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Short communication

"Pecorino di Filiano" cheese as a selective habitat for the yeast species, *Debaryomyces hansenii*

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ABSTRACT

The composition of yeast microflora in artisanal "Pecorino di Filiano" cheese, a typical product of the Basilicata region of Southern Italy, was studied during ripening. The isolates were identified by restriction analysis of the 18S rDNA amplified region with the combined use of Hinf I and Cfo I enzymes. The majority of the isolates were identified as *Debaryomyces hansenii*, whereas two yeasts were identified as *Kluyveromyces lactis* and one as *Dekkera anomala*. To evaluate natural biodiversity, *D. hansenii* "Pecorino di Filiano" isolates were submitted to genetic and technological characterization. RAPD-PCR analysis with P80 (5CGCGTGCCCA3) primer revealed significant polymorphism among *D. hansenii* isolates. About 30% of the isolates showed single molecular profiles, whereas the other *D. hansenii* yeasts were separated into three main patterns, differing for both the ripening time and the isolation source. Furthermore, the yeasts showed significant variability in their, "proteolytic activity". This work demonstrated the high predominance of *D. hansenii* among the yeast population of "Pecorino di Filiano" cheese, probably in consequence of the traditional salting process, which was selected for this salt tolerant species. This preliminary study allowed us to isolate autochthonous *D. hansenii* yeasts potentially useful as starters for the production of this artisanal cheese.

1. Introduction

"Pecorino di Filiano" is an artisanal hard cheese produced from raw whole ewe's milk in the NorthWest area of the Basilicata region of southern Italy. The occurrence of yeasts in artisanal ewe's and goat's milk cheese has been investigated by different authors (Carreira et al., 1998; Pereira-Dias et al., 2000; Cosentino et al., 2001; Fadda et al., 2004), but little information is available on the yeasts associated with ewe's milk cheese of the Basilicata region.

The recovery of yeasts in high numbers from cheeses (e.g. 10^6–10^9 CFU/g) and their ability to hydrolyse milk fat and proteins have suggested that they influence the organoleptic characteristics of cheese (Jakobsen and Narvhus, 1996; Roostita and Fleet, 1996). Even in cheeses inoculated with bacterial starters, yeasts may be detected at counts as high as 10^8 CFU/g. The functions of yeasts during cheese production and their influence on cheese quality have been, in general, poorly explored. Investigations of yeast flora composition revealed a large diversity of species belonging to the genera *Candida*, *Yarrowia*, *Debaryomyces*, *Cryptococcus*, *Trichosporon*, *Zygosaccharomyces*, *Geotrichum*, *Kluyveromyces*, *Rhodotorula*, *Saccharomyces*, and *Torulaspora* (Viljoen and Greyling, 1995; Wyder and Puhan, 1999). Although the prevalence of different yeast species depends on the type of the cheese considered, *D. hansenii* is the most common species found in all types of cheese (Fleet, 1990). This is due to its ability to grow in the presence of salt at low temperature and to metabolize lactic and citric acids. *D. hansenii* is involved in the fermentation of several types of surface-ripened cheeses, such as Limburger, Tilsitter, Port Salut, Trappist, Brick and Danish Danbo. Yeasts species, such as *D. hansenii* and *Y. lipolytica*, are particularly strong users of lactic acid, especially at the surface of the curd, causing an increase in pH which enables the growth of less acid tolerant species, such as micrococci and coryneform bacteria. *D. hansenii* also produces proteolytic and lipolytic activities that contribute to the ripening process (Jakobsen & Narvhus, 1996; Guerzoni et al., 2001; Ferreira & Viljoen, 2003). In addition, this species may produce factors useful for bacterial growth, as well as aroma components. Another positive effect of *D. hansenii* is its inhibition of cheese spoilage bacteria of the genus, *Clostridium* (Fatichenti et al., 1983).

Taking into account that, within the same cheese type, the yeast species composition depends on the cheese factory, the part of the cheese analyzed and the ripening stage, this study analyzed the yeast population of "Pecorino di Filiano" cheese. The origin of "Pecorino di Filiano" cheese dates back some centuries to about 1600. The milk produced on sheep-farms is transformed directly in the same factory by following technological practices handed down from one generation to another. The milk is coagulated with lamb or kid rennet paste without the addition of starter cultures. Therefore, the ripening process occurs only with the natural flora originating from the milk and the environment. The cheese is ripened (matured) in natural

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caves or in a ripening room for a minimum of 180 days at 14 °C, and at a humidity of 70–80%. This cheese is produced under EU regulations and has been awarded Protected Designation of Origin (PDO) (Reg. CEE N. 1485/2007).

Due to a very limited production area and the absence of inoculated starter cultures, "Pecorino di Filiano" cheese represents not only a typical regional product, but also an important source of natural yeast microflora that could be further exploited in cheese processing. The objective of this study was to identify the yeast species present throughout the ripening process of "Pecorino di Filiano" artisanal ewe's cheese of the Basilicata region and to characterize them for genotypic variation and some technological properties.

2. Materials and methods

2.1. Yeast strains and isolation

In this research 78 yeasts were isolated from samples of "Pecorino di Filiano" cheese produced by traditional methods at two different factories in the Basilicata region. The pH of analyzed cheese was 5.5, whereas NaCl and moisture content were 2% (w/w) and 32% (w/w), respectively. Yeasts were isolated from the interior part of the cheese (curd), which were coded with letters A and B, and the cheese rind, which were coded with letters E and T, at different ripening times (0, 30 and 120 days of maturation). Other yeasts were isolated from the air of the ripening room, and coded with letters F and J. The air of the ripening room was sampled by using Surface Air System sampler (SAS). YPL agar (1% yeast extract; 2% peptone; 2% lactose; 2% agar) added with 0.1 g/l chloramphenicol (Merck, Darmstadt, Germany) was used for the enumeration and isolation of yeasts. Plates were incubated at 25 °C for 5–7 days. Yeast counts ranged from 10^4 to 10^9 CFU/g, with the higher counts observed in the rind of the cheese and at the start of ripening (0 day of maturation). The isolates are listed in Table 1 and were maintained on slants of YPD agar (1% yeast extract; 2% peptone; 2% glucose; 2% agar) at 4 °C.

Reference strains obtained from the Centraalbureau voor Schimmelcultures (CBS, Utrecht, Netherlands) and the Industrial Yeast Collection DBVPG (Dipartimento di Biologia Vegetale Università di Perugia (Perugia, Italy) were used: *Debaryomyces hansenii* DBVPG 6017 and DBVPG 6050, *Candida famata* (imperfect form of *D. hansenii*) CBS 767, *Kluyveromyces lactis* var. *lactis* DBVPG 6305 and DBVPG 6075, *K. marxianus* DBVPG 6165 and CBS 712, *P. membranifaciens* DBVPG 6720 and CBS 107, *Zygosaccharomyces rouxii* CBS 732 and DBVPG 6187, *Yarrowia lipolytica* DBVPG 7070 and CBS 6124, *Saccharomyces cerevisiae* DBVPG 6173, and *Dekkera anomala* DBVPG 4075.

2.2. DNA extraction

DNA was extracted from exponential phase cultures of the strains, grown for 24 h in YPD medium (Oxoid), by following the modified method of Hoffman and Winston (1987). A loopful of yeast cells was suspended in 200 µl of lysing buffer, containing 2% (v/v) Triton X-100 (Sigma), 1% (v/v) SDS (Sigma), 100 mM NaCl (Sigma), 1 mM EDTA (Sigma), 10 mM Tris–HCl pH = 8. To the mixture, 0.3 g of glass beads (diameter 425–600 µm) (Sigma) and 200 µl of buffered phenol, chloroform and isoamyl alcohol (ratio 25:24:1 v/v) were added; afterwards, the mixture was vortexed for 1 min and centrifuged at maximum speed for 5 min. The upper phase was recovered and the DNA was precipitated by the addition of 1 ml of cold ethanol (96%) (Carlo Erba). The precipitated DNA was collected by centrifugation and the pellet was suspended in 50 µl of TE buffer. The RNA was digested by the addition of RNase A (10 mg/ml) (Sigma) and incubation at 37 °C for 2 h. The DNA was recovered by precipitation with cold ethanol and centrifugation; the dried pellet was re-suspended in 50 µl of TE buffer and stored at −20 °C until analysis.

Table 1
Isolation origin and codes of "Pecorino di Filiano" cheese wild yeasts.

Ripening time (d)	Strain code		
	Curd	Rind	Air
0	A1, A2, A3, A4, B1, B3, B5, B10	E1, E3, E5, T1, T2, T3	F1, F2, F3, F4, F5, F7,
30	A11, A12, A13, A14, A16, A17, A20, A23, B11, B13, B23, B24	E11, E15, E16, E21, E22, T11, T19, T20, T22	F8, F9, J4, J5, J6, J7, J8, J9, J10
120	A29, A33, A34,A39, A40, A41, A43, A47, B31, B31', B34, B37, B40, B42, B43,B48	E31, E32, E39, E40, E42, E43, E44, E47, T32, T40, T47, T49	

Codes A, E, F = yeasts isolated from cheese sample I; codes B, T, J = yeasts isolated from cheese sample II.

2.3. Yeast identification

The isolates were identified at species level by amplification and restriction analysis of 18S rDNA and ITS regions. The 18S rRNA genes were amplified using the primers P108 and M3989, following the protocol reported by Capece et al. (2003). Amplification products were analyzed on 1.2% agarose gel. PCR products were submitted to restriction analysis using the endonucleases Hae III, Rsa I, Hinf I, Dde I, Msp I and Cfo I separately. In addition, the enzymes Hinf I and Cfo I were used together.

The reaction mixture contained 5 µl of amplified rDNA, 1 µl of specific enzyme buffer (Promega), and 0.25 µl of enzyme (10 U/µl) (Promega), in the case of enzyme used separately. In the case of the combined use of Hinf I and Cfo I, 0.125 µl of each enzyme was mixed and water was added to reach a final volume of 10 µl. The reaction mixture was incubated overnight at the specific temperature for each enzyme and the restriction fragments were separated on a 2% (w/v) agarose gel (Euroclone).

Amplification of the ITS region, followed by restriction analysis with Hae III enzyme, was performed by following the protocol reported by Jeyaram et al. (2008).

2.4. Genetic polymorphism in D. hansenii

Genomic DNA isolated from natural and type strains of *D. hansenii* were submitted to RAPD-PCR analysis using the primer P80 (CGCGTGCCCA), following the protocol reported by Capece et al. (2005). Amplification products were analyzed on 1.2% agarose gel and the DNA bands were sized against the DNA molecular weight marker VI (Roche Diagnostics). The gel was captured by the Gel Doc 2000 system (Bio-Rad) and analyzed by Diversity Database 2.2.0 software. Similarities among combined fingerprints were calculated using the Pearson product–moment correlation coefficient. Cluster analysis of the pairwise values was generated using unweighted pair group algorithm with arithmetic averages (UPGMA).

2.5. Evaluation of proteolytic activity in D. hansenii

The proteolytic activity in *D. hansenii* isolates was determined on a synthetic medium with the following composition: 0.67% YNB (Promega), 0.5% casein (Merck), 0.5% glucose (Carlo Erba), 2% agar (Oxoid), pH 6.5. The strains were incubated at 26 °C for 5 days, using a strain of *Y. lipolytica* as positive control. After incubation, the plates were covered by a solution of HCl 0.1 N (Carlo Erba). The formation of a clarification halo around the colonies indicated the presence of proteolytic activity, which was expressed in centimetres of the cleared, diffusion zone.

3. Results

3.1. Identification of yeasts isolated from "Pecorino di Filiano"

Yeasts (78 isolates) from "Pecorino di Filiano" cheese, and the 15 reference strains of 8 different species were submitted to amplification and restriction analysis of the 18S rDNA.

To differentiate all the species analyzed, we used a combination of restriction enzymes, by choosing those which could be employed at the same reaction conditions. The enzymes selected were Hinf I, which determined the highest species discrimination, and Cfo I, which complemented the action of Hinf I due to its capacity to differentiate *D. hansenii* from *K. marxianus*. The combined use of these two enzymes resulted in species-specific patterns for all the isolates tested.

With regard to the 78 yeasts isolated from "Pecorino di Filiano", amplification of the 18S rDNA region yielded a single PCR product. The size of these amplicons was similar for all the isolates, ranging between 1600 and 1700 bp (data not shown). For identification of these isolates, their amplification products were submitted to digestion with the combination of the two enzymes, Hinf I and Cfo I.

The majority of the isolates (75) showed the restriction pattern exhibited by *D. hansenii* reference strains and, therefore, identified as *D. hansenii*. Only three isolates, A2 and A20 (isolated from the curd of the cheese) and T3 (from the cheese rind) exhibited different restriction profiles. The isolate A2 was identified as *D. anomala*, whereas A20 and T3 exhibited the same pattern of *K. lactis*. All the yeasts isolated from the air of the ripening room were identified as *D. hansenii*.

These identifications were confirmed by restriction analysis of the amplified ITS region with Hae III enzyme (Esteve-Zarzoso et al., 1999).

3.2. Genetic polymorphism in D. hansenii

Genetic polymorphism in *D. hansenii* isolates from the cheese was evaluated by RAPD-PCR analysis with the P80 primer in comparison to the reference strains. The molecular profiles obtained revealed the existence of a high degree of intraspecific variability. Among the 75 *D. hansenii* yeasts analyzed, unique molecular patterns were observed for many strains. However, some predominant profiles were found for other isolates. In particular, 48 strains were distributed in three genetic profiles, coded as P1, P2 and P3 (Fig. 1).

It is interesting to underline that all the yeasts showing the profile P3 were isolated at the last sampling time (120 days of ripening), whereas those exhibiting P2 and P1 were isolated during all the sampling (times 0, 30 and 120 days). Fig. 2 reports the distribution of the predominant profiles among the strains as a function of the isolation source (curd and rind of the cheese, air of the ripening room). Strains with the P2 profile were widespread. On the contrary, the P1 pattern was distributed at a low percentage in *D. hansenii* isolated from the curd and the rind of the cheese, while it was absent among isolates from the air of the ripening room. About 70% of yeasts isolated from the curd of the cheese were distributed among the three

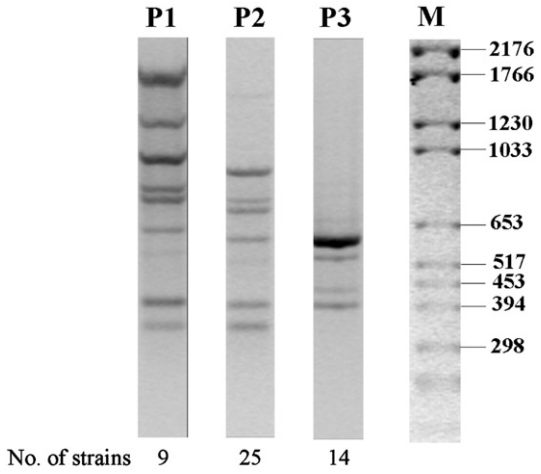

Fig. 1. Predominant genetic patterns obtained by RAPD-PCR analysis with P80 primer (CGCGTGCCCA) and their frequency among indigenous *Debaryomyces hansenii* population.

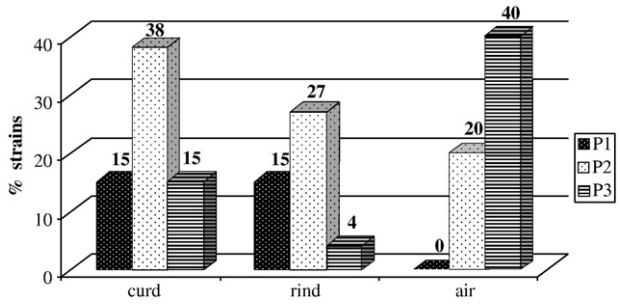

Fig. 2. Distribution of genetic predominant patterns P1, P2, and P3 among wild *Debaryomyces hansenii* isolates in relation to isolation source.

predominant profiles (P1, P2 and P3), with P2 as the prevalent one. Less than 50% of the *D. hansenii* isolates from the rind were spread among the three profiles, always with P2 as the prevalent one. With regards to the *D. hansenii* isolates from air, about 60% belonged to the P2 and P3 profiles. No isolates from the air exhibited the P1 profile but the P3 profile was dominant.

Of the three *D. hansenii* reference strains, two exhibited profile P2 (DBVPG 6050 and CBS 767) and one gave profile P3 (DBVPG 6017).

The RAPD-PCR profiles generated with primer P80 for the 75 *D. hansenii* isolates and for the reference strains produced the UPGMA dendrogram shown in Fig. 3.

The cheese isolates were grouped in 3 major clusters (A, B and C) with a similarity level of about 75%. Cluster A includes all the strains showing the P3 genetic profile. At a similarity level of about 80%, clusters B and C were subdivided in further sub-clusters, which, in some cases, contain *D. hansenii* isolated from the same source. Thus, sub-cluster B1 includes isolates from the rind of the cheese, sub-cluster B2 includes a group composed of about 50% of the yeasts isolated from the air of the ripening room. In cluster C, two sub-clusters were distinguished (C1, C2), other than the single strain F3.

3.3. Proteolytic activity in D. hansenii yeasts

The cheese isolates gave remarkable variability for proteolytic activity on casein. In particular, the isolates were distributed in four classes on the basis of proteolytic activity, measured as the dimension of the clarification halo around the colonies. About 45% of *D. hansenii* isolates did not exhibit proteolytic activity, showing no clarification halo around the colonies. Among the other isolates, proteolytic activity was expressed at different levels, with about 15% of them showing activity at the maximum level.

Expression the proteolytic activity was correlated to the molecular profiles P1, P2, and P3. Isolates possessing the P3 profile were characterized generally by the highest level of proteolytic activity, whereas the majority of yeasts exhibiting the P1 profile displayed negative proteolytic activity. *D. hansenii* isolates belonging to the P2 profile gave significant variability in the levels of proteolytic activity.

4. Discussion

This study has reported the yeast microflora present in a unique artisinal cheese of the Basilicata region, "Pecorino di Filiano". In particular, this cheese has been produced without the addition of any selected starter cultures from ancient times. Consequently, the yeast population developing in this cheese represents the natural resident microflora which, during time, has been selected by this habitat.

D. hansenii was the main yeast isolated from these chesses throughout maturation and this correlates with the low pH, high salt content and low water activity of this product. This finding is consistent with many other studies that report the predominance of this species in cheeses during maturation (Roostita and Fleet, 1996;

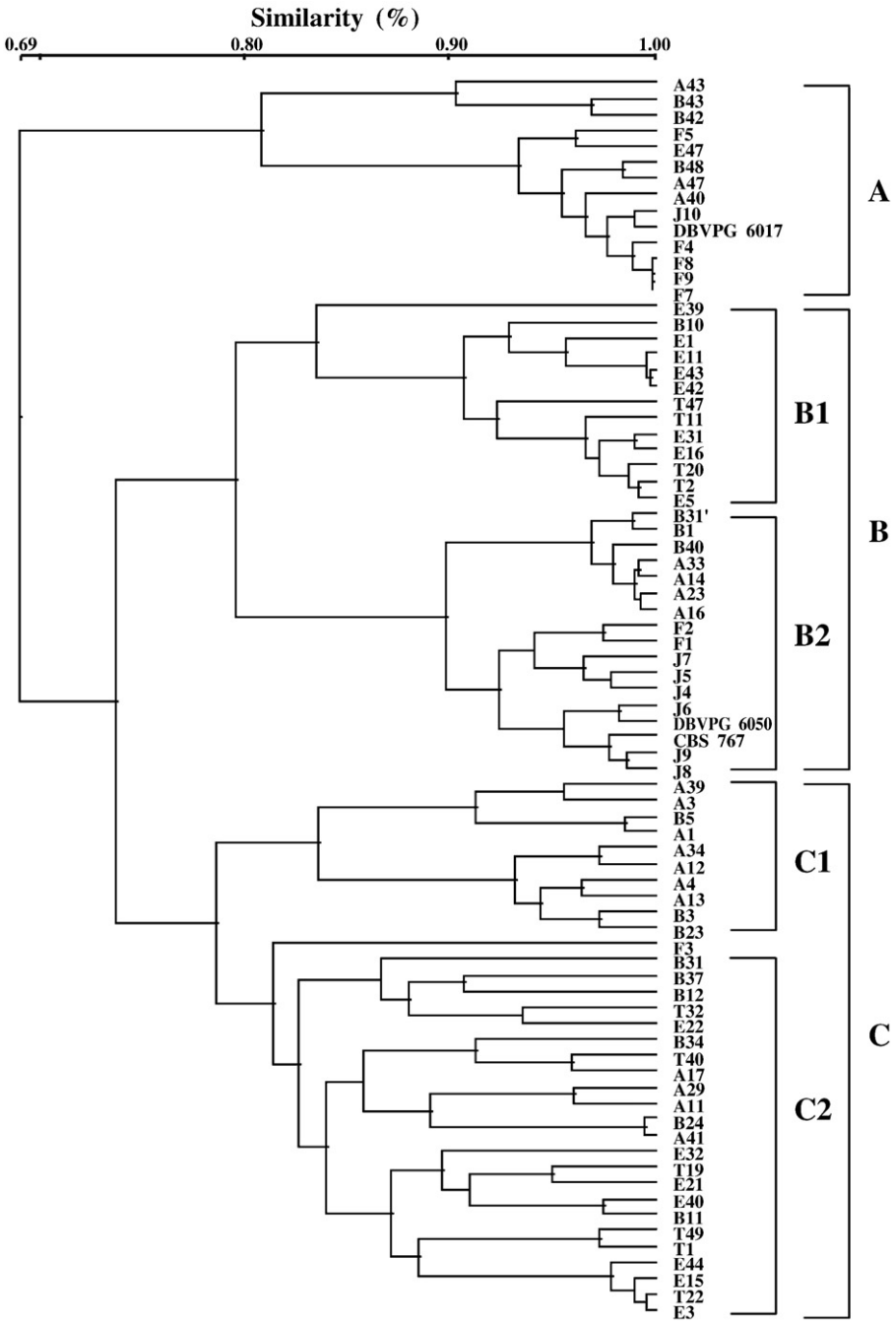

Fig. 3. Cluster analysis of RAPD-PCR patterns generated with P80 primer (CGCGTGCCCA) of *Debaryomyces hansenii* isolates.

Welthagen and Vilijoen, 1998), including some artisanal cheeses (Cosentino et al., 2001; Fadda et al., 2004).

Strains of this species have the ability to grow at high salt concentrations and therefore indigenous *D. hansenii* represent a surviving population in cheese treated with salt. According to Viljoen et al. (2003), the high yeast counts in 5 h brined cheese curds as well as the high loads of this yeast in brine, indicated the salting procedure as the main source of this organism.

There was a notable absence of *Y. lipolytica* and a very low presence of *Kluyveromyces* spp. in the "Pecorino di Filiano" cheeses examined, which contrasts to a more abundant presence of these species in cheeses, generally (Deak and Beuchat, 1996). *Y. lipolytica* has been isolated from several types of artisanal Portuguese ewes' cheeses (Freitas et al., 1996; Carreira and Loureiro, 1998). Its absence from the cheeses analyzed may be explained by its aerobic growth that is not favoured under the preferential anaerobic conditions present in the interior of semi-hard Pecorino cheese. Regarding *Kluyveromyces* spp., other authors reported the absence or low frequency of this species (Carreira et al., 1998; Roostita and Fleet, 1996; Pereira-Dias et al., 2000), and its low frequency could be related to the specific physical–chemical characteristics of Pecorino cheeses such as the high salt content.

The isolates of *D. hansenii* exhibited notable genetic polymorphism and production of extracellular proteolytic activity. Literature reporting proteolytic activity of *D. hansenii* species is variable. Numerous studies reported the absence of this activity in this species (Roostita and Fleet, 1996; Welthagen and Vilijoen, 1998; Van den Tempel and Jakobsen, 2000). However, our findings seem to suggest the existence of a relationship between the isolate phenotype and its source. Thus, the highest level of proteolytic activity was exhibited by isolates

belonging to the P3 biotype, which were mainly isolated at the end of the ripening process. This result might be due to the selective pressure of environmental parameters on the yeast population.

In conclusion, this preliminary study allowed us to demonstrate the predominant yeast microflora of "Pecorino di Filiano" cheese, which is characterized by a unique production process and of typical taste. Taking into account the trend to improve product quality together with maintenance of product typicality, the use of a well-defined starter culture of *D. hansenii* for controlled fermentation becomes advantageous. Among the autochthonous yeasts isolated during this research, some *D. hansenii* strains characterized by the P3 biotype could be considered as a "starter candidate" for the production of "Pecorino di Filiano" cheese. The use of these indigenous selected strains could assure cheese quality and typicality, and help safeguard the traditional process of this product.

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