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Coagulation of Camel Milk using Dromedary Gastric Enzymes as a Substitute of the Commercial Rennet

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ABSTRACT

Camel milk is recognised to furnish important components including vitamin C, niacin and riboflavin. It is also known to provide health protective functions such as anti-diabetic, anti-infectious, anti-stress and its effects against stomach-ache to name a few. However, its valorisation is still very limited. The particular composition of this milk makes its conservation and transformation very difficult. Investigation on the conservation possibilities of camel milk, thus, its transformation into derived products such as cheese so as the population gets the full benefits from its nutritional and therapeutic virtues, is hereby undertaken. However, previous reports showed its weak coagulation propriety, which is the key to its transformation into derived products. In order to remedy this obstacle, a variety of techniques have been proposed including the use of dromedary gastric enzymes. The data showed that the GEC from the older camels gave the best results significantly ($p \leq 0.05$) for both milk clotting activity and flocculation time of both bovine and camel milk compared with the other tested enzyme preparations. The optimum flocculation time was obtained at pH 5.8 and 42°C for the camel milk and at pH 6.0 and 37°C for bovine milk.

Key words: Dromedary, milk, protein, cheese, pepsin, chymosin, coagulation

INTRODUCTION

The dromedary (*Camelus dromedarius*) is an animal particularly adapted to the aridity of its environment especially in the steppe and desert zones of the Algerian Sahara (Wilson, 1984). In spite of these extreme agro-climatic conditions, the species produces milk which is recognized as complete food for human beings because it contains most of the essential nutrients (Singh and Sachan, 2011). Not only particularly rich in lipids, proteins and glucides but also in vitamins (especially vit. C) and minerals. This milk of which the conservation period at ambient temperature is prolonged by some days, because the animal udder is endowed with a sophisticated antibacterial system (Al-Humaid *et al*., 2010), has nonetheless weak transformation aptitudes into derived products. This is considered as a limiting factor as for the technological transformation of this milk, in spite of its important quantitative and qualitative production that can easily answer the population requirements in these regions (Ramet, 2001).

Within the context of finding a solution to such a hindrance, many studies were carried out during the last few decades, all of which have the ultimate goal of improving the technological aptitudes of the milk, notably the one of cheese-making (Ramet, 1985, 1994, 2001; Farah, 1993; Farah *et al*., 1990; Laleye *et al*., 2008).

The coagulation phase has been particularly investigated testing a large variety of enzymes (rennet, pepsin, microbial enzymes, etc.) However, coagulation techniques using rennet have not been conclusive (Mehaia, 1993; Bayoumi, 1990; Ramet, 1997), the test carried out using bovine pepsin camifloc commercial enzymes or dromedary gastric enzymes provided interesting results (Wangoh *et al*., 1993; Ramet, 1994; Elagamy, 2000b; Siboukeur *et al*., 2005; El-Zubeir and Jabreel, 2008; Saliha *et al*., 2011).

For a better control and understanding of these preparations, we propose in the current study to optimize the flocculation duration using gastric protease extracted from the stomach of various aged dromedaries.

MATERIALS AND METHODS

Materials: The experiments were conducted during 2010 to 2011 at the University of K. Merbah, Ouargla Algeria.

The camel abomasa samples were collected over two years and the laboratory experiments were carried out during the year 2010-2011.

Abomasal tissues: The camel abomasal tissues were obtained from camel slaughterhouse of Ouargla, Algeria. The abomasa (sing. abomasum) were obtained from camels of different ages (young animals suckling, fed mixed and adults. The abomasal tissues were cleaned with running water, defatted, cut in slices, packaged in plastic bags and frozen at -8°C.

Commercial enzymes: Bovine pepsin in powder form and bovine rennet containing 80% chymosin and 20% pepsin were purchased from Texel-Poulenc (France).

Milk samples: The camel milk was collected early morning from a free range camel herd (Camelus dromedarius), breed Sahraoui, in good health, living in the South-East Ouargla region (Algeria). The milk samples were collected in sterile bottles and delivered in a cooler with ice to the laboratory (Laboratory of Ecosystems Protection in Arid and Semi-Arid Regions, Department of Biology, Merbah-Ouargla University, Algeria).

Methods

Extraction of gastric enzymes from camel abomasal tissues: The method of gastric enzymes extraction from bovine abomasal tissue as described by Valles and Furet (1977) was used with minor modifications. The steps involved were: (1) soaking of a known weight of sliced abomasal tissue in 1.25 volume of 0.2 M HCL at 42°C temperature for 60 min and filtration through a paper filter, (2) clarification: of the extract using 1% (v/v) of 1 M solution of Al_2SO_4 and 5% of a 1 M solution of Na_2SO_4 (1 M) heated to 42°C, After filtration a yellowish clarified solution was obtained and (3) concentration: A double solution of saturated NaCl containing 1% (w/w) of concentrated HCl was added to the known weight of the abomasal tissue. After mixing, the mixture was put to rest for one h, centrifuged at 2100 g for 20 min, the supernatant was discarded and the wet weight was recorded followed up by adding 10% (w/v) of distilled water. The pH of the concentrated

filtrated was adjusted to 5.5 with Na_2HPO_4 at 42°C. The extracted camels' gastric enzymes obtained were assigned the labels GEC S for young animals Suckling; GEC FM for animals Fed Mixed and GEC A for Adults' animals. The fresh GEC analyzed and some samples were stored at 4°C with the addition of 10% (v/v) of thymol and 10% NaCl for preservation purpose.

Protein analysis of the GEC: The method of Lowry *et al.* (1951) was used to determine the protein content of the gastric enzyme extracts of camels. The amount of proteins (g mL^{-1}) was obtained using a standard curve based on Bovine Serum Albumin (BSA).

Clotting activities of the GEC: The method of Berridge (1952) was used. The main steps were the following.

The standard substrate was the "low heat" milk powder at 10% (w/v) solution in $CaCl_2$ (0.01 M) solution and the pH was adjusted to 6.5 with 0.1 N NaOH The GEC was added at 1/10 mL^{-1} of standard substrate and mixed manually and incubated in a water bath at 30°C. After thoroughly mixing three times, the clotting time zero started. The clotting activity equation as reported by Berridge (1952) in rennet units (RU) was used:

$$RU = \frac{10 \times V}{Tc \times Q}$$

Where:
RU: Rennet Unit
V: Volume of standard substrate (mL)
Q: Volume of GEC (mL)
Tc: Time of clotting (sec)

Clotting strength: The clotting activity of the GEC was also reported in clotting strength of Soxhlet (F) based on the equation of Bourdier and Luquet (1981):

$$F = \frac{RU}{0.0045}$$

Where:
F: Clotting strength of Soxhlet

Proteolytic activity: The method of Bergere and Lenoir (1997) for the proteolytic activity of the GEC was used. In addition the clotting activity was optimized by using the method of Shamet *et al.* (1992).

Coagulation of camel milk by the GEC: Camel and bovine milk coagulation was carried out by using the method of Ramet (1997). However, the flocculation time was measured visually by the method of Lenoir *et al.* (1997) at different pH and temperatures. The flocculation time is the time between the addition of coagulating enzyme of the appearance of flakes visible to the naked eye. This method consists in the introduction of 10 mL of milk in a test tube with a fixed concentration of $CaCl_2$ solution and incubated at the desired temperature after the addition of a coagulant enzyme concentration. The concentration of the enzyme is such that the flocculation of milk to pH = 6.3 (a concentration of 0.01 M $CaCl_2$ and 30°C) occurs after about 15 min.

Optimization of the flocculation time: The flocculation time (ft) of camel and bovine milk was optimized at four different pHs (5.8, 6.0, 6.3 and 6.6) and at three different temperatures (30, 37 and 42°C). The mixture is adjusted to the desired pH with 0.1 M HCl, incubated at the desired temperature (Lenoir *et al*., 1997).

Statistical analysis: All experiments were performed with three replicates each. All data are reported as means with standard deviations. An analysis of variance (ANOVA) was applied to assess differences among the "Gastric Enzyme Extract from Camels" (GEC) and the commercial enzymes by using SPSS (Statistical Package for the Social Sciences) software version 18.0.

RESULTS AND DISCUSSION

Characterization of gastric extract enzymes: The protein average in g L^{-1} of the GEC was 1.26, 1.38 and 1.44 for GECS, GEC FM and GEC A, respectively. Means are significantly different at $p \leq 0.05$. There was an increase in protein content with the age of the camels (Table 1).

Clotting activity: The clotting activity was the highest for the GEC A (older camels) compared to the GEC FM and GEC S. In addition, the commercial bovine rennet had higher clotting activity compared to the commercial bovine pepsin, however, the clotting activity for all gastric enzyme extracts from camels at different ages and the two commercial enzymes, rennet and pepsin bovine, were significantly different at $p \leq 0.05$ (Table 2).

Proteolytic activity: The proteolytic activity of the GEC and commercial enzymes were measured on both camel milk and bovine milk and all data were significantly different ($p \leq 0.05$) (Table 3). The proteolytic activity of each of the GEC was higher than those of bovine enzymes on both milks. An increase in absorption at this wavelength (280 nm) could be fully attributed to the formation of unspecific cleavage products. Kappeler *et al*. (2006) observed that the proteolytic activity of enzymes increased with higher incubation temperatures and also when the pH of the assays was decreased.

Table 1: Protein content of GEC

Gastric enzyme extracts	Protein content (g L^{-1})
GEC S	1.26±0.02[a]
GEC FM	1.38±0.02[b]
GEC A	1.64±0.02[c]

Means followed by different letters are significantly different at $p \leq 0.05$, GEC S: Gastric enzyme extract of camel suckling, GEC FM: Gastric enzyme extract of camel with Fed Mixed, GEC A: Gastric enzyme extract old camel adult

Table 2: Change in clotting activity (rennet unit: RU)

Enzymatic preparations	Clotting activity (RU)
GEC S	0.135±0.002[a]
GEC FM	0.255±0.001[b]
GEC A	0.410±0.020[c]
Pepsin bovine (Pb)	0.123±0.002[d]
Rennet bovine (Rb)	0.164±0.002[e]

Means followed by different letters are significantly different at $p \leq 0.05$, GEC S: Gastric enzyme extract of camel suckling, GEC FM: Gastric enzyme extract of camel with Fed Mixed, GEC A: Gastric enzyme extract old camel adult, Pb: Pepsin bovine, Rb: Rennet bovine

Table 3: Proteolytic activity of the enzymatic preparations on camel milk and bovine milk

Enzymatic preparations	Proteolytic activity (Camel milk)	Proteolytic activity (Bovine milk)
GEC S	1.68±0.020[a]	1.44±0.020[a]
GEC FM	1.28±0.020[b]	1.24±0.020[b]
GEC A	0.79±0.020[c]	0.84±0.015[c]
Pepsin bovine (Pb)	0.63±0.025[d]	0.84±0.020[d]
Rennet bovine (Rb)	0.58±0.026[e]	1.34±0.015[e]

Means followed by different letters are significantly different at $p \leq 0.05$, GEC S: Gastric enzyme extract of camel suckling, GEC FM: Gastric enzyme extract of camel with Fed Mixed, GEC A: Gastric enzyme extract old camel adult, Pb: Pepsin bovine, Rb: Rennet bovine

GEC A showed a lower proteolytic activity than GEC S and GEC FM. However GEC A showed a proteolytic activity close to that of the bovine pepsin; the proteolytic activity of GEC A was 0.89 on camel milk and 0.85 on bovine milk compared to the proteolytic activity of bovine pepsin of 0.79 and 0.94 on camel milk and bovine milk, respectively. In cheese production, the desired enzyme should have high clotting activity and low proteolytic activity (Ramet, 1997). GEC A could be considered as a good source of enzyme for cheese making compared to GEC S and GEC FM. The high clotting activity and low proteolytic activity as shown by GEC A are a pre-requisite for an acceptable rennet substitute (Fox, 1969; Elagamy, 2000a) particularly for making cheese from camel milk.

Except for the bovine rennet (Fig. 1), the time required to reach the flocculation of the camel milk was shorter than that of the bovine milk. These results are in agreement with those of Kappeler *et al.* (2006).

In addition the flocculation time decreased with the age of camels from which the GEC were extracted. In the same experimental conditions, the flocculation time of bovine pepsin was shorter for camel milk than for cow milk but higher than GEC A. This finding is in agreement with other researchers who reported that the use of bovine pepsin could coagulate camel milk (Ramet, 1994; Siboukeur *et al.*, 2005). In order to define the affinity of the enzyme preparations to the two substrates (camel and cow milk), the ratio between the flocculation time of bovine milk and camel milk (ftb/ftc) was determined. Figure 2 shows that the GEC demonstrated an affinity for both substrates with a ratio over 1.0 compared to 0.17 for the bovine rennet. The latter has less affinity for camel milk. The ratio for the bovine pepsin was the highest at 3.32, indicating that this enzyme would be suitable for camel milk coagulation, as reported by other researchers (El-Abbassy and Wahba, 1986; Mehaia, 1987; Wangoh *et al.*, 1993; Ramet, 1994). The large variations in the ability of cow rennet to coagulate camel milk reported in literature (Bayoumi, 1990; Farah and Bachmann, 1987; Mohamed, 1990; Ramet, 1985) may be explained by differences in the pepsin content of the rennet used. The better coagulation of camel milk by camel rennet could be the result of better suitability of camel rennet for coagulating camel milk.

Effect of pH on the flocculation time: Milk clotting activity was influenced by the pH of the milk at the stage. All enzyme preparations exhibited almost a linear curve with an increased pH from 5.8-6.6. The optimum pH for clotting camel milk for all GECs was at 5.8 and the flocculation time increased with the age of the camels (GECS, GECFM and GECA). Also it appeared that GEC A was less affected by the increased pH (Fig. 3). The pH of the milk for rapid flocculation is very important during cheese making since the acidification by the lactic acid bacteria helps the enzyme activity in which the enzyme is a protease having an optimum activity around pH 5.5. This contributes to the destabilization of the casein micelles (Ramet, 1994). With regards to bovine milk

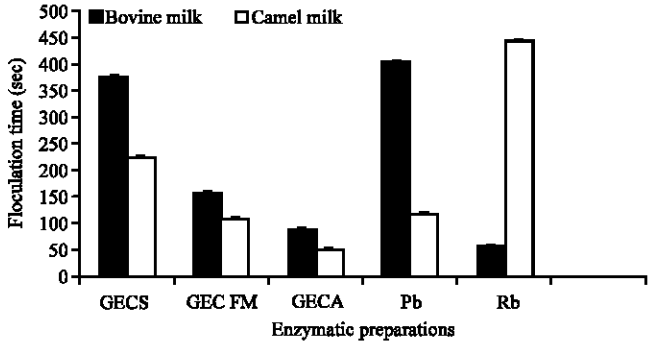

Fig. 1: Effect of the enzymatic preparations on the flocculation time of bovine and camel milk, Means followed by different letters are significantly different at $p \leq 0.05$, GEC S: Gastric enzyme extract of camel suckling, GEC FM: Gastric enzyme extract of camel with Fed Mixed, GEC A: Gastric enzyme extract old camel adult, Pb: Pepsin bovine, Rb: Rennet bovine

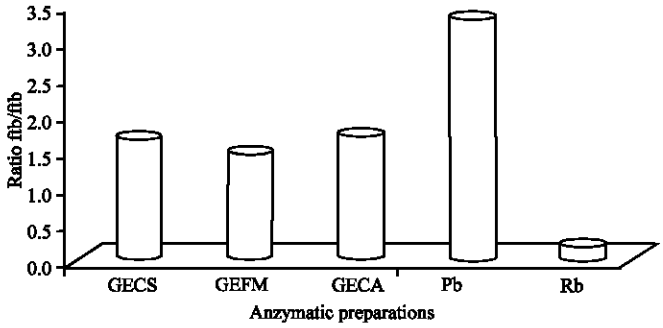

Fig. 2: Effect of the enzymatic preparations on the ratio of flocculation time of bovine milk (ftb) and camel milk (ftc), GEC S: Gastric enzyme extract of camel suckling, GEC FM: Gastric enzyme extract of camel with Fed Mixed, GEC A: Gastric enzyme extract old camel Adult, Pb: Pepsin bovine, Rb: Rennet bovine

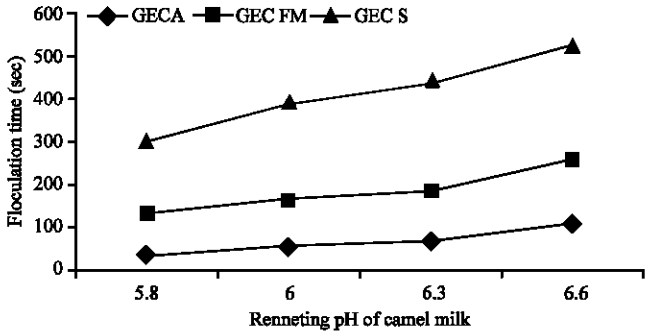

Fig. 3: Effect of renneting pH of camel milk on the flocculation time, GEC S: Gastric enzyme extract of camel suckling, GEC FM: Gastric enzyme extract of camel with Fed Mixed, GEC A: Gastric enzyme extract old camel adult

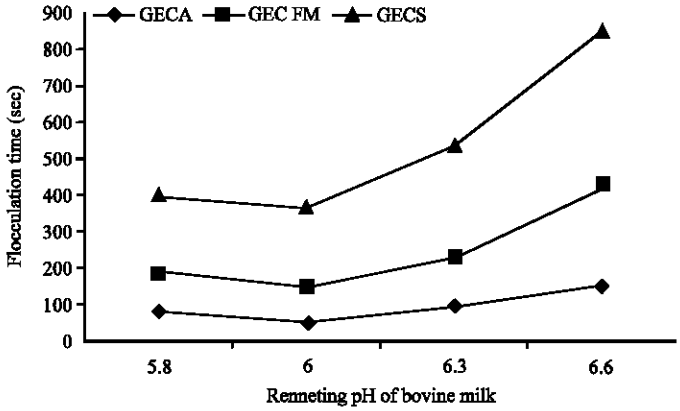

Fig. 4: Effect of renneting pH of bovine milk on the flocculation time, GEC S: Gastric enzyme extract of camel suckling, GEC FM: Gastric enzyme extract of camel with Fed Mixed, GEC A: Gastric enzyme extract old camel adult

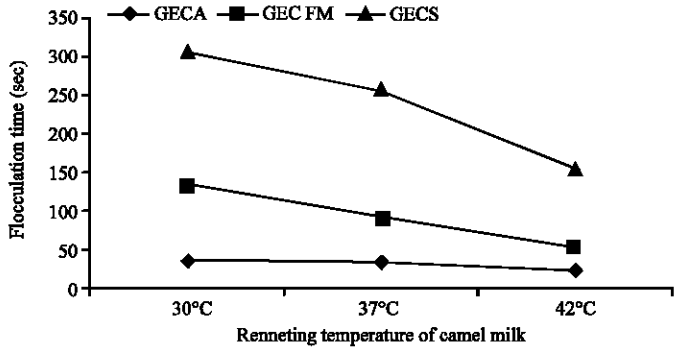

Fig. 5: Effect of renneting temperature of camel milk on the flocculation time, GEC S: Gastric enzyme extract of camel suckling, GEC FM: Gastric enzyme extract of camel with Fed Mixed, GEC A: Gastric enzyme extract old camel adult

(Fig. 4), the optimum pH for GEC FM and GEC A was 6.0 but GEC S showed a similar pH at 5.8 with both camel and bovine milk. Figure 4 shows that both GEC FM and GEC A were less sensitive to pH variation. Based on these results, bovine and camel milk appears to behave differently in function of the pH at renneting, probably due to the difference in the protein fractions of the two milks (Attia *et al*., 2000). In fact, Lenoir *et al*. (1997) and Chazarra *et al*. (2007) found that the effect of pH of milk on flocculation was very sensitive and apparent, thus the flocculation time is further reduced if the renneting pH is far below the normal pH of milk. This is in agreement with the findings of Ramet (1985, 1993), that all clotting cheese enzymes are acid protease, that their activity is optimum at pH 5.5 and that the kappa casein presents stability at pH 5.6. This is not the case for camel milk since the slow pH drop in camel milk is not conducive to the clotting activity (Ramet, 1985, 2001).

The effect of temperature on the flocculation time of the various GEC on camel milk is shown in Fig. 5.

Increased temperatures (30, 37 and 42°C) led to a decrease in flocculation time by all GEC. However, the shortest flocculation time was obtained with GEC A, indicating that GEC could be the more stable enzyme but all the GEC showed an optimum activity and flocculation time at 42°C (Fig. 5). This is in line with the data reported by Ramet (1985, 1993), that the optimum

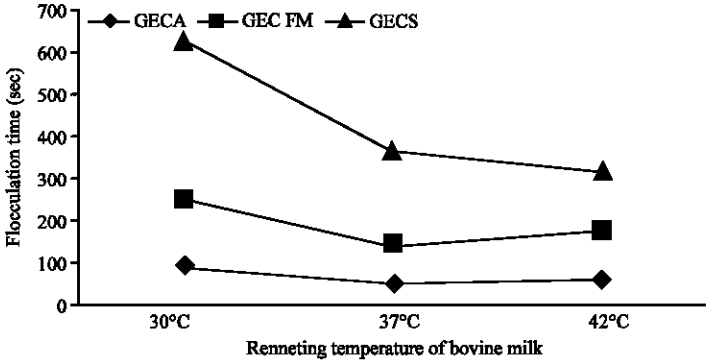

Fig. 6: Effect of temperature of bovine milk on the flocculation time, GEC S: Gastric enzyme extract of camel suckling, GEC FM: Gastric enzyme extract of camel with Fed Mixed, GEC A: Gastric enzyme extract old camel adult

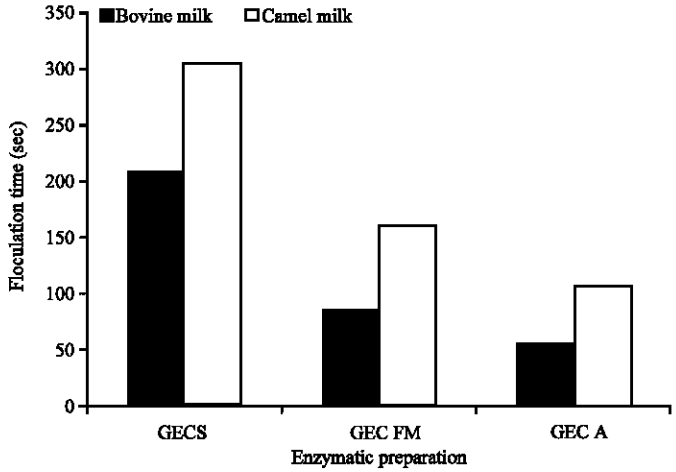

Fig. 7: Effect of enzymatic preparations on the flocculation time of camel milk and bovine milk, Means followed by different letters are significantly different at $p \leq 0.05$, GEC S: Gastric enzyme extract of camel suckling, GEC FM: Gastric enzyme extract of camel with Fed Mixed, GEC A: Gastric enzyme extract old camel adult

temperature of most clotting enzymes were around 40-50°C, but beyond these values there was a progressive denaturation of the enzyme and at 65°C there was no activity. Similarly, Mohanty *et al.* (2003) reported that the proteolytic activity of buffalo chymosin treated at different temperatures exhibited a relatively stable proteolytic activity curve up to a temperature of 55°C after which there was a decline of the activity. Measuring the flocculation time at different temperature suggested which enzyme would be more suitable for camel milk clotting in a short period well as the manufacture of various type of cheeses, such as soft, semi-hard and hard cheese.

The effect of temperature on flocculation time of the various GEC on bovine milk is shown in Fig. 6. In contrast with camel milk, all GECs showed their optimum flocculation time at 37°C and the shortest flocculation time was obtained with GECA indicating that this enzyme had some affinity with both milk substrates (Fig. 7). In addition, the age of camels had a significant ($p \leq 0.05$) influence on the flocculation time, the older camels gave the shorter flocculation time. Ramet (1985) reported that pepsin is the minor component of rennet but its secretion increases after the lactation

period. On the other hand, Wangoh *et al.* (1993) did not conduct their study with different age of camels but their study recommended the use of gastric enzyme extracted from the abomasa of camels rather than from other species. Based on all data, the short flocculation time obtained with the GEC were probably due to the presence of pepsin which was in higher amount in GEC A compared to GEC FM and GEC S. Similarly, Ramet (1994) reported that the use of bovine pepsin provided a rapid flocculation time in camel milk compared to bovine milk. Therefore, this suggested that the content of pepsin was higher in the older camels (GECA), as previously reported by Wangoh *et al.* (1993). This finding was in contrast with the case of bovine chymosin which is extracted in younger calves. It can be concluded that the pepsin content in older camels (GECA) has more coagulating activity than proteolytic activity in camel milk due to the molecular difference in camel proteins and bovine proteins, such as the distribution and size of the casein micelles, various fractions of the casein, sites of the potential cleavage etc. It is because coagulation time varies with the micelle size and reaches an optimum in the medium and small size micelles. This appears to be related to the availability of k-casein. The content of k-casein decreases with increasing micelle size (Ekstrand *et al.*, 1980; Walstra and Jenness, 1984; Farah and Ruegg, 1989). Overall, based on the data reported and in line with other researchers' reports (Farah and Bachmann, 1987; Mehaia, 1992; Ramet, 1993; Desmazeaud, 1990; Thouvenot, 1997), this study proposes an optimum temperature at 42°C and a pH of 5.8 for an optimum clotting activity and flocculation time using gastric enzymes extracted from camels. From the results obtained it can be concluded that the crude enzymes extracted from old camels coagulate better camel milk than cow milk. Elsewhere the results of the study undertaken by Bansal *et al.* (2009), suggest that camel chymosin can be used successfully to make cheddar cheese with lower levels of proteolysis but with good flavor.

This study focused primarily on the coagulation step that represents the key step in making cheese but additional studies are necessary on the performance and characteristics of the cheese obtained with these enzymes.

CONCLUSION

The non-purified enzyme preparations (GEC) obtained from the older camels showed better coagulation activity on both milks. Flocculation time data showed that the GEC and bovine pepsin had good specificity towards bovine casein and camel casein. In addition, the short flocculation time obtained for GEC A of older camels at an optimum temperature at 42°C and a pH of 5.8 was encouraging since older camels are more available for slaughter in Algeria. Therefore, the production of GEC from older camels could be an excellent substitute for the commercial chymosin for cheese making using either bovine or camel milk. It was recommended that additional research be conducted to purify the extract, to characterize the extract using electrophoreses and finally for the production of various type cheeses from camel milk.

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