Usefulness of *Moringa oleifera* seed extract as Coagulant for the production of fresh camel cheese

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Abstract

The current study aimed to enhance the coagulation properties of camel milk using enzymatic extracts derived from *Moringa oleifera* seeds. Experiments were conducted to evaluate the clotting activities of *Moringa* extract on camel milk and compare them with cow milk. Our results regarding the characterisation of the enzymatic extract showed an extraction yield of 54.3±1.8%. The optimum coagulation conditions were determined to be pH 5 and a temperature of 55°C. In addition, the enzymes exhibited substantial clotting activity (2.54 RU) on both camel and cow milk. The cheese samples showed significant oxidative and antibacterial activity.

Keywords: camel milk; cheese; clotting activity; extraction yield; MSE

Introduction

Camel milk is popular for its traditional anti-infective properties (Morrin *et al.*, 2021) as well as its anti-cancer properties (Krishnankutty *et al.*, 2018), anti-diabetic (Sboui *et al.*, 2022; Ashraf *et al.*, 2021). In addition, it is values more generally as a restorative agent for convalescent patients. These benefits are attributed to components such as antimicrobial factors such as Lactoferrin, Lysozyme, Lactoperoxidase, and Immunoglobulin (Konuspayeva *et al.*, 2004).

Camel milk is mainly consumed raw after milking or fermented, but it is rarely processed into cheese (Konuspayeva & Faye, 2016). Compared to cow milk, camel milk contains higher levels of whey protein, lower levels of αs1-casein, and a very low κ- to β-casein ratio. According to Bekele (2023), these factors affect the technological characteristics of milk’s acidic or enzymatic coagulation process, resulting in final curds that are weak, brittle and have an open body and texture. Hence, several studies have focused on the functional and coagulation properties camel milk’s proteins (Fguiri *et al.*, 2021; Mbye *et al.*, 2020). Despite the above difficulties, satisfactory cheese can be produced when cheese-making procedures are tailored to the unique characteristics of camel milk (Ramet, 2001). Much work was dedicated to improving the coagulum formation process using various proteolytic enzymes from animal, plant, and microbial sources.

Milk coagulation is the primary step in manufacturing most cheeses, and calf rennet has been the most widely used milk-clotting enzyme preparation (Mohamed *et al.*, 2009a). However, the last decades have witnessed a population explosion that increased cheese production and consumption demand. On top of this, the price of calf rennet significantly increased along with the reduced supply of natural calf rennet (Mohamed *et al.*, 2010). In addition, the use of animal rennet is limited by religious
and safety considerations, change in diet (vegetarianism), or fear of consumption of foods containing genetically engineered products (Roseiro et al., 2003). All these factors have necessitated the search for new proteases with high specific milk clotting activities and low general proteolytic activities to be used as a rennet substitute. Accordingly, interest has been directed toward discovering a milk-clotting enzyme that would satisfactorily replace calf rennet in cheese manufacturing, and numerous enzyme preparations of animal, microbial, and plant origin have been studied (Jacob et al., 2011). Microbial rennets produced by genetically engineered bacteria have proven to be suitable substitutes for animal rennet, but increasing attention has been directed towards natural rennet extracted from plants such as Ananas comosus, Carica papaya, Calotropis procera, Ficus carica, Calm viscera, Cynara cardunculus (Roseiro et al., 2003), Cynara scolymus (Sidrach et al., 2005), and Solanum dubium (Mohamed et al., 2009a,b) among others. Unfortunately, most of these plant rennets were found to be inappropriate because they possess high general proteolytic activity, which leads to the production of short peptides responsible for the defect in the flavour and texture of cheese (Anusha et al., 2014). An exception to this general rule is represented by the aqueous extract of Cynara cardunculus containing two aspartic acid-type proteases, named cardosin A and B (Verissimo et al., 1995), which has been used for years for the manufacture of sheep milk cheese in several areas of Portugal and Spain. However, the flowers of Cynara cardunculus are not used to produce cow-milk cheeses as they tend to produce a bitter taste because of the formation of several peptides identifiable in the digests of isolated bovine β- and α-casein (Macedo et al., 1996). Thus, searching for a rennet substitute from plant sources with a high ratio of milk clotting to general proteolytic activity was highly needed to overcome the abovementioned problems.

*Moringa oleifera* is grown in rural regions of Mexico, and its different parts, such as leaves, flowers, and seeds, are edible. It is a source of protein, calcium, iron, carotenoids, and phytochemicals, and it is employed for several applications in developing countries (Idris et al., 2016). Previously, it has been reported that *M. oleifera* is an interesting source of milk clotting enzymes. Pontual et al. (2012) reported the caseinolytic and milk-clotting activities of *M. oleifera* flowers using azocasein and skim milk as substrates, respectively. *M. oleifera* seed extract was also used as a milk-clotting agent, and the resulting curd was white and firm (Tajalsir et al., 2014). Despite the studies above on milk-clotting enzymes from *M. oleifera*, a deep evaluation of this potential source of a rennet substitute is still absent. Thus, this research aimed to determine the potential ability of *M. oleifera* seeds to coagulate camel milk and to investigate the use of *M. oleifera* in the production of soft cheese. In this study, we used cow milk as a control to assess the efficiency of the coagulation activity of *M. oleifera* seeds extract.

### Materiel and Methods

#### Milk samples

Fresh camel milk was collected from female camels (*Camelus dromedarius* belonging to the Arid Land Institute (IRA Medenine, Tunisia). Cow milk samples were collected from a private farm. Samples were brought to the laboratory in an isotherm container and were analysed and processed upon arrival.

#### Collection and preparation of Moringa Oleifera seed samples

Samples of *Moringa oleifera* seeds were obtained from a private farmer in the South of Tunisia. All samples were collected and dried at room temperature in the laboratory. Thereafter, each sample was ground using an all-purpose high-speed smashing machine and stored in closed containers in a freezer at -20°C until use (Amna et al., 2014).

#### Preparation of extract

*Moringa* seed extract (MSE) was prepared according to the methods described by Tajalsir et al. (2014) and Mohamed Ahmed et al. (2010). Briefly, 5 g of the obtained powder was macerated in 50 ml NaCl solution (5%) by stirring at 37°C for 4 h. The mixture was filtered (filtration tissue) and then centrifuged for 10 minutes at 3500 rpm and 4°C. The sediment was neglected, and the filtrate was taken and used to estimate the enzymatic activity according to the method.

#### Moringa seed extract characterisation

##### Extraction yield

The extraction yield R constitutes the ratio of the volume of the extract obtained and the total volume of the solution prepared for extraction. This yield is given as a percentage according to the following formula:

\[
R = \frac{\text{Extracted volume}}{\text{Total volume}} \times 100
\]

##### Physicochemical characterisation

The pH and dry matter of the enzymatic extract were determined using International standard methods (Afnor, 1993). The protein content was determined...
Moringa oleifera seed extract as coagulant

of milk, which can be coagulated by 1 ml of coagulant preparation in 100 seconds and at 30°C (Bengana, 2001). The formula is as follows:

\[
MCA = \frac{10 \times V}{Tf \times Q}
\]

MCA: unit of coagulant activity
V: volume of coagulant activity
Q: volume of milk used
Tf: flocculation time

Cheese-making process
Fresh camel and cow milk were pasteurised at 65°C for 30 minutes and then cooled to 40°C. The extract was then added to 10% milk and mixed thoroughly. The mixture was incubated at 55°C until coagulation. After coagulation, the whey was drained, and the obtained camel (CAMSE) and cow (CMSE) cheeses were kept at 4°C for further analysis.

Cheeses characterization
The cheese yield was calculated using the following formula:

\[
\text{Yield (\%) } = \frac{W_{\text{cheese}}}{W_{\text{milk}}} \times 100
\]

With:

\[
W (\text{Weight}) \text{ milk } = \text{volume of milk (ml)} \times 1.03
\]

The physicochemical characteristics were determined using International standard methods (Afnor, 1993).

As microbiological analysis, the total viable counts were determined on a plate count agar (Oxoid et al., UK) at 30°C for 72 h; total coliforms on violet red bile agar (Oxoid) at 30°C for 24 h; Staphylococcus on Bair Parker (Oxoid) at 37°C for 24 h; Lactic acid bacteria on MRS agar (Oxoid) at 30°C for 48h under anaerobiosis; and yeasts and moulds on Sabouraud chloromphenicol agar (Oxoid) at 30°C for 72h. Results were expressed as log colony-forming units per ml of milk or gram of cheese.

The fat-soluble vitamins and sugar content were determined by LC-MS chromatography, according to Albala Hurtado et al. (1997). GC-MS determined the fatty acids profile. The milk fat was extracted by centrifugation of camel milk (3500 rpm, 20 min, 4°C). Then, the milk fat was subjected to methylation using a methanol KOH (2 N) solution. The solution was mixed with hexane to extract the fatty acids methyl ester (FAME), and the supernatant was analysed using gas chromatography QP2010 Shimadzu (Tokyo, Japan) coupled with mass
The BHI (Brain Heart Broth) was inoculated with the pathogenic strains and then incubated for 24 hours at 37°C.

Barefoot et al. (1983) recommended methods include bringing extract and cheese into contact with the pathogenic indicator strain. Wells (4.5mm) were dug under sterile conditions on Muller-Hinton agar inoculated with the inducing (pathogenic) strain. The wells are filled with 80 μl of extract and cheese. The Petri dishes were put at a temperature of +4°C/4 h to allow good diffusion of the antimicrobial substance (Doumadji et al., 2010). The plates were incubated at 37°C. The inhibition zone formed around the wells was examined after 24 to 48 h of incubation (Hwanhlem et al., 2011).

The reading was done by measuring the diameter in mm of the inhibition zones (Zi). Inhibition was considered positive if the diameter was greater than 2 mm (Thompson et al., 1996). The measurement of the inhibition diameter Zi was carried out according to the following formula:

$$Zi (\text{mm}) = \text{inhibition zone diameter (mm)} - \text{Wells diameter (4,5 mm)}$$

**Statistical study**

All tests were replicated three times, and the data are expressed as the mean ± standard deviation (SD). The present work’s statistical analysis was carried out using XLSTAT software (2014.5.03, Addinsoft, Pearson edition, Waltham, MA, USA). Differences are considered significant at p < 0.05.

**Result and Discussion**

**Characterisation of enzymatic extract**

The physicochemical characteristics of MSE are presented in Table 1.

The extraction yield can be considered very interesting. The yield of a vegetal extract is influenced not only by the type of initial material but also by the extraction spectrometry. The Fatty acids were quantified and identified using FAME internal standards (2013).

**Antioxidant activity of cheese**

**Ferric-reducing power test**

The FRAP test is a direct test of total antioxidant power. An antioxidant can reduce ferric iron (Fe³⁺) to ferrous iron (Fe²⁺), according to the reaction described by (Oyaizu et al., 1986).

One ml of the extract was mixed with 2.5 ml of 1% potassium ferrocyanide (K₃Fe) solution and 2.5 ml of 0.2 M phosphate buffer pH 6.6. The mixture was incubated at 50°C for 20 min in a water bath. The reaction was stopped by adding 2.5% trichloroacetic acid (TCA). After centrifugation at 3000 rpm for 10 min, 2.5 ml of supernatant was collected and mixed with 3 ml of distilled water and 0.5 ml of 0.1% iron chloride (FeCl₃) solution. The positive control is represented by a solution of a standard antioxidant: ascorbic acid. The absorbance was measured for all samples at 700 nm. An increase in absorbance corresponds to an increase in the reducing power of the sample (Wang, 2012).

**DPPH radical scavenging activity**

The determination of antioxidant activity by the DPPH test was carried out using the method described by (Brand-Williams et al., 1995), slightly modified by (Pyo et al., 2006). A DPPH (0.1 mM) solution was prepared by dissolving 3.94 mg of this product in 100 ml of methanol. Then, add 1 ml of extract or 1g of cheese and 1.5 ml of methanol. After incubation for 24 h, the extracts were centrifuged at 3500 rpm for 20 min at 4°C, then 1 ml of the DPPH solution and 1.5 ml of methanol were added to 0.5 ml of supernatant. The control tube contains 1.5 ml of the DPPH solution and 1.5 ml of methanol. The absorbance was measured at 517 nm after 30 minutes of incubation in the dark. The antioxidant activity linked to the trapping effect of the DPPH˙ radical was expressed as inhibition percentage (IP) using the following formula:

$$IP = \frac{A_b - A_c}{A_a} \times 100$$

$A_a$: absorbance of DPPH

$A_c$: Sample absorbance

$A_b$: absorbance of the white tube

**Antibacterial activity**

The antibacterial activity of the selected isolates was determined by agar diffusion assay. The indicator bacteria used in this study were E. coli, Klebsiella pneumonia: Kp, S. aureus, Salmonella tipi, Listeria inocula, Pseudomonas aeruginosa, and Enterococcus fecalis (Lab collection).
Moringa oleifera seed extract as coagulant conditions (extraction solution, extraction duration, pH, temperature, etc.) (Benyahia, 2013).

MSE presents a high total protein content (15.42 g/l), which seems higher than that found by Muñoz et al. (2017), who showed a protein content in the same type of extract equal to 10.3±0.45mg/ml.

Polyphenol contents

MSE presented a polyphenol content of 148.5 μgGAE/ml. Furthermore, the leaves of M. oleifera were known for their richness in phenolic compounds more than Seeds (Kasolo et al., 2010; Moyo et al., 2011; Baba et al., 2015). Indeed, it has been reported that the phytochemical composition of plants depends on several variables, such as the age of the plant, the harvest season, the harvested part and the climatic conditions (Miliauskas et al., 2004; Tomas-Menor et al., 2013).

Optimum conditions of the enzymatic extract

As shown in Figure 1, the temperature for the optimal clotting activity of the enzymatic extract of Moringa Seeds was 55°C. The effect of temperature proceeds mainly on the secondary phase of coagulation, specifically the aggregation step. This is due to the importance of hydrophobic interactions in the aggregation of hydrolysed micelles (Boudjneh, 2012)

The optimal clotting activity of MSE was at pH 5. Ramet (1989) reported that all enzymes used in cheese manufacture are acidic proteases, and their activity is generally optimal at pH values close to 5.5. Few studies on milk-clotting substitutes for calf rennet applied to camel milk are available. This agrees with Yonas et al. (2014), who reported that the highest clotting activity was observed at pH 5.0.

CaCl₂ concentration has no significant effect on clotting activity. Similar results were mentioned by Castillo et al. (2002), who considered that the main effect of CaCl₂ is important in aggregation and firming. Thus, it is usually added as a texturing agent. Indeed, the presence of ionised calcium is essential for the achievement of the secondary phase of milk coagulation since Ca²⁺ ions neutralise negative casein micelle residues to form a firm curd in the second phase of the coagulation process (Barrett et al., 1998).

Milk clotting activity (MCA)

The obtained milk clotting activity for MSE was 2.54 RU, which was higher than that reported by Boudjinah et al. (2011) and Siboukeur et al. (2005). Their studies found

Figure 1. Optimal coagulation conditions of MSE. (A) optimum temperature; (B) optimum pH; (C) calcium chloride concentration.
maximum coagulant activities of 0.360 RU and 0.174 RU while working on the camel abomasum and 0.149 RU using commercial cow rennet, respectively.

**Physicochemical characteristics of curd cheese**

The physicochemical characteristics of camel and cow cheese with enzymatic extracts of Moringa Seeds are shown in Table 2.

Table 2 showed that CAMSE was more acidic and richer in dry, ash and fat matter than CMSE. Fresh cheeses of both types of milk were characterised by low and minimal mineral content due to the significant losses that occur during coagulation at the whey level. This is still in the range of 60–70% reported for fresh cheese (Lobato-Calleros et al., 2006; Torres-Llanez et al., 2006).

The major fat-soluble vitamin in prepared cow cheeses is vitamin A, which is described as an indicator of good cheese quality. In addition, the variation in the fat-soluble vitamin content of cheeses depends on the fat content of the milk used as raw materials, the addition of cream and the dry matter concentration achieved during draining (Bouraoui, 2014). Vitamin K is found in cow and camel cheeses.

**Fatty acid content**

The relative proportions of the different fatty acids obtained by gas chromatography are indicated in the following table (Table 3).

The composition of CAMSE revealed a low content of short and medium-chain fatty acids (from C4 to C12) and a relatively high content of long-chain fatty acids. These results support the good digestibility of this fat and a relatively important nutritional criterion (Gnan & Sheriha, 1986; Farah & Ruegg, 1989; Gorban & Izzeldin, 2001). The level of palmitic acid (C16:0) in CAMSE (32.393%) was similar to that reported in camel milk (31.45 %, according to (Attia et al., 2000).

CAMSE contained 61.868% of saturated fatty acids and 38.132% of unsaturated fatty acids.

**Sugar composition**

The sugar contents of the obtained cheeses are mentioned in Table 4.

CMSE are richer in lactose than camel cheeses. This richness can be attributed to cow milk’s higher lactose content than camel milk (Konuspayeva et al., 2008).
Microbiological quality

The microbiological quality of cow and camel cheeses is shown in Table 5.

The microbiological quality of the cheeses shows that cow cheese is richer in total aerobic mesophilic flora than camel cheese. This may be due to antibacterial proteins in camel milk (Jrad et al., 2013). Indeed, the total flora is a usual criterion for the hygiene of processes in factories. According to Martin (2012), their evidence in foods means inadequate or too careful conservation of raw materials. Despite the high load of total mesophilic flora, the number of germs found remains below the thresholds indicated by the Tunisian standards relating to the microbiological specifications of milk and derived products (NT 16.40. 1988) (10^4 CFU/ml)).

The bacterial flora present in cheeses can originate from the starting milk, the equipment, and the production environment or from the addition of lactic ferments during manufacturing (Ozturkoglu-Budak et al., 2017; Pyz-Lukasik et al., 2018).

Antioxidant activity

The results of the antioxidant activity of MSE and the camel and cow cheeses are represented in Figure 2. The MSE presents a high antioxidant activity (33.64%), which agrees with the results of Dalei et al. (2016). With different methods (DPPH and Frap test), camel cheeses’ anti-radical activity is higher than cow cheeses. This can be attributed to the richness of camel milk in vitamin C.

Antibacterial activity

Figure 3 represents the results of the antibacterial activity of an enzymatic extract of Moringa Seeds and camel and cow cheeses.

The MSE shows an inhibitory effect on all strains tested with an inhibition diameter varying between 11 mm ±1 and 15.5 mm ±0.5.

Indeed, in plants, contamination by pathogenic microorganisms leads to a sharp increase in the levels of phenolic compounds, which corresponds to establishing the plant’s defence mechanism (Macheix et al., 2006; Meziani

Table 4. Composition of cheeses obtained in sugars (g/L).

<table>
<thead>
<tr>
<th></th>
<th>Camel cheese</th>
<th>Cow cheese</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose</td>
<td>0.14±0.03</td>
<td>0.17±0.00</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.14±0.00</td>
<td>0.15±0.00</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.17±0.01</td>
<td>0.15±0.05</td>
</tr>
<tr>
<td>Lactose</td>
<td>7.04±0.70</td>
<td>9.33±3.10</td>
</tr>
</tbody>
</table>

Table 5. Microbiological quality of cow and camel cheeses with Moringa seeds.

<table>
<thead>
<tr>
<th></th>
<th>FMAT (UFC/g)</th>
<th>CT (UFC/g)</th>
<th>MY (UFC/g)</th>
<th>Staphylococcus (UFC/g)</th>
<th>LAB (UFC/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Camel cheese</td>
<td>2.38±0.11 × 10^3</td>
<td>6.18±0.04 × 10^2</td>
<td>0</td>
<td>3.54±0.06 × 10^2</td>
<td>2.58±0.15 × 10^5</td>
</tr>
<tr>
<td>Cow cheese</td>
<td>6.81±0.26 × 10^3</td>
<td>9.54±0.18 × 10^2</td>
<td>0</td>
<td>2.50±0.16 × 10^2</td>
<td>1.23±0.23 × 10^5</td>
</tr>
</tbody>
</table>

FMAT: total aerobic mesophilic flora; CT: Total coliforms MY: Molds and Yeast; LAB: Lactic Acid Bacteria.

Figure 2. Antioxidant activity of MSE and obtained cheese. (A) DPPH test. (B) Frap test with MSE: enzymatic extract of Moringa seeds; CAMSE: Camel cheese with Moringa seeds extract; CMSE: Cow cheese with MSE; AA: ascorbic acid.
et al., 2015). Some authors have noted that the antibacterial activity of phenolic compounds is probably due to their ability to combine with extracellular soluble proteins and, thus, with bacterial cell walls (Tsuchiya et al., 1996).

Figure 4 shows camel cheese has strong activity against Pseudomonas aeruginosa, Candida albicans, and Micrococcus luteus, with an inhibition zone ranging from 12±0.5 to 14±2mm. It also has middling activity against Streptococcus pyogenes, Escherichia coli, and Staphylococcus aureus (inhibition diameter between 9±1 and 10±0.5mm) and low activity against Listeria inocula, Salmonella typhi, and Enterococcus faecalis.

The antibacterial activity of camel cheese was higher than that of cow cheese, which can be due to the combination of MSE seeds and the antibacterial activity of camel milk (Jrad et al., 2013).

Conclusion

The extract of Moringa oleifera seeds revealed a high milk-clotting activity on camel milk and high antioxidant and antibacterial effects.

Moringa oleifera seed extract can be used as an alternative to rennet to produce camel milk cheese. However, further studies are requested to complete the purification and characterisation of this promising extract.

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