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Microbiological characterization of protected designation of origin Azeitão cheese

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Abstract

Azeitão is an appreciated Portuguese protected designation of origin (PDO) cheese produced following artisanal procedures using ovine raw milk, *Cynara cardunculus* L. flower and salt. Although its origins trace back to the early 1800s, limited research regarding the characterization of its microbiota is available. So, this work aimed to perform a microbiological characterization of Azeitão PDO cheeses and raw materials obtained from a certified producer throughout the 2020/2021 production campaign, focusing on Lactic Acid Bacteria (LAB) composition. The results showed that LAB content in cheeses exceeded 8.8 log CFU/g. Overall, lactococci and lactobacilli predominated over enterococci and *Leuconostoc* spp. strains. A Multiple Correspondence Analysis also identified *L. curvatus*, *L. mesenteroides*, *L. plantarum*, *L. paracasei* and *L. lactis* as crucial constituents of these cheeses´ microbiota. Milk matrices were associated with enterococci and *L. rhamnosus* strains, while curd shared a higher similarity with cardoon samples due to their content on *Weissella* spp. and enterococci strains. In the end, this characterization contributes to a deeper understanding of the scientific community, producers and the public in general, of the microbiota, especially LAB, that is associated with a PDO cheese, whose knowledge is so far scarce.

1. Introduction

Cheese is a millennial food product that is thought to have emerged over 8000 years ago (Fox and McSweeney, 2017). The primordial cheese making craft was passed out from generation to generation, evolving until it became well-established in ancient Middle Eastern, Egyptian, Greek and Roman empires, using sheep, goat, cow, mare and jenny milk (Fox and McSweeney, 2017; Guiné and Florença, 2020).

The Roman Empire is considered the main vector of cheese dissemination within the controlled territories adjacent to the Mediterranean Sea, including Portugal (Morales *et al.,* 2017; Guiné and Florença, 2020). Here, the regional edaphoclimatic conditions potentiated the development of the Portuguese cheese making craft, which accounts nowadays for almost 100 traditional cheese varieties made from cow, goat and ovine milks or milk mixtures (Reis and Malcata, 2011; Guiné and Florença, 2020). In a valorization and protection effort, to this date, eleven traditional Portuguese cheeses were awarded with the Protected Designation of Origin (PDO) status (McSweeney *et al.,* 2017). Of those, Azeitão, Castelo Branco, Serpa and Serra da Estrela are the most popular ovine raw milk cheeses (Reis and Malcata, 2011). The origin of Azeitão cheeses dates to the first quarter of the 19th century from the efforts of Gaspar Henriques de Paiva, a native of the Serra da Estrela region, to reproduce its hometown cheese (Guiné and Florença, 2020; Cardinali *et al.,* 2021). Despite producing cheeses somewhat similar to the ones from Serra da Estrela, they presented unique organoleptic characteristics. The manufacturing process was perfected over the years and is nowadays characterized by a flat cylinder, featuring a clean, smooth, slightly spicy, acidic and salty flavored cheese, with a thin, soft, uniform yellow rind and a white or slightly yellowish, semi-soft, buttery, creamy and unctuous paste with none too few little eyes (Guiné and Florença, 2020; Cardinali *et al.,* 2021).

Cheese manufacture is a complex biochemical and

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biological process, comprehending a finely orchestrated series of consecutive and concomitant events, that are ultimately responsible for the development of cheese organoleptic characteristics (Montel *et al.,* 2014; Fox and McSweeney, 2017; Kilcawley and O'Sullivan, 2017; Jonnala *et al.,* 2018). Most cheese types rely, at least at some point, on the microbial machinery to perform the fundamental biochemical reactions required for high quality cheese production (Montel *et al.,* 2014; Cotter and Beresford, 2017; Kilcawley and O'Sullivan, 2017; Parente *et al.,* 2017; Jonnala *et al.,* 2018). Cheese is throughout its manufacture a highly selective three−dimensional dynamic microbial ecosystem composed of bacteria, yeasts and/or moulds (Cotter and Beresford, 2017; Irlinger *et al.,* 2017). Of those, lactic acid bacteria (LAB) have a preponderant role in cheese making, namely as starter cultures, involved in early lactose metabolization and as secondary and/or adjunct cultures, involved in the biochemical changes that occur at the ripening stage, responsible for the development of the organoleptic characteristics intrinsic to each type of cheese (Montel *et al.,* 2014; Cotter and Beresford, 2017; O'Sullivan and Cotter, 2017; Parente *et al.,* 2017).

Traditional Portuguese cheeses have been extensively studied over the years regarding their microbiological, biochemical and organoleptic characteristics (Reis and Malcata, 2011). However, the characterization of Azeitão PDO cheeses is restricted to few available reports (Vasconcelos, 1990; Mimoso *et al.,* 1992; Cardinali *et al.,* 2021; Rocha *et al.,* 2022). So, the present work aimed to perform a characterization of the microbial community associated with Azeitão PDO cheeses and raw materials across the 2020/2021 production campaign, specially focused on LAB.

2. Materials and methods

2.1 Sampling strategy and cheese manufacture

The microbial characterization was performed on cheese, curd, ovine raw milk and cardoon samples collected throughout the 2020/2021 production campaign and distributed by three manufacturing periods: 1) December – January; 2) February – March; 3) March – April, as depicted in Figure 1. Comprehending an assessment of early, mid and late productions, considering that artisanal Azeitão cheese manufacture elapses from November until May.

The Azeitão cheeses were manufactured by a certified PDO cheese producer from Palmela, Portugal. Milk and cardoon samples were collected immediately before use, while curd samples were collected prior to the molding stage. Standard, 250 g Azeitão cheeses were produced following standard procedures with a total ripening period of 20 days. All samples were transported and kept under refrigeration (≤4°C) before processing.

2.2 Microbial characterization analysis

The viable microbial load of Azeitão cheese, curd, milk and cardoon samples were analyzed by culture plating and colony counting into a selection of non-selective, selective, elective and differential media. To that end, 25 g of cheese, curd and milk samples were aseptically transferred to a homogenization bag with a strainer and homogenized in 225 mL of Buffered Peptone Water (Liofilchem srl, Italy) using a paddle blender (Seward Ltd, UK) for 1 min. Cardoon samples were homogenized as previously reported by Rocha *et al.* (2021). Briefly, 8 - 10 g of dried thistle flower (*C. cardunculus* L.) samples were suspended in 100 mL of a sterile solution containing 10% (v/v) Buffered Peptone Water (Liofilchem srl, Roseto degli Abruzzi, Italy) and 0.01% (v/v) Tween 80® (PanReac AppliChem). Subsequently, these suspensions were subjected to an ultrasonic bath for 30 s (Jet Program option) (Soltec, Milan, Italy) followed by 150 rpm orbital agitation for 30 mins at room temperature (B. Braun Biotech International, Melsungen, Germany) and finally poured into a homogenization bag with strainer.

Serial decimal dilutions in maximum recovery diluent (Liofilchem srl, Roseto degli Abruzzi, Italy) were prepared with the filtered homogenizations and used for the microbial characterizations. To that end, 100 µL of bacterial suspension dilutions were inoculated in duplicate for the enumeration of presumptive: 1) total lactic acid bacteria on Man, Rogosa and Sharpe Agar

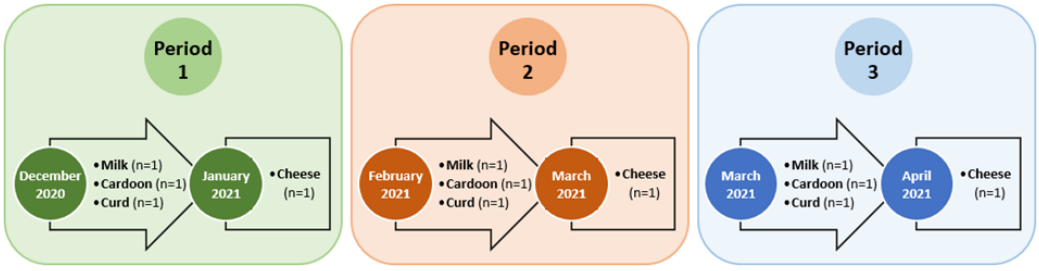

Figure 1. Schematic representation of the sampling strategy used in this study for the microbial characterization of ovine raw milk, cardoon, curd and Azeitão PDO cheese. The letter n refers to the number of samples analyzed of the corresponding sample in that specific period.

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(MRSA [VWR International, Leuven, Belgium]); 2) lactococci on M17 Agar (Liofilchem srl, Roseto degli Abruzzi, Italy); 3) lactobacilli on Rogosa Agar (RA [Biokar Diagnostics, Beauvais, France]); 4) enterococci on Slanetz Bartley Agar (SBA [VWR International, Leuven, Belgium]), 5) *Leuconostoc* spp. on Mayeux, Sandine and Elliker Agar (MSE [Biokar Diagnostics, Beauvais, France)] and psychrophilic microbiota on Nutrient Agar (NA [Biokar Diagnostics, Beauvais, France]). The inoculated agar plates of MRSA, M17 and RA were incubated anaerobically (Thermo Scientific, UK), while SBA, MSE, NA and a second set of MRSA plates were incubated aerobically at 30°C for 48 to 72 hrs, except for SBA plates that were incubated at 37°C and NA plates incubated at 10°C for 10 days. The grown colonies were sorted according to their phenotypic characteristics and three representative LAB specimens were isolated onto MRSA plates for biochemical confirmation, including Gram-staining, catalase (Sigma-Aldrich GmbH, Steinheim, Germany) and cytochrome oxidase activity (Merk KGaA, Darmstadt, Germany) tests. Milk, cardoon, curd and cheese samples were also screened for the enumeration of 1) microorganisms at 30°C according to International Organization for Standardization (ISO) 4833-1:2013; 2) β-glucuronidase positive *Escherichia coli* according to ISO 16649-2:2001; 3) *Enterobacteriaceae* according to ISO 21528-2:2017 and 4) yeasts and moulds according to ISO 21527 -1:2008. Finally, cheese samples were also screened for the enumeration of 1) *Bacillus cereus* according to ISO 7932:2004; 2) coagulase-positive staphylococci according to ISO 6888-1:1999; 3) *Listeria monocytogenes* and *Listeria* spp. according to ISO 11290 -2:2017 and 4) for the detection of *Salmonella* spp. according to ISO 6579-1:2017.

For each sample, the mean values of viable counts and respective standard deviation errors were estimated and expressed as the logarithm of colony forming units per gram of product (log CFU/g).

2.3 Microbial characterization analysis

The biochemically confirmed LAB isolates obtained from anaerobically incubated MRSA plates were subjected to genetic identification through the amplification and sequencing of the 16S rRNA gene. To that end, the DNA of each isolate was extracted by boiling a cell suspension in 50 µL 0.05M NaOH solution (Biochem Chemopharma, Cosne sur loire, France) on a heating block (Stuart Scientific, Staffordshire, UK) at 98°C for 15 mins. The lysed suspensions were centrifuged at 10 000×*g* for 5 mins and the lysate (supernatant) was transferred to a new tube and kept in a cooling block prior to PCR amplification stage. PCR amplifications were conducted in a CFX96 thermocycler (BioRad Inc., Hercules, USA) with a final reaction volume of 25 µL, containing 1× iTaq™ Universal SYBR® Green Supermix (2×) (BioRad Inc., USA), 200 nM of forward primer 27F (5' - AGAGTTTGATCCTGGCTCAG - 3') (STAB VIDA Lda., Lisboa, Portugal), 200 nM of reverse primer 1492R (5' - GGTTACCTTGTTACGACTT - 3') (STAB VIDA Lda., Lisboa, Portugal) and 2 µL of lysate as template. The amplification protocol consisted of a primary denaturation step at 95°C for 5 mins, followed by 45 cycles of denaturation in 20 s at 95°C, annealing in 20 s at 55°C and extension in 90 s at 72°C and a final extension at 72°C for 10 mins.

The amplified products were purified and Sanger sequenced at STAB VIDA Lda. (Lisboa, Portugal). The resulting chromatograms were analyzed using UGENE program (version 38.1, Unipro, Novosibirsk, Russia) for the extraction of high-quality fragments, that were aligned and compared against the National Center for Biotechnology Information (NCBI) database using the nucleotide Basic Local Alignment Search Tool (BLAST) function with the default parameters (https:// blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4 Statistical analysis

A multiple correspondence analysis (MCA) was applied using the overall frequencies of identified LAB isolates to determine the overall relationship between LAB isolates and the food matrix. This multivariate exploratory method uses nominal or categorical data, designed to find correspondence between two or more categorical variables by reviewing the distances between variables, allowing a pattern analysis of several categorical dependent variables (Alves *et al.,* 2020; Xia, 2020). MCA was calculated using frequency distribution through the Burt table to distribute all variables across the computed dimensions. Variables with the lowest distance are considered as those with the highest degree of similarity in the corresponding dimension. MCA analysis was performed using TIBCO® Statistica® (version 14.0.0, TIBCO Software Inc, Palo Alto, CA, USA).

3. Results and discussion

3.1 Microbial characterization of Azeitão protected designation of origin cheeses and raw materials

LAB are ubiquitous bacteria, frequently associated with the dairy environment. In cheese production they are a key part of its microbiota, either acting as starter cultures, including strains of *Lactococcus lactis*, *Streptococcus thermophilus*, *Lactobacillus delbrueckii* and *Lactobacillus helveticus*, responsible for early

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lactose metabolization and pH decrease. Or as secondary or adjunct cultures, such strains of heterofermentative lactobacilli, *Leuconostoc* spp., *Pediococcus* spp. and *Enterococcus* spp., that in conjugation with other bacteria, yeasts and moulds, are ultimately responsible for the acquisition of the intrinsic organoleptic characteristics of each cheese type during the ripening stage (Montel *et al.,* 2014; Cotter and Beresford, 2017; Irlinger *et al.,* 2017; O'Sullivan and Cotter, 2017; Parente *et al.,* 2017).

As expected, LAB constitute a major fraction of Azeitão cheese microbiota, averaging 8.8 and 9.0±0.2 log CFU/g, for total aerobic and anaerobic counts, respectively. Of those, lactococci and lactobacilli predominated over enterococci and *Leuconostoc* spp. strains (Table 1). These results are in line to previous microbiological characterizations of Azeitão cheeses (Freitas and Malcata, 2000; Reis and Malcata, 2011; Cardinali *et al.,* 2021; Rocha *et al.,* 2022) and other PDO ovine milk cheeses such Castelo Branco, Serpa and Serra da Estrela (Freitas and Malcata, 2000; Roseiro *et al.,* 2003; Inácio *et al.,* 2019). On the contrary, LAB colonization of cardoon samples is considerably less expressive, ranging from absent up to 3 log CFU/g (Table 1). These results are also in line with the few reports available, concerning the microbiological characterization of cardoon samples (Fernández-Salguero *et al.,* 1999; Gómez *et al.,* 2001). Concerning ovine raw milk and curd samples, their LAB content in absolute figures, is somewhat similar between both matrices, ranging between 5 and 6.5 log CFU/g (Table 1). Also, the here reported LAB figures found in milk samples are similar to the ones reported in previous studies on the subject (Quigley *et al.,* 2013).

Furthermore, viable counts of *B. cereus*, coagulase-positive staphylococci and *L. monocytogenes* were not found, while *Salmonella* spp. was not detected in any of the cheese samples analyzed (Table 1). Concerning the total mesophilic microorganisms, their presence ranged from 5 up to 9 log CFU/g, with the lowest concentrations being respective to cardoon samples and the highest ones associated with cheese matrices. This arises fundamentally from the high load of LAB colonizing the dairy matrices analyzed in this study, as reported above in this section. Psychrophiles were found ranging from 5 to 7 log CFU/g, with the highest concentrations associated with milk and cheese matrices. The yeast and mould load ranged from 2 to 5 log CFU/g (Table 1). Overall, yeasts were found at higher concentrations than moulds in all analyzed matrices. The here reported yeast and mould content on Azeitão cheeses is in line with previous assessments (Reis and Malcata, 2011; Cardinali *et al.,* 2021; Rocha *et al.,* 2022) and other ovine milk cheeses, such as Castelo Branco, Serpa and Serra da Estrela (Freitas and Malcata, 2000; Reis and Malcata, 2011).

Finally, *Enterobacteriaceae* were found in all analyzed matrices, averaging between 3 to 5 log CFU/g, with a minimal contribution of *E. coli* to the overall *Enterobacteriaceae* content in the case of cardoon matrices. On the contrary, *E. coli* constitutes half or the majority of the total *Enterobacteriaceae* fraction found in milk/curd and cheese matrices, respectively (Table 1). Overall, the here reported results in terms of *Enterobacteriaceae* content are in line with previous assessments on Azeitão cheeses (Freitas and Malcata, 2000; Reis and Malcata, 2011; Cardinali *et al.,* 2021) and other ovine milk cheeses, such Castelo Branco (Freitas and Malcata, 2000), Serpa (Roseiro *et al.,* 2003) and Serra da Estrela (Macedo *et al.,* 1996; Tavaria and Malcata, 1998; 2000). According to the European Union Guideline (EC) No 2073/2005 of 15 November 2005, establishing the microbiological criteria for foodstuffs and ready-to-eat food products, the here analyzed Azeitão cheeses presented an unsatisfactory *E. coli* content, exceeding the reference limit of 3.0 log CFU/g in all analyzed samples. Despite the high aromatic potential of *Enterobacteriaceae* strains (Montel *et al.,* 2014; Irlinger *et al.,* 2017), their presence above the admissible limits is generally indicative of defective and/or inefficient hygiene measures and cross-contamination issues.

3.2 Multiple correspondence analysis

The isolation of viable LAB strains recovered from MRSA plates from each matrix and their genetic identification through 16S Sanger sequencing allowed the execution of a MCA to the data (Figure 2).

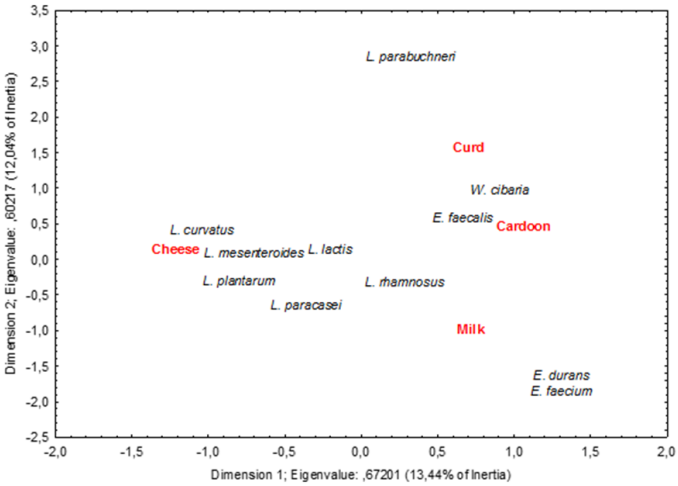

Figure 2. Multiple correspondence analysis plot displaying the association between matrix and the identified viable Lactic Acid Bacteria grown on Man, Rogosa e Sharpe Agar in Azeitão PDO cheese and raw materials.

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Table 1. Microbial abundance data obtained for ovine raw milk, cardoon, curd and Azeitão cheese samples analyzed from the 2020/2021 production campaign. The mean values of viable counts and respective standard deviation errors, under brackets, are presented for each microbial group/species as the logarithm of colony forming units per gram of product (log CFU/g).

	Milk (n = 3)	**Cardoon** (n = 3)	**Curd** (n = 3)	**Cheese** (n = 3)
Microorganisms at 30°C acc. ISO 4833-1:2013	7 (1)	5 (1)	6.6 (0.9)	9.05 (0.04)
Escherichia coli acc. ISO 16649-2:2001	1.8 (0.3)	n.d.	1.8 (0.6)	5.0 (0.2)
Enterobacteriaceae acc. 21528-2:2017	4 (1)	3 (1)	4.5 (0.8)	5 (1)
Bacillus cereus acc. ISO 7932:2004	ND	ND	ND	n.d.
Coagulase-positive staphylococci acc. ISO 6888-1:1999	ND	ND	ND	n.d.
Listeria monocytogenes acc. 11290-2:2017	ND	ND	ND	n.d.
***Listeria* spp.** acc. 11290-2:2017	ND	ND	ND	1 (1)
***Salmonella* spp.** acc. ISO 6579-1:2017	ND	ND	ND	Absent (in 25g)
Yeasts acc. ISO 21527-1:2008	3.6 (0.1)	3.3 (0.8)	4.9 (0.5)	4.4 (0.3)
Moulds acc. ISO 21527-1:2008	2.6 (0.3)	2.6 (0.5)	2.6 (0.5)	3.3 (0.8)
Psychrophiles	7 (1)	5.2 (0.6)	6.5 (0.7)	7.4 (0.7)
Presumptive **Lactic Acid Bacteria** (total aerobic counts)	5 (1)	3 (3)	6 (1)	8.8 (0.2)
Presumptive **Lactic Acid Bacteria** (total anaerobic counts)	6 (1)	2 (2)	6 (1)	9.0 (0.2)
Presumptive **Lactococci**	5.2 (0.4)	3 (3)	6.5 (0.5)	9.0 (0.3)
Presumptive **Lactobacilli**	6 (1)	3 (3)	6.0 (0.9)	8.8 (0.2)
Presumptive **Enterococci**	5.5 (0.9)	3 (3)	5.3 (0.6)	8.1 (0.4)
Presumptive ***Leuconostoc* spp.**	5 (1)	3 (3)	6 (1)	8.2 (0.4)

ND: Not determined, n.d.: not detected.

Microbial sampling is mostly performed blindly about environmental heterogeneity, and the abundance of target species is generally determined without systematically analyzing associated environmental parameters. Therefore, MCA was used to determine the general relationships between LAB species and matrices through nominal or categorical data designed to match two or more categorical variables, using the distances between variables and allowing the analysis of patterns of several categorical dependent variables. Frequencies were used to distribute all variables by the calculated dimensions. The variables with the smallest distance are those with the highest degree of similarity in the corresponding dimension. Thus, in Figure 2, certain associations are noticeable, namely, the approximation of heterofermentative *Latilactobacillus curvatus*, *Leuconostoc mesenteroides*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei* and homofermentative *L. lactis* strains to cheese matrices. These heterofermentative strains belong to the group of secondary or adjunct cultures. Active during cheese ripening, are involved in cheese flavor and aroma profile development, through residual sugar metabolization, peptide hydrolysis and amino acid conversion (Irlinger *et*

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al., 2017). *L. lactis* is considered a starter culture, whose main function relies on the metabolization of lactose to lactate in the early stages of cheese manufacture (Cotter and Beresford, 2017). However, previous microbial characterization reports of other ovine raw milk cheeses, found that these strains can also persist through the ripening stages (Macedo *et al.,* 1995; Rocha *et al.,* 2021). Some strains were found to display proteinase, peptidase, lipase activities and the ability to convert metabolization products of other LAB into aromatic and flavor compounds (Macedo and Malcata, 1997; Quigley *et al.,* 2013; Irlinger *et al.,* 2017).

Concerning ovine raw milk matrices, this analysis showed an association with enterococci and *L. rhamnosus* strains. Enterococci are a controversial group within LAB, that depending on the strain, can be considered starter culture, probiotic, spoilage or pathogenic bacteria (Bhardwaj *et al.,* 2009). They compose the natural microbiota of raw milk, present an adaptable metabolism, capable of growing under different conditions and substrates, surviving refrigeration, high-temperature and high-salinity environments (Quigley *et al.,* 2013). *Lacticaseibacillus rhamnosus*, another heterofermentative LAB, is frequently found associated to cheese microbiota and involved in flavor and aroma profile development (Irlinger *et al.,* 2017). However, its close association with ovine milk matrices, as shown in Figure 2, points towards a minor contribution/importance during Azeitão cheese ripening, in comparison to other heterofermentative LAB species, as previously stated.

Unexpectedly, this analysis showed higher microbial similarities from curd matrices to cardoon rather than ovine milk samples (Figure 2). Despite the abundance patterns presented in Table 1, seemed to point otherwise. Overall, curd and cardoon matrices were associated with LAB strains of *Enterococcus faecalis*, *Weissella cibaria* and *Lentilactobacillus parabuchneri*, with the last one being curd specific. Enterococci and *Weissela* spp. strains are widely distributed in the environment, commonly isolated from soil, water, vegetation, animals, faeces, fermented foods and as part of human microbiota of the skin, oral cavity, urogenital and gastro-intestinal tracts (Byappanahalli *et al.,* 2012; Fusco *et al.,* 2015). As with *L. rhamnosus*, its close association with cardoon and curd matrices suggests a minor contribution/ importance during ripening of Azeitão cheeses. Nonetheless, *W. cibaria* could exert a potential role in the early stages of cheese manufacture, due to their antimicrobial and exopolysaccharide synthesis capability (Fusco *et al.,* 2015). Lastly, *L. parabuchneri* is an obligatory heterofermentative LAB known to produce CO_2 from lactate metabolization, which leads to the appearance of visually undesired features such as split defects and eye formation (Fröhlich-Wyder *et al.,* 2015; Wechsler *et al.,* 2021). Moreover, this species encodes an enzyme, histidine decarboxylase (EC 4.1.1.22), that produces histamine in the presence of the amino acid histidine. Histamine is a biogenic amine, that in high concentrations can lead to food poisoning episodes, particularly in sensitive or intolerant individuals (Wechsler *et al.,* 2021). Despite the potential health problems associated with the presence of *L. parabuchneri*, in this study, its presence was solely identified in one curd sample (Table 2). Nonetheless, a histamine assessment in Azeitão PDO cheeses twenty years ago, revealed a relatively high concentration, around 450 mg Kg^{-1} of dry matter (Pinho *et al.,* 2001), in contrast to the average values of 20.6 - 61 mg Kg^{-1} reported by the European Food Safety Authority (EFSA) in a survey to the same food category (EFSA 2011). Considering this information, further studies are required to on the one hand determine the histamine concentration in current Azeitão PDO cheese manufacturers, and on the other hand assess the load of obligatory heterofermentative lactobacilli strains, like *L. parabuchneri*, in raw materials up to the finished product.

4. Conclusion

In this work, samples of Azeitão PDO cheese, curd and raw materials, ovine raw milk and rennet (dried flowers of *Cynara cardunculus* L.) gathered across the manufacturing season of 2020/2021 were microbiologically characterized concerning their LAB, yeasts, moulds, *Enterobacteriaceae*, psychrophilic and mesophilic microorganisms' content. Overall, the highest abundances found on dairy matrices, *i.e.,* ovine raw milk, curd and cheese samples, were related to mesophilic, psychrophilic and LAB microbiota.

In the specific case of LAB, this study showed that cheese matrices presented the highest abundances, followed equally by raw milk and curd and finally cardoon presented the lowest LAB figures. In cheese, lactococci and lactobacilli predominated over other LAB and strains regarded as responsible for flavor and aroma development, such as the heterofermentative *L. curvatus*, *L. mesenteroides*, *L. plantarum*, *L. paracasei* and homofermentative *L. lactis*, were identified by a multivariate correspondence analysis to be characteristic of the here analyzed Azeitão PDO cheese samples.

Despite the here documented findings and other available literature, a complete Azeitão PDO cheese product characterization is still far from completion. Consequently, a deeper analysis at a regional level and across the entire production chain is required. Including,

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Table 2. Data identification of Lactic Acid Bacteria isolates recovered from cheese, curd, ovine raw milk and cardoon samples, resulting from 16S Sanger sequencing.

Period	Matrix	Phenotype	Colony	Biochemical confirmation tests			Fragment size used (bp)	Homology (%)	Identification
				Catalase	Cytochrome oxidase	Gram staining			
December/January (Autumn)	Ovine raw milk	2	1	-	-	Gram-positive rods	697	100	*Lacticaseibacillus rhamnosus*
December/January (Autumn)	Ovine raw milk	3	1	-	-	Gram-positive rods	823	100	*Lacticaseibacillus rhamnosus*
December/January (Autumn)	Ovine raw milk	3	2	-	-	Gram-positive rods	773	100	*Lacticaseibacillus rhamnosus*
December/January (Autumn)	Ovine raw milk	3	3	-	-	Gram-positive rods	778	100	*Lacticaseibacillus rhamnosus*
December/January (Autumn)	Cardoon	-	-	NF	NF	NF	NF	NF	NF
December/January (Autumn)	Curd	1	1	-	-	Gram-positive rods	683	100	*Weissella cibaria*
December/January (Autumn)	Curd	1	2	-	-	Gram-positive rods	766	100	*Lentilactobacillus parabuchneri*
December/January (Autumn)	Curd	1	3	-	-	Gram-positive cocci	904	100	*Enterococcus faecalis*
December/January (Autumn)	Cheese	1	1	-	-	Gram-positive rods	744	100	*Lacticaseibacillus paracasei*
December/January (Autumn)	Cheese	1	2	-	-	Gram-positive rods	833	99.16	*Lactiplantibacillus plantarum*
December/January (Autumn)	Cheese	1	3	-	-	Gram-positive rods	788	99.87	*Latilactobacillus curvatus*
December/January (Autumn)	Cheese	2	1	-	-	Gram-positive cocci	866	100	*Leuconostoc mesenteroides*
December/January (Autumn)	Cheese	2	2	-	-	Gram-positive cocci	888	99.89	*Lactococcus lactis*
December/January (Autumn)	Cheese	2	3	-	-	Gram-positive cocci	899	100	*Lactococcus lactis* subsp. *lactis*
December/January (Autumn)	Cheese	3	1	-	-	Gram-positive rods	1014	99.90	*Lactiplantibacillus plantarum*
December/January (Autumn)	Cheese	3	2	-	-	Gram-positive rods	868	100	*Lactiplantibacillus plantarum*
December/January (Autumn)	Cheese	3	3	-	-	Gram-positive rods	979	99.90	*Lactiplantibacillus plantarum*
February/March (Winter)	Ovine raw milk	1	1	-	-	Gram-positive rods	885	99.77	*Lacticaseibacillus paracasei*
February/March (Winter)	Ovine raw milk	1	2	-	-	Gram-positive rods	899	100	*Lacticaseibacillus paracasei*
February/March (Winter)	Ovine raw milk	1	3	-	-	Gram-positive rods	784	100	*Lacticaseibacillus paracasei*
February/March (Winter)	Ovine raw milk	2	1	-	-	Gram-positive rods	845	100	*Leuconostoc lactis*
February/March (Winter)	Ovine raw milk	2	2	-	-	Gram-positive rods	796	100	*Leuconostoc lactis*
February/March (Winter)	Ovine raw milk	2	3	-	-	Gram-positive rods	858	100	*Leuconostoc lactis*
February/March (Winter)	Cardoon	1	1	-	-	Gram-positive rods	817	100	*Leuconostoc mesenteroides*
February/March (Winter)	Cardoon	1	2	-	-	Gram-positive rods	870	100	*Weissella cibaria*
February/March (Winter)	Cardoon	1	3	-	-	Gram-positive rods	746	100	*Leuconostoc mesenteroides*
February/March (Winter)	Curd	1	1	-	-	Gram-positive cocci	1056	100	*Lactococcus lactis* subsp. *lactis*
February/March (Winter)	Curd	1	3	-	-	Gram-positive rods	858	100	*Weissella cibaria*
February/March (Winter)	Cheese	1	1	-	-	Gram-positive rods	981	99.80	*Lactiplantibacillus plantarum*
February/March (Winter)	Cheese	1	2	-	-	Gram-positive rods	1015	99.90	*Lactiplantibacillus plantarum*

NF: Not Found

https://doi.org/10.26656/fr.2017.9(3).173

Table 2 (Cont.). Data identification of Lactic Acid Bacteria isolates recovered from cheese, curd, ovine raw milk and cardoon samples, resulting from 16S Sanger sequencing.

Period	Matrix	Phenotype	Colony	Biochemical confirmation tests			Fragment size used (bp)	Homology (%)	Identification
				Catalase	Cytochrome oxidase	Gram staining			
February/March (Winter)	Cheese	1	3	-	-	Gram-positive rods	919	100	*Lactiplantibacillus plantarum*
February/March (Winter)	Cheese	2	1	-	-	Gram-positive rods	980	100	*Lactiplantibacillus plantarum*
February/March (Winter)	Cheese	2	2	-	-	Gram-positive rods	941	99.79	*Weissella cibaria*
February/March (Winter)	Cheese	2	3	-	-	Gram-positive rods	920	100	*Latilactobacillus curvatus*
February/March (Winter)	Cheese	3	1	-	-	Gram-positive rods	1083	100	*Lactiplantibacillus plantarum*
February/March (Winter)	Cheese	3	2	-	-	Gram-positive rods	905	100	*Lactiplantibacillus plantarum*
February/March (Winter)	Cheese	3	3	-	-	Gram-positive rods	856	100	*Lactiplantibacillus plantarum*
March/April (Spring)	Ovine raw milk	1	1	-	-	Gram-positive rods	909	100	*Lactiplantibacillus plantarum*
March/April (Spring)	Ovine raw milk	1	2	-	-	Gram-positive cocci	891	100	*Enterococcus faecium*
March/April (Spring)	Ovine raw milk	1	3	-	-	Gram-positive rods	815	100	*Lacticaseibacillus paracasei*
March/April (Spring)	Ovine raw milk	2	1	-	-	Gram-positive cocci	882	100	*Enterococcus faecalis*
March/April (Spring)	Ovine raw milk	2	2	-	-	Gram-positive cocci	921	100	*Enterococcus faecalis*
March/April (Spring)	Ovine raw milk	2	3	-	-	Gram-positive cocci	814	100	*Enterococcus durans*
March/April (Spring)	Cardoon	2	1	-	-	Gram-positive cocci	840	100	*Weissella cibaria*
March/April (Spring)	Curd	1	1	-	-	Gram-positive rods	856	100	*Weissella cibaria*
March/April (Spring)	Curd	1	2	-	-	Gram-positive rods	868	100	*Weissella cibaria*
March/April (Spring)	Curd	1	3	-	-	Gram-positive rods	941	100	*Weissella cibaria*
March/April (Spring)	Cheese	1	1	-	-	Gram-positive rods	1027	99.81	*Lactiplantibacillus plantarum*
March/April (Spring)	Cheese	1	2	-	-	Gram-positive rods	866	100	*Lacticaseibacillus paracasei*
March/April (Spring)	Cheese	1	3	-	-	Gram-positive rods	869	99.77	*Lactiplantibacillus plantarum*
March/April (Spring)	Cheese	2	1	-	-	Gram-positive rods	767	100	*Leuconostoc mesenteroides*
March/April (Spring)	Cheese	2	2	-	-	Gram-positive rods	818	100	*Leuconostoc mesenteroides*
March/April (Spring)	Cheese	3	1	-	-	Gram-positive rods	797	100	*Leuconostoc mesenteroides*
March/April (Spring)	Cheese	3	2	-	-	Gram-positive cocci	903	100	*Lactococcus lactis* subsp. *lactis*
March/April (Spring)	Cheese	3	3	-	-	Gram-positive cocci	900	100	*Lactococcus lactis*

NF: Not Found

but not limited to, an expansion of the scope of these characterizations, accessing spatial and temporal intra and inter-variability in the region and a comprehensive characterization of the production processes from raw materials up to the finished product. Nonetheless, this study contributes with an updated microbiological characterization complemented with a multiple correspondence analysis of a very appreciated cheese product whose knowledge from previous studies was scarce.

Conflict of interest

The authors declare no conflict of interest.

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