Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Improvement of camel milk-clotting: Usefulness of crude extract from green pods of carob (*Ceratonia siliqua*. L) as a substitute for commercial rennet

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ARTICLE INFO

Original paper

Article history:
Received: April 15, 2023
Accepted: September 07, 2023
Published: December 10, 2023

Keywords:

Camel milk, crude carob extract, clotting activity, proteolytic activity, commercial rennet, electrophoresis

ABSTRACT

Camel milk transformation into cheese remains an objective to be improved today. This study aimed to improve camel milk clotting using a crude extract from green pods of carob as a substitute for commercial rennet. The composition of the crude carob extract was determined for dry matter and protein content. Milk clotting conditions were studied at different temperatures, pH and $CaCl_2$ concentrations. Milk clotting properties were assessed by milk clotting activity, specific activity and proteolytic activity. Enzymatic hydrolysis of camel milk caseins by crude carob extract and its inhibition were demonstrated by SDS-polyacrylamide gel electrophoresis. Crude carob extract analysis showed a protein and dry matter content of 23.26±0.5 mg/ml and 30.66±0.5 g/l, respectively. Optimal milk clotting activity was observed at 53.6 °C, pH 4.5, and 0.09 M $CaCl_2$. The crude carob extract showed a high milk clotting activity (4.97 U/ml) and a low proteolytic activity (0.04U/ml) with camel milk. The cheese yield of curd produced from camel milk using crude carob extract was the highest (23.95%) compared with that of Camel chymosin (20.5%). The high ratio of milk-clotting to proteolytic activity shows the potential of this extract as a substitute for commercial rennet in the dairy industry.

Doi: http://dx.doi.org/10.14715/cmb/2023.69.13.14

Introduction

The dromedary (*Camelus dromedarius*) is perfectly adapted to the drylands, which are continually expanding. Camel milk is the most specific product consumed by rural communities' populations in dry regions of Africa and the Middle East. It is characterized by its richness in all essential nutrients (proteins, carbohydrates, and fatty acids), and therapeutic interest, such as anti-cancer activity, hypoallergenic activity, and antidiabetic activity (1,2). Because of these various advantages, the interest in camel milk products has progressively increased to valorize this milk on a large commercial scale (3). The main challenge is to succeed in camel milk processing into dairy products. It is a complex process compared to bovine and goat's milk (4). According to several comparative studies with bovine milk, some specificities related to this limited coagulation ability have been identified, including low k-casein concentration (5) and more marked physical stability towards coagulation by acidification (6). Several studies have provided important information on the use of commercial enzymes (rennet and chymosin), crude gastric enzymes extracted from camel abomasum, transgenic camel chymosin, and the enzymatic extract of the kaolin layer (EKL) of chicken gizzards in camel milk coagulation. (7,8). Moreover, the increase in cheese production, coupled with a decrease in the supply of natural animal rennet and leads to an increasing claim for alternative substitutes to commercial rennet. For this reason and various other factors (religious beliefs, vegetarianism, etc.), attention focused on the use of coagulants extracted from plants (9). Therefore, some plant extracts have been used as clotting agents for some types of milk as ovine and bovine milk (10,11). However, few references are available for using plant crude extracts for camel milk cheese making (12-14). Several methodologies were previously performed regarding the preparation of plant coagulant extracts. However, the parameters influencing the efficiency of aqueous extraction have received little attention, although its application is of prime interest to the food industry (15). Depending on the extraction solvent and ripening stage of carob pods, the phytochemical contents and the antioxidant activity of the aqueous extract of carob pods were higher at the unripe stage and lower at the ripe stage (16,17). Therefore, the aqueous crude extract of carob green pods can serve as an excellent source of health-promoting phytochemicals and an interesting additive in functional food formulations by using it as a camel milk clotting agent.

Thus, the present study merits the usefulness of the aqueous crude extract from green pods of carob (*Ceratonia siliqua*. L) as a novel source of proteases for clotting camel milk and its suitability to substitute commercial rennet.

Materials and Methods

Experimental materials

Samples of Camel Milk (CM) were collected from herds reared in the livestock and wildlife laboratory at

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Arid Regions Institute (IRA) (Medenine, Tunisia). Fresh samples of green pods of carob (*Ceratonia siliqua*. L) were collected from the region of Medenine, Tunisia. Camel Chymosin (CC) FAR-M stick (Chr. Hansen A/S, Horsholm, Denmark) was used to coagulate the milk for comparison with the Crude Carob Extract (CCE). Calcium chloride ($CaCl_2$, 98% purity, MW: 147.02 g/mol) and sodium hydroxide (NaOH, 98% purity, MW: 40 g/mol) were obtained from Loba Chemie, Mumbai, India. Hydrochloric acid (HCl, 99% purity MW: 36.46 g/mol) and trichloroacetic acid (TCA, 98% purity, MW: 163.39 g/mol) were obtained from SRL Chemicals, Mumbai, India. Bovine serum albumin (BSA, 96% purity, MW: 66 kDa) was purchased from Merck. Bradford protein assay reagents were obtained from Bio-Rad Laboratories, Munich, Germany.

Experimental methods

Preparation of CCE

The water extraction method was used for the preparation of CCE. Crude carob extract was prepared from fresh green pods (100 g) mixed with 200 ml of distilled water using a Moulinex (Blender LM2421EG, Egypt). The extract was centrifuged at 5000 rpm for 30 min at 4 °C and the supernatant was stored at 4 °C in sterile and dark-colored flasks to prevent oxidation.

Characterization of CCE

Extraction yield

Extraction yield (%) was defined as the ratio of the weight of juice extracted to the total weight of wet bagasse. It was calculated by the following equation described by Tressler and Joslyn (18):

$$\text{Extraction yield (\%)} = ((\text{Je(g)}) \,/\, ((\text{Je (g)}) + \text{Wr (g)})) \times 100$$

Where: Je = Weight of extracted CCE juice (g); Wr = Weight of residue (g)

Protein content

Total protein concentration was determined by the Bradford method (1976), using BSA as the standard for protein quantification at concentrations ranging from 10 mg/ml to 80 mg/ml.

Dry matter content

Dry matter content was determined after drying 10 ml of CCE at 105°C for 24 hours to a constant mass (19):

$$\text{DM (g/l)} = ((\text{M1} - \text{M0}) \,/\, \text{V}) \times 1000$$

Where: DM (g/l): dry matter, M0: mass in (g) of the empty crucible, M1: mass in (g) of the crucible with residue after drying and cooling, V: volume in (ml) of sample

Optimal coagulation temperature, pH and $CaCl_2$ concentration

The response optimization option in the DOE (Design Of Experiments) module of the Minitab 17 statistical software was used to optimize the optimal milk coagulation temperature. After setting the temperature at 30 °C, the optimal pH was determined by varying the pH of Berridge Substrate (BS) from 4.5 to 8 with a range of 0.5 using 0.1 M NaOH and 0.1 M HCl solutions. In each pH, 1 ml of CCE was added to 10 ml of BS and the flocculation time was noted for each pH. The optimal calcium chloride, $CaCl_2$ concentration was determined by varying the $CaCl_2$ ion concentration of Berridge Substrate (BS) (12 g of skimmed milk powder in 100 ml of a 0.01 M $CaCl_2$ solution (Berridge 1952) from 0.01 M to 0.09 M with a range of 0.01 M at 30 °C and pH 6.6. The flocculation time was recorded for each concentration.

Milk-Clotting Activity (MCA)

The MCA was measured according to the Berridge method (20), modified by Collin et al. (21): 1 ml of CCE was added to 10 ml of the substrate (BS and CM) and recording the clotting time at 35 °C. The MCA was calculated using the following equation (22):

$$\text{MCA (U/ml)} = (2400 \,/\, \text{t}) \times (\text{S} \,/\, \text{E})$$

Where t: clotting time (sec), S: volume of milk (ml), E: volume of enzymatic solution (ml)

Specific milk-clotting Activity (SA)

The SA was defined by the ratio of MCA to protein content:

$$\text{SA (U/mg)} = (\text{MCA (U/ml)}) \,/\, (\text{Protein content (mg/ml)})$$

Proteolytic Activity (PA)

The PA of the CCE was determined using BS and CM as substrates (23). Briefly, 1 ml of substrate solution was mixed with 1 ml of CCE, and the mixture was incubated at 35°C for 60 minutes. The reaction was stopped by the addition of trichloroacetic acid, TCA (12%) (w/v), then the absorbance was measured at 280 nm. One unit of the PA was defined as the amount of protein (mg) required to increase the absorbance by one unit.

Enzymatic hydrolysis of caseins by CCE and its inhibition

Camel milk casein powder was dissolved (1%) in 100 mM sodium phosphate buffer, pH 6.8 for 2 h at room temperature. The hydrolysis profile of camel caseins by CCE proteases was assayed by incubating a mixture of 350 µl of CCE and 3 ml of sodium caseinate solution at 53.6°C for 1h, 3h, 6h and 9h. The hydrolysis was stopped by heating the samples at 100°C for 5 minutes. The hydrolysis of camel caseins was inhibited by incubating the CCE in a protease inhibitor solution for 60 minutes, using protease inhibitor cocktail tablets (one tablet is sufficient to inhibit a very high proteolytic activity in 25 ml of extraction solution).

SDS–polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed, using the method of Laemmli (24), with a 15% (w/v) acrylamide separating gel in 0.125 M Tris–HCl buffer, pH 6.8 and a 5% (w/v) acrylamide stacking gel in 0.38 M Tris–HCl buffer, pH 8.8 containing 0.1% (w/v) SDS. Samples were mixed in equal volumes in sample buffer (0.125 M Tris-HCl buffer (pH 6.8), 2 % (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.1% (w/v) bromophenol blue). After heating the mixture at 100 °C for 5 min, 10 µL of the sample was loaded in the gel and electrophoresis was carried out at a current vol-

tage of 150V for 2h in electrophoretic tris–glycine buffer containing 0.1% SDS, pH 8.16. After migration, the gel was stained for 3h in coloration solution (Coomassie Blue R-250 (0.1%), acetic acid (10%), ethanol (30%) and distilled water (50%), followed by destaining into discoloration solution (acetic acid (10%), ethanol (30%), and distilled water (60%)).

Cheese making process

Camel milk cheese was produced by the traditional method. After receiving the raw milk, pasteurization was performed at 65 °C for 30 min. After cooling to 40 °C, $CaCl_2$ (0.2 g/l), CCE or CC were added to the CM. After coagulation, the whey was drained and the camel milk cheese was stored at 4 °C until analysis.

Cheese yield

After draining for one night at 4-6°C, the obtained curd was used to determine the cheese yield. This parameter was calculated by the following formula described by Sulieman et al. (25):

$$\text{Cheese yield (\%)} = ((\text{weight of cheese}) / (\text{weight of milk})) \times 100$$

Statistical analysis

Statistical analysis and graphs were performed using the XLSTAT 2019 (Addinsoft 2019, Paris, France) and GraphPad Prism 7.0 (GraphPad Prism software Inc, California, USA), and the experiments were conducted in triplicate. The correlation was conducted with Pearson's test. The values were expressed in terms of mean ± standard deviation, and means were compared using Tukey's multiple comparison tests. Results were considered significant if the P-value <0.05.

Results

In this study, the extraction yield obtained from carob green pods was 71.05±1.8%. The CCE showed a protein content of 23.26±0.5 mg /ml and a dry matter content of 30.66±0.5 mg /ml.

The optimal clotting activity of CCE with CM obtained using the MINITAB 17 statistical software (Minitab, Inc. State College, Pennsylvania, USA) was at 53.6 °C. A significant negative correlation (r=-0.763, p<0.0001) was found between pH and MCA and a significant positive correlation (r=0.918, p<0.0001.) was found between $CaCl_2$ concentration and MCA (Figure 1).

As shown in Table 1, the MCA of CCE with CM was significantly higher (4.97±0.03 U/ml, P<0.0001) than with BS (3.58 ±0.01 U/ml). The SA of CCE with CM was significantly (P<0.0001) higher (0.214±0.00 U/mg) than with BS (0.154 ±0.00 U/mg). The PA of CCE is significantly higher especially with CM (P<0.05) (0.04±0.00 U/ml) in comparison with BS (0.03±0.00 U/ml). Also, the results indicate that CCE showed a significantly higher ratio of MCA/PA with CM (P<0.0001) compared with BS.

The hydrolysis profile of camel caseins by CCE at different stages of coagulation is shown in Figure 2. The casein hydrolysis was assessed by SDS-PAGE using a standard molecular weight marker. Camel caseins migrated as a large band (at approximately 27 kDa) (Figure 2, lane 1 and lane 6). The highest molecular weight band was identified as α-casein, while the largest band was represented as β-casein, just above the κ-casein.

In the first hours of coagulation (1h-3h), κ-casein was preferentially hydrolyzed, while α-casein and β-casein bands were slightly reduced (Figure 1, lanes 2-3). After later coagulation hours (6h-9h), the κ-casein and α-casein bands were not visible, indicating complete hydrolysis and the β-casein band was greatly reduced (Figure 2, lanes 4-5).

The cheese yield obtained by CM clotting with CCE was significantly (P<0.0001) higher (23.9%) than that obtained with CC (20.5%).

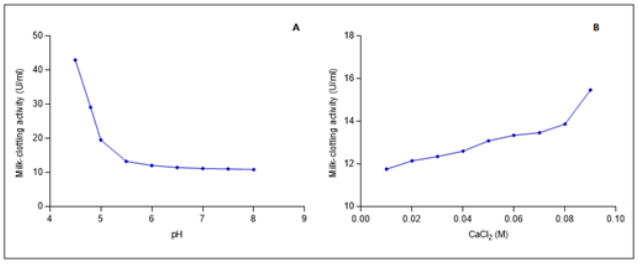

Figure 1. Effect of pH and $CaCl_2$ concentration on the milk-clotting activity of crude carob extract.

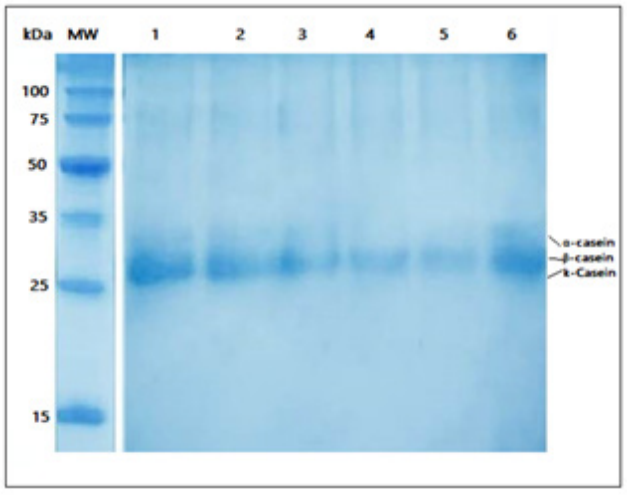

Figure 2. SDS-PAGE patterns of camel milk caseins hydrolysis by CCE proteases. MW: molecular weight marker. Lane 1: camel milk caseins; lanes 2-5: camel milk caseins incubated with CCE for 1h, 3h, 6h and 9h, respectively; Lane 6: Camel milk caseins incubated with CCE for 9h after inhibition of its proteolytic activity by protease inhibitors cocktail.

Table 1. Effect of CCE on BS and CM coagulation.

Type of milk	MCA (U/ml)	SA (U/mg)	PA (U/ml)	Ratio MCA/PA
BS	3.58[a]	0.15[a]	0.03[a]	97,54[a]
CM	4.97[b]	0.21[b]	0.04[b]	122,91[b]

MCA: Milk Clotting Activity; PA: Proteolytic Activity; SA: Specific Activity; CCE: Crude Carob Extract; BS: Berridge Substrate; CM: Camel Milk.

Discussion

Characterization of CCE

Extraction yield of CCE

The extraction yield depends on the species and water content of the plant and the extraction methods (26). Akinloye & Adewumi (27) reported similar high yields for aqueous extracts of papaya (*Carica papaya*) (75.8%) and Sodome apple (*Calotropis procera*) (89.5%) used in the manufacture of cow's and sheep's milk cheese. A study carried out by Hachana et al. (28) using caprifig tree extract as a substitute for calf rennet in goat's fresh cheese, showed that the extraction yield was 36.5 %, using a Moulinex (JU610D10) centrifugal extractor without using any chemicals.

Protein content

For the protein content, similar results were obtained with the crude extract of fig tree latex (*Ficus carica*) with a protein content of 22 mg/ml, which is higher than that obtained with its purified extract (6 mg/ml) and that of rennet (1.23 mg/ml) (29). In addition, the coagulant fruit extract of *Withania somnifera* L. had a lower protein content (2.47 mg/ml) than that obtained with CCE (30).

Dry matter content

Few references were found in the literature about the dry matter content of crude plant extracts. Grek et al. (31) showed that Plantago major juice had a dry matter content of 4.5±0.23%. Some studies have shown that the type and concentration of coagulant did not affect the dry matter content of various types of cheese (32,33). In contrast, López et al. (9) found that the type of coagulant influenced the dry matter content of cheeses, and the highest dry matter values correspond to the cheeses made with vegetable coagulants.

Optimal coagulation conditions

Each coagulant extract has its temperature susceptibility. For example, Liang et al. (34) showed an increase in SA in pineapple peels as the temperature increased, with a maximum value of 55 °C. Similar results were obtained with *Moringa oleifera* flower and *Opuntia ficus-indica* fruit extracts with optimal clotting activity at 50 °C and 55 °C, respectively (35,36).In addition, the high temperature (above the normal pasteurization temperature) can lead to undesirable proteolysis, resulting in loss of flavour and bitter taste (37). Thus, the optimal coagulation temperature of CCE (53.6 °C) suggested that this extract could be a safe and useful coagulant agent in dairy applications. The pH affects both the enzymatic and protein aggregation phases of the milk coagulation process, although the influence of pH on protein aggregation is more profound (38). Amira et al. (39) found that rennet extracts from *Cynara cardunculus* are more active at acidic pH, using the reconstituted bovine skimmed milk. Hashem. (40) also observed that an increase in the pH of the reaction mixture was associated with a loss of milk coagulation activity (at pH 7), which is in agreement with our results. In the present study, the flocculation that occurs at pH 4.5 using CCE as a coagulant agent may be caused by the presence of an acidic protease in the CCE that could also influence the coagulation kinetics. The variations observed with $CaCl_2$ concentration could be due to a direct effect on casein micelles dissociation because the addition of $CaCl_2$ reduces the pH of milk, resulting in an increased protein aggregation rate (41). Moreover, $CaCl_2$ is known to be a determining factor in the clotting ability of CM because the addition of calcium ions, in the form of calcium chloride, significantly improves the CM coagulation (coagulation time and firmness) with a subsequent increase in the cheese yield (42,43).

Milk coagulation study (MCA, SA and PA)

Similar results were obtained by Moreno-Hernández et al. (44) who found that crude extract from *Bromelia pinguin* L. fruit showed an efficient capacity to clot milk in a short time with a maximum MCA of around 3.99 U/ml.

In addition, Fguiri et al. (13) proposed some plant extracts as CM coagulants with an important MCA, particularly with the enzymatic extract of kiwi (*Actinidia arguta*) compared to the enzymatic extracts of pineapple (*Ananas comosus*) and ginger rhizome (*Zingiber officinale Roscoe*). Thus, further studies should be conducted to identify the nature of proteases present in CCE. Plant coagulants have long been suggested as possible alternatives to chymosin in the cheese-making process, but their suitability for use depends on their specificity, and catalytic properties (45). The CCE has a higher SA with the CM than that of BS. This result shows that the ability of the coagulant to form a curd is dependent on its specificity for milk proteins and the type of substrate. The SA of CCE observed with CM was higher than the specific protease activity of ginger rhizome juices from the Tosataichi variety (0.080 U/mg) and Ogon Kokuzo II variety (0.017 U/mg) (47). In the cheese industry, the coagulation enzymes used are always sought to have a high coagulant activity and a low proteolytic activity (48), so CCE seemed to be the best because excessive proteolysis can lead to a decrease in cheese yield due to loss of peptides in the whey and defects in taste such as bitterness and texture of the ripened cheese (49). The PA values in this study were lower than those of other studies such as that of *Withania coagulans* fruit extract (30) and *Bromelia pinguin* fruit extract (44) with 1.06 U/mg-and 2.0 U/mg respectively. The MCA/PA ratio is one of the most important properties of the enzymes used in cheese making, which is the index of coagulation efficiency and suitability of the extract to be used as a substitute for rennet (50). In general, chymosin has a higher MCA/PA ratio than most microbial and plant coagulants (46). However, in the present study, CCE showed a significantly higher MCA/PA ratio with CM than with SB, implying that the proteases present in this extract have proteolytic specificity without excess hydrolysis of other proteins. A high ratio is better able to form curd with a higher yield and to develop less bitterness during the transformation of the cheese, while a low ratio can lead to a lower yield and firmness of the curd and the release of bitter peptides that affect the sensory properties of the final product (39).

Caseins hydrolysis by CCE

During different incubation times of camel caseins with CCE, there is a reduction in the intensity of major protein bands, indicating the hydrolysis of casein proteins. The milk clotting properties of proteases are dependent on their activity and hydrolysis preference for κ-casein compared to other milk proteins during the early stages of milk coa-

gulation (44). Moreover, the primary phase of rennet action implicates the enzymatic hydrolysis of κ-casein, while the secondary phase involves the aggregation of rennet-altered micelles into the gel, its proteolytic specificity of milk coagulation (38). Despite the fact that plant enzymes have long been studied as rennet substitutes, few findings on the specificity of the action of plant proteases on κ-casein have been reported. Therefore, α- and β-caseins seemed to be initially more resistant to CCE hydrolysis than κ-casein, which is in agreement with the results reported by Li et al, (51), using a crude tamarillo fruit extract. Plant coagulants may have other important cleavage sites on κ-casein hydrolysis, which could assist milk clotting in the same way as rennet. The inhibition of camel milk casein hydrolysis by a protease inhibitor cocktail increased the intensity of the casein bands (Figure 1, lanes 6), which confirmed the enzymatic effect of CCE by the presence of proteases. To further identify CCE milk coagulation enzymes, the effect of specific protease inhibitors needs to be studied.

Cheese yield

On the economic scale, the high cheese yield is an important parameter. The decrease in cheese yield of milk coagulated with plant extracts is attributed to the high proteolytic nature of these coagulants (52). In contrast, the improvement in cheese yield obtained with CCE may be related to its low proteolytic activity, which gives a specific advantage to this extract. In comparison to other coagulant extracts such as crude extract of ginger (*Zingiber officinale*) and coagulant agent containing transgenic camel chymosin (9), CCE was found to be the best in terms of cheese yield. In addition, cheeses made with a vegetable coagulant showed better hardness and chewing ability (12). Similar results were reported for kiwi, pineapple and ginger extracts, which also showed a cheese yield that was significantly different from that obtained with chymosin (13). Cheese yield also depends on factors such as type of cheese, milk quality, composition, and heat treatment.

In conclusion, the results of the present study revealed the possibility of making cheese from camel milk with a high yield by using the crude extract from green pods of carob as a substitute for commercial rennet. However, the high specificity of crude carob extract with camel milk in terms of highly clotting and specific activities and minimal proteolytic activity could improve the use of this extract in the cheese industry. The high ratio of milk clotting to the proteolytic activity of crude carob extract contributed to the higher cheese yield. Therefore, the crude extract from green pods of carob is suitable for cheese making and can be considered an appropriate commercial rennet substitute. Camel milk cheese made with crude carob extract could be a novel functional food with commercial applications to diversify camel milk dairy products. Further research is recommended to purify and identify the proteases responsible for camel milk coagulation.

Acknowledgements

The authors would like to acknowledge the project of the Quality Support Program, PAQ because this study is part of a partner project titled "Valorization of camel milk by promoting a value chain based on a public-private partner ship (PPP)".

Interest conflict

The authors declare no conflict of interest.

Consent for publications

The author read and proved the final manuscript for publication.

Availability of data and material

All data generated during this study are included in this published article

Authors' contribution

All authors had equal roles in study design, work, statistical analysis and manuscript writing.

Funding

This article was achieved based on the material and equipment of the Arid Land Institute (IRA, Medenine), University of Gabes.

Ethics approval and consent to participate

No humans or animals were used in the present research.

References

1. Bulca S, Sarikoç E. Composition of Camel Milk and Some Functional Properties of the This Milk and Application of These Properties in Dairy Technology. J Tekirdag Agric Fac. 2016; 30: 9.
2. Sboui A, Atig C, Khabir A, Hammadi M, Khorchani T. Camel Milk Used as an Adjuvant Therapy to Treat Type 2 Diabetic Patients: Effects on Blood Glucose, HbA1c, Cholesterol, and TG Levels J Chem. 2022; 2022:1–6.
3. Khalesi M, Salami M, Moslehishad, Winterburn J, Moosavi-Movahedi AA. Biomolecular content of camel milk: A traditional superfood towards future healthcare industry. Trends Food Sci. 2017; 62: 49–58.
4. Bornaz S, Sahli ALI, Attalah A, Attia H. Physicochemical characteristics and renneting properties of camels' milk: a comparison with goats', ewes' and cows' milks. Int J Dairy Technol. 2009; 62:505–513.
5. Kappeler S, Farah Z, Puhan Z. Sequence analysis of Camelus dromedarius milk caseins. J Dairy Res. 1998; 65:209–222.
6. Attia H, Kherouatou N, Nasri M, Khorchani T. Characterization of the dromedary milk casein micelle and study of its changes during acidification. Le Lait. 2000; 80:503–515.
7. Saliha BH, Louis LC, Farida MM, Saliha SA, Nasma M, Elkhir SO, Abderrahmane M. Comparative study of milk clotting activity of crude gastric enzymes extracted from camels' abomasum at different ages and commercial enzymes (rennet and pepsin) on bovine and camel milk. EJFA. 2011; 23: 301-310.
8. Sboui A, Fguiri I, Dbara M, Hammadi M, Khorchani T. Improvement of Camel Milk Coagulation: Usefulness of an Enzymatic Extract from the Kaolin Layer of Chicken Gizzard. J Agric Vet Sci. 2021; 1:1–10.
9. López M. Effect of vegetable coagulant, microbial coagulant and calf rennet on physicochemical, proteolysis, sensory and texture profiles of fresh goat's cheese. Dairy Sci Technol. 2012; 92:691–707.
10. Ibhaze GA. Comparative Evaluation of Nutritional Quality of Soft Cheese Made from Four Plant Sources. Thai J Agric Sci. 2018; 51:118–124.
11. Roseiro LB, Barbosa M, Ames JM, Wilbey RA (2003) Cheesemaking with vegetable coagulants—The use of Cynara L. for the production of ovine milk cheeses. Int J Dairy Technol. 2003; 56:76–85.

12. Hailu Y, Seifu E, Yilma Z. Physicochemical properties and consumer acceptability of soft unripened cheese made from camel milk using crude extract of ginger (*Zingiber officinale*) as coagulant. Afr J Food Sci. 2014; 8:87–91.
13. Fguiri I, Atigui M, Sboui A, Marzougui C, Dbara M, Hammadi M et al. Camel Milk-Clotting Using Plant Extracts as a Substitute to Commercial Rennet. J Chem. 2021; 2021:1–8.
14. Bouazizi A, Felfoul I, Attia H, Karoui R. Characterization of nettle leaves (Urtica dioica) as a novel source of protease for clotting dromedary milk by non-destructive methods. Colloids Surf B. 2022; 211:112312.
15. Antoniou C, Kyratzis A, Rouphael Y, Stylianou S, Kyriacou MC. Heat-and ultrasound-assisted aqueous extraction of soluble carbohydrates and phenolics from carob kibbles of variable size and source material. Foods. 2020; 9:1364.
16. Benchikh Y, Louaileche H, George B, Merlin A. Changes in bioactive phytochemical content and in vitro antioxidant activity of carob (*Ceratonia siliqua* L.) as influenced by fruit ripening. Ind Crops Prod. 2014; 60:298-303.
17. Ben Othmen K, Garcia-Beltrán JM, Elfalleh W, Haddad M, Esteban MA. Phytochemical compounds and biological properties of carob pods (*Ceratonia siliqua* L.) extracts at different ripening stages. Waste Biomass Valoriz. 2021; 12:4975-4990.
18. Tressler DK, Joslyn MA. Fruit and Vegetable Juice Processing Technology. Westport, Connecticut, USA, 1961, pp. 453-457.
19. AFNOR (1993) Contrôle de la qualité des produits alimentaires. In Laits et produits laitiers (ed) Analyses Physicochimiques. Association Française de Normalisation, Paris, 1993, pp. 220-251.
20. Berridge N J. Some observations on the determination of the activity of rennet. Analyst. 1952; 77:57–62.
21. Collin JC, Grappin R, Legraet Y. Etude de la méthode de mesure selon Berridge du temps de coagulation du lait additionné d'une solution enzymatique. Rev lait fr. 1977; 355:389–439.
22. Upasana AC, Tripathi ACY. Assessment of milk clotting activities of plant latex. AJHS. 2013; 8:456–460.
23. Bergere JL, Lenoir J. Les accidents de fromagerie et les défauts des fromages. In: A. Eck and J. C. Gillis (ed) Le fromage. Technique et Documentation Lavoisier, Paris, 1997, pp. 509-541.
24. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; 227: 680–685.
25. Sulieman AME, Ali RAM, Razig KAA. Production and effect of storage in the chemical composition of Mozzarella cheese. Int j food sci nutr. 2012; 2:21-26.
26. Jing PU, Giusti MM. Effects of extraction conditions on improving the yield and quality of an anthocyanin-rich purple corn (*Zea mays* L.) color extract. J Food Sci. 2007; 72:C363–C368.
27. Akinloye AM, Adewumi OO. Effects of local coagulants on the yield of cheese using cow and sheep milk. Int J Dev Sustain. 2014; 3:150–161.
28. Hachana Y, Aloui O, Fortina, R. Use of caprifig tree extract as a substitute for calf rennet in goat's fresh cheese production. Small Rumin Res. 2021;199:106382.
29. Nouani A, Dako E, Morsli A, Belhamiche N, Belbraouet et al. Characterization of the purified coagulant extracts derived from artichoke flowers (*Cynara scolymus*) and from the fig tree latex (*Ficus carica*) in light of their use in the manufacture of traditional cheeses in Algeria. J Food Technol. 2009; 7:20–29.
30. Kazemipour N, Salehi Inchebron M, Valizadeh J, Sepehrimanesh M. Clotting characteristics of milk by Withania coagulans: Proteomic and biochemical study. Int J Food Prop. 2017; 20:1290–1301.
31. Grek O, Krasulya O, Chubenko L, Tymchuk, A. The investigation of the potentional comlex from Plantago major to coagulate milk proteins. Food Environ Saf J. 2018; 2:165–175.
32. Kandarakis I, Moschopoulou E, Anifantakis E. Use of fermentation produced chymosin from E. coli in the manufacture of Feta cheese. Milchwissenschaft. 1999; 54:24–25.
33. Yasar K, Guzeler N. Effects of coagulant type on the physicochemical and organoleptic properties of Kashar cheese. Int J Dairy Technol. 2011; 64:372–379.
34. Liang HH, Huang HH, Kwok KC. Properties of tea-polyphenol-complexed bromelain. Food Res Int. 1999; 32:545-551.
35. Lo Piero AR, Puglisi I, Petrone G. Characterization of "Lettucine", a serine-like protease from Lactuca sativa leaves, as a novel enzyme for milk clotting. J Agric Food Chem. 2002; 50:2439–2443.
36. Pontual EV, Carvalho BE, Bezerra RS, Coelho LC, Napoleão TH, Paiva PM. Caseinolytic and milk-clotting activities from Moringa oleifera flowers. Food Chem. 2012; 135:1848–1854.
37. Vishwanatha KS, Appu Rao AG and Singh SA. Production and characterization of a milk-clotting enzyme from Aspergillus oryzae MTCC 5341. Appl Microbiol Biotechnol. 2010; 85:1849-1859.
38. Fox PF, Guinee TP, Cogan TM, McSweeney PL, Fox PF, Guinee TP et al. Enzymatic coagulation of milk. Fundamentals of cheese science. 2017; 185-229.
39. Amira AB, Makhlouf I, Petrut RF, Francis F, Bauwens J et al. Effect of extraction pH on techno-functional properties of crude extracts from wild cardoon (*Cynara cardunculus* L.) flowers. Food Che. 2017; 225:258-266.
40. Hashem AM (2000) Purification and properties of a milk-clotting enzyme produced by Penicillium oxalicum. Bioresour Technol. 2000; 75:219-222.
41. Philippe M, Gaucheron F, Le Graet Y, Michel F, Garem A. Physicochemical characterization of calcium-supplemented skim milk. Le lait. 2003; 83:45-59.
42. El Zubeir IE, Jabreel SO. Fresh cheese from camel milk coagulated with Camifloc. Int J Dairy Technol. 2008; 61(1), 90-95.
43. Konuspayeva G, Camier B, Gaucheron F, Faye B. Some parameters to process camel milk into cheese. EJFA. 2014; 26:354-358.
44. Moreno-Hernández JM, Pérez M de JB, Osuna-Ruiz, Salazar-Leyva JA, Ramirez-Suarez JC. Exploring the milk-clotting properties of extracts from *Bromelia pinguin* fruit. J Microbiol Biotechnol Food Sci. 2017; *7*(1), 62
45. Mazorra-Manzano MA, Perea-Gutiérrez TC, Lugo-Sánchez ME, Ramirez-Suarez JC, Torres-Llanez MJ, González-Córdova AF et al. Comparison of the milk-clotting properties of three plant extracts. Food Chem. 2013; 141(3), 1902-1907.
46. Vallejo JA, Ageitos JM, Poza M & Villa TG. A comparative analysis of recombinant chymosins. J Dairy Sci. 2012; 95(2), 609-613.
47. Yamada N, Kokean Y, Umetani K, Shimizu J, Kurita O, Tsumura K. Protease activity and gel-forming ability of ginger rhizome juice. J Food Res. 2021; 9(2), 1-30.
48. Ramet J. Les agents de transformation du lait; la présure et les enzymes coagulantes. In: A. Eck (ed) Le fromage. Technique et Documentation Lavoisier, Paris, 1997, pp. 101-107.
49. Fox PF, McSweeney PLH. Dairy chemistry and biochemistry. London, 1998, UK.
50. Merheb-Dini C, Gomes E, Boscolo M, da Silva R. Production and characterization of a milk-clotting protease in the crude enzymatic extract from the newly isolated Thermomucor indicae-seudaticae N31:(Milk-clotting protease from the newly isolated Thermomucor indicae-seudaticae N31). Food Chem. 2010; 120:87–93.
51. Li Z, Scott K, Hemar Y & Otter D. Protease activity of enzyme extracts from tamarillo fruit and their specific hydrolysis of bovine caseins. Food Res Int. 2018; 109: 380-386.
52. Mahajan RT, Chaudhari GM (2014) Plant latex as vegetable source for milk clotting enzymes and their use in cheese preparation. Int j adv res. 2014; 5:1173-1181.