Physicochemical and rheological changes of acidified camel milk added with commercial low methoxyl-pectin

Donia Zidi [a], Adem Gharsallaoui [b], Coralie Dupas-Farrugia [b], Hamadi Attia [a], Mohamed Ali Ayadi [a,*]

[a] *Laboratoire Analyses, Valorisation et Sécurité des Aliments, Ecole Nationale d'Ingénieurs de Sfax, Université de Sfax, Route Soukra, Sfax 3038, Tunisia*
[b] *Univ Lyon, Université Claude Bernard Lyon 1, ISARA Lyon, BioDyMIA, Equipe Mixte d'Accueil n°3733, rue Henri de Boissieu, F-01000 Bourg en Bresse, France*

ABSTRACT

The influence of the addition of low methoxyl amidated pectin (LMA) on acid milk gels from whole camel milk (WCM) on physicochemical and rheological proprieties were studied. The zeta potential, particle size, viscosity, dynamic oscillatory rheology and isothermal titration calorimetry were monitored. The presence of LM-pectin in milk had an impact on the average size of the casein micelles and a large and dominant influence on rheological behavior during acidification. Zeta potential and viscosity of gels with 0.5% pectin were not affected. However, milk gels containing 1%, 1.5% and 2% of LM-pectin showed highest values of particle size at pH 4. This modification of the structure of the casein micelles induces a significant improvement ($p < 0.05$) on its acid gelation behavior. Therefore, the addition of pectin enhanced the rheological proprieties. Higher pectin concentration led to a strong gel with higher G′ values. This result could be attributed to the formation of complexes and the mechanical spectra prove the hypothesis that pectin forms strands with caseins micelles. Isothermal titration calorimetry results showed that pectin concentration had a marked influence on the gels structure and that this polysaccharide stabilizes caseins micelles in acidified camel milk gel due to electrostatic interaction and steric repulsion.

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1. Introduction

According to the recent statistics of FAO for 2016, the worldwide production of dromedary milk was around 2700 thousand tons in 2016, of which only 1092 tons were produced in Tunisia (FAOSTAT, 2016). Nowadays, camel milk production is in progress in the regions where climatic conditions make it difficult to produce bovine milk. Camel milk has specific properties like its long shelf life without treatment compared to cow's milk. In fact, in addition to its higher content of essential fatty acids, camel milk contains antimicrobial agents that prevent the proliferation of microorganisms thus increasing its stability over time [1]. Moreover, previous studies have reported a unique camel milk health benefit in diabetic patients [2] and therapeutic properties which are widely exploited for human health in several countries [3].

The chemical composition of camel milk has been studied [4,5]. Total protein content ranges from 2.4 to 5.3% and is divided into caseins (CN) and whey proteins. The casein fraction composes 52–98%, and distributes into four fractions: αs1-, αs2-, β-, and κ-CN [6]. The β-CN is the main camel milk casein followed by αs1-CN, and constitutes about 65% and 21% of total casein, respectively compared with 36% and 38% in bovine milk, respectively [7]. Only 3.47% of the total casein corresponds to in camel milk compared with 13% in bovine milk [8]. It was

found that the casein structure of camel milk is similar to that of bovine milk, and with only few pronounced differences were noticed [9]. Those differences were highly noticeable in the primary structure of αs1-CN, whereas similarities were observed in the secondary structure of caseins when both compared with bovine casein structure. Camel milk κ-CN was found to have different site for hydrolysis by chymosin compared with bovine milk κ-CN. Chymosin is known to hydrolyze bovine milk k-CN at the Phe105-Met106 bond, whereas its hydrolysis site on camel milk k-CN is Phe97-Ile98 [10].

Camel milk is consumed as fresh or soured beverage. Therefore, to extend its shelf life, different treatments such as pasteurization or fermentation may be applied to camel milk. Lactic acid fermentation is the most used acidification process to coagulate milk during the manufacturing of dairy products. Despite the uniqueness of camel milk in terms of therapeutic and nutritional proprieties [11,12], it failed to form gel-like structure by lactic acid fermentation process. The fermented camel milk has a fragile structure and watery consistence [13]. This finding was attributed to the presence of antibacterial agents such as lactoferrin and lysozyme, the large size of casein micelles [14], the absence of β-lactoglobulin in camel milk [15], and the relative distribution of protein fractions into casein micelles [4]. These reports show the difficulty to produce fermented camel milk with an acceptable consistence due to the problems associated with milk coagulation.

However, not many studies on camel milk coagulation process could be found. Only a few studies comparing the effect of ingredients,

including polysaccharides on gel proprieties have been reported [15] and these have focused only on the texture and sensory proprieties of the final product. Therefore, the objective of this research was to better understand the behavior of camel milk during acidification and to assess the effect of a polyanionic polysaccharide (low methoxyl pectin) on the rheological properties of acidified camel milk gels formed at different pHs.

Pectin is a water soluble polysaccharide widely used in food manufacturing for its thickening and gelling properties. Native pectins are high methoxyl pectin in which the majority of carboxylic acid group are esterified by methanol and consequently non ionizable: therefore in the present work, LM pectin having a higher proportion of free ionized carboxylic acid groups was considered in order to favor electrostatic interactions with positively charged caseins. Pectin has been shown to strongly interact with proteins under different physicochemical conditions. Thus, it has been demonstrated that, at pH values below their pI, pea protein [16], gelatin [17] and sodium caseinate [18] are able to form complexes with pectin chains. The formation of casein/pectin complexes has been shown to depend on several factors such as pH, thermal history and protein/polysaccharide ratio.

However, to the best of our knowledge, few works have studied the interactions between camel milk protein and pectin. In this study, we used acidified milk as a model allowing the separation of the acidification and the gelation process, and also in order to understand the mechanisms involved in electrostatic complexation.

2. Materials and methods

2.1. Materials

Whole camel milk (WCM) was collected from reared camels (*Camelus dromedarius*) from local farm located in south Tunisia and was stored untreated at −20 °C until use. Whole cow milk was provided from local farm. Low methoxyl amidated pectin (Unipectine TM OF 305 C SB, degree of esterification from 22% to 28% and degree of acetylation from 20% to 23%) was supplied by Cargill (Baupte, France). Analytical grade imidazole ($C_3H_4N_2$), sodium hydroxide (NaOH), acetic acid and hydrochloric acid (HCl) were purchased from Sigma-Aldrich Chimie (St Quentin Fallavier, France). All solutions were prepared using distilled water.

2.2. Milk samples preparation

WCM was used to prepare gels. Before adding LM pectin, milk was homogenized with an ULTRA-TURRAX homogenizer (IKA T18 basic, Germany) at 22,000 rpm for 5 min. A series of LM pectin concentrations (final pectin: 0.5, 1.0, 1.5, and 2.0% (w/w)) were studied. A sample with no stabilizer was used as control. Mixtures of WCM and LM pectin were magnetically stirred at room temperature for 2 h before analysis. Acidification was done by decreasing gradually the pH to 3.0 with addition of hydrochloride acid (1 mol·L^{-1}). The pH values were recorded for all the samples using a pH meter (Model pH 150, EUTECH instruments (France)).

2.3. Zeta potential measurement

The zeta potential (ζ-potential) of camel milk with or without LM pectin was measured using a Zetasizer NanoZS90 (Malvern Instrument, Malvern, UK). Imidazole-acetate buffer adjusted to the desired pH value was used to prepare diluted samples (0.5% (w/w)). The mean values of ζ-potential (ZP) and the standard deviations (±SD) were obtained from the instrument.

2.4. Particle size measurement

Particle size distributions of milk particles after acidification and pectin addition were measured by a laser diffraction instrument (Malvern Mastersizer S, Malvern Instruments, Worcs., UK) at 23 ± 1 °C. To avoid multiple scattering effects, milk samples were diluted with imidazole/acetate buffer (5 mmol·L^{-1}) prior to the measurements. The solutions were stirred continuously throughout the measurement to ensure the samples were homogeneous. Droplet size measurements are reported as average diameters, d_{43}, with d_{43} being defined as:

$$d_{43} = \sum n_i d_i^4 / \sum n_i d_i^3$$

where n_i is the number of particles with diameter d_i. Mean particle diameters were calculated as the average of duplicate measurements.

2.5. Apparent viscosity

Apparent viscosity of gels was measured in a BROOKFIELD extra DV2T (USA) viscometer. Whole milk gels were gently stirred and 40 mL samples were placed in the viscometer. All samples were maintained at 25 °C. The measurements were carried out on the speed 200 rpm for 1 min. The given viscosity (mPa·s) was calculated as the average of triplicate measurements.

2.6. Dynamic rheology measurements

Dynamic rheological measurements were carried out in a stress-controlled rheometer (HAAKE Viscotester IQ, rheometer, Germany) using a 1 mm gap parallel-plate sensor. An aliquot (0.5 mL) of milk gel sample was transferred to the rheometer bottom plate. The top plate was slowly lowered until the gap was 1 mm and a cone (3.5 cm diameter; 2° angle) was used. The plate temperature was maintained at 25 °C. To ensure that viscoelastic measurement were carried out in the linear viscoelastic region (LVR), strain sweep tests were conducted from 0.01% to 50% at frequency 1 Hz. After that, samples were submitted to a frequency sweep, from 0.01 Hz to 20 Hz at a constant shear strain in the LVR, fixed at 0.1%. The elastic modulus (G′) and the viscous modulus (G″) were recorded as a function of frequency. In all rheological experiments, each measurement was performed at least in duplicate.

2.7. Isothermal titration calorimetry (ITC)

Energetic and binding parameters from the complexion between camel milk protein and LM pectin were investigated by ITC. For this experiment, LM pectin solution (10 g/L) and camel milk (diluted to obtain a final protein concentration of 1 g/L) were prepared in imidazole-acetate buffer (5 mM) at different pH. The isothermal titration calorimetry experiments were performed at 25 °C using an ITC microcalorimeter (VP-ITC, Microcal, Northampton, MA). All solutions were degassed for 5 min at 20 °C prior to ITC measurements. Camel milk solution was loaded into a 1.42 mL calorimetric cell and titrated by LM pectin solution placed in 200 μL syringe. The titration was performed with 29 successive 10 μL injections of LM pectin solution while the first injection was 2 μL and did not collected into final result. The stirring speed was set at 307 rpm. Each injection lasted 20 s with an interval of 200 s between consecutive injections. Data analysis was performed with Origin 7.0 software Microcal LLC. Measurements were carried out in duplicate and were highly reproducible.

The results were reported as the change of enthalpy per g of LM pectin injected into the reaction cell as a function of the LM pectin/protein mass ratio. The raw ITC data were fitted using "Sequential Binding Site" model based on two different types of binding sites, as provided by the software. Thermodynamic parameters, binding constant (K), enthalpy (ΔH) and entropy (ΔS) were calculated.

2.8. Statistical analysis

All experiments were performed using at least three samples. The results presented are the average and the standard deviation that were calculated from replicate measurements. Statistical differences between samples were calculated using Student's *t*-test for independent samples.

3. Results and discussion

3.1. Influence of LM pectin addition on zeta potential and particle size of acid gels from WCM

3.1.1. Zeta potential

The ζ-potential measurements were used in this study to provide some information about changes in particle surface charge during the acidification. Fig. 1.a shows that the ζ-potential of whole camel milk changed from negative to positive as the pH was decreased, indicating that the isoelectric point of bovine and camel milk were, respectively, about 4 and 4.2. Our result for cow milk and camel milk are in commitment with previous finding in dairy research [9,19]. Zeta potential has been used as an indicator of the electrical charge of milk fat globules and of casein micelles/particles. And it is obvious that the measurements technique and experimental conditions influences the results significantly.

The zeta potential of milk particles after addition of different ratios of LM pectin is presented in Fig. 1.b. As illustrated in Fig. 1.b, important variations of zeta potential were observed when milk was supplemented with anionic LM pectin for the three studied pHs. The effect of pH on the interactions between pectin and milk proteins can be explained by the changes in the ionization degree of some side groups carried by the biopolymers (i.e. amino and carboxylic groups) [20]. The electrical charge changed from 7.6 ± 0.14 mV (0% pectin) to -2.6 ± 0.21 mV (0.5% pectin) at pH 4. The ζ-potential changed from positive to negative as the LM pectin concentrations was increased. The anionic polysaccharides molecules are negatively charged between pH 7.0 and 3.0, whereas casein micelles reach a net zero charge near pH 4 and become positively charged at lower pH values. Therefore, a net attraction between polysaccharides and casein micelles could be driven by electrostatic interactions [21]. In the same way, Tholstrup Sejersen et al. [22], observed changes in zeta potential with varying pectin concentrations.

3.1.2. Particle size measurement

The average size of casein micelles in different milk samples is presented in Fig. 2.a. This figure shows that bovine caseins having a mean size higher than 100 μm were formed at pH 4.5 whereas camel caseins were aggregated and had an average size (d_{43}) <45 μm. The obtained results showed that camel milk presented a dispersed casein fragments with small size [23].

The association of pectin with camel milk protein allowed homogenous samples. It is known that low-methoxyl pectin does not form complex with casein micelles at neutral pH, where both biopolymers carried negative charges [24]. On further lowering the pH down to pH 3.5, the mean diameter of aggregates increased (Fig. 2.b) and reached its maximum value at 2% LM pectin concentration (at pH 4). It is important to note that for 1% pectin, the binding sites between pectin and caseins were saturated and the caseins were always repulsed which reflected a smaller diameter. The results obtained here coordinate with an increasing content in pectin, where pectin started to adsorb onto casein micelles [25].
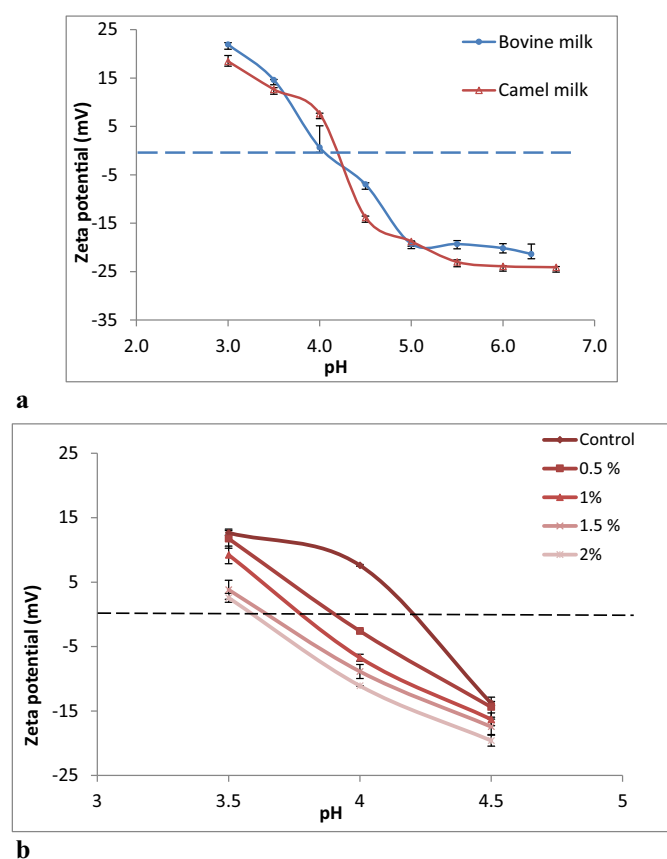

a

b

Fig. 1. a. ζ-Potential of whole bovine (•) and camel milk (Δ) as function of pH (5 mmol/L imidazole-acetate buffer, 25 °C). b. ζ-Potential of whole camel milk at different concentrations of LM pectin (5 mmol/L imidazole-acetate buffer, 25 °C).
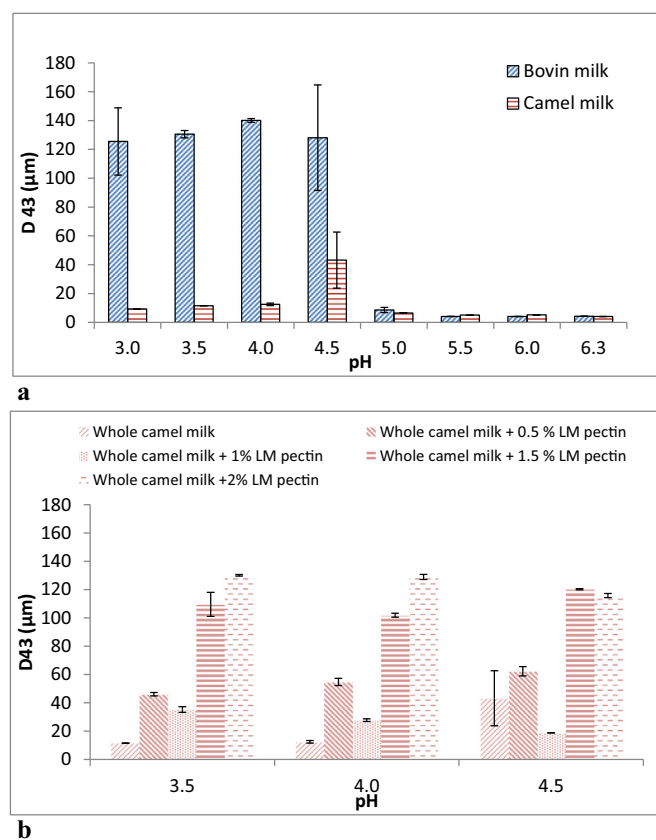

a

b

Fig. 2. a. Dependence of volume-weighted distribution mean diameter (D43) of bovine and camel whole milk on pH (imidazole-acetate buffer, 5 mmol/L). b. Dependence of volume-weighted distribution mean diameter (d_{43}) of whole camel milk on LM pectin concentration (imidazole-acetate buffer, 5 mmol/L, 25 °C).

3.2. Influence of LM pectin on the rheological properties of acid gels from WCM

3.2.1. Apparent viscosity

The effect of pH on the apparent viscosity was studied. After a slight and progressive decrease, the apparent viscosity increased until the last measured point (Fig. 3.a). The maximum of viscosity for camel milk was observed at pH 3.5. At 200 rpm, the apparent viscosity of WCM gels was 13.91 mPa·s (SD = 0.02) against 21.13 (SD = 0.08) for bovine milk gels. This increasing in viscosity could be due to the destruction of bonds between the casein particles. Camel milk presented lower viscosity than bovine milk which related to a reduction in the quantity of suspended particles and to a fragile structure for protein aggregates for camel milk [23]. This confirms the hypothesis of a late release of micelle minerals and thus explains the higher stability of camel milk toward increased acidity.

The influence of using LM pectin in WCM gels on the apparent viscosity was studied. The fortification of milk with pectin impacted positively on the viscosity of gels (Fig. 3.b). The lag period, where no significant changes in apparent viscosity of samples were observed, was not modified by the addition of LM pectin ($p < 0.05$). However, viscosity was increased by addition of 1, 1.5 and 2% LM pectin to milk samples during acidification. In the other hand, no effect was observed at 0.5% of pectin. The apparent viscosity presented a significant increment and reached the maximum value with 2% (28.11 ± 0.04 mPa·s) at pH 3.5 ($p < 0.05$).

A clear influence of the addition of 2% of LM pectin is observed and the global extreme is found at pH 3.5 ($p < 0.05$). The viscosity of fortified gels with 2% of pectin was considerably higher than that of gels formed from bovine milk (Fig. 3.a). The addition of LM pectin induced an increase in apparent viscosity similar to what was described with microbial acidified gels fermented with a ropy strain [26–28].
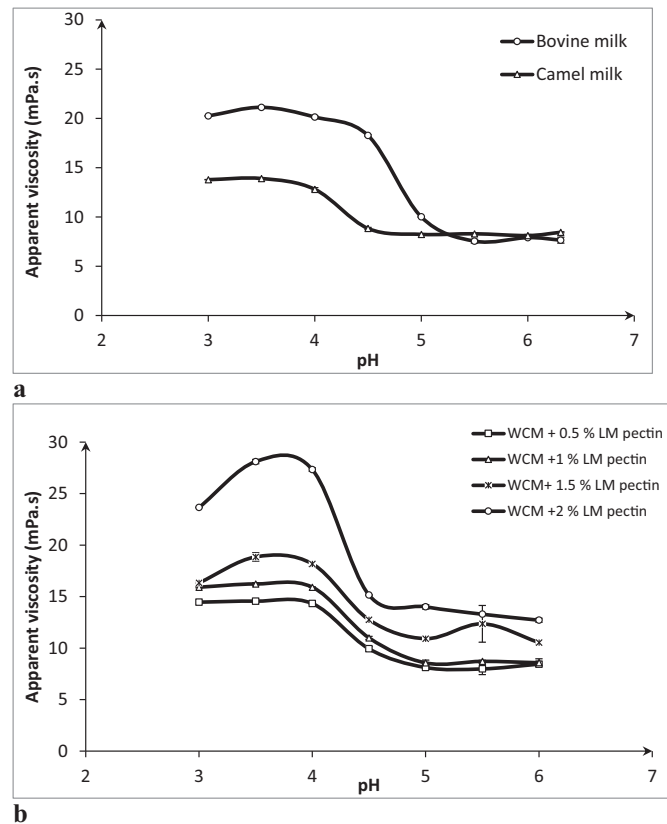
Moreover, from initial pH to pH 4.5 no significant changes in the viscosity were observed ($p < 0.05$). When further reduction in pH to 4 the viscous character became more pronounced with high values of viscosity ($28,11 \pm 021$ mPa·s). El-Agamy et al. [15] reported that the viscosity of the acid-pectin gelation network increases and influenced by the formation of junctions zones. Otherwise, according to [17], this network is governed by hydrogen bonds by the carbonated carboxyl group of the pectin molecules. This significant change of the viscosity of pectin-fortified gels could suggest that the interaction between the pectin chains was more intensive, due to less repulsion and led to a higher number of binding sites [29].

3.2.2. Rheology

Sweep strain tests SST were made to ensure that frequency sweep tests FST were conducted within the region of linear viscoelasticity and to assess the nonlinear behavior. Fig. 4.b shows a typical result for the dependence of G′ and G″ of whole bovine milk during acidification. Results showed that an important and significant increment of both elastic (G′) and viscous (G″) moduli was observed for bovine milk gels. G′ increased rapidly and surpassed G″ from pH 5 until the end of acidification, indicating elastic behavior of acid gel (Fig. 4.a). The elastic modulus (G′) for bovine milk gel had a maximum mean value of 1974 $\pm$ 380 Pa at pH 4 which has been shown by Pang et al. [19], while camel milk gels without pectin addition showed a non-viscoelastic behavior (Fig. 5.a). The viscoelastic proprieties remain a Newtonian liquid. No significant difference with viscous modulus (76 ± 2 Pa) and storage modulus (77 ± 10 Pa) at pH 4.5 could be observed.

The effect of added LM pectin on the rheological properties of camel acid milk gels was investigated. The study was performed at different pH levels to investigate conditions above and below the pI of caseins, as the charge on caseins varies with pH and, thus, the type of interaction with LM pectin could vary. When milk samples were fortified by pectin,
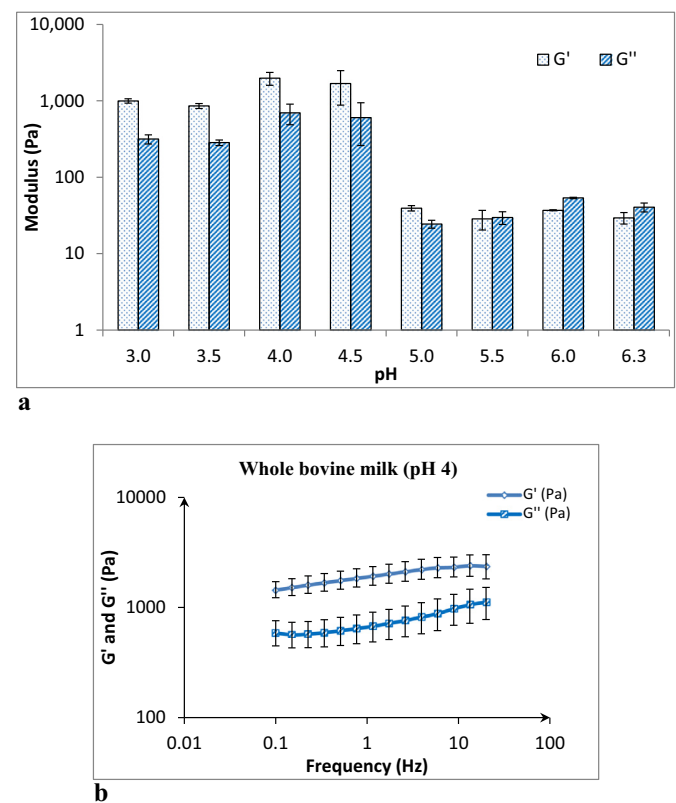

a

b

a

b

Fig. 3. a. Changes in the viscosity (200 rpm; 20 °C) of bovine and camel whole milk during acidification (HCl 1 M). b. Changes in the viscosity of whole camel milk with different concentrations of LM pectin during acidification (200 rpm, 25 °C).

Fig. 4. a. Changes in the storage modulus (G′) and in the loss modulus (G″) during the acidification of whole bovine milk. b. Elastic modulus (G′) and viscous modulus (G″) as a function of oscillatory frequency for whole bovine milk gel at pH 4.
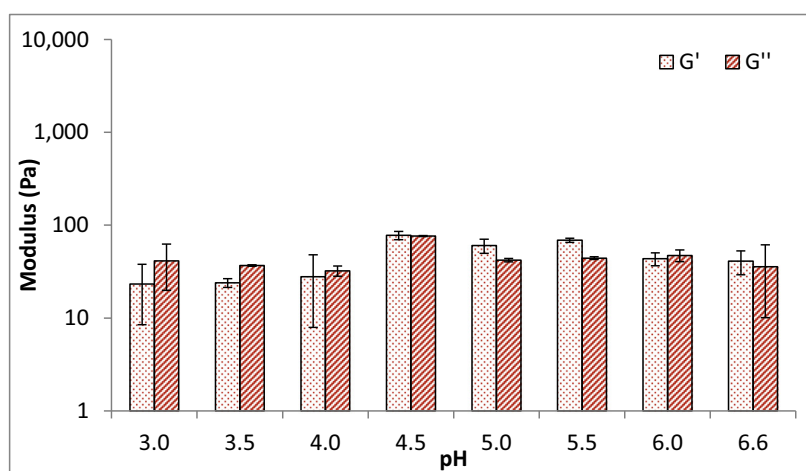
a
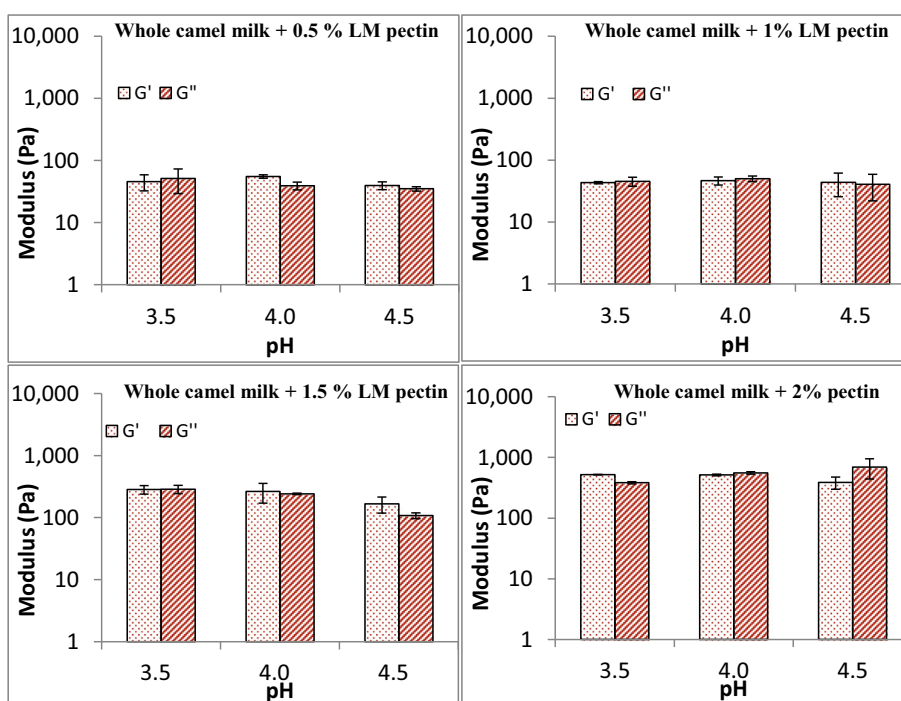
b

Fig. 5. a. Changes in the storage modulus (G′) and in the loss modulus (G″) during the acidification of whole camel milk. b. Effect of the LM pectin concentrations on the storage modulus (G′) and the loss modulus (G″) of acid milk gels at 25 °C.

the rheological behavior was clearly different (Fig. 5.b). It could be observed that the pectin adding modified the mechanical spectra, that G′ was higher than G″ with which was significantly different in comparison with milk control (Fig. 5.a) (p < 0.05). The G′ increased from the beginning of acidification stage, at much lower increased rate than for control milk. As the pectin concentration was increased, the storage modulus (G′) increased up to 264 Pa for 1.5% pectin at pH 4 and to 515 Pa for 2% pectin at the same pH, which indicated casein micelle fusion, and casein dissociation and rearrangement, as discussed by Pang et al. [30].

Matia-Merino et al. [31] observed an increase in storage modulus up to 120 Pa with an increase of pectin concentration of 1.0% (w/v). The increment in G′ may be attributed to interaction between pectin and milk constituents. Storage modulus (G′) values depend on the number and strength of bonds between the caseins particles, on

the structure of the latter and on the strands making up these particles [32]. The sharp increase in the elastic modulus of aggregated system was consistent with the thickening function of pectin in acid dairy product. Ionic calcium migration from casein micelles into the milk serum during acidification promoted interactions among LM-pectin strands before the bioelectrical aggregation of the casein micelles occurred [18,29].

As the pH is reduced, an increasing amount of the calcium ions will be available for binding to the pectin, hence promoting its aggregation. The carboxylic groups of pectin become less ionized under acidification, promoting conformational ordering and intermolecular association [27]. It is also well established that pectin will adsorb onto the casein micelles as a result of an electrostatic interaction at around and below pHi between the positively charged bovine casein and the negatively charged pectin [25].

In a like manner, whey protein-pectin electrostatic interactions will also take a place under acidification as shown for pure protein system [33]. Consequently, the final acid gel will be the result of all these processes taking place at the same time.

4. Molecular interactions

ITC is the most direct method to measure the heat change during the formation of a complex at constant temperature. The magnitude and type of the energies involved in the complexion process between protein and polysaccharides were studied using ITC [34,35]. Heat flow versus time profiles resulted from the titrating of 10 g/L LM pectin solution into a reaction cell containing diluted camel milk (final protein concentration: 1 g·L^{-1}) camel milk protein solution at pH 6.0, 5.0 and pH 4.0 were shown in Fig. 6A, B and C, respectively. The fact that exothermic and endothermic processes were observed suggested that at least two processes occurred in the binding mechanism (Fig. 6A and B). Since the only potential interacting sites on the pectin are the carboxylic groups [36], the two different processes were rather suggested to be two different physicochemical phenomena in the camel milk protein-LM pectin interaction.

From these profiles, the total enthalpy changes (ΔH) per gram of LM pectin versus LM pectin/camel milk protein mass ratio were obtained. The heat generated at each injection of the LM pectin solution aliquot decrease gradually with each additional injection yielding a typical titration isotherm. The negative enthalpy change (ΔH < 0) at pH 6.0, 5.0 and pH 4.0 revealed that the interaction between LM pectin and camel milk proteins was exothermic process. Aberkane et al. [37] reported that complexion is mainly driven by electrostatic interaction which supports this result. In the case of this study, it appears that, at the first step, enthalpically driven, is mainly due to the direct interaction between the carboxylic groups of the pectin and positively charged groups of the camel milk caseins. The second step, entropically driven, could be mainly assigned to the association of the caseins-pectin aggregates involving a molecular rearrangement of one or both components and/or a release of hydration water molecules or ions [38].
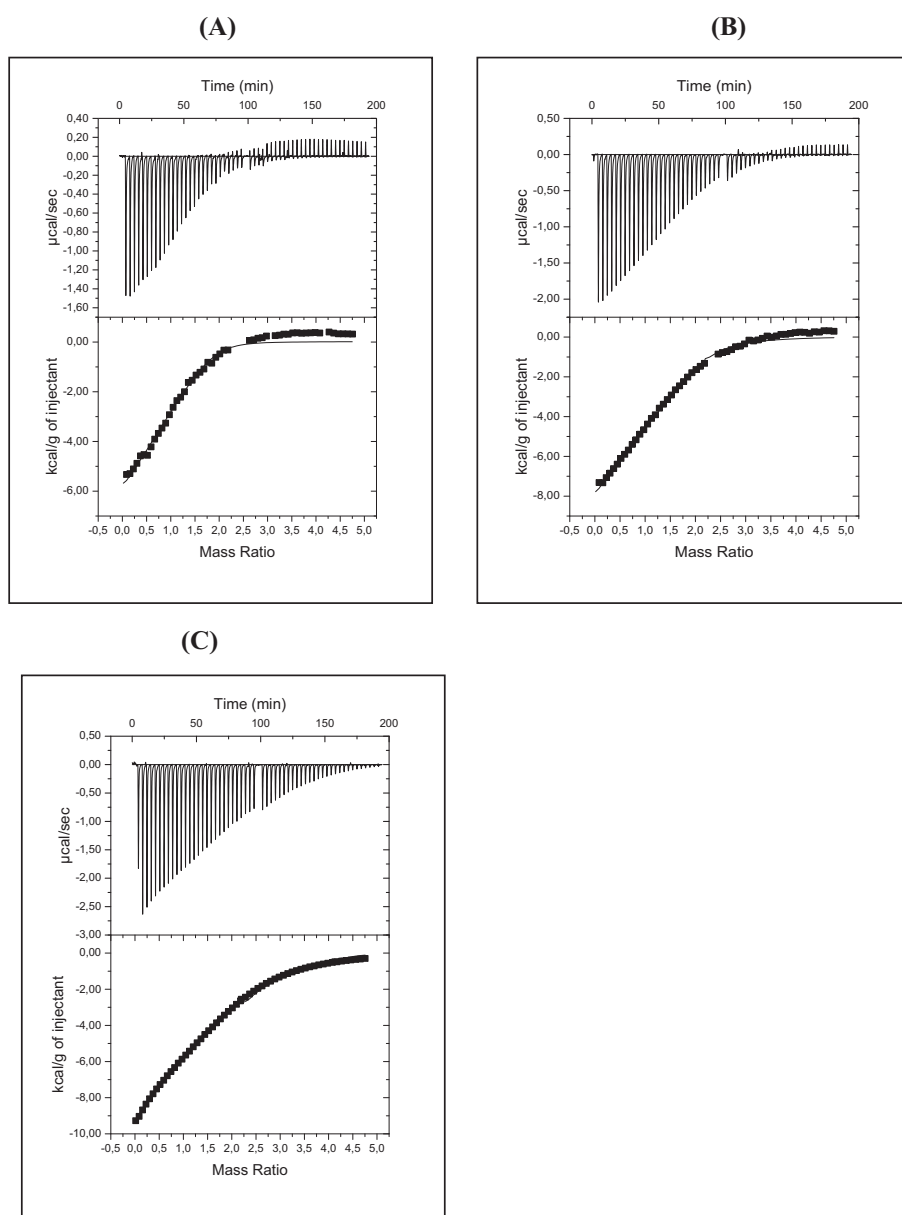
(A)

(B)

(C)

Fig. 6. Representative raw data (top panel) and binding isotherm (bottom panel) of the titration of WCM (1 g/L) with successive injections of LM pectin (10 g/L) stock solution. The experiment was carried at 25 °C in 5 mM imidazole-acetate buffer, pH 6 (A), 5 (B) and 4 (C) respectively.

To find an exothermic ΔH at pH 4.0 was reasonable, because the global charges of camel milk protein and LM pectin are opposite; rendering them attracted each other electrically. However, the negative ΔH at pH 6.0 and pH 5.0 somewhat unexpected since both LM pectin and camel milk caseins were negatively charged and expelled each other. The electrostatic attractions may come from the short range interactions between the positively charged patches on the surfaces of camel milk caseins and the negatively charged LM pectin. This was compatible with the observations of low ΔH and the absence of insoluble complex. Indeed, the enthalpy changes (ΔH) were -2.6 ± 0.02 cal/g at pH 4 and -1.4 ± 0.01 at pH 6 which were significantly different ($p < 0.05$) [21]. However, the binding entropy was negative at pH 4.0, which were mainly derived from the change in biopolymer conformational freedom after complex conservation [39].

5. Conclusion

According to the results of this study, the presence of LM pectin in whole camel milk had an impact on the casein particles size, apparent viscosity and an important influence on its rheological proprieties during the formation of acid milk gels. The increment in the elastic modulus (G′) of aggregated system was consistent with the thickening function of LM pectin in acid dairy products. During acidification, interactions among LM pectin and milk caseins were prompted. Otherwise, the final G′ values of the mixed gels, such as 1% pectin, were significantly higher than those without pectin. Adding pectin increased the viscosity of all gels, except the addition of 0.5% pectin, which, significantly, did not affect the apparent viscosity. Mechanical spectra proved the hypothesis that pectin forms strands with caseins micelles. The rheological properties depend on the concentration of LM pectin. Therefore, pectin can enhance the rheological behavior and the viscosity of acid milk gels of camel milk, which could be valuable in yogurt manufacturing.

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