A review on microbiological and technological aspects of Serpa PDO cheese: an ovine raw milk cheese

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ABSTRACT

Serpa is a PDO cheese considered one of most popular and relevant traditional Portuguese cheeses due to its unique aroma and flavor, which are very much-appreciated characteristics. The special and recognized sensorial attributes of Serpa cheese are a result of the ingredients used, coupled with the manufacturing process, specifically raw ovine milk and extracts of *Cynara cardunculus* L. as coagulant, without addition of any starter culture or milk pasteurization. Serpa quality and safety issues, linked to the high susceptibility and heterogeneity of its final sensorial attributes fosters the importance of large-scale studies focused on biochemical and microbial aspects. Despite the scientific relevance of this traditional product, Serpa studies are still very scarce and limited with the cheesemaking procedure lacking in regulation. Accordingly, the present work emphasizes the current knowledge on Serpa PDO cheese, giving an overview and critical analysis of existing studies and discussing Serpa technological process.

1. INTRODUCTION

In southern Europe production of artisanal cheeses is not only economically important but also part of a strong cultural heritage, since these are very ancient practices and manufacturing often takes place in the rural and less-favored regions (Freitas & Malcata, 2000). Besides being of great importance for the local agriculture, these products hold a high benefit for their special and recognized sensorial characteristics that are widely appreciated by consumers (Freitas, Macedo & Malcata, 2000; Roseiro, Wilbey & Barbosa, 2003d). As such, several European cheeses benefit from the Protected Designation of Origin (PDO) label in order to promote the quality and preservation of regional products (Freitas et al., 2000; Randazzo, Caggia & Neviani, 2009).

Particularly in the Iberian Peninsula, cheesemaking dates to the Roman occupation and preserve a strong tradition regarding use of raw milk in the process. The PDO seal protects thirteen traditional Portuguese cheeses, all manufactured with raw milk (Freitas et al., 2000; Freitas & Malcata, 2000). Given the long-lasting cultural heritage of Portuguese PDO cheeses regarding the technological process and final sensorial attributes as well as the issues related to their quality and safety, the study of Portuguese traditional cheeses is crucial to improve the cheese characteristics while preserving tradition.

Accordingly, the present review focuses on Serpa PDO cheese, an ewe's milk cheese coagulated with extracts of *Cynara cardunculus* L.. Serpa is considered one of most popular and relevant traditional Portuguese cheeses due to its unique organoleptic attributes (Roseiro et al., 2003d). Several parameters such as, distinct milk composition and variations in manufacturing processes among producers influence final flavor and texture properties of raw milk cheeses, which may result in a final product considerably heterogeneous and, in some cases, with organoleptic or safety defects (Montel et al., 2015). The high biodiversity of the microbial population and susceptibility of final sensorial features of Serpa cheese raise the importance of large-scale chemical and microbiological studies and implementation of regulation in the technological procedure.

Despite the scientific relevance of Serpa cheese, studies focusing on this PDO cheese are very scarce and limited, prompting the discussion on identification of potential gaps and limitations and how to overcome these hurdles. In this context, all phases of the technological process as well as recent improvements in the Serpa cheesemaking process will be discussed. The present review also gives an overview and critical analysis regarding Serpa PDO studies. Future research on biochemistry and microbiology of this traditional product are also suggested in order to contribute to the improvement of Serpa quality and safety.

2. SERPA PDO CHEESE

The production of Serpa cheese dates to immemorial times but it is believed that Serpa cheese originates from Serra da Estrela cheese during the transhumance period due to the similar manufacturing method and ingredients used. Serra da Estrela is also an ovine raw milk cheese coagulated with *C. cardunculus* L., being produced in a mountainous region in the north of the country with the same name. It is also a famous and extremely appreciated Portuguese PDO cheese (Freitas et al., 2000; Roseiro, García-Risco, Barbosa, Ames & Wilbey, 2003b; Roseiro et al., 2003d). Although there are similarities in the production and ingredients of both cheeses, the cheesemaking area is different and the milk for their production has distinct origins. Classically, the milk for Serra da Estrela cheese was produced from a native sheep breed named Bordaleira, while for Serpa cheese, milk from Merino sheep breeds was originally used. This distinct animal species of origin, coupled with different pastures, milking and cheesemaking areas of the two cheeses contributes to a diverse cheese microbiota, resulting in a final product that is substantially different in terms of flavor and texture (Roseiro et al., 2003d).

The demarcated area of Serpa cheese production is located in the Alentejo region, in the south of the country (Decreto Regulamentar 39/87, 1987; Freitas et al., 2000; Roseiro et al., 2003d). Serpa is a full fat cheese with a semi-soft and creamy texture, possessing a characteristic strong and exquisite flavor considered slightly hot and spicy (Freitas et al., 2000; Roseiro, Gómez-Ruiz, García-Risco & Molina, 2003c). The fat content on dry matter basis (DM) of this Alentejo cheese varies between 45 and 60%, while the protein content (DM) is approximately 40% (Decreto Regulamentar 39/87, 1987; Roseiro et al., 2003d). Serpa traditional cheese is considered a salty cheese with a typical NaCl concentration in moisture being ca. 40 g kg^{-1}, with Na^{+} and Ca^{2+} as the most incident minerals (Roseiro et al., 2003d). Regarding the minimum maturation index (soluble nitrogen in relation to the total nitrogen), it is ca. 45% and its moisture content varies between 61 and 69% (Decreto Regulamentar 39/87, 1987; Roseiro et al., 2003d).

Typically, Serpa PDO cheese possesses a light-yellow color with a smooth and slightly wrinkled rind as well as a flat cylindrical shape with few or no eyes. The characteristic weight of this ripened product ranges between 0.2 and 2.5 kg and its diameter and height between 10-30 cm and 3-8 cm, respectively (Decreto Regulamentar 39/87, 1987; Freitas et al., 2000). The pH of the ripened cheese is approximately 5.41, while the acidity (lactic acid) is characteristically between 7.5-9.8 g kg^{-1}. Its ash content is around 84 g kg^{-1} and water activity is approximately 0.97 (Roseiro et al., 2003d). The main Serpa PDO specifications and sensorial attributes as well as biochemical parameters are presented in Table 1 and 2, respectively.

The unique organoleptic attributes of Serpa cheese are a result of the type of milk and rennet used, coupled with the processing technology (Roseiro et al., 2003c). In Serpa cheesemaking process, raw ewe's milk and *C. cardunculus* L. vegetable rennet are used without the addition of any starter culture or milk pasteurization (Dos Santos, Benito, de Guía Córboba, Alvarenga & Herrera, 2017; Dos Santos et al., 2018; Roseiro, et al., 2003c).

3. *CYNARA CARDUNCULUS L.* FLOWER AS MILK COAGULANT

Several plant extracts such as, *Cynara* sp., *Carica papaya* and *Ficus* sp. exhibit the capacity to coagulate milk due to their proteolytic activity (Roseiro, Barbosa, Ames & Wilbey, 2003a). The extracts prepared from wild cardoon flowers (mainly *C. cardunculus* and *C. humilis*) are reported as very proteolytic and consequently highly effective in the coagulation of ewe's milk. In addition, use of vegetable coagulants in the cheesemaking process allows the commercialization of these traditional products in lacto-vegetarian and ecological markets (Gomes et al., 2019).

Accordingly, in the production of ovine PDO Portuguese cheeses, *C. cardunculus* L. is extensively used as the coagulant. All of these cheeses are coagulated with plant extracts except Terrincho that uses animal rennet. *C. cardunculus* L. is also used in the manufacture of some Spanish PDO cheeses as well as in some French and Italian ovine cheeses (Conceição et al., 2018; Fernández-Salguero & Sanjuán, 1999; Roseiro et al., 2003d).

C. cardunculus L. species is found in southern and western Mediterranean regions, Canary Islands and Portugal. In Portugal, the cardoon plant grows spontaneously in the southwest regions and on the Madeira Island. Typically, the violet flowers of this species possess the proteolytic enzymes (Fernández-Salguero & Sanjuán, 1999; Roseiro et al., 2003a). This plant contains acid proteinases, belonging to the cyprosins, cardosins or cynarase groups, with similar characteristics to other aspartic proteinases used in cheese manufacture (Conceição et al., 2018; Fernández-Salguero & Sanjuán, 1999; Pino, Prados, Galán, McSweeney & Fernández-Salguero, 2009; Silva & Malcata, 2005).

The action of cardoon proteinases promotes milk coagulation, cleaving specific bonds of casein protein that results in a formation of a casein gel (Conceição et al., 2018). The caseins are the most prevalent proteins in milk and can be divided in calcium-sensitive (α_{s1}- and α_{s2}-caseins and β-casein) and calcium-insensitive (κ-casein) caseins (Park, Juárez, Ramos & Haenlein, 2007). Although the vegetable enzymes possess lower specificity than chymosin (animal enzyme), they exhibit higher proteolytic activity as well as higher secondary proteolytic specificity regarding the hydrolysis of α_s- and β-caseins (Ben, Besbes, Attia & Blecker, 2017; Conceição et al., 2018; Pino et al., 2009; Pires et al., 1994). This intense proteolytic action has impact on Serpa cheese ripening, resulting in the development of its unique texture and flavor attributes, mainly the softer texture of Serpa cheese (Ben et al., 2017; Conceição et al., 2018; Pires et al., 1994).

4. SERPA CHEESEMAKING PROCESS

The manufacture of most PDO cheeses occurred mainly on farm scale, however, in the last decades, some artisanal dairies have gradually been modernizing to respond to the market demands, improving processing and hygiene conditions (Freitas et al., 2000; Roseiro et al., 2003c). Therefore, Serpa has also been produced on a semi-industrial scale, with some amendments to the traditional approach. Nevertheless, they must comply with the certification recommendations to be considered a PDO cheese. Several regional cheesemaking industries produce cheeses similar to Serpa cheese, but only seven of these produce under the PDO denomination requirements and only those can benefit from the seal. Nowadays, all Serpa PDO certified dairies produce this traditional cheese using a semi-industrial approach (Dos Santos et al., 2017; Dos Santos et al., 2018).

The PDO specifications (Table 1) require the use of pure raw ovine milk produced in the demarcated geographic area, without addition of any starter culture or milk treatment and coagulated with aqueous extract of *Cynara cardunculus* L., followed by a minimum maturation period of 30 days. Although the native sheep breed for Serpa production is Merino, other breeds can be used, either alone or in combination. All the cheesemaking procedures also must occur in the demarcated area of the PDO cheese. Only dairies authorized by the Serpa producers' group can produce this traditional cheese, following its specific rules of production and submitting the cheeses to the control and certification system for the PDO seal. Cheeses produced with different technological processes or not approved by the control and certification system may be marketed as ewe's milk cheeses without the PDO label (Decreto Regulamentar 39/87, 1987; Council

Regulation EEC2081/92, 2017). The Serpa PDO specifications are summarized in Table 1.

Variations in the pre-processing of milk, preparation of the coagulant, curd working, whey drainage, salting and ripening processes among dairies directly affect the final product characteristics (Randazzo et al., 2009). In this context, in the following topics, all phases of Serpa PDO cheese manufacture will be discussed, highlighting some variations according to dairies. Several manufacturing stages are also summarized in Figure 1.

4.1 OVINE MILK

In Portugal, the last statistical data regarding small ruminant's milk production point to an annual production of about 69.9 million liters of sheep's milk alone. Most of the milk from small ruminants is used for cheesemaking, with a milk yield approximately of 20%. According to the same statistical data, in the ovine sector 11,700 tons of cheese were produced in 2018 (Conceição et al., 2018; INE, 2019). In the demarcated area of Serpa cheese production were produced 72,225 kg of ovine cheese in 2017 (INE, 2018).

Ewe's milk possesses a higher protein, fat and mineral content than cow's or goat's milk, this higher nutritional value is extended to ovine cheeses. Contrary to the bovine sector, the composition of ovine milk depends strongly on the season of the year. Similarly, physiological factors such as, age and lactation period, management systems, for instance, nutrition, milking conditions and sheep genetics largely influence the chemical and microbiological milk composition, resulting in significant variations in final product texture and flavor (Bencini & Pulina, 1997; Park & Haenlein, 2006; Park et al., 2007).

Animal genetics (breed and genotype) is the most determinant factor in the quality and yield of milk. Usually, sheep breeds with higher milk production, will yield milk with lower protein and fat content (Bencini & Pulina, 1997). Some milk producers have been replacing traditional ovine breeds with breeds with higher milk yield such as, the Lacaune breed. Even though some traditional breeds, such as the Merino, are less productive, their milk possesses a richer composition (Roseiro et al., 2003d). Use of milk with higher fat and protein content has a positive impact in the nutritional properties and affects the final sensorial features (Bencini & Pulina, 1997).

In the demarcated areas of Serpa manufacture, production of ovine milk is usually seasonal, restricted from October to June due to the high temperatures typically registered in summertime (Alvarenga, Canada & Sousa, 2008). In summer, the poorer nutritive content of pastures results in high variations in milk composition and yield (Bencini & Pulina, 1997). During the other seasons, milk collection frequently occurs twice a day and automatic technologies may be used to improve the milking process (Roseiro et al., 2003d).

All management systems and equipment used during both milk collection and cheese manufacture are sources of contaminating microorganims (Montel et al., 2014). In the last decades, the high market value of small ruminant's milk coupled with market demands has led to the implementation of automatic systems in ovine and caprine milk collection. However, several studies propose some differences between milk composition from manual and mechanic collection, suggesting a higher incidence of mastitis in machine-milking collection (Reinmann, 2017).

Roseiro et al. (2003d) found a significant presence of *Staphylococcus aureus* in Serpa milk and cheese produced in a semi-industrial dairy compared with milk and cheese from a traditional dairy (Roseiro et al., 2003d). The presence of some *Staphylococcus* and

Streptococcus species in milk and cheese may be associated to mastitis, possibly resulting from mechanical milking (Dos Santos et al., 2018; Guerreiro et al., 2013). Dos Santos and co-workers (2018) only found the presence of *S. aureus* in cheeses from a non-PDO certified dairy and, in addition to mastitis, poorer hygiene practices may be associated with the presence of this bacterial species (Guerreiro et al., 2013; Dos Santos et al., 2018).

Although an evolution in the cheesemaking practices and hygiene conditions was verified, a stricter control of the chemical and microbiological composition of raw milk is still required. Guerreiro et al. (2013) carried out molecular screening to detect subclinical mastitis in three distinct ovine breeds, living in the southern region of Portugal. The authors focused on the importance of implementing new approaches for mastitis detection in order to control milk composition (Guerreiro et al., 2013). In addition to the importance of early mastitis detection, it may be crucial to implement simple solutions for a stricter control of milk composition, for instance, screen for some physical and chemical milk parameters to a rapidly assess of milk quality and safety.

4.2 MILK TREATMENT

The first step of Serpa cheese manufacture consists in milk filtration in order to remove the impurities (Figure 1), typically performed with cotton cloths (Roseiro et al., 2003d). In the manufacture of Serpa pre-processing treatments of milk and starter culture inoculation are not permitted (Figure 1), resulting in a high microbial biodiversity that contributes to the development of an exceptional flavor and texture (Bachmann et al., 2011; Montel et al., 2014). However, as described previously, special control of microbiological and chemical quality of the raw milk is required as well as application of a minimum ripening period to obtain a final product with required organoleptic and safety characteristics (Montel et al., 2014).

Given the high number of parameters that influence the chemical and microbiology of milk, Roseiro et al. (2003d) studied in detail the milk used in Serpa cheese production in two different dairies (artisanal and semi-industrial), finding some significant differences among some biochemical parameters, for instance, acidity, protein and ash. Regarding microbiology, significant differences in lactic acid bacteria counts were also found in milk from distinct industries. Significant variations in Serpa milk from artisanal and semi-industrial dairies corroborates the high heterogeneity of milk composition and, consequently, large variation in the features of traditional cheeses produced with raw milk without addition of any starter culture or milk pasteurization (Roseiro et al., 2003d).

4.3 MILK COAGULATION

Serpa is a vegetable coagulated cheese and, during its production dairies prepare an aqueous extract of *C. cardunculus* L. on the day prior to cheese production. Depending on the producer, vegetable coagulant preparation may differ considerably; employing distinct preparation methods and quantities of dried flowers added (Roseiro et al., 2003b). The cardoon flowers are placed in a water infusion and, then, typically macerated in a mortar (Alvarenga et al., 2008; Roseiro et al., 2003d). Nowadays, the coagulant is typically triturated in an electric blender and filtered with a thin cotton cloth. The resultant aqueous extract possesses a purplish or brown color. The filtered milk is usually soft heated (ca. 30 ºC) to aid the coagulation (Figure 1). Milk coagulation occurs within approximately an hour with a temperature around 30 ºC (Roseiro et al., 2003d; Alvarenga et al., 2008).

For suitable coagulant activity, the amount of cardoon extract necessary per liter of ovine milk frequently ranges between 0.2 and 0.6 g. Sometimes during coagulant maceration and preparation, some cheese producers add salt to improve extraction of the proteinases (Conceição et al., 2018). In addition to proteolytic enzymes, the filtered extract of cardoon possesses other components such as, phenolic compounds, which are not involved in the milk coagulation or cheesemaking process but affect enzymatic activities during ripening process (Conceição et al., 2018; Jervis et al., 1989).

4.4 CURD WORKING

Following coagulation, curd is placed in perforated molds to facilitate air and whey drainage and there is a continuous whey drainage and decrease in curd volume approximately during 10 min, leaving the curd more compact. Afterwards, the curd is removed from perforated molds and the cheese is cleaned of curd leftovers and placed in molds again. Cheeses are turned over several more times, to drain remaining whey (Figure 1; Alvarenga et al., 2008; Roseiro et al., 2003d). In the described stage, distinct modifications have been introduced at the semi-industrial scale of Serpa cheese production such as, the molds are placed in a hydraulic press to facilitate whey drainage (Roseiro et al., 2003d).

In these stages of the cheese-making procedure, the microbial population is dominated by lactic acid bacteria (LAB), such as *Lactococcus* spp., *Enterococcus* spp. and *Lactobacillus* spp. (Beresford, Fitzsimons, Brennan & Cogan, 2001; Jany et al., 2008; Quigley et al., 2011). LAB acidification coupled with the cheese manufacturing process promote both curd syneresis and whey drainage (Beresford et al., 2001).

4.5 SALTING

Beyond milk and coagulant, salt addition is another important ingredient in cheese processing and the quantity and stage at which it is added may be different between dairies. Salt incorporation can occur for instance, at the same time as coagulant addition and/or following whey drainage (Figure 1), resulting in considerably distinct salt concentrations in the final product (Alvarenga et al., 2008; Roseiro et al., 2003d). Typically, in Serpa PDO cheese manufacture, approximately 1500 g of salt are added to 100 L of milk (Alvarenga et al., 2008).

4.6 RIPENING PROCESS

During ripening of Serpa cheese, traditionally manufactured cheese ripened occurred under ambient conditions, while semi-industrial dairies use ripening rooms under controlled conditions (Figure 1; Roseiro et al., 2003b; Roseiro et al., 2003d). Under controlled ripening two successive controlled rooms are used: the first stage lasts about two weeks in a controlled temperature (8–9 °C) and relative humidity (92-97%) room and; the second stage of three or four weeks between 10 and 13 °C of temperature and 85 and 90% relative humidity (Alvarenga et al., 2008; Freitas et al., 2000).

During the ripening period, cloth bandages are placed around the cheese to avoid deformation of the rind. The cheeses are repeatedly turned and washed to provide a uniform maturation and to remove the viscous layer on the cheese surface (Roseiro et al., 2003). The ripening time adopted may be variable as well but, as previously appointed, a minimum maturation period is required to ensure the product's safety. In the case of Serpa cheese legislation, a minimum ripening time of 30 days is applied, typically ranging between 30 and 40 days (Figure 1; Alvarenga et al., 2008; Silva et al., 2000).

Evolution of the microbial population during the fermentation and ripening processes influences the metabolic activities of bacteria and fungi and, consequently, their evolution affects the content and composition of fat, protein, sugar and minerals (Randazzo et al., 2009). Throughout maturation, pH decreases due to lactic acid production and plays an important role in final product properties and safety, since this reduction contributes to the production of several aroma and taste related compounds and inhibits the growth of pathogenic microorganisms, respectively (Alvarenga et al., 2008; Beresford et al., 2001).

Specifically, proteolytic and lipolytic activities, as well as production of volatile compounds by bacteria and fungi present on the cheese surface enables development of both flavor and texture properties in the final product (Schornsteiner, Mann, Bereuter, Wagner & Schmitz-Esser, 2014). Roseiro et al. (2003b) evaluated the proteolysis and maturation index of Serpa cheeses from a traditional and semi-industrial dairy, suggesting that proteolytic activity is dependent on the technological process, mainly on the coagulant activity, salting and ripening conditions. The results suggested a higher proteolysis of cheeses from the traditional dairy, probably resulting from the lower salt concentration and higher moisture. Higher proteolysis results in a softer texture (Roseiro et al., 2003b).

During fermentation the production of antimicrobial compounds occurs (e.g. lactic acid, ethanol, carbon dioxide, hydrogen peroxide, diacetyl and bacteriocins), which contributes to inhibition of growth and proliferation of undesired microorganisms. In parallel, this inhibition throughout maturation period lead to a reduction of several toxic compounds, such as aflatoxins, which may be produced by the undesired microorganisms and may be present in the cheese environment (Giraffa, 2004).

5. SERPA CHEESE SHORTCOMES

Despite the unique sensorial profile of Serpa cheese, several parameters might impact the physical, chemical and microbiological characteristics and directly impacted the cheese sensory profile (Montel et al., 2015; Randazzo et al., 2009). These variations may result in a final product considerably heterogeneous and, in some cases, with organoleptic defects (Montel et al., 2015).

There is great controversy around consumption of raw milk cheeses, raising questions regarding quality and safety due to the possible presence of some undesired microbial groups in raw milk such as, pathogenic microorganisms. In the last decades, a relatively small number of food-borne outbreaks in the dairy industry have been reported, however these occurrences were linked to unpasteurized or defective pasteurized milk and/or post-contamination, rending commercialization of raw milk cheeses in more demanding markets rather difficult (Montel et al., 2015).

Implementation of the use of an autochthonous starter culture in traditional cheese manufacture constitutes a possible way to overcome these problems and promote a consistent quality in the final product, reducing variability as well as minimizing safety risks. However, one of the challenges in development of such a starter culture is selection of one or a group of technological relevant strains. These must be well adapted to the cheesemaking process, optimizing the fermentative process and ensuring that the authenticity of the final product is maintained as much as possible (Montel et al., 2014; Silvetti et al., 2017).

The understanding of the microbial community's evolution over time will lead to a better understanding of the role of different microbial groups during cheese manufacture and ripening (Justé, Thomma & Lievens, 2008; Silvetti et al., 2017). Accordingly, it is pivotal to quantify and identify the evolution and activity of such a microbial community throughout the manufacturing process and understand their technological importance, in order to select the most relevant autochthonous strains (Silvetti et al., 2017).

6. MICROBIOLOGY OF SERPA CHEESE

Despite the importance of cheese microbiology, Serpa microbiological studies are very scarce and limited (Dos Santos et al., 2018). In this context, it is important to discuss the current knowledge of Serpa cheese microbiology in order to recognize the existing gaps and propose alternative studies that would complement the current information on the Serpa microbial community. Available Serpa studies will be discussed next and are summarized in Table 3, being divided into culture-dependent works and studies that coupled both culture-dependent and -independent approaches.

6.1 CULTURE-DEPENDENT SERPA STUDIES

In a study by Roseiro and Barbosa (1996), they evaluated qualitatively the microflora of Serpa milk and cheese. According to these authors, the microbial communities are similar, being dominated by lactobacilli. Throughout the maturation period, a decrease in coliforms and streptococci bacteria was reported together with an increase in proteolytic bacteria (Roseiro & Barbosa, 1996). In another study focused on the microbiology of Serpa and Serra da Estrela cheeses, enterococci and mesophilic LAB were reported as the main groups with *Lactococcus* and *Leuconostoc* reported as the most prevalent genera (Barbosa, 2000).

One parameter that significantly contributes to chemical and microbiological variations in milk and cheese is the distinct cheesemaking procedures used in different dairies (Montel et al., 2014). Roseiro et al. (2003d) characterized microbiologically the milk and Serpa cheese from two dairies, finding significative differences between the final products from artisanal and semi-industrial manufacture. These results suggested a distinct microbial composition of milk and cheese between producers. In terms of the microbiological profile, it was reported that LAB, yeasts, molds and coliforms as well as some pathogenic species, such as *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus* were present (Roseiro et al., 2003d).

During the last decades, Serpa cheese microbiota was investigated using culture-dependent approaches, which involves the cultivation and isolation of a microbial population on selective media (Jany & Barbier, 2008; Quigley et al., 2011; Randazzo et al., 2009). These methods involve a complex and long experimental period prior to identification based on phenotypic or genotypic characteristics of the microorganisms (Jany & Barbier, 2008; Ndoye, Rasolofo, LaPointe & Roy, 2011). Moreover, culturing techniques may be inaccurate in the microbial diversity estimation since less abundant microbial species are often outcompeted by the more abundant ones. Furthermore, some species are unable to grow *in vitro* (Dolci et al., 2015; Ndoye et al., 2011; Neviane et al., 2013; Quigley et al., 2011). Therefore, culture-dependent approaches typically underestimate microbial diversity of cheese and other habitats (Jany & Barbier, 2008; Ndoye et al., 2011). These studies were based on the dominant groups under specific conditions of manufacture, or were developed in a perspective of final product safety, aiming at quantifying some microbial groups and detecting presence of key microorganisms, both in milk or cheese, namely pathogenic or spoilage bacteria and fungi (Barbosa, 2000; Roseiro & Barbosa, 1996; Roseiro et al., 2003d).

6.2 CULTURE-DEPENDENT AND -INDEPENDENT SERPA STUDIES

With the development of culture-independent approaches, the microbiology of food and other ecosystems has been revolutionized, enabling the study of microbiota faster and more exhaustively. These techniques are based on direct analysis of DNA or RNA, not requiring the cultivation and isolation of microorganisms present in the target habitat (Dolci et al., 2015; Ndoye et al., 2011; Neviane et al., 2013). Culture-independent methods have been reported as an effective and reliable way to explore the dynamic of the cheese microbial community, contributing to an understanding of the technological role of microorganisms during cheese manufacture and ripening (Neviane et al., 2013).

Recently, two microbiological studies of Serpa cheese were done coupling culture dependent and -independent methods. The first study explored the yeast community of Serpa cheese (Dos Santos et al., 2017) while, in the other work the authors studied the bacterial community of this traditional product (Dos Santos et al., 2018). In both cases, PDO and non-PDO certified cheeses from two different batches and seasons were analyzed, after the minimum maturation period (30 days), coupling culture-dependent and -independent approaches (Dos Santos et al., 2017; Dos Santos et al., 2018).

The studies of Serpa yeast and bacterial community suggested a wide diversity of genera and species as well as indicated significant differences between the microbial population from PDO and non-PDO cheeses (Dos Santos et al., 2017; Dos Santos et al., 2018). These variations proposed a lack of regulation in the Serpa cheesemaking process. Additionally, in samples from one of the non-PDO certified industries, *S. aureus* was found (Dos Santos et al., 2017) and, as previously mentioned, this species may be associated with foodborne disease. The authors suggested that the use of longer ripening periods in Serpa cheese would be favorable (Dos Santos et al., 2017), also a revision in the manufacture procedures and implementation of stricter hygiene practices as well as a better control of milk composition would help to overcome safety issues.

The prevalent genera and species reported in these studies are among the most predominant groups occurring in other raw milk cheeses studies, for instance, Serra da Estrela cheese (Dahl, Tavaria & Malcata, 2000; Tavaria & Malcata, 1998, 2000), being reported as technologically safe and relevant for the development of key sensorial attributes during ripening (Dos Santos et al., 2017; Dos Santos et al., 2018). In the first study, *Debaryomyces* and *Kluyveromyces* were reported as the most prevalent yeast genera in the core of all cheeses analyzed, however, culture-independent approaches also revealed a high prevalence of *Galactomyces* genus (Dos Santos et al., 2017). Regarding bacterial community, the culture-independent analysis reported *Lactococcus*, *Leuconostoc* and *Lactobacillus* as the most prevalent genera, while the culturing analysis pointed *Lactobacillus paracasei/ casei* as the main species in PDO cheeses and *Lactobacillus brevis* in non-PDO cheeses (Dos Santos et al., 2018).

Altogether, these results contribute to the current knowledge on Serpa microbiology, identifying the most prevalent microbial group on Serpa thirty-day old cheeses. The culture-independent data complemented the results obtained by culturing methods, identifying a high range of yeast and bacterial genera (Dos Santos et al., 2017; Dos Santos et al., 2018). Nevertheless, the culture-dependent approaches allowed the discrimination of microbial isolates at a deep taxonomic level (species level) and their culturing and isolation may allow for determination of several parameters, related to their technological and functional attributes. The culture-dependent approach may therefore be crucial in contributing to the selection of isolates of a tailor-made autochthonous starter culture to improve cheese quality.

These results may be useful to improve the ripening conditions, favoring some desirable microbial species involved in the maturation phase. However, these microbiological works lack, an overall, understanding of the microbial evolution as well as the technological role of the microbial communities during manufacture and ripening. Exploration of the microbial community dynamics throughout cheese manufacture and ripening will complement these results and allow for recognition of the impact of distinct groups in the various cheesemaking phases.

7. CONCLUSIONS AND FUTURE DIRECTIONS

Even though pasteurized cow milk cheeses currently dominate the cheese market, traditional cheeses are the most representative of Southern Europe. In Portugal these artisanal products are mainly manufactured from small ruminant's milk, mainly ovine (Roseiro et al., 2003d). Both the processing technology and ingredients used in Serpa cheese production result in a final product characterized by its high microbial biodiversity, allowing for development of unique sensorial attributes. The high susceptibility of raw milk cheeses to the milk composition and manufacturing procedures, may result in a final product heterogeneous in terms of flavor and texture characteristics. The safety risk of raw milk consumption is also a relevant issue.

The major impact that microorganisms play in the organoleptic and safety features make it mandatory to increase the knowledge of the microbiological profile of this cheese. Recent studies have shed some light on the microbial ecosystem of ripened Serpa cheese, nevertheless with a higher number of analyzed samples and monitoring of the microbial dynamics throughout cheese manufacture and ripening, could provide clearer insights about the technological and functional impact of the most relevant bacterial and yeast strains. Moreover, core and rind are distinct environments and some studies and ancient data reported significant differences in the microbial community of the two environments (Schornsteiner et al., 2014) and, thus, study of rind microbiota may be important to complement the results as well.

Advances in these directions will certainly allow for improvement and implementation of better practices, assuring better control of Serpa cheese manufacture and ripening and consequently, contribute to the upgrading of its quality and safety. In parallel, identification of microbial strains with technological and organoleptic relevance will guide the selection and development of an autochthonous starter culture for this traditional cheese. Even though in the Portuguese legislation of PDO cheeses a starter culture inoculation is not permitted, the development of a starter culture may contribute to introduction into the market of a cheese with a similar organoleptic profile of Serpa PDO cheese. This will allow enhanced marketability of a widely appreciated Portuguese cheese, both in the country of origin and in other markets with stricter hygiene and food safety regulations.

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Table 1. Serpa PDO cheese specifications (Decreto Regulamentar 39/87, 1987; Council Regulation EEC2081/92, 2017).

PDO specification of Serpa cheese	
Type of milk	Pure raw ovine milk produced in the demarcated area
Milk Treatment	No milk treatment or addition of starter culture
Coagulant	Aqueous extract from *Cynara cardunculus* L.
Ripening conditions	30 days (minimum), temperature between 6-12 ºC and relative humidity between 85-90%
Conservation conditions	0-5 ºC in the dairy industry; 0-10 ºC in the transport and retailer
Dimensions	Weight: 0.2-2.5 kg; Height: 3-8 cm; Diameter: 10-30 cm
Maturation index (WSN/TN)	45% (minimum)
Moisture (FFB)	61-69%
Fat (DM)	45-60%
Surface	Malleable consistence; whole and well-formed aspect, slightly rough and thin; uniform light-yellow color
Interior	Buttery texture, with easily deformable cutting zone; unctuous aspect, with few or no eyes; light-yellow color; strong and slightly spicy flavor

***** DM – Dry matter; WSN – Water soluble nitrogen; TN – total nitrogen

Table 2. Serpa PDO biochemical composition.

Biochemical composition of Serpa cheese		Reference
pH	4.95-5.7	Roseiro et al., 2003d; Alvarenga et al., 2008; Dos Santos et al., 2017; Dos Santos, et al., 2018
Acidity	7.5-9.8 g kg^{-1} lactic acid	Roseiro et al., 2003
Protein (DM)	36-41 g kg^{-1}	Roseiro et al., 2003
Total nitrogen (m/m)	3.5%	Alvarenga et al., 2008
Non-protein nitrogen (NPN/TN)	2.3%	Alvarenga et al., 2008
Aminoacidic nitrogen	3.7%	Alvarenga et al., 2008
Salt in Moisture (S/M)	36.1-44.3 g kg^{-1} NaCl	Roseiro et al., 2003
Ash	80.9-86.3 g kg^{-1}	Roseiro et al., 2003
Water activity (a_w)	0.96-0.98	Dos Santos et al., 2017; Dos Santos et al., 2018
Hardness	7.05 N	Alvarenga et al., 2008
Ca^{2+} content (DM)	12.48-13.11 g kg^{-1}	Roseiro et al., 2003
K^{+} content (DM)	1.94-2.14 g kg^{-1}	Roseiro et al., 2003
Mg^{2+} content (DM)	0.69-1.26 g kg^{-1}	Roseiro et al., 2003
Na+ content (DM)	12.58-13.32 g kg^{-1}	Roseiro et al., 2003

*****DM – Dry matter; NPN – Non-protein nitrogen; TN – total nitrogen

Table 3. Serpa cheese microbiology studies.

Type of study	Main purpose and results	Predominant microbial groups	Examples of microbial groups and counts	Reference
Culture dependent	Similar microbiota between Serpa milk and cheese. Decrease in coliforms and streptococci and increase in proteolytic bacteria during ripening.	Lactobacilli	Coliforms and streptococcus ca. 10^7 cfu g^{-1} of cheese in fresh cheese and proteolytic bacteria ca. 10^5 cfu g^{-1} in ripened cheese	Roseiro and Barbosa (1996)
	Microbiological investigation of Serpa cheese.	Enterococci and mesophilic LAB	Mesophilic LAB ca. 10^8 cfu g^{-1} of cheese and enterococci ca. 10^7 cfu g^{-1} of cheese; *Lactococcus spp*. and *Leuconostoc spp.*	Barbosa (2000)
	Significant biochemical and microbiological differences between artisanal and semi-industrial dairies.	LAB, yeasts and coliforms	LAB ca. 2×10^8, yeasts ca. 8×10^4 and coliforms ca. 4×10^5	Roseiro et al. (2003d)
Culture dependent and independent	Significant differences between bacterial and yeast community of PDO and non-PDO cheeses. High diversity of bacteria and yeast genera and species.	*Galactomyces*, *Debaryomyces* and *Kluyveromyces* genera	*Debaryomyces hansenii*, *Kluyveromyces lactis*, *Candida zeylanoides*, *Pichia fermentans*, *Cryptococcus oeirensis* and *Yarrowia lypolytica*	Dos Santos et al. (2017)
		Lactococcus, *Leuconostoc* and *Lactobacillus* genera	*Lb. paracasei*, *Lb. plantarum*, *Lb. brevis*, *Lb. pentosus*, *Lb. curvatus*, *L. mesenteroides*, *Lc. Lactis*, *E. faecalis*, *E. faecium*, *E. hirae* and *Hafnia alvei*	Dos Santos et al. (2018)

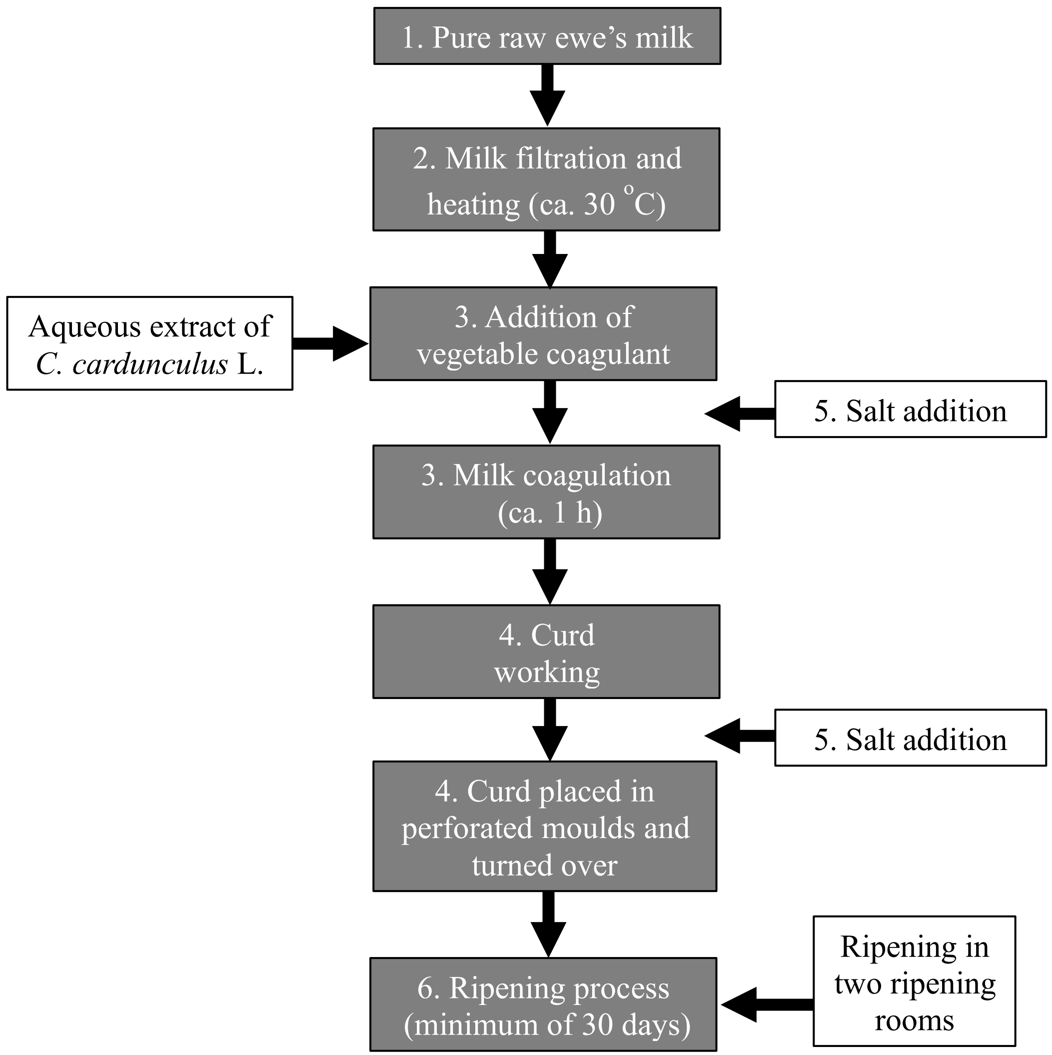
1. Pure raw ewe's milk
2. Milk filtration and heating (ca. 30 °C)
Aqueous extract of *C. cardunculus* L.
3. Addition of vegetable coagulant
5. Salt addition
3. Milk coagulation (ca. 1 h)
4. Curd working
5. Salt addition
4. Curd placed in perforated moulds and turned over
Ripening in two ripening rooms
6. Ripening process (minimum of 30 days)

Figure 1. PDO Serpa cheesemaking procedure.