Bacterial Communities in Serpa Cheese by Culture Dependent Techniques, 16S rRNA Gene Sequencing and High-throughput Sequencing Analysis

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Abstract: Serpa cheese is one of the traditional regional Portuguese cheeses having the Protected Denomination of Origin (PDO) designation. This study investigated the bacterial community in the traditional Portuguese Serpa cheese. The microorganisms identified at the end of ripening (30 days) mainly were lactic acid bacteria (LAB). *Lactobacillus paracasei/Lactobacillus casei* was the main species in cheese from PDO registered industries, whereas in non-PDO registered industries *Lactobacillus brevis* was highlighted, among other LAB. *Enterobacteriaceae* species were detected at 20% to 40% of the total isolates. The results obtained by high-throughput sequencing analysis confirmed that LAB was the main microbial group, with *Lactococcus* genus contributing to approximately 40% to 60% of the population, followed by *Leuconostoc* and *Lactobacillus*. The *Enterobacteriaceae* family was also important. The differences between bacterial communities from PDO and non-PDO registered industries suggest that the lack of regulation of the cheese-making practices may influence unfavorably. The new knowledge about bacterial diversity in Serpa cheese could be useful to set up new ripening conditions, which favor the development of desirable microorganisms.

Keywords: cheese, microbial population, traditional cheese

Practical Application: The control of the manufacturing process of traditional cheeses can be improved through the knowledge of the bacterial diversity that develops. Thus, the growth of desirable microorganisms can be promoted to homogenize the final product.

Introduction

Serpa is an artisanal ripened Portuguese cheese granted the Protected Designation of Origin (PDO) label (Council Regulation EEC 2081/92), with six industries making cheese under this designation, although there are also other producers in the area without following the PDO regulation. It is produced within the Alentejo province (south of Portugal) from raw ewes' milk using aqueous infusion of the dried flowers from *Cynara cardunculus* L. plant as coagulant and without the addition of a starter culture. The absence of thermal process and starter microorganisms means that its quality and characteristics depend mainly on the endogenous microbiota, which arises primarily from the raw milk, vegetable coagulant and the cheese dairy environment (Aquilanti et al., 2011; Bokulich & Mills, 2013; Ordiales et al., 2013b; Pereira, Graça, Ogando, Gomes, & Malcata, 2010a).

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Most of the microbial community present in raw milk are lactic acid bacteria (LAB) (*Lactococcus* spp., *Lactobacillus* spp., and *Enterococcus* spp.), but microorganisms, such as *Enterobacteriaceae*, coliforms, *Staphylococcus aureus*, *Pseudomonas* spp., or even *Listeria monocytogenes* may also be present, and this raises the potential of public health risks (Pereira et al., 2010a). Hence, controlling the microbial communities during cheese production is the main factor to ensure food safety and the sensorial properties of the final product (Coton et al., 2012). During cheese ripening, the microbiota is mainly dominated by various LAB species, and to a lesser extent by cocci Gram-positive catalase-positive, diverse Gram-negative bacteria (*Citrobacter* spp., *Enterobacter* spp., *Pseudomonas* spp., *Serratia* spp. and *Hafnia* spp.), yeasts and molds (Abriouel, Martín-Platero, Maqueda, Valdivia, & Martínez-Bueno, 2008; Ordiales et al., 2013a; Dos Santos, Benito, Córdoba, Alvarenga, & Ruiz-Moyano, 2017). The contribution of LAB to the cheese final organoleptic characteristics is a consequence of their ability to ferment lactose and their proteolytic activity (Menéndez, Centeno, Godínez,& Rodríguez–Otero, 2000).

The properties of the resulting cheese can be expected to vary between 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 & Mills, 2013; Guiné, Tenreiro, Correia, Correia, & Barracosa, 2016; Pereira

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et al., 2010b). A thorough microbial survey of similar cheeses regarding their origin and production technology, as PDO and non-PDO cheeses, could be very important for the dairy industry, as the PDO regulation enables knowledge of the production technology and milk production conditions to guarantee the quality of the product.

Accurate identification of microorganisms requires a culture-dependent approach combined with mainly DNA-based molecular techniques (Bokulich & Mills, 2013; Ordiales et al., 2013a). Sequencing the 16S rRNA gene in bacteria is a suitable tool for describing the microbial diversity of food process. Identifying live microorganisms along the cheese making process may contribute to establish strategies to control the microbial population and their influence on the final cheese characteristics (Ordiales et al., 2013b). However, the advent of culture independent technique such as high-throughput sequencing (HTS) technology has revolutionized the study of microbial ecosystems, including food fermentations. High-throughput sequencing enables comprehensive microbial surveys with detection sensitivities and throughputs several orders of magnitude greater than earlier molecular techniques via massive parallel sequencing of short amplicons of universally conserved DNA fragments, typically the 16S rRNA gene in bacteria (Bokulich & Mills, 2013). Thus, HTS tools have superior sequence coverage (live and death cells during the whole process) and lower sequencing cost, but the shorter fragment length results in lower taxonomic resolution (Bokulich & Mills, 2012).

Although Serpa cheese is considered one of the most appreciated Portuguese cheeses for its sensorial characteristic, little is known about the microbial diversity of this traditional cheese. Therefore, the aim of the present work was to study the bacterial community and foodborne pathogens presence in Serpa cheese with PDO label in comparison with similar non-PDO registered cheeses of the area by culture dependent and independent methods.

Materials and Methods

Serpa cheese samples

Samples were taken at the end of the ripening process (30 days) from 5 different dairy industries located in the geographical area of production. Three industries, identified as A, C, and G belonged to PDO "Serpa cheese," while the non-PDO registered industries were designated as V and B. Serpa cheeses were manufactured after raw ewes' milk coagulation with a *Cynara cardunculus* L. flowers infusion as described Alvarenga, Canada, and Sousa (2011). Ripened cheeses have a cylindrical shape with around 800 g each, 15 cm diameter and 5 cm height.

The ripening process was performed in 2 stages. In PDO registered industries, the cheeses were ripened in rooms with temperature and relative humidity controlled. The first stage was at temperature around 8 to 9 °C and relative humidity between 92% and 97% during 8 to 10 days, followed by second period until the end of ripening at temperature around 10 to 13 °C and relative humidity between 85% and 90%. In non-PDO registered industries, the cheese-making process is more artisanal, and the production is limited to winter season. In the initial stage, the ripening was performed in a room with temperature around 12 to 13 °C and relative humidity of 90% during 8 to 10 days, followed by a second period until the end of the ripening in a room at ambient conditions. Consequently, the temperature and relative humidity varied depending on the weather conditions, with values ranging from 8 to 18 °C and relative humidity between 65% and 95%.

Two different batches by season, winter and spring, were analyzed for the PDO industries, whereas only winter was used in the non-PDO industries. Each physicochemical and microbiological assay was performed in three different cheeses by batch ($n = 48$), making each determination in triplicate.

Physicochemical analysis

The moisture content of the samples was determined by dehydration at 102 ± 2 °C to a constant weight according to the official method of the International Organization for Standardization protocols ISO 5534:2004E. 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).

Culture dependent analysis: microbial counts and pathogens detection

For the isolates and microbial counts, each cheese sample (10 g) was placed aseptically into a sterile plastic bag with 90 mL 1% peptone water (Pronadisa, Alcobendas, Madrid, Spain), and homogenized for 120 s in a Stomacher instrument (Lab-Blender 400 Seward Lab., London, England). The cheese homogenates were serially diluted and aliquots from each dilution were inoculated onto agar plates. Mesophilic aerobic bacteria were counted on plate count agar (PCA; Oxoid, Hampshire, UK) after incubation at 30 °C for 48 hr. Mesophilic lactobacilli and lactococci were enumerated on de Man, Rogosa and Sharpe (MRS; Oxoid) agar acidified to pH 5.6 with acetic acid (10%) and M17 agar (Oxoid), respectively, under 10% CO_2 atmosphere at 30 °C after 48 hr. Enterococci, typical pink or dark red colonies with a narrow whitish border, were counted on Slanetz and Bartley (SB; Oxoid) agar at 35 °C for 48 hr. The transparent and gelatinous colonies of *Leuconostoc* spp. were enumerated on Mayeux, Sandine, and Elliker (MSE) agar medium (Biokar Diagnostic, Beauvais, France) at 21 °C after 72 hr. The growth of black staphylococci colonies on Baird-Parker agar (BP; Oxoid) supplemented with potassium tellurite and egg yolk emulsion was assessed after incubation at 37 °C for 48 hr. Enterobacteria (Gram-negative and cytochrome oxidase negative), identified as rose-coloured colonies surrounded by a halo of purple precipitate, were counted on violet red bile glucose agar (VRBG; Oxoid), after incubation at 30 °C for 24 hr. *Escherichia coli* were selectively grown on chromogenic tryptone bile X-glucuronide (TBX) agar (Serco, Mexico) and the typical blue-green colonies, indicating the presence of ß-D-glucuronidase activity, were enumerated after incubation at 44 °C for 24 hr. For the enumeration of sulfite-reducing *Clostridium* spp., 10 mL aliquots of 1:10 diluted cheese samples were heated at 80 °C for 10 min in water bath to kill vegetative forms. Then, 0.1 and 1 mL aliquots of the suspensions were mixed with 9.9 and 9 mL of sulfite–polymyxin–sulfadiazine agar (SPS, Oxoid), respectively, while a 5 mL aliquot was mixed with 5 mL of SPS agar 2X. After solidification of the media, another 2 to 3 mL of SPS agar was overlaid and the test tubes were incubated under anaerobic conditions at 44 °C for 72 hr. Colonies with appearances similar to black cotton wool and approximately 2 to 3 mm in size were counted as suspected sulfite-reducing *Clostridia*.

Finally, for *Listeria* spp. and *Salmonella* spp. analysis, 25 g of each cheese sample were placed aseptically into sterile plastic bags and homogenized in 225 mL of buffered peptone water and incubated at 30 °C for 24 hr. Then, *Listeria* spp. and *Salmonella* spp. were detected by ISO 11290–2:1998+A1:2004 (1998) and ISO 6785:2001 (2001), respectively.

Microbial identification of isolates by DNA sequencing analysis

Various colonies isolated from the cheeses were identified by rRNA 16S gene sequencing analysis. From each plated medium with colonies in the highest dilutions, 10 colonies were isolated at random on nutrient agar (Oxoid) and finally grown in 5 mL of nutrient broth (Oxoid). The genomic DNA of the pure isolates was obtained and the 16S rRNA gene sequences analyzed as described by Benito et al. (2008a, b). The sequences were compared with the EMBL/GenBank database using the BLAST algorithm. The isolates were confirmed based on the highest identity score (highest sequence homology).

For pathogens identification, presumptive *Listeria* spp. or *Salmonella* spp. isolates and *S. aureus* colonies from BP agar with a black appearance and surrounded by a clear zone and tested for catalase and coagulase activity (Staphylex, Oxoid) were confirmed by sequencing the rRNA 16S gene as above. Finally, presumptive *E. coli* from TBX plates were transferred to tubes of brilliant green lactose bile broth (Oxoid) and incubated at 35 °C for 24 to 48 hr. The confirmed *E. coli* colonies were transferred to eosin-methylene-blue lactose sucrose agar (Oxoid) before identification by 16S rRNA sequencing. Enterohemorrhagic *E. coli* serotype O157:H7 was then detected using a multiplex PCR for verotoxins as described by Fratamico, Bagi, and Pepe (2000). The primers used in the multiplex PCR and the sizes of the expected PCR products were as follows: Shiga 1 toxin (210 bp) stx1F (5′-TGTAACTGGAAAGGTGGAGTATACA-3′) and stx1R (5′-GCTATTCTGAGTCAACGAAAATAAC-3′); and Shiga 2 toxin (484 bp) stx2F (5′-GTTTTTCTTCGGTATCCTATTCC-3′) and stx2R (5′-GATGCATCTCTGGTCATTGTATTAC-3′). Primers for 16S rRNA gene amplification were used as the reaction control and the verotoxin-producing *E. coli* CECT4267 as the positive control.

Identification of bacterial community by HTS of the 16S rRNA gene

Cheese (5 g) was homogenized in 45 mL of a 2% trisodium citrate buffer (VWR, Dublin, Ireland). A 2 mL aliquot of the homogenate was enzymatically lysed with lysozyme (1 mg/mL), mutanolysin (50 U/mL), and proteinase K (800 μg/mL), followed by incubation at 55 °C for 1 hr, as previously described by Quigley, O'Sullivan, Beresford, Ross, and Fitzgerald (2011). After, 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 V3V4 region with specific primers and further re-amplified in a limited-cycle PCR reaction to add a sequencing adaptor and dual-indexed barcodes. The initial PCR reactions were performed for each sample using 2X KAPA HiFi HotStart ready mix, 0.2 μM of each PCR primer: forward primer Bakt_341F 5′-CCTACGGGNGGCWGCAG-3′' and reverse primer Bakt_805R 5′-GACTACHVGGGTATCTAATCC-3' (Herlemann et al., 2011; Klindworth et al., 2013) and 12.5 ng of template DNA. The PCR conditions involved denaturation at 95 °C for 3 min, followed by 35 cycles of 98 °C for 20 s, 55 °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 indexes and sequencing adaptors to both ends of the amplified target region by the use of 2X KAPA HiFi HotStart ready mix, 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 and 72 °C for 30 s and a final extension at 72 °C for 5 min. The amplicons were quantified by fluorimetry with PicoGreen dsDNA quantitation kit (Invitrogen, Life Technologies, Carlsbad, CA, 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 & 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 less than 150 bases were discarded. The forward and reverse reads were merged by overlapping paired-end reads using the AdapterRemoval v2.1.5 (Schubert, Lindgreen, & Orlando, 2016) software 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, Haas, Clemente, Quince, & Knight, 2011) against the Greengenes v13.8 database (DeSantis et al., 2006) (script identify_chimeric_seqs.py, QIIME). Operational taxonomic units (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 Greengenes v13.8 database (DeSantis et al., 2006). The remaining merged reads were then clustered at 97% similarity against the same databases. 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).

Statistical analyses

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

Results and Discussion

Physicochemical data

The physicochemical characteristics of the cheeses (Table 1) revealed some differences among samples from different industries and seasons. The cheese samples had a pH range between 4.95−5.49, with the highest values for industry V and A in spring season. These pH data are in concurrence with those reported for soft cheeses (Alvarenga, Silva, Garcia, & Sousa, 2008; Ordiales et al., 2013a; Roseiro, Wilbey, & Barbosa, 2003). At 30 days of ripening, the moisture content of the cheeses ranged from 45.02 to 48.76% ($p < 0.05$) for PDO industries and 39.10-51.90% for non-PDO registered industries, while the a_w values were around 0.90 to 0.98. Significant differences in moisture and a_w between samples from industry V (non-PDO registered) and the rest of the industries were observed. Overall the results obtained in PDO registered industries agreed with the findings presented by Alvarenga et al. (2008) in Serpa Portuguese cheese.

Table 1–Mean values of pH, moisture and a_w values in cheese samples.

Serpa Cheese			Physicochemical parameters		
Season	PDO registered	Industries	pH Mean ± SD*	Moisture (%) Mean ± SD	a_w Mean ± SD
Winter	Yes	A	5.08 ± 0.09^{a}	48.76 ± 0.62^{a}	0.96 ± 0.02^{a}
		C	5.03 ± 0.03^{a}	47.71 ± 1.53^{ab}	0.98 ± 0.01^{a}
		G	4,95 ± 0.01^{a}	47.21 ± 0.96^{ab}	0.97 ± 0.03^{a}
	No	V	5.49 ± 0.04^{c}	39.10 ± 1.34^{c}	0.90 ± 0.03^{b}
		B	5.02 ± 0.09^{a}	51.90 ± 0.99^{d}	0.96 ± 0.01^{a}
Spring	Yes	A	5.48 ± 0.04^{c}	47.25 ± 1.37^{ab}	0.98 ± 0.01^{a}
		C	4.99 ± 0.16^{a}	45.02 ± 3.69^{a}	0.98 ± 0.01^{a}
		G	5.36 ± 0.10^{b}	46.6 0 ± 1.17^{ab}	0.98 ± 0.01^{a}
P values			***0.000***	***0.038***	***0.046***

^{a,b,c}For a given determination (column), values with different superscript numbers are significantly different ($P < 0.05$).

Table 2–Mean values of the microbiological counts (log cfu/g) in Serpa cheese from different PDO and non-PDO registered industries.

Season	Winter					Spring		
PDO registered	Yes			No		Yes		
Industries	A	C	G	V	B	A	C	G
Mesophilic aerobic bacteria (PCA*)	8.52	8.65	8.61	8.20	8.53	8.51	8.59	8.92
Lactobacilli (MRS)	8.03^{b}	8.53^{bc}	9.54^{d}	7.81^{b}	7.33^{a}	8.19^{bc}	8.32^{bc}	8.63^{b}
Lactococci (M17)	8.23^{ab}	8.44^{ab}	8.56^{bc}	8.46^{ab}	8.02^{a}	8.04^{a}	8.57^{bc}	9.01^{d}
Leuconostoc (MSE)	2.24^{b}	2.83^{b}	7.96^{c}	n.d.**^{a}	7.21^{c}	7.01^{c}	7.82^{c}	7.20^{c}
Enterococci (SB)	6.54^{ab}	7.15^{c}	6.32^{a}	7.65^{d}	6.66^{ab}	6.83^{bc}	7.04^{c}	6.51^{ab}
Staphylococci (BP)	n.d.^{a}	n.d.^{a}	n.d.^{a}	3.04^{b}	n.d.^{a}	6.01^{b}	4.78^{b}	2.18^{a}
***Staphylococcus aureus* (BP)**	n.d.^{a}	n.d.^{a}	n.d.^{a}	3.04^{b}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}
Enterobacteria (VRBG)	7.28^{b}	5.20^{b}	6.33^{b}	5.97^{b}	5.30^{b}	2.55^{a}	6.47^{b}	6.89^{b}
***Escherichia coli* (TBX)**	3.93^{d}	2.20^{b}	2.44^{bc}	3.88^{d}	3.12^{abc}	0.86^{a}	3.55^{cd}	3.14^{abc}
Sulfite-reducing Clostridia (SPS)	n.d.^{a}	n.d.^{a}	1.88^{b}	n.d.^{a}	n.d.^{a}	0.88^{b}	n.d.^{a}	0.83^{b}

^{a,b,c,d}For a given determination, values (row) with different superscript letters are significantly different ($P < 0.05$).
*PCA: Plate count agar; MRS: de Man, Rogosa and Sharpe Agar; M17: M17 agar; MSE: Mayeux, Sandine and Elliker agar; SB: Slanetz and Bartley agar; BP: Baird-Parker agar; VRBG: Violet Red Bile Glucose agar; TBX: chromogenic tryptone bile X-glucuronide agar; SPS: sulfite–polymyxin–sulfadiazine agar.
**n.d. not detected.

Microbial counts and identifications. The microbiological counts revealed some significant differences among cheeses from different industries and seasons (Table 2). Total aerobic bacteria counts ranged from 8.20 to 8.92 log cfu/g at the end of ripening process. Overall, the microbiota of raw milk cheese without the addition of starter is commonly dominated by a limited number of LAB genera, which have important roles in the organoleptic properties of artisanal cheeses (Feutry, Oneca, Berthier, & Torre, 2012; Ordiales et al., 2013a, b). The presumptive lactobacilli ranged from 7.33 to 9.54 log cfu/g, with the lower counts among the samples for non-PDO industries, V and B (7.33 and 7.81 log cfu/g respectively). The counts of lactococci were similar to lactobacilli, whereas the levels of *Leuconostoc* spp. were lower in all samples. This last LAB group showed the most variation between the industries. The enterococci population was also detected at a considerable level, with counts ranging from 6.32 to 7.65 log cfu/g. The presence of high numbers of enterococci is typical of artisan raw milk cheese, which, due to their ubiquitous presence, can easily contaminate the raw milk during its collection or at various stages of cheese making, due to poor manufacturing practices (Ordiales et al., 2013a, b; Ortigosa et al., 2008). However, they are considered a common member of cheese microbiota, with important influence in the ripening process (Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006).

For secondary microbiota, staphylococci were detected at a significantly lower level in winter cheeses compared to the spring samples. The staphylococci population is common in artisanal cheese with high variability in the counts (Galan, Cabezas, & Fernández-Salguero, 2012; Ordiales et al., 2013a).

Finally, the enterobacteria counts were between 5.20 and 7.28 log cfu/g with no significant differences between the industries, except for industry A in the spring, which scored statistically lower counts at 2.55 log cfu/g. A high level of enterobacteria is usually considered an indication of poor hygienic practices. However, the aforementioned values are in agreement with the range found by other workers for similar soft cheeses at around 30 days of ripening (Ordiales et al., 2013a, b; Tavaria & Malcata, 1998). Importantly, the presence of enterobacteria in high number at the end of the ripening may also be involved in the development of the sensory properties of the final cheese. Conversely, some species of this microbial group are considered spoilage organisms or even may be human pathogens, such as *E. coli* or *Salmonella* spp. Therefore, the level of *E. coli* was also investigated. Although much lower counts were detected, *E. coli* followed a similar tendency to the enterobacteria counts between the industries, with industry A in spring displaying the lowest counts.

The isolates from the PCA, MRS, M17, MSE, and SB plates were further investigated for their identification at the species level

Table 3–Identification of the isolates from PCA, MRS, M17, MSE, and SB plates and percent of distribution of each species by industry.

Identification	Industry							
	Winter					Spring		
	PDO			Non-PDO		PDO		
Sequencing of 16S rRNA gene Species-Accession no. (% identity)	A	C	G	V	B	A	C	G
					% of isolates on PCA*			
Lb. paracasei – CP013921.1/ *Lb. casei* – /KT897918.1 (100%)	26.6	71.4	61.8	30.1		47.2	35.4	32.5
Lb. plantarum – KT946604.1 (100%)	26.6		12.4		16.7			32.5
Lb. brevis – KP793171.1 (100%)				15.1	50.2		6.4	
L. mesenteroides – KP742818.1 (99%)						5.9	30.1	8.1
E. faecalis – KU321632.1 (100%)	17.8	5.1			8.3	6	5.9	
E. faecium – CP014449.1 (100%)						17.8		
E. hirae – LC122277.1 (100%)				15.1				
Hafnia alvei – KT767875.1 (100%)	29.2	23.6	25.9	21.7	13.5	23.1	23.1	26.8
Escherichia coli – CP015076.1 (99%)				18.1	11.3			
					% of isolates on MRS			
Lb. paracasei – CP013921.1/ *Lb. casei* – /KT897918.1 (100%)	37.5	100	71.4	40		61.3	50	40
Lb. plantarum – KT946604.1 (100%)	37.5		14.3		22.2			40
Lb. pentosus – KU945826.1 (100%)			14.3					
Lb. curvatus – KT763326.1 (100%)								10
Lb. brevis – KP793171.1 (100%)				20	66.7		8.3	
L. mesenteroides – KP742818.1 (99%)						7.7	41.7	10
E. faecalis – KU321632.1 (100%)	12.5				11.1	7.7		
E. faecium – CP014449.1 (100%)	12.5			20		23.2		
E. hirae – LC122277.1 (99%)				20				
					% of isolates on M17			
Lb. paracasei – CP013921.1 /*Lb. casei* – /KT897918.1 (100%)						50	14.3	
Lb. plantarum – LC125266.1 (100%)							14.3	
Lb. brevis – KT757228.1 (100%)	25							
L. mesenteroides – KP742818.1 (99%)			100			25	57.1	100
Lc. lactis – KT757263.1 (100%)							14.3	
E. faecalis – KU321632.1 (100%)	75	50		20	50	25		
E. faecium – CP014449.1 (100%)				40				
E. hirae – LC122277.1 (100%)		50		40	50			
					% of isolates on MSE			
L. mesenteroides- KP742818.1 (99%)	100	100	100	100	100	100	100	100
					% of isolates on SB			
E. faecalis – KU321632.1 (100%)	87.5	71.4			100	87.5	100	57.2
E. faecium – CP014449.1 (100%)		14.3						42.8
E. hirae – LC122277.1 (100%)	12.5	14.3	16.7	75				

*PCA: Plate count agar; MRS: de Man, Rogosa and Sharpe Agar; M17: M17 agar; MSE: Mayeux, Sandine and Elliker agar; SB: Slanetz and Bartley agar.

Table 4–Identification of the isolates from BP, VRBG, and TBX plates and percent of distribution of each species by industry.

Identification	Industry							
	Winter					Spring		
	PDO			Non-PDO		PDO		
Sequencing of 16S rRNA gene Species-Accession no. (% identity)	A	C	G	V	B	A	C	G
					% of isolates on BP*			
S. epidermidis – KT989845.1 (100%)						25	100	
S. caprae – KT387321.1 (100%)						12.5		
S. hominis – LN774575.1 (100%)						12.5		
S. warneri – KC139448.1 (100%)						37.5		33.3
S. simulans – CP014016.1 (100%)						12.5		
S. cohnii – KX023361.1 (100%)								66.7
S. aureus – CP015173.1 (100%)				100				
					% of isolates on VRBG			
H. alvei – KT767875.1 (100%)	100	100	100	30	60	100	100	100
E. coli – CP015076.1 (99%)				60	40			
K. oxytoca – KU761531.1 (100%)				10				
					% of isolates on TBX			
E. coli – CP015076.1 (100%)	100	100	100	100	100	100	100	100

*BP: Baird-Parker agar; VRBG: Violet Red Bile Glucose agar; TBX: chromogenic tryptone bile X-glucuronide agar.

(Table 3). Counts on the PCA medium provided a guide to the prevalence of live bacteria in the final cheese. The microorganisms identified in the PCA medium mainly corresponded to LAB and to a lesser extent, enterobacteria. *Lactobacillus paracasei/Lb. casei* were the main species in cheese samples from PDO industries (A, C and G) in both seasons. *Lb. casei* and *Lb. paracasei* are closely related species and, therefore, difficult to distinguish, with 99.4% similarity (Öztürk & Meterelliyöz, 2015). In cheeses from non-PDO registered industries, *Lb. brevis* and *Lb. paracasei/casei* were the dominant species in industries B and V, respectively. Other LAB identified were *Lb. plantarum, Leuconostoc mesenteroides,* only identified in spring season, and species belonging to the genus *Enterococcus*. *E. faecium* appeared in cheeses from industries A and V, while *E. faecalis* has a higher prevalence in A and B. These genera and species are among the most common LAB found in raw milk cheese (Feutry et al., 2012; Fuka et al., 2013; Ordiales et al., 2013a; Picon, Garde, Ávila, & Nuñez, 2016). Finally, some species from the *Enterobacteriaceae* family were detected, which contributed to around 20 to 40% of the total isolates identified. The species, *Hafnia alvei*, was identified at around 20 to 30% in all the industries. This species has demonstrated its ability to survive during the ripening process of soft cheese and other types of cheese with a long maturation process (Abriouel et al., 2008; Coton et al., 2012; Ordiales et al., 2013a; Tabla et al., 2016). Moreover, it is known to positively contribute to the aromatic properties of cheeses (Irlinger et al., 2012). The most disturbing was the presence of *E. coli* in the final product at appreciable level, particularly those obtained from industries V and B (both non-PDO registered industries), which may be due to poor milking hygiene or poor hygienic processing as consequence of different manufacturing practices suggested by PDO "Cheese Serpa".

These results differ slightly from the results obtained with HTS (Figure 1). During whole cheese processing, the predominant bacteria comprised *Lactococcus*, *Lactobacillus*, and *Leuconostoc* genera. Most of the identifications matched with those obtained in PCA, except for *Lactococcus*. Studies to assess the persistence and viability of this microorganism throughout manufacturing and ripening of cheeses have been done (Ruggirello, Cocolin, & Dolci, 2016; Ruggirello, Dolci, & Cocolin, 2014). The authors observed that at the end of ripening, lactococci were detected at a minimal amount and this discrepancy was explained with the knowledge that during the ripening process, lactococci enter in a stressed physiological state (viable not culturable, VNC), which might cause their inability to grow on synthetic medium despite their viability in the cheese matrix.

The identification of microorganisms isolated from the specific media MRS, M17, MSE and SB for various LAB genera is shown in Table 3. In MRS, corresponding mainly to *Lactobacillus* spp., *Lb. paracasei/Lb. casei* was the most common species in this medium, followed by sporadic identification of *Lb. platarum*, *Lb. brevis*, *Lb. pentosus* and *Lb curvatus*. In particular, *Lb. brevis* was present at a relatively high percentage in samples from V and B (both non-PDO registered industries). *L. mesenteroides*, *E. faecalis*, *E. faecium* and *E. hirae* were also identified in MRS agar but not in all samples. *L. mesenteroides* was also detected in PCA agar but was also only associated with spring season samples.

In M17, only *Lactococcus lactis* was detected in samples from industry C. This is in discordance with the HTS results (Figure 1B), where *Lactococcus* was the most abundant genera in the cheeses. The viability of *Lactococcus* at the end of ripening is minimal because they succumb to a stressed physiological state during the ripening process (Ruggirello et al., 2014, 2016). On the other hand, it has been reported that M17 medium it is not such a selectivity for lactococci species growth (Ruggirello et al., 2014).

All the isolates identified in MSE and SB medium corresponded to *Leuconostoc* spp. and *Enterococcus* spp., respectively, showing both media were highly selective for enumerating these genera of LAB (Table 3).

Leuconostoc spp. isolates were found in all industries and were identified as *L. mesenteroides* (Table 3). This species is usually found as a subdominant LAB species with raw milk cheese (Aquilanti et al., 2011; Fuka et al., 2013; Picon et al., 2016) and, alongside *Leucnostoc lactis,* is the most relevant species of this genus in the dairy industry (Hemme & Foucaud-Scheunemann, 2004). Although these species grow poorly in milk, they are more competitive in the late stage of the cheese ripening process. In particular, it is known that these bacteria contribute to the peculiar aroma of traditional cheeses, mainly due to their ability to metabolize citrate and lactate (McSweeney & Sousa, 2000). Additionally, they may influence the texture of cheese by the synthesis of dextrans. These valuable technological properties are the reason some researchers have considered them as starters for cheese manufacture (Alegría, Delgado, Flórez, & Mayo, 2013; Nieto-Arribas, Seseña, Poveda, Palop, & Cabezas, 2010) and may positively influence the organoleptic properties in Serpa cheese from spring season.

The enterococci population was dominated by *E. faecalis*, followed by *E. faecium* and *E. hirae*, which was only identified in winter samples and was the predominant species in industry V (Table 3). *E. faecalis* and *E. faecium* are common enterococci species found in raw milk cheese, whereas *E. hirae* is more sporadically present at an important level (Feutry et al., 2012; Ordiales et al., 2013a). Among the LAB genera, enterococci raise some controversy because some members, particularly *E. faecalis*, have been linked to various virulence factors, antibiotic resistance and gene transfer mechanisms related to human pathogenesis (Moreno et al., 2006). Moreover, they also present important tyrosine decarboxylase activity, responsible for tyramine accumulation in cheese (Picon et al., 2016). However, in many artisanal cheeses, their presence has been correlated with a positive contribution to the ripening process, influencing the development of the typical organoleptic properties, and inhibiting foodborne pathogens by producing the bacteriocins, enterocins (Giraffa, 2003; Ordiales et al., 2013b).

Table 4 shows the species identified in the BP, VRBGA and TBX plates. *S. aureus* is a foodborne pathogen and was the unique species identified in the samples originating from industry V, which is a non-PDO registered. *Staphylococcus epidermidis*, *S. warneri* and *S. cohnii* were mainly identified in cheeses prepared during spring. *Staphylococcus* spp. has been detected in most cheese varieties as a major component of the secondary flora that remains throughout ripening, probably due to their resistance to salt and dehydration (Little et al., 2008; Ordiales et al., 2013a).

The predominance of the *Enterobacteriaceae* family was also observed by HTS analysis during the ripening process (Figure 1A). *Enterobacteriaceae* have frequently been found during cheese manufacture (Coton et al., 2012; Ordiales et al., 2013a). The enterobacteria identified were mainly dominated by *H. alvei*, except for industries V and B (both non-PDO registered). For these two industries *E. coli* was also identified at a high level (Table 4), some strains of this species are considerate important foodborne pathogens. *H. alvei* has often been described as the dominant Gram-negative bacteria in cheeses (Abriouel et al., 2008; Coton et al., 2012; Ordiales et al., 2013a). These bacteria have been shown to display high proteolytic activities, which

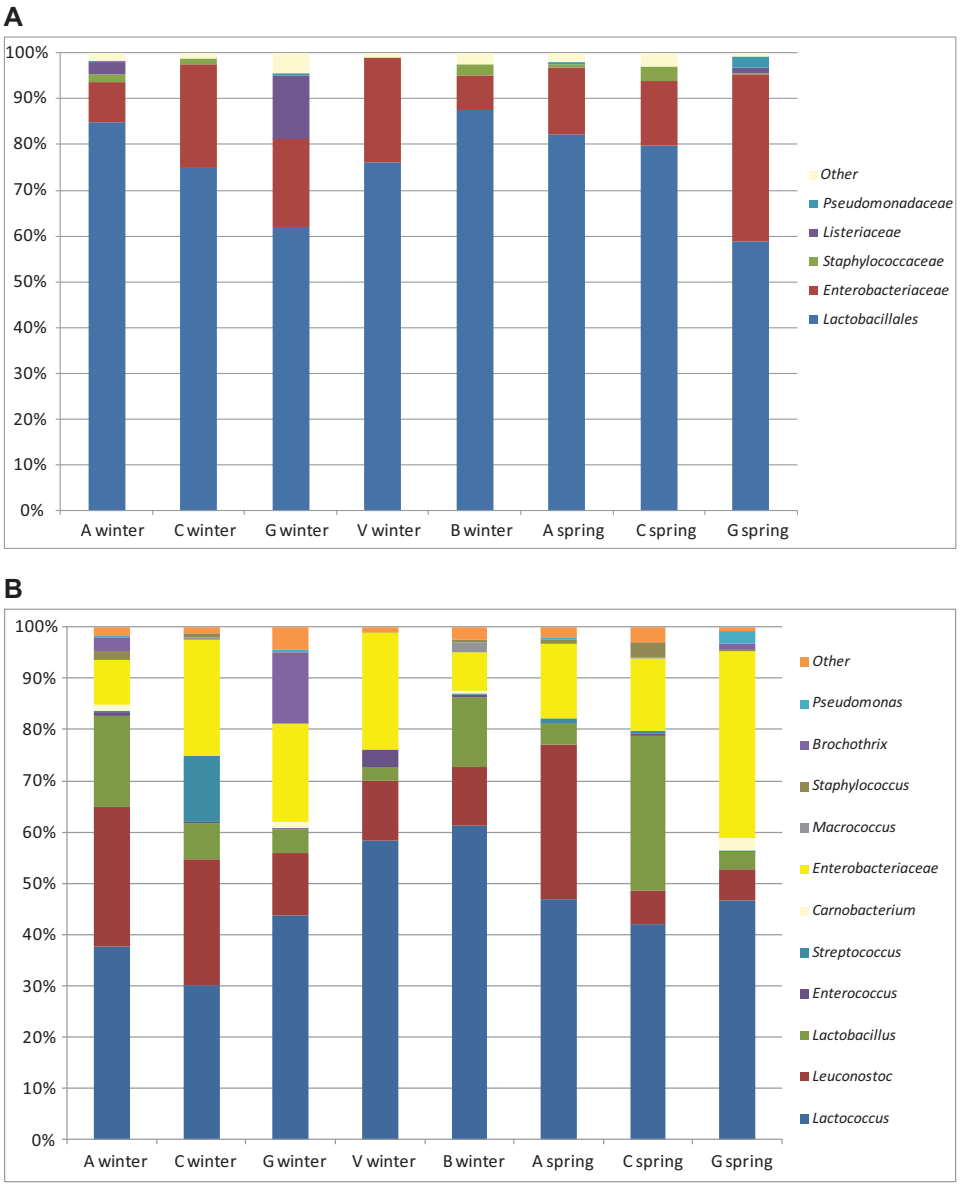

Figure 1–Mean relative abundance of bacteria in cheeses from different industries (capital letters) analyzed in two different seasons (winter and spring) using high-throughput sequencing of the 16S rRNA gene. (A) at the family level; (B) at the genus level.

could affect the sensory quality of cheese, such as creamy cheeses (Ordiales et al., 2013b).

Pathogen detection

The most relevant foodborne pathogens in raw milk cheeses, *Listeria monocytogenes*, *Salmonella* spp. and enterohemorrhagic *E. coli* were not detected in any of the cheese samples after 30 days of ripening. The absence of these pathogens in the cheese indicates the safety quality of the final product. The bacterial populations at the end of the ripening time depend on the adaptation of the microorganisms to the stress conditions found during the maturation process, such as salt concentration, pH decrease and competitive interaction with the microbiota present (Donnelly, 2004).

E. coli was found in all cheese samples at 30 days of ripening (Table 4) and all the isolates from the TBX medium were identified as *E. coli* by sequencing. This could have been due to the use of contaminated milk. *E. coli* is also used as an indicator of direct or indirect fecal contamination of foods, suggesting possible presence of enteric pathogens. In cheese, the presence of *E. coli* may indicate poor hygiene conditions (Kornaki & Johnson, 2001). Little et al. (2008) found initial levels of *E. coli* ranging from 1.1 $\times$ 10^5 to 4.6 $\times$ 10^6 cfu/g in raw milk cheese, and Ordiales et al. (2013a) found *E. coli* at 30 days of ripening in soft cheese "Torta del Casar," but these were not detected at the end of ripening process (60 days). So, these reports highlight the importance of the ripening period in raw milk cheese to ensure the safety of the final product, particularly considering the ability of some pathogens, such as *E. coli* O157:H7 and *Listeria monocytogenes* to tolerate periods of similar maturity to Serpa cheese (Almeida et al., 2007; Montet et al., 2009). Although no relevant foodborne pathogens were detected at 30 days, the *E. coli* level found in all samples at the end of the ripening (Table 2), highlighted its identification among

the dominants species on PCA agar on non-PDO industries (Table 3), suggests that a longer period, close to 60 days of ripening, would be recommended.

S. aureus was only found in cheeses from industry V (non-PDO registered) (Table 3). In raw milk cheese, *S. aureus* contamination can be caused by raw milk produced by animals suffering from mastitis, contaminated milk tank, and poor hygiene practices or the cheese handlers who are *S. aureus* carriers (Guerreiro, Velez, Alvarenga, Matos, & Duarte, 2013; Rola, Czubkowska, Korpysa-Dzirba, & Osek, 2016). *S. aureus* has been reported in cheese made from raw milk with a higher incidence than other foodborne pathogens (Little et al., 2008; Ordiales et al., 2013a). However, although *S. aureus* can produce an enterotoxin that causes illness, high numbers of the organism are necessary to produce the toxin in sufficient quantities to be a threat to public health (Le Loir, Baron, & Gautier, 2003). *S. aureus* can grow during cheese processing, but once a_w and pH are decreased, its growth is generally inhibited (Stewart et al., 2002).

Sulfite-reducing clostridia are widespread in the environment and can contaminate milk and cheese. They were detected in some cheese samples from industries G and A, but at low levels (Table 2). Clostridia include pathogenic representatives, such as *Clostridium perfringens, C. botulinum, C. difficile*, and *C. tetani*, as well as the spoilage species, *C. tyrobutyricum*, which is the main cause of the late-blowing defect in cheese, responsible for relevant financial losses in the dairy industry (Brändle, Domig, & Kneifel, 2016).

Conclusions

The data reported in this study showed higher differences among the identified microorganisms isolated from cheeses obtained from PDO and non-PDO industries. Hence, the production regulation gives guarantees of standardization to the product. The microorganisms identified at the end of ripening were LAB and to a lesser extent, enterobacteria, with *Lb. paracasei/Lb. casei* being the main species in cheese from PDO industries, while in non-PDO industries *Lb.brevis* was also identified at high level. However, by culture independent methodology (HTS), the predominating genera were mainly *Lactococcus*, followed by *Lactobacillus*, and *Leuconostoc* genus. Finally, although high level of enterobacteria were found, the main foodborne pathogens, *L. monocytogenes*, *Salmonella* spp. and enterohemorrhagic *E. coli* were not detected in cheese samples at the end of maturation, which guarantee the microbiological safety of the final product. Moreover, the study of technological and functional properties of autochthonous strains of lactic acid bacteria could be also an important tool, starter cultures to control the ripening process and ensure the final quality and safety of the cheese.

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Authors' Contributions

Maria Teresa P. Gonçalves carried out experiments, interpreted the results and drafted the manuscript. María José Benito designed the study, interpreted the results and wrote the manuscript. María de Guía Córdoba collaborated in designing experiments and drafted the manuscript. Conceição Egas developed high-throughput sequencing analysis and drafted the manuscript. Almudena V. Merchán carried out experiments and participated in data analysis. Ana I. Galván carried out experiments and participated in data analysis. Santiago Ruiz-Moyano participated in designing the study and experiments, interpreted results and drafted the manuscript.

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