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Effect of coagulants from aerial parts of Tunisian *Moringa oleifera* on cheese properties

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ABSTRACT. The present study aimed to produce vegetable clotted cheese using coagulants from aerial parts of Tunisian *Moringa oleifera* as calf rennet substitute. The *Moringa* enzymatic extracts were recovered, partially purified and characterized for their proteolytic and milk-clotting activities. The obtained cheeses were subjected to physicochemical, textural, microbiological and sensory analysis during refrigerated storage. The extracted proteins from *Moringa* aerial parts were fractionated with ammonium sulfate at various concentrations (from 20 to 60%). Highest milk clotting activities were observed in the 40 and 60% fractions, for seed and leaf extracts, respectively, showing that these fractions contain the enzymatic coagulants. Thus, seed enzymatic extract showed to be more active than those recovered from the other aerial parts. During cheese manufacturing, results revealed no significant difference in the kinetic parameters when substituting calf rennet with *Moringa* milk clotting enzymes. Moreover, the characterization of produced cheeses showed that the use of partially purified milk clotting enzyme improved cheese yield and its properties during refrigerated storage. In fact, cheese clotted with *Moringa* seed coagulant presented the highest hardness value (3.60 N) and dry matter content (53.86%), at the end of storage period. Also, the addition of vegetable coagulants contributed to the development of an intense color and flavor, which can result in satisfactory cheese properties.

Keywords: *Moringa oleifera* lam; enzymatic extracts; milk-clotting activity; cheese quality.

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Introduction

Enzymatic coagulation of milk is a crucial step in cheese-making process. After rennet addition, this step begins with an enzymatic cleavage of the phenylalanine$_{105}$–methionine$_{106}$ peptide bond of κ-casein (Wang et al., 2022). Coagulation using calf rennet is the most used procedure. However, the worldwide increase in cheese production, coupled with reduced supply and increasing prices of calf rennet, has led to a search for alternative milk-clotting enzymes as suitable substitutes for rennet (Badgujar and Mahajan, 2012). Several milk-clotting enzymes of microbial origin have been commercialized and used in cheese making. However, these products are partially suitable for cheese making due to excessive proteolytic activity (PA), and the microorganisms must be genetically modified in order to reduce their thermostability effect (Yegin et al., 2013).

In this context, vegetable rennet considered as a substitute for calf rennet has become a subject of growing interest in cheese industry, due to its easy availability and simple purification processes. Evaluation of enzymatic activities: mainly milk-clotting activity (MCA) and proteolytic activity (PA) is a crucial step in the selection of an appropriate substitute of calf rennet during cheese manufacturing. Rheological and sensorial properties of final products are related to the values of MCA/PA ratio (Abdeen et al., 2021; Anusha et al., (2014). A high value of this ratio reflects an excellent product with desirable firmness and no release of bitter flavors', which is typical for plant proteases (Ben Amira et al., 2018).

In this regard, this study recovered milk clotting enzymes from various aerial parts of *Moringa oleifera* cultured in Tunisia. Moreover, a fresh cheese clotted by *Moringa oleifera* enzymatic extracts was produced and then, subjected to analysis of physico-chemical, organoleptic aspects and microbiological properties, which were then compared to those of a cheese clotted by animal rennet.

Material and methods

Raw material

Leaves and seeds of *Moringa oleifera* were collected from the North of Tunisia (Morneg, Ben Arous) between August and October 2022. Fresh material of *Moringa* aerial parts were cleaned and washed under tap water to remove dust and were then air-dried for one week at room temperature until reaching constant weight (Terefe et al., 2017). *Moringa* seed husk were removed manually from the whole seed to prepare the shelled white kernel. Then, all samples were stored in closed containers and kept in freezer at -20°C until further use.

Extraction and partial purification of milk clotting enzymes (MCE) from Moringa oleifera aerial parts

Enzymatic crude extracts from different *Moringa oleifera* aerial parts were recovered according to the procedure of Benkahoul et al. (2016), with some modifications. In fact, *Moringa* leaves and seeds were ground to fine powders using mortar and pestle under liquid nitrogen (MSA animal health in Sidi Thabet, Tunisia). The obtained powders were dissolved in acetate buffer (50 mM, pH 5) containing NaCl (5%) with a ratio of 1:10 (w/v). Crude enzymatic extracts were obtained using magnetic stirring for 4 h at 4°C, followed by filtration using medical gauze and centrifugation for 20 min at 10.000 rpm and at 4°C (Ohaus Frontier$^{T.M}$5718R Multi Pro Centrifuge). The recovered supernatants were considered as crude enzymatic extracts and were partially purified using fractionation by graded saturated ammonium sulfate (Sigma Aldrich, France) ranging from 20 to 60% saturation degree, as described by Terefe et al. (2017). Fractions with intense milk clotting activity were considered as partially purified MCE and were used for cheese manufacturing.

Characterization of Moringa oleifera MCE

Protein content determination

The protein content of *Moringa* enzymatic extracts was estimated using the Bradford method, as described by Banik et al. (2018). Enzymatic extract was diluted in distillated water, and then, 200 µL of Bradford reagent (Sigma Aldrich, France) were added. Next, the whole mixture was incubated in the dark for 10 min and the absorbance was determined at 595 nm with reference to the standard curve prepared with BSA (Sigma Aldrich, France) standard solutions.

Proteolytic activity evaluation

The proteolytic activity (PA) of *Moringa oleifera* enzymatic extracts was determined according to Terefe et al. (2017), using 1% casein solution dissolved in a 50 mM potassium phosphate buffer pH = 6.5 as a substrate. One unit of proteolytic activity (U) was defined as the amount of enzyme required to cause an increase of 0.1 in absorbance at 280 nm under the assay conditions, and it was calculated as follows:

$$PA\ (U\ mL^{-1}) = Abs_{280nm} \times 0.5 \times \text{dilution factor} / E \times t$$

Abs 280 nm is the variation of absorbance between assay and control; **E** is the volume of partially purified extract solution, and t is the reaction time.

Milk-clotting activity (MCA) evaluation

The milk clotting activity (MCA) of *Moringa* enzymatic extracts was determined as described by Tajalsir et al. (2014), using the Berridge substrate (10% skimmed milk in 0.01 M $CaCl_2$, pH 6.5). MCA unit is defined as the amount of enzyme that coagulates 1 mL of the substrate in 40 min at 35°C, which is expressed in Soxhlet unit (SU) and calculated as follows:

$$MCA\ (SU\ mL^{-1}) = 2400 \times V / T \times v$$

V: volume of substrate (mL); v: volume of the enzymatic solution (ml); T: clotting time (sec).

The MCA to PA ratio was also calculated.

Fresh cheese manufacturing

Fresh cow milk was purchased from an agricultural farm located in Bizerte city, from Northern Tunisia. Then, the milk was pasteurized at 72°C for 15 sec and then cooled at 37°C. After that, 0.005% of $CaCl_2$ and 0.0005% of mesophilic starter culture were added. The inoculated milk was divided into three batches, as

follows: 1) Coagulated milk with calf rennet (25 mL $100L^{-1}$) (CMR), 2) Coagulated milk with *Moringa* MCE extracted from seeds (CMS), and 3) Coagulated milk with *Moringa* MCE extracted from leaves (CML). The added dose of *Moringa* coagulant was equivalent to the Soxhlet units necessary to clot the milk with calf rennet. Milk coagulation was performed at 40°C for 75min for all samples. The obtained cheese curds were wrapped in cheese cloth in perforated rectangular moulds (≈300 g). Finally, control and vegetable clotted cheeses were stored at 4°C. The cheeses were sampled at 0, 4, 8 and 12 days of storage.

Coagulation kinetic parameters

In order to compare the cheese coagulation process performed using calf rennet as a control and *Moringa* enzymatic extracts, the evaluations of physico-chemical parameters (pH and acidity) were performed each 15 min during milk clotting. The pH was measured using a digital pH meter according to Ong et al. (2007), with maceration of 1g of the cheese sample in 10 mL of distilled water. Also, titratable acidity was determined according to AOAC (2005).

Cheese yield determination

The cheese yield is the expression of the amount of cheese obtained from a given amount of milk (often 100 L or 100 kg). It was determined as described by Adesina et al. (2022), as follows:

Yield (%) = [weight of cheese produced (g)/weight of milk used (g)] x 100.

Determination of physico-chemical properties

Moisture, ash, protein and lipid contents were determined according to AOAC (2005), as well as titratable acidity**.** The pH was also performed as described before.

Determination of color parameters

The colorimetric parameters L* (Lightness on a scale from 0 (blank) to 100 white), a* (negative and positive values indicate, respectively, green and red) and b* (negative and positive values indicate, respectively, blue and yellow color) were performed on all samples, using a colorimeter (Minolta Chroma Meter, CR-300, Tokyo, Japan) (Mahmoudi et al., 2021).

Texture profile analysis

The textural profile analysis on different cheese samples was performed with a texture analyzer (Perten Instruments, North Ryde BC, NSW, Australia), using a circular compression plate (75 mm in diameter) with a speed of 0.5 mm s^{-1} and a compression ratio of 5 mm with a rest time of 5s. The generated plot of force (N) versus time (s) was recorded. Thus, hardness, elasticity, cohesiveness, chewiness and gumminess were calculated from the obtained curves (Wang et al., 2018).

Sensory evaluation

The sensorial properties of the clotted cheeses with animal and vegetable rennet were analyzed. Cheeses were served to the panelists into coded plates in a randomized order. Descriptive sensory evaluation was performed on cheese samples by a panel of 20 trained persons from the Higher Institute of Food Industries of Tunisia. Thus, the main tested attributes were color, flavor, taste, texture and overall acceptability, using a six-point scale ranging from 0 (low intensity) to 5 (high intensity) for each descriptor (ISO 13299, 2016).

Microbiological analysis

Total viable counts were enumerated on Plate Count Agar (PCA) (Biokar Diagnostics, France) after incubation at 30°C for 48 h. The number of coliform bacteria was determined on Violet Red Bile Lactose Agar (Biokar Diagnostics, France) after incubation at 37°C for 24 h. Yeasts and molds were enumerated on Yeast Glucose Chloramphenicol Agar (Biokar Diagnostics, Beauvais, France), which was incubated for 72 h at 25°C.

Statistical analysis

All analyses were performed in triplicate and results were expressed as X ± SD, (X: mean; SD: standard deviation). An analysis of variance (ANOVA) was performed using Duncan's multiple range tests at a significance level of 5%, in order to highlight significant differences among the produced samples and during storage time. The statistical analysis was performed using SPSS software.

Results and discussion

Characterization of partially purified Moringa oleifera MCE

Enzymatic activities of *Moringa oleifera* seeds and leaves proteases were performed. The results related to proteolytic activity (PA), milk clotting activity (MCA), protein content, specific activity and MCA/PA ratio are shown in Table 1.

Table 1. Characterization of partially purified *Moringa oleifera* proteases.

Moringa aerial parts	Ammonium sulfate fraction	PA (U mL^{-1})	MCA (SU mL^{-1})	Protein (mg mL^{-1})	Specific activity (SU mg^{-1})	MCA/PA
Seeds	ASP 20-40%	1.71 ± 0.01^{b}	80 ± 0.57^{b}	1.70 ± 0.01^{b}	47.11 ± 0.02^{b}	46.70 ± 0.31^{b}
Leaves	ASP 40-60%	0.43 ± 0.03^{a}	14.81 ± 0.01^{a}	1.16 ± 0.01^{a}	12.73 ± 0.01^{a}	34.72 ± 0.06^{a}

ASP: Ammonium sulfate precipitation; PA: Proteolytic activity; MCA: Milk clotting activity; Specific activity = Enzyme activity/Protein content; Specific activity of purified enzyme/Specific activity of crude enzyme; Data are expressed as mean ± standard deviation, n = 3. Means with different superscripts are significantly different ($p < 0.05$). Lowercase letters (a, b, c) represent the statistical difference between samples.

The results showed that the leaves enzymatic extract has low milk clotting activity. Also, fractionated proteases with ammonium sulfate at various concentrations revealed highest milk-clotting activities in the 40 and 60% fractions for seed and leaf extracts, respectively (Table 1). This result was similar to that of Abdeen et al. (2021), who reported that the highest MCA of *Moringa* seed extract was registered at 40% of ammonium sulfate saturation. The ammonium sulfate precipitation represents an economic purification procedure (Terefe et al., 2017) and leads to the concentration of the enzyme to a workable volume, having a good potential for use in cheese making industry. Besides, it also facilitates the effective removal of the characteristic colors of raw materials in the crude extract. The recorded proteolytic activities of partially purified *Moringa oleifera* seed (1.71 ± 0.01 U mL^{-1}) and leaf (0.43 ± 0.03 U mL^{-1}) coagulants were different from those found by Tajalsir et al. (2014) and Terefe et al. **(**2017) on cow (2.5 U mL^{-1}) and camel (0.35 U mL^{-1}) milks, respectively. This variation may be due to the inherent characteristics of the substrate (milk) (Terefe et al., 2017).

Thus, in the current study, partially purified MCE recovered from *Moringa oleifera* seeds showed higher milk clotting activity to proteolytic activity (MCA/PA) ratio, when compared to *Moringa* leaf MCE. This finding confirmed that *Moringa* seeds MCE is highly active, which can result in satisfactory cheese properties as reported by Tajalsir et al. (2014), suggesting that the low ratio leaded to a remarkable bitterness in ripening cheese.

Effect of Moringa MCE on coagulation kinetics

Evolution of pH and acidity values during milk coagulation using various *Moringa oleifera* MCE and commercial calf rennet are shown in Table 2.

Table 2. Evolution of coagulation kinetic parameters.

Coagulation time (min)	Coagulated milk samples					
	CMR		CMS		CML	
	pH	Acidity °D	pH	Acidity °D	pH	Acidity °D
0	6.74 ± 0.06^{aA}	14 ± 0.33^{aA}	6.74 ± 0.02^{aB}	14 ± 0.14^{aA}	6.74 ± 0.10^{aC}	14 ± 0.25^{aA}
15	6.59 ± 0.28^{aA}	17 ± 0.17^{aB}	6.58 ± 0.06^{aA}	17.5 ± 0.23^{aB}	6.56 ± 0.04^{aB}	18.6 ± 0.65^{bB}
30	6.58 ± 0.11^{aA}	17.5 ± 0.32^{aB}	6.56 ± 0.01^{aA}	18.5 ± 0.25^{bB}	6.54 ± 0.07^{aA}	18.9 ± 0.29^{bB}
60	6.57 ± 0.24^{aA}	18 ± 0.11^{aC}	6.54 ± 0.02^{aA}	19 ± 0.25^{bB}	6.47 ± 0.03^{aA}	19.5 ± 0.37^{bB}
75	6.40 ± 0.08^{aA}	20 ± 0.49^{aD}	6.50 ± 0.08^{aA}	19.9 ± 0.03^{aC}	6.45 ± 0.06^{aA}	20 ± 0.85^{aB}

CMR: Control coagulated milk with animal rennet; CMS: Coagulated milk with *Moringa* seeds MCE; CML: Coagulated milk with *Moringa* leaves MCE. Data are expressed as mean ± standard deviation, n = 3. Means with different superscripts are significantly different ($p < 0.05$). Lowercase letters (a, b, c) represent the statistical difference between samples; Uppercase letters (A, B, C) represent the statistical difference between results of the same sample during coagulation time.

Initially, no significant difference ($p < 0.05$) was observed between the pH values of all fermented milk samples. Then, the pH values decreased significantly ($p < 0.05$) with the coagulation time. This result was attributed to the action of the lactic starter culture having an action on lowering the pH (Ben Moussa et al., 2019). Also, the acidity increased significantly ($p < 0.05$) in all samples. Indeed, coagulated processed milk reached the same value of acidity (20°D) after 1h 15 min. for all produced cheeses.

Effect of Moringa MCE on cheese properties

Variation in cheese yield

In order to compare the effect of the use of various *Moringa oleifera* MCE during the manufacturing of fresh cheese to that of calf rennet on cheese characteristics, cheese yield was calculated and results are shown in Table 3.

Table 3. Variation in cheese yield.

Parameter	Storage period (day)	Cheeses samples		
		CR	CS	CL
Yield (%)	0	25.13 ± 0.28^{b}	17.98 ± 0.06^{a}	36.83 ± 0.19^{c}

CR: Control clotted cheese with animal rennet; CS: Clotted cheese with *Moringa* seeds MCE; CL: Clotted cheese with *Moringa* leaves MCE. Data are mean ± standard deviation, n = 3. Means with different superscripts are significantly different ($p < 0.05$). Lowercase letters (a, b, c) represent the statistical difference between samples.

The cheese yield is a parameter of great interest in the cheese industry. In this study, the obtained cheese yields showed significant differences ($p < 0.05$) between all analyzed samples with values of about 25.13, 17.98 and 36.83%, for CR, CS and CL cheeses, respectively. Similar yield (18.19 %) was found by Mahami et al. (2012) for traditional soft cheese obtained with latex and *Moringa* seed extracts. Besides, a curd made using kiwi (17.8%), melon (15.1%), and ginger (15.4%) extracts presented lower yields than chymosin curd (20.2%) (Mazorra-Manzano et al., 2013), which are in agreement with the results of (Bruno et al., 2010) who found lower *Bromelia hieronymi* cheese yield (14.52%), when compared to that of chymosin (16.05%). These findings are confirmed by previous studies, suggesting that most aspartic proteases from plants cannot successfully replace calf rennet, because of the great loss of protein during cheese making (Mazorra-Manzano et al., 2013). However, no significant ($p < 0.05$) difference was observed in the study of (Ben Amira et al., 2021), who reported similar curd yields derived from cardoon extract (18.16%) and calf chymosin (18.62%). This difference can be attributed in part to the difference in the dry matter content between the obtained clots.

Variation in biochemical properties

Protein and lipid contents of control and *Moringa* clotted cheeses are shown in Table 4.

Table 4. Biochemical properties of control and vegetable clotted cheeses.

Parameter	Storage period (day)	Cheeses samples		
		CR	CS	CL
Protein (%)	0	18.17 ± 0.26^{a}	18.7 ± 0.18^{c}	18.25 ± 0.05^{b}
Lipid (%)	0	16.02 ± 0.06^{a}	16.78 ± 0.14^{c}	16.5 ± 0.07^{b}

CR: Control clotted cheese with animal rennet; CS: Clotted cheese with *Moringa* seeds MCE; CL: Clotted cheese with *Moringa* leaves MCE. Data are expressed as mean ± standard deviation, n=3. Means with different superscripts are significantly different ($p < 0.05$). Lowercase letters (a, b, c) represent the statistical difference between samples.

The respective protein and lipid contents were about 18.7 ± 0.18% and 16.78 ± 0.14 %, respectively, for vegetable clotted cheese with seed MCE. These contents were higher than those noted for clotted cheese with calf rennet (18.17 ± 0.26% and 16.02 ± 0.06 %, respectively). These findings were assigned to the fatty acid composition and the richness of *Moringa* extracts in nutritional components, as reported by Waheed et al. (2017). In fact, the obtained values were attributed not only to protein and fat contents in raw milk, but also to the concentration of the extracted coagulants. These results are partially in accordance with those obtained by Abebe and Emire (2020), and García et al. (2012), who reported lipid contents varying between 13.04% and 19.37%, and protein contents ranging from 14.77 to 22.82%. In contrast, Uaboi-Egebenni et al. (2010) and Adetunji and Olutayo (2011), reported lower protein and lipid contents for cheeses clotted with apple leaf extracts from *Calotropis procera,* in the range of 12.56 and 14.43%, respectively. Other results found by Salih et al. (2020) showed higher fat (25.53 -25.92%) and protein (16.50-16.97%) contents in cheese prepared with *Moringa* seed extracts. It is important to note that, in the research conducted by Abdeen et al. (2021), no significant difference was observed in terms of protein content between veal rennet clotted cheese and the one produced using plant coagulant.

Variation in sensorial properties

Sensory evaluation of control and vegetable clotted cheeses at the beginning of storage is presented in Table 5.

Table 5. Sensory properties of control and vegetable clotted cheeses.

	Cheese samples		
Sensory property	CC	CS	CL
Color	4.42 ± 0.16^{b}	4.00 ± 0.15^{b}	1.22 ± 0.17^{a}
Texture	3.75 ± 0.23^{a}	3.94 ± 0.15^{a}	4.64 ± 0.15^{b}
Taste	0.80 ± 0.21^{a}	1.55 ± 0.33^{a}	1.00 ± 0.20^{a}
Flavor	3.72 ± 0.08^{a}	4.36 ± 0.10^{b}	4.16 ± 0.04^{b}
Overall appreciation	3.60 ± 0.27^{b}	3.86 ± 0.18^{b}	2.47 ± 0.32^{a}

CC: Control clotted cheese with animal rennet; CS: Clotted cheese with *Moringa* seeds MCE; CL: Clotted cheese with *Moringa* leaves MCE. Data are expressed as mean ± standard deviation, n = 3. Means with different superscripts are significantly different ($p < 0.05$). Lowercase letters (a, b, c) represent the statistical difference between samples.

In the present study, the results showed that sensory properties were significantly different ($p < 0.05$) between all analyzed cheeses. In fact, the highest color score was recorded for the control cheese (4.42), followed by clotted cheese using *Moringa* seed coagulant (4.00). In addition, *Moringa* seed coagulant affected the flavor of cheese samples. The highest flavor score ($p < 0.05$) was attributed to cheese with *Moringa* seed MCE, and the lowest value was registered for the control sample. Regarding the taste, although there were no significant differences ($P > 0.05$), the highest score was assigned to CS cheese, while control cheese was the less preferred in terms of this descriptor. No significant difference ($p < 0.05$) was also observed between cheese samples in terms of texture. Furthermore, cheese clotted with *Moringa* seed coagulant had the highest overall consumer acceptability. In contrast, cheese clotted with *Moringa* leaf coagulant was the less preferred. These findings are in agreement with the literature (Salih et al., 2020; Abdeen et al., 2021), reporting that the total sensorial scores of white cheeses with 4% *Moringa* seed extract and goat soft cheeses coagulated with partial purified *Moringa* MCE were higher than those attributed to other cheeses.

Effect of Moringa MCE on the evolution of cheese properties during refrigerated storage

Physico-chemical cheese properties

The evolution of biochemical and physico-chemical properties of clotted fresh cheeses using commercial calf rennet or partial purified *Moringa* MCE during refrigerated storage are shown in Table 6.

Table 6. Changes of physico-chemical properties of control and vegetable clotted cheeses during refrigerated storage.

Parameter	Storage period (day)	Cheeses samples		
		CC	CS	CL
pH	0	6.37 ± 0.03^{aD}	6.55 ± 0.01^{bA}	6.34 ± 0.04^{aD}
	4	6.23 ± 0.02^{bC}	6.45 ± 0.04^{bA}	5.51 ± 0.15^{aC}
	8	5.66 ± 0.04^{bB}	6.42 ± 0.07^{cA}	5.28 ± 0.03^{aB}
	12	5.36 ± 0.06^{bA}	6.36 ± 0.07^{cA}	5.14 ± 0.06^{aA}
Titrable acidity (%)	0	1.35 ± 0.03^{aA}	1.26 ± 0.03^{aA}	1.44 ± 0.02^{bA}
	4	1.80 ± 0.05^{aB}	1.71 ± 0.03^{aB}	1.89 ± 0.02^{bB}
	8	2.52 ± 0.07^{aC}	2.25 ± 0.05^{aC}	2.70 ± 0.12^{bC}
	12	4.05 ± 0.12^{bD}	3.60 ± 0.05^{aD}	4.50 ± 0.04^{cD}
Total solids (%)	0	30.07 ± 0.10^{bA}	41.49 ± 0.60^{cA}	28.33 ± 0.45^{aA}
	4	39.77 ± 0.20^{aB}	42.26 ± 0.10^{aA}	39.40 ± 0.68^{aB}
	8	44.47 ± 0.14^{aC}	50.17 ± 0.60^{aB}	49.51 ± 0.89^{aC}
	12	47.61 ± 0.25^{aC}	53.86 ± 0.59^{bB}	52.77 ± 0.19^{bC}
Ash (%)	0	4.61 ± 0.15^{bD}	4.08 ± 0.07^{aD}	5.48 ± 0.14^{cD}
	4	3.30 ± 0.07^{aB}	3.96 ± 0.08^{bC}	4.99 ± 0.17^{cC}
	8	3.31 ± 0.05^{aC}	3.24 ± 0.07^{aB}	3.35 ± 0.05^{aB}
	12	2.71 ± 0.18^{bA}	2.99 ± 0.17^{cA}	2.30 ± 0.18^{aA}

CC: Control clotted cheese with animal rennet; CS: Clotted cheese with *Moringa* seeds MCE; CL: Clotted cheese with *Moringa* leaves. Data are expressed as mean ± standard deviation, n = 3. Means with different superscripts are significantly different ($p < 0.05$). Lowercase letters (a, b, c) represent the statistical difference between samples; Uppercase letters (A, B, C) represent the statistical difference between the same sample during coagulation time.

Initial pH values noted on CL and CS cheeses were 6.34 ± 0.04 and 6.55 ± 0.01, respectively. These values corresponded to acidity of about 1.44 ± 0.02 and 1.26 ± 0.03°D, respectively. These results are in perfect agreement with those reported by Salih et al. (2020) and Akinloye and Adewumi (2014) on cheeses made with *Moringa* seed and *C. procera* extracts, respectively. During storage, it was shown that pH values decreased significantly ($p < 0.05$) to reach, after 12 days of storage, mean values of 5.14 ± 0.06 and 6.36 ± 0.07 for CL and

CS, respectively. These results are in line with those obtained by El-Siddig et al. (2018), who recorded a pH of 5.13 ± 0.03 in *Moringa* leaf clotted cheese after one month of storage under the same conditions of the control (5.50 ± 0.05). Indeed, this evolution of pH is probably due to differences in the degree of proteolysis of different cheeses, and therefore by the variation of free amino acids quality (Mankai et al., 2012). Moreover, the acidity of all cheese samples increased significantly ($p < 0.05$) during cold storage. These findings are in agreement with those noted by Abdeen et al. (2021).

Furthermore, initial total solids contents varied significantly ($p < 0.05$) from 28.33 ± 0.45 in CL to 41.49 ± 0.60 % in CS cheeses. These results are in line with those found by Uaboi-Egebenni et al. (2010) and Abebe and Emire (2020), who reported dry matter contents of about 36 and 43.66%, respectively, for cheeses made with *Calotropis procera* extracts. In this study, the obtained values increased significantly during refrigerated storage, up to the highest dry matter content (53.86 ± 0.59), found in clotted cheese with seed MCE. These levels are higher than that reported by Toro et al. (2016) for fresh cheese (42.75%). This finding is attributed essentially to the decrease in the water content resulting from the continuous expulsion of whey. In fact, this result could be explained by the fact that the high salt content promotes hydrophobic interactions and can cause a faster expulsion of serum from brined cheese, leading to a decrease in cheese moisture content (McMahon et al., 2009). Indeed, the lowering of water activity has an inhibitory effect on the microbial flora, accompanied by a decrease in the activity of water-soluble enzymes, as described by Toro et al. (2016).

For ash contents, the highest initial value (5.48 ± 0.14%) was registered in CL cheese, when compared to the other analyzed samples. Ash contents decreased significantly and reached values of 2.71 ± 0.18, 2.99 ± 0.17 and 2.30 ± 0.11, for CC, CS and CL cheeses, respectively, at the end of storage period. These findings are in agreement with that reported by Abdeen et al. (2021).

Evolution of color parameters

The evolution of color parameters of clotted fresh cheeses using commercial calf rennet or partial purified *Moringa* MCE during refrigerated storage are shown in Table 7.

Table 7. Evolution of color parameters of control and vegetable clotted cheeses during refrigerated storage for 12 days.

Color parameters	Storage period (day)	Cheese samples		
		CC	CS	CL
L*	0	93.26 ± 0.63^{cC}	90.31 ± 0.42^{bC}	78.02 ± 0.26^{aB}
	4	87.57 ± 0.67^{cB}	84.24 ± 0.53^{bB}	78.41 ± 0.24^{aC}
	8	86.80 ± 0.48^{cA}	82.07 ± 0.71^{bA}	74.31 ± 0.07^{aA}
a*	0	-2.26 ± 0.06^{aB}	-1.83 ± 0.04^{bB}	-2.27 ± 0.06^{aB}
	4	-2.75 ± 0.07^{aA}	-2.81 ± 0.05^{aA}	-3.09 ± 0.19^{bA}
	8	-2.61 ± 0.04^{bA}	-2.69 ± 0.01^{bA}	-2.94 ± 0.03^{aA}
b*	0	8.67 ± 0.08^{aA}	8.84 ± 0.10^{aA}	11.63 ± 0.17^{bA}
	4	9.35 ± 0.16^{aB}	9.92 ± 0.15^{aB}	14.00 ± 0.18^{bB}
	8	9.59 ± 0.07^{aB}	12.72 ± 0.21^{bC}	15.35 ± 0.10^{cC}

CC: Control clotted cheese with animal rennet; CS: Clotted cheese with *Moringa* seeds MCE; CL: Clotted cheese with *Moringa* leaves MCE. Data are expressed as mean ± standard deviation, n = 3. Means with different superscripts are significantly different ($p < 0.05$). Lowercase letters (a, b, c) represent the statistical difference between samples; Uppercase letters (A, B, C) represent the statistical difference between the same sample during coagulation time.

It was observed that control cheese presented the better luminosity L* during all storage periods, when compared to vegetable clotted cheeses. Besides, L* parameter decreased significantly ($p < 0.05$) for all analyzed cheese samples during refrigerated storage. This finding can be explained mainly by the proteolysis of milk caseins during storage, as reported by Tokuşoğlu et al. (2013). In addition, the a* values showed a dominance of the green color in cheeses produced using *Moringa* coagulants. This parameter increased significantly ($p < 0.05$) during storage until reaching very close values ($p > 0.05$) for all analyzed samples. This result is in perfect agreement with that of Shokery et al. (2017), who found that produced yoghurt using *Moringa* leaf extract is darker, yellowish and greenish, when compared to control. Also, b* values revealed the dominance of yellow color. These values increased significantly ($p < 0.05$) during storage for all analyzed cheeses, with the lowest values noted on control cheese.

Evolution of textural properties

The evolution of textural characteristics of control and vegetable clotted fresh cheeses during refrigerated storage are shown in Table 8.

Table 8. Evolution of textural parameters of control and vegetable clotted cheeses during refrigerated storage.

Textural Properties	Storage period (day)	Cheeses samples		
		CC	CS	CL
Hardness (N)	0	1.92 ± 0.01^{bA}	2.23 ± 0.04 ^{cA}	1.72 ± 0.03 ^{aA}
	12	3.32 ± 0.05 ^{aB}	3.60 ± 0.03 ^{aB}	4.59 ± 0.05^{bB}
Cohesiveness	0	0.90 ± 0.01 ^{bA}	0.87 ± 0.01 ^{aA}	0.87 ± 0.01 ^{aA}
	12	0.90 ± 0.03 ^{aA}	0.88 ± 0.03 ^{aA}	0.96 ± 0.003^{bB}
Springiness (mm)	0	0.74 ± 0.02 ^{aA}	0.78 ± 0.004 ^{aA}	0.77 ± 0.01 ^{aA}
	12	0.80 ± 0.00 ^{bB}	0.82 ± 0.01 ^{bB}	0.78 ± 0.01$^{a\,A}$
Gumminess (N)	0	2.13 ± 0.22 ^{cA}	1.94 ± 0.03 ^{bA}	1.49 ± 0.04 ^{aA}
	12	3.01 ± 0.11 ^{aB}	3.20 ± 0.12 ^{aB}	4.43 ± 0.05^{cB}
Chewiness (N.mm)	0	1.59 ± 0.20 ^{bA}	1.58 ± 0.03 ^{bA}	1.15 ± 0.04 ^{aA}
	12	2.40 ± 0.10 ^{aB}	2.45 ± 0.08 ^{aB}	3.44 ± 0.09 ^{bB}

CC: Control clotted cheese with animal rennet; CS: Clotted cheese with *Moringa* seeds MCE; CL: Clotted cheese with *Moringa* leaves MCE. Data are expressed as mean ± standard deviation, n = 3. Means with different superscripts are significantly different ($p < 0.05$). Lowercase letters (a, b, c) represent the statistical difference between samples; Uppercase letters (A, B, C) represent the statistical difference between the same sample during coagulation time.

The obtained results showed a significant ($p < 0.05$) difference in terms of hardness between produced cheeses regarding the use of different *Moringa* coagulants. The highest hardness value (2.23 N) was observed in cheese clotted with *Moringa* seed MCE. This result was lower than that (5.10 N) mentioned by Abdeen et al. (2021) in goat soft cheese with partial purified MCE from *Moringa oleifera* seed. For springiness, gumminess and chewiness characteristics, no significant differences ($p > 0.05$) were observed between control and *Moringa* seed coagulant cheeses. However, *Moringa* leaf coagulant affected significantly ($p < 0.05$) the textural properties of produced cheese. Furthermore, the gumminess and springiness values for clotted cheese using partially purified *Moringa* MCE (CS) were the highest ones, when compared to cheese clotted with partially purified *Moringa* leaf enzyme. It was noted that texture properties of all analyzed cheeses increased during storage period, which might be due to the moisture content, as reported by Calvo et al. (2007) and Awad et al. (2009). Besides, these findings are partially in agreement with those reported by Abdeen et al. (2021), who suggest that differences in texture profiles are related to cheese making process, texture measurements methods, plant extracts used, and to the moisture contents in the produced curds (Ben Amira et al., 2017).

Evolution of microbiological properties

The evolution of microbial flora counts in produced cheeses using calf rennet or *Moringa oleifera* proteases during refrigerated storage are shown in Table 9.

Table 9. Evolution of microbial counts (log CFU g^{-1}) of control and vegetable clotted cheeses during refrigerated storage.

Cheeses samples	Parameters	Storage period (days)		
		0	8	12
CC	Total bacterial count	2.38 ± 0.24^{cA}	2.81 ± 0.15^{bB}	3.27 ± 0.01^{bC}
	Total coliforms	< 1	< 1	< 1
	Yeasts and molds	< 1	< 1	1.45 ± 0.04^{b}
CS	Total bacterial count	2.08 ± 0.12^{bA}	2.43 ± 0.21^{aB}	2.65 ± 0.18^{aC}
	Total coliforms	< 1	< 1	< 1
	Yeasts and molds	< 1 ^{a}	< 1 ^{a}	1.26 ± 0.01^{b}
CL	Total bacterial count	1.96 ± 0.06^{aA}	2.38 ± 0.08^{aB}	2.51 ± 0.17^{aB}
	Total coliforms	< 1	< 1	< 1
	Yeasts and molds	< 1 ^{a}	< 1 ^{a}	1.31 ± 0.06^{b}

CC: Control clotted cheese with animal rennet; CS: Clotted cheese with *Moringa* seeds MCE; CL: Clotted cheese with *Moringa* leaves. Data are expressed as mean ± standard deviation, n = 3. Means with different superscripts are significantly different ($p < 0.05$). Lowercase letters (a, b, c) represent the statistical difference between samples; Uppercase letters (A, B, C) represent the statistical difference between the same sample during coagulation time.

The observed results showed significant differences ($p < 0.05$) between cheese samples in terms of microbial quality. The highest initial total bacterial count (2.38 CFU mL^{-1}) was noted for the control sample. This number reduced significantly ($p < 0.05$) in cheese produced using *Moringa* seed and leaf MCE, with respective values of 2.08 ± 0.01 and 1.96 ± 0.01 CFU g^{-1}. These results are lower than those enumerated by Salih et al. (2020).

Coliforms, yeasts and molds were not found in any of the examined samples at the beginning of storage, indicating that the cheeses' microbiological quality was satisfactory.

During storage, total bacterial count increased in all fresh cheeses' samples, and reached 3.27 ± 0.01 log CFU g^{-1} after 12 days of storage in control cheese. However, total coliforms remained absent during all storage

period in all cheese samples. This finding is in line with that of (Awad et al., 2009), who reported the absence of coliforms during the storage of cheese samples using lemon orange and grapefruit juices as coagulants.

In addition, yeast and molds counts remained absent until the final day of storage, when reached the highest count (1.45 ± 0.04 log CFU g^{-1}) in control cheese. In fact, yeasts and molds are the most common contaminants of dairy products, and are responsible for odor and flavor defects, as mentioned by Mileriene et al. (2021).

Conclusion

Vegetable milk clotting enzymes were extracted from Tunisian *Moringa oleifera* aerial parts and then, were partially purified in order to substitute calf rennet during cheese making.

Moringa seed extract was very active and it revealed the highest MCA and MCA/PA ratio when compared to other extracted proteases. Moreover, animal and vegetable coagulants presented similar effect on coagulation kinetic parameters during cheese manufacturing.

The data showed that *Moringa* seed MCE improved the biochemical, microbiological and textural properties of fresh cheese. Besides, cheese clotted with *Moringa* seed coagulant showed the highest overall acceptability when compared to cheeses clotted using calf rennet and leaf extract. Thus, *Moringa* seed protease could be used as a good substitute to animal rennet due to its easy availability and simple protease purification process, which could provide great potential for large scale food production.

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References

Abdeen, E. M. M., Ibrahim, O. A., & Kholif, A. M. M. (2021). Utility of *Moringa oleifera* waste as a coagulant in goat soft cheese production. *Heliyon*, *7*, e07536.

Abebe, B., & Emire, S. (2020). Manufacture of fresh cheese using east African *Calotropis procera* leaves extract crude enzyme as milk coagulant. *Food Science & Nutrition*, *8*(9), 4831-4842.

Adesina, R. A., Olugbenga, A. B. O., Ngozi, U. A., & Zacchaeus, S. O. (2021). Optimization of the production of local cheese from cow milk processed with the seed of *Moringa oleifera*. *Journal of Food Processing and Preservation*. https://doi.org/10.1111/jfpp.16189

Adetunji, V., & Olutayo, B. (2011). A comparative assessment of the nutritional contents of 'wara' a west African soft cheese using *Calotropis procera* and *Cymbopogon citratus* as coagulants. *African Journal of Food, Agriculture, Nutrition and Development*, *11*(7), 5573–5585. https://doi.org/10.4314/ajfand.v11i7

Akinloye, A., & Adewumi, O. (2014). Effects of local coagulants on the yield of cheese using cow and sheep milk. *International Journal of Development and Sustainability*, *3*(3), 150-161.

Anusha, R., Singh, M. K., & Bindhu, O. S. (2014). Characterisation of Potential Milk Coagulants from *Calotropis gigantea* Plant Parts and Their Hydrolytic Pattern of Bovine Casein. *European Food Research and Technology*, *238*, 997–1006.

AOAC International. (2005). *Official Methods of Analysis of AOAC International*. AOAC International.

Awad, K., Razig, A., & Babiker, N. A. (2009). Chemical and Microbiological Properties of Sudanese White Soft Cheese Made by Direct Acidification Technique. *Pakistan Journal of Nutrition*, *8*(8), 1138-1143. https://doi.org/10.3923/pjn.2009.

Badgujar, S. B., & Mahajan, R. T. (2012). Comparison of cysteine proteases of four laticiferous plants and characterization of *Euphorbia nivulia* Buch-Ham. latex glycosylated cysteine peptidase. *Indian Journal of Natural Products*, *3*(2), 152-160.

Banik, S., Biswas, S., & Karmakar, S. (2018). Extraction, purification, and activity of protease from the leaves of *Moringa oleifera*. *F1000Research*, *7*, 1151. https://doi.org/10.12688/f1000research.15642.1

Ben Amira, A., Besbes, S., Attia, H., & Blecker, C. (2017). Milk-clotting properties of plant rennets and their enzymatic, rheological, and sensory role in cheese making: A review.[3] *International Journal of Food Properties*, *20*(sup1), S76-S93.

Ben Amira, A., Blecker, C., Richel, A., Arias, A. A., Fickers, P., Francis, F., & Attia, H. (2018). Influence of the ripening stage and the lyophilization of wild cardoon flowers on their chemical composition, enzymatic activities of extracts and technological properties of cheese curds. *Food Chemistry*, *245*, 919-925.

Ben Amira, A., Arias, A. A., Fickers, P., Jaouadi, B., & Attia, H. (2021). Effect of brine concentration on physico-chemical characteristics, texture, rheological properties and proteolysis level of cheeses produced by an optimized wild cardoon rennet. *Journal of Food Science and Technology*, *58*, 1331–1340. https://doi.org/10.1007/s13197-020-04643-8

Benkahoul, M., Benchiheub, M., Bellil, I., Khelifi, D., & Maza, A. M. (2016). Physical and chemical properties of the acid protease from *Onopordum acanthium*: Comparison between electrophoresis and HPLC of degradation casein profiles. *African Journal of Biotechnology*, *15*(9), 331-340.

Ben Moussa, O., Mzoughi, M., Chouaibi, M., Boulaares, M., & Hassouna, M. (2019). The combined effect of phytosterols and lactulose supplementation on yoghurt quality. *Journal of Food and Nutrition Research*, *7*(4), 261-269.

Bruno, M. A., Lazza, C. M., Errasti, M. E., López, L. M., Caffini, N. O., & Pardo, M. F. (2010). Milk clotting and proteolytic activity of an enzyme preparation from *Bromelia hieronymi* fruits. *LWT - Food Science and Technology*, *43*(4), 695-701.

Calvo, M. V., Castillo, I., Díaz-Barcos, V., Requena, T., & Fontecha, J. (2007). Effect of a hygienized rennet paste and a defined strain starter on proteolysis, texture and sensory properties of semi-hard goat cheese. *Food Chemistry*, *102*(3), 917-924.

El-Siddig, E. E. S., Abdelgadir, W. M., Kabeir, B., Koko, M., & Ibrahim, R. (2018). Quality of White Cheese Made Using *Moringa oleifera* Leaf Extract. *Journal of Academic Industrial Research*, *7*(7), 7-11.

García, C., Rovira, R., Teruel, S., Boutoial, K., & Rodríguez, M. (2012). Effect of vegetable coagulant, microbial coagulant and calf rennet on physicochemical, proteolysis, sensory and texture profiles of fresh goat's cheese. *Dairy Science & Technology*, *92*(6), 691-707.

ISO 13299. (2016). *Sensory Analysis-Methodology-General guidance for establishing a sensory profile*. https://www.iso.org/standard/58042.html

Mahami, T., Ocloo, F. C. K., Odonkor, S. T., Owulah, C., & Coffie, S. A. (2012). Preliminary study on the influence of moringa seed extracts supplementation on the yield and quality of cottage cheese.[4] *International Journal of Recent Trends in Science and Technology*, *2*, 4-8.

Mahmoudi, I., Telmoudi, A., Ben Moussa, O., Chouaibi, M., & Hassouna, M. (2021). Quality characteristics of goat yogurt containing *Lactobacillus* probiotic bacteria. *Journal of Agricultural Science and Technology*, *23*(1), 83-96.

Mankaï, M., Boulares, M., Ben Moussa, O., Karoui, K., & Hassouna, M. (2012). Effet du stockage réfrigéré du lait cru sur la qualité physico-chimique et microbiologique du fromage tunisien semi-dur de type Gouda au cours de l'affinage. *Journal International de la Technologie Laitière*, *65*, 25-29.

Mazorra-Manzano, M. A., Perea-Gutierrez, T. C., Lugo-Sanchez, M. E., Ramirez-Suarez, J. C., Torres-Llanez, M. J., Gonzalez-Cordova, A. F., & Vallejo-Cordoba, B. (2013). Comparison of the milk-clotting properties of three plant extracts. *Food Chemistry*, *141*(3), 1902–1910.

McMahon, D. J., Motawee, M. M., & McManus, W. R. (2009). Influence of brine concentration and temperature on composition, microstructure, and yield of feta cheese. *Journal of Dairy Science*, *92*(9), 4169-4179.

Mileriene, J., Serniene, L., Henriques, M., Gomes, D., Pereira, C., Kondrotiene, K., & Malakauskas, M. (2021). Effect of liquid whey protein concentrate–based edible coating enriched with cinnamon carbon dioxide extract on the quality and shelf life of Eastern European curd cheese. *Journal of Dairy Science*, *104*(2), 1504-1517.

Ong, L., Henriksson, A., & Shah, N. P. (2007). Proteolytic pattern and organic acid profiles of probiotic Cheddar cheese as influenced by probiotic strains of *Lactobacillus acidophilus*, *Lb. paracasei*, *Lb. casei* or *Bifidobacterium sp. International Dairy Journal*, *17*(1), 67-78.

Salih, Z., Abdelsalam, H., Aburigal, A., & Sulieman, A. (2020). Physicochemical, Microbiological and Sensory Characteristics of White Cheese Made by Adding *Moringa Oleifera* Seeds Extract. *Academic Journal of Life Sciences*, *6*(7), 76-82.

Shokery, E. S., El-Ziney, M. G., Yossef, A. H., & Mashaly, R. I. (2017). Effect of green tea and *Moringa* leave extracts fortification on the physicochemical, rheological, sensory and antioxidant properties of set-type yoghurt. *Journal of Advanced Dairy Research*, *5*(179), 2.

Tajalsir, A. E., Ebraheem, A., Abdallah, A. M., Khider, F. J., Elsamani, M. O., & Ahmed, I. A. (2014). Partial purification of milk-clotting enzyme from the seeds of *Moringa oleifera*. *Journal of Microbiology, Biotechnology and Food Sciences*, *4*(1), 58-62.

Terefe, M. A., Kebede, A., & Kebede, M. (2017). Clotting activities of partially purified extracts of *Moringa oleifera* L. on dromedary camel milk. *East African Journal of Sciences*, *11*(2), 117-128.

Tokuşoğlu, Ö. (2013). The physicochemical, microbiological, organoleptic properties and antioxidant activities of functional cream cheeses fortified with lutein. *International Journal of Dairy Technology*, *66*(4), 527-534.

Toro, E. E. B., Valencia, J. U. S., & Molina, D. A. R. (2016). Characterization of a processed cheese spread produced from fresh cheese (quesito antioqueño). *Revista Facultad Nacional de Agronomía Medellín*, *69*(2), 8015-8022.

Uaboi-Egebenni, P. O., Okolie, P. N., Akintunde, T. I., Bisi-Johnson, O., Enwe, L., & Bessong, P. O. (2010). Proximate Analysis and Microbiological Quality of Cheese Produced from Raw cow milk obtained from Fulani Settlement in Ogun State Nigeria, Using Lactic Acid Bacteria and extract from Sodom Apple Leaf (*Calotropis procera*). *Pakistan Journal of Nutrition*, *9*(9), 920-925. https://doi.org/10.3923/pjn.2010.920.925

Waheed, M., Hussain, M. B., Majeed, M., Rehman, T. U., Khan, M. U., Shariati, M. A., & Glinushkin, A. P. (2017). Quality evaluation of cheese prepared by plant coagulants: *Carica papaya* and *Moringa oleifera* leaves. *Russian Journal of Agricultural and Socio-Economic Sciences*, *64*(4), 232-239.

Wang, X., Zhao, Q., He, L., Shi, Y., Fan, J., Chen, Y., & Huang, A. (2022). Milk-clotting properties on bovine caseins of a novel cysteine peptidase from germinated *Moringa oleifera* seeds. *Journal of Dairy Science*, *105*(5), 3770-3781.

Wang, Y., Yuan, C., Cui, B., & Liu, Y. (2018). Influence of cations on texture, compressive elastic modulus, sol-gel transition and freeze-thaw properties of kappa-carrageenan gel. *Carbohydrate Polymers*, *202*, 530-535.

Yegin, S., & Dekker, P. (2013). Progress in the field of aspartic proteinases in cheese manufacturing: structures, functions, catalytic mechanism, inhibition, and engineering. *Dairy Science & Technology*, *93*(5), 565-594.