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Impact of different *Cynara cardunculus* L. extracts on the physicochemical, microbial, and sensory properties of Serpa cheese

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ABSTRACT

This study aimed to evaluate the impact of different *Cynara cardunculus* L. extracts on the physicochemical, microbial, and sensory properties of PDO Serpa cheese, including moisture content, texture, nitrogen fractions, fatty acid profile, microbial characteristics, and overall acceptance. Cheese samples were produced in two dairies (C and G) with three cardoon coagulants (J, C, and G) and analyzed at the beginning of ripening (0 days) and after 30 days.

Initially, all samples showed similar moisture, pH, titratable acidity, and trichloroacetic acid-soluble nitrogen (TCA-SN). After ripening, moisture decreased (1.78%–2.82%), with higher levels in cheeses made with cardoon J. Water activity declined more significantly in dairy G's samples, especially with cardoon J. pH and acidity decreased without notable differences between cardoon types or dairies. Nitrogen fractions stabilized after 30 days, indicating microbial activity as the key driver of proteolysis. Fatty acid analysis revealed palmitic, myristic, oleic, and capric acids as predominant, with dairy G's samples showing higher monounsaturated fatty acids (MUFA) and lower short-chain fatty acids, like butyric acid.

Principal Component Analysis (PCA) highlighted ripening time as the main factor influencing cheese characteristics and, at the end of the ripening process, samples were separated by factory. Microbial analysis showed increased mesophilic and lactic acid bacteria during ripening, while fungi and Enterobacteriaceae counts remained stable. Sensory evaluation indicated higher ratings for dairy G's cheeses in flavour and acceptance, regardless of cardoon type.

This study demonstrates how cardoon type and dairy practices shape cheese quality.

1. Introduction

The production of traditional cheeses is of major importance in southern European countries, not only for economic reasons but also as part of a deep cultural heritage, relying mostly on an oral tradition passed down through generations. In fact, such traditional cheeses are bounded to a terroir, legacy and techniques that cannot be replicated elsewhere. Most of them benefit from geographical indication protection created by the European Commission in 1992, namely Protected Designation of Origin (PDO) and Protected Geographical Indication (PGI) (Council Regulation No. 2081/92). The PDO Serpa cheese is part of the eleven PDO cheeses recognized in Portugal, using exclusively raw sheep's milk coagulated with a *Cynara cardunculus* L. flower extract (eAmbrosia, n.d.) and produced inside a delimited area part of the Baixo Alentejo province (Decreto Regulamentar No. 39/87).

The sensory attributes of Serpa cheese include a thin, malleable crust

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that is slightly rough and light straw-yellow color. The cheese interior e has few or no holes, yellowish-white color and buttery consistency. The aroma and flavor are generally strong and sharp (Decreto Regulamentar No. 39/87).

Milk coagulation is the first chemical step in cheesemaking, significantly impacting the production process and sensorial profile of the cheese. In the industrial cheesemaking, the use of gastric proteinases as rennet (from calves, kids or lambs) and microbial milk-clotting enzymes produced by fungi and bacteria (Di Rosa et al., 2024) are the most common. However, some regions of southern Europe – particularly Portugal, Spain and Italy - maintain a strong tradition of using vegetable extracts, primarily *C. cardunculus* L., in the production of sheep cheeses (Conceição et al., 2018; Rampanti et al., 2023). This practice is documented in the classical Roman *Res Rustica* and references from the 16th and 18th centuries (Dias, 2022). The use of cardoon (*C. cardunculus* L.) as a coagulant is mandatory for certain Portuguese cheeses that hold the status of PDO status recognized by the European Union (EU) (Comission Regulation No. 1107/96).

C. cardunculus L. is an herbaceous perennial diploid plant, distributed throughout the Mediterranean basin, Madeira and Canary Islands. According to Gostin and Waisundara (2019), three sub-species can be identified: wild cardoon (*C. cardunculus* var. *sylvestris*), cultivated cardoon (*C. cardunculus* var. *altilis*) and globe artichoke (*C. cardunculus* var. *scolymus* L.). The wild cardoon grows spontaneously in marginal areas of the countryside or along paths (Conceição et al., 2018), and pistils of the flower are manually collected from ripe plants in June and July (Alavi & Momen, 2020).

The capacity of aqueous extracts from the pistils for cheesemaking results from the presence of aspartic proteases identified as cardosins A, B, E, F, G, and H, with A and B being the most representative. Cardosin A is primarily found in the upper parts of the pistils, while cardosin B is exclusively present in the lower part (Barracosa et al., 2021). The ratio between cardosins A and B varies among different populations of cardoons and is considered a cause of the heterogeneity within *C. cardunculus.* Like chymosin, cardosins A and B specifically cleave κ-casein at the Phe105-Met106 bond (Galán et al., 2008); However, it is now well established that chymosin possesses additional active cleavage sites beyond its primary specificity (Bijl et al., 2014). The lower ratio between the specific milk-clotting activity and the non-specific enzymatic proteolytic activity of the cardosins has been identified as the cause for the softer texture and greater bitterness in cheeses made with cardoon extracts. Nonetheless, this characteristic is also an identifying mark of PDO sheep cheeses produced in Spain and Portugal (Agboola et al., 2004). Different factors can affect the enzymatic activity of cardoon extracts and, consequently, the sensory properties of cheeses, including maceration, drying conditions, plant variety, ripening stage and moisture content (Gostin & Waisundara, 2019). The application of cardoon flower in large-scale cheese production faces limitations such as limited and seasonal supply, along with a lack of standardization (Barracosa et al., 2021). Regarding plant variability, Gomes et al. (2019) identified substantial diversity in the technological properties of cardoon flowers from the Alentejo region in Portugal, revealing clear distinctions between clusters of ecotypes based on these properties. These variations are crucial as they can significantly influence cheese manufacturing processes and directly impact the quality and attributes of the resulting cheeses. Recognizing these differences highlights the importance of carefully selecting suitable cardoon ecotypes to refine cheesemaking techniques and enhance the overall quality of cheese products (Gomes et al., 2019). The variety of rennets and other milk coagulants available on the market has significantly increased over the last three decades. This expansion has heightened the necessity for thorough analysis and comparison of the various commercially available products to ensure optimal milk coagulation properties, as these influence cheesemaking processes (Beux et al., 2017).

The main objective of this study was to evaluate the impact of different *Cynara cardunculus* L. extracts on the physicochemical, microbial, and sensory properties of PDO Serpa cheese, including moisture content, texture, protein content, nitrogen fractions, fatty acid profile, microbial characteristics, and overall acceptance, in order to understand how variations in cardoon coagulants influence cheese quality.

2. Materials and methods

2.1. Plant material

The flower samples used in this study were the pistils from three cardoon (*Cynara cardunculus* L.) populations located inside the Baixo Alentejo region of southern Portugal. During the planning phase, three *Cynara cardunculus* L. flower extracts were selected for use in the factories: C, G, and J. This selection was based on the following rationale: the study was conducted in an industrial setting, where cheese producers used their routine coagulants (C or G) to reflect standard production practices. To ensure comparability and introduce a standardized control, a third coagulant (J) was included. This approach enabled the study to be conducted on an industrial scale. The cardoon extract was prepared using the traditional method commonly employed in cheesemaking and described by Gomes et al. (2019).

2.2. Milk-clotting activity

The milk-clotting activity (MCA) of the extracts were evaluated following ISO 23058:2006 / IDF 199:2006 (2006) standard method. A standard low-heat, skim spray-dried milk powder (Actalia Cecalait, Poligny, France) was reconstituted to 11% with 0.5 g/L $CaCl_2$ solution (Actalia Cecalait) and a final pH of 6.5, serving as the substrate. For MCA calculations, a calf rennet reference standard powder, featuring a MCA of 987 IMCU/g (Calf Rennet Reference/Pouch BP Chr. Hansen, Hørsholm, Denmark) was used (Gomes et al., 2019; ISO 23058:2006 / IDF 199:2006, 2006). All assays were conducted in duplicate.

2.3. Monitoring of enzymatic coagulation

The enzymatic coagulation properties were assessed using an Optigraph equipment (Alliance, Frépillon, France), which calculates coagulation parameters in real-time, including coagulation time, firmness evolution, optimum curd cutting time, and organization speed. All tests were conducted over a period of 120 min, measuring the following parameters: R (clotting time, measured in seconds); 0K20 (micellar aggregation speed, determined as the time required to reach a standard clot firmness, measured in seconds) and A20, A40, AR and A2R (firmness measures after 20 or 40 min from the start of the trial or after two or three times R, respectively, measured in volts) (Gomes et al., 2019). All analyses were performed in duplicate.

2.4. Serpa cheeses producion

Cheese samples were obtained from two artisanal cheese factories (identified as dairy C and dairy G), using raw sheep milk coagulated with three types of cardoon extracts as shown in Fig. 1.

The production starts with the filtration of milk, usually using wool or cotton filters, followed by heating to 30–31 °C in a double wall tank (Alvarenga et al., 2021). Salting is made by placing NaCl over the filters in a proportion of 1.5 kg salt per 100 L. The coagulation is made through the incorporation of an aqueous extract of *Cynara cardunculus* L. dried flowers (0.3 g/L milk) at such temperature, maintaining this temperature for 45–60 min (Conceição et al., 2018). Afterward, the coagulum is cut into 25–30 mm pieces, after 15 min whey was drained off, and curd is distributed into cylindrical moulds 12 cm of diameter and 7 cm of height (Decreto Regulamentar No. 39/87). After 8 h, the fresh cheeses are placed in the first ripening room for 15 days, at temperature of 8–9 °C and 92–97% relative humidity. Then, cheeses are transferred to

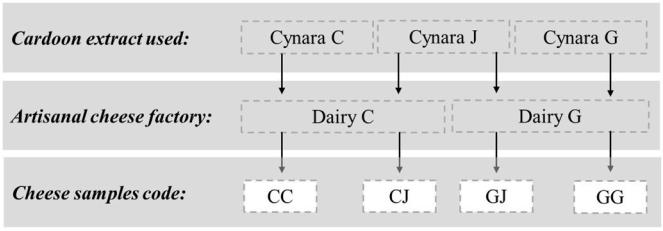

Fig. 1. Schematic presentation of the Serpa cheese samples obtained using different types of *Cynara cardunculus* L. extracts from two dairies (C and G). Each type of sample represents four independent samples: two production vats and two samples per vat (n = 4).

the second ripening room for an additional 15-day period, at a higher temperature of 10–13 °C and a lower relative humidity of 85–90%. During this period, cheeses are turned daily to ensure even ripening. During the transfer from the first to the second ripening room, cotton bandages are wrapped around the cheese to prevent deformation (Araújo-Rodrigues et al., 2020). Two samples from each factory were analyzed at the start (0 days) and after 30 days of ripening. Sensory analysis was only carried out on 30-day samples.

2.4.1. Physicochemical analysis

The moisture was performed according to ISO 5534:2004 / IDF 4:2004 (2004), titratable acidity was performed according to AOAC 920.124-1920 (1920) and pH was evaluated with a penetration electrode at 20 ± 1 °C (HI981032 Cheese pH Tester, Rhode Island, USA).

The water activity (a_w) was determined using a hygrometer at 20 °C ± 0.2 °C (HP-23-AW, Rotronic, Switzerland). Initially, the sample was ground and then placed inside the plastic box of the sensor. The sensor was placed in a chamber at 20 °C for 15 min, after which the value was recorded.

Protein content, water-soluble nitrogen (WSN), and trichloroacetic acid-soluble nitrogen (TCA-SN) were determined using ISO 17837 (2008) and ISO 27871:2011 / IDF 224:2011 (2011). Using the digestion block (Tecator, 2020 Digestor, Foss, Hillerød, Denmark) and steam distillation with colorimetric endpoint detection (Kjeltec 2300 Analyzer Unit, Foss, Denmark) (Monteiro et al., 2023). Samples were evaluated in duplicate and expressed as a mass percentage. WSN was quantified by performing an aqueous extraction of the N-components, followed by nitrogen determination by the micro-Kjeldahl method. TCA-SN was determined by the N-component precipitation with a 12% trichloroacetic acid solution and N determination on the filtrate (filter paper Whatman No. 42), using the micro-Kjeldahl method. The protein content is calculated using the factor 6.38.

2.4.2. Fatty acids

Cheese samples (4 g) were hydrolyzed with 50 mL of 4 N hydrochloric acid at 100 °C for 60 min. The mixture was filtrated through Whatman No. 4 filter paper rinsed with approximately 150 mL of distilled water. The dried fat hydrolysate was weighed and extracted using the Soxtec ST 255 (Foss, Hillerod, Denmark), with petroleum ether as solvent.

Fatty acids were converted into the corresponding fatty acid methyl esters (FAME) according to the ISO/IDF standard method (ISO 15884 / IDF 182, 2002). For this purpose, 100 mg of extracted fat was placed inside a sealed tube, and 5 mL hexane and 1 mL of a surrogate fatty acid (C17:0–10 mg/mL in methanol; a surrogate recovery of about 70% was obtained at the end of FAME synthesis) were added to dissolve it. The tube content was shaken vigorously until the fat was completely dissolved. Next, 0.2 mL of 2 M methanolic potassium hydroxide solution was added. The tube was vigorously mixed in the vortex mixer for 1 min and then left for 5 more minutes at room temperature (approximately 20 °C). After that time, 0.25 g of sodium hydrogen sulphate-monohydrate was added, and the mixture spun for 3 min (approximately 3000 rpm). The top layer of prepared methyl esters was taken for gas chromatographic (GC) analysis.

Chromatographic separation was performed using a 7820A Gas Chromatograph (Agilent Technologies, Münster, Germany) with a flame-ionization detector (FID) and a capillary column with a length of 30 m and an internal diameter of 0.25 mm. The stationary phase was HP-5 (Agilent Technologies, Middelburg, Netherlands) with a film thickness of 0.2 μm. The conditions of separation were as follows: carrier gas: helium; gas flow 1.5 mL/min, column temperature from 100 to 120 °C (1 °C/min), 120 to 220 °C (4 °C/min) and 220 to 270 °C (10 °C/min); the detector temperature 250 °C and injector temperature 200 °C. Sample injection volume was 1 μL (split: 10:1). FAMEs were identified according to their retention times, which were then compared with the retention time of standard FAME mixtures (Supelco 37 FAME Mix, Sigma-Aldrich, Missouri, USA). Amounts of fatty acids were expressed as grams of fatty acids per 100 g of lipids (g FA/100 g lipids).

2.4.3. Color and texture properties

The setup for the acquisition of digital images and post-processing was performed according to Dias et al. (2021). The conversion from RGB to CIELab color system was made using an algorithm based on Minz and Saini (2021). The degree of color difference (ΔE*) of rind and interior of fresh cheese and ripened cheese was calculated using Equation (1) (Minz & Saini, 2021).

$$\Delta E^{*} = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}} \quad \text{(Equation 1)}$$

A TA.XT Plus100 texture analyzer (Stable Micro Systems, Godalming, UK) was used for the instrumental texture analyses at 20 ± 1 °C, adapted from Alvarenga et al. (2021). Tests were conducted with a 100 N load cell equipped with a 10 mm Ø aluminum cylindrical probe, penetration depth of 10 mm, and crosshead speed of 0.2 mm/s. The dimensions of the tested cheeses was 3 cm in height and 16–17 cm in diameter. A 0.5 cm layer was cut off from the upper surface to expose the inside for texture determinations. Five replicates per cheese sample were performed, evenly distributed across the surface. Finally, the force vs time texture graphs were used for calculating parameters, namely: (i) hardness (N), maximum force; and (ii) adhesiveness (−N.mm), the negative surface of the graph.

2.4.4. Microbiological analysis

From each sample unit under analysis, 10 g fractions, representative of the cheese core, were collected and prepared for analysis, under sterile conditions. Each fraction was homogenized for 120 s, in 90 mL of a sterile sodium citrate solution (2% w/v), in a laboratory blender 400 (Seward Lab, London, UK). The initial suspensions were then decimally diluted with Ringer solution and duplicate samples of each appropriate dilution were plated for total or selective colony counting. Microbiological analyses were performed in triplicate, and the media and supplements used were Biokar Diagnostics (Pantin, France).

Total aerobic mesophilic bacteria were counted on Plate Count Agar (PCA) medium incubated at 30 °C for 72 h, and total mesophilic lactic acid bacteria (LAB) on de Man, Rogosa and Sharpe (MRS) agar acidified to pH 5.6, after 72 h at 30 °C; Fungi counts were performed in Rose Bengal Chloramphenicol Agar (RBC), at 25 °C for 72 h; *Enterobacteriaceae* were estimated in Violet Red Bile Glucose Agar (VRBG) after 24 h at 37 °C, with only pink colonies surrounded by a halo of purple precipitate, confirmed as Gram-negative and oxidase negative, being counted.

2.4.5. Sensory analysis

The sensory analysis was conducted following the methodology described by Alvarenga et al. (2018), with modifications to suit the study's specific requirements. A hedonic panel consisting of 16 trained tasters (9 females and 7 males), aged between 25 and 60 years, was assembled from employees at the National Institute for Agricultural and

Veterinary Research (INIAV). The panel employed a five-point hedonic scale, ranging from "1 - Dislike Extremely" to "5 - Like Extremely," to assess key sensory parameters such as color, texture, odor, flavor, and overall acceptance.

The evaluations were carried out in a sensory analysis room constructed in compliance with the standards outlined in ISO 8589:2007/Amd 1:2014 (2014). Each taster was seated in an individual booth under uniform white lighting, at room temperature to ensure consistency across evaluations. Samples were presented to the panelists in Petri dishes, each labeled with a randomly assigned three-digit code to prevent any bias. This analysis was only performed on 30-day samples.

2.5. Statistical analysis

The results obtained were analyzed using the STATISTICA software (StatSoft Inc., Tulsa, OK, USA). Analysis of variance (ANOVA) was conducted to compare means, employing the Scheffé test to ensure a 95% confidence interval ($p < 0.05$). The data analysis included calculations of the mean, standard deviation, and variance. Additionally, a Principal Component Analysis (PCA) was performed to evaluate differences between samples by considering multiple attributes simultaneously (Alvarenga et al., 2011).

3. Results and discussion

3.1. Evaluation of the enzymatic coagulation

Milk-clotting activity of the three cardoons used in the experiments is reported in Table 1.

Cynara G was the fastest and exhibited higher MCA, followed by Cynara J and Cynara C. However, these results are lower than those obtained by Gomes et al. (2019), who evaluated ecotypes from the Alentejo region in Portugal where MCA values ranging from 57 to 128 IUMC/g were reported, demonstrating significant variability in flower properties within the same region.

Regarding the coagulation properties, the coagulation time (R) followed a pattern similar to the MCA results, with Cynara G exhibiting the lower clotting-time (R) value. This finding is consistent with Barracosa et al. (2021), who reported that the cardoon with the highest coagulating activity also demonstrated the lowest R value. Cynara C, which showed a higher 0K20 (indicating lower micellar aggregation rates), produced a firmer curd, as evidenced by higher AR and A2R values. Cynara G also achieved high 0K20 values but showed increased curd firmness at higher A40 values. This discrepancy may be attributed to distinct coagulant action profiles and non-specific proteolytic activity influencing the progression of coagulation firmness (Barracosa et al., 2021). For curd firmness at 20 min (A_{20}), values for the three cardoon flower samples were zero since the clotting-time (R) was around that period, resulting in no curd formation at that.

Table 1
Means and standard deviation (within brackets) of MCA and coagulation properties of the three types of *Cynara cardunculus* L. coagulants (J, C, and G).

	Cynara J	Cynara C	Cynara G
MCA (IMCU/g^{-1})	38(8)	34(1)	47(1)
R (s)	1523(52)	1629(59)	1277(78)
AR (V)	2.66(0.15)	2.68(0.57)	2.25(0.07)
A2R (V)	3.91(0.22)	3.96(0.82)	3.28(0.06)
A40 (V)	1.88(0.14)	1.60(0.49)	2.05(0.11)
0K20 (s)	1363(132)	1534(566)	1520(35)

MCA - milk clotting activity; IMCU - International Milk Clotting Unit; R - coagulation time; A40 - curd firmness at 40 min.after coagulant addition; AR and A2R - curd firmness after two and three times the coagulation time (R) and after coagulant addition, respectively; 0K20 - speed of micellar aggregation, measured as time to reach a standard clot firmness; s – seconds; V – volts.

However, the results from evaluating technological properties and MCA did not consistently translate into cheese characteristics. This discrepancy likely reflects the impact of milk composition and variations in cheesemaking processes. Differences in physicochemical compositions, as well as variations in temperature and handling during cheesemaking, can significantly influence the final product, potentially overshadowing the inherent properties of the coagulants used.

3.2. Physicochemical properties of serpa cheeses

The average values of the physicochemical parameters, including pH, titratable acidity, moisture, a_w, protein content and nitrogen fractions, for the samples are presented in Table 2.

The pH parameter demonstrated an acidification of curd throughout ripening process, with an average decrease of 0.8 units. This acidification, observed after 30 days of ripening, is further confirmed by an average increase of 3.29 ml NaOH 0.1N/100 g in titratable acidity. The acidification process is an inherent aspect of the ripening process in this type of cheeses and is widely reported in the literature (Araújo-Rodrigues et al., 2023). No significant differences were observed in these two parameters between the different cheese dairies or among the types of cardoon used.

The reduction in water content observed during cheese ripening is supported by the analysis of water-related parameters in the samples. At the onset of the study (day 0), the average moisture content across all cheese samples was 55.98%, with no statistically significant differences between samples, indicating uniformity in initial moisture levels.

After 30 days of ripening, the data revealed that cheeses made using cardoon J exhibited a higher moisture content compared to those produced with other cardoons. This is particularly evident in the samples from dairy C, which showed a moisture content decrease of only 2.82%, whereas dairy G reported a reduction of 1.78%. These moisture content values align with those found in existing literature for this type of cheese (Gonçalves Dos Santos et al., 2017).

The CC samples, both at day 0 and after 30 days, did not show significant differences in a_w, indicating stable water content retention throughout the curing period. In contrast, the CJ sample exhibits a slight reduction in a_w of 0.03, highlighting a minor but measurable decline.

Remarkably, samples from cheese factory G experienced the most substantial decrease in a_w after the 30-day ripening period. Specifically, the sample produced with cardoon J showed a reduction in a_w by 0.13, whereas the sample made with cardoon G exhibited a decrease of 0.10. According to Araújo-Rodrigues et al. (2023), cheeses that achieve optimal quality and receive high acceptability from tasters or consumers typically present an a_w between 0.90 and 0.92. Among the analyzed samples, three exhibited a_w values below this ideal range (CJ, GJ, and GG), with the CC sample remained within the average parameters referenced for Serpa cheese. These findings underscore the importance of a_w and moisture content as critical determinants of the cheese maturation process. They influence textural qualities, flavor development, and microbial dynamics. As ripening progresses, both parameters play a significant role in shaping the final product's quality, highlighting their relevance in cheese production and evaluation.

Proteolysis plays a crucial role in the characteristics of this type of cheese influencing the final product's quality and flavor profile (Fox et al., 2017). At the beginning of the ripening period, significant differences in protein content, were verified. The cheeses from dairy C had an average protein content of 17.98%, and this was highest in cheesemaking with cardoon J. On the other hand, in dairy G, lower protein content was obtained in cheesemaking with cardoon J when compared to cheesemaking with cardoon G, presenting a difference of 0.10%. Such differences are expected due to different raw materials and processing conditions, namely coagulation temperature, dosage of coagulant, ecotype of cardoon (Conceição et al., 2018), and ways of handling the cheese curd (Roseiro et al., 2003). On the 30th day of ripening the protein content differences decreased: CJ presented the highest nitrogen

Table 2
Means and standard deviation (within brackets) of the physicochemical parameters analyzed, including protein content and nitrogen fractions (water-soluble nitrogen and trichloroacetic-soluble nitrogen), of the cheese samples at 0 days and 30 days, produced in the two dairies (C and G) using three types of *Cynara cardunculus* L. coagulants (J, C, and G).

	0 days				30 days			
	Dairy C		Dairy G		Dairy C		Dairy G	
	Cynara J	Cynara C	Cynara J	Cynara G	Cynara J	Cynara C	Cynara J	Cynara G
pH	6.33^{c} (0.11)	6.25^{c} (0.06)	6.01^{bc} (0.30)	6.24^{c} (0.14)	5.26^{a} (0.24)	5.38^{a} (0.06)	5.53^{ab} (0.14)	5.40^{a} (0.27)
Titratable acidity (mL NaOH 0.1N/ 100g)	1.55^{a} (0.10)	1.35^{a} (0.10)	1.55^{a} (0.62)	1.65^{a} (0.25)	5.33^{b} (0.59)	5.10^{b} (0.43)	4.33^{b} (0.32)	4.51^{b} (0.81)
Moisture % (m/m)	56.20^{a} (0.49)	57.07^{a} (0.36)	55.42^{a} (0.65)	55.24^{a} (0.72)	44.70^{bc} (3.52)	41.88^{b} (1.31)	46.54^{c} (1.50)	44.76^{bc} (0.7)
a_W	0.91^{abc} (0.02)	0.95^{ab} (0.01)	0.96^{a} (0.00)	0.95^{ab} (0.01)	0.88^{bcd} (0.04)	0.94^{ab} (0.00)	0.83^{d} (0.04)	0.85^{cd} (0.05)
Protein Content %(m/m)	18.18^{ab} (0.68)	17.78^{a} (0.55)	19.23^{abc} (0.26)	20.47^{c} (0.35)	20.86^{c} (0.35)	20.27^{bc} (0.49)	19.61^{abc} (0.62)	20.66^{c} (1.76)
TCA-SN/TN %(m/m)	0.48^{a} (0.05)	0.50^{a} (0.05)	0.34^{a} (0.01)	0.34^{a} (0.01)	1.37^{b} (0.05)	1.47^{b} (0.06)	1.30^{b} (0.05)	1.46^{b} (0.33)
WSN/TN %(m/m)	25.18^{c} (5.46)	21.92^{bc} (2.26)	10.38^{a} (2.02)	13.40^{ab} (3.09)	34.35^{d} (3.73)	36.31^{d} (1.40)	39.75^{d} (0.99)	38.55^{d} (3.61)

Means in the same row and same ripening days marked with different letters are significantly different ($p < 0.05$, $n = 4$, Scheffé test); a_W: water activity; TCA-SN/TN: ratio of the soluble nitrogen content in trichloroacetic acid to total nitrogen; WSN/TN: ratio of water soluble nitrogen to total nitrogen.

content, 20.86% and GJ samples had the lowest protein content, with a 1.5% difference despite using the same cardoon type. These results fall within the ranges reported in existing literature, confirming their validity (Alvarenga et al., 2008; Araújo-Rodrigues et al., 2022).

The ratio of water soluble nitrogen to total nitrogen (WSN/TN) content also displayed significant variation at the initial stage (0 days). This variation does not appear to be dependent on the type of cardoon used, as the samples with the highest (25,2 % WSN/TN) and lowest (10.4 % WSN/TN) content were both produced using cardoon J. However, at the end of the ripening process, the WSN/TN ratio becomes more similar between samples, ranging from 34.4% to 39.8%. The differing ripening dynamics of raw sheep cheeses across cheese factories have been previously reported in the literature (Alvarenga et al., 2008). By day 30, this parameter showed no significant differences, indicating a leveling effect over time. The ratio of the soluble nitrogen content in trichloroacetic acid to total nitrogen (TCA-SN/TN) content showed no significant differences between samples at 30 days, with average values of 37.24%. This consistency suggests that, regardless of the initial cardoon type, the TCA-SN/TN content stabilizes over the ripening period.

The similarity in the nitrogen fractions, WSN and TCA-SN, across all samples at 30 days, regardless of the cardoon type, suggests that the type of cardoon does not significantly impact these parameters. Instead, microbial activity likely plays a more substantial role in the proteolysis process. This observation supports the findings of Araújo-Rodrigues et al. (2023) who suggested that microbial dynamics are pivotal in cheese maturation, emphasizing the microbiological processes over the difference in coagulant origin.

3.3. Fatty acids profile of Serpa cheeses

Table 3 shows mean values and standard deviations for individual FA concentrations in the sample cheeses.

The most abundant FAs were palmitic (C16:0), myristic (C14:0), oleic (C18:1) and capric (C10.0) acids, which together accounted for roughly 75% of total FAs. These results are aligned with those found in

Table 3
Means and standard deviation (within brackets) of the fatty acids identified in the cheese samples at 0 days and 30 days, produced in in two dairies (C and G) using three types of *Cynara cardunculus* L. coagulants (J, C, and G).

	0 days				30 days			
	Dairy C		Dairy G		Dairy C		Dairy G	
	Cynara J	Cynara C	Cynara J	Cynara G	Cynara J	Cynara C	Cynara J	Cynara G
C4:0	3.36(0.66)^{bc}	3.85(0.71)^{c}	3.29(0.44)^{bc}	3.27(0.75)^{bc}	1.76(0.58)^{ab}	1.36(0.42)^{a}	1.13(0.31)^{a}	1.11(0.39)^{a}
C6:0	4.54(0.96)^{a}	3.48(0.54)^{a}	3.70(0.33)^{a}	3.57(0.35)^{a}	3.58(0.33)^{a}	3.50(0.38) ^{a}	4.04(0.24)^{a}	3.57(0.19)^{a}
C8:0	4.34(0.88)^{a}	3.24(0.49)^{a}	3.37(0.26)^{a}	3.18(0.3)^{a}	3.37(0.29)^{a}	3.32(0.34)^{a}	3.71(0.3)^{a}	3.25(0.13)^{a}
C10:0	9.28(0.84)^{a}	8.88(1.75)^{a}	8.97(0.52)^{a}	8.36(0.90)^{a}	9.47(0.89)^{a}	9.34(0.95)^{a}	9.34(0.22)^{a}	8.82(0.28)^{a}
C12:0	6.26(0.38)^{ab}	7.72(1.52)^{b}	5.30(0.47)^{a}	4.94(0.48)^{a}	6.08(0.79)^{ab}	6.28(0.61)^{ab}	5.41(0.40)^{a}	5.35(0.07)^{a}
C14:0	10.56(2.76)^{a}	11.62(1.69)^{a}	12.13(0.61)^{a}	11.03(1.29)^{a}	13.06(1.39)^{a}	13.14(1.32)^{a}	12.77(0.22)^{a}	12.21(0.19)^{a}
C14:1	0.08(0.01)^{a}	0.08(0.02)^{a}	0.06(0.01)^{a}	0.06(0.01)^{a}	0.06(0.01)^{a}	0.06(0.01)^{a}	0.06(0.01)^{a}	0.05(0.01)^{a}
C15:0	0.13(0.03)^{a}	0.11(0.03)^{a}	0.12(0.03)^{a}	0.10(0.01)^{a}	0.13(0.03)^{a}	0.12(0.02)^{a}	0.14(0.01)^{a}	0.13(0.03)^{a}
C16:0	24.71(0.97)^{a}	23.82(3.18)^{a}	26.88(1.56)^{a}	24.60(2.78)^{a}	25.44(2.48)^{a}	25.53(2.55)^{a}	27.52(0.86)^{a}	26.89(0.43)^{a}
C16:1n-7 cis 9	0.13(0.03)^{a}	0.11(0.01)^{a}	0.11(0.01)^{a}	0.10(0.01)^{a}	0.11(0.01)^{a}	0.12(0.02)^{a}	0.12(0.03)^{a}	0.10(0.00)^{a}
C18:0	1.77(0.36)^{a}	1.60(0.17)^{a}	1.66(0.14)^{a}	1.51(0.15)^{a}	1.61(0.15)^{a}	1.63(0.15)^{a}	2.06(0.36)^{a}	1.65(0.02)^{a}
C18:1n-9 trans 9	0.12(0.02)^{a}	0.10(0.03)^{a}	0.18(0.01)^{abc}	0.19(0.03)^{abc}	0.12(0.02)^{ab}	0.16(0.03)^{a}	0.27(0.07)^{c}	0.22(0.03)^{bc}
C18:1n-9 cis 9	8.8(1.61)^{a}	10.62(1.39)^{ab}	11.42(0.74)^{ab}	11.37(1.48)^{ab}	10.44(0.94)^{ab}	10.43(1.11)^{ab}	12.39(0.67)^{b}	11.4(0.2)^{ab}
C18:2n-6 cis 9,12	2.97(0.15)^{a}	3.11(0.25)^{a}	2.75(0.62)^{a}	2.23(0.19)^{a}	2.91(0.2)^{a}	2.90(0.41)^{a}	2.58(0.32)^{a}	2.24(0.08)^{a}
C20:0	(*)	(*)	(*)	(*)	0.12(0.02)^{a}	0.14(0.01)^{a}	0.14(0.02)^{a}	0.12(0.01)^{a}
SFA	64.96(3.49)^{a}	64.32(5.87)^{a}	65.42(4.33)^{a}	60.55(6.08)^{a}	64.61(5.96)^{a}	64.36(6.52)^{a}	66.27(1.34)^{a}	63.10(1.65)^{a}
MUFA	9.12(1.59)^{a}	10.90(1.42)^{ab}	11.77(0.77)^{ab}	11.72(1.53)^{ab}	10.73(0.97)^{ab}	10.77(1.14)^{ab}	12.84(0.73)^{b}	11.77(0.19)^{ab}
PUFA	2.97(0.15)^{a}	3.11(0.25)^{a}	2.75(0.62)^{a}	2.23(0.19)^{a}	2.91(0.20)^{a}	2.90(0.41)^{a}	2.58(0.32)^{a}	2.24(0.08)^{a}

Means in the same row and same ripening days marked with different letters are significantly different ($p < 0.05$, $n = 4$, Scheffé test); SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids; (*): Not detected - below the detection limit of 0.0004 mg/mL and the quantification limit of 0.001 mg/mL.

other sheep milk cheeses (Estrada et al., 2019).

Generally, the FA composition did not change significantly between samples regarding to polyunsaturated fatty acids (PUFAs) and saturated fatty acids (SFAs). The cheeses displayed low content of PUFAs (2.23–3.11 g/100 g lipids) and a high content of SFAs (60.55–66.27 g/100 g lipids). A moderate content of monounsaturated fatty acids (MUFA, 9.12–12.84 g/100 g lipids) was observed, with significant differences noted for JG samples after 30 days of ripening, where MUFA concentrations increased compared to other cheese samples. This increase is attributed to higher levels of oleic acid in the cheeses. The rise in oleic acid content may result from the inherent differences in fat composition among cheeses made with different cardoon types. Consequently, each type of cardoon contributes unique microbial and biochemical factors that influence the ripening process, leading to significant alterations in the fatty acid profile of the final cheese product. Conversely, a decrease in short-chain butyric acid was observed after 30 days of ripening, likely due to microbial degradation and enzymatic activity that facilitate the transformation of butyric acid into other flavor compounds during the maturation process.

Although the total FA composition remained relatively stable, it is the free fatty acids that are crucial for flavor development during ripening. Variability in the FA content may arise from differences in milk composition and microbial activity, influences by factors such as sheep breed, stage of lactation, season, and feeding practices. Numerous studies on cheeses produced at different dairy plants corroborate this variability (Boutoial et al., 2013).

FAs are categorized into short-chain fatty acids (C4:0 - C8:0, SCFAs), medium-chain fatty acids (C10:0 - C14:0, MCFAs), and long-chain fatty acids (C16:0 - C18:3, LCFAs) (Ibrahim et al., 2023). LCFAs have a higher perception threshold and contribute minimally to cheese flavor, while MCFAs did not show significant variation between cardoon types, dairies, or ripening periods. It is important to note that while MCFAs are present, their contribution to flavor is limited due to their relatively low volatility when they exist primarily in triglycerides.

However, the proportion of SCFAs, particularly butyric acid, decreased after 30 days of ripening, with no significant differences observed between cardoon types or dairies. SCFAs are generally considered significant for developing characteristic cheese flavors (Collins et al., 2003). According to Collins et al. (2003), in triacylglyceride molecules, SCFAs are predominantly located in the sn-3 position, and the lipases involved in cheese ripening are specific to this position, leading to the primary hydrolysis of SCFAs. It is well-established in the literature that free fatty acids released during cheese ripening can undergo further transformations into methyl ketones, lactones, secondary alcohols, and other volatile compounds that contribute to cheese flavor (Collins et al., 2003). Thus, the observed decrease in butyric acid after 30 days could be attributed to these transformations.

3.4. Color and texture properties of Serpa cheeses

The results of the digital images converted to CIE L*a*b* colorimetric system are presented in Table 4.

At the beginning of the ripening process, both rind and interior exhibited similar color parameters: L* values ranged from 88.4 to 93.9, a* value from −4.2 to −2.8, and b* values from 7.2 to 10.6, indicating a light grey-ivory color. After 30 days of ripening, the rinds's color shifted to lower L* values (76.5–79.9) and higher b* values (44.2–46.7), reflecting a yellowish hue due to the drying process, which primarily affected the rind, as noted in previous studies (Buffa et al., 2001; Dias, Lage, Alvarenga, et al., 2021; Juric et al., 2003). Consequently, the color difference (ΔE*) was more pronounced in the rind (38.31–39.74) compared to the interior (5.11–11.81). No significant change was observed in the a* parameter, which remained between −4.8 and −3.4.

Initial hardness values ranged from 2.94 to 4.33 N, decreasing from 1.34 to 2.25 N after ripening period, primarily due to proteolysis (Sousa et al., 2001) and lipolysis mechanisms (Araújo-Rodrigues et al., 2020). These changes are linked to biochemical reactions affecting fatty acids and proteins, as observed in the significant variations in fatty acid composition, particularly the decrease in short-chain fatty acids like butyric acid. These alterations contribute to the development of aroma and flavor in the cheese (Araújo-Rodrigues et al., 2022). No significant differences in rheological parameters were observed based on the type of cardoon or dairy technology used.

3.5. Microbiology evaluation of Serpa cheese

Counts of the main microbial groups in cheeses manufactured in two dairies (C and G), with different cardoon extracts (J, C and G), at 0 and 30 days of ripening, are shown in Table 5.

As reported, total mesophilic bacteria were the dominant microbial group both at the beginning of process (0 days) and after 30 days of ripening. LAB emerged as the second prevalent group, constituting the majority within the mesophile group. In fact, total LAB counts in MRS are very close to the total mesophilic bacteria counts, with both groups initially recording values around 7.00 log CFU/g. These counts increased to approximately 9.00 log cfu/g after 30 days of repining. This progression mirrors the trends observed in other raw ewes' milk cheeses without starter cultures (Alvarenga et al., 2021; Dias, Lage, Alvarenga, et al., 2021; Freitas & Xavier Malcata, 2000; Gonçalves et al., 2018)

This evolution translates into significant differences in mesophilic counts between the two controlled ripening times (0 and 30 days). Conversely, for each of these times points, no significant differences were observed between total mesophilic bacteria or LAB counts in cheeses from different dairies (C or G), or made with different types of cardoon (J, C or G). This suggests that the manufacturing process or the type of cardoon used does not impact on these total counts. However,

Table 4
Means and standard deviation (within brackets) of the color and texture properties analyzed of the cheese samples at 0 days and 30 days, produced in the in two dairies (C and G) using three types of *Cynara cardunculus* L. coagulants (J, C, and G).

	0 days				30 days			
	Dairy C		Dairy G		Dairy C		Dairy G	
	Cynara J	Cynara C	Cynara J	Cynara G	Cynara J	Cynara C	Cynara J	Cynara G
Hardness (N)	3.20^{bc} (0.29)	2.94^{bc} (0.23)	3.76^{c} (0.63)	4.33^{c} (0.19)	2.25^{ab} (0.95)	1.34^{a} (0.20)	1.80^{ab} (0.42)	1.46^{a} (0.40)
Adhesiveness (-N.mm)	3.15^{a} (0.46)	2.80^{a} (0.59)	4.41^{a} (0.66)	5.62^{ab} (0.46)	8.74^{b} (2.95)	5.79^{ab} (0.73)	3.84^{a} (0.38)	3.36^{a} (0.45)
L (rind)	93.1^{b} (0.2)	93.9^{b} (0.1)	91.5^{b} (0.4)	90.4^{b} (0.1)	78.7^{a} (4.5)	76.5^{a} (0.9)	79.7^{a} (0.7)	79.9^{a} (0.9)
a (rind)*	-3.1^{ab} (0.1)	-2.8^{b} (0.0)	-3.3^{ab} (0.2)	-3.5^{ab} (0.2)	-3.8^{ab} (1.4)	-3.4^{ab} (0.5)	-4.4^{ab} (0.3)	-4.8^{b} (1.0)
b (rind)*	8.8^{a} (0.3)	7.9^{a} (0.1)	8.1^{a} (1.1)	9.0^{a} (0.4)	44.2^{b} (5.6)	44.9^{b} (4.3)	46.0^{b} (1.0)	46.7^{b} (3.1)
ΔE* (rind)	–	–	–	–	38.31 (6.10)	39.50 (2.32)	39.74 (2.29)	39.74 (2.62)
L (paste)	92.9^{c} (0.1)	92.5^{bc} (0.4)	88.4^{ab} (0.2)	88.5^{ab} (0.2)	88.8^{abc} (3.9)	87.9^{a} (0.8)	90.6^{abc} (1.1)	90.6^{abc} (0.7)
a (paste)*	-2.8^{a} (0.1)	-3.0^{bc} (0.1)	-4.0^{ab} (0.3)	-4.2^{ab} (0.1)	-5.0^{ab} (1.7)	-5.1^{ab} (0.9)	-4.8^{ab} (0.8)	-4.7^{ab} (0.4)
b (paste)*	7.2^{a} (0.2)	7.4^{a} (0.8)	9.9^{ab} (1.3)	10.6^{ab} (0.1)	17.5^{b} (6.7)	18.0^{b} (3.3)	15.2^{ab} (2.6)	15.0^{ab} (1.2)
ΔE* (paste)	–	–	–	–	11.81 (6.12)	11.81 (3.71)	6.24 (2.13)	5.11 (0.97)

Means in the same row and same ripening days marked with different letters are significantly different ($p < 0.05$, $n = 4$, Scheffé test).

Table 5
Means and standard deviation (within brackets) of the log counts of different microbial groups analyzed in the cheese samples at 0 days and 30 days, produced in the in two dairies (C and G) using three types of *Cynara cardunculus* L. coagulants (J, C, and G).

Microbial groups	0 days				30 days			
	Dairy C		Dairy G		Dairy C		Dairy G	
	Cynara J	Cynara C	Cynara J	Cynara G	Cynara J	Cynara C	Cynara J	Cynara G
Total mesophilic bacteria (log CFU/g)	6.99^{a} (0.32)	7.41^{a} (0.19)	7.50^{a} (0.24)	7.53^{a} (0.13)	9.25^{b} (0.40)	9.29^{b} (0.06)	9.03^{b} (0.27)	9.09^{b} (0.04)
Total mesophilic LAB (log CFU/g)	6.82^{a} (0.31)	7.05^{a} (0.24)	7.23^{a} (0.24)	7.33^{a} (0.18)	9.13^{b} (0.34)	9.22^{b} (0.08)	8.78^{b} (0.31)	8.98^{b} (0.06)
***Enterobacteriaceae* (log CFU/g)**	5.85^{ab} (0.60)	6.17^{abc} (0.19)	6.91^{a} (0.39)	6.85^{bc} (0.13)	5.73^{a} (0.39)	6.51^{abc} (0.41)	6.51^{abc} (0.41)	6.77^{abc} (0.23)
Yeasts (log CFU/g)	3.60^{a} (0.29)	3.50^{a} (0.42)	2.67^{a} (0.47)	3.43^{a} (0.36)	3.06^{a} (0.88)	2.55^{a} (0.64)	6.67^{a} (0.23)	2.08^{a} (1.41)

Means in the same row and same ripening days marked with different letters are significantly different ($p < 0.05$, $n = 4$, Scheffé test); LAB: Lactic Acid Bacteria.

this generalized assessment does not account for potential influences on the quantitative and qualitative evolution of specific strains, particularly among LAB.

The *Enterobacteriaceae* family ranks next in terms of representation among the cheese microflora, with counts remaining consistently close throughout ripening, regardless of producer or cardoon type. These counts are generally average around 6.00 log cfu/g, which, although high, aligns with those observed in other raw milk cheeses (Dias, Lage, Alvarenga, et al., 2021; Gonçalves et al., 2018; Tabla et al., 2016; Tavaria & Malcata, 1998).

The microbial group of fungi, predominantly consisting of yeasts, shows the lowest counts (close to 3.00 log cfu/g) and no significant differences between the various conditions studied. A slight decrease of approximately one logarithmic unit is noted by the end of ripening, consistent with findings from other studies (Alvarenga et al., 2021). The counts of Gram-negative bacteria from the *Enterobacteriaceae* family and fungi appear unaffected by the manufacturing process or the type of cardoon. In particular, for yeast, it would be valuable to use next-generation molecular methods to differentiate the strains present and assess their potential impact at this level.

3.6. Sensory evaluation of Serpa cheeses

The sensory analysis data were statistically processed and summarized in Fig. 2.

Among the parameters evaluated, color showed the most consistent ratings across all four samples, with no significant differences detected between the cheese manufactories or the types of cardoon used. In contrast, the remaining parameters exhibited heterogeneity in their evaluations. The samples produced by dairy C received lower ratings compared to those from dairy G. For the odor parameter, the GJ sample received the highest rating, while CC sample received the lowest. Regarding the flavor parameter, tasting panel preferred the GG sample, whereas the CJ sample had the lowest acceptability. In terms of texture, the differences between samples produced with the same type of cardoon were not as pronounced, with samples from dairy G showing the highest acceptance. The overall acceptance demonstrated a greater acceptance of the samples produced by dairy G, with no significant difference attributed to the type of cardoon used.

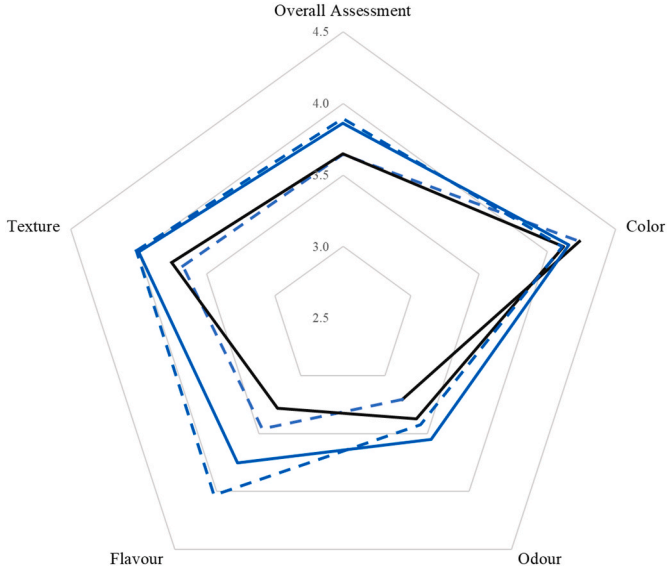

Fig. 2. Radar graph showing the average values of the sensory parameters evaluated for samples CJ (——), CC (— —), GJ (——), and GG (— —). Scale: 1 - "I hated it" to 5 -"I really liked it" (the farther from the centre of the graph, the greater the samples acceptance).

3.7. Principal Component Analysis

Principal component analysis (PCA) was carried out to integrate results from different dairies, coagulants and ripening time. The parameters used in this analysis were pH, titratable acidity (acidity), moisture, hardness, protein content and nitrogen fractions, SFAs and MUFAs.

The similarity map defined by the first two principal components accounted for 89.1% of the total variance. The first component (PC1) condensed 65.8%, while the second component (PC2) represented 23.3% of the total variance.

The PC1 was negatively correlated with pH, moisture and hardness and positively correlated with acidity, protein content, TCA-SN, and WSN. The PC2 shows a negative correlation with SFAs and positive correlation with MUFAs. Fig. 3 shows the projection of the samples onto

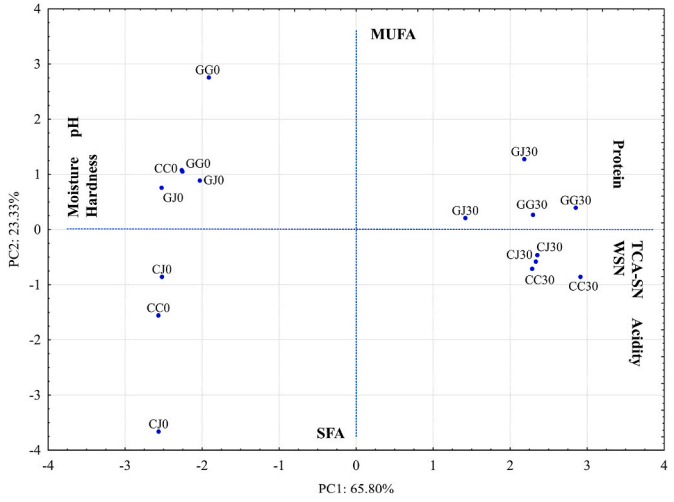

Fig. 3. Principal component analysis: PC1 vs. PC2 projection of samples (n = 2); Code of samples: the first letter represents the cheesemaker, the second letter represents the coagulant, and the number represents the ripening time; The most important variables for the definition of the two components are shown in each axis, indicating the direction in which each variable grows.

the PC1/PC2 plane.

Through PCA, the results from different dairies, coagulants, and ripening times were integrated, allowing to confirm and formulate new conclusions. PC1 is primarily explained by the ripening time, clearly separating 0-day samples from those at 30 days along the first axis. A significant dispersion is observed at the beginning of ripening, with samples clustering closer together at the end of the 30day ripening. This suggests an initial influence of coagulants on curd properties, with enzymes produced by the microbiome becoming more dominant as ripening progresses (Gobbetti et al., 2015; González et al., 2010). PC2 is explained by fat quality, with higher unsaturated fat content samples located in in the upper plane and those with greater saturated fat content in the lower plane. As previously mentioned, by the end of ripening, the samples are closer together in the plane defined by the two principal components. At this stage, the main difference between the samples is explained by PC2, which is related to fat quality. Generally, cheeses from dairy G have higher levels of unsaturated fat, while samples from dairy C have more saturated fat. According to the literature (Renes et al., 2020), cheese fat composition largely depends on milk composition, which is heavily influenced by animal feed. Therefore, the notable difference between the samples at the end of curing can be attributed to the differing animal nutrition practices of the two dairies.

4. Conclusion

This study aimed to assess the impact of different *Cynara cardunculus* L. aqueous extracts on the physicochemical, microbial, and sensory properties of PDO Serpa cheese, including moisture content, texture, nitrogen fractions, fatty acid profile, and overall acceptance. The findings demonstrate how variations in cardoon coagulants influence key cheese quality attributes.

The results confirmed that ripening time plays a dominant role in shaping the cheese's sensory and physicochemical characteristics, with fat composition emerging as a key differentiator between cheese samples. Although variations in cardoon coagulants did influence some attributes, factors such as dairy practices and animal nutrition also contributed to the differences observed in the final product quality.

Overall, this study highlights the dynamic interaction between coagulant type, ripening time, and production practices in artisanal cheese, underscoring the complexity of factors that contribute to the final quality of PDO Serpa cheese. Understanding these interactions can inform strategies for optimizing cheese production processes and improving consistency in sensory qualities.

CRediT authorship contribution statement

N. Alvarenga: Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization. **J. Fernandes:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **S. Gomes:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **T. Baltazar:** Formal analysis. **V. Fiates:** Formal analysis. **L.G. Fidalgo:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **T. Santos:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **C. Conceição:** Writing – review & editing. **J. Dias:** Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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