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**Yeast and mold dynamics in an Italian specialty cheese coagulated with an aqueous extract of *Carlina acanthifolia* All.**

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**ABSTRACT**

*Caciofiore della Sibilla* is a specialty ewe's milk cheese traditionally manufactured in a foothill area of the Marche region (Central Italy) with a crude extract of fresh leaves and stems of *Carlina acanthifolia* All. subsp. *acanthifolia*. as a coagulating agent. The fungal dynamics and diversity of this specialty cheese were investigated throughout the manufacturing and 20-day ripening process, using a combined PCR-DGGE approach. The fungal biota of a control ewe's milk cheese manufactured with the same batch of milk coagulated with a commercial animal rennet was also monitored by PCR-DGGE, in order to investigate the contribution of the peculiar vegetable coagulant to the fungal diversity and dynamics of the cheese.

In both cheese manufactures eumycetes occurred at a high level and a succession of species was seen, with the taxa (f.i. *Candida parapsilosis*, *Rhodotorula mucillaginosa*) dominating the early cheese-making stage being progressively replaced by taxa which are more adapted to the cheese environment (f.i. *Penicillium* sp., *P. kudriavzevii*, and *Rhizopus stolonifer*). Some species stably occurring throughout the cheese-making processes (f.i. *Debaryomyces hansenii* and *Candida zeylanoides*) were also found together with occasional cheese contaminants (*Cladosporium cladosporioides* and *Cryptococcus wieringae*).

Based on the overall results collected, a potential qualitative rather than quantitative contribution of thistle rennet to the fungal diversity and dynamics of *Caciofiore della Sibilla* cheese was assumed, although the raw milk and the dairy environment represented the main sources of fungal contamination.

**Keywords:** raw ewe's milk cheese, *Caciofiore della Sibilla*, thistle rennet, yeast, mold, PCR-DGGE.

Introduction

The term *Caciofiore* refers to Italian soft cheeses traditionally manufactured with raw ewe’s milk and a vegetable coagulant mostly obtained from dried flowers of herbaceous perennial plants, native to the western and central Mediterranean area, which are commonly known as “thistles” and scientifically ascribed to different genera within the composite Asteraceae family, namely *Cynara* (artichoke or cardoon), *Scolymus* (golden thistle or oyster thistle), *Carlina* (carline thistle) or *Silybum* (milk thistle or Marian thistle).

The exploitation of crude extracts from thistles, which dates back to Roman times, as documented by Lucius Junius Columella in his treatise *De Re Rustica* (c. 50 bc), and the peculiar procedure needed for the preparation of these extracts are strictly linked to the territory and have been handed down from father to son for generations. In ancient times, thistle flowers were collected during the summer transhumance, the seasonal movement of shepherds with their livestock between fixed pastures. They were then left to dry in the sun, dipped in cold water for a variable period of time, and roughly filtered through a cloth; the resulting aqueous extract was hence added to the milk for coagulation, the latter occurring within ~30-60 min (Roseiro et al., 2003; Roseiro et al., 2005).

Currently, at artisan level, thistle flowers are collected from wild plants grown in uncultivated foothill or mountainous areas, air-dried, and used by local cheese-makers for the preparation of milk coagulants, whereas at industrial level liquid or powdered thistle rennet is directly purchased from large-scale ingredient suppliers. Members of this botanical family are also cultivated and exploited for the extraction of food additives and the production of nutraceuticals, by reason of the numerous health benefiting nutrients contained in these plants, including anti-oxidants, fiber, vitamins and minerals (Christaki et al., 2012).

In the Mediterranean area, the exploitation of thistle rennet, especially obtained from *Cynara cardunculus* L., *C. scolymus* L. and *C. humilis* L., is not confined to Italy, where renowned specialty cheeses, such as *Caciofiore Aquilano* and *Caciofiore di Columella*, are traditionally manufactured

in Abruzzo and Lazio, respectively. The use of this type of vegetable coagulant is also particularly widespread in western Africa (García et al., 2012) and the Iberian Peninsula, the latter boasting a large number of Protected Designation of Origin (PDO) cheeses (Roseiro et al., 2003). Even in Latin American countries, such as Argentina and Chile, where “*cardo de Castilla*” (*Cynara cardunculus* L.) grows vigorously, thistle rennet is used for the manufacture of a variety of ewe’s milk cheeses (Fox, 1999).

Crude extracts from flowers or even leaves of thistles are thermostable and characterized by a high proteolytic activity (Fox, 1999) for the occurrence of aspartic proteases, known as cardonsins (or cynarases and cyprosins), with a high specificity for caseins (Roseiro et al., 2003). As the number of flowers used for the preparation of the coagulant largely depends on the cheesemaker’s experience, the amount of coagulant to be added to the milk is also often empirical.

The impact of vegetable coagulants derived from thistles on milk clotting and the physico-chemical, sensory and textural traits of cheese has been thoroughly investigated (Sanjuán et al., 2002; Tejada et al., 2007; García et al., 2012; Llorente et al., 2014; Ordiales et al., 2014) whereas, to date, the contribution of these plant extracts to cheese microbial diversity and dynamics has undoubtedly been less explored, with only a few studies carried out for this specific purpose (Sousa et al., 1997; Vioque et al., 2000; Gomez et al., 2001; Roseiro et al., 2003; Tejada et al., 2003; Aquilanti et al., 2011; Galán et al., 2012). This is especially true for yeast and molds, whose importance in cheese manufacturing is being increasingly acknowledged, due to the many beneficial activities of these microorganisms, including the excretion of growth factors (especially vitamins), the metabolization of lactate/lactose, which enhances the growth of a secondary microflora constituted by acid-sensitive bacteria, the production of alkaline metabolites and aromatic compounds, the inhibition of spoilage microorganisms, the enhancement of cheese flavor and aroma through proteolysis and lipolysis, and even probiosis (Beresford et al., 2001; Jacques and Casaregola, 2008; Panelli et al., 2013; Padilla et al., 2014; Cardoso et al., 2015). On the other hand, eumycetes can also act as spoilage microorganisms, causing cheese defects, such as cheese softening, superficial

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discoloration, early blowing and the development of off-flavors (Fleet, 1990; Jakobsen and Narvhus, 1996; Carreira et al., 1998; Wyder et al., 1999). Finally, toxicogenic molds can produce secondary metabolites generally referred to as mycotoxins that, once ingested, have serious consequences on consumers' health (Creppy 2002; Sengun et al., 2008).

The fungal biota is widely distributed in dairy environments, originating from multiple sources, including the raw milk, the indoor air, the processing equipment, brine, starter cultures (Banjara et al., 2015) as well as deliberately added ingredients, such as milk coagulants. Based on these premises, the aim of the present study was to investigate the eumycetes diversity and dynamics of a central Italian specialty raw ewe's milk cheese, locally known as *Caciofiore della Sibilla*, throughout its ripening, as affected by milk coagulation with a crude extract of stems and leaves of *Carlina acanthifolia* All. subspecies *acanthifolia*. To that end, the fungal communities of both *Caciofiore della Sibilla* and a control ewe's milk cheese manufactured using commercial animal rennet were analyzed through a combined culture-dependent and -independent (PCR-DGGE) approach, relying on the analysis of the fungal DNA extracted directly from samples and the bulk of cells harvested from selected agar dilution plates.

**Materials and Methods**

*Yeast and mold reference strains*

Two DGGE ladders, referred to as Mix' and Mix'', were constructed using 5 yeast and 3 mold reference strains, respectively; these were: *Wickerhamomyces anomalus* DBVPG 6613, *Starmerella bombicola* DBVPG 3827, *Candida humilis* CBS 6897<sup>T</sup>, *Saccharomyces cerevisiae* CBS 1171<sup>T</sup>, *Kazachstania exigua* DBVPG 6481, *Alternaria alternata* M9, *Cladosporium* spp. M5 and *Mucor racemosus* M12. Yeasts were purchased from the Industrial Yeasts Collection (DBVPG, University of Perugia, Italy, <http://www.dbvpg.unipg.it/index.php/en/>) and the Centraalbureau voor

Schimmelcultures (CBS, Utrecht, the Netherlands, <http://www.cbs.knaw.nl/index>), whereas molds had previously been isolated and molecularly identified in our laboratory (Garofalo et al., 2012).

### *Cheese-making and sampling*

Cheese-making trials were conducted in a family-run dairy farm located in the Monti Sibillini National Park (Marche region, Central Italy), following a traditional manufacturing method (the flow chart is shown in the supplementary material, Fig. S1), without any addition of starter culture.

The raw milk obtained from ewes of the *Sopravissana* breed during one milking day in September 2014 was split into two batches. The first batch, labeled “CF”, was coagulated with a crude extract from fresh leaves and stems of *C. acanthifolia* All. subsp. *Acanthifolia*. The crude extract was prepared as follows: the stem and petioles of leaves from 4 plants of *C. acanthifolia* All. subsp. *acanthifolia* harvested from an uncultivated foothill area of the Monti Sibillini National Park were carefully peeled, chopped with a mezzaluna knife and macerated in an aqueous solution of wine vinegar (1:1 ratio) for ~18 h at room temperature. The preparation was filtered through a muslin cloth and ~100 mL of the crude extract were used to clot ~10 L of raw ewe’s milk. The second batch, labeled “C”, was coagulated with commercial powdered calf rennet (Caglifacio Clerici, Cadorago, CO, Italy; 1:10000). For each batch, filtered raw milk was poured into a food grade steel coagulation vat, heated at ~35-36 °C, added with the coagulant and gently stirred.

After clotting (ca. 30’), the both curds were manually broken into rice-sized grains, then transferred into 8x8 cm plastic perforated molds and manually pressed to drain the whey. Molded cheeses were held at 12-13 °C for 4 h, dry salted and ripened for 20 days at 12-13 °C and 70% relative humidity.

During cheese-making and ripening, the following samples were collected and subjected to analysis: raw ewe’s milk, stems and leaves of *C. acanthifolia* All. subsp. *acanthifolia*, aqueous crude extract, curds, and cheeses after 1, 3, 6, 10 and 20 days of ripening. After collection, samples were transported to the laboratory under refrigerated conditions (+ 4 °C) and analyzed within 24 h.



*pH measurements*

All the collected samples, except for the stems and leaves of *C. acanthifolia* All. subsp. *acanthifolia*, underwent pH measurement using a pH-meter (model 300, Hanna Instruments, Padova, Italy) equipped with a solid electrode (model HI2031). For each sample, three independent measurements were performed and the mean values  $\pm$  standard deviations were calculated.

*Viable cell counting and bulk formation*

Aliquots of solid samples (10 g) were homogenized in 90 mL of sterile peptone water (0.1 % peptone) with a Stomacher apparatus (400 Circulator, International PBI, Milan, Italy) for 2 min at 260 rpm. Liquid samples (raw ewe's milk and crude extract) and the homogenates were serially diluted and an aliquot (100  $\mu$ L) of each dilution was inoculated in duplicate on Rose Bengal Agar base (RBA, Oxoid) supplemented with chloramphenicol (0.1 g/L) and incubated at 25  $^{\circ}$ C for 48 h. Viable counts were expressed as means of the Log colony forming units (cfu) per gram or mL of sample  $\pm$  standard deviations. Bulk cells were obtained by harvesting colonies from selected RBA dilution plates used for viable counting. In more detail, colonies were suspended in 2 mL of saline solution and glycerol (0.85% NaCl, 50% glycerol), harvested with a sterile pipette and stored at -20  $^{\circ}$ C. For all the samples except cheeses, bulk cells were prepared from plates spiked with both the highest and lowest dilution, whereas for cheeses, the sole plates spiked with the highest dilution were analyzed.

*DNA extraction*

Direct extraction of fungal DNA from samples collected during cheese-making and ripening was performed using a commercial kit (PowerFood<sup>TM</sup> Microbial DNA Isolation Kit, Mo Bio Laboratories, Carlsbad, USA). Briefly, 1.5 mL of raw milk, thistle rennet, curd and cheese homogenates were transferred into sterile 2 mL-tubes and centrifuged at 12.000 g for 10 min. For milk and cheese samples, after centrifugation, the fat layer on the top was removed with a sterile

cotton tip before discarding the supernatant. Extraction of fungal DNA from bulk cells and from the pure reference strains was performed using the method proposed by Makimura et al. (1999). All the DNA suspensions were subjected to optical readings at 260, 280 and 234 nm with a UV-Vis spectrophotometer (Shimadzu Corporation, Kyoto, Japan) for the assessment of quantity and purity.

#### *PCR-DGGE analysis*

Amplification of the D1-D2 regions of the 26S rRNA gene was performed using primers NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3'), with the GC clamp described by Sheffield et al. (1989) added at the 5' end, and LS2 (5'-ATT CCC AAA CAA CTC GAC TC-3') (Cocolin et al., 2000). Two  $\mu$ L of fungal DNA (adjusted to 50 ng/ $\mu$ L) were amplified as reported by Osimani et al. (2015) in the thermal cycler My Cyclor (Bio-Rad Laboratories, Hercules, CA, U.S.A); aliquots (5  $\mu$ L) of the PCR products were routinely checked for positive amplification on 1.5% agarose (w/v) gels, prior to further DGGE analysis.

PCR products were then separated in 8% polyacrylamide (acrylamide/bis-acrylamide mix 37.5:1, w/v) gels with a denaturant gradient made of urea/formamide from 30 to 60% (w/v) using the DGGE Bio-Rad D-code™ apparatus (Bio-Rad Laboratories). Gels were subjected to a constant voltage of 130 V for 4 h at 60 °C. After electrophoresis, gels were stained in 1X TAE buffer containing SYBR Green I Stain 1X (Lonza, Walkersville, MD, USA) and photographed under UV transillumination, using the Complete Photo XT101 system (Explera, Jesi, Italy).

Selected DGGE bands (labeled with unique and progressive numeric codes) were excised from the gels with sterile pipette tips and transferred into microtubes containing 50  $\mu$ L of sterile deionized water for elution of the DNA. After overnight incubation at 4 °C, 5  $\mu$ L of the DNA suspension were re-amplified with the same primer set (the forward primer deprived of the GC clamp) and sent to Beckman Coulter Genomics (Hope End, Takeley, United Kingdom) for purification and sequencing. The sequences obtained in FASTA format were compared with those deposited in the GenBank DNA database (<http://www.ncbi.nlm.nih.gov/>) using the basic BLAST search tools

(Altschul et al., 1990). A sequence identity equal to or higher than 97% was chosen as a threshold for unambiguous assignation into species. The sequences longer than 200 bp have been deposited in the NCBI GenBank data library under the accession numbers from KU196966 to KU197012.

*Statistical analysis*

Viable counts and pH data recorded during cheese ripening at 5 selected time points (1, 3, 6, 10 and 20 days of maturation) were analyzed by a multivariate analysis of variance (MANOVA) for repeated measures. For each sampling time, viable counts and pH of both batches were compared according to the Student's t test ( $\alpha = 0.05$ ).

All statistical analyses were performed using JMP statistical software version 11.0.0 (SAS Institute Inc., Cary, NC, USA).

**Results**

*pH measurement and viable counting of eumycetes*

Results from the MANOVA demonstrated a significant effect of the cheese batch, Cf or C, ( $p < .0001$ ) and ripening time ( $p < .0001$ ) on fungal viable counts. A significant effect of both cheese batches ( $p = .0410$ ) and ripening time ( $p = .0319$ ) on pH was also seen, although for the latter parameter the variation during ripening was not consistent over time ( $p = .0295$ ) (Table 1).

The results of pH measurement and viable counting are reported in Table 2. With regard to pH, a drop was seen throughout the two cheese-making processes with significant differences occurring between curds and cheeses from batches Cf and C, until the 10<sup>th</sup> day of ripening. By contrast, after 20 days of maturation, *Caciofiore della Sibilla* and the control ewe's milk cheeses reached comparable pH values.

Regarding the microbiological analyses, in both cheese manufactures the eumycetes progressively increased from the raw milk ( $2.42 \log \text{CFU mL}^{-1} \pm 0.04$ ) to cheese until the 10<sup>th</sup> day of maturation,

whereas after 20 days, the load of this microbial group decreased in both cheese manufactures. Fungal viable counts were consistently higher on C<sub>f</sub> compared to C throughout the whole process. Relatively high fungal loads were also found in the phyllosphere of *Carlina acanthifolia* All. ( $5.35 \pm 0.01 \log \text{CFU g}^{-1}$ ) and in the crude extract from crushed leaves and stems ( $3.54 \pm 0.10 \log \text{CFU g}^{-1}$ ) (Tab.2) .

#### PCR-DGGE analyses

The results of the alignments of selected DGGE bands are reported in the supplementary material (Table S1) whereas the overall taxa identified are listed in Table 3. The DGGE profiles obtained from the analysis of the fungal DNA extracted from the samples and the bulk cells are shown in Fig. 1 and Fig. 2. The analysis of the fungal microbiota characterizing the phyllosphere of *C. acanthifolia* All. and the vegetable coagulant revealed the occurrence of *Debaryomyces hansenii* and *Candida zeylanoides*, respectively. Other yeast species were also detected in these samples: *Candida parapsilosis* plus *Galactomyces candidus*, which is better known by the anamorphic name *Geotrichum candidum*, in the vegetable coagulant and *Rhodotorula mucilaginosa* plus *Cladosporium coralloides* on the phyllosphere of *C. acanthifolia* All., respectively; in the latter sample, the mold species *Fusarium oxysporium* was also found.

As regards the raw milk, curds and cheeses sampled during ripening, a higher biodiversity of eumycetes was seen with respect to milk, with numerous yeasts and molds detected. A few taxa were exclusively found in the raw milk (*Saccharomyces cerevisiae*) or in the curd obtained by milk coagulation with commercial animal rennet (*Cladosporium cladosporioide*, *Cryptococcus wieringae*), whereas others occurred in different samples, as was the case for *Candida membranifaciens*, *Candida parapsilosis*, *Candida zeylanoides*, *Debaryomyces hansenii*, *G. candidum*, *Kluyveromyces marxianus*, *Meyerozyma guilliermondii*, *Penicillium* sp., *Candida inconspicua* (teleomorph *Pichia cactophila*), *Pichia kluyveri*, *Pichia kudriavzevii*, *Rhizopus stolonifer*, *Rhodotorula mucilaginosa* and *Yarrowia lipolytica*. Among these, some taxa could be

detected exclusively in the raw milk and during the early stage of maturation (*C. Parapsilosis*, *R. mucillaginosa*), whereas others occurred occasionally (f.i. *C. Zelanoydes*, *G. candidum*) or stably (*D. hansenii*, *P. Kudriavzevii*, *R. stolonifer*) throughout the cheese-making and ripening period. Furthermore, species apparently associated to one cheese manufacture rather than the other were also encountered, such as *M. guilliermondii* and *K. marxianus*, which were found exclusively in the *Caciofiore della Sibilla* and control ewe's milk cheese, respectively.

**Discussion**

Numerous studies have previously been focused on the effect of thistle coagulants, especially made from *Cynara cardunculus*, *C. scolymus* or *C. humilis*, on the microbiological properties of traditional cheeses (Vioque et al., 2000; Roseiro et al., 2003; Fernández-Salguero et al., 2002), but to the author's knowledge, this is the first attempt to shed light on the contribution of thistle coagulants to the fungal diversity and dynamics of the cheese. Furthermore, this is the first report describing the exploitation of the botanical species *C. acanthifolia* All. in cheese-making. This research also contributes to the assessment of the eumycetes load and diversity in thistle phyllosphere and raw ewe's milk which are two poorly investigated ecosystems.

Concerning the latter ecosystem, comparable or slightly lower viable counts were seen compared with those reported by other authors for raw milk (Vioque et al., 2000; Roseiro et al., 2003; Pangallo et al., 2014; Gardini et al., 2006; Tejada and Fernández-Salguero 2003). As has previously been elucidated, the relatively low contamination of raw milk with eumycetes can be mainly ascribed to the competitive interaction for growth substrates by psychrotrophic bacteria or the excretion of inhibiting metabolites by these microorganisms (Viljoen, 2001).

By contrast, the phyllosphere of *C. acanthifolia* All. was found to be colonized by a relatively high load of yeasts and molds, thus supporting the assumption of an effective role of the vegetable coagulant in the contamination of the cheese milk with these microorganisms. However, no significant differences ( $p = .8026$ ) were observed in the curds made with vegetable and animal

rennet, respectively, thus suggesting a qualitative rather than quantitative contribution of thistle rennet to the cheese fungal diversity.

As regards cheeses in the early stage of maturation, after 1 day of ripening, viable counts were almost 3 and 2 Log units higher than those initially found in the raw milk, respectively; in both cheese manufactures (batches Cf and C), yeasts and molds reached the highest load after 10 days of maturation. However, a significantly higher evolution of the eumycetes community was seen throughout the ripening of *Caciofiore della Sibilla* compared with the control ewe's milk cheese. In other studies, carried out on traditional Iberian cheeses manufactured with vegetable coagulants from thistle flowers, a relatively poorer occurrence of eumycetes was seen in cheeses after 15 days of maturation, with viable counts approximately 2 or 3 orders of magnitude lower (Gomez et al., 2001; Vioque et al., 2000; Tejada and Fernández-Salguero, 2003).

The PCR-DGGE fingerprinting of samples collected during cheese-making and ripening allowed the evolution of the fungal microbiota to be traced and the potential sources of curd and cheese contamination with yeasts and molds to be identified. These microorganisms can grow either in the cheese core or on the rind, being favored by the peculiar physico-chemical properties characterizing the cheese environment, such as the low pH, the reduced water activity, the high salt concentration and the low storage temperature (Padilla et al., 2014; Cheong et al., 2014). The composition of the cheese fungal biota has been found to change among cheeses of different varieties, cheeses of the same variety from different producers and even cheese batches of the same variety from the same producer (Banjara et al., 2015).

In the present study, a wide range of yeast species was identified with the double PCR-DGGE approach, whose usefulness in depicting cheese ecosystems has recently been elucidate (Aquilanti et al. 2016); most of these taxa had previously been found in raw milk, curd or cheese, including *K. marxianus* (Macedo et al., 1995; Lavoie et al., 2012; Quigley et al., 2013), *D. hansenii*, *S. cerevisiae* and *Rhodotorula* spp. (Capece and Romano, 2009; Cosentino et al., 2001; Fadda et al., 2001). Of these, *K. marxianus* is known to have a positive impact on cheese quality, either as a result of the



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3 310 development of flavor compounds during maturation or because of partial deacidification due to  
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5 311 lactate/lactose consumption, the latter enhancing the growth of acid-sensitive bacteria (Corsetti et  
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7 312 al., 2001). *D. hansenii* has also been reported to play a key role during cheese-ripening, for the  
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9 313 ability of this yeast, growing in high salt concentrations and low pH (Beresford et al., 2001; Büchl  
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11 314 and Seiler, 2011; Padilla et al., 2014), to utilize several carbon and nitrogen sources and to produce  
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13 315 proteolytic and lipolytic enzymes capable of metabolizing milk proteins and fat (Banjara et al.,  
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15 316 2015; Cardoso et al., 2015). A high proteolytic and lipolytic activity also characterizes the majority  
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17 317 of strains ascribed to *R. mucilaginosa* (Fadda et al., 2010; Cardoso et al. 2015), a further yeast  
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19 318 species frequently occurring in raw milk and cheese (Fadda et al., 2010; Pereira-Dias et al., 2000;  
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21 319 Corbo et al., 2001; Cocolin et al., 2002; Borelli et al., 2006; Quigley et al., 2013; Lavoie et al.,  
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23 320 2012).

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27 321 Other species, such as *C. membranifaciens*, *G. candidum* and *P. inconspicua* have more rarely been  
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29 322 identified in milk-based products, whereas, to the authors' knowledge, this is the first report of *M.*  
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31 323 *guilliermondii* in raw ewe's milk cheese. To date, this yeast, which is commonly isolated from fruit  
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33 324 surfaces (Pelliccia et al., 2011), where it shows a great potential for the post-harvest control of  
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35 325 spoilage fungi (Corte et al., 2015) has been detected in goat's and cow's milk cheeses, as well as in  
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37 326 butter milk, yogurt and the dairy environment (Büchl and Seiler, 2011; Giannino et al., 2011;  
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39 327 Callon et al., 2006). Its capacity to produce flavor compounds in fermented food products is  
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41 328 acknowledged (Wah et al., 2013).

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45 329 Other interesting evidence emerged by comparing the fungal dynamics in the *Caciofiore della*  
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47 330 *Sibilla* and the control ewe's milk cheeses. Firstly, the fingerprinting of the fungal biota occurring  
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49 331 in samples collected at different steps during the two cheese-making processes allowed a microbial  
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51 332 succession to be observed in both cheese manufactures, with some taxa occurring in the sole raw  
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53 333 milk (*S. cerevisiae*.), others in the early (*C. parapsilopsis* and *R. mucilaginosa*) or late (*Penicillium*  
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55 334 sp.) stages of fermentation and maturation, and further species occurring throughout the whole  
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57 335 process (*C. zeylanoides* and *D. hansenii*). In addition to the species that are well adapted to the  
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cheese environment and stably detected in cheeses during maturation, such as *C. membranifaciens*, *P. kluyveri*, *P. kudriavzevii*, *R. stolonifer* and *Y. lipolytica*, other species occurring as occasional contaminants were also sporadically found, such as *C. cladosporioides* and *C. wieringae*.

Among the overall taxa identified, only *M. guilliermondii* and *K. marxianus* were exclusively found in the *Caciofiore della Sibilla* and control ewe's milk cheese, respectively.

As regards cheese contamination, multiple sources, such as the vegetable coagulant, the raw milk and the dairy-environment can be assumed to have played a role; in more detail, *C. parapsilosis*, *C. zeylanoides*, *K. marxianus* and *R. mucillaginosa* feasibly originated from the raw milk, whereas for *C. membranifaciens*, *C. incospicua*, *P. kluyveri*, *P. kudriavzevii*, *R. stolonifera*, and *Y. lipolytica*, cheese contamination from the dairy environment can be hypothesized.

Although no species occurring in the vegetable coagulant were found to distinctively characterize the *Caciofiore della Sibilla* cheese, the joint contribution of thistle rennet together with the raw milk and the dairy environment to the fungal diversity of this specialty cheese cannot be denied.

### Acknowledgements

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**Table 1.** MANOVA (Multivariate Analysis of Variation) on repeated measures for fungal viable counts and pH values testing the effect of cheese batch, ripening time and their interaction.

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Effect	DF	F	Fungal viable counts	F	pH
Cheese batch	1	61592.9	<.0001*	8.8469	0.0410*
Ripening time	4	1088.8	<.0001*	550.73	0.0319*
Cheese batch x ripening time	4	84.24	0.0815	647.17	0.0295*

**Table 2.** pH and viable counts of eumycetes, expressed as mean values  $\pm$  standard deviations, of samples collected during cheese-making and ripening of *Caciofiore della Sibilla* and control ewe's milk cheese. Mean separation test of pH and viable counts of eumycetes throughout ripening: different letters on the same column indicate significant differences over time according to Tukey's test ( $\alpha = 0.05$ ). P values  $< 0.05$ ; (\*) indicates the occurrence of significant differences due to cheese batch (C or Cf) according to the Student's t test.

Sample	<i>Caciofiore della Sibilla</i> (Cf)	Control cheese (C)	p
pH			
raw milk	6.39 $\pm$ 0.02		
vegetable coagulant	3.79 $\pm$ 0.03	n.a.	
curd	5.14 $\pm$ 0.02	6.43 $\pm$ 0.01	<.0001*
1-day ripened cheese	5.22 $\pm$ 0.01 <sup>c</sup>	5.84 $\pm$ 0.03 <sup>a</sup>	<.0001*
3-day ripened cheese	5.31 $\pm$ 0.02 <sup>b</sup>	5.04 $\pm$ 0.02 <sup>b</sup>	<.0001*
6-day ripened cheese	5.38 $\pm$ 0.01 <sup>a</sup>	5.08 $\pm$ 0.02 <sup>b</sup>	<.0001*
10-day ripened cheese	5.23 $\pm$ 0.01 <sup>c</sup>	4.97 $\pm$ 0.03 <sup>b</sup>	.0002*
20-day ripened cheese	5.02 $\pm$ 0.03 <sup>d</sup>	4.94 $\pm$ 0.15 <sup>b</sup>	0.4169
Fungal viable counts (Log CFU g <sup>-1</sup> or mL <sup>-1</sup> )			
raw milk	2.42 $\pm$ 0.04		
<i>Carlina acanthifolia</i> All	5.35 $\pm$ 0.01	n.a.	
vegetable coagulant	3.54 $\pm$ 0.10	n.a.	
curd	3.50 $\pm$ 0.03	3.51 $\pm$ 0.09	0.8026
1-day ripened cheese	5.13 $\pm$ 0.02 <sup>d</sup>	4.65 $\pm$ 0.01 <sup>c</sup>	<.0001*
3-day ripened cheese	7.12 $\pm$ 0.03 <sup>c</sup>	6.03 $\pm$ 0.00 <sup>c</sup>	<.0001*
6-day ripened cheese	7.41 $\pm$ 0.09 <sup>b</sup>	6.71 $\pm$ 0.06 <sup>b</sup>	.0003*
10-day ripened cheese	7.89 $\pm$ 0.08 <sup>a</sup>	6.83 $\pm$ 0.02 <sup>a</sup>	<.0001*
20-day ripened cheese	7.43 $\pm$ 0.01 <sup>b</sup>	5.42 $\pm$ 0.08 <sup>d</sup>	<.0001*

n.a. not applicable.

**Table 3.** Yeasts and molds identified by PCR-DGGE analysis during the manufacture and ripening of *Caciofiore della Sibilla* and control ewe's milk cheeses.

		<i>Candida inconspicua</i>	<i>Candida membrunifaciens</i>	<i>Candida parapsilosis</i>	<i>Candida zeylanoides</i>	<i>Cladosporium coraloides</i>	<i>Cladosporium cladosporioides</i>	<i>Cryptococcus wieringae</i>	<i>Debaryomyces hansenii</i>	<i>Fusarium oxysporium</i>	<i>Geotrichum candidum</i>	<i>Kluyveromyces marxianus</i>	<i>Meyerozyma guilliermondii</i>	<i>Penicillium sp.</i>	<i>Candida inconspicua</i>	<i>Pichia kluyveri</i>	<i>Pichia kudriavzevii</i>	<i>Rhizopus stolonifer</i>	<i>Rhodotorula mucilaginosa</i>	<i>Yarrowia lipolytica</i>	<i>Saccharomyces cerevisiae</i>
<i>Caciofiore della Sibilla</i> cheese	Raw milk			●	●			●				●							●		□
	Phyllosphere of <i>Carlina acanthifolia</i> All.				○	●	■		●	●	■								●		
	vegetable coagulant			●	○				●	■									●		
	curd	●		●	○				●	■	■		●		●				●		
	cheese t1	●	●		●				●	■					●	●	●	■	●		
	cheese t3	●							■	■	■		●	■	●	●	●	■	●		
	cheese t6								●	■				■			●	■		●	
	cheese t10								●	■				■			●	■			
	cheese t20	●			■				■		■	■	●		●	●	●	■		●	
Control cheese	curd		●	●	●		■	●	●			●				●			●		
	cheese t1		●		●				●	■		●				●	●	■	●	●	
	cheese t3	●			●				●	■		●			●		●	■			
	cheese t6								●	■				●			●	■			
	cheese t10		●		●	■			●	■	●			■				■			
	cheese t20				●				●	■	●	●		■			●	■			



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516 ● DGGE bands showing  $\geq 97\%$  sequence identity with reference species resulting from the analysis of the DNA extracted from the bulk of colonies  
517 harvested from selected Rose-Bengal chloramphenicol Agar (RBA) dilution plates; ○ DGGE bands showing  $< 97\%$  sequence identity with  
518 reference species resulting from the analysis of the DNA extracted from the bulk of colonies harvested from selected Rose-Bengal chloramphenicol  
519 Agar (RBA) dilution plates; ■ DGGE bands showing  $\geq 97\%$  sequence identity with reference species resulting from the analysis of the DNA  
520 extracted directly from samples collected during cheese-making; □ DGGE bands showing  $< 97\%$  sequence identity with reference species resulting  
521 from the analysis of the DNA extracted directly from samples collected during cheese-making. t1: cheese sampled after 1 day of maturation; t3:  
522 cheese sampled after 3 days of maturation; t6: cheese sampled after 6 days of maturation; t10: cheese sampled after 10 days of maturation; t20:  
523 cheese sampled after 20 days of maturation.

## LEGENDS TO FIGURES

**Fig. 1.** Fungal DGGE profiles of the DNA extracted directly from the bulk of colonies harvested from the Rosa Bengal chloramphenicol Agar (RBA) plates spiked with the highest and lowest dilutions (lane I and lane II) and of the DNA extracted directly from the samples (lane III) of: raw ewe's milk (rm); thistle (*Carlina acanthifolia* All.) rennet (tr); curd obtained by milk coagulation with thistle rennet (ct); leaves and stems of *Carlina acanthifolia* All. subsp. *acanthifolia* (ca); curd obtained by milk coagulation with commercial animal rennet (cc). Ladder (Mix'): *Wickerhamomyces anomalus* DBVPG 6613 (Y1), *Starmerella bombicola* DBVPG 3827 (Y2), *Saccharomyces cerevisiae* CBS 1171T (Y3), *Kazachstania exigua* DBVPG 6481 (Y4), *Candida humilis* CBS 6897T (Y5). Ladder (Mix''): *Alternaria alternata* M9, *Cladosporium* spp. M5, *Mucor racemosus* M12.

**Fig. 2.** Fungal DGGE profiles of the DNA extracted from the bulk of colonies harvested from selected Rosa Bengal chloramphenicol Agar (RBA) plates (panel a) and samples of *Caciofiore della Sibilla* cheese (batch Cf) and control raw ewe's milk cheese (batch C) (panel b) collected at 1, 3, 6, 10 and 20 days of ripening (Cf<sub>1</sub>, Cf<sub>3</sub>, Cf<sub>6</sub>, Cf<sub>10</sub>, Cf<sub>20</sub> and C<sub>1</sub>, C<sub>3</sub>, C<sub>6</sub>, C<sub>10</sub>, C<sub>20</sub> respectively). Ladder (Mix'): *Wickerhamomyces anomalus* DBVPG 6613 (Y1), *Starmerella bombicola* DBVPG 3827 (Y2), *Saccharomyces cerevisiae* CBS 1171T (Y3), *Kazachstania exigua* DBVPG 6481 (Y4), *Candida humilis* CBS 6897T (Y5). Ladder (Mix''): *Alternaria alternata* M9, *Cladosporium* spp. M5, *Mucor racemosus* M12.

**Fig. S1.** Flow chart of the traditional manufacturing process of *Caciofiore* cheese.

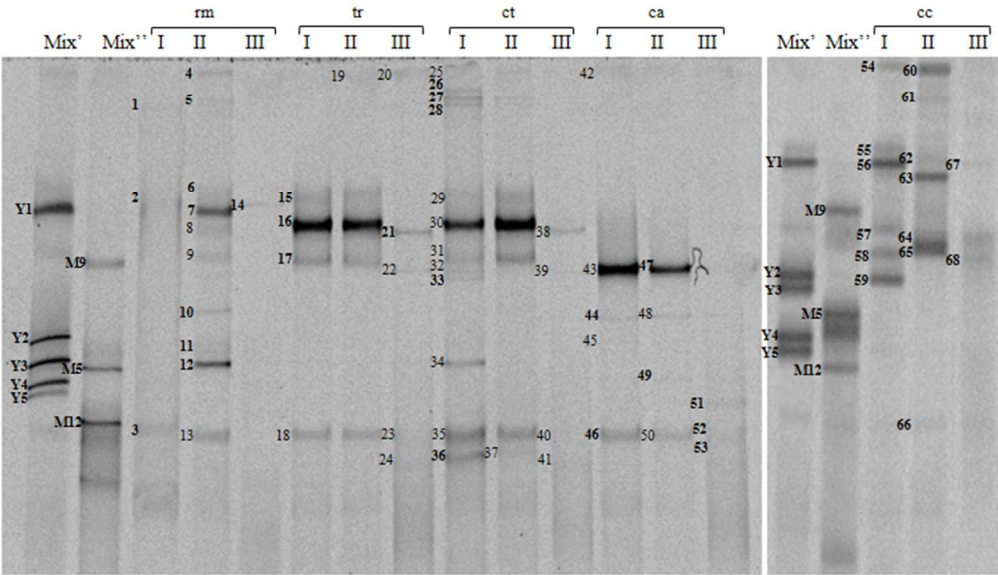


Fig. 1. Fungal DGGE profiles of the DNA extracted directly from the bulk of colonies harvested from the Rosa Bengal chloramphenicol Agar (RBA) plates spiked with the highest and lowest dilutions (lane I and lane II) and of the DNA extracted directly from the samples (lane III) of: raw ewe's milk (rm); thistle (*Carlina acanthifolia* All.) rennet (tr); curd obtained by milk coagulation with thistle rennet (ct); leaves and stems of *Carlina acanthifolia* All. subsp. *acanthifolia* (ca); curd obtained by milk coagulation with commercial animal rennet (cc). Ladder (Mix'): *Wickerhamomyces anomalus* DBVPG 6613 (Y1), *Starmerella bombicola* DBVPG 3827 (Y2), *Saccharomyces cerevisiae* CBS 1171T (Y3), *Kazachstania exigua* DBVPG 6481 (Y4), *Candida humilis* CBS 6897T (Y5). Ladder (Mix''): *Alternaria alternata* M9, *Cladosporium* spp. M5, *Mucor racemosus* M12.

198x114mm (96 x 96 DPI)

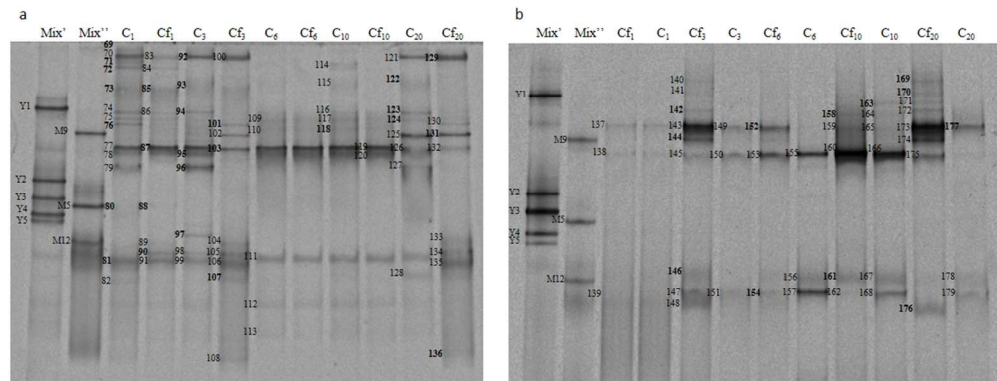


Fig. 2. Fungal DGGE profiles of the DNA extracted from the bulk of colonies harvested from selected Rosa Bengal chloramphenicol Agar (RBA) plates (panel a) and samples of Caciofiore della Sibilla cheese (batch Cf) and control raw ewe's milk cheese (batch C) (panel b) collected at 1, 3, 6, 10 and 20 days of ripening (Cf1, Cf3, Cf6, Cf10, Cf20 and C1, C3, C6, C10, C20 respectively). Ladder (Mix'): *Wickerhamomyces anomalus* DBVPG 6613 (Y1), *Starmerella bombicola* DBVPG 3827 (Y2), *Saccharomyces cerevisiae* CBS 1171T (Y3), *Kazachstania exigua* DBVPG 6481 (Y4), *Candida humilis* CBS 6897T (Y5). Ladder (Mix''): *Alternaria alternata* M9, *Cladosporium* spp. M5, *Mucor racemosus* M12.

303x116mm (96 x 96 DPI)

**Table S1.** Results from the sequencing of the bands cut from the DGGE gels.

Band(s)	Closest relative	% Ident. <sup>a</sup>	Acc.no. <sup>b</sup>	Acc.no. <sup>c</sup>
1	<i>Candida zeylanoides</i>	96%	KJ413172	*
2	<i>Candida zeylanoides</i>	99%	JQ965864	KU196966
3	<i>Candida zeylanoides</i>	97%	KJ413172	*
4, 19, 20, 25, 42	<i>Candida zeylanoides</i>	94%	JQ965864	*
5	<i>Candida zeylanoides</i>	97%	KJ413172	*
6	<i>Candida zeylanoides</i>	97%	KJ413164	*
7	<i>Candida zeylanoides</i>	99%	KC442252	KU196967
10	<i>Kluyveromyces marxianus</i>	98%	KC512907	KU196968
11, 45	<i>Rhodotorula mucilaginosa</i>	99%	KM222349	KU196969
12, 34	<i>Rhodotorula mucilaginosa</i>	99%	KC160628	KU196970
14	<i>Saccharomyces cerevisiae</i>	88%	JX867131	*
15, 29	<i>Candida parapsilosis</i>	99%	JQ965833	KU196971
8, 16, 30	<i>Candida parapsilosis</i>	99%	JQ965866	KU196972
9, 17, 31	<i>Candida parapsilosis</i>	99%	JQ965835	KU196973
21, 38	<i>Galactomyces candidum</i>	99%	KM391959	*
24, 41	Failed	-	-	-
26	<i>Candida parapsilosis</i>	99%	FJ746058	*
27	<i>Candida parapsilosis</i>	98%	KF214402	*
28	<i>Debaryomyces hansenii</i>	94	JX068679	*
33	<i>Meyerozyma guilliermondii</i>	98%	JX423569	KU196974
36, 37	<i>Candida inconspicua</i>	100%	KC512909	KU196975
44, 48	<i>Cladosporium coralloides</i>	98%	JQ388759	KU196976
13, 18, 23, 35, 40, 46, 50	<i>Debaryomyces hansenii</i>	98%	KJ794669	KU196977
22, 32, 39, 43, 47	<i>Debaryomyces hansenii</i>	99%	JX068679	KU196978
49	<i>Fusarium oxysporum</i>	99%	EF363781	KU196979
51	Failed	-	-	-
52	Failed	-	-	-
53	Failed	-	-	-
54	<i>Candida zeylanoides</i>	95%	KC442252	*
55	<i>Candida zeylanoides</i>	98%	FN554768	KU196980
56	<i>Candida zeylanoides</i>	100%	KC442252	KU196981
57	<i>Candida zeylanoides</i>	99%	KC442252	KU196982
58	<i>Kluyveromyces marxianus</i>	100%	KJ491106	KU196983
59	<i>Rhodotorula mucilaginosa</i>	99%	KP737852	KU196984
60	<i>Candida membranifaciens</i>	93%	JQ991936	*
61	<i>Debaryomyces hansenii</i>	93%	KF214428	*
62	<i>Candida parapsilosis</i>	100%	KF214407	KU196985
63	<i>Candida parapsilosis</i>	97%	KF214404	KU196986
64	<i>Debaryomyces hansenii</i>	98%	KC692228	KU196987
65	<i>Cryptococcus wieringae</i>	97%	HM627074	KU196988
66	<i>Candida parapsilosis</i>	95%	JQ965833	*
67	Failed	-	-	-
68	<i>Cladosporium cladosporioides</i>	99%	KP780460	KU196989
69	Failed	-	-	-
71, 114	<i>Candida membranifaciens</i>	96%	JQ991936	*
72, 84	<i>Debaryomyces hansenii</i>	98%	KC692228	KU196990
73	<i>Debaryomyces hansenii</i>	98%	JQ965865	KU196991

75	Failed	-	-	-
76	<i>Yarrowia lipolytica</i>	98%	JQ690257	*
80	<i>Rhodotorula mucilaginosa</i>	92%	FJ468469	*
81, 91, 99, 106, 135	<i>Pichia kluyveri</i>	100%	KC510043	*
85	<i>Debaryomyces hansenii</i>	99%	JX068679	KU197002
77, 87, 119	<i>Debaryomyces hansenii</i>	99%	KF214439	KU197003
88	<i>Rhodotorula mucilaginosa</i>	97%	FJ468469	*
90, 98, 105, 134	<i>Candida inconspicua</i>	99%	HQ641283	KU197004
70, 92, 121	<i>Debaryomyces hansenii</i>	98%	HQ641266	KU196992
93	<i>Debaryomyces hansenii</i>	97%	KJ794669	*
74, 86, 94	<i>Candida zeylanoides</i>	99%	EU131538	KU196993
78, 95, 120	<i>Debaryomyces hansenii</i>	100%	HQ641266	KU196994
79, 96, 127	<i>Kluyveromyces marxianus</i>	99%	KJ491105	KU196995
89, 97	<i>Pichia kluyveri</i>	100%	KF738157	KU196996
101, 130	<i>Yarrowia lipolytica</i>	100%	JN021559	KU197005
103, 132	<i>Meyerozyma guilliermondii</i>	100%	KM103033	KU197006
104, 133	Failed	-	-	-
82, 107, 128	<i>Pichia kudriavzevii</i>	95%	KM234442	*
111	<i>Debaryomyces hansenii</i>	99%	KF214439	KU197011
112	<i>Debaryomyces hansenii</i>	100%	EU816305	KU197012
113	<i>Pichia kudriavzevii</i>	81%	KJ472906	*
110, 118	<i>Debaryomyces hansenii</i>	99%	KF214439	KU196997
115 122	<i>Debaryomyces hansenii</i>	98%	JQ965865	KU196998
116, 123	<i>Candida zeylanoides</i>	99%	EU131538	KU196999
109, 117, 124	<i>Galactomyces candidus</i>	96%	KM391959	*
126	<i>Debaryomyces hansenii</i>	100%	HQ641266	*
83, 100, 129	<i>Pichia kudriavzevii</i>	98%	KM234448	*
102, 125, 131	<i>Galactomyces candidus</i>	99%	KM115152	KU197007
108, 136	<i>Pichia kudriavzevii</i>	100%	KM234442	KU197008
139	Failed	-	-	-
142, 172	<i>Galactomyces candidus</i>	98%	KM391959	*
144, 174	<i>Galactomyces candidus</i>	99%	KM391959	*
146	<i>Penicillium sp.</i>	98%	KM249089	*
138, 145, 150, 153, 155, 160, 166, 175	<i>Debaryomyces hansenii</i>	99%	JX068679	KU197000
147, 151, 154, 157, 162, 168, 179	<i>Penicillium sp.</i>	99%	KJ443120	KU197001
158, 164	<i>Debaryomyces hansenii</i>	99%	JQ965865	KU197009
156, 161, 167, 178	<i>Debaryomyces hansenii</i>	99%	JX068679	KU197010
163, 171	<i>Candida zeylanoides</i>	98%	JQ965864	*
140, 169	<i>Galactomyces candidus</i>	99%	KM115152	*
141, 170	<i>Galactomyces candidus</i>	99%	KM391959	*
148, 176	<i>Galactomyces candidus</i>	99%	KC967683	*
137, 143, 149, 152, 159, 165, 173, 177	<i>Rhizopus stolonifer</i>	98%	AB250195	*

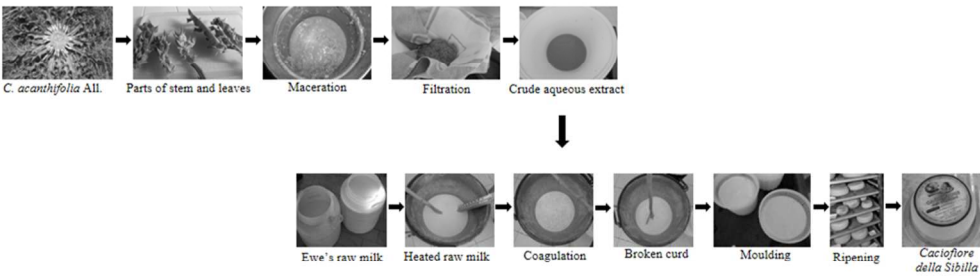
<sup>a</sup> Percentage of identical nucleotides in the sequence obtained from the DGGE band and the sequence of the closest relative found in the GenBank DNA database.

<sup>b</sup> Accession number of the sequence of the closest relative found by BLAST search.

<sup>c</sup> Accession number of the sequence deposited in the NCBI GenBank data library.

\* Sequences not deposited in GenBank as shorter than 200 bp.

Fig. S1. Flow chart of the traditional manufacturing process of *Caciofiore* cheese.



88x60mm (300 x 300 DPI)