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ORIGINAL PAPER

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OPTIMIZATION OF CAMEL MILK COAGULATION: THE USE OF COAGULANTS OF MICROBIOLOGICAL AND PLANT ORIGIN

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ABSTRACT

Background. Due to its low casein-k content (only 3%, compared to 13% for cow's milk), processing camel milk into cheese is a critical and challenging step.

Materials and methods. The objective of the present study was to determine the optimal time for camel milk clotting using a microbial enzyme (*Rhizomucor miehei*) and plant extract (*Cynara cardunculus* L.) at two different concentrations (1 and 2 IMCU) while investigating the effect of physicochemical parameters, namely, pH, temperature, and calcium chloride ($CaCl_2$). This was achieved by developing a mathematical equation for each coagulant, where each model is related to the physicochemical composition, acidity of the analyzed milk, and clotting properties of the used coagulant.

Results. Based on the conducted experiments, the two mathematical models were established to exploit all the physicochemical parameters that have a significant level (p value < 5%). The two obtained models using the microbial enzyme and the plant extracts explained 98.14% and 99.68% of the variability of the response data around their means, respectively. The Pareto analysis identified temperature as the most influential parameter on the clotting time by the microbial enzyme. Afterwards, the combination of pH and temperature appeared as the second most significant parameter, followed by pH and enzyme concentration, while calcium chloride content was found to be the least effective parameter on clotting time among all the studied parameters. In the case of the plant extracts, the temperature induced the highest effect, followed by pH, the combination between pH*temperature, the concentration of the used coagulant and, finally, the $CaCl_2$ concentration. Hence, by using the plant extracts (*Cynara cardunculus* L.), the obtained results revealed that adding a low concentration of $CaCl_2$ is associated with a minor effect on camel milk-clotting time.

Conclusion. A mathematical model was developed to optimize the parameters that affect the clotting time of camel milk for each coagulant. As a result, the used microbial enzyme and the *Cynara cardunculus* L. flower extracts showed excellent coagulating properties and immense potential as coagulants for cheese production using camel milk.

Keywords: camel milk, coagulation, clotting time, optimization, microbial coagulant, vegetable coagulant

INTRODUCTION

Camels (*Camelus dromedarius*) are a domesticated species commonly reared in desert regions. They play a crucial role in meeting the nutritional requirements of communities residing in arid and semi-arid zones (Oselu et al., 2022). Despite being suitable for both meat and milk productions, camels are mainly raised

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for milk production due to their ability to produce milk for extended periods, particularly in arid regions where other ruminants may not thrive (Wayua et al., 2012).

Due to its nutritional value and health benefits, camel milk is quite popular and considered superior to bovine dairy mainly in dry zones across the Middle East and Arabian territories (Ho et al., 2022; Swelum et al., 2021). Indeed, as recently reported by Ait El Alia and co-workers, 77.31% of the Moroccan population expressed a more favorable impression towards camel milk consumption once they had received information regarding its health benefits (Ait El Alia et al., 2023).

The consumption of camel milk in its raw or fermented form is considerably more prevalent than its use in processed products such as cheese (Konuspayeva and Faye, 2016). This is mainly explained by the low amount of κ-casein in camel milk, which typically ranges between 1.5% and 3.5%, compared to around 13% in bovine milk and 13% to 20% in buffalo milk. Furthermore, the cleavage sites of camel κ-casein for hydrolysis are different from those in bovine milk, and this limits its use for cheese production. Another characteristic of camel milk that hinders its rennetability is the larger size of its casein micelles being approximately 380 nm, which is almost double that of bovine milk casein micelles (Ho et al., 2022).

Several studies have been conducted for improving the coagulation properties of camel milk. For instance, camel milk proteins' functional and coagulation properties have already been investigated (Fox et al., 2015; Hailu et al., 2016). Other studies have been devoted to improving milk coagulation using various proteolytic enzymes of animal, microbial, and plant origin. Recombinant camel chymosin has successfully been used to coagulate camel milk (Al-zoreky and Almathen, 2021). Recombinant camel chymosin showed a ratio of milk clotting activity (C) to proteolytic activity (P) seven times higher than bovine chymosin; this was explained by variations in surface charge, camel chymosin mobility, and better substrate binding (Langholm Jensen et al., 2013).

Microbial coagulants are simple to manufacture by fermentation; they can be produced in large quantities at a lower cost than animal rennet. In addition, there is no risk of disease transmission from ruminants, and lacto-vegetarians agree with their use. Proteases from *Rhizomucor miehei*, *Rhizomucor pusillus*, and *Cryphonectria parasitica* are the most widely used microbial coagulants. *R. miehei* has already been used as an alternative to animal rennet for almost 40 years (El-Tanboly et al., 2013; Jacob et al., 2011).

For many years, plant extracts have been widely used for milk clotting during the production of traditional cheeses, especially in Asia, the Mediterranean region, and West Africa. Simple purification procedures can isolate plant proteases from a variety of readily available plants, such as kiwifruit (*Actinidia* L.) (Fguiri et al., 2021), nettle leaves (*Urtica dioica*) (Bouazizi et al., 2022), rhizome of ginger (*Zingiber officinale*) (Hailu et al., 2014), and wild cardoon flowers (*Cynara cardunculus* L.) (García et al., 2012). Only a few types of research have shown the use of plant proteases in camel milk clotting and cheese production. For instance, Hailu and coworkers found that camel milk could be coagulated using an extract of raw ginger (Hailu et al., 2014). Likewise, the ability of partially purified *Moringa oleifera* grain extract to coagulate camel milk and form a firm curd has also been reported (Terefe et al., 2017). Fguiri and colleagues (2021) found that kiwi fruit proteases acquire chymosin-like properties in cheese production from camel milk, such as milk clotting activity and textural properties of curd fees obtained (Fguiri et al., 2021). It has also been demonstrated that camel milk cheese produced using a combination of *withania* extract and camel chymosin exhibited a higher quality when compared to chymosin alone (Mbye et al., 2021). *Urtica dioica* extract can also coagulate camel milk with a non-specific action (Bouazizi et al., 2022).

Other than camel milk composition and rennet's type and concentration, the physicochemical parameters (temperature, pH, concentration of $CaCl_2$) also affect the coagulation of this milk (Baig et al., 2022). Heat treatment leads to many physicochemical changes in milk components (Fox et al., 2015). In a stimulant example, it has been reported that the coagulation time of camel milk rennet in the presence of different concentrations of $CaCl_2$ (0 to 20 mg/100 ml) increased with increasing temperature (Hattem et al., 2011). Meanwhile, Hailu et al., (2016) showed that by increasing the gelation temperature, the chymosin content of camel milk, and lowering the pH, it was possible to maximize the coagulation of camel milk.

Decreasing the pH during cheese processing enhances rennet action, facilitates charge neutralization of colloidal calcium phosphate (CCP), and improves calcium solubilization (Baig et al., 2022; Fox et al., 2015). Lowering the pH of milk to 5.6 for the addition of bovine rennet and raising the cooking temperature to 42°C were already used for camel milk cheese production (Oumelkheir Siboukeur et al., 2005).

Within this context, the objective of the present contribution was to determine the optimal clotting time of camel milk from the Guerzni breed, using a microbial coagulant (*Rhizomucor miehei*) and vegetable coagulant (*Cynara cardunculus* L.) under the effect of different physicochemical parameters (pH, temperature, concentration of enzyme and calcium chloride $CaCl_2$) by developing a mathematical equation of milk clotting time.

MATERIALS AND METHODS

Milk samples

10 samples of fresh camel milk from the Guerzni breed were collected in January 2022 from 10 different camels aged between 8 and 12 years, each of which had given birth 2 to 3 times. These camels were from herds owned by nomads in the Béni Mellal region, Morocco, and were fed a mixture of grasses and pasture, especially the *Cynara cardunculus* plant. Before their analysis, the milk samples were stored at 4°C. All analyses were carried out in triplicate on the same day of milking

Physico-chemical analysis

The physico-chemical analysis was carried out by Milko-Scan (Foss 5000 combi, Foss Electric, Hillerod, Denmark). Milk acidity was determined by acid-base titration with sodium hydroxide (NaOH) and phenolphthalein as a color indicator, as mentioned by (Guiraud, 1998). The results were expressed as a percentage of lactic acid.

Coagulants

Two coagulants were utilized to coagulate milk. The first coagulant was a microbial coagulant (Hannilase XP 200 Chrhansen, Denmark) sourced from *Rhizomucor miehei*, with a coagulant activity of 200 IMCU/mL. The recommended dosage for this coagulant was 33–66 IMCU/L of milk. The second coagulant used was of plant origin and obtained in the form of dry samples of wild cardoon flowers (*Cynara cardunculus* L.) from a local market. The extraction of the plant coagulant was achieved by suspending 20 g of ground flowers in 80 mL of water for 30 minutes. Then, the extract was filtered through cheesecloth and centrifuged at 4000 rpm for 10 minutes. The supernatant was filtered through filter paper and stored at 4°C for no longer than 2 days before use. The Berridge method standardized the coagulants to have the same milk clotting activity in standard milk as specified by the IDF (García et al., 2012).

Clotting time determination

The Berridge method, acknowledged by the International Dairy Federation, is the widely accepted technique for determining clotting time(García et al., 2015; López et al., 1999). For microbial enzymes and other coagulants, comparable approaches have been developed (Tabayehnejad et al., 2012). In the present work, a predetermined volume of milk and calcium chloride solution was mixed, and then milk coagulation enzyme solution was added. The mixture was maintained at a consistent temperature in a water bath while the stopwatch was started simultaneously. The clotting time was determined by recording the duration between the addition of the enzyme solution and the initial detection of flocculation on the tube wall (López et al., 1999).

Factorial model

The Minitab 18 software employs a factorial design for optimizing cheese production by investigating the impact of physicochemical parameters on coagulation time. A four-factor design with three replicates was used, which allows the effects of multiple factors to be studied. In this experimental design, the pH of milk was modified between 5.2 and 6.6 using 88% extra pure lactic acid (LOBA CHEMIE PVT. LTD), and its values were measured using a HI 2211 pH/ORP pH meter. Additionally, the temperature range was set between 30°C and 46°C. The calcium chloride concentration was varied between 0.5 mM and 3 mM and produced using calcium chloride dihydrate (Sigma-Aldrich, Germany) to create a calcium chloride solution with a concentration of 510 g/L. The coagulant

enzyme concentration was adjusted between 1 IMCU and 2 IMCU for 20 mL of milk volume, as reported in previous studies (Fagan et al., 2007; García et al., 2015).

RESULTS AND DISCUSSION

Camel milk composition

The physicochemical composition of the 10 tested samples of fresh milk is presented in Table 1. The analysis revealed that the camel milk samples were homogeneous, and thus suitable for blending.

As shown in Table 1, the mean values of fat, protein, lactose, dry matter contents, and density in the studied samples were 2.724 ±0.518% w/v, 3.214 ±0.136% w/v, 4.774 ±0.192% w/v, 11.422 ±0.786% w/v, and 1.030 ±0.001 g/cm^3, respectively. In the case of pH and titratable acidity, the obtained values were 6.440 ±0.063 and 0.184 ±0.010, respectively.

The fat content mean value of camel milk was found to be comparable to the values reported by Alaoui Ismaili et al. (2019) and Kouniba et al., (2005), which were 2.72% w/v and 2.7% w/v, respectively. The dry matter percentage was slightly higher than the previous values reported for Moroccan camel milk by Alaoui Ismaili et al., (2019) and Kouniba et al., (2005), which were 10.42% w/v and 10.8% w/v, respectively. Lactose percentage was also found to be considerably higher than the values reported by Alaoui Ismaili et al., (2019) and Kouniba et al., (2005), which were 4.37% w/v and 4.1% w/v, respectively. The protein content was higher than the values reported by Alaoui Ismaili et al., (2019) and comparable to the values reported by Kouniba et al., (2005), which were 2.554% w/v and 3.3% w/v, respectively. The density of the milk sample had a mean value of 1.030 ±0.001 g/cm^3, which is similar to the values 1.026 ±0.003 g/cm^3 found in other Moroccan camel milk samples (Alaoui Ismaili et al., 2019), but higher than the value 1.023 g/cm^3 reported for Algerian camel milk (Siboukeur, 2007). Differences in camel watering frequency can affect camel milk densities (Rahli et al., 2013). The pH and acidity values were in good agreement with the literature (Alaoui Ismaili et al., 2019; Kouniba et al., 2005). The observed variations in the chemical composition of camel milk could be attributed to several factors, such as camel breed, age, stage of lactation, herd management techniques, and environmental circumstances (Al Haj and Al Kanhal, 2010).

Table 1. Physico-chemical composition of camel milk samples

Variable	Mean	Standard deviation
pH	6.440	0.063
Titratable acidity, lactic acid %	0.184	0.01
Fate content, %	2.724	0.518
Dry matter, %	11.422	0.786
Density, g/cm^3	1.03	0.001
Freezing point, °C	0.549	0.026
Protein content, %	3.214	0.136
Lactose, %	4.774	0.192
Salts, %	0.71	0.029

Factorial design

The design table (Table 2) shows the experimental conditions for each factor corresponding to the Factorial Design. A total of 24 tests were correctly performed, and the clotting times of both coagulants (microbial and plant) results are presented in Table 2.

The employment of microbial enzymes yields a shorter clotting time in camel milk when compared to the utilization of vegetable extracts. However, a different trend was obtained when these coagulants were employed in goat milk (García et al., 2012).

Clotting time prediction

An uncoded unit regression equation of clotting time as a function of the four physicochemical factors (pH, temperature, and enzyme and $CaCl_2$ concentration) for each coagulant was developed based on the experimental approach. The R-squared calculation was used to evaluate the model fit (Onyutha, 2020). The results are reported in Table 3.

The R-squared in our study was 99.68% for the plant coagulant and 98.14% for the microbial coagulant. Therefore, the model could have explained more than 0.32% and 1.86% of the total variation,

Table 2. Parameters of each factor and clotting time results

Run order	pH	T, °C	$CaCl_2$, mM	E, IMCU	Cl.t by microbial enzyme, s	Cl.t by vegetal coagulant, s
1	5.2	30	3	2	18	480
2	5.2	30	0.5	1	70	515
3	5.2	30	0.5	1	60	490
4	6.6	46	3	2	14	187
5	6.6	46	3	2	12	173
6	6.6	30	3	1	82	1 380
7	5.2	30	0.5	1	62	530
8	6.6	30	3	1	71	1 440
9	5.2	46	0.5	2	11	50
10	5.2	30	3	2	16	380
11	6.6	46	0.5	1	19	350
12	6.6	46	0.5	1	20	360
13	5.2	46	0.5	2	23	49
14	6.6	46	3	2	13	162
15	6.6	30	0.5	2	76	1 060
16	6.6	30	0.5	2	65	1 080
17	5.2	30	3	2	17	420
18	5.2	46	3	1	19	135
19	6.6	46	0.5	1	19	338
20	5.2	46	0.5	2	16	47
21	6.6	30	0.5	2	72	1 030
22	6.6	30	3	1	68	1 320
23	5.2	46	3	1	16	130
24	5.2	46	3	1	20	144

T – temperature; E – enzyme concentration; Cl.t – clotting time.

demonstrating how well the model fits the data. In addition, the fitted R-squared (R-sq(adj) = 99.54% for the plant coagulant; R-sq(adj) = 97.33% for the microbial enzyme) is strong, showing that the model is robust. Similarly, the projected R-sq(pred) is high (99.28% and 95.82%, respectively), indicating that each model can anticipate the new data.

The coded coefficients for the analysis of the variability of the regression equation for each coagulant are represented in Tables 4 and 5.

The impact of a factor means the expected change in the average response as the element progresses from the lower to the higher level. After analyzing the obtained results for the coded coefficients of each

Table 3. Uncoded unit regression equations for clotting time

	Regression Equation in Uncoded Units	R-sq	R-sq(adj)	R-sq(pred)
Microbial enzyme	Cl.t = –49,8 + 35.74 pH + 6.301 T – 39.77 $CaCl_2$ – 98.6 E – 1.488 pH*T + 5.90 pH*$CaCl_2$ + 14,29 pH*E	98.14%	97.33%	95.82%
Vegetable coagulant	Cl.t = –6237 + 1450.2 pH + 111.10 T – 107.4 C + 103.8 E – 25.89 pH*T + 20,76 pH*C – 23.29 pH*E	99.68%	99.54%	99.28%

Table 4. Coefficients coded for the analysis of variability in the regression equation obtained by the microbial enzyme

Term	Effect	Coef	SE Coef	P-Value	VIF
Constant		36.667	0.886	0.000	
pH	15.333	7.667	0.886	0.000	1.00
T	–39.667	–19.833	0.886	0.000	1.00
$CaCl_2$	–12.333	–6.167	0.886	0.000	1.00
E	–14.333	–7.167	0.886	0.000	1.00
pH*T	–16.667	–8.333	0.886	0.000	1.00
pH*$CaCl_2$	10.333	5.167	0.886	0.000	1.00
pH*E	10.000	5.000	0.886	0.000	1.00

Table 5. Coefficients coded for the analysis of variability in the regression equation obtained by the vegetable coagulant

Term	Effect	Coef	SE Coef	P–Value	VIF
Constant		510.42	6.25	0.000	
pH	459.17	229.58	6.25	0.000	1.00
T	–666.67	–333.33	6.25	0.000	1.00
$CaCl_2$	37.67	18.83	6.25	0.008	1.00
E	–167.83	–83.92	6.25	0.000	1.00
pH*T	–290.00	–145.00	6.25	0.000	1.00
pH*$CaCl_2$	36.33	18.17	6.25	0.010	1.00
pH*E	–81.50	–40.75	6.25	0.000	1.00

enzyme, it was found that the main negative effect for the microbial enzyme is the temperature, as the clotting time decreases when it rises from 30 to 46°C. The second effect was linked to the combination of pH*temperature. The pH was the main positive effect. When increased from 5.2 to 6.6, the latter parameter affects the direction in which the response (clotting time) increases. Similar outcomes were already reported (Hailu et al., 2016). The pH of the coagulant has also shown its primary effect on camel milk clotting. Another recent study on camel milk-clotting using camel chymosin and *Urtica dioica* extract

prepared at different pH levels (3–6) showed that the extract prepared at a pH of 4 induced the best action on casein micelles compared to other pH levels (Bouazizi et al., 2022). The other factors can be ordered as follows: microbial enzyme concentration, calcium chloride, pH*concentration of $CaCl_2$, and finally, pH*concentration of enzymes. An increase in these factors results in a decrease in clotting time. Similarly, Hailu et al., 2016 found that clotting time was lower at 40°C than at 30°C, increasing coagulant addition and decreasing pH. On the other hand, Genene et al., (2019) showed that camel milk's clotting time increases when the temperature exceeds 65°C for 30 minutes. Also, adding calcium salts lowers the pH, which helps to reduce the clotting time of milk and improve the coagulation of rennet (Li and Zhao, 2019; Lin et al., 2018).

Based on the evaluation of the result of the coded coefficients of the *C. carduclus* extract, the temperature was found to exhibit the main effect, followed by the pH, the combination of the two pH*T, the coagulant concentration, the $CaCl_2$ concentration, and the pH*$CaCl_2$. Therefore, it can be deduced that adding a low concentration of $CaCl_2$ has no significant effect on camel milk-clotting time when using the plant coagulant. This is in accordance with the recently reported results by (Fguiri et al., 2021).

The estimate was accurate for all terms since each coefficient's standard error was less than 0.886 for microbial enzyme and 6.25 for vegetable coagulant (the magnitude of the coefficient is half that of the effect). Furthermore, the *p*-value was below the significance level of = 0.05 (Zine-Eddine et al., 2021a, 2021b). Therefore, it seems to be truthful to state that there is a statistically significant correlation between the terms and the response variable (clotting time). A lack of multicollinearity between the predictors was also indicated by the fact that the variance inflation factor (VIF) is equal to 1, which facilitates the determination of statistical significance.

To evaluate the importance of the effect terms, we have used the Pareto chart (Fig. 1–2). The Pareto chart displays the absolute values of the standardized effects in descending order. The terms in the diagram were statistically significant, exceeding the 95% confidence interval baseline. In these experiments, the variables that most influenced the clotting time of camel milk by

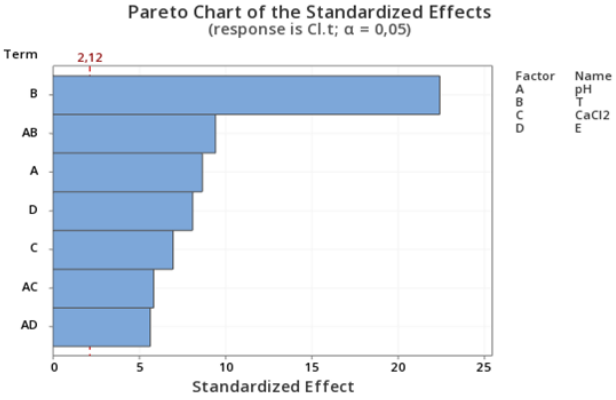

Fig. 1. Pareto chart of the absolute values of the normalized effects using microbial enzyme

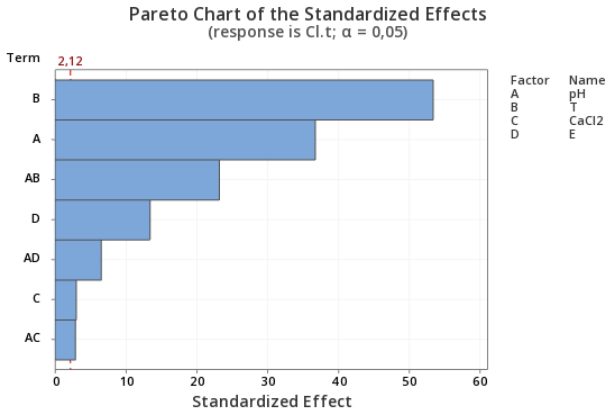

Fig. 2. Pareto chart of the absolute values of the normalized effects using vegetable coagulant

using the microbial enzyme were: temperature, pH*T, and pH. For the plant extracts, the temperature was found to be the most affecting parameter, followed by pH, and the combination between pH*T.

The main effects graph for clotting time was used to study how the terms affect the response (clotting time), as shown in Figures 3 and 4. The clotting time using the microbial enzyme decreases as the pH decreases from 6.6 to 5.2, resulting in a shorter clotting time. In addition, as the temperature increases and the concentration of the enzyme and $CaCl_2$ increases, the coagulation time decreases. These findings corroborated the previous ones.

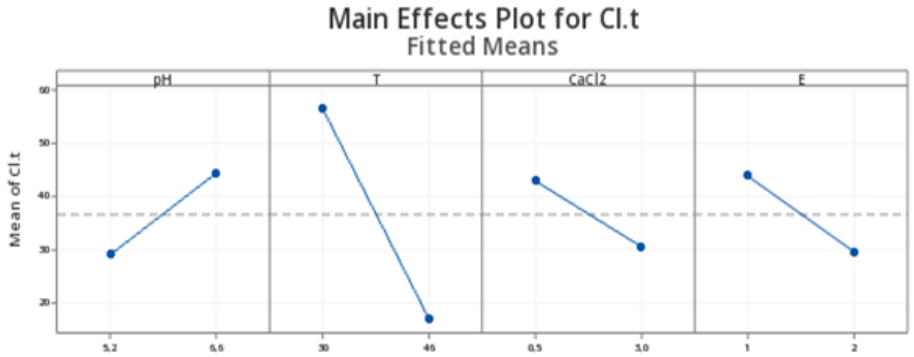

Fig. 3. Main effects plot for clotting time using the microbial enzyme

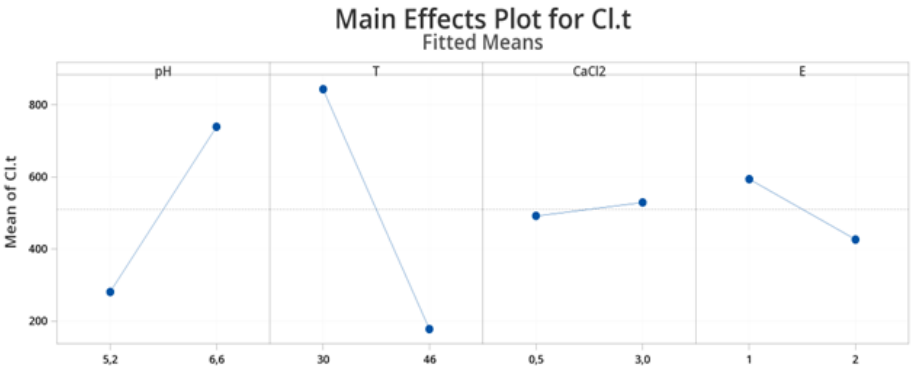

Fig. 4. Main effects plot for clotting time using the vegetable coagulant

Similarly, for the plant extracts, lowering the pH and increasing the temperature in the presence of an increasing dose of the enzyme from 1 to 2 mMol results in a reduction of the clotting time; however, concerning the $CaCl_2$ concentration, it has no significant effect on the clotting time of camel milk. These results confirmed the previous ones.

The cube plot (fitted averages) was used to illustrate the relationship between the input variables (physicochemical parameters) and the output (clotting time). Figures 3 and 4 represent the obtained results for the used microbial enzyme and plant extract. Considering all possible combinations of the variables pH, coagulation temperature (T), amount of $CaCl_2$, and coagulants concentration (E), they display the clotting time values obtained using the multiple linear regression equation presented in Table 3.

Figures 5a and 6a show the response when the coagulant concentration is 1 IMCU, and Figures 5b and 6b illustrate the response when the coagulant concentration is 2 IMCU. Regarding the microbial enzyme (Fig. 5), the parameters of pH = 5.2, temperature = 46°C and $CaCl_2$ = 3 mMol in both coagulant doses demonstrate the short clotting times. The high concentration shows the fastest clotting times. Therefore, the clotting time was shortened by reducing the pH and by increasing the temperature and enzyme concentration, while increasing $CaCl_2$ levels reduce the clotting time.

For the *C. carduclus* extract, the shortest clotting time was obtained at pH = 5.2 and temperature = 46°C for both doses of the plant extracts. An opposite tendency was observed for the $CaCl_2$, as increasing its concentration from 0.5 to 3 mMol led to a prolongation

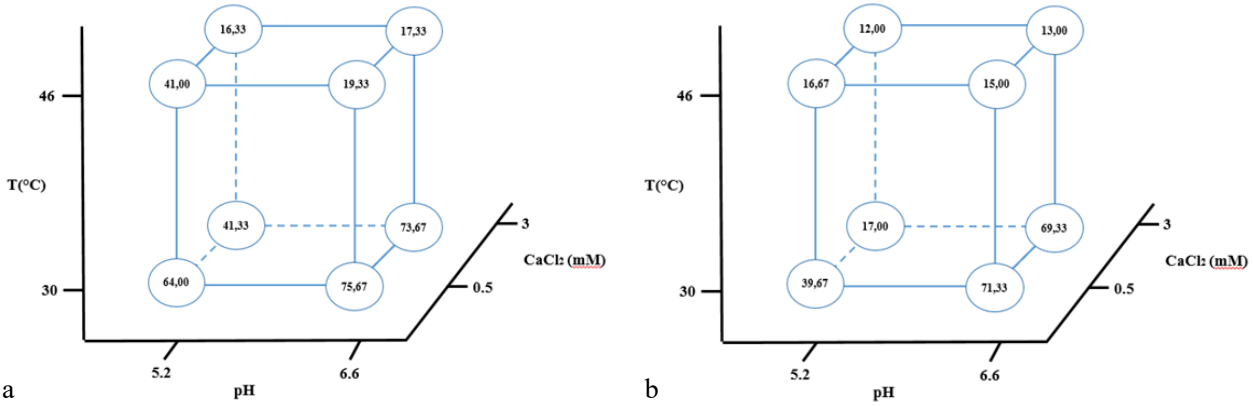

Fig. 5. Cube plot (adjusted means) for clotting time; a – microbial enzyme = 1 IMCU, b – microbial enzyme = 2 IMCU

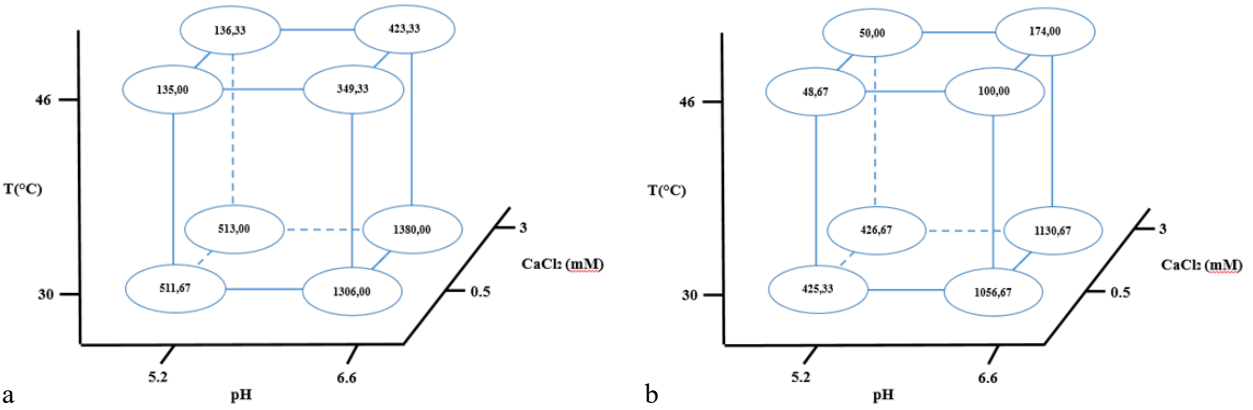

Fig. 6. Cube plot (adjusted means) for clotting time; a – vegetable coagulant = 1 IMCU, b – vegetable coagulant = 2 IMCU

in the clotting time of camel milk. Many authors have highlighted the role of Ca^{2+} ions on the enzymatic coagulation process of milk (Chazarra et al., 2007; Nouani et al., 2009), which confirmed that the addition of $CaCl_2$ does not affect camel milk clotting time using coagulant form *C. cardanculus* flower extract.

CONCLUSION

In the current work, two regression equations were developed to study the effect of physicochemical parameters on camel milk-clotting time using microbial enzyme and plant extract. The parameters affecting the clotting time can be ordered as follows: temperature, pH*T combination, pH, enzyme concentration, and $CaCl_2$ concentration for microbial enzyme. The factors affecting clotting time for plant extract were temperature, pH, and pH*T combination. However, $CaCl_2$ concentration did not affect clotting time. Hence, to improve the coagulation process of camel milk using plant extracts, it is essential to purify cardoon flower crude extracts and better understand its coagulant properties. These findings are believed to play a crucial role in optimizing camel milk's coagulation by using plant-based extracts leading to an improved processing efficiency for cheese production.

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