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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.

the International Organisation for Standardisation (ISO Norm R-1442., 1979). The water activity (a_w) was determined using a GBZ FA-St/1 apparatus (Scientific Instruments, Romans-sur-Isère, France). The pH was measured using a Crison mod. 2002 pH meter (Crison Instruments, Barcelona, Spain).

2.3. Yeast counts and identification

For yeast counts, 10 g of each cheese sample was aseptically placed into a sterile plastic pouch with 90 mL of 1% peptone water (Pronadisa, Alcobendas, Madrid, Spain), followed by homogenisation for 120 s in a stomacher (Lab Blender 400, Seward Medical, London, UK). The cheese homogenates were serially diluted and aliquots from each dilution were inoculated on rose bengal chloramphenicol agar plates (RBC; Oxoid, Hampshire, UK). The enumeration was performed after incubation at 25 °C for 72 h.

2.3.1. PCR-RFLP analysis of the ribosomal internal transcribed spacers (ITS)

Ten colonies from each agar plate containing the highest dilutions were isolated at random, on RBC agar (Oxoid) and finally grown in 5 mL of yeast extract peptone dextrose broth (YPD, Pronadisa) at 25 °C for 24 h and stored at − 80 °C in 25% glycerol, until they were sufficiently grown to allow species identification.

Genomic DNA of the pure isolates was obtained by centrifugation (10,000 *g* for 5 min at 4 °C) of 1 mL of 24 h culture in YPD broth. The supernatant was discarded. The cell pellet was ground with a motor-driven pestle in 0.6 mL of hot (65 °C) extraction buffer (50 mM Tris-HCl, pH 8; 50 mM EDTA; 3% SDS; 1% β-mercaptoethanol) at 65 °C. Then, 50 μL proteinase K (10 μg/mL) was added, followed by incubation at 65 °C for 1 h, cooling on ice and, finally, extraction with phenol–chloroform–isoamyl alcohol (25:24:1). This suspension was centrifuged at 3000 *g* for 5 min. The upper phase, containing DNA, was washed with phenol–chloroform–isoamyl alcohol (25:24:1), transferred to a 1.5-mL centrifuge tube and precipitated by addition of 3 M sodium acetate to a final concentration of 10% (*w/v*) and two volumes of cold ethanol. Then, the tubes were mixed gently by inversion and overnight incubate at − 20 °C. After centrifugation, the pellet was washed with ethanol (70% w/v), dried under vacuum at 37 °C, and suspended in 50 μL of sterile water. Finally, the DNA was incubated with 20 μL of RNAse A (10 μg/mL) at 37 °C for 30 min.

Afterwards, yeast isolates were identified by PCR-RFLP of the ITS1-5.8S rRNA-ITS2 region, using the restriction enzymes *Taq*I, *Sau*3AI, *Dde*I and *Hae*III (Thermo Fisher Scientific, Waltham, MA, USA), as previously described (Gallardo et al., 2014). The resulting fragments were separated on 1.5% agarose gels and PCR fragment sizes were estimated by comparison with the GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific), using GeneTools image analysis software (SynGene, Cambridge, UK). Fig. 1. show an example of PCR-RFLP profiles obtained after digestion with enzymes *Taq*I and *Sau*3 AI. The fragment profiles obtained were grouped into operational taxonomic units (OTUs).

2.3.2. Sequencing analysis of the 26S rRNA region

Five representative isolates of each OTU were identified to the species level, by sequencing the D1/D2 domain of the 26S LSU rRNA, using the primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) (O'Donnell, 1993), according to the PCR conditions described by Gallardo et al. (2014). The PCR products were purified with NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany), sequenced by the Facility of Applied Bioscience Techniques (STAB) at the University of Extremadura (Badajoz, Spain) and edited with BioEdit software v7.2.5 (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html). The sequences were compared with the EMBL/GenBank database, using the BLAST algorithm. The taxonomic isolate identification was confirmed, based on the highest identity score (highest sequence homology).

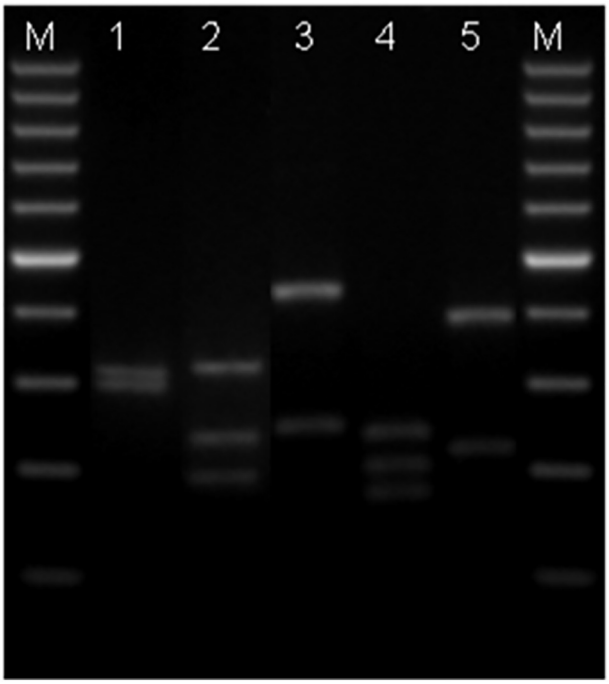

Fig. 1. Restriction profiles of ITS1-5.8S rRNA-ITS2 region with enzyme *Taq*I of *D. hansenii* profile T1 (lane 1) and *K. lactis/marxianus* profile T3 (lane2) and with enzyme *Sau* 3AI of *K. lactis/marxianus* profile S2 (lane 3), *Cryptococcus oeirensis* profile S10 (lane 4) and *D. hansenii* profile S1 (lane 5). Lanes M correspond to the GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific) from 100 bp to 1000 bp.

2.4. Identification of fungal community by HTS of the ITS rRNA gene

Cheese (5 g) was homogenised in 45 mL of 2% trisodium citrate buffer (VWR, Dublin, Ireland). A 5-mL aliquot of the homogenate was centrifuged at 10000 *g* for 5 min and the supernatant discarded. The cell pellet was suspended in lysis buffer and disrupted with 400 − 600 μm silica grinding beads in 1600 MiniG® (SPEX SamplePrep, Metuchen, NJ) at 1500 rpm for 2 min, followed by enzymatic lysis with lysozyme (1 mg/mL), mutanolysin (50 U/mL) and proteinase K (800 μg/mL) at 55 °C for 1 h, as previously described (Quigley et al., 2011). Next, the DNA was extracted using a PowerFood Microbial DNA Isolation kit (MoBio Laboratories Inc., Carlsbad, CA, USA).

The genomic DNA was amplified for the hypervariable ITS region with specific primers and further reamplified in a limited-cycle PCR reaction, to add a sequencing adapter and dual-indexed barcodes. The initial PCR reactions were performed for each sample using 2X KAPA HiFi HotStart ReadyMix, 0.2 μM of a pool of forward primers: ITS3NGS1_F 5′-CATCGATGAAGAACGCAG-3′, ITS3NGS2_F 5′-CAACGATGAAGAACGCAG-3′, ITS3NGS3_F 5′-CACCGATGAAGAACGCAG-3′, ITS3NGS4_F 5′-CATCGATGAAGAACGTAG-3′, ITS3NGS5_F 5′-CATCGATGAAGAACGTGG-3′, and ITS3NGS10_F 5′-CATCGATGAAGAACGCTG-3′ and reverse primer ITS3NGS001_R 5′-TCCTSCGCTTATTGATATGC-3′ (Tedersoo et al., 2014) and 12.5 ng of template DNA. in a total volume of 25 μL. The PCR conditions involved denaturation at 95 °C for 3 min, followed by 30 cycles at 98 °C for 20 s, 65 °C for 30 s and 72 °C for 30 s and a final extension at 72 °C for 5 min. Negative controls were included for all amplification reactions. Second PCR reactions added the indices and sequencing adapters to both ends of the amplified target region, by the use of 2X KAPA HiFi HotStart ReadyMix, 5 μL of each index (i7 and i5) (Nextera XT Index kit, Illumina, San Diego, CA) and 5 μL of the first PCR product, in a total volume of 50 μL. The PCR conditions involved a 95 °C denaturation for 3 min, followed by 8 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 5 min. The amplicons were quantified by fluorimetry with

Table 2
Restriction band profile of ITS1-5.8S rRNA-ITS2 with the various restriction enzymes.

Dde I[a]		*Hae* III[a]		*Sau* 3AI[a]		*Taq* I[a]	
Profile	PCR RFLP bands (bp)	Profile	PCR RFLP bands (bp)	Profile	PCR RFLP bands (bp)	Profile	PCR RFLP bands (bp)
D1	462 + 216	H1	421 + 176 + 81	S1	397 + 221	T1	319 + 300
D2	587 + 171	H2	646 + 112	S2	448 + 250	T2	300 + 211 + 108
D3	301 + 199 + 155	H3	628 + 113	S3	390 + 215	T3	329 + 235 + 192
D4	453	H4	453	S4	246 + 107	T4	312 + 294
D5	557	H5	400 + 143	S5	325 + 172	T5	267 + 186
D6	514	H6	390 + 124	S6	350 + 104	T6	251 + 191
D7	288 + 183	H7	366 + 70	S7	301 + 101	T7	209 + 183
D8	521 + 160	H8	422 + 91	S8	272 + 101	T8	188 + 177
D9	405	H9	540 + 86	S9	363 + 125	T9	228 + 144
D10	539 + 65	H10	405	S10	239 + 206 + 175	T10	367 + 255
D11	336 + 130	H11	381 + 144 + 79	S11	144 + 112 + 82 + 67	T11	237 + 168
D12	493	H12	358 + 90 + 72	S12	229 + 163 + 152	T12	303 + 301
		H13	493	S13	280 + 129	T13	208 + 115 + 81
				S14	298 + 119	T14	295 + 198

[a] By columns, letters with a different number indicate different sizes of PCR RFLP bands, or different restriction profiles.

PicoGreen dsDNA Quantitation kit (Invitrogen, Life Technologies, Carlsbad, California, USA), pooled at equimolar concentrations and pair-end sequenced with MiSeq® V3 chemistry, according to the manufacturer's instructions (Illumina, San Diego, CA, USA) at Genoinseq (Cantanhede, Portugal).

Sequenced reads were demultiplexed automatically by the Illumina® MiSeq® sequencer, using the CASAVA package (Illumina, San Diego, CA, USA) and quality-filtered with PRINSEQ software (Schmieder and Edwards, 2011), using the following parameters: 1) bases with average quality lower than Q25 in a window of 5 bases were trimmed, and 2) reads with < 150 bases were discarded. The forward and reverse reads were merged by overlapping paired-end reads, using the AdapterRemoval v2.1.5 software (Schubert et al., 2016) with default parameters. The QIIME package v1.8.0 (Caporaso et al., 2010) was used for OTU generation, taxonomic identification, sample diversity and richness indices calculation. Sample identifications were assigned to the merged reads and converted to FASTA format (split_libraries_fastq.py, QIIME). Chimeric merged reads were detected and removed, using UCHIME (Edgar et al., 2011) against UNITE/QIIME ITS v12.11 database (Abarenkov et al., 2010) (script identify_chimeric_seqs.py, QIIME). The OTUs were selected at 97% similarity threshold, using the open reference strategy. First, merged reads were pre-filtered by removing sequences with a similarity lower than 60%, against the UNITE/QIIME ITS v12.11 database (Abarenkov et al., 2010). The remaining merged reads were then clustered at 97% similarity against the same databases listed above. Merged reads that did not cluster in the previous step, were *de novo* clustered into OTUs at 97% similarity. A representative sequence of each OTU was then selected for taxonomy assignment (pick_rep_set.py, assign_taxonomy.py; QIIME).

2.5. Statistical analyses

SPSS for Windows 21.0 (SPSS Inc. Chicago, IL, USA) was used. The physicochemical data were analysed by a one-way analysis of variance (ANOVA), and a three-way ANOVA was used for the microbiological data. The means were separated by Tukey's honestly significant difference test.

3. Results and discussion

3.1. Physicochemical changes and yeast counts

The cheese samples had a pH range between 4.95 − 5.49. These pH data concur with those reported for other soft body cheeses (Alvarenga et al., 2008; Ordiales et al., 2013a). At 30 days, the moisture content of the cheeses ranged from 45.02 − 48.76%, while the a_w values were around 0.90 − 0.98. Among the samples, those from industry V, non-PDO registered, showed the highest pH value and a significantly lower a_w and moisture ($P < 0.05$) (Table 1). Overall, these results agree with the findings presented by Roseiro et al. (2003) in Serpa Portuguese cheese made using a semi-industrial process.

The mean RBC yeast counts ranged between 4.24 − 5.66 log CFU/g in the winter, with non-PDO producers having the lowest mean values. In spring, the counts for producers A and C were significantly lower than in the winter season, whereas samples from producer G showed a contrary tendency (Table 1). The observed counts in the core of the cheese at the end of the process (30 days) were slightly higher than that reported by other authors in similar types of cheeses at around 30 days of ripening (Ordiales et al., 2013a; Tavaria and Malcata, 1998). Moreover, the differences in the yeast counts in the final cheese between producers and seasons are expected, due to several factors, such as the initial population of the raw materials, the hygiene practices during the cheese-making process and the industry environment, which have a strong influence on the final cheese fungal populations (Bokulich and Mills, 2013; Ordiales et al., 2013a, 2013b; Quigley et al., 2013).

3.2. Culture-dependent identifications by PCR-RFLP analysis of ITS region

The isolates from the RBC plates were further investigated for their identification at the species level (Table 2). The 471 yeast isolates from the core cheese samples were grouped by PCR-RFLP analysis of the ITS1-5.8S rRNA-ITS2 and identified by subsequent sequencing of the 26S LSU rRNA D1/D2 domains. This provided a guide to the prevalence of live yeast in the final cheeses from different industries and seasons.

Based on the molecular identification of the ITS region by RFLP, the endonucleases *Dde*I, *Hae*III, *Sau*3AI and *Taq*I showed different results in the restriction analyses (Table 2). Twelve different restriction profiles were obtained with *Dde*I (D1 − D12), while thirteen were observed with *Hae*III (H1 − H13) and fourteen with *Sau*3AI (S1 − S14) and *Taq*I (T1 − T14). The high numbers of restriction profiles obtained and the different sizes of the PCR products from the ITS region, means a wide diversity of yeast species exist in Serpa cheese (Tables 2 and 3). The combination of restriction analyses using these four enzymes, allowed separation of 471 isolates into seventeen different OTUs. The sequencing of the 26S LSU rRNA and BLAST sequence comparison of representatives isolates from each OTU obtained by PCR-RFLP, allowed their identification at the species level (Table 3). The PCR-RFLP profiles P1, P2, P3 and P4, contained the majority of the isolates, approximately 71.1%, which belonged to *Debaryomyces hansenii* (OTUs 1 and 2), *Kluyveromyces lactis* and *Kluyveromyces marxianus*, respectively. The remaining 28.9% of the total isolates comprised twelve different species

Table 3
Identification of the yeast isolated from Serpa cheese samples by a culture-dependent method.

			Restriction analysis of ITS1-5.8S rRNA-ITS2				Identification		
PCR-RFLP profiles (OTUs)	% of isolates	PCR ITS size (bp)	*Dde*I[a]	*Hae*III[a]	*Sau*3AI[a]	*Taq*I[a]	26S rRNA sequencing	Accession numbers	% identification
P1	30.5	678	D1	H1	S1	T1	*Debaryomyces hansenii*	KY107562.1	100%
P2	5.3	678	D1	H1	S1	T2	*Debaryomyces hansenii*	KY107562.1	100%
P3	18.7	756	D2	H2	S2	T3	*Kluyveromyces lactis*	KY108048.1	100%
P4	16.6	756	D2	H3	S2	T3	*Kluyveromyces marxianus*	KY108098.1	100%
P5	9.1	665	D3	H1	S3	T4	*Candida zeylanoides*	KY106915.1	100%
P6	1.9	453	D4	H4	S4	T5	*Candida pararugosa*	HE660073.1	100%
P7	1.9	557	D5	H5	S5	T6	*Candida parapsilosis*	KY106676.1	100%
P8	1.3	514	D6	H6	S6	T7	*Candida cabralensis*	NG_055163.1	99%
P9	4.4	470	D7	H7	S7	T8	*Pichia fermentans*	KY108815.1	100%
P10	1.3	470	D7	H7	S8	T8	*Pichia fermentans*	KY108815.1	100%
P11	1.3	551	D5	H8	S9	T9	*Pichia kudriavzevii*	KY108855.1	100%
P12	3.2	681	D8	H9	S10	T10	*Cryptococcus oeirensis*	AF181519.1	100%
P14	1.9	405	D9	H10	S11	T11	*Yarrowia lipolytica*	KY110199.1	100%
P15	0.6	604	D10	H11	S12	T12	*Cyberlindnera jadinii*	KC844835.1	100%
P16	1.3	520	D11	H12	S13	T13	*Moniliella suaveolens*	LC004102.1	100%
P17	0.6	493	D12	H13	S14	T14	*Magnusiomyces capitatus*	NG_055400.1	100%

[a] By columns, letters with a different number indicate different sizes of PCR bands, or different restriction profiles.

from seven different genera. Of these, *Candida zeylanoides* and *Pichia fermentans* represented around 9.1 and 5.7% of the total, whereas the remaining species were minor contributors (Table 3). Overall, the majority of the species found are very common in different cheese variety (Álvarez-Martín et al., 2007; Atanassova et al., 2016; Banjara et al., 2015; Gardini et al., 2006; Padilla et al., 2014; Pereira-Dias et al., 2000; Tofalo et al., 2014).

For *D. hansenii*, two different profiles for the restriction enzyme *Taq*I were obtained, generating two OTUs (P1 and P2). Gallardo et al. (2014) also acquired different profiles using the *Taq*I enzyme, for isolates from *D. hansenii*. Likewise, two different OTUs were ascribed to *P. fermentans* species (P9 and P10) due to different restriction profiles obtained by *Sau*3AI (Table 3). Thus, the restriction analysis of the ITS-5.8S rRNA, combining the enzymes *Taq*I and *Sau*3AI, allows an accurate and simple way to identify common yeast species from cheese.

Regarding the distribution of yeast species according to industry and season, Table 4 shows the percentage of isolates from each species detected. The microbiota of yeast in cheese samples from PDO registered industries, were dominated by *D. hansenii* and *Kluyveromyces* spp., with different percentages depending on the season. In industry A, *D. hansenii* was the only species present in spring samples, while, in winter, although *D. hansenii* was dominant, other yeast species commonly associated with cheese including *Cryptococcus oeirensis*, *P. fermentans*, *Pichia kudriavzevii* and *Candida pararugosa*, were also found at noticeable levels. The appearance of the ubiquitous species *Magnusiomyces capitatus* (teleomorph form of *Saprochaete capitata*), previously named *Geotrichum capitatum*) in samples from industry A, is concerning, given this organism has been associated with human pathology in immunocompromised patients (Brunetti et al., 2016). However, in the remaining two PDO registered industries, the yeast community was composed mainly of species from the genera *Kluyveromyces*. In particular, *K. marxianus* was mainly found in winter samples and *K. lactis* in spring. Moreover, *P. fermentans* in cheese from industry G in winter and *C. zeylanoides* in cheese produced by industry C in spring, were also present at important levels, with minor percentages of the other isolated yeast species.

On the contrary, the yeast population in non-PDO industries (V and B) was different to PDO industries. In industry V, *C. zeylanoides* was the major species, with *Candida parapsilosis* and *Moniliella suaveolens* present to a lesser extent, whereas in industry B, although around 60% of the isolates belonged to *D. hansenii* and *K. marxianus*, remarkable levels of three other species were also present. Two species, *Candida cabralensis* and *M. suaveolens*, were only associated with non-PDO industries. The presence of these species in cheese have been reported sporadically, with *C. cabralensis* found in traditional Cabrales cheese (Flórez et al., 2010), and *M. suaveolens* in artisanal Fiore Sardo cheese (Fadda

Table 4
The percentages of each yeast species identified in Serpa cheese samples according to the producer and season.

Season		Winter					Spring		
Industries		PDO			Non PDO		PDO		
		A	C	G	V	B	A	C	G
Identified species	*Debaryomyces hansenii*	25.0				58.4	100.0		6.0
	Kluyveromyces marxianus		71.5	32.0		8.3		28.5	
	Kluyveromyces lactis	5.0	24.0	26.0				28.5	88.0
	Pichia fermentans	15.0		33.0					
	Pichia kudriavzevii	15.0							
	Candida pararugosa	15.0							
	Candida cabralensis					16.7			
	Candida zeylanoides				63.0			35.0	6.0
	Candida parapsilosis			9.0	25.0				
	Yarrowia lipolytica					8.3		8.0	
	Cryptococcus oeirensis	20.0							
	Magnusiomyces capitatus	5.0							
	Moniliella suaveolens				12.0	8.3			
	Cyberlindnera jadinii		4.5						

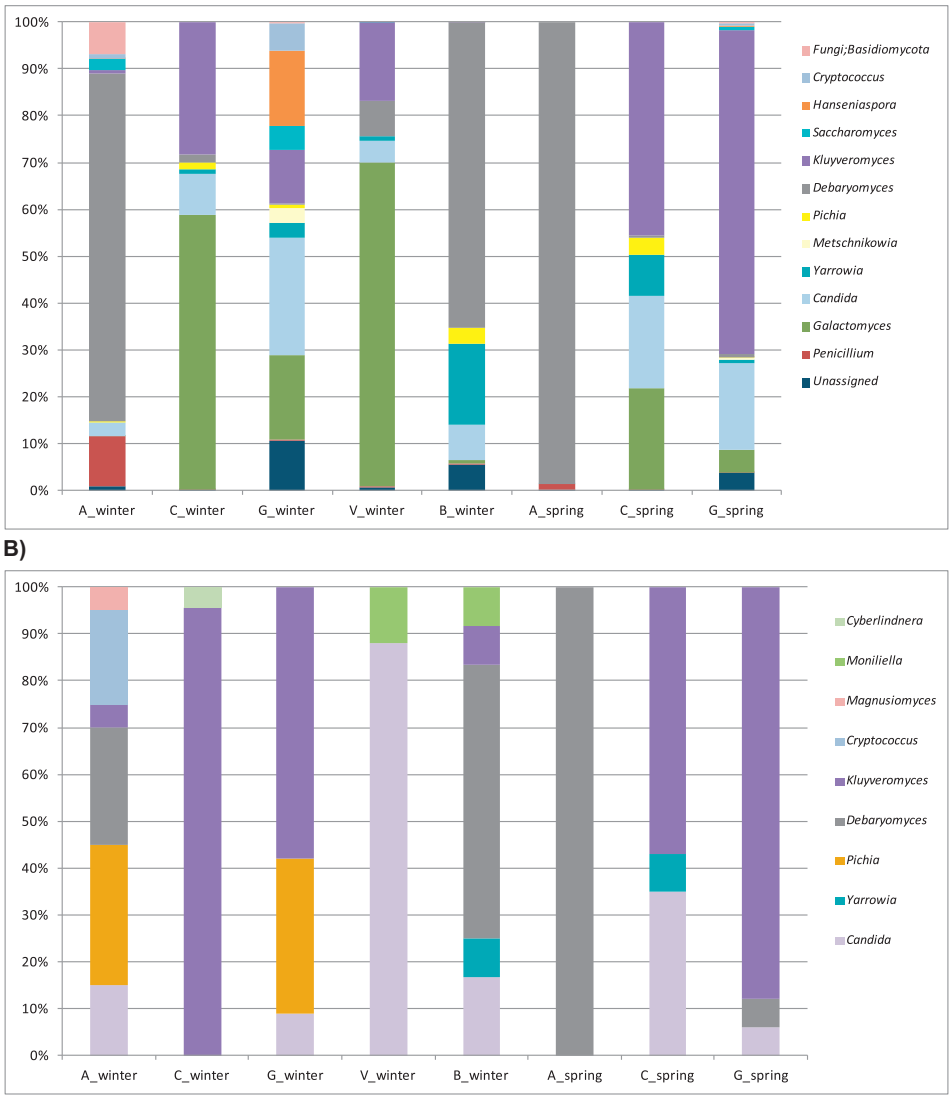

Fig. 2. Mean relative abundance of yeast in cheeses from different industries (capital letters) analysed in two different seasons (winter and spring) using high-throughput sequencing of the ITS rRNA gene at the genus level (A), and culture dependent methods (B).

et al., 2004) and Mexican artisanal Cotija cheese (Chombo-Morales et al., 2016). Furthermore, although yeasts are rarely associated with foodborne infections, *C. parapsilosis* is among the most common yeast pathogens and responsible for various mycoses (Jacques and Casaregola, 2008). This species was isolated in winter, at a low level in cheese obtained from industry G (PDO registered) but it was found at a higher level in cheese produced by industry V (non-PDO registered). However, *C. parapsilosis* has been detected in various types of cheese and infection for the arising from the consumption of food contaminated with this species, has not yet been documented (Banjara et al., 2015; Padilla et al., 2014; Pereira-Dias et al., 2000).

3.3. Culture-independent identification by HTS of the ITS rRNA gene

The HTS identifications at the genera level, of the yeast community in the cheeses obtained from the various industries and different seasons, are presented in Fig. 2A. In agreement with the culture-dependent tools, a wide diversity of yeasts was found. The sequenced isolates were clustered into 11 main OTUs. Low percentages of sequences were associated with the phyla Basidiomycota and this was mainly found in cheese from industry A in winter. Most of the sequences were assigned to phyla Ascomycota and it is remarkable that between 4 and 10% of the sequences (industry G in both seasons and industry B) were not matched to any yeast genera of the UNITE/QIIME database. These species may be entrenched in the environment of these industries and it could be of interest to perform isolates from these buildings to obtain information about these unknown yeasts.

Regarding the yeast OTUs identified, most of the yeast species detected by the culture-dependent approach belonged to the genera found by HTS (Table 4; Fig. 2). Among the new OTUs, *Galactomyces* spp. (= *Geotrichum* spp.) was found at an important level in cheeses from industries C, G and V in winter and from industry C in spring. Among the species belonging to the genera *Galactomyces*, *G. candidus* (anamorph *Geotrichum candidum*) is a ubiquitous yeast species commonly found in cheese and with important technological application in the cheese-making process (Boutrou and Guéguen, 2005; Ceugniez et al., 2017). However, the salt sensitivity of this species is well known and, in general, its growth is limited at levels above 1% (Wyder, 1998). HTS of target genes from genomic DNA, cover live and dead microorganisms. Hence, *G. candidus* was probably dominant at the beginning but was out-competed by other yeast species, such as those detected by the culture-dependent method, due to the decrease in moisture and consequent increase in the salt concentration with the progression of cheese ripening. In addition, another three genera detected by HTS, *Metschnikowia*, *Saccharomyces* and *Hanseniaspora*, were also not found among the dominant isolates.

Among the industries, although slight differences in the abundance of each genus, except *Galactomyces*, were found, overall, a high correlation between both approaches used in this work to study the Serpa cheese yeast community, was obtained (Table 4 and Fig. 2). Regarding the PDO registered industries, *Debaryomyces* spp. was dominant in cheese from industry A and *Kluyveromyces* spp. from industry C, in both seasons. However, in cheese from industry G, although *Kluyveromyces* spp. was also the most important yeast genera in spring, in winter, although HTS showed an important level of this organism, its abundance was lower than *Candida* spp. that was isolated from culture media at a lower level. A similar tendency was observed for the non-PDO industries, where *Debaryomyces* spp. was dominant in cheese from industry B in agreement with the culture-dependent method, however, in industry V, *Candida* spp. was not the major yeast and it was out-competed by other yeast genera.

The results of this study demonstrate that the yeast community of Serpa cheese is composed of a wide diversity of species, similar to the results reported in the literature in other artisanal cheese (Atanassova et al., 2016; Padilla et al., 2014; Pereira-Dias et al., 2000; Tofalo et al., 2014). The most common species detected, belonged to *Debaryomyces* and *Kluyveromyces* genera and *Galactomyces* may also be important during the initial stage of ripening. These species are among the most frequent yeasts found in cheese. In addition, they are recognised as safe and contribute positively to the ripening and sensorial characteristics (Fleet, 2011). Interestingly, in general, the same yeast genera were prevalent in PDO registered industries in both seasons, which, despite the expected differences in the yeast community of the raw materials, indicates that the cheese-making environment may be an important source of yeast with relevant functions during the ripening period. These results confirmed the evidence reported by other authors, who studied the influence of the industry environment (Bokulich and Mills, 2013; Gori et al., 2013; Stellato et al., 2015).

4. Conclusion

The data reported in this study showed the complex community of yeasts in artisanal Serpa cheese. The differences between the identified yeast isolated from cheeses obtained from PDO and non-PDO registered industries, reveal that the lack of regulation of the cheese-making practice may unfavourably influence the final yeast microbiota. The combination of culture-dependent and -independent techniques, demonstrated that the prevalent yeast belonged to *Debaryomyces* and *Kluyveromyces* genera. In addition, the high levels of *Galactomyces* detected by HTS, which may be not viable at the end of the process, indicate its importance during the early stages of cheese ripening. The new knowledge 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, *D. 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 to develop an autochthonous starter culture, to ensure the final quality and safety of the cheese.

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