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Yeast community in traditional Portuguese Serpa cheese by culture-dependent and -independent DNA approaches

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ABSTRACT

This study investigated the yeast community present in the traditional Portuguese cheese, Serpa, by culture-dependent and -independent methods. Sixteen batches of Serpa cheeses from various regional industries registered with the Protected Designation of Origin (PDO) *versus* non-PDO registered, during spring and winter, were used. Irrespective of the producer, the yeast counts were around 5 log CFU/g in winter and, overall, were lower in spring. The yeast species identified at the end of ripening (30 days), using PCR-RFLP analysis and sequencing of the 26S rRNA, mainly corresponded to *Debaryomyces hansenii* and *Kluyveromyces marxianus*, with *Candida* spp. and *Pichia* spp. present to a lesser extent. The culture-independent results, obtained using high-throughput sequencing analysis, confirmed the prevalence of *Debaryomyces* spp. and *Kluyveromyces* spp. but, also, that *Galactomyces* spp. was relevant for three of the five producers, which indicates its importance during the early stages of the cheese ripening process, considering it was not found among the dominant viable yeast species. In addition, differences between the identified yeast isolated from cheeses obtained from PDO and non-PDO registered industries, showed that the lack of regulation of the cheese-making practice, may unfavourably influence the final yeast microbiota. The new knowledge provided by this study of the yeast diversity in Serpa cheese, could be used to modify the cheese ripening conditions, to favour desirable yeast species. Additionally, the prevalent yeast isolates identified, *Debaryomyces hansenii* and *Kluyveromyces* spp., may have an important role during cheese ripening and in the final sensorial characteristics. Thus, the study of their technological and functional properties could be relevant, in the development of an autochthonous starter culture, to ensure final quality and safety of the cheese.

1. Introduction

Serpa is an artisanal ripened Portuguese cheese granted the Protected Designation of Origin (PDO) label (Council Regulation (EEC) No 2081/92, 2017), with six industries making cheese under this designation, although there are also many producers in the area that make it without following the PDO regulation. It is produced within the Alentejo province (south of Portugal) from raw ewes' milk, using an aqueous infusion of the dried flowers from *Cynara cardunculus* L. as rennet and without the addition of a starter culture. Cheese ripening is a complex fermentation process involving a wide range of biochemical reactions. Industrial-scale cheese production usually applies a thermal treatment to standardise the microbial diversity, followed by starter culture inoculation to ensure the safety and reduce the variability in the final product but affecting the original sensorial characteristics (Montel et al., 2014). In contrast, there is no standardisation for the thermal process and starter microorganisms application in traditional raw milk cheese, which, therefore, possess a complex microbial community that arises primarily from the raw milk, vegetable rennet and the cheese dairy environment (Aquilanti et al., 2011; Bokulich and Mills, 2013; Ordiales et al., 2013a, 2103b; Pereira et al., 2010; Roseiro et al., 2003), leading to desirable sensorial properties highly appreciated by consumers. The development of the initial microbial population during the cheese-making process and ripening period, strongly contribute to the quality and safety of the cheese, through their metabolic activities (Montel et al., 2014). Most of the microbial community present in raw

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milk are lactic acid bacteria (*Lactococcus* spp., *Lactobacillus* spp. and *Enterococcus* spp.) and their importance in cheese ripening is well recognised (Beresford et al., 2001). Additionally, the yeast population is also important in raw milk (Delavenne et al., 2011; Quigley et al., 2013) and is associated with the secondary microbiota of diverse types of cheeses, where they play an important role during the ripening.

Artisanal cheeses possess a large assortment of yeast species, mainly belonging to the genera *Debaryomyces*, *Geotrichum* (= *Galactomyces*), *Kluyveromyces*, *Candida*, *Pichia* and *Yarrowia* (Banjara et al., 2015; Binetti et al., 2013; Ceugniez et al., 2015; Padilla et al., 2014), although the prevalence of certain yeast species can be influenced by the type of cheese (Dugat-Bony et al., 2016). Yeasts contribute to the cheese ripening by lactate consumption, alkaline metabolite formation, lactose fermentation, lipolysis, proteolysis, production of aromatic compounds and by their positive or negative interactions with other members of the microbial cheese community, which are important for the typical characteristics of cheese (Jakobsen and Narvhus, 1996; Rossi et al., 1997). Conversely, yeasts can also cause cheese spoilage, by generating undesirable flavours, texture losses, excessive gas formation, acidity increase and brown surface discolourations (Carreira et al., 1998; Jakobsen and Narvhus, 1996; Liu and Tsao, 2009; Wyder et al., 1999).

Differences in artisanal cheese can be expected among producers, due to small differences in the cheese-making technology and slight variations in the chemical and microbial composition of the milk associated with the conditions of milk production, such as hygiene, geographical area, animal breed, season and the microbial population of the cheese-making environment (Alessandria et al., 2016; Bokulich and Mills, 2013; Guiné et al., 2016). Thus, considering the relevance of the microbial community, particularly, the fungi, on the organoleptic properties, as well as in the safety of cheese, the various genera and species present must be identified and quantified, to determine their influence on maturation, alteration or deterioration of cheese. A thorough microbial survey of similar cheeses, regarding their origin and production technology, as PDO and non-PDO cheeses, could be highly valuable for the dairy industry, as the PDO accreditation enables knowledge of the production technology and milk production conditions, to guarantee the quality of the product.

For several decades, the diverse composition of cheese fungi has been investigated by the application of conventional culture-dependent approaches combined with molecular tools based on the polymerase chain reaction (PCR) of the short non-coding ribosomal ITS regions (ITS I and ITS II), which are extremely variable spacers in both sequence and length that provide an excellent tool to differentiate between and within species, and the D1/D2 domain of the large subunit ribosomal RNA (LSU rRNA) (Álvarez-Martín et al., 2007; Blackwood et al., 2003; Tofalo et al., 2014). However, the development of culture-independent molecular methods has changed the approach to study microbial communities during food fermentations processes. In particular, high-throughput sequencing (HTS) technology, has revolutionised the study of microbial ecosystems (De Filippis et al., 2017). HTS enables comprehensive microbial surveys, with detection sensitivities and throughputs several orders of magnitude greater than earlier molecular techniques (Bokulich and Mills, 2013). Among HTS possibilities, PCR amplification and sequencing of universally conserved DNA fragments, typically the ITS gene in fungi, is the most common approach exploited in food microbiology ecology studies (De Filippis et al., 2017). The advantages of this HTS approach, are its superior sequence coverage (live and dead cells throughout the process) and lower sequencing cost, however, it is restricted by primer amplification bias and by short read length, which results in a lower taxonomic resolution (Bokulich and Mills, 2012). Culture-dependent and -independent molecular biology techniques are complementary rather than contradictory. Thus, an in-depth study of the fungal diversity involved in the cheese maturation process by combining these approaches, may contribute to better understand the metabolic activities of this microbial community and their possible interactions with other members, to establish strategies to control the microbial population.

Most of the studies of microbial ecology using HTS technologies in cheese have addressed bacterial communities, whereas, scarce have investigated the fungal ecology, despite the relevance of yeasts during cheese ripening (Bokulich and Mills, 2013; Ceugniez et al., 2017; De Filippis et al., 2017; Stellato et al., 2015; Wolfe et al., 2014). Although Serpa cheese is highly valued for its sensorial characteristics, little is known about its yeast diversity. In this context, the present study aimed to compare the yeast community in Serpa cheese with a PDO label with similar non-PDO registered cheeses of the area, by culture-dependent and -independent methods.

2. Materials and methods

2.1. Serpa cheese samples

Samples were taken from the core of ripened cheeses (30-days-old) produced by five different dairy industries located in the geographical area of production. Three industries, identified as A, C and G, belonged to PDO “Queijo Serpa”, while the non-PDO registered industries were designated V and B. Two different batches and seasons, winter and spring, were analysed for the PDO industries, whereas only samples produced in winter were considered for the non-PDO industries (Table 1). Each assay was performed in three different cheeses by batch (n = 48), making each determination in triplicate.

2.2. Physicochemical analysis

The moisture content of the samples was determined by dehydration at 104 °C to a constant weight, according to the official method of

Table 1
Mean values of pH, moisture, water activity (a_w) and yeast counts (log CFU/g) in the core of cheese samples.

Serpa cheese			Physicochemical parameters			Yeast count
Season	PDO registered	Industries	pH Mean ± SD*	Moisture (%) Mean ± SD	a_w Mean ± SD	(log CFU/g) Mean ± SD
Winter	Yes	A	5.08 ± 0.09^{a}	48.76 ± 0.62^{a}	0.96 ± 0.02^{a}	5.66 ± 0.11^{d}
		C	5.03 ± 0.03^{a}	47.71 ± 1.53^{ab}	0.98 ± 0.01^{a}	5.81 ± 0.45^{d}
		G	4,95 ± 0.01^{a}	47.21 ± 0.96^{a,b}	0.97 ± 0.03^{a}	4.62 ± 0.20^{c}
	No	V	5.49 ± 0.04^{c}	39.10 ± 1.34^{c}	0.90 ± 0.03^{b}	4.44 ± 0.79^{b,c}
		B	5.02 ± 0.09^{a}	51.90 ± 0.99^{d}	0.96 ± 0.01^{a}	4.20 ± 0.19^{b}
Spring	Yes	A	5.48 ± 0.04^{c}	47.25 ± 1.37^{ab}	0.98 ± 0.01^{a}	2.55 ± 0.63^{a}
		C	4.99 ± 0.16^{a}	45.02 ± 3.69^{a}	0.98 ± 0.01^{a}	3.93 ± 0.27^{b}
		G	5.36 ± 0.10^{b}	46.60 ± 1.17^{ab}	0.98 ± 0.01^{a}	5,80 ± 0.10^{d}
P values			*0.000*	*0.038*	*0.046*	*0.000*

^{a,b,c}For a given determination (column), values with a different superscript number are significantly different ($P < 0.05$).
*SD: standard deviation.