

The Technology, Chemistry, and Microbiology of Serra Cheese: A Review¹

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ABSTRACT

This paper comprehensively reviews fundamental and applied aspects of the manufacture of Serra cheese, its composition, the biochemical reactions that take place during coagulation and ripening, and the microbial ecology. Serra cheese is the most traditional cheese manufactured in Portugal. Aspects that make it unique are 1) its manufacture by the coagulation of raw ewe milk using a vegetable rennet (cardoos flower) and 2) its final buttery texture and flavor.

The wide variation of the final quality of this "Appellation d'Origine Contrôlée" cheese has been explained by the intrinsic variabilities of raw materials, cheese-making practices, and maturation. The available studies pertaining to Serra cheese have indicated that 1) the coagulant activity of cardoon flower extract on ewe milk is higher and is more affected by pH and salt concentration than that of animal rennet, 2) the best coagulation temperature is 27 to 29°C, 3) the best ripening conditions are 8°C and 90% relative humidity, 4) the lactic acid fermentation results mainly from the action of lactic acid-type *Streptococcus*, *Leuconostoc*, and *Lactobacillus* species, 5) a yellow to reddish surface viscous material (mainly composed of yeasts) is important in the ripening process, 6) maturation is essentially surface-driven, and 7) the low temperatures prevailing during winter help to control the extensive microbial contamination that occurs from the poor sanitary conditions during cheese manufacture. These points are

described and critically discussed in light of the principles of dairy science.

(Key words: Serra cheese, raw ewe milk, vegetable rennet)

Abbreviation key: AOC = Appellation d'Origine Contrôlée.

INTRODUCTION

Serra da Estrela cheese, often called Serra cheese, is economically and organoleptically the most important variety of traditional cheese manufactured in Portugal. This type of cheese is obtained by coagulation of raw ewe milk with an extract of the cardoon flower (*Cynara cardunculus* L.).

Dating from the Roman occupation of the Iberian Peninsula, the empirical techniques associated with the manufacture of Serra cheese have been passed through generations of families of shepherds. Although genuine Serra cheese is increasingly harder to find (because of the exodus of the younger generations from the farms to the cities), the unique characteristics of this cheese have given it the status of a gourmet delicacy; hence, extensive research efforts, including applied and fundamental characterization, have been initiated to explain its properties. Another focus for research is the ongoing discussion of the hygienic and safety aspects of using raw milk in cheese production.

Serra cheese is named for the highest mountains of Portugal, the Serra da Estrela (ca. 2000 m high). These mountains (currently bearing the status of natural reserve for purposes of environmental protection) possess a special microclimate that supports the growth of natural pastures that are used throughout most of the year to feed the flocks. During winter, the snow and strong wind prevent grazing on the slopes, so the shepherds bring their flocks to the valleys nearby. For good milk production, in both quantity and quality, the pastures need

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to be free of ice and as varied as possible in location. A typical flock consists of either black or white varieties of Bordaleira da Serra da Estrela, an ovine breed that is well adapted to the prevailing geoclimatic conditions at Serra da Estrela. This breed, the best native breed in Portugal for milk production, is able to produce between 150 and 200 L of milk during a typical lactation (usually 210 to 240 d, from October to May) (37).

The estimated annual overall production of ewe milk in Serra da Estrela is ca. 12 million L. The corresponding estimated annual production of Serra cheese is ca. 1200 tonnes, which, when the cheese is sold at an average price of US \$16.5/kg [the National Breeders Association for Serra da Estrela Sheep (ANCOSE), personal communication, 1992], gives a total sales income of about US \$19,800,000/yr. This income constitutes a major source of wealth for the farmers of the regions where Serra cheese is produced.

Several dairies have tried to produce a standardized industrial product resembling Serra cheese from pasteurized ewe milk (with or without the addition of goat or cow milk). Although the shelf-life is longer and more predictable, the resulting product (which cannot legally bear the label Serra cheese) is much less appealing organoleptically.

The major focus of this review is the local techniques of artisanal cheese making (i.e., from the milk of a flock of ca. 40 sheep, which corresponds to an average of four 1.4-kg wheels per d of cheese), coupled with the fundamental knowledge available on enzymatic and microbial activities during cheese manufacture and ripening.

Serra cheese exists in two major types: buttery (the genuine Serra cheese) and aged. The usual ripening period of the former is 30 to 45 d, whereas the latter requires an aging process of at least 6 mo. The present review concentrates on aspects of the buttery Serra cheese.

DEFINITION

According to Portuguese legal standards (22), Serra cheese has a flat-cylinder shape without sharp edges; bulging is considerable on the lateral sides and slight on the upper

surface. A typical cheese has a diameter of 15 to 20 cm, a height of 4 to 6 cm, and a weight of 1 to 1.7 kg. The rind is thin, uniform, smooth, and well formed, and it possesses a soft, straw-yellow color. The cheese has no eyes or only a few small eyes, and its color ranges from ivory to white. Serra cheese has a buttery texture, which leads to spontaneous and rapid deformation upon slicing, and possesses a strong aroma and a clean, smooth, slightly acid flavor. The moisture content ranges from 61 to 69% (fat-free basis), and the fat content ranges from 45 to 60% (DM basis).

Serra cheese bears the status of "Appellation d'Origine Contrôlée" (AOC) cheese and, by legal definition, can be made only from raw milk produced at farms located in the region of Serra da Estrela using an extract of the cardoon flower (*Cynara cardunculus* L.) as rennet. The AOC region of Serra da Estrela, which has been established via a governmental act (23), is depicted in Figure 1. The regions of major production of Serra cheese are Manteigas, Gouveia, Seia, Celorico da Beira, Oliveira do Hospital, and Guarda (40). Recently, the government of Portugal has entrusted FAPROSERRA (the Federation of Cheese Producers of Serra da Estrela) with the power for certification of Serra cheese (24). Criteria for certification (the denomination seal is reproduced in Figure 2) include compliance with minimum standards of hygiene during fabrication, shape, organoleptic characteristics, and chemical and microbial contents of the final product.

CHEESE MANUFACTURE

Although it has undergone extensive improvements because of governmental inspection and enforcement according to results from extensive field studies (1, 2, 13, 14, 34, 35), Serra cheese has been traditionally manufactured under very poor conditions of hygiene and is subject to wide variation among farmhouses, cheese makers, and even days. The major operations associated with the manufacture of this type of cheese (some of which may, at times, be absent) are 1) milk handling; 2) coagulation; 3) cutting, working of the curd, and draining of the whey; 4) pressing; 5) salting; and 6) ripening.

Milk Handling

Milking is done twice a day: at sunrise, before the flock is walked to the pasture, and at sunset, after the flock is returned to the stable. Milking is done manually by the shepherd into a small open vessel (*ferrada*). During this process, milk is subjected to several kinds of contamination from the mammary glands

and the wool of the ewes, the hands of the shepherd, the soil, and the collection vessel. The milk is accumulated in larger containers, which are brought to the cheese-making area in the house and kept warm for 30 to 60 min before setting (4).

The ewe milk is then filtered through a fine, clean cloth to remove particulate impurities (e.g., hair and dust) and poured into a coagulation vat (usually a domestic pan with a capacity of ca. 20 L).

Coagulation

To achieve and to maintain the desired temperature for coagulation, the pan is usually placed by the fireplace or, less frequently, in a water bath. Temperature is controlled empirically by the cheese maker, who evaluates the temperature via finger tips.

After the desired temperature has been reached, the vegetable rennet (cardoos) is added. Although the technique of addition differs greatly, most cheese makers have elected one of the following methods: 1) direct addition of the dry cardoon flower to the warm milk, followed by efficient mixing for a few minutes and filtration of the milk through a fine, clean cloth; 2) maceration of the dry cardoon flowers with small amounts of water and salt until a paste results, dilution of the paste in more

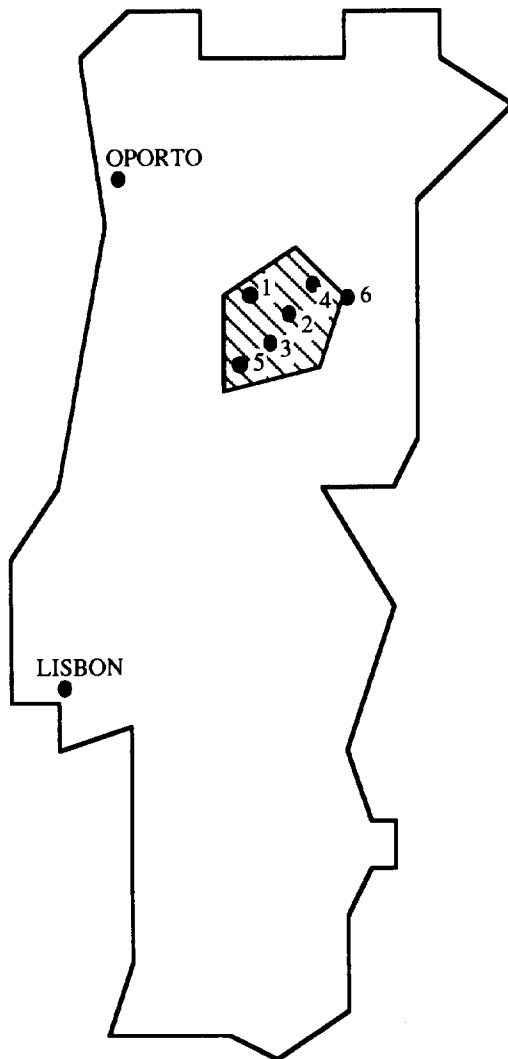


Figure 1. Map of Portugal with the indication of the "Appellation d'Origine Contrôlée" region of Serra da Estrela cheese (shaded zone): 1) Manteigas, 2) Gouveia, 3) Seia, 4) Celorico da Beira, 5) Oliveira do Hospital, and 6) Guarda.



Figure 2. Official label of certified Serra cheese.

water to aid in extracting the enzymes, filtration of the extract through a fine, clean cloth, and addition of this crude extract directly to the milk; and 3) maceration of the dry cardoon flower with salt and water, recovery of this paste inside a cloth with closed ends (*boneca*), followed by submersion in the milk, agitation, and squeezing.

The milk is then allowed to rest in the closed pan for a time interval that depends on the empirical knowledge of the cheese maker. The end of coagulation is confirmed by slight agitation of the pan to evaluate empirically the consistency of the gel. The average times and temperatures of coagulation have been measured in various farmhouses in the AOC region of Serra cheese: 30 to 45 min at 20 to 30°C (14), 28 to 240 min at 17 to 40°C (2), 25 to 63 min at 28 to 32°C (35), 37 to 90 min at 27 to 31°C (13), about 60 min at 27 to 29°C (4), 20 to 360 min (1), 45 to 240 min (34), and 30 to 40 min (27). The amount of crude cardoon extract added to the milk was measured as .11 to .22 g/L of milk (2) and .32 to .40 g/L of milk (13).

Cutting and Working the Curd and Draining the Whey

The cutting of the curd consists of manually stirring the coagulum for ca. 10 to 15 s (2) with the bare hand or with a cup, knife, or kitchen spoon. The curd thus obtained is very irregular in shape and size (13).

Some cheese makers prefer to work the curd before molding it. In this case, after preliminary cutting of the curd in the coagulation pan, the curd pieces are poured into special cheese molds made from a flexible, perforated metal plate in the form of an open-ended cylinder with adjustable diameter (*cincho*). After being placed in the mold, the curd is hand cut into smaller pieces and pressed with both open hands so as to fit better in the mold and to release as much whey as possible. Finally, the curd is lined with a fine cloth. The simultaneous operations (work of the curd and drainage) are performed on the top of a round, sloped table (*francela*), which facilitates the drainage of the whey into an open vessel [where the whey is collected prior to undergoing heat treatment to precipitate the soluble proteins and to produce curd cheese (*requeijão*)]. This operation can take up to 1 h per

cheese, and the final texture of the cheese is heavily dependent on the technique used.

The curd can be also worked in a well-washed cloth bag. The open ends of the bag are brought together, and the bulk is carefully pressed to expel the whey, which permeates the cloth. This process, which can take from 10 min (2) to 45 min (35), transforms the curd into a homogeneous paste, which can then be placed in the molds. Recently, the use of fixed diameter, perforated plastic molds has increased, although this type of mold requires preliminary wrapping of the cheese with a fine cloth to facilitate unmolding. The drainage is usually completed via light pressing of the curd while it is in the mold.

Pressing

Whenever employed, pressing takes place for 3 to 24 h with a 6- to 20-kg stone (2) placed on top of the curd, which is resting in the mold.

Salting

The most common process of salting is to add crude, unrefined kitchen salt to milk before addition of the vegetable rennet or to add a concentrated brine that also contains the crude cardoon extract. Other cheese makers prefer alternatively to rub the top and bottom surfaces of the pressed cheese with crystals of salt, usually waiting from 6 h (13) to 24 h (35) between consecutive rubbings. The entire amount of salt used for either process is ca. 30 g/L of milk (2) or .5 to .9 g/cm² on the top surface of cheese and .5 to .6 g/cm² on the bottom surface (13), respectively.

Ripening

After salting and pressing, the cheeses are taken to the maturation room, which often is next to the manufacturing room (to prevent excessive temperature fluctuations), and are placed in the open air on wooden shelves previously covered with cloth. Usually, there is no artificial control of the temperature and humidity of the room. Each cheese is turned upside down once per day. After 8 to 15 d (2, 13, 35), a white to reddish viscous smear (*reima*) appears on the exposed surfaces of the cheeses. (According to experienced cheese

makers, this coloration is an indication of a potentially good final cheese). The cheese is then washed with warm water, whey, or plain raw milk for the first time. Then, a band of linen or cotton cloth is wound around the cheese and tied up with a small knot. This band is essential to prevent the loss of cylindrical shape of the cheese, which may occur in the more advanced stages of maturation.

At this time, the cheese is moved to a second maturation room, usually cooler than the first (although, once again, the weather directly determines the prevailing maturation environment conditions). The cheeses are turned upside down according to the same pattern as before, and the cloth band is adjusted as necessary. The cheeses are washed once per week (or more often, depending on the rate of production of viscous smear).

The entire ripening period for buttery Serra cheese ranges from 30 to 45 d (1, 4, 14, 35, 42). From October to May, the observed temperatures in the maturation rooms of various farmhouses located in the region of the Serra da Estrela ranged from 19 to 30°C (1), 15 to 30°C (13), 4 to 16°C (2), and 4 to 12°C; the relative humidities ranged from 75 to 95% (2), 42 to 98% (13), and 90 to 95% (42).

TECHNOLOGICAL STUDIES

Different characteristics can be imparted to the cheese, depending on the manufacturing technique employed and on the environmental conditions prevailing during coagulation, draining, pressing, salting, and ripening. For example, the rheological properties of the coagulum depend on 1) amount of coagulant enzyme, 2) pH, 3) acidification rate, and 4) temperature. In addition, the characteristics of the curd depend not only on the coagulum properties but also on the nature and intensity of the mechanical work that follows and on the rate of drainage. The texture, flavor, and aroma of cheese are directly dependent on the composition (moisture, protein, fat, and mineral contents) and pH of the curd as well as on the maturation conditions (temperature, percentage of humidity, and extension of microbial contamination in the bulk and on the surface of the cheese). Some of these effects are discussed herein. It should be emphasized that acidification depends entirely on the in-

digenous microflora of raw milk because starter cultures are not used in the manufacture of Serra cheese.

Coagulation

Of the various operating parameters, temperature and time of coagulation prevailing during cheese making have received the most interest in the past. Three periods (January to February, March to April, and May to June), which correspond to distinct weather, pasture, and handling conditions, have been arbitrarily considered (35) in temperature and time of coagulation. The mean coagulation temperatures for 14 farmhouses in each of the three bimonthly periods were 28.2, 30, and 30.9°C, respectively, although percentages of difference (up to 100% of the average coagulation temperature) were also significant between some farmhouses (2). This variability partly accounted for the fact that, at the 5% significance level, the temperature of coagulation was not different in different periods; the coagulation could be characterized by a temperature of $29.9 \pm 4.2^\circ\text{C}$. For each farmhouse, the mean coagulation temperature ranged between 27.6 and 31.6°C during each period and decreased 1°C during the overall time interval of coagulation. According to Barbosa (4), cheeses are organoleptically better when the coagulation temperature is maintained between 27 and 29°C; higher temperatures usually lead to losses in the softness of the final cheese.

The coagulation time ranged from 28 to 57 min during the first period (January to February), from 25 to 63 min during the second period (March to April), and from 25 to 54 min during the third period (May to June) (35). Student's *t* test showed no differences between periods at the 5% significance level; hence, coagulation could be characterized by a coagulation time of 38.2 ± 19.1 min. Local variations in the time of coagulation may be attributed to differences in amounts of cardoon extract employed and possible differences in the native acidity of the milk. (Because Serra cheese is made from raw milk, which, in some cases, is not immediately used after milking, reductions in the coagulation time may be derived from microbial-driven increases in acidity.)

Pressing

The effect of pressing on the final texture of Serra cheese has been studied by Lopes et al. (26), who concluded that pressing the curd avoids extensive development of cracks in the cheese, which is a condition for good final quality.

Maturation

The temperature and humidity of the maturation room are directly dependent on the exterior weather conditions.

Vieira de Sá et al. (42) concluded that a temperature of 10°C and a relative humidity of 90 to 95% are the best conditions for cheese ripening. However, good yields of high quality cheese were obtained for 80 to 90% relative humidity. Saramago et al. (35) and Antunes and Santos (2) concluded that constant temperature and relative humidity ensure better final quality than periodic (or random) fluctuations in these parameters. The natural maturation conditions during the winter in Serra da Estrela were well mimicked by a constant temperature of 8 to 11°C and a relative humidity of 95% (35). Those conditions produced similar final products, although the cheeses produced in the farmhouses during March and April were less buttery than those kept under controlled conditions (35). Barbosa (4) observed that the best cheeses were produced between December and March. Vieira de Sá et al. (42) observed that the moisture content of cheese ($45.3 \pm 4.4\%$) remained much more constant during maturation under controlled environmental conditions than in the farmhouses. As expected, the decrease in the moisture content during maturation was accelerated by high temperatures, coupled with low relative humidities (42). Forced ventilation prevents regular development of the smear (*reima*) and damages the solid appearance of the rind by helping in the formation of cracks (42).

Some authors (42) have suggested that the sanitary condition of the raw milk, coupled with the technique of cheese making, has a stronger effect on the final quality of the cheese than the ripening conditions employed. However, all studies mentioned herein suggest that appropriate control of temperature and relative humidity in the maturation room is a

key factor in ensuring constant quality; therefore, this issue merits further study.

Yield

All studies performed on Serra cheese indicated that the cheese yield (i.e., the amount of cheese obtained from a given amount of milk), compared with that of other cheese varieties made from ewe milk, is low (2, 13, 35). Cruz (13) determined that the amount of milk necessary to produce 1 kg of cheese after the regular ripening period of 45 to 50 d ranges from 5.5 to 6.0 L for a final yield of ca. 17%.

According to Antunes and Santos (2), the low yields are partly due to the loss of curd during cheese making (around 7 to 8% of the total curd is lost during the operations of breaking and molding the curd) because the highly particulated curd is easily drained out with the whey. Other reasons for these yields include the temperature and relative humidity in the maturation room. Saramago et al. (35) observed that, from January to June, the yield tends to decrease, although the decrease is more significant in February and March and, to a lesser extent, from April to June. This decrease in yield was explained by the different weather conditions during the different months. Statistical analyses of the data collected by those authors (35) indicated that, at the 5% significance level, temperature and relative humidity influence yield. Similar analyses showed that the cheese yields (in both fresh and cured states) obtained during the first period (January to February) were significantly higher from those from the second (March to April) and third periods (May to June). Yields can be increased by use of a controlled environment (2).

Cheese-Making Standardization

Vieira de Sá and Barbosa (40) assessed the influence of mechanized processing of milk on the quality of the resulting Serra cheese. Cheeses were made from raw ewe milk on average, of 6% fat, .26% acidity, and pH 6.5. The cardoon extract was added to milk kept at 27°C, and, after 1 h, the curd was cut and stirred very slowly. After drainage, the curd was salted (2%), scooped into the molds, and lined with a fine cloth. Drainage was completed by light pressing. Ripening took place

in a maturation chamber maintained at 8°C and 90% relative humidity for 45 d. From the 25 cheeses produced by those authors (40), 96% presented good textural and organoleptic characteristics, comparable with the best cheeses produced traditionally in the Serra da Estrela region; more extensive studies are, nevertheless, required for more quantitative conclusions.

CHEMICAL STUDIES

The texture, flavor, and aroma of any cheese are associated with its chemical composition and pH.

Composition

Composition is, in general, a complex function of the quality of the raw milk and the process of transformation of milk into cheese.

As with other species bred for their milking characteristics, Bordaleira da Serra da Estrela sheep produce milk that is generally characterized by high protein (ca. 6%) and fat (ca. 8%), as shown in Table 1. Usually, high cheese yields require high concentrations of both fat and casein.

The reported chemical composition of Serra cheese differs greatly (Table 2); nominal values are ca. 40% water, 30% fat, 20% casein, 3% sugar, 5% ash, and 2.5% salt. This variation can be attributed to the lack of standardization of raw milk and to the lack of standardized procedures for the manufacture and ripening of Serra cheese. Variation in the composition of cheeses of the same age throughout the cheese-making period (from January to June) was studied by Saramago et al. (35). The moisture content of cheese decreased because

of increases in the average ripening temperature and because of a decrease in the average relative humidity during the aforementioned 6 mo (35). Vieira de Sá et al. (42) observed that a 5-d increase in ripening time (for a total of 45 d) caused a 5% decrease in the moisture content of the cheese. Because each cheese maker has an individualized method of adding salt in amounts fixed by past experience and personal taste, the salt content of cheese (on a dry basis) can vary almost twofold among cheese makers (35).

pH

Based on studies of the pH variation of cheese during the processes of manufacture and ripening, Saramago et al. (35) confirmed that (as is widely accepted today) the first step of coagulation (the hydrolysis of κ -casein) is a result of enzymatic action rather than acid action. Student's *t* tests at the 5% significance level showed that 1) the pH of raw milk ($6.69 \pm .16$) in the coagulation vat is statistically equal to the pH of fresh cheese ($6.62 \pm .23$); 2) the pH significantly decreases within the 7 d thereafter (to a final value of $4.90 \pm .51$); and 3) the following weeks are characterized by a stabilization of the pH: $4.78 \pm .46$ after 15 d and $4.84 \pm .69$ after 30 d. These results agree with those reported by Vieira de Sá et al. (42), who found that cheese pH and acidity decreased during early stages of ripening and did not change significantly during later stages. Furthermore, those authors (42) observed that the pH and the acidity of cheeses produced in different farmhouses were similar. Studies of the variation of pH in three different zones of the cheese (i.e., the surface and two distinct bulk zones) indicated higher acidity in the interior (34).

TABLE 1. Chemical composition of milk of the Bordaleira da Serra da Estrela sheep.¹

Water	Fat	Protein	Lactose	Ash	Reference
		(%)			
80.0 ¹ ± 2.7	7.8 ± 1.6	6.0 ± 1.3	4.4 ± .5	NA ²	(2)
80.9 ± 1.6	7.4 ± 1.5	6.0 ± .9	4.6 ± .5	.9 ± .1	(13)
NA	8.6 ± 2.0	5.0 ± .7	NA	NA	(35)
81.2	6.8	6.8	5.1	.9	(41)

¹Mean ± 95% confidence interval.

²Not available.

TABLE 2. Chemical composition of Serra cheese.

Moisture	Fat	Casein	Sugar	Ash	Salt	Reference
(%)						
48.8	28.8	19.9	NA ¹	4.4	2.6	(4)
46.7 to 48.8	28.1 to 30.7	19.2 to 20.4	NA	4.1 to 4.3	2.2 to 2.6	(5)
31.9	40.1	22.2	2.2	3.4	.9	(21)
39.4 ² ± 19.3	27.9 ± 14.4	NA	3.9 ± 4.0	5.8 ± 4.1	2.6 ± 2.9	(28)
34.0 to 48.8	30.6 ± 7.3	NA	NA	NA	2.9 ± 1.2	(35)
NA	23.0 to 40.0	18.0 to 23.0	NA	NA	NA	(36)

¹Not available.²Mean ± 95% confidence interval.

MICROBIOLOGICAL STUDIES

The ewe milk used to produce Serra cheese is not subject to any type of physical or chemical treatment prior to coagulation with the vegetable rennet (except for macrofiltration). Therefore, the native flora of raw milk can play an important role during the maturation of Serra cheese. The total microbial count (millions per cubic centimeter) and associated 95% confidence intervals for milk just before use were $.98 \pm 3.95$ (2) and $.53 \pm 1.21$ (13). These values are not particularly high, given the poor sanitary condition of the equipment and the absence of heat treatment, possibly because the low temperatures prevailing in winter in the Serra da Estrela region help to prevent extensive microbial multiplication. The unusually large amplitude of the 95% confidence intervals can be attributed to the high variability in the characteristics of the flock, the procedures of milking and handling, and the hygienic conditions of different farmhouses. The yeast count was $.62 \pm 1.42$ (2) and $.8 \pm .8$ (13). Plant cells and epithelial cells are seldom found in raw ewe milk (2, 13).

Because the milk is not submitted to any chemical or physical treatment, the natural variability of the qualitative and quantitative native microbial composition, hygienic conditions, and manufacturing techniques of different farmhouses strongly affects the microbial profile of Serra cheese. The total microbial count (millions per gram) reported for ripened cheese was 31 to 460 (34), 169 ± 264 (2), and 17.9 ± 45.7 (13), whereas, for proteolytic bacteria, the count was lower than .8 (34) and $.3 \pm .8$ (2); for lipolytic bacteria, microbial count was 11 to 424 (34), 111 ± 450 (2), and $5.9 \pm$

22.6 (13). Aerobic and anaerobic spore-forming microorganisms and anaerobes that do not form spores were not detected (2, 13, 34).

Studies focusing on the bacterial and fungal flora of Serra cheese indicated the presence of cocci (isolated or grouped as diplococci and streptococci) (2, 13), rods (2, 13), yeasts (2, 13, 34), and molds (2, 13, 34). Chromogenic bacteria and *Lactobacillus* able to hydrolyze tributyrin were also observed (20). The lactic acid bacteria in Serra cheese have been classified as *Streptococcus lactis* (2, 13), *Leuconostoc* (20), *Lactobacillus helveticus* (2, 13), *Lactobacillus acidophilus* (2, 13), *Lactobacillus casei* (2, 13, 20), and *Lactobacillus plantarum* (20). Other bacteria were identified as *Enterococcus* (42), *Escherichia coli* (2, 13, 42), *Escherichia freundii* (2, 13), *Streptococcus faecalis* ssp. *liquefaciens* (2, 13), *Streptococcus durans* (2, 13), *Pseudomonas convexa* (2, 13), *Micrococcus epidermidis* (2, 13), *Micrococcus candidus* (2, 13), *Micrococcus cremoris* ssp. *viscosi* (2, 13), *Bacillus subtilis* (2, 13), and *Bacillus mesentericus* (2, 13). According to Hiscox et al. (20), the lactic acid fermentation (and the typical taste and aroma) of Serra cheese results from the action of a group of homofermentative bacteria, including lactic *Streptococcus*, *L. plantarum*, and *L. casei*, the dominant flora in cheese. Two families of yeasts with an action on milk were found (2, 13) in Serra cheese, namely, Rhodotorulaceae and Torulopsoideae. Molds identified (2, 13) include *Geotrichum* sp., *Penicillium* sp., and *Aspergillus* sp. From these, *Geotrichum candidum* and *Penicillium roqueforti* possess beneficial action on the manufacture of some types of cheese. The yeast count (millions per gram)

for ripened cheese was <220 (34) and 60 ± 246 (2), whereas the mold count was <143 (34), 75 ± 132 (2) and $.15 \pm .55$ (13).

A better understanding of the role of certain microorganisms in cheese ripening is possible by studying the strains present in the cardoon flower extract, whey, and smear or *reima* (the viscous material formed on the surface of cheese during the first days of ripening). Lactobacilli, proteolytic and lipolytic bacteria, and some enterococci were found in the crude extract of cardoon flowers (20). The dominant flora in whey were reported (34) to be mainly composed of *Strep. lactis* and some *Bacillus* sp., although the latter have been implicated with postprocessing contamination (13). The microbial flora in the smear is mainly composed of yeasts (spherical and ellipsoidal in shape) and, to a lesser extent, of cocci (proteolytic bacteria with different dimensions) and rods (13, 34). After the inoculation of sterilized milk with the smear, Santos (34) observed that the acid-driven coagulation of milk occurred after 24 h at 30°C. In the beginning, the culture was essentially composed of cocci, a few rods, and yeasts. After 20 d, the culture consisted only of yeasts. Suspecting that the environment may contribute to the microflora of cheese, Santos (34) analyzed the air in the maturation room and the cloth bands wrapped around the ripening cheeses. Those analyses indicated the presence of yeasts and coccus in the air of the maturation room, as well as rods and red-pigmented yeasts in the cloth bands.

The variation of the microbial profile with maturation time and location within the cheese was studied by Santos (34) and Cruz (13). Those studies indicated that cocci were located essentially in the central and middle zones of the cheese, whereas yeasts were essentially located in the peripheral zones of the cheese (13). Furthermore, although the dominant flora of fresh cheese were mainly cocci and, to a lesser extent, rods and yeasts, they were not uniformly distributed throughout cheese and ripening time (34): the numbers of cocci and rods tended to increase with time in both the bulk and the surface of the cheese, but the number of yeasts tended to increase on the surface of the cheese and to decrease inside.

Santos (34) studied the effect of addition of some cultures that were previously isolated from the cheese (e.g., lactic-type *Streptococ-*

cus, acid-proteolytic *Micrococcus*, and proteolytic yeasts) to sterilized whole cow milk and to cheese immediately after pressing (via surface rubbing). Upon inoculation, the dominant flora on the cheese surface were no longer composed solely of yeasts. Unfortunately, the effect of this addition on the organoleptic characteristics of the final cheese was not ascertained.

Given that Serra cheese is made from raw milk, under poor conditions of hygiene, and without starter culture and that it has a high initial pH and moisture content, microbiological safety is of critical concern. However, no relevant outbreaks of food poisoning by consumption of Serra cheese have been reported to official health and sanitation institutions, possibly because the cheese is consumed after typical ripening of ca. 45 d at temperatures around 10°C; most pathogenic microorganisms cannot survive this long subjected to the conditions prevailing in the bulk of the cheese (in the US, ripening of not less than 60 d at a temperature not less than 1.4°C is required for cheese made from raw cow milk before microbial safety can be ensured). Although microbiological studies have not focused on microbial safety, past experience indicates that the most common microorganisms with harmful properties after 45 d are coliforms, and many inhabitants of Portugal seem to have developed immunity against them because of the traditional lack of heat treatment (especially pasteurization) in several common foods. Rapidly changing habits and diets of the indigenous population and the interest in Serra cheese by foreign markets, however, indicate significant need to examine microbial safety issues. Furthermore, the coming of a single European market has created controversy regarding all cheeses made from raw milk. The extensive experience of the French indicates that some cheeses, as a food delicacy, require the use of raw milk. However, a general concern for public safety can jeopardize the future of raw milk cheeses.

To promote adequately safe products for the future, the following sequential actions will be essential: identification of the level and source of microbial pathogenic contamination of Serra cheese and the development of processing operations to prevent or to control such contaminations (e.g., physical processes, such as

microfiltration, or microbial processes, such as addition of high levels of starter). Furthermore, development of good starter cultures and focus on identification and selection work in the indigenous microflora will be necessary so that maximum quality can be obtained if, in the future, the use of raw milk becomes difficult because of legislative regulation; however, little work has been done in these areas.

BIOCHEMICAL STUDIES

The most important biochemical studies pertaining to Serra cheese have been conducted on two major topics: 1) the characterization of the vegetable rennet action and 2) the ripening of Serra cheese as related to protein breakdown and liberation of volatile fatty acids and free amino acids.

Coagulation

In the manufacture of many traditional Portuguese cheeses, including Serra cheese, the coagulation of milk caseins is effected by a crude extract from the dry cardoon flowers.

Cardoon (*cardo*), the common name of *C. cardunculus* L., is a composite culinary vegetable (related to the globe artichoke) with purple flowers (see Figure 3). The cardoon grows wild in large amounts in the dry, rocky, and uncultivated regions of southern Portugal (mainly Estremadura, Ribatejo, Alentejo, and Algarve), although it can also be cultivated. The cardoon flowers are picked and dried in the open air, in the shade, without any special treatment. When dry, they are stored in cloth or plastic bags in a dry place and sold at local markets. Because no standard conditions exist for the cutting and drying processes, the activity of the cardoon flower extract is extremely variable, depending on the variety, the stage of maturity, the part of the flower used, the drying time, and the final moisture content (3, 11).

Several studies (9, 11, 16) indicated that the maximum proteolytic activity is associated with the proteases in the stylet. The protease isolated from dry flowers exhibits at least four different peaks by ion-exchange chromatography, which is in contrast with the single peak obtained for the fresh flower counterpart, thus suggesting that the drying process is a vector

for increased enzyme heterogeneity (16). The crude extracts of cardoon from various southern regions of Portugal show different proteolytic and coagulant activities (11), which might be the result of variable chemical compositions, especially in moisture, sugar, and cellulose contents of different cultivars (3) (Table 3).

Purification and partial characterization of the milk-clotting proteases from the flowers of *C. cardunculus* L. (termed cynarins) has been performed by several authors, but their conclusions are not entirely consistent. Heimgartner et al. (19) isolated three cynarins (arbitrarily denoted as C1, C2, and C3), each formed by two subunits with different molecular mass (C1, $16.5 \pm .5$ and 32.5 ± 1.0 kDa; C2, $16.5 \pm .5$ and 32.5 ± 1.0 kDa; and C3, $13.5 \pm .5$ kDa and 35.5 ± 1.0 kDa, respectively). Cynarin C3 showed proteolytic and coagulant activities that were significantly greater than that of the other two proteases. Other studies (8) showed

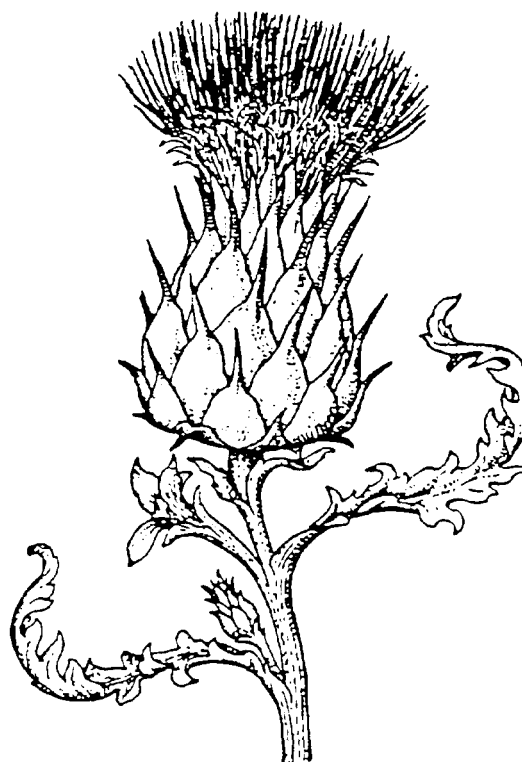


Figure 3. *Cynara cardunculus* L.

that the cynarin obtained by salting out with 50% saturated ammonium sulfate (composed of six subunits with molecular mass of 17.3, 24.1, 27.0, 32.0, 38.0, and 60.0 kDa) has the maximum ratio of coagulant activity to proteolytic activity (.43) compared with the other two cynarins (precipitated at 40 and 80% saturation in ammonium sulfate). The aforementioned ratio is similar to that for *Mucor miehei* protease. Barbosa (3) obtained five enzyme fractions, of which only one (with a molecular mass of 50 kDa) showed significant proteolytic and coagulant activity toward casein. More recent studies (16, 19) indicated that cynarin is a glycoprotein formed only by two glycosylated subunits with high concentrations of mannose (average total carbohydrate content of 11%) of different molecular mass, 31 and 16 kDa (or 27.5 and 14.4 kDa, respectively, in deglycosylated form) (16). The smaller subunit is not a proteolytic fragment, or a monomer, of the larger subunit (16). The coupling between the subunits is maintained through noncovalent interactions, which can only be broken under the influence of strong denaturing conditions (16).

The cynarins display their maximum proteolytic activity at acidic pH 5.1 (19), 5.7 (8), or 6.0 (16), at temperatures ranging from 37°C (8) to 50°C (16), and under ionic strengths equivalent to .1 to .6 M in NaCl (16). The maximum proteolytic activity at acidic pH suggests that the cynarins belong to the class of acidic proteases (16), specifically to the aspartic acid type (19). Studies conducted on the

specificity of the cynarins indicated that these proteases can act both as endopeptidases (16, 19) and exopeptidases (16). Cynarins prefer peptide bonds that contain apolar amino acids, i.e., of the form (Phe, Leu, Ile)-X where X is preferentially Val and Tyr (16). Although some authors (3, 8, 10, 12) reported that the protease or proteases from the cardoon flower have a broad specificity in relation to peptide bonds, more recent work (16, 31) showed that (as with other enzymes used for cheese making) the protease of *C. cardunculus* L. starts the coagulation process by cleaving the bond Phe₁₀₅-Met₁₀₆ of κ -casein, producing only para- κ -casein and Met₁₀₆-Val₁₆₉ TCA-soluble macropeptide. The cynarins undergo extensive denaturation at temperatures above 60°C and tolerate the presence of urea, Triton X-100, reducing agents, and some organic solvents at low concentrations, but they are inhibited by guanidine chloride and SDS and have a high potential to produce proteic fragments with biological activity (16).

Comparing the proteolytic activities of animal rennet, *M. miehei* rennet, and cardoon dry flower extract, all acting on κ -casein isolated from cow milk, Morgado (31) concluded that the cynarins and *M. miehei* rennet display identical activities, which are lower than the clotting activity of the proteases of animal rennet. However, in the presence of κ -casein isolated from ewe milk, the proteolytic activity of the proteases from cardoon and animal rennet are identical and higher than that of *M. miehei* (31). Similar studies (31) on the action of those enzymes on the α_s - and β -caseins isolated from cow milk and from ewe milk indicated that the three proteases differ in their specificity. In particular, the cynarins exhibit the overall highest activity for α_s - and β -caseins, and this effect is more relevant for the ewe milk caseins. The high proteolytic activity of the cynarins was also implied (38, 39) by the milky color that whey presents when drained, which can be a result of extensive protein breakdown. Morgado (31) suggested that the lower proteolytic action of the proteases isolated from animal rennet, cardoon extract, and *M. miehei* in whole milk, compared with that on pure caseins (especially α_s - and β -caseins), is due to the protection of the labile peptide bonds by the lipid fraction of milk.

TABLE 3. Chemical composition of the native cardoon flower and crude extract.¹

Compounds	Native	Extract
Fat and resins, %	27.2 ± 11.4	NA ²
Lignin, %	16.6 ± 10.1	NA
Sugar, %	16.2 ± 6.6	42.5
Protein, %	11.7 ± 1.9	26.0
Humidity, %	11.3 ± 11.5	5.0
Cellulose, %	13.3 ± 8.6	NA
Ash, %	5.0 ± .8	NA
Tannin, %	NA	4.5
Chloride, %	NA	9.5
pH	NA	5.8

¹Adapted from Barbosa (3).

²Not available.

The maximum clotting activity of cynarins is achieved at a temperature of $32 \pm 1^\circ\text{C}$ (39). This observation may explain why, when cardoon extract is used, the coagulation time is longer than when rennet is used (3). The clotting activity of the cardoon flower extract is affected by the milk pH, amount of crude extract added, and salt concentration more than are animal rennets (3, 39). Although the total time necessary to obtain curd from cardoon flower extract with the same firmness as curd from animal rennet is the same, the difference between the time at which coagulation starts and the time at which the curd is ready for cutting is higher for the cardoon flower extract than for animal rennet in the presence of cow milk. For ewe milk, the clotting activity of the cardoon flower extract is slightly higher than that of animal rennet, but the curds acquire a firmness that is about three times greater than that of the curds generated from cow milk (3, 39).

Several other works pertaining to the applications of cardoon extract in cheese making were reviewed by Roseiro (33). Special mention should be made of the studies of Barbosa et al. (6, 7), who tried to substitute cardoon extract for animal rennet in the manufacture of cheese made with cow milk, namely, Italian cheeses (Grana, Provolone, and Bel Paese) (6) and French cheeses (Camembert and Gruyère) (7). Major problems encountered included slightly more acid and bitter flavors (6, 7) and softer curds (with concomitantly higher tendencies for loss of shape) (6, 7, 29). Therefore, the cynarins are good substitutes for chymosin in cheese making using ewe milk, but, for cow

milk, chymosin is preferable because of its higher activity on κ -casein.

Large-scale biomass production of cardoon flower cells has been achieved using cell cultures (15, 18, 25). The suspended cells showed clotting and proteolytic activities similar to those of the native, wild-type. This fact, coupled with low doubling time, indicates the aptitude of cell cultures as potential sources for the commercial production of plant proteases for cheese manufacture (17, 18). The influence of the inoculum density and degree of aeration on the rate of biomass growth was also studied (25). The results obtained by Esquivel et al. (15) demonstrated the feasibility of using immobilized, proteolytically active plant cells in a fixed-bed reactor for the continuous enzymatic hydrolysis of κ -casein (the step that precedes the purely physical step of aggregation of the disrupted casein micelles).

Ripening

Experimental results on the form of nitrogen in Serra cheese are listed in Table 4. Santos (34) studied the extent and rate of protein breakdown as a function of time and location in the cheese. After determination of the soluble nitrogen in three arbitrarily defined zones (central, middle, and surface), Santos (34) concluded that 1) caseins are rapidly solubilized and 2) the degrees of protein breakdown at the center and at the surface of the same cheese are considerably different. The higher protein breakdown at the surface probably accounts for the differences in texture and color between the interior and rind of the cheese (34). As a consequence of his observa-

TABLE 4. Percentage of total nitrogen, water-soluble nitrogen, and amino acid nitrogen in cheese, and values of the corresponding maturation coefficients.

Total	Nitrogen		Maturation coefficient ¹	Reference
	Soluble	Amino acids		
————— (%) —————				
3.1 to 4.2	1.0 to 2.0	.1 to .3	32 to 56	(2)
2.8 to 4.0	1.1 to 1.7	.1 to .4	35 to 48	(30)
$3.6^2 \pm .7$	$1.2 \pm .4$	NA ³	32 ± 13	(35)

¹Defined as the percentage of soluble nitrogen relative to total nitrogen.

²Mean \pm 95% confidence interval.

³Not available.

tions, Santos (34) hypothesized for Serra cheese a centripetal maturation; i.e., changes in the cheese associated with ripening progress from the surface toward the center. Analyses of Serra and Cheddar cheeses of approximately the same age showed that the release of products resulting from protein hydrolysis (20) and of volatile fatty acids (20, 42) is similar.

The amount of volatile acids in the fat fraction of Serra cheese (ca. 16 mmol/g of cheese) was about one-half of that in Roquefort cheese (20), which may be a consequence of the very small number of lipolytic and proteolytic bacteria in Serra cheese compared with the number of lipolytic and proteolytic molds in Roquefort cheese (20). Vieira de Sá et al. (42) concluded that cheese quality is higher when amounts of volatile fatty acids, especially butyric and isovaleric acids, are higher. They (42) also concluded that bitter flavors (which typically arise in aged cheeses) are due to the presence of propionic acid.

In a study on the liberation of free amino acids (42), Phe, Leu, and Ile were the dominant free amino acids present in fresh cheese. During maturation, the total amount of the free amino acids increased, although the relative increase of each free amino acid was not established. However, Phe, Leu, and Ile continued to be the dominant free amino acids in ripened cheese. A clear relationship between the free amino acid profile and the quality of the cheese cannot be established from these results.

CONCLUSIONS

Although extensive field work and microbial and biochemical determinations have been done relating to Serra cheese, more *in vitro* fundamental studies and *in vivo* applied studies are necessary for causal relationships to be completely established.

For example, the proteolytic system of the vegetable rennet has been thoroughly characterized; however, the importance of its action on the extent of protein breakdown of the final cheese compared with the action of the proteolytic and lipolytic systems of the microorganisms found in cheese remains unknown. In soft, quickly ripened cheeses (such as Serra cheese), most of the proteolysis is brought about by extracellular proteases released by

various microorganisms that grow on the surface of the cheese (32). Furthermore, the role of the various microbial strains must be assessed following studies of ecological evolution in cheese during ripening. Such studies will eventually lead to the development of starter cultures for standardized manufacture of Serra cheese from raw (or pasteurized) milk. Only in this way can Serra cheese enter the demanding European Economic Community and American markets, which are characterized by increasingly strict hygienic and sanitary regulations.

It is hoped that, via the presentation of the available scientific and technical information on Serra cheese in a consistent fashion suitable for the international scientific community, Serra cheese can generate sufficient scientific interest to merit the further research efforts required for its complete, fundamental characterization, which will undoubtedly help in its eventual preservation as a food delicacy. Results of such studies may also help cheese makers to improve the quality of their products, which is an important asset for today's market.

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