Artichoke cv. Francés flower extract as a rennet substitute: effect on textural, microstructural, microbiological, antioxidant properties, and sensory acceptance of miniature cheeses

Running title: *Cheese manufactured with Francés varietal artichoke flower as rennet substitute*

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Abstract

BACKGROUND

The most common milk-clotting enzymes in the cheese industry are recombinant chymosins. Food naturalness is a factor underpinning consumers' food choice. For consumers that avoid food with GMO-derived ingredients, the use of vegetable-based rennet substitute in the cheese formulation may be a suitable solution. Artichokes that deviate from optimal products, when allowed to bloom due to flower protease composition, are excellent as raw material for vegetable rennet preparation. As enzymatic milk-clotting exerts a significant impact on the characteristics of the final product, this product should be carefully studied.

RESULTS

Mature flowers from unharvested artichokes (*Cynara scolymus* cv. Francés) that did not meet aesthetic standards for commercialization were collected and used to prepare a flower extract. This extract as coagulant preparation enabled the manufacture of cheeses with distinctive characteristics compared with cheeses prepared with chymosin. Rennet substitution did not affect actual yield but led to significant changes in dry matter yield, humidity, water activity, protein content, and color and conferred antioxidant activity to the cheeses. The rennet substitution promoted significant modifications in springiness, and in the microstructure of the cheese with a more porous protein matrix and an increment in the size of the fat globules. Both formulations showed a similar microbiota evolution pattern with excellent microbiological quality and a good sensory acceptance.

CONCLUSIONS

The studied rennet substitute produced a cheese adapted to specific market segments that demand more natural and healthier products made with a commitment to the environment but well accepted by a general cheese consumer.

Keywords: Plant rennet, *Cynara scolymus*, Microbiota evolution, Cheese microstructure, Cheese texture, Antioxidant activity

1. Introduction

Taste, healthiness, and price remain the strongest drivers of food choice, but also ethically sourced ingredients, social consciousness, and environmental considerations are important determinants of food choices[1,2]. Reducing food waste is one of the most discussed topics in the societal change towards a more sustainable future. One significant source of food waste is the reluctance of supply chains and consumers to sell, purchase, or consume products that deviate from optimal products based on only cosmetic specifications[3]. Initiatives aiming to promote sustainable food consumption should ensure a high sensorial quality of sustainable products[1].

The continuous expansion of the market niche of vegetarians as well as the growth of the market for Kosher and Halal foods has increased the search for vegetable rennet substitutes (RS), to generate products adapted for these specific market segments[4]. Enzymatic clotting of milk is one of the most critical steps in cheesemaking, and it exerts a significant impact on the characteristics of the final product[5]. The most traditional milk-clotting enzyme is chymosin, with its synthetic versions being by far the most common forms of milk clotting enzymes used in cheese industry[6]. RS currently used in cheese production are aspartic endopeptidases (AP) from microbial and vegetable origin, with not exactly the same proteolytic properties of chymosin[7]. An adequate RS is characterized by a high specific hydrolytic activity and a low general proteolytic activity[8]. In general, APs with milk clotting activity (MCA) meet both conditions, and consequently become favorite candidates on the search for RS[9].

In the Mediterranean basin, extracts from dried flowers of *Cynara cardunculus* L. are traditionally used to produce many varieties of sheep and goat cheeses. This coagulant produces cheeses with a soft texture, intense aroma, and slightly piquant flavor[9,10]. Natural variability of the plant extract concerning enzyme activity, as well as the flower shortage limit the use of *C. cardunculus* wildflower extract as an RS to artisanal production[4,11]. Artichoke flower (*C. scolymus*) has the advantage of being a cultivated species with less variability than wildflowers for the preparation of an RS. Artichokes from La Plata (Argentina) possess a geographical indication (GI) sign; this seal

identifies and guarantees the particular quality and reputation linked to the geographical origin of the product (https://news.agrofy.com.ar/noticia/176503/alcaucil-platense-estrella-invierno)[12] turning the flowers grown in this area an excellent material for the study of this RS. In addition, the use of small inflorescences that would not reach the artichoke commercial quality could represent a benefit for small and medium producers from the region.

The objectives of this work were to exploit artichokes that do not meet aesthetic standards for commercialization of vegetables in the preparation of flower extract and to use it as an RS. Miniature cheeses from bovine milk were produced with *C. scolymus* extract from flowers of Francés varietal. The physicochemical parameters, textural attributes, sensory acceptance, *in-vitro* antioxidant activity (AA) of cheeses, as well as the evolution of microstructure and microbiota during ripening were investigated and compared with those of cheeses curdled with chymosin.

2. Materials and methods

2.1. Flower-enzyme extract

Mature inflorescences of *C. scolymus* L. cv. Francés were collected from farm plants grown in La Plata in December (2018). Enzyme extract (EE) was obtained according to the method described by Colombo et al.[10]; the flowers were ground in a mortar under liquid nitrogen, homogenized in 25 mM citric acid, 25 mM sodium citrate buffer (pH 3.0) at 1 g per 3 mL, stirred for 30 min. and centrifuged at 5,000 × *g* for 20 min at 4 °C.

2.2. Formulation of miniature cheese from bovine milk

MCA of 100 µL of EE or 100 µL of commercial rennet dilutions were evaluated by mixing with 1 mL of reconstituted skim milk powder (Ilolay, 2300 Rafaela, Argentina) at 125 g kg^{-1} total solids in 30 mM $CaCl_2$ and at 37°C. The time until a firm coagulum was formed was measured for sextuplicates of each sample[12]. The commercial rennet used was chymosin: Chy-Max Extra rennet powder (Chr. Hansen Argentina S.A.I.C, 1107 Buenos Aires, Argentina) with 2080 international milk-clotting units per gram (IMCU).

Cheeses (20 g) were produced following the method described by Colombo et al.[10] with minor modifications employing ultra-pasteurized whole milk (Armonía, Mastellone Hnos SA, 1748 General Rodríguez, Argentina). A direct vat set culture Mesophilic Chr-Hansen R-703 (Chr. Hansen Argentina S.A.I.C) was used as starter culture at ~~a rate of~~ 0.013 g L^{-1}, and $CaCl_2$ was added to milk at a final concentration of 30 mM; the mix was placed in a water bath at 37°C until pH 5.4 was reached. Two coagulants were used: chymosin (1/400 dilution from stock solution with 50 IMCU/mL Chy-Max Extra rennet powder) and EE. Each preparation had equal total MCA; eighteen miniature Cheddar-like cheeses were produced for each coagulant preparation. Cheeses were produced in wide-mouth centrifuge bottles (100 mL); 100 mL of milk were inoculated with rennet; a firm coagulum was formed in 10 min. The coagulum was manually cut and allowed to rest (5 min) and subsequently stirred slowly (5 min). Incubation for 35 min was performed increasing gradually the temperature from 33°C to 42°C. The bottles were then centrifuged at room temperature (1700 × *g*, 30 min, 25°C) to allow the separation of the curds from the whey. Curds were then incubated at 36°C (15 min), cheeses were inverted and centrifuged again (1700 × *g*, 30 min).

The cheeses were dipped in brine (NaCl 200 g L^{-1}, 25°C, 5 min), vacuum-packed and ripened for one month (4-5°C).

2.3. Analysis of cheeses

Cheeses were analyzed for total protein content (TN) by micro-Kjeldahl method, moisture by oven drying, water activity by dew-point hygrometry (Aqualab series 3, Decagon Devices Inc., USA), ash content by incineration at 550°C, and internal pH by direct contact of the cheese with a lance tip pH electrode (Hanna Instruments, C1229 ACK Bs As, Argentina). Actual cheese yield was expressed as the weight of cheese in kg produced from 100 kg of milk (percentage yield, Ya) and cheese solid yield (Ys) as dry matter in kg produced from 100 kg of milk to take into account the differences in moisture content.

Surface color measurements were made using the Commission Internationale de l'Éclairage L*, a*, and b* parameters with a handheld chroma meter CR-400 (Konica Minolta, Sakura-machi, Hno-shi191-8511 Tokyo, Japan)[13]. Each analysis was performed in triplicate. The total color difference (ΔE*) between Q- and Cs-cheeses was calculated as[14]:

$$\Delta E^* = [\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}]^{½}$$

2.4. Antioxidant activity

The quencher procedure was followed with slight modifications[15]. The samples, 5, 10, or 50 mg of Cs– and Q–cheeses, were mixed with 10 mL of 2,2'-azino-bis (3-ethyl benzothiazoline-6-sulphonic acid; $ABTS^{+}\cdot$; Sigma-Aldrich, 63103 St. Louis, Mo., USA) or 2,2-diphenyl-1-picrylhydrazyl (DPPH·; Sigma-Aldrich). Trolox (6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid; Sigma-Aldrich) was used as a reference substance (0.5-0.85 mg mL^{-1}). The results were exhibited as TEAC (Trolox equivalent antioxidant capacity) employing values calculated from Trolox calibration plot and expressed as μmol of Trolox g^{-1} of cheese. Five replicates were performed for each sample.

2.5. Texture profile analysis (TPA)

TPA was conducted using a CT3 Texture Analyzer (Brookfield Ametek, Middleboro, MA 02346, USA). A cylinder probe of 38.1 mm D/20 mm (probe TA4/1000) was attached to the moving crosshead. Cylindrical cheese pieces (diameter: 20 mm, height: 30 mm) were placed on the platform (TA-BT-KIT). Samples were compressed in two-cycle tests at a speed of 0.5 mm s^{-1} with a 25% deformation from the initial height of the sample. The textural parameters hardness (gf), cohesiveness, chewiness (mJ), and springiness (mm), were determined after 30 days of maturation. Parameters were recorded or calculated according to Frau et al.[16]. Measurements were performed for three independent samples for each treatment.

2.6. Confocal laser scanning microscopy (CLSM) and image analysis

The microstructure of cheeses was observed every week during ripening using a dual labeling approach and an inverted confocal laser scanning microscope (Leica SP5, Leica Microsystems,

35578 Wetzlar, Germany), fluorescent dyes (Sigma-Aldrich) were used to visualize fat globules (0.31 mM acetonic Nile Red, λex: 543 nm, λem: 550-640 nm) and protein matrix (0.51 mM ethanolic FITC, λex: 488 nm, λem: 500-530 nm). Images collected on each day and trial were processed using the Leica LAS AF Lite software (Leica Microsystems).

Image analysis of three micrographs for each trial was performed using ImageJ software (Research Services Branch, Natl. Inst. of Health, Md., U.S.A.). The true radius (R) was estimated according to Everett and Olson[17] as:

$$R = R_{app} \times \sqrt{3}$$

Where R_{app} was the radius measured from image analysis of CLSM. This correction was made to account the possibility that a planar sectional image may be observed at some distance from the equatorial plane of a sphere.

The circularity was calculated according to the same authors as:

$$C = \frac{4\pi A}{2L}$$

A is the measured area of the fat globule while L is the perimeter length. C=1 indicates a sphere, and C<1 an increasing degree of distortion.

2.7. Microbiological analysis

To study the evolution of the microbiological characteristics of Cs–cheeses during the maturation process samples were taken every week since cheese manufacture. Approximately 10 g of cheese samples were diluted in 90 mL of tryptone water and homogenized in a Stomacher 400 (Seward Ltd., BN14 8HQWest Sussex, UK) for 15 min at medium speed. Then the suspension was inoculated onto a range of agars and liquid broths designed to be selective for predominant bacteria. Bacterial counts were determined by plate count on plate count agar (Oxoid Ltd., RG24 8PW Basingstoke Hampshire, England) for total mesophilic and psychrotrophic bacteria, MRS-agar for lactobacilli (Biokar Diagnostics, 60000 Allonne, France), and YGC agar (yeast extract glucose chloramphenicol) for molds and yeasts (Oxoid Ltd.). Coliforms were determined by plate counts in VRBD agar (violet red bile glucose agar) (Oxoid Ltd.) for counts above 1000 CFU/g and by MPN

(most probable number) in brilliant green bile (20 g L^{-1}) broth (Oxoid Ltd.) when counts were less than 1000 CFU/g. Cultures were incubated during 48 h in aerobic conditions at 37°C for lactobacilli, total mesophilic microbiota, and coliforms; yeast and molds were incubated at 30°C for 5 days, and the psychrotrophic microbiota was incubated during 15 days at 10°C.

2.8. Sensorial acceptance test

A sensorial acceptance test of cheeses was performed by an untrained panel of 18 judges following the method described by Colombo et al.[10]; the participants reported liking cheese and consuming it frequently. Informed consent was obtained from all subjects. One sample of each product was presented to each panelist at room temperature (24°C) and under normal lighting conditions. The appearance, texture, aroma, flavor and general acceptability of the cheeses were evaluated at the end of the ripening period. A 5-point hedonic scale (from dislike extremely = 1 to like extremely = 5) was used to yield data on the magnitude of liking/disliking of sensation. Precautions were taken to randomize the samples, to mask the identity, and to minimize the effects of contrast and adaptation. Panelists were also asked if as consumers they would buy the cheeses; a 5-point scale was used to measure their likelihood of purchase.

2.9. Statistical analysis

All data were reported as the means ± the standard deviations of three or five technical replicates, except for sensory data reported as the means ± the variances. Results of physicochemical and textural parameters, sensory acceptance, purchasing intention, were evaluated by one-way analysis of variance (ANOVA) using InfoStat software[18], while results of pH were evaluated by two-way ANOVA. Fishers' Least Significant Difference test was applied in order to determine which mean values were significantly different at the 95% confidence level. Microbiological analysis, apparent radius from CLSM images, and AA data analysis were subjected to one-way ANOVA, followed by Student-Newman-Keuls method, Tukey test and Dunnet test, respectively, to compare the means. Differences of the mean at p-value <0.05 were considered significant.

3. Results

3.1. Physicochemical parameters

In the present work we obtained an EE at pH 3.0 from frozen artichoke flowers of the Francés varietal, with MCA (expressed as time for coagulation) < 2 min, and we manufactured bovine-milk miniature cheeses. Both the plant extract and the selected dilution of stock commercial rennet coagulated milk in about 10 min. Regardless of the rennet used, the curd was firm and elastic and the whey produced was clear and slightly yellow. In Table 1, yield and main physicochemical properties of those cheeses are compared. Ya, as well as ash content, did not exhibit significant differences for both rennets. Cs–cheeses contained higher humidity and lower water activity than Q–cheeses; both parameters were significantly different. In view of the differences in moisture between cheeses, yield was also expressed in terms of dry matter; in fact, Ys was significantly lower for Cs–cheeses. TN was significantly lower for Cs–cheeses than for Q–cheeses. The ratio of moisture-to-protein of Q–cheeses was significantly lower compared to Cs–cheeses. Changes in pH due to the time of ripening were significant regardless of the rennet used. In terms of the color parameters, a* was less negative when RS was the coagulant, while b* and L* showed no significant differences. According to ΔE* value, the sample and control cheeses had two different colors.

3.2. Microbiological analyses

The predominant microbiota in both kinds of cheese was the lactic acid bacteria (LAB) coming from the starter culture (Table 2). LAB only suffered 1 log decrease of their viable counts during the ripening period. In both cheese formulations, the average concentration of coliforms was very low at the beginning. The number of viable counts of coliforms significantly decreased after 7 days and was no detectable by plate counts on day 15. The exact concentration of coliforms until day 28 was determined by the MPN, being below zero values (data not shown). The mesophilic microbiota had a concentration of 1×10^4 CFU g^{-1} at the beginning of the ripening and decreased 2 logs on day 28 with no differences between cheese formulations. On the other hand, psychrotrophic microbiota

remained low and constant during all the ripening period. The viable number of molds and yeast increased during the ripening period. Their concentration significantly stepped up after 28 days of ripening. Even when Cs–cheeses exhibited a low extend rise of fungi microbiota their concentration was 2 logs under Q–cheeses at day 28.

3.3. TPA and CLSM

Textural parameters are shown in Table 1. Q–cheeses had a higher springiness than Cs–cheeses. On the contrary, hardness, cohesiveness, and chewiness parameters did not show significant differences for both formulations.

The changes in the microstructure of cheeses that occurred during ripening were followed by CLSM. Fig. 1 images are representative of what we observed between replicates (Supplementary Figures 1 and 2 and Table 1). The oil- and water-soluble zones did not overlap in both formulations. The microstructure of the cheeses showed a continuous and porous protein matrix being a little more compact in the control cheeses, even before ripening. Numerous fat globules of variable size and more or less spherical shape were uniformly distributed in both kinds of cheese after 7 days of manufacture. In Cs–cheeses small fat globules prevailed after 7 days of maturation, 92% of the fat globules had a corrected radius ≤ 2.5 μm, mean corrected radius and mean circularity of the fat globules of Q–cheeses after 7 days of maturation were significantly greater than that of Cs–cheeses. During cheese maturation, regardless of the rennet used, the rough appearance of the protein network became more porous, and through the formation of larger spaces, the fat globules coalesced. After 30 days of maturationin Cs–cheeses, fat particles could still be seen as individual small globules embedded in the protein matrix — 62% of the fat globules had a corrected radius ≤ 2.5 μm — or as more massive and more irregularly shaped fat globules — 17 % of the fat globules were>4 μm. Regarding the control cheeses, although coalescence of fat globules was observed, globules were significantly smaller in size at the end of ripening than the globules in Cs–cheeses. Large globules had ~~low or~~ medium circularity and a large dispersion of size distribution is evident

for Cs–cheeses with 30 days of maturation, even though the mean circularity did not differ significantly from control cheeses.

3.4. Antioxidant activity

The results obtained by both methods employed showed absorbance values significantly higher than those in the negative control group (Dunnett´s test). The comparison among the data obtained for both kinds of cheese showed a slightly higher value for Cs–cheeses than for Q–cheeses (Fisher's test). In the case of $ABTS^{+}\cdot$ method, TEAC values were 52.65 ± 1.73 and 37.39 ± 1.99 µmol of Trolox/g of sample, for Cs– and Q–cheeses, respectively. Otherwise, 0.35 ± 0.02 and 0.24 ± 0.02 µmol of Trolox/g of sample were exhibited by using the DPPH method. These last values were lower than those obtained by $ABTS^{+}\cdot$ method, which is expected since it only recovers the activity of soluble ethanol compounds, and this assay was performed in the resultant ethanolic supernatant after a centrifugation step.

3.5. Sensory acceptance

Most of the evaluators considered that all the attributes evaluated in the two types of cheeses satisfied them or did a lot (Supplementary Figure 3); no significant differences were observed in both formulations for each attribute. Dispersion between appreciations for the two types of cheeses was observed with respect to the aroma, where more than a third of the evaluators were indifferent to the Q– and Cs–cheeses. Purchasing intention was also evaluated with no significant differences observed between both formulations. Approximately 78% of the panelists would probably or certainly buy the Cs–cheeses.

4. Discussion

We aimed to test a crude aqueous extract from flowers of artichoke cv. Francés with MCA as potential RS. For this, we used mature flowers from unharvested artichokes that did not meet aesthetic standards for commercialization. Flower aqueous extract enabled the manufacture of miniature cow-milk cheeses adapted to specific market segments that demand more natural and

healthier products made with a commitment to the environment but well accepted by a general cheese consumer.

The MCA of artichoke flowers is likely due to the presence of AP belonging to the cardosin group[18]. Extract of *C. cardunculus* wildflower, ancestor of artichoke, has been used traditionally as coagulant; literature indicates that the activity of *C. cardunculus* extract depends on numerous factors including thistle flower ecotype. As a result of this, *C. cardunculus* extracts are non-standardized and may possibly fluctuate in terms of clotting and proteolytic activities, eventually affecting production yield and final cheese properties[4,19]. Some of these natural variations in the source of AP could be overcome or minimised by using a specific varietal of a cultivated plant. Cheeses manufactured with *C. cardunculus* exhibit a bitter taste in the final product, which is strongly perceived in cow's cheeses. Thus, the use of this extract is limited to goat and ewe cheeses[11]. Llorente et al.[18] demonstrated that flower extracts of *C. scolymus* cv. Green Globe obtained at pH 6.0 are suitable for the manufacturing of Gouda-type cheese, but prolonging the salting time before ripening was a necessary operational change for preventing over-proteolysis and avoiding the development of background bitterness. However, this undesirable bitter taste was not perceived in our formulations. The lack of bitterness in these cheeses could be associated with one or several of the following factors: the extraction pH (3.0), the artichoke varietal, or the cheese formulation. For *C. Cardunculus*, due to its significant impact on the level of cardosin extracted, extraction pH is the most influential factor on MCA; pH 3.0 is the most efficient pH for extraction and which produce better gel firmness than other extraction conditions[20]. In what regards to artichoke variety a wide phenotypic and genetic diversity is observed within varietal groups[21] which could lead to differential enzyme expression and proteolytic profile.

EE did not produce an impact on Ya, but influenced the protein content in the gel and the levels of moisture expulsed from the curd. Even if a coagulant is highly specific, nonspecific breakdown of casein fractions may occur. The resulting loss of small degradation products into the whey impacts on cheese yield[22]. Differences found in the protein content and water activity between sample and

control cheeses, suggested a higher proteolytic activity from the RS in comparison to chymosin what may have affected the Ys of Cs–cheeses. Residual rennet is retained in the curd and is the major source of primary proteolytic activity in cheeses made with mesophilic starters and low cooking temperatures like the ones reported in this paper. Proteolysis would lead to the further production of low molecular weight molecules with water-binding capacity[23], what would explain the lower water activity. As the water activity decreases, the bacteria are predisposed to cell lysis, the release of intracellular enzymes and a subsequent breakdown of casein[24].

Cheese texture is affected by the composition and interactions between components. The structural arrangement between the casein molecules, the state of water (bulk, or bound to the casein matrix) would affect cheese texture[25]. The higher ratio of moisture-to-protein observed for Cs–cheeses indicated a higher relative degree of casein hydration on Cs–cheeses than Q–cheeses and was probably responsible for differences observed on texture profile and microstructure between cheeses. When the water content is high protein particles are more swollen and offer less resistance to deformability, what would explain the lower springiness in Cs–cheeses. Noteworthy, Cs–cheeses did not turn out to be soft like traditionally produced cheeses which are soft-bodied[26].

After clotting casein micelles are freely present in the milk; during cheese processing, they begin to fuse to form a dense protein matrix in which other components are distributed[27]. The less compact protein matrix observed in Cs–cheeses was probably due to an increased breakdown of casein even before ripening. Fat globules participate in cheese microstructure by binding to the casein matrix or as inert filler material partially disrupting the casein matrix[27,28]. Fat globule coalescence was possibly due to the fusion of the casein micelles in both kinds of cheese added to the protein hydrolysis in Cs–cheeses what could explain differences in fat globule mean radius. The large irregular ‘fat globules’ observed can be pools of fat within voids in the casein matrix or clusters of emulsified globules[17].

The use of EE made the cheeses redder and less green than chymosin as the renneting agent, with that difference possibly being attributable to the violet color of the EE. The same dissimilarity in

chromaticity was observed for cheeses manufactured with coagulant blends of flower enzyme extract of *Silybum marianum* (Cardueae tribe) and chymosin[10]. However, differences in lightness are also detected for those cheeses, being the cheeses curdle with the coagulant blend darker than control cheeses.

Coliforms, mesophilic, and especially fungi microbiota are the main responsible groups for microbiological spoilage of dairy products[29]. All these populations were in low concentration at the manufacture and then decreased during the ripening, with the exception of molds and yeasts. The low quantity of microorganisms in the whole process of manufacture and ripening suggests a good prognosis of Cs–cheese quality during storage.

The use of "quencher" protocol for the determination of AA allows the release of antioxidant compounds trapped in the matrix of aggregated proteins[30]. The higher AA detected in Cs–cheeses could be assigned to the presence of antioxidant peptides released by the RS peptidases. Bioactive peptides with several bioactivities including AA are released from bovine casein hydrolysates obtained using artichoke flower extracts[30]. Furthermore, the release of bioactive peptides in cheeses by peptidase-action is well-known in several kinds of cheese[31]. Additionally, part of AA could be due to the presence of polyphenols from artichoke EE[32]. Regardless the source of the AA, we propose that Cs-cheese is a valuable product since it could contribute to consumer health and its self-conservation as a food product.

C. scolymus–flower extract from cultivated flowers of Francés varietal, as coagulant preparation, was suitable for replacing rennet in the production of cheeses at laboratory-scale and in so doing resulted in the development of the distinctive textural, appearance, microstructural and functional properties, as well as good microbiological quality. Further investigations need to be undertaken to ascertain if the particular proteolytic specificity of the RS is associated with the overall differences observed in the cheeses, and if so, specific features can be further improved by fine-tuning of proteolysis. Our results anticipate the potential of Francés varietal artichoke-based rennet.

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Conflict of Interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

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Figure legends

Fig. 1. Confocal laser scanning micrographs of cheeses made with plant rennet (panels A to D) or chymosin (panels E to H) at day 7, 14, 21, and 30 (A and E, B and F, C and G, and D and H, respectively) were captured using 63.5 × objective lens. White scale bars are 10 µm in length. Fat is colored in red (Nile Red) and protein in green (FITC); small dark areas correspond to free serum. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1. Comparison of yield, physicochemical and textural parameters of miniature cheeses.

Physicochemical parameters	Q	Cs
Ya[†] [kg (100 kg milk)$^{-1}$]	17.2 ± 0.7[a]	16.6 ± 0,7[a]
Moisture (g kg^{-1})	530 ± 19[a]	591 ± 7[b]
Ys[‡] [dry matter kg (100 kg milk)$^{-1}$]	7.6± 0.3[a]	6.6 ± 0.1[b]
Water activity	0.978 ± 0.002 [a]	0.971 ± 0.002 [b]
Ash (g kg^{-1})	37 ± 2[a]	38 ± 1[a]
pH^{7d}	4.69 ± 0.04[a]	4.69 ± 0.16[a]
pH^{30d}	4.52 ± 0.06[c]	4.46 ± 0.06 [c]
Color [§] L*	98.48 ± 0.09[a]	98.11 ± 0.04[a]
a*	-3.00 ± 0.01[a]	-2.40 ± 0.01[b]
b*	17.03 ± 1.22[a]	16.67 ± 0.08[a]
Protein (g kg^{-1})	158 ± 2[a]	150 ± 3[b]
Moisture-to-protein ratio	3.40 ± 0.13[a]	3.94 ± 0.05[b]
Textural parameters		
Springiness (mm)	4.82 ± 0.29[a]	3.90 ± 0.29[b]
Hardness (gf)	472.67 ± 44.38 [a]	667.33 ± 172.02 [a]
Cohesiveness	0.78 ± 0.02[a]	0.76 ± 0.07[a]
Chewiness (mJ)	17.43 ± 2.25[a]	19.27 ± 5.31[a]

All the cheeses were made on the same day from the same milk and the analysis performed after 1 month of ripening except for the pH that was also measured 7 days after manufacturing (pH^{7d}). The results represent the mean ± SD. Treatments followed by the same letter do not differ significantly ($p > 0.05$). Q: Cheese renneted with chymosin; Cs: Cheese renneted with *C. scolymus* flower extract

[†] Cheese percentage yield

[‡] Cheese solid yield

[§] Commission Internationale de l'Éclairage parameters: L* indicates lightness and a* and b* the respective red-green and yellow-blue coordinates.

Table 2. Changes in log CFU g^{-1} of main microbial groups of Q– and Cs–cheeses during ripening.

		Storage period (days)			
	sample	0	7	15	28
Coliforms	Q	2.60 ±1.40^{a}	1.54 ±0.71^{a}	<0^{b}	<0^{b}
	Cs	3.34 ±0.14^{a}	1.95 ±1.50^{a}	<0^{b}	<0^{b}
Lactic acid bacteria	Q	9.06 ±0.17^{a}	8.49 ± 0.70 ^{a}	9.03 ±0.11^{a}	7.92 ±0.14^{b}
	Cs	9.02 ± 0.31^{a}	8.64 ±0.21^{a}	9.12±0.06^{a}	8.00 ±0.07^{a}
Mesophilic microbiota	Q	3.77 ±0.14^{a}	1.69 ±1.40^{b}	2.33 ±0.35^{a}	1.18 ±0.71^{b}
	Cs	4.07 ±0.34^{a}	2.32 ±0.42^{a}	2.27 ±0.07^{a}	1.29 ±1.10^{b}
Moulds and Yeast	Q	2.17 ±0.14^{a}	1.30 ±1.40^{a}	1.65 ±0.72^{a}	4.35 ±0.07^{b}
	Cs	0.74 ±1.00^{a}	1.00 ±0.01^{a}	0.74 ±2.00^{a}	2.60 ±1.41^{a}
Psychrotrophic microbiota	Q	2.40 ±1.00^{a}	2.34 ±0.28^{a}	2.47 ±0.64^{a}	1.17 ±0.71^{a}
	Cs	1.80 ±0.28^{a}	1.98 ±2.00^{a}	1.84 ±2.10^{a}	2.23 ±0.21^{a}

Log CFU g^{-1} are expressed as average values ± standard deviation. Comparison of means was tested using analysis of variance with the Student-Newman-Keuls method, and if $p < 0.05$, the difference was considered statistically significant. Different letters represent significant differences ($p < 0.05$). Q: Cheese renneted with chymosin; Cs: Cheese renneted with *C. scolymus* flower extract.