Full Length Article

Mineral fortification modifies physical and microstructural characteristics of milk gels coagulated by a bacterial enzymatic pool

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ABSTRACT

An enzymatic pool from the Amazonian bacterium Bacillus sp. P7 was used as milk coagulant. Discovery of novel coagulants is of great interest in dairy industry for the development of new textures in cheese. Color, mechanical and microstructural characterization of milk gels induced by the bacterial enzymatic pool was carried out. Effect of mineral fortification on these characteristics was studied. Whiter gels with smaller pore diameters were obtained in the presence of Ca^{2+} or Mg^{2+}. These characteristics seemed to be influenced by the effect of ionic strength on casein structure which was also evidenced by digital texture features analysis. On the other hand, specific affinity of the assayed cations for milk proteins showed to be important in the development of the mechanical texture of the gels. Firmness and fracture force of milk gels obtained in the presence of Zn^{2+} or Ca^{2+} were higher than in the presence of Mg^{2+} and Na^{2+}.

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1. Introduction

Milk gelation is the first step in both cheese and yogurt manufacture, making this process of great economic importance. Gelation can be induced by enzyme action, acidification, and (or) heat treatment of milk [1]. The first clotting enzyme used was animal rennet but since its demand started exceeding the supply, interest in finding new sources of milk coagulants has grown up. Nowadays, fermented produced chymosin (FPC) comprises 70–80% of the global market for coagulants [2]. However, limitations to use animal rennet as religious reasons (e.g., Judaism and Islam) and diet (vegetarianism) or consumer concern regarding genetically engineered foods, has focused research on finding alternative coagulants [3–7]. The enzymatic pool produced by the keratinolytic Bacillus sp. P7

(EPP7) has been proposed as milk coagulant. EPP7 consist mainly of serine proteases and its optimal temperature and pH for milk protein aggregation are 44 °C and 7.4, respectively [8,9]. In a previous work, milk protein aggregation by EPP7 in the presence of nutritionally essential minerals was characterized [9].

Mineral ions have essential functions in the human body as the regulation of enzyme activities, the maintenance of acid-base balance and osmotic pressure, and the facilitation of membrane transport of essential nutrients, among others. Nevertheless, some studies confirm that most people consume foods that have less than two-thirds of one or more essential minerals [10]. Because of this, food fortification results an effective approach to prevent mineral deficiency [11]. The success of fortification depends on many points: firstly, the development of a product acceptable to consumer, taking into account food habits of the population. At this point, dairy products are good candidates for mineral fortification due to their worldwide consumption by all groups at risk of mineral deficiency. Secondly, what mineral salt is best to use and how much of it is necessary to add to ensure enough concentration in the food product. As this second issue has been previously discussed [9], now we focus on the influence of the selected mineral salts on

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the optical and mechanical properties of EPP7-induced milk gels. To achieve this, experiments of confocal scanning laser microscopy (CSLM), penetrometry and color analysis were carried out.

Obtainment of milk gels using a novel bacterial coagulant, fortified with certain minerals (Ca^{2+}, Mg^{2+}, Zn^{2+} and Na^+), could be the base of a product with different appearance and textural properties leading to an innovation in food industry. Therefore, the aim of this work is to study the overall visual appearance, microstructure, and mechanical texture of milk gels fortified with minerals and coagulated using EPP7.

2. Materials and methods

2.1. Skim milk solution

Skim milk powder (Milkaut, Franck, Argentina) was reconstituted at 20% w/v in 5 mM $CaCl_2$ (Cicarelli SRL, San Lorenzo, Argentina). The dispersion was stirred for about 1 h at 25 °C before each experiment to allow equilibration. Subsequent dilutions, for the fortification using minerals, were carried out using 10 mM Tris–HCl buffer pH 7.4 (Sigma-Aldrich Co., St Louis, USA) [9].

2.2. Reagents

Stock solutions of $CaCl_2 \cdot 2H_2O$ (50 mM), $MgCl_2$ (50 mM), $ZnCl_2$ (10 mM) and NaCl (500 mM) were prepared by dissolution of the solid drugs (Cicarelli SRL, San Lorenzo, Argentina) in distilled water. Complete dissolution of $ZnCl_2$ was achieved by addition of 0.1 M HCl (Merck, Darmstadt, Germany) drops. Milk suspensions at 10% w/v were enriched with the cations at different final concentrations: 7.5 or 12.5 mM $CaCl_2$, 5 or 10 mM $MgCl_2$, 0.125 or 0.250 mM $ZnCl_2$ and 50 or 100 mM NaCl.

Rhodamine B (Fluka, Buchs, Switzerland) was used to stain milk proteins.

2.3. Color analysis

Milk gels were formed (using 2.5 IU of EPP7) in plastic cylindrical containers of 3 cm diameter and 3 cm height and were incubated overnight at 44 °C. Three gels were prepared per assayed condition. Digital images of milk gels were obtained in a white wooden box with a uniform illumination system using a matte black background and stored in TIFF format. Camera (EOS-Rebel T3, Canon, USA) settings were fixed as follows: manual mode with lens aperture at f/8, exposure time 1/200 s, 35 mm zoom, no flash, ISO sensitivity 400, maximum resolution (3648 × 2736 pixels).

Color analysis of the images was carried out applying the CIELAB color system using Photoshop CS6 (Adobe Systems Inc., USA) [12]. The model consist of a luminance component (L^*) and two chromatic components, a^* (from green to red) and b^* (from blue to yellow). An IT8 calibration card (Wolf Faust, Germany) was photographed under the same conditions as the samples and was used to obtain a color profile [13]. Finally, whiteness index (WI) was calculated using the equation $WI = L^* - 3b^*$ [14].

2.4. Textural analysis

After obtaining the images, milk gels were subjected to a penetration test at room temperature. Each container was penetrated 10 mm using a single-column Universal Testing Machine (Multitest 2.5-d, Mecmesin, UK) with a 25 N load cell and a 2 cm diameter cylindrical probe operating at a speed of 10 mm/min. Three parameters were obtained from the force-distance curves: firmness (FI, N/mm) defined as the initial slope (during first 12 s) of the penetration profile, fracture force (FF, N) defined as the force at the first significant break and displacement (mm) which is the distance at

fracture [15]. Three independent repetitions of each condition were carried out.

2.5. Microstructure

Before addition of coagulant, hydrophobic dye Rhodamine B was added (0.002 mg/mL) to each skim milk suspension (final concentration 10% w/v). After EPP7 addition, samples were placed in Lab-Tek chamber slides (80 μL/well) (Thermo Fisher Scientific, Rochester, USA), which were incubated in a water bath at 44 °C for 90 min.

All images were collected with a Nikon C1 plus confocal on a Nikon TE2000-E2 inverted microscope equipped with 60× oil Plan Apo NA 1.4 objective (Nikon Instruments, Inc., Melville, USA). Images were acquired with Nikon EZ-C1 3.9 software with a resolution of 512 × 512 pixels. 55 z-series optical sections were collected with a step size of 100 nm and were finally used to make a stack [16].

Digital analysis of all images was carried out using ImageJ software, version 1.47 v (National Institutes of Health, Bethesda, Maryland, USA). Images were processed using the plugin BoneJ version 1.3.12 and two parameters were determined: pore diameter (PD) and degree of anisotropy (DA). This plugin was primarily designed to measure bone geometry but, according to Doube et al. [17], parameters are not limited to bone structure and can be generalized to different systems, such as particles or pores in food products [17]. Measurement of PD was carried out by the Local Thickness method [18], whereas measurements of DA are based on the Mean Intercept Length method [19]. Binarization of images (considering white objects on black background) was necessary for the determination of these parameters. To achieve this, the original method of auto thresholding available in ImageJ, which is a variation of the IsoData algorithm [20], was used. Textural features were determined applying the Grey-Level Co-occurrence Matrix (GLCM) texture plugin [21]. In this case, images were converted to 8-bit greyscale (grey objects on black background) before the analysis and two features were calculated: Angular second moment (ASM) and entropy (E). E measures the amount of grey levels and ASM quantifies grey level transitions, being a measurement of homogeneity (H) of the image [22]. After the construction and normalization of the co-occurrence matrix ($P(k, l)$), texture features were calculated as follows [23]:

$$ASM = \sum_k \sum_l P^2(k, l) \tag{2}$$

$$E = -\sum_k \sum_l P(k, l) \log(P(k, l)) \tag{3}$$

2.6. Statistical analysis

Data were reported as mean values ± standard deviations. As data fulfilled normal distribution, student's t-test was used to determine significant differences ($p < 0.05$) between results obtained under the different conditions assayed. In the correlation analysis, strength of linear relationship between variables was calculated using Pearson coefficient of correlation (r), being the level of significance $p < 0.05$. The software used for data analysis was SigmaPlot 12 (Systat Software Inc., USA).
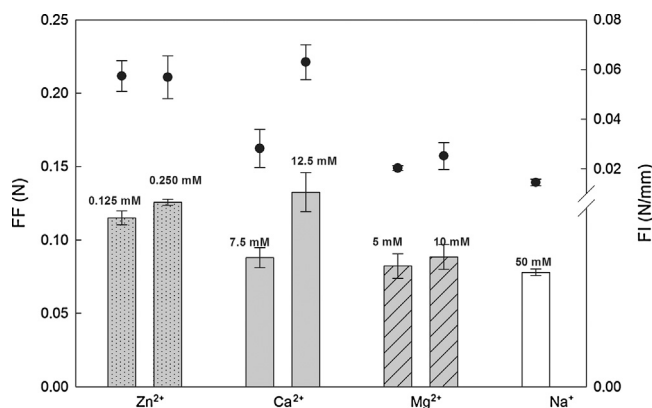
3. Results and discussion

3.1. Color analysis

The mineral-fortified milk gels were initially subjected to color analysis and the results are shown in Fig. 1. Milk gels fortified with Ca^{2+} or Mg^{2+} had higher WI than the ones fortified with Zn^{2+} or
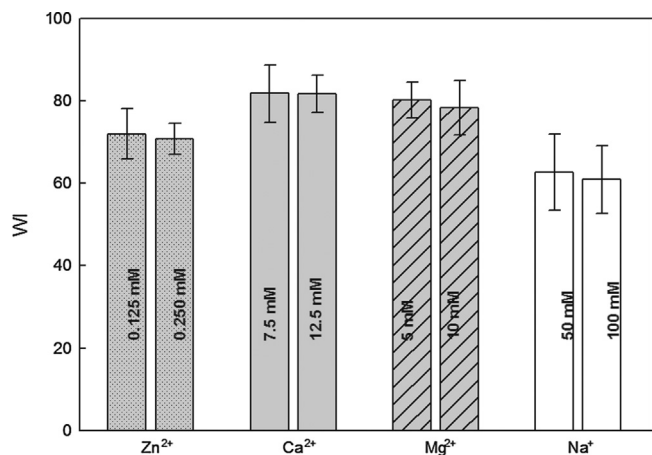
Fig. 1. Effect of salt concentration on whiteness index (WI) of EPP7-induced milk gels.

Fig. 2. Effect of salt concentration on mechanical texture of EPP7-induced milk gels. Bar graph corresponds to FF values. Filled circles represent the FI values.

Na$^+$ at both concentrations assayed for each cation ($p < 0.001$). It is known that the addition of mineral salts to milk involves a differential distribution of ions between micellar and aqueous phases. An increase of mineral concentration in the aqueous phase respect to micellar phase of milk at neutral pH has been reported [24,25], affecting the ionic environment of caseins, their physicochemical characteristics and thereby the micelle structure. The effect of fortifying milk systems with selected minerals (iron, magnesium, zinc, manganese, molybdenum, chromium and selenium) affected color development in yogurt [10]. Thus, it was proposed that changes in micelle structure can lead to changes in the opacity of milk suspensions and thereby in the WI. In the presence of Ca^{2+} or Mg^{2+}, EPP7-induced milk gels resulted whiter (higher WI) than the gels obtained in the presence of Zn^{2+} or Na$^+$. This could be related to the negative correlation found between WI and pore diameter (PD) ($r = -0.943$; p value = 0.001). According to this, more compact gels (smaller pore diameter) had whiter color.

3.2. Textural features

The values of FI and FF determined from force-distance curves are shown in Fig. 2. These values were slightly lower than those obtained in rennet-induced milk gels [26]. Weakness of EPP7-induced milk gels could be explained taking into account the usual low ratio of clotting activity to proteolytic activity of microbial coagulants [2]. In addition, high temperatures are known to affect milk gel texture [27]; thus, the relatively high temperature used for milk coagulation by EEP7 may be also affecting the FI and FF

Table 1
Distance at fracture of milk gels fortified with cations and coagulated by EPP7.

Cation	Concentration (mM)	Displacement (mm)
Zn^{2+}	0.125	2.2 ± 0.2
	0.25	2.3 ± 0.2
Ca^{2+}	7.5	2.7 ± 0.9
	12.5	2.2 ± 0.3
Mg^{2+}	5	3.8 ± 0.4
	10	3.6 ± 0.8
Na$^+$	50	5.2 ± 0.6
	100	–

of the resulted gels compared to rennet-induced gels obtained at 35 °C. Milk gels fortified with 12.5 mM CaCl$_2$ or ZnCl$_2$ were the strongest. Under the other conditions assayed, significantly lower values of FI and FF were measured ($p < 0.001$). It has been reported that the order of association of the assayed cations with caseins is: Zn^{2+} > Ca^{2+} > Mg^{2+} [28]. Specific affinity of each cation for some groups in milk proteins could be playing a more important role than plain electrostatic interaction, *i.e.* the ionic strength effect [9,28]. As a conclusion, stronger gels were developed in the presence of cations with higher percentage of casein-association.

Values of FI and FF could not be determined from milk samples containing 100 mM Na$^+$ since extremely weak gels were formed. This could be related to the decrease in the concentration of calcium bound to casein molecules observed when NaCl was added to milk [24]. It has been already shown that a reduction in levels of Ca^{2+} bound to casein slows down coagulation rate, resulting in weaker gels [29]. Displacement results are shown in Table 1. The strongest milk gels obtained in the presence of Zn^{2+} or 12.5 mM Ca^{2+} resulted also less deformable. Because of this, less distance was elapsed by the probe until the gel breaks.

3.3. Microstructure

As an example, stack of images of milk gels fortified with calcium are shown in Fig. 3. To characterize the microstructure of milk gels under all the assayed conditions a quantitative analysis was performed, determinations of PD, DA and digital textural features were carried out. Although CSLM have been previously used for pore size determination in milk protein gels [15,16,30], a new application of a plugin from ImageJ is presented to calculate pore diameter.

Degree of anisotropy (DA) is a measure of how highly oriented substructures are within a volume, DA = 0 corresponds to randomly orientated substructure (isotropy), whereas DA = 1 corresponds to totally anisotropic substructure. The mechanical properties of a system depend on the orientation of its substructure [19,31], so it is important to characterize the DA when mechanical properties are being studied. Besides, anisotropic structures were observed in protein-rich foods conformed by micelles [32,33].

It is important to consider that human visual system can distinguish a digital texture spontaneously only if it differs in second order spatial statistics of grey scale [34]. Thus, the determination of textural features is an essential step for interpretation.
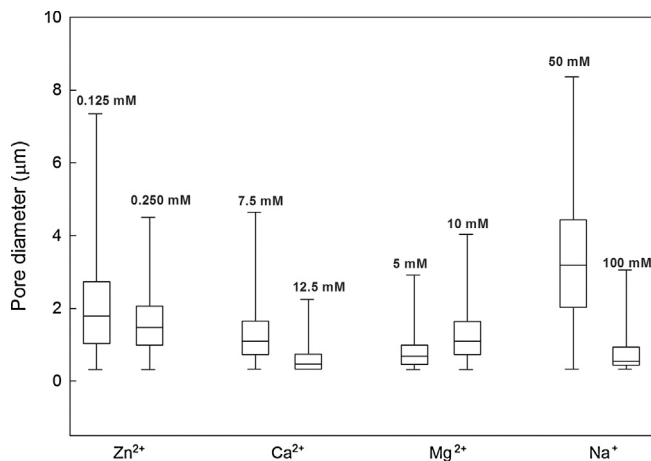
3.3.1. Pore diameter

As a result of the Local Thickness method, a PD distribution was obtained per stack of images. Asymmetrical distributions were obtained under all the conditions assayed, because of this, data were displayed in box plots. Distributions of PD in milk gels fortified with different concentrations of each cation are shown in Fig. 4.

At pH 7.4, caseins have net negative charge, which is screened by the added cations leading to bigger casein micelles [9]. EPP7-induced coagulation of milk suspensions in the presence of high concentration of divalent cations would lead to more compact milk
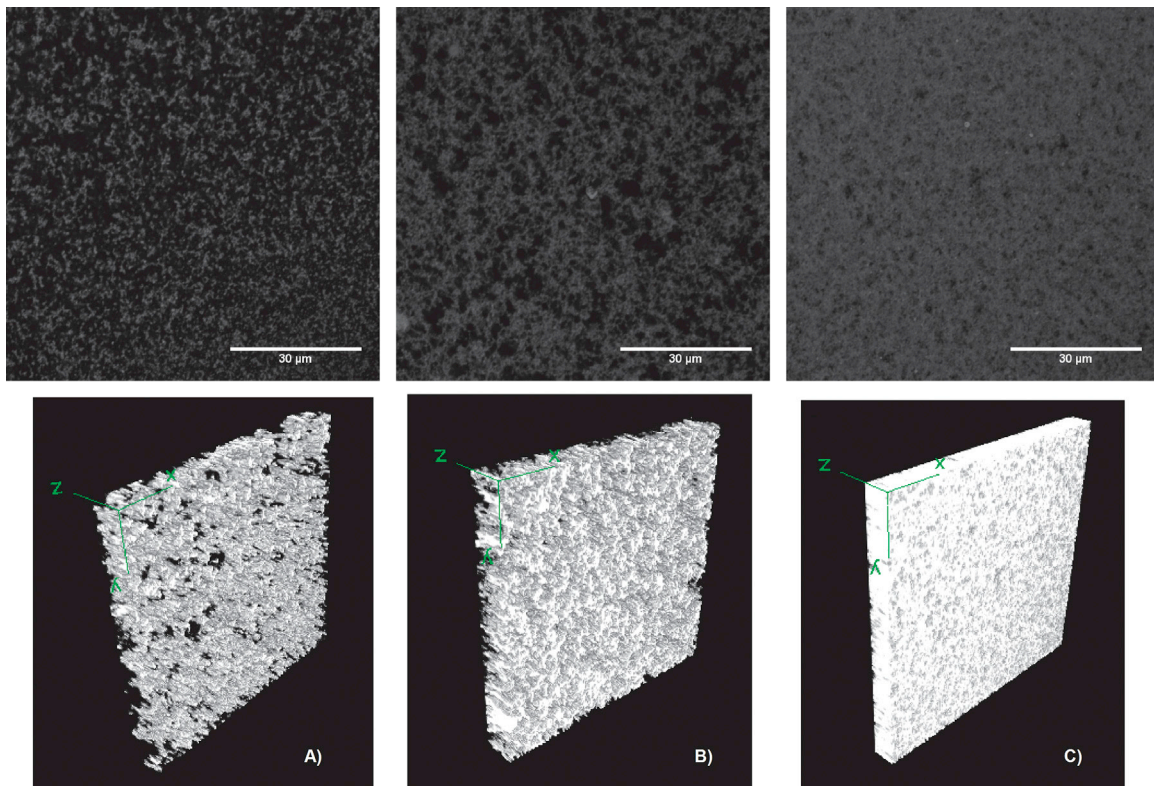
Fig. 3. Stack of images and tridimensional images of EPP7-induced milk gels fortified with (A) 2.5 mM, (B) 7.5 mM, (C) 12.5 mM CaCl$_2$. Conditions for milk coagulation: Tris–HCl buffer 10 mM pH 7.4; T = 44 °C.

Fig. 4. Pore diameter distribution measured from stacks of images of milk gels obtained using EPP7 as coagulant. Effect of milk fortification with Zn^{2+}, Ca^{2+}, Mg^{2+}, or Na$^+$. Conditions for milk coagulation: Tris–HCl buffer 10 mM pH 7.4; T = 44 °C.

gels with smaller PD. In fact, more compact gels were obtained in the presence of Ca^{2+} or Mg^{2+}, highlighting that the effect of ionic strength would be more important than specific affinity of the assayed cations on the PD of milk gels. As Na$^+$ is a monovalent cation, more concentration was needed to obtain more compact gels.

3.3.2. Degree of anisotropy

No significant differences for DA values were obtained in most of the assayed conditions (DA = 0.76 ± 0.03) However, in the presence of 12.5 mM CaCl$_2$ a significantly lower value (p = 0.015) of DA was obtained (DA = 0.3 ± 0.1). As it is shown in Fig. 3, milk gels fortified with 12.5 mM CaCl$_2$ showed less fibrous structure, which is

responsible for anisotropy [32]. The less fibrous structure in these gels in related to the smallest pore diameter measured. By digital analysis, images of more compact gels are considered less fibrous as the surface became more uniform.

3.3.3. Digital texture

In 8 bit images there are 256 grey levels that can be included in a pixel, varying from black (weak intensity) to white (strong intensity) [35]. Each pixel of an image contains two types of information: intensity values and spatial locations. Digital texture is related to both because it can be defined as a function of the spatial variation in pixel intensities. Grey level co-occurrence matrix (GLCM), related to second order statistics of pixel intensity, is one of the most popular methods in digital texture analysis [36]. Several textural features can be calculated from GLCM. Among the most commonly used, we have chosen Entropy (E) and Angular Second Moment (ASM). E measures the amount of grey levels, whereas ASM quantifies grey level transitions and is a measure of homogeneity (H) of the image [22].

Milk gel images obtained from Ca^{2+} or Mg^{2+} treatments exhibited lower H and higher E, when compared to milk gel images obtained from Zn^{2+} and 50 mM Na$^+$ treatments. However, when Na$^+$ concentration was increased to 100 mM, the effect was just the opposite, as shown in Fig. 5. This would be related to the loss of Ca^{2+} bound to casein micelles in the presence of NaCl as was discussed in Section 3.2. Both textural features were correlated to PD and also to WI. A negative correlation (r = − 0.851; p value = 0.015) between PD and E was found, whereas a positive correlation was found between PD and H (r = 0.941, p value = 0.002).

During digital analysis, black background was considered in all the images whereas the proteins (originally red, because of the rhodamine) were converted to shades of grey. Therefore, an increase of the amount of grey levels (E) would be related to changes in the protein structure of gels. According to this, a decrease in H would
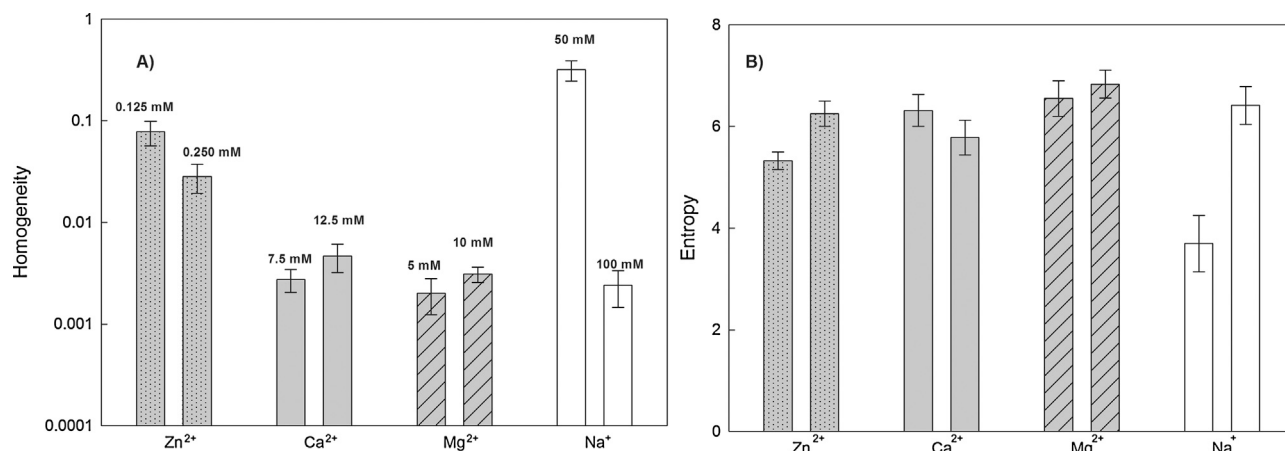
Fig. 5. Effect of salt fortification of EPP7-induced milk gels on digital texture features from stack of images. Conditions for milk coagulation: Tris–HCl buffer 10 mM pH 7.4; T = 44 °C.

involve the appearance of new grey level transitions, which could refer to the new electrostatic interactions between proteins in milk gels in the presence of Ca^{2+} and Mg^{2+}.

4. Conclusions

Characterization of color, mechanical and microstructural properties of milk gels fortified with essential minerals and coagulated by a bacterial enzymatic pool was carried out. Different types of interactions among cations and milk proteins showed to be important in setting the studied properties. Electrostatic interactions between cations and caseins gave place to micelles with higher size that, after coagulation by EPP7, formed more compact and whiter gels. On the other hand, the mechanical texture of the gels showed to be controlled by the specific affinity of each cation for milk proteins ($Zn^{2+} > Ca^{2+} > Mg^{2+}$), resulting stronger and firmer milk gels in the presence of Zn^{2+} or Ca^{2+}. As Na^+ is a monovalent cation, its behavior was completely different. Higher concentration of this mineral was necessary to reach more compact milk gels by electrostatic interaction. In addition, a decrease in the concentration of Ca^{2+} bound to casein in the presence of NaCl is probably making the coagulation process slower. As a conclusion, it would be possible to use the EPP7 as coagulant to induced gelation of mineral-fortified bovine milk. However, mineral concentrations should be adjusted depending on the characteristics of the desired final product. The results presented in this work would have great impact in dairy industry as they could be applied in the manufacture of a novel product, although we know and concern about further research that has to be done before this.

Acknowledgments

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