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CHARACTERIZATION OF MILK CLOTTING PROPERTIES OF ADULT CATTLE RENNET MODIFIED WITH *Moringa oleifera* SEEDS AND ITS SUITABILITY AS CALF RENNET ALTERNATIVE IN CHEESE MAKING

Hesham A. Ismail*

Dairy Sci. Dept., Fac. Agric., New Valley Univ., Egypt

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ABSTRACT: The present study focused on the mixing of adult cattle rennet with Moringa seeds during the extraction process to improve its strength and use it as an alternative for the calf rennet in cheese production. The *Moringa oleifera* seeds powder were mixed with adult cattle rennet at a ratio of 2%. Crude and partially purified extracts (using ammonium sulphate 60% concentration) were used. The study include six different milk coagulants; adult cattle rennet extract (ARE), mix of adult cattle rennet with Moringa seeds (AME), partially purified adult cattle rennet extract (ARP), partially purified adult rennet mixed with Moringa seeds (AMP), commercial rennet (RRE) and calf rennet extract (CRE). The flocculation time, clotting time, clotting activity, proteolytic activity, the optimum pH and temperature for clotting activity, curdling quality (RCQ) and protein profile of curd formed were determined (SDS-PAGE). The physical properties include water release (WR) and water holding capacity (WHC), yield and sensory attributes of Domiati cheese made by using different coagulant extracts were estimated. The results showed that CRE has shorter flocculation and clotting time and higher milk clotting activity but ARE had the opposite, in the midst were treatments AME, ARP and AMP. The pH range of 5.5 to 6.5 and temperature between 50 - 60°C were suitable for milk clotting activity of milk coagulants. The best (RCQ) was found to be for the curd formatted by CRE, AMP, ARP and RRE. Physical properties gave the lowest (WR) and highest (WHC) in CRE curd cheese, followed by AMP, RRE, ARP and AME cheese, whilst it was observed the contrary in ARE. Electrophoretic patterns show that the levels of degradation αs-casein, κ-casein and β-casein differed with milk coagulant types. Using Moringa seeds and partially purified by ammonium sulphate improved yield and cheese sensory with characteristic; coherent texture and creamy taste. In conclusion mixing of adult cattle rennet with Moringa seeds powder during the extraction process and/ or partially purified gave an opportunity to be used as an alternative to calf rennet in cheese making.

Key words: *Moringa oleifera* seeds, adult cattle rennet, calf rennet; ammonium sulphate, commercial rennet, curd properties, Domiati cheese, sensory evaluation.

INTRODUCTION

Coagulation of milk is a basic step in the manufacture of most cheeses and can be achieved by a number of proteolytic enzymes from various sources, such as different animal rennet, microbial proteinases and proteinases extracted from fruits and plants **(Vioque *et al.*, 2000)**. Animal rennet is traditionally manufactured by extraction from the fourth stomach abomasum. The enzymes extracted from abomasum consist mainly of two enzymes maxing chymosin and pepsin. The relative proportion of the two enzymes varies with the age of the animal at slaughtering time. Calf rennet, originally obtained from the abomasum of recent born ruminant calves contains chymosin as the major, milk-clotting components of 88 to 94%, and pepsin to a lesser extent but adult cattle rennet may contain 90 to 94% of pepsin and only 6 to 10% of chymosin **(Broome and Limsowtin, 1998)**. Chymosin has been extensively used in

* **Corresponding author: Tel. :** +201092550623
E-mail address: h_alshnety@yahoo.com

the cheese making to produce a stable curd with good flavour due to its high specificity for cleaving the k-casein Phe 105- Met 106 bond (**Rao *et al*., 1998**). Whereas pepsin is much less specific and hydrolyses casein coupled with certain beneficial effect during ripening of cheese **(Papoff *et al.* 2004).** Compared to chymosin, pepsin has number of difficulties such as longer clotting time, forming a soft curd, and an undesirable taste. The clotting activity affects the properties of the curd, such as firmness or softness, during processing **(Walstra *et al.*, 2005)**. However, the worldwide increase in cheese production, coupled with reduced supply and increasing prices of calf rennet, some religious factors (Islam and Judaism) and others related to vegetarianism of some consumers, have encouraged the search for alternatives sources of milk-coagulants to be used as appropriate rennet substitutes. Research effects have been directed for new proteases with high specific milk-clotting activity and low general proteolytic activity to resemble animal rennet properties **(Shah *et al*., 2014).** Accordingly, some plant proteases similar chymosin are able to cleave few sites at αs_1 and β caseins, which may occur in maintaining the micelles stability (**Silva *et al.,* 2002**). **Pontual *et al.* (2012)** confirmed that *Moringa oleifera* is a new source of proteases with potential use for cheese production on account of promoting extensive hydrolysis of k-casein and low degradation of α_s- and β-caseins compared with other plant coagulants. It has been concluded that *Moringa oleifera* plant is an exciting source of milk clotting enzymes such as aspartic enzyme in different parts (**Pontual *et al*., 2012**). Agreeing to **Tajalsir *et al.* (2014)**, the milk clotting activity of *M. oleifera* was only found in the seeds extract while the other parts were either deficient or has very low milk clotting activity. *Moringa oleifera* seeds extract are rich in proteases having specific clotting activity 200 times higher than that of flower extract**.** Approving to these results, it is concluded that the seed extract of *M. oleifera* generates suitable milk clotting activity for cheese making **(Sánchez-Muñoz *et al*., 2017). Mahami *et al.* (2012)** proved that *Moringa oleifera* seed extracts used to prepare cottage cheese with increasing in the yield of cheese. On the same line, sensorial properties including texture, appearance, flavour, taste and colour of the produced cheeses were improved by used *Moringa oleifera* seeds as coagulant (**Orhevba and Taiwo, 2016**).

Precipitation with ammonium sulphate is extensively used in processing of biological products, such as proteins. According to **Kent (1999)** protein purification using ammonium sulphate has three main advantages; it is a rapid and inexpensive method, it does not affect the structure and function of proteins and the salt can be easily removed from the protein solution by dialysis. Sedimentation with ammonium sulphate could be an effective way to produce substantial amounts of active purified proteases, and concentrated the enzyme to a workable volume that could efficiently be used for milk coagulation in cheese making industry **(Tajalsir *et al.*, 2014)**.

The present study was undertaken to evaluate the milk clotting properties of crude and partially purified adult rennet modified by mixing with *Moringa oleifera* seeds during the process of extraction. The suitability of the resultant extracts as calf rennet alternatives in cheese making was given considerable attention

MATERIALS AND METHODS

This study was carried out during November 2017 to August 2018 in the Dairy Technology laboratory of Dairy Department, Agricultural Faculty, New Valley University, Egypt.

Materials

Fresh abomasum of adult cattle and calf were brought from one butcher in El-kharga City, New Valley Governorate, Egypt. The dried pods in *Moringa oleifera* were collected from the local farm in El-Kharga City, New Valley Governorate, Egypt. Raw cow's milk (total solid 12.8%, 3.5% protein, 3.7% fat, 5.0% lactose and pH 6.7) was obtained from private animal farm in El-kharga City, New Valley Governorate, Egypt. Low heat skim milk powder imported from USA (Dairy America California). Commercial liquid calf rennet was purchased from Mifad, Misr Food Additives, Egypt. Sodium chloride, ammonium sulphate, sodium acetate, and other chemicals were purchased from Gomhoria Company, Assiut Governorate, Egypt.

Experimental Procedure

Preparation of experimental crude rennet extracts

Rennet of adult cattle (ARE) and rennet of calf (CRE) extracts were prepared according to the method of **Ahmed *et al.* (2013)**, with some modification. After removing the internal contents, abomasum tissues were cleaned with tap water, as well veins and fat contents were external removed. Then they were inflated with air just like a balloon, 20 g sodium chloride were externally sprayed and dried in an open air for about one week followed by storage for one month. The abomasum was then cut into thin pieces. Weight (100g) of dried abomasum was soaked in 1000 ml solution containing (60 g sodium chloride, 2 g sodium benzoate, 0.5 g sorbic acid in distilled water), and adjust the pH to 5.4. The solution was stored at room temperature for one week with daily stirring. The extract was filtered through muslin cloth, clarification by addition 8g aluminum potassium sulphate and free enzyme by addition 16 g disodium phosphate (10% solution) with stirring for 3 min. The extract was again filtered through Whatman filter paper No. 1 and centrifuged at a speed of 5000 rpm for 20 min, then the pellet was discarded while the supernatant was collected and designate as crude enzyme extract.

Preparation of crude adult cattle rennet modified by Moringa seed

Manually the seeds were removed from pods and removed seed coats from seeds to obtain clean seed kernels. The white kernels were then crushed to a powder using an electric grinder to obtain a fine powder. About 20g of seeds powder was blended in 1000 ml crude enzyme of adult cattle rennet. Then it stirred rapidly for sixty minutes, after every interval the samples were allowed to rest for 60 minutes and were filtered through Whatman filter paper No1. Then centrifuge at a speed of 5000 rpm for 20 min. The pellet was discarded and the supernatant was collected designate crude enzyme mix of adult cattle rennet with Moringa seeds (AME).

Partial purification of crude adult cattle rennet extracts

The crude enzyme of adult cattle rennet without and with added *Moringa oleifera* seeds powder were partially purified by ammonium sulphate precipitation technique. The crude enzyme extract (50 ml) was partially purified by adding of solid ammonium sulphate to 60% concentration at 4°C for 30 min according to **Ahmed *et al.* (2009)**, with some modifications. The sedimentary protein was collected by centrifugation at 5000 rpm at room temperature for 20 min. The supernatant was discarded while the precipitate was re-dissolved in 5 ml of 0.1 M sodium acetate buffer (pH 5), and then dialyzed overnight at 4°C against the same buffer with several change of the dialysis buffer. The extracts obtained were named ARP and AMP for partially purified for adult cattle rennet without and with added Moringa seeds powder, respectively.

Methods of Analysis

Determination of rennet flocculation and clotting times

The rennet flocculation time (RFT) is the time period between the additions of coagulant enzyme and the appearance of flakes visible to the naked eye (**Kappeler *et al.*, 2006)**. The rennet clotting time (RCT) is the time period between the application of the coagulant enzyme to the milk at 35°C and the aggregation of milk. The rennet curdling quality (RCQ) is evaluated after the curd formed and tipped out into Petri dish to assay the appearance and firmness of the curd and the appearance of the whey as investigated by **Kuchtík *et al.* (2008)**.

Determination of milk-clotting activity (MCA)

The milk clotting activity of coagulant enzyme was performed as described by **Badgujar and Mahajan (2009)**. The enzyme extract (0.2 ml) was added to 2 ml of substrate solution of reconstitute 10% skim milk powder with 0.01M $CaCl_2$. The time needed for the formation of curd fragment at 35°C was measured. Milk clotting activity is expressed in term of Soxhlet unit.

$$\text{Soxhlet units (U/ml)} = \frac{M \times 2400}{E \times t}$$

Where:

M: the volume of substrate (ml).

E: the amount of enzyme extract (ml).

t : the clotting time (sec).

Determination protein content and proteolytic activity (PA)

Total protein was determined in milk coagulants by the standard micro- Kjeldahl method of the **AOAC (1995)**. A nitrogen conversion factor of 6.25 was used for calculation of protein. Proteolytic activity of coagulant extracts was evaluated by comparing non protein nitrogen (NPN) contents of raw reconstituted milk and rennet coagulated milk **(Ahmed *et al*., 2013)**. Briefly 0.2 ml of enzyme extract was added to 2 ml of 10% (*W/W*) of reconstituted skim-milk powder containing 0.01 M calcium chloride and incubated at 35°C for 40 min. Non-protein nitrogen (NPN) content was determined according to the method of International Dairy Federation **(IDF, 1993).** The specific activity was determined by dividing the MCA on protein content (mg).

Optimum temperature and pH for milk-clotting activity

Aliquots of reconstituted spray-dried skim milk (10 g in 100 ml 0.01 M calcium chloride) were adjusting to different pH values (5.5, 6.0, 6.5, 7.0, 7.5 and 8.0), and incubated at different temperatures (30, 35, 40, 45 50, 55, 60, 65 and 70^0C) . Adjusted to the desired pH with 0.1 M HCl or Na OH and incubated at the desired temperature, according to **Corrons *et al.* (2012)**.

Electrophoretic Analysis (SDS–PAGE)

Soluble proteins were extracted from curd formed by ARE, AME, ARP, AMP, RRE and CRE coagulants. Crushing 1.0 g of the sample in 1ml of extraction buffer (0. 1 M Tris-HCl, 2 mM EDTA, 2% glutathione, pH 7.8). The mixture was centrifuged for 20 min at 13000 rpm and 4°C. A mixture of the supernatant and the sample application buffer (2.5 mM Tris-HCl pH 6.8, 10.0% glycerol, 4.0% SDS, 0.02% bromophenol blue and 10% mercaptoethanol) was prepared with a ratio of (1:1) and then, the mixtures were incubated in water path at 100°C for five min. for protein denaturation, and then loaded in the electrophoresis apparatus. Electrophoresis was carried out according to the method of **(Laemmli, 1970)**. Electrophoresis was performed in a vertical slab gel apparatus "SE 600, vertical slab gel" at 10°C in a 12% polyacrylamide separating gel, 3.5% stacking gel, denaturing conditions using 1% sodium dodecyl sulphate, at 30mA, until the dye band reached the bottom of the separating gel. For the detection of protein bands, the gels were stained with commassie brillant blue R for one hour. Then, the destining was performed by repeated immersion in a mixture of methanol/ acetic acid/water (1: 1:8, by volume).

Cheese making

Domiati cheese treatments were made according to **Abou-Donia (1986)** with some modulation**.** Fresh cow milk (3.7% fat and 3.5% protein) was heated to 72°C for 2 min. and then cooled to 45°C, followed by addition of skim milk powder, $CaCl_2$ and NaCl at the rate of 5%, 0.02% and 6% respectively. The milk was divided into six equal portions .Milk coagulants; ARE, AME, ARP, AMP and CRE were added at the ratio of 2 ml/l milk and that of RRE concentration was 0.6 ml/l of milk. The resultant cheeses were refereed as called ARE, AME, ARP, AMP, CRE and RRE cheese. The milk was mixed and left until coagulation was completed. After coagulation; the curd was cut with a sharp knife. The curd was poured into small stainless steel holes molds overnight. Cheese samples were taken for analyses after one day.

Estimation of cheese curd properties

Water release **(**WR) analyses were performed using the method reported by **Lemes *et al.* (2016)**. 10 g of curd cheese were centrifuged at 5000 rpm for 20 min at room temperature. Water release **(**WR) was defined as follows:

WR (%) = 100 (Initial weight - final weight) / (initial weight).

Water holding capacity (WHC) was calculated according to **Farnsworth *et al.* (2006)**.

WHC (%) =100 (curd cheese weight– serum weight) / curd cheese weight

Yield calculation

Cheese yields were calculated as a weight of cheese divided by weight of milk expressed as a percentage (**Nelson *et al*., 2004**).

Sensory attributes

Organoleptic properties of fresh Domiati cheese were assessed by taste panel included mainly researchers and graduate students of the Dairy Science Department, Faculty of Agriculture, New Valley University. Fresh cheese samples were evaluated for flavour (50 points), body and texture (40 points) and appearance (10 points) according to **Bodyfelt *et al.* (1988).**

Statistical Analysis

SPSS (Version 11.0) software was used for all statically analysis. The effects of crude extract, purified enzyme, commercial rennet and calf rennet on curd properties yield and sensory attributes of Domiati cheese were estimated using analysis of variance (ANOVA). Significant means were compared using Duncan's test on the level of $P \leq 0.05$.

RESULTS AND DISCUSSION

Flocculation and Clotting Times

The coagulation properties of milk are of great importance as they influence cheese yield and quality. Fig. 1 clears that the flocculation time (RFT) of ARE, AME, ARP, AMP, RRE and CRE was found to be 169.67, 133.67, 122.67, 82.33, 56.00 and 28.00 seconds respectively. Clotting time (RCT) was 217, 177.67, 152.00, 119.33, 83.67 and 53.67 seconds, respectively. From these results, it could be concluded that crude and partially purified adult cattle extract containing *Moringa oleifera* seeds had shorter flocculation and clotting time compared with the corresponding extract prepares without Moringa seeds. Shorter flocculation and clotting time were found to be for the partially purified adult rennet extract with added Moringa seeds. Whereas their values were comparable to those of both calf rennet extract and commercial rennet. These results could be explained on the basis that *Moringa oleifera* seeds contained one or more enzymes with rennet like activity (**Sánchez-Muñoz *et al.*, 2017**). Partial purification using ammonium sulphate has led to more concentrated milk clotting proteases. This may be due to molecular rearrangements in protein structure can lead to increase of enzyme activity (**Purich, 2010**). Moreover **Castillo *et al.* (2002)** showed that the use of calf rennet as milk coagulant led to faster increase in reflectance due to a higher specific activity to κ-casein. Short clotting time is imported in the manufacture of various type of cheeses, such as soft, semi-hard and hard cheese, could be due to increase in rate of aggregation as a result of decrease in volume of the aqueous phase **(Mehaia and El-Khadragy 1998)**.

Milk Clotting Activity (MCA)

Milk clotting activity (MCA) is the most important property of proteases used in cheese production. Accordingly, the highest milk clotting activity (MCA) was recorded for the CRE as illustrated in Table 1. While, ARE had the lowest milk clotting activity. This indicated that the clotting activity is related to the enzyme's capacity to calving k-casein at the Phe105-Met106 peptide bond, which is the starting point of enzymatic cheese making. **Guinee and Wilkinson (1992)** showed that the clotting activity of rennet relies on its ability to degrade casein micelles. The action is dependent on the chymosin and pepsin content of the rennet. Therefore, this suggested that the content of pepsin was higher in adult cattle rennet extract **(Broome and Limsowtin, 1998)**, compared with rennet extracted from younger calves containing higher chymosin **(Akin, 1996)**. Normally commercial rennet contains 70% chymosin and 30% pepsin. The standard ratio is approximately 80% chymosin and 20% pepsin **(Irigoyen *et al.*, 2001)**. However, adult cattle rennet may contain up to 90 to 94% of pepsin and only 6 to 10% of chymosin **(Broome and Limsowtin, 1998)**. Good quality rennet should possess a constant clotting activity and contain no other enzymes than chymosin (**Çakmakçi and Boroğlu, 2004)**. It was also observed from Table 1 that the milk clotting activity increased nonetheless decreasing clotting time with mixing adult cattle rennet with Moringa seeds powder during the process of extraction, indicating that *M. oleifera* is an exciting source of milk clotting enzyme with an excellent clotting property (**Sánchez-Muñoz *et al.*, 2017**). Partial purification by ammonium

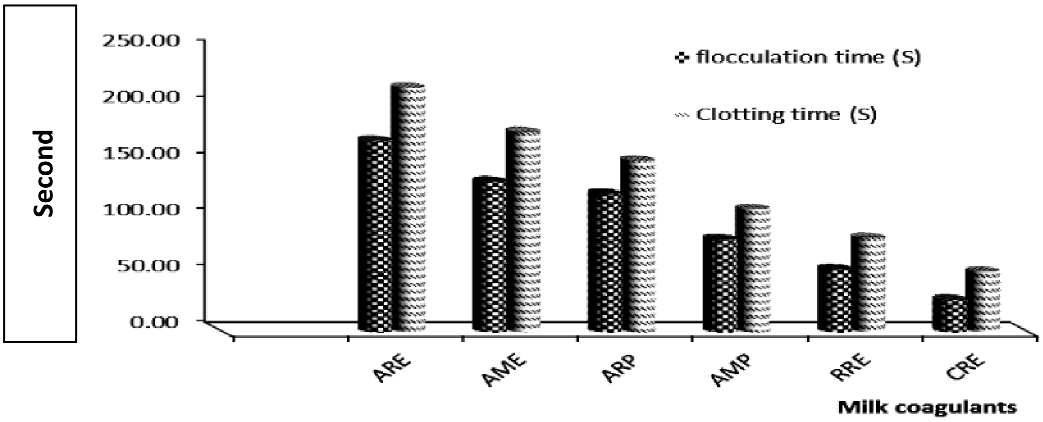

Fig. 1. Flocculation and clotting time of the different milk coagulants

ARE (Adult cattle rennet extract)
AME (mix of adult cattle rennet with Moringa seeds)
ARP (partially purified of adult cattle rennet extract)
AMP (partially purified adult rennet mixed with Moringa seeds)
RRE (commercial rennet)
CRE (calf rennet extract)

Table 1. Milk clotting properties of different milk coagulants

Milk coagulant	Protein content (g/l)	Milk clotting activity (U/ml)	Specific activity (U/mg)	Proteilytic activity (U/ml)	MCA/PA
			Crude extract		
ARE	1.53±0.05 [a]	110.74±4.91 [d]	72.54±3.37 [d]	0.88±0.05[d]	125.84±10.44[d]
AME	1.45±0.08 [ab]	135.44±8.40 [d]	93.54±6.80 [d]	0.93±0.08[d]	145.63±6.14[cd]
RRE	1.31±0.03 [de]	288.22±23.76 [b]	220.41±14.38 [b]	0.47±0.05[b]	613.23±20.95[b]
CRE	1.27±0.03 [e]	455.71±79.23 [a]	358.96±51.52 [a]	0.31±0.05[a]	1470.03±346.64[a]
		Partially purified extracts using aammonium sulphate precipitation			
ARP	1.42±0.03 [bc]	157.26±3.63[b]	111.06±4.35 [d]	0.64±0.06[d]	245.72±20.97[cd]
AMP	1.36±0.05 [cd]	219.79±9.70 [c]	162.27±12.12 [c]	0.55±0.09[c]	399.95±96.61[bc]

Means followed by different letters are significantly different at $P < 0.05$.
ARE (adult cattle rennet extract)
AME (mix of adult cattle rennet with Moringa seeds)
ARP (partially purified of adult cattle rennet extract)
MCA (milk clotting activity)
AMP (partially purified adult rennet mixed with Moringa seeds)
RRE (commercial rennet)
CRE (calf rennet extract)
PA (proteolytic activity)

sulphate of adult cattle rennet (ARP) without or with mixing with Moringa seeds increased the clotting activity of the resultant extracts approaching that of commercial rennet (RRE). There were significant differences ($p \leq 0.05$) in milk clotting activity of crude and purified extracts. Similar results were reported by **Purich (2010) and Lemes *et al.* (2016).** The milk clotting properties are important both with regard to quality and yield of cheese. It has been submitted that a firmer curd at cutting is positively linked to yield of cheese **(Wedholm *et al.*, 2006)**.

Protein Content, Proteolytic Activity and Ratio Milk Clotting Activity (MCA) to Proteolytic Activity (PA)

Evaluation of enzymatic activities and proteolytic activities (PA) is a crucial step in the selection of an appropriate substitute of calf rennet. Table 1 shows the results of protein content, proteolytic activities (PA) and MCA/

PA for different milk coagulants. The protein contents (g/l) were 1.53, 1.45, 1.31, 1.27, 1.42 and 1.36 for ARE, AME, RRE, CRE, ARP and AMP, respectively. There were significant differences (P≤0.05), between coagulants. However, an increase in protein content decreases milk clotting activity. In addition the results indicated that CRE was lower than ARE in proteolytic activity. This may be due to pepsin naturally present in rennet, which has a lower specific milk-clotting activity with a broader proteolytic activity than chymosin **(Slamani *et al*., 2018).** The breakdown depends on synergistic enzymatic action of chymosin and pepsin contained in animal rennet. The low proteolytic activity as shown in trials AMP followed ARP and AME are pre-requisite for an acceptable rennet substitute. The ratio of milk-clotting activity (MCA) to proteolytic activity (PA) of the enzyme is a useful indicator for its quality as a rennet substitute for cheese making **(Sánchez-Muñoz *et al*., 2017)**. The results clear that ARP and AMP extract had higher ratio (MCA/PA), in agreement with the findings of **Barros *et al*. (2001)**. Therefore an enzyme with higher ratio of milk-clotting to proteolyic activity is able to form curd, get higher yield and less bitterness development during cheese processing. While, low ratio may developed lower curd recovery, weak curd firmness and release bitter peptides that affect the sensory properties of the final product **(Mazorra-Manzano *et al*., 2013**). For this reason, the evaluation of enzyme activities and their comparison with commercial rennet is an important first step in choosing a suitable plant coagulant. From the results found, it can be concluded that, the milk clotting enzymes present in AMP, ARP and AME are promising candidates for application in industrial scale for production of cheese. **Tajalsir *et al.* (2014)** showed that partially purified enzyme from Moringa seeds showed higher ratio of milk clotting activity to proteolytic activity compared to that of calf rennet that is extensively used in cheese making industry.

Optimum pH and Temperature for Milk-Clotting Activity

The effect of pH and temperature on milk clotting activity is shown in Fig 2. Nearly all milk coagulants preparation; ARE, AME, ARP, AMP, RRE and CRE exhibited almost a linear milk clotting activity with an increased pH from 5.5 up to 6.5. Milk clotting activity significantly enhanced at pH 5.5 but activity was lowered above pH 6.5. Milk clotting activity decreased gradually as pH increased. The milk clotting activity of AME, ARP, AMP, RRE, and CRE was found to be high at pH 6.0 and ARE at pH 6.5.These results agree with those of **Tajalsir *et al*. (2014**), who used the same conditions. Furthermore, AME, RRE and CRE reached less activity up to pH 7.0. Also ARP and AMP showed complete inactivation up to pH 7.0. These results are in accordance with **Elagamy (2000)**. **Lamas *et al*. (2001)** showed that most plant protease are unstable at alkaline pH value. Calf rennet showed similar conduct, acting better in acid (pH range of 5.5–6.0) than in alkaline reaction medium. The pH of the milk for rapid milk clotting activity is very important during cheese making. **Ramet (2001)** showed that all clotting cheese enzymes are acid protease, with optimum activity at pH 5.5 and also that the kappa casein presents stability at pH 5.6. **Lenoir *et al.* (1997)** 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 below the normal pH of milk. **Formaggioni *et al.* (2001)** reported that milk clotting properties were better when associated with decreasing values of pH and increasing values of titratable acidity.

On the other side, the influence of temperature was most pronounced between the 55 - 60°C for milk clotting activity of milk coagulants. Similarly **(Kumari *et al*., 2012)** reported that the purified serine protease (clotting enzymes) from *Ficus religiosa* had optimum temperature around 55 - 60°C, but beyond these values there were a progressive denaturation of the enzyme and at 65°C there were no activity. The highest milk clotting activity was recorded for ARE, AME and ARP at 50 - 60°C, and for AMP at 40 - 50°C, CRE at 50 - 60°C and RRE at 55 - 65°C. The temperature of maximum activity for milk coagulants AMP and CRE was 50°C likewise, milk coagulants ARE, AME and ARP was 55°C furthermore, milk coagulant RRE was 60°C. The clotting activity began to decrease when temperature increased to 70°C, which in the line

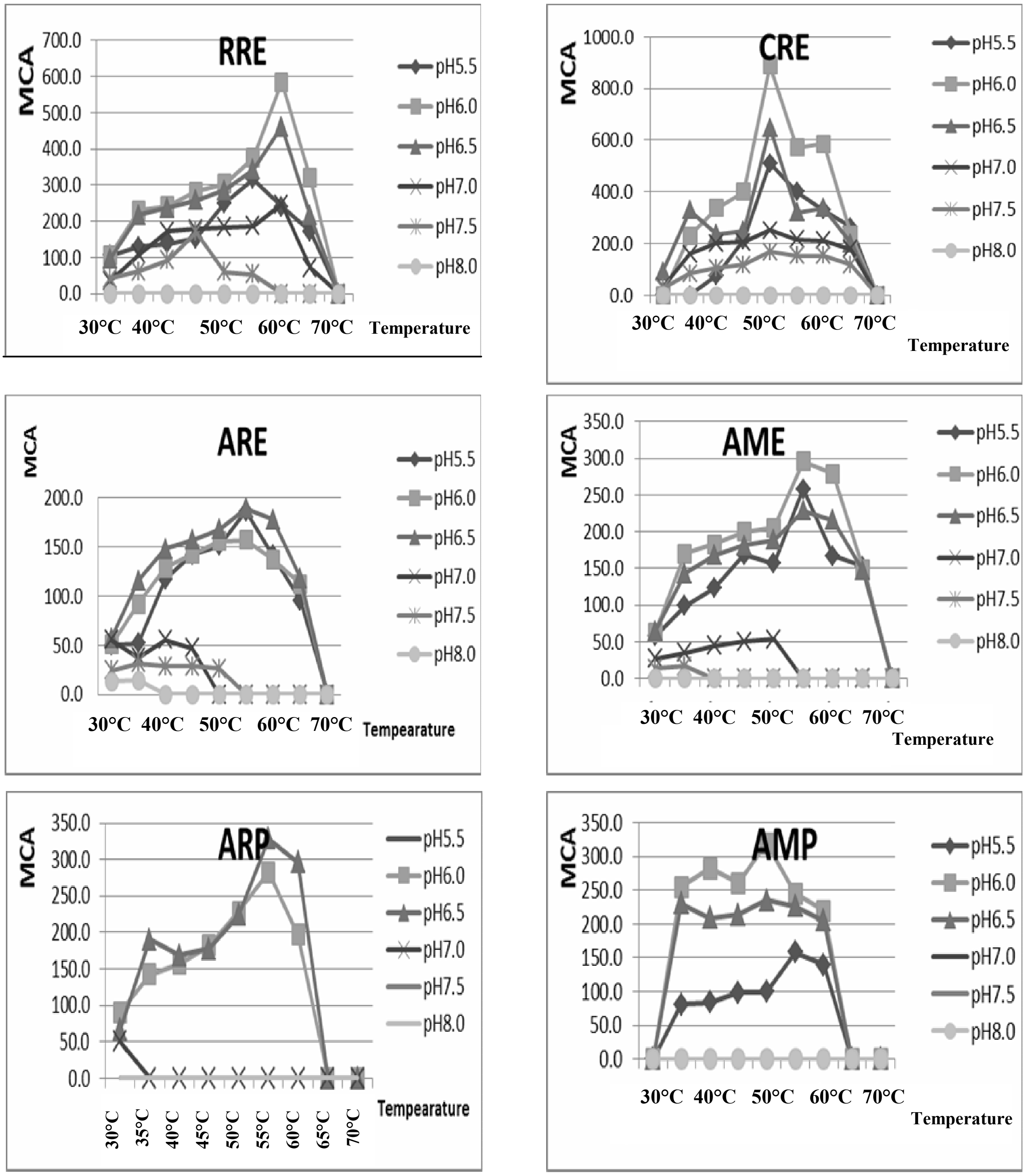

Fig 2. Optimum temperature and pH for milk-clotting activity of different milk coagulants

ARE (adult cattle rennet extract)

AME (mix of adult cattle rennet with Moringa seeds)

ARP (partially purified of adult cattle rennet extract)

MCA (milk clotting activity)

AMP (partially purified adult rennet mixed with Moringa seeds)

RRE (commercial rennet)

CRE (calf rennet extract)

to the activity described by **Ahmed *et al.* (2009)**. It is well known that the reaction rate of enzyme increases with increasing temperature, but after a certain temperature, the rate of reaction decrease due to denaturation and consequently the coagulation time is lengthened **(Hashim *et al*., 2011). Shindo and Arima (1979)** reported that cattle chymosin was relatively stable up to 50°C and a relative decline in the milk-clotting activity of almost 55% was observed at 55°C, there being a total loss of activity at 60°C. The optimum temperature of most clotting enzymes are around 40-50°C, but further than these values there are a progressive denaturation of the enzyme and at 65°C there was no activity **(Ramet, 1985).** On the other hand, **Balcones *et al*. (1996**) reported that the decrease in clotting activity as a result of increasing temperature over certain limit could be explained by the changes in salt equilibrium and the complex between k-casein and β- lactoglobulin.

The Evaluation of the Rennet Curdling Quality

The rennet curdling quality (RCQ) of crude extract, purified enzyme, commercial rennet and calf rennet is shown in Table 2. The curd formatted by CRE was very good and hard without separated whey. AMP had good hard curd, keeping its shape without appearing whey. However ARP had good curd, with little whey of yellow-greenish colour, RRE had hard curd, without appearing whey. AME, had softer creaminess curd, keeping its shape quite perfectly and whey was yellow. ARE, had, weak curd, not keep its shape, whey was milky white. In the same case **Slamani *et al.* (2018)**, found that curd produced using ovine pepsin was less firm than that made with chymosin. Chymosin, an aspartyl proteinase, is used for coagulating of milk and fabrication of cheese. The results indicated that the best (RCQ) was found in the curd formatted by CRE followed by AMP, ARP and AME. Whilst, the worst (RCQ) was found in the curd shape by ARE coagulant. So, the curd formation from CRE coagulant exhibited the fastest and ARE had the lowest. Results from this study indicated that mix adult cattle rennet with Moringa seeds and its partially purified enzyme demonstrated the highest clotting activity compared with adult cattle rennet. These results are in agreement with those of **Tajalsir *et al.* (2014**).

Electrophoretic Profile of the Curd Format by Different Milk Coagulants

Fig. 3 shows SDS-PAGE of curd formed by ARE, AME, ARP, AMP, RRE and CRE extract. The applied electrophoretic method separated curd cheese proteins into multiple components with molecular weights ranged from 10 kDa - 250 kDa, values were similar to those reported by **Manzo *et al.* (2017)**. There were notable differences in electrophoretic patterns among curds shaped by different coagulant extracts. All coagulant extracts hydrolysis κ-casein but in varying proportions. CRE coagulant characters appeared κ-casein. AME similar CRE had clear hydrolysis of κ-casein. On the other hand, there hydrolysis products (α, β -casein) were found in curd cheeses made with AME coagulant. The results agree with those of **García *et al.* (2012).** Electrophoresis profile found that β-casein disappeared from curd formed by ARP curd. Partial purification from *M. oleifera* seeds (AMP) is a potentially useful tool in cheese production processes, since it did not promote extensive hydrolysis of α_s- and β-caseins. This is in line with the results reported by **Bruno *et al.* (2010).**The result also found in ARE coagulant similar hydrolysis casein to that of AME coagulant. RRE coagulant had higher proteolytic activity against κ-casein than α- and β-casein (**Ordiales *et al*., 2012**)

Physical Characteristics of Cheese Curd

Physical characteristics of curd were assessed by determination of water release (WR) and water holding capacity (WHC).

Water Release and Water Holding Capacity

The results of water release (WR) and water holding capacity (WHC) of cheese curd are shown in Table 3. The results showed that CRE cheese curd had the lowest water release followed by AMP, RRE, ARP and AME, whilst, in turn, ARE had the highest ($p \leq 0.05$), possibly

Table 2. Evaluation of curdling quality (RCQ) clotting by different milk coagulants

Coagulant	Appearance, firmness of curd and appearance of whey
ARE	Weak curd, partly liquefaction and loss of shape, whey milky white colour.
AME	Well curd but a softer creaminess, keeping its shape quite perfectly.
ARP	Good curd, a little whey, of yellow-greenish colour.
AMP	Good curd and hard, keeping its shape without appearing whey
RRE	Good curd and hard, without appearing whey.
CRE	Very good curd and hard, without separated whey.

ARE (Adult cattle rennet extract)
AME (mix of adult cattle rennet with Moringa seeds)
ARP (partially purified of adult cattle rennet extract)
AMP (partially purified adult rennet mixed with Moringa seeds)
RRE (commercial rennet)
CRE (calf rennet extract)

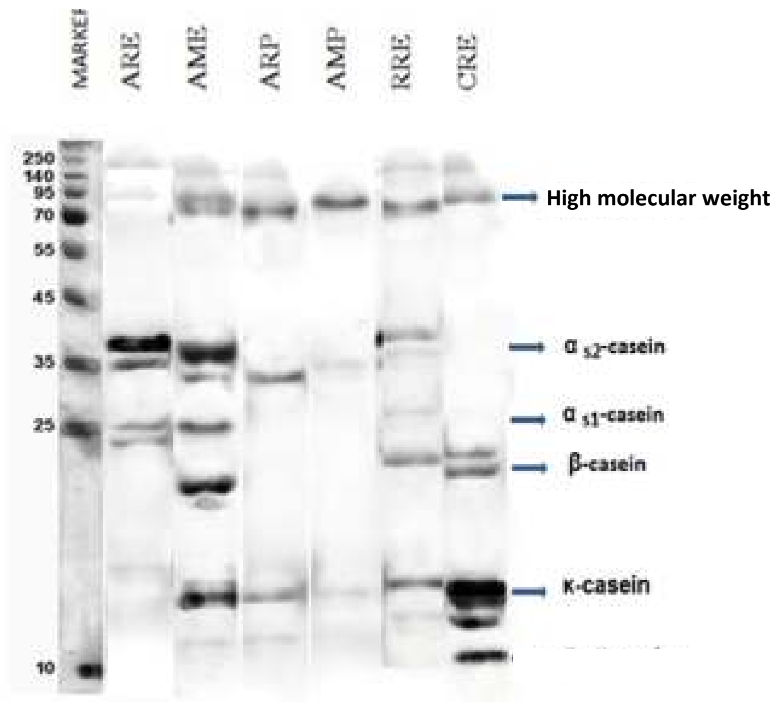

Fig. 3. SDS-PAGE profile of curd formed with different milk coagulants

ARE (Adult cattle rennet extract)
AME (mix of adult cattle rennet with Moringa seeds)
ARP (partially purified of adult cattle rennet extract)
AMP (partially purified adult rennet mixed with Moringa seeds)
RRE (commercial rennet)
CRE (calf rennet extract)

Tables 3. Physical properties of Domiati cheese curd formed by different milk coagulants

Coagulant	Water release (WR)	Water holding capacity (WHC)
ARE	77.10±2.07 [a]	23.43±2.97 [b]
AME	73.23±6.31 [ab]	27.27±6.62 [ab]
ARP	72.93±1.62 [ab]	27.65±1.35 [ab]
AMP	69.83±3.19 [b]	31.07±3.15 [ab]
RRE	70.17±3.91 [ab]	30.80±4.66 [ab]
CRE	68.97±2.60 [b]	31.87±2.95 [a]

Means followed by different letters are significantly different at $P < 0.05$.

ARE (Adult cattle rennet extract) AMP (partially purified adult rennet mixed with Moringa seeds)

AME (mix of adult cattle rennet with Moringa seeds) RRE (commercial rennet)

ARP (partially purified of adult cattle rennet extract) CRE (calf rennet extract)

because of the porous and loose gel in ARE curd. This may be due to that chymosin has high specificity for the k-casein and pepsin has low specificity which affects the properties of the curd, such as firmness or softness **(Walstra *et al.*, 2005)**. Pepsin as a single coagulating agent is not preferred in cheese making because of its high proteolytic activity **(Temiz *et al.*, 2008)**. A decrease in the water release causes the syneresis process, which refers to the serum that is out from the gel structure and accumulates on the surface. An increase in the syneresis process was observed in the ARE, which can be verified by reduction the values of water holding capacity (WHC). This occurrence was more evident when the samples were estimated by centrifugation, indicating a greater water release from gel structure. These results were markedly, and it may depend on the more water release and non-stable structure. Syneresis is one of the most important processes in cheese-making because it directly affects cheese yield and quality through its effect on moisture, mineral and lactose content of curd **.**It is also imperative to cut the curd at an appropriate firmness so that syneresis takes place properly and loss of milk solids in the whey can be minimized **(Bynum and Olson, 1982)**. There were significant differences (P≤ 0. 05) between the coagulant activities obtained for crude ARE and CRE comparative of other milk coagulants. The results showed that CRE, AMP and RRE curd cheese samples had a higher (WHC). Per cent of (WR) and (WHC) of ARP and AMP gels was similar. The clots formed by milk coagulants; AME and ARP showed the same characteristics; stability and transparent whey exudate. These results showed that both the mix adult cattle rennet with Moringa seeds and partial purified enzyme could be used as alternative of commercial rennet.

Yield and Sensory Evaluation of Fresh Domiati Cheese

Yield of cheese

The casein fraction of milk protein is the dominant factor affecting curd firmness, syneresis rate, moisture retention, and ultimately affecting cheese quality and yield **(Lawrence, 1993)**. Table 4 shows the yield of Domiati cheese made by different coagulants. It could be noticed that the cheese made by CRE had the highest yield, white cheese made by ARE had the lowest yield compared with cheese made by other coagulants. Furthermore, highly significant differences (p ≤ 0.05) were observed between all fresh cheeses except ARP similar AMP cheese. The speed of hydrolysis of caseins influences in yield, and slow degradation of α s-and β-caseins is guarantee production of a firm curd, which is occurs when chymosin is used **(Bruno *et al.,* 2010)**. Most rennet substitutes are more proteolytic than calf rennet and cause diminished yields of casein and fat. The increase in syneresis was probably due to the decreasing water holding capacity that led

Table 4. Yield and sensory attributes of fresh Domiati cheese made from different milk coagulants

Item	ARE	AME	ARP	AMP	RRE	CRE
Yield	15.86±0.65^{d}	16.86±0.05^{cd}	17.12±0.1^{cd}	17.89±0.58^{bc}	18.48±0.93^{ab}	19.56±1.14^{a}
			Sensory Properties			
Appearance (10)	5.82±0.75a^{c}	6.91±0.70^{b}	7.60±0.74^{a}	7.73±0.47^{a}	8.00±0.78^{a}	8.30±0.82^{a}
Flavour (50)	44.27±1.49^{c}	46.87±1.49^{b}	46.30±1.16^{b}	47.55±1.04^{a}	46.55±1.13^{ab}	46.10±0.88^{b}
Body and texture (40)	34.45±2.81^{c}	35.36±1.29^{bc}	37.10±5.13^{ab}	37.40±1.73^{ab}	36.91±1.81^{ab}	38.30±0.68^{a}
Overall scores (100)	84.54±2.81^{c}	89.14±1.81^{b}	91.00±5.95^{a}	92.68±2.15^{a}	91.46±2.42^{a}	92.70±0.82^{a}

Means followed by different letters are significantly different at $P < 0.05$.

ARE (Adult cattle rennet extract)

AME (mix of adult cattle rennet with Moringa seeds)

ARP (partially purified of adult cattle rennet extract)

AMP (partially purified adult rennet mixed with Moringa seeds)

RRE (commercial rennet)

CRE (calf rennet extract)

to more release of whey. The whey drainage indicated weakness of the curd, probably decline the yield of cheese. Obviously, this submitted that Moringa seeds may have accounted for the increase in yield. These results are in agreement with the remark of **Mahami *et al.* (2012)** who used Moringa seeds in improving the yield and quality of cottage cheese. Results indicated that mixing adult cattle rennet with Moringa seeds or purified by ammonium sulphate resulted in decreasing the coagulation time, decreasing the curd syneresis and increasing the water holding capacity compared with adult cattle rennet only. Differences in the cheese yield among the different types of coagulants might be due to their proteolytic specificity, as highly specific coagulants provide higher cheese yields. Milk with short coagulation time, with lesser water retention and rise water holding capacity is expected to give more cheese yield **(Kübarsepp *et al.*, 2005)**.

Sensory evaluation of cheese

Sensory attributes included appearance, flavour, body and texture as well as overall scores of Domiati cheese made using different rennet sources are illustrated in Table 4. The results show that general acceptability of Domiati cheese as total scores indicated that CRE cheese gained the highest acceptability and that ARE cheeses gained the lowest scores being significantly ($p \leq 0.05$) different from other cheese treatments. Chymosin is the major enzyme of calf rennet, and it has been widely used in the dairy industry to produce a stable curd with good flavour due to its high specificity for the Phe105-Met106 bond of k-casein **(Rao *et al.*, 1998).** Acceptability increased with mixing Moringa seeds and partial purification. Some studies reported that vegetable coagulants lead to a soft and pasty cheese texture, partly to liquefaction and loss of shape **(Galán *et al.*, 2008)**. As well as short peptides produced by its high proteolytic activity affect the flavour, which resulted in an excessively acidic and bitter cheese **(Öner and Akar, 1993)**. On the present study appear**ed** the status contradictory, the cheese made by used mix of adult cattle extract with Moringa seeds and partial purified had better sensory with characteristic; coherent texture and buttery taste. **Tajalsir *et al.* (2014)** reported that *M. oleifera* seed extracts were used as a milk-clotting agent, and the resulting curd was white and firm. The use of a low amount of plant coagulants in milk gelation at low pH, may avoid the negative impact of excessive proteolysis of caseins on the texture and flavour of cheeses **(Esteves *et al.*, 2003)**. Generally cheese made by CRE had the higher overall scores followed AMP, RRE, ARP and AME. The results reveal that mixing Moringa seeds and its partial purification (by ammonium sulphate) are a new source of proteases with potential use for cheese making, since it promotes extensive hydrolysis of k-casein and low degradation of α_s- and β-caseins resulting. These results agree with **Pontual *et al.* (2012)**. ARE cheese had the lowest scores; appearance, body and texture as well as flavour. Total sensory parameter of the cheeses clotting by mix Moringa seeds and purified enzyme were close to the RRE and of CRE cheese and both of them had higher sensory scores than ARE cheese.

Conclusion

Clotting studies are important in the manufacture and ripening of cheeses. Good milk-clotting enzyme is characterized by a high specific activity and a low proteolytic activity, later the proteolysis strongly affects the textural and sensory properties of cheese. However, a better understanding of the effect of plant coagulants on rheological properties, sensory characteristics (appearance, flavour, body and texture, taste as well as colour) in addition yield of the cheese is also of great importance when choosing the best substitute of calf rennet. This study focused primarily on maxing adult cattle rennet with Moringa seeds powder during extract processing to increase the rennet strength for milk clotting activity which effect on organoleptic scores and yield of cheeses. The characteristics of the final product could strongly resemble to those of the same cheese made with calf rennet.

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توصيف خصائص تجبن اللبن بمنفحة الماشية الكبيرة وتعديلها ببذور المورينجا لتكون بديلا مناسبا لمنفحة العجول الرضيعة في تصنيع الجبن

هشام عبدالرحمن اسماعيل

قسم الألبان – كلية الزراعة – جامعة الوادى الجديد – مصر

سلطت هذه الدراسة الضوء على خلط منفحة الماشية الكبيرة مع مسحوق بذور المورينجا أثناء الاستخلاص لتحسين قوتها التجبنية واستخدامها كبديل لمنفحة العجول الرضيعة في صناعة الجبن، خلط مسحوق بذور المورينجا مع مستخلص منفحة الماشية الكبيرة بنسبة ٢%، واستخدم المستخلص الخام والمنقى جزئيا باستخدام كبريتات الامونيوم تركيز ٦٠%، تضمنت الدراسة ستة أنواع من مجبنات اللبن كالتالى: مستخلص منفحة الماشية الكبيرة (ARE)، خليط مستخلص منفحة الماشية الكبيرة وبذور المورينجا (AME)، مستخلص منفحة الماشية الكبيرة المنقى جزئيا (ARP)، مستخلص منفحة الماشية الكبيرة و خليط بذور المورينجا المنقى جزئيا (AMP)، مستخلص المنفحة التجارية (RRE) وأخيرا مستخلص العجول الرضيعة (CRE)، تم تقدير زمن بداية وتمام التجبن والنشاط التجبنى والتحللى لجميع المجبنات ودرجات الحرارة والحموضة المناسبة للنشاط التجبنى وجودة الخثرة (RCQ) والتفريد البروتينى للخثرة المتكونة باستخدام (SDS-PAGE)، وأيضًا الخواص الفيزيائية مثل انسياب الماء (WR) وسعة الاحتفاظ بالماء (WHC) والتصافى والخواص الحسية للجبن الدمياطى المصنع باستخدام مختلف المجبنات التى بصدد الدراسة، أوضحت النتائج أن المجبن CRE احتاج إلى وقت بداية التجبن وتمام التجبن قصير وارتفاع النشاط االتجبنى بينما المجبن ARE جاء على النقيض ووقع بينهما المجبنات AME وARP وAMP، تراوحت حدود درجة الحموضة ما بين ٥,٥-٦,٥ ودرجات الحرارة ما بين ٥٠-٦٠م هى المناسبة للنشاط التجبنى للمجبنات، وجودة الخثرة (RCQ) المكونة من المجبنات CRE, AMP, ARP, RRE كانت أفضل والخواص الفيزيائية أظهرت انخفاضاً ملحوظاً لانسياب الماء وارتفاع لسعة الاحتفاظ بالماء فى الخثرة المكونة بواسطة المجبن CRE يليه الخثرة الناتجة من المجبنات AME ARP , RRE, AMP بينما العكس فى الخثرة المتكونه من المجبن ARE كان الاعلى فى انسياب الماء والأقل فى سعة الاحتفاظ به، التفريد الكهربائى للخثرة المتكونة من المجبنات المختلفة أوضحت اختلاف فى مستوى تحلل كلا من αs –casein وκ-casein وβ-casein، استخدام مستخلص منفحة الماشية الكبيرة مع بذور المورينجا وكذلك المستخلص من تنقيتها جزئيا حسن التصافى والخواص الحسية للجبن الناتج، حيث أعطت القوام المتماسك والطعم الدهنى، الخلاصة أوضحت الدراسة أن المستخلص الناتج من خلط مستخلص منفحة الماشية الكبيرة مع بذور المورينجا أثناء الاستخلاص والمستخلص الناتج من تنقيتها جزئيا مناسب جدا لاستخدامه كبديلا لمنفحة العجول الصغيرة فى صناعة الجبن.

المحكمـون :

١- أ.د. محمـــد بـديـــر الألفـي أستاذ الألبان المتفرغ – كلية الزراعة بمشتهر – جامعة بنها.

٢- أ.د. عطية عبدالمعطي عبدالباقي أستاذ الألبان المتفرغ – كلية الزراعة – جامعة الزقازيق.