*	99%	EU377824
f	*Sphingomonas* sp.	98%	EU400649

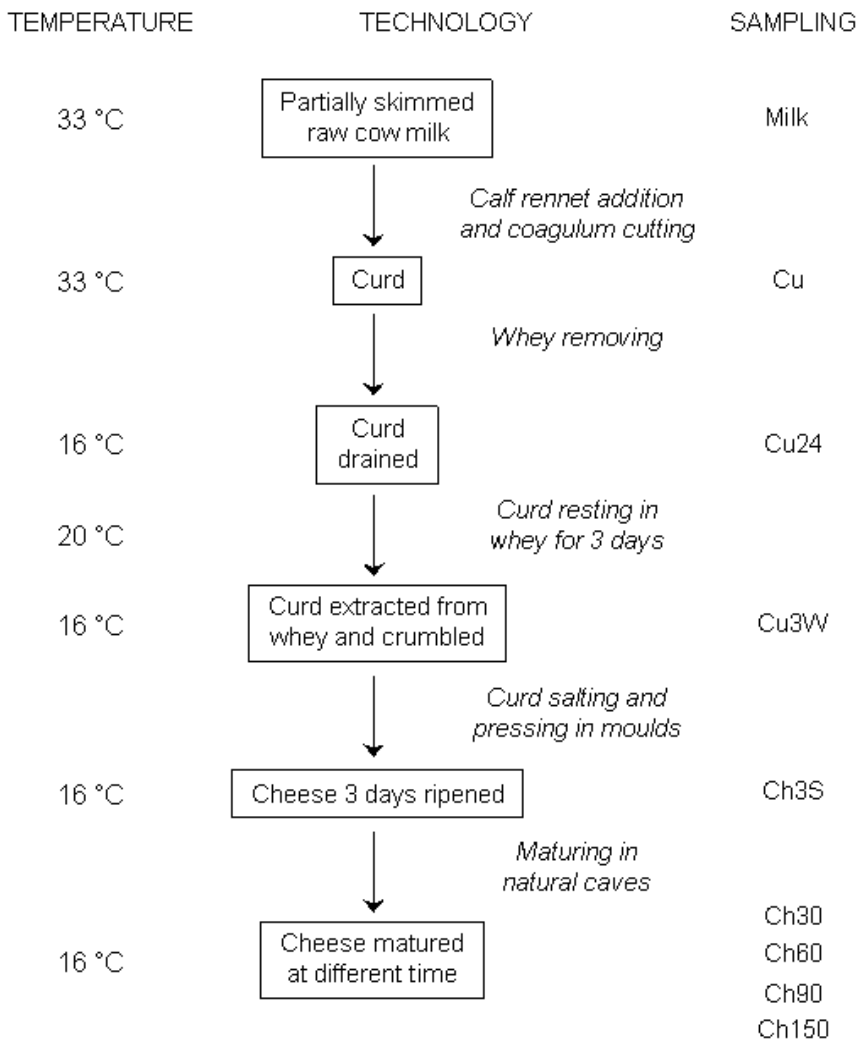
*Referred to figure 2.

Figure 1. Flow scheme of Castelmagno PDO cheese technology with a focus on relative temperatures and sampling points carried out in this study.

Figure 2. PCR-DGGE (a) and RT-PCR-DGGE (b) profiles of bacterial communities by direct nucleic acid analysis of Castelmagno PDO samples. The identification of bands “a” to “f” has been reported in table 3.

M: manufacturing (referred to samples from milk to the curd after 3 days of rest in whey); R: ripening (referred to samples of cheese from 3 to 150 days of ripening).
*Sample DGGE profiles reported as example of manufacturing and ripening banding patterns

100 Figure 1.



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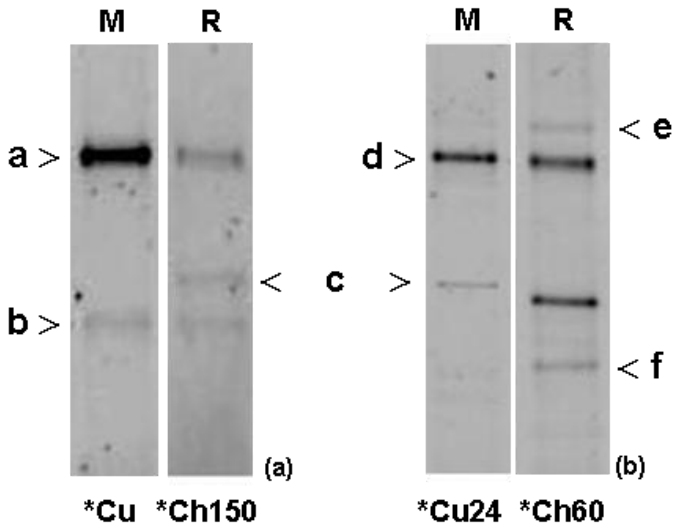
104 Figure 2.



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