Impact of preacidification of milk and fermentation time on the properties of yogurt

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ABSTRACT

Casein interactions play an important role in the textural properties of yogurt. The objective of this study was to investigate how the concentration of insoluble calcium phosphate (CCP) that is associated with casein particles and the length of fermentation time influence properties of yogurt gels. A central composite experimental design was used. The initial milk pH was varied by preacidification with glucono- δ -lactone (GDL), and fermentation time (time to reach pH 4.6 from the initial pH) was altered by varying the inoculum level. We hypothesized that by varying the initial milk pH value, the amount of CCP would be modified and that by varying the length of the fermentation time we would influence the rate and extent of solubilization of CCP during any subsequent gelation process. We believe that both of these factors could influence casein interactions and thereby alter gel properties. Milks were preacidified to pH values from 6.55 to 5.65 at 40°C using GDL and equilibrated for 4 h before inoculation. Fermentation time was varied from 250 to 500 min by adding various amounts of culture at 40°C. Gelation properties were monitored using dynamic oscillatory rheology, and microstructure was studied using fluorescence microscopy. Whey separation and permeability were analyzed at pH 4.6. The preacidification pH value significantly affected the solubilization of CCP. Storage modulus values at pH 4.6 were positively influenced by the preacidification pH value and negatively affected by fermentation time. The value for the loss tangent maximum during gelation was positively affected by the preacidification pH value. Fermentation time positively affected whey separation and significantly influenced the rate of CCP dissolution during fermentation, as CCP dissolution was a slow process. Longer fermentation times resulted in greater loss of CCP at the pH of gelation. At the end of fermentation (pH $\sim$ 4.6), virtually all CCP was

dissolved. Preacidification of milk increased the solubilization of CCP, increased the early loss of CCP cross-links, and produced weak gels. Long fermentation times allowed more time for solubilization of CCP during the critical gelation stage of the process and increased the possibility of greater casein rearrangements; both could have contributed to the increase in whey separation.

Key words: yogurt, gelation, pH, fermentation time

INTRODUCTION

Yogurt is a cultured dairy product made by fermentation of heated milk with lactic acid bacteria, which convert lactose into lactic acid resulting in a reduction in pH. In spite of the long history of yogurt production, the mechanisms involved in the formation of acid milk gels remain poorly understood. The majority of the research work carried out on yogurt has been technological in nature, mainly directed at process optimization rather than at achieving any fundamental understanding of the underlying mechanisms of gel formation through which greater control of product texture could be achieved (Horne, 1999).

Casein micelles are the building blocks of acid milk gels, so the nature and type of casein interactions are very important for the properties of acid milk gels. The integrity of casein micelles is controlled by a localized balance between hydrophobic interactions and electrostatic repulsion (Horne, 1998; Lucey, 2002), such that if electrostatic repulsion decreases (e.g., by a decrease in pH), then the balance is altered in favor of hydrophobic interactions (Horne, 1998). Modification of the attractive or repulsive interactions modulates casein interactions and micelle integrity (Horne, 1998). Colloidal calcium phosphate (CCP) is a structural unit within the casein micelle acting as cross-linking bridge by neutralizing negatively charged phosphoserine groups (Horne, 1998). Colloidal calcium phosphate is a major component responsible for maintaining the integrity of the micelle because its removal disrupts the micelle (Walstra, 1990).

Rheological and physical properties of yogurt gels, including whey separation (the appearance of whey on the surface of yogurt), play an important role in quality

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and consumer acceptance (Lee and Lucey, 2004). In commercial stirred yogurts, gelling hydrocolloids (e.g., gelatin or low methoxyl pectins) or nongelling stabilizers (e.g., starch) are added to alter the texture, viscosity, and susceptibility to whey separation (Tamime and Robinson, 1999). Rheological parameters characterizing casein gels depend on the number and strength of bonds between the casein particles, on the structure of the latter, and the spatial distribution of the strands making up the network (Roefs and van Vliet, 1990). The dynamic moduli indicate the strength and number of bonds in the network, the yield stress (σ_{yield}) indicates the susceptibility of the strands to breakage, and loss tangent (**LT**), which is the ratio of viscous to elastic properties, indicates the relaxation behavior of bonds (van Vliet et al., 1991). The extent of rearrangements can be related to the dynamics of the protein-protein bonds, as expressed in terms of the yielding stress of the casein strands (van Vliet et al., 1997).

Microstructure studies on yogurt gels have demonstrated that these gels consist of a coarse particulate network of casein particles linked together in clusters, chains, and strands (Kalab et al., 1983). The network has pores or void spaces where the aqueous phase is confined. The microstructure of acid milk gels has been studied with scanning electron microscopy (Harkwalkar and Kalab, 1980) and confocal scanning laser microscopy (Lee and Lucey, 2004). Choi et al. (2007) used fluorescence microscopy (**FM**) to observe the microstructure of rennet-induced gels. However, the detailed microstructural studies of acid milk gel using FM has not been reported. We proposed a method of milk sample preparation for FM to help visualize the microstructure of acid milk gels.

The properties of acid gels are affected by many factors including incubation temperature, inoculum level, and milk pH, all of which directly affect the acidification process. The inoculum level has an important impact on the acidification process, and changing inoculum level modifies the fermentation time, and thus the acidification rate. Lower inoculation levels resulted in an increase in the time of achieving the maximum acidification rate (Sebastiani et al., 1998; Kristo et al., 2003), an increase in maximum acidification rate (Champagne et al., 2000; Kristo et al., 2003), a decrease in maximum storage moduli (G'), and an increase in LT values (Kristo et al., 2003). However, the reason for differences in yogurt properties with various inoculum levels is not very clear. In previous research (Lee and Lucey, 2004), the effects of inoculum level and incubation temperature on the physical properties and microstructure of yogurt gels were studied. At the same incubation temperature, higher G' and σ_{yield} values were observed in yogurt gels made with a higher inoculum

level. The permeability, pore size, and whey separation of yogurt gels increased with a decrease in the inoculum level. These results suggested that aggregation/gel formation was modified by a fast acidification process caused by a high inoculum level; however, acidification rate probably affects various factors including the rate and extent of solubilization of CCP during fermentation and the time available for particle rearrangement and fusion during critical stages of the fermentation process.

The physicochemical basis of the textural properties of yogurt gel; that is, detailed knowledge about the nature and types of protein interactions involved, is not well understood. Yogurt is a complex system, with many types of possible interactions such as hydrophobic and electrostatic interactions, hydrogen bonding, steric repulsion, and dissolution of CCP, which collectively have a strong influence on the physical and rheological properties of yogurt. Casein gels are dynamic by nature (van Vliet et al., 1997). The strength of each of these interactions is highly dependent on the pH, temperature, and time. There is very little information related to the effect of solubilization of CCP during acidification on the properties of acid milk gels, despite the importance of CCP in maintaining the integrity of the casein micelles. Solubilization of CCP in milk during acidification is considered a slow process (Walstra and Jenness, 1984).

Prefermentation of yogurt has been investigated by several groups mainly for the purposes of accelerating the fermentation process and making a more continuous yogurt manufacturing process (Driessen et al., 1977; Otten et al., 1996). With this 2-stage method, milk was prefermented at 45°C to a pH of 5.7 in the first stage and then cooled to 37°C for further acidification and final formation of yogurt texture (Driessen et al., 1977). A high inoculation percentage of fed-batch prefermented culture that was in the exponential growth phase was developed to accelerate the yogurt production (Otten et al., 1996). The kinetics of the prefermentation process were investigated in these previous papers; however, the detailed effect of any prefermentation on gel development and yogurt texture has not been studied in detail.

We wanted to determine if the main reason for the differences in yogurt textures with various inoculum levels (acidification rates as reported by Lee and Lucey, 2004) was the change in the amount of time allowed for the solubilization of CCP. The objectives of this project were to study the effects of preacidification pH (before inoculation) and the fermentation time (from preacidification pH to pH 4.6) on the rheology, whey separation, permeability, and microstructure of yogurt gels. To control the CCP content in milk we altered

Table 1. Independent variables in coded and actual values for the second-order central composite experimental design

Treatment ¹	Coded values ²		Actual values	
	Preacidification pH	Fermentation time (min)	Preacidification pH	Fermentation time (min)
1	α	0	6.55	375
2	0	0	6.10	375
3	0	$-\alpha$	6.10	250
4	0	0	6.10	375
5	-1	-1	5.78	285
6	-1	1	5.78	465
7	0	α	6.10	500
8	$-\alpha$	0	5.65	375
9	1	-1	6.42	285
10	1	1	6.42	465

¹Experiments were performed in random order.² $\alpha = 1.414$.

the initial milk pH by preacidification. Changing the preacidification pH will alter the amount of CCP associated with casein before addition of starter culture and allow us to control the nature of the casein particles in milk before fermentation. Independently varying the time from the addition of culture to reach pH 4.6 allows us to understand if the time allowed for gel development and complete solubilization of CCP also directly influences the properties of yogurt gel.

MATERIALS AND METHODS

Materials

Low-heat skim milk powder with 7.42 mg/g (wt/wt) of undenatured whey protein nitrogen (Bradley et al., 1992) was obtained from Dairy America (Fresno, CA). A commercial yogurt starter culture (YC-087, Chr. Hansen Inc., Milwaukee, WI) was used, which mainly consisted of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* with a few other probiotic strains. Glucono- δ -lactone (**GDL**) was supplied by Sigma-Aldrich (minimum 99.0%, St. Louis, MO).

Preparation of Reconstituted Skim Milk and Preacidification

Reconstituted skim milks were prepared by dispersing 12 g of low-heat skim milk powder into 100 g of deionized water (10.7% wt/vol) and stirring using a magnetic stirring unit for 3 h at room temperature. Reconstituted skim milks were heat-treated in a thermostatically controlled water bath at 85°C for 30 min and then stored in a refrigerator (~8–10°C) overnight before use. Some milk samples were used for chemical analysis; 0.02% (wt/wt) sodium azide was added to these samples to prevent bacterial growth. Reconsti-

tuted skim milk (after heat treatment) was preacidified with GDL, where the hydrolysis of GDL to gluconic acid resulted in a reduction in milk pH. Preacidification pH was controlled by adding various amounts of GDL at 40°C and milk was equilibrated for 4 h at the desired preacidification pH value. We used preacidification pH values of 6.55, 6.42, 6.10, 5.78, and 5.65 (Table 1).

Yogurt Fermentation

Starter culture was prepared using the method described by Lee and Lucey (2004). Briefly, stock cultures were prepared by transferring 130 mg of the freeze-dried culture to 1,000 g of autoclaved reconstituted skim milk, and incubating at 35°C for 10 h (pH ~4.80) before frozen storage at -80°C. Working cultures were made by thawing frozen stock cultures, transferring 0.8 mL of thawed stock culture to 79.2 g of autoclaved reconstituted skim milk (10.7% wt/wt), and incubating at 35°C for 10 h. Fermentation times were defined as the time from these preacidification pH values to a final pH of 4.6. Based on preliminary experiments, we were able to vary the fermentation time from 250 to 500 min by using various amounts of working culture (i.e., 0.005 to 3.5%). Skim milk, which had been already preacidified with GDL, was inoculated with various amounts of working culture and incubated at 40°C until the yogurt reached pH 4.6. A model PCM 700 Orion Sensor Link system (Orion Research Inc., Beverly, MA), connected to a personal computer, was used to continuously monitor pH changes during the preacidification and fermentation steps.

Turbidity Measurements

Preacidified milk sample was diluted by a factor of 5 with ultrafiltration permeate (10 kDa molecular weight

cut-off) from milk. The reference sample was prepared after adding sufficient 10% EDTA (EDTA, 99%, Acros Organics, Morris Plains, NJ) to completely dissociate the particles. Turbidity was measured on UV-visible spectrophotometer (UV-1601 PC, Shimadzu, Tokyo, Japan) using a cell with a 1-cm light path. Experiments were performed in triplicate. Particle size was measured by using the wavelength dependence of turbidity method described by Holt et al. (1975). A wavelength of 600 nm was used for the single wavelength turbidity measurements reported.

Acid-Base Buffering Properties

The resistance to pH change in milk is controlled by the acid-base buffering properties. Acid-base titrations were performed as described by Lucey et al. (1993). Samples were titrated with a Mettler Toledo DL50 Autotitrator (Mettler Toledo, Greifensee, Switzerland). Preacidified milks were titrated from their initial pH to 3.0 with 0.5 *N* HCl and then back titrated from pH 3.0 to 9.0 with 0.5 *N* NaOH. Buffering indices (dB/dpH) were calculated according to Van Slyke (1922) as follows and buffering curves were drawn by plotting the buffering index as a function of pH:

$$\frac{\text{dB}}{\text{dpH}} = \frac{\text{normality of acid or base} \times \text{mL of acid or base added}}{\text{volume of sample} \times \text{pH change produced}}. \quad [1]$$

Ca Analysis

Ultrafiltration permeates of preacidified skim milks were obtained using a Prep/Scale-TFF membrane (Millipore, Billerica, MA) at 25°C, which had a 10 kDa molecular weight cut-off (Ozcan-Yilsay et al., 2007). Separation of the soluble phase of milk gels was achieved by centrifugation of milk gels at $3,000 \times g$ for 10 min at 10°C. After centrifugation the supernatant was carefully removed and kept at 4°C until Ca analysis. The Ca contents in the milk samples and the Ca in the permeate and supernatant were determined using inductively coupled plasma optical emission spectrometry (Vista-MPX Simultaneous ICP-OES, Varian Inc., Palo Alto, CA). Wavelength used for Ca analysis was 317.9 nm (Park, 2000). Casein-bound Ca in milk was calculated based on equation [2] (White and Davies, 1958) and casein-bound Ca in milk gels was calculated based on equation [3]:

$$\begin{aligned} \text{Casein-bound Ca in milk} &= \text{total Ca} \\ &- \text{Ca in UF permeate;} \end{aligned} \quad [2]$$

$$\begin{aligned} \text{Casein-bound Ca in gels} &= \text{total Ca} \\ &- \text{Ca in supernatant.} \end{aligned} \quad [3]$$

Rheological Properties

Yogurt gel formation was monitored using a Universal Dynamic Spectrometer (Paar Physica UDS 200 controlled stress rheometer, Physica Messtechnik GmbH, Stuttgart, Germany) with a cup-and-bob geometry consisting of coaxial cylinders (inner diameter 25 mm; outer diameter 27.5 mm). During fermentation, yogurts were subjected to oscillation at frequency of 1 Hz and with an applied strain of 1%, which did not disrupt the development of the gel network (van Marle and Zoon, 1995). Parameters measured included G' and LT . Fourteen milliliters of preacidified milk was inoculated with the desired amount of working culture and transferred to the rheometer. A few drops of vegetable oil were added to the milk surface to prevent evaporation. Measurements were taken every 5 min until pH 4.6 was reached. Gelation was arbitrarily (instrumentally) defined as the moment when the G' values of gels were >1 Pa (Lucey et al., 1997a). The large deformation properties of yogurt gels formed in situ were determined by applying a single constant shear rate (0.01 s^{-1}); σ_{yield} was defined as the point when shear stress started to decrease, and yield strain was the strain value at the yield point (Lucey et al., 1997a).

Whey Separation

Whey separation, which refers to the appearance of liquid (whey) on the surface of a milk gel, was determined using the volumetric flask method described by Lucey et al. (1998b). Immediately after inoculation, 220 g of milk was transferred to a 250-mL volumetric flask and incubated in a temperature-controlled incubator at 40°C until pH reached 4.6. Eight flasks were used for each treatment. Any free whey expelled on the top of gel during fermentation was gently poured out and collected. After letting the flask sit for approximately 1 min, any further free whey was poured out and then weighed. The degree of whey separation was expressed as a percentage of the total weight of milk.

Permeability

The permeability coefficient (B) of yogurt gels was measured using the "tube" method described by Roefs and van Vliet (1990) and Lucey et al. (1997b). Yogurt gels were formed in glass tubes with open ends (length of 25 cm and inner diameter of 4 mm). Immediately

after the pH reached 4.6, the glass tubes were placed in a rack, which was located in a vat filled with acid whey (pH 4.6, 30°C, and viscosity of 0.95 mPa·s). A model 2202 digital cathetometer (Precision Tool and Instrument, Bexhill-on-Sea, UK) was used to measure the height of whey in the tubes. The B was calculated using equation [4]:

$$B = - \left[\ln \frac{(h_{\infty} - h_{t2})}{(h_{\infty} - h_{t1})} \right] \eta H / [\rho g (t_2 - t_1)], \quad [4]$$

where B is the permeability coefficient, h_{∞} is the height of whey in the reference tube, h_{t1} is the height of whey in tube at time t_1 , h_{t2} is the height of whey in tube at time t_2 , η is the viscosity of whey, H is the length of gel, ρ is the density of whey, and g is due to gravity acceleration.

Fluorescence Microscopy

The microstructure of yogurt gel was evaluated in fluorescence mode as recently reported by Choi et al. (2007). Acridine orange (Sigma Chemical Co.) was used as fluorescent protein dye. One hundred milliliters of preheated and preacidified skim milk was inoculated with working culture and then mixed with 700 μ L of acridine orange dye solution (0.2%, wt/wt). A few drops of the mixture was transferred to a slide with concavity and covered with a lifter slip cover glass (size 22 $\times$ 40 mm, Electron Microscopy Sciences, Washington, PA). The innovative design of the lifter slip has a unique raised edge with a bar width of 0.75 mm and can hold up to 50 μ L volume, which allows for the even dispersal of material between the slide and coverslip. The mixture was incubated at 40°C until the pH reached 4.6. The microstructure of yogurt gels was observed using a fluorescent microscope (Axioskop 40, Carl Zeiss Light Microscopy, Gottingen, Germany) equipped with motorized stage (z-drive; Axioskop z mot plus, Carl Zeiss Inc., New York, NY). To eliminate out-of-focus light in FM, deconvolution was applied (Carl Zeiss Vision, 2000) as described by Choi et al. (2007). An excitation wavelength of 450 nm and an emission wavelength of 515 nm was used (Choi et al., 2007). After many fields were observed, representative micrographs were reported.

Experimental Design and Statistical Analysis

The effects of preacidification pH value and the length of the fermentation time on the properties of yogurt gels were investigated using a 2-level central

composite rotatable experimental design (Lee and Lucey, 2004; Lucey et al., 1998b) with 2 star points ($\alpha = 1.414$), 10 treatments, and 2 replicates of the central point (Table 1). Experiments were performed in triplicate and resulted in 30 runs. Results were analyzed by response surface methodology and multiple (step-wise) regression using the Statgraphics program, (version 6.0, Manugistics, Rockville, MD). The models were simplified by step-wise regression eliminating insignificant terms ($P > 0.05$) and selecting the terms with the highest sequential F -values ($F > 4$).

RESULTS

Turbidity Measurement

Turbidity of milk was greatly affected by preacidification pH, as shown in Figure 1. The absorbance value of preacidified milk at 600 nm initially showed a slight decrease with a decrease in preacidification pH as CCP was removed from casein particles during acidification. Turbidity increased at low preacidification pH values, especially when at pH values <6, which might be due to aggregation of particles that occurred before gel formation was detected by the rheometer (Figure 1).

Acid-Base Titration

The acid-base buffering curves for preacidified skim milk samples are shown in Figure 2. The buffering peak at pH $\sim$ 5.0 was caused by the solubilization of CCP (Lucey et al., 1993). The area between pH 5.8 and 4.1 can be used to estimate the amount of CCP in milk (Lucey et al., 1993). The size of this buffering peak decreased with a decrease in preacidification pH because preacidification resulted in the solubilization of CCP. These results are in agreement with previous work on solubilization of CCP in milk with various pH values (Dalglish and Law, 1989). When an acidified sample was back-titrated with NaOH, buffering at pH 5.0 was low, because CCP was already solubilized. As expected, there were no differences in the back-titration buffering curves.

Ca Analysis

The soluble Ca contents in milks as a function of preacidification pH are shown in Figure 3. Total calcium content of milk was 128 ± 0.6 mg/100 g. The preacidification pH value of milk had a statistically significant effect ($P < 0.001$) on the solubilization of CCP. The soluble Ca content increased almost linearly as the preacidification pH decreased from 6.55 to 5.50. More than

Figure 1. Influence of preacidification of milk on the turbidity of milk sample. Turbidity was measured as absorbance (arbitrary units) at 600 nm as a function of preacidification pH. Results are the means of triplicates with error bars for standard deviation.

50% of CCP in milk was dissolved at a preacidification pH value of 5.65.

The soluble Ca contents in the supernatant of yogurt gels at pH 4.6 are shown in Figure 3b. There was no significant difference in the Ca content in the superna-

tants of milk gels made from different preacidification pH values and different fermentation times.

The soluble Ca content in the supernatants of yogurt gels at pH 5.0 as a function of fermentation time is shown in Figure 3c. When all yogurts were compared at

Figure 2. Acid-base buffering curves of preacidified skim milk samples that were preacidified to pH values of 6.55 (□), 6.42 (×), 6.10 (Δ), 5.78 (○), and 5.65 (—). Results are the means of triplicates. Arrows indicate the direction of titration.

Figure 3. Calcium content in (a) ultrafiltration permeate of milks as a function of preacidification pH, (b) supernatant measured at pH 4.6 of yogurt gels made with different preacidification pH values and fermentation times measured at pH 4.6, (c) supernatant of yogurt gels measured at pH 5.0. Yogurts were made from milks with different preacidification pH values and fermentation times as a function of time. Results are the means of triplicates with error bars for standard deviations.

the same pH value of 5.0 during the fermentation process, the soluble Ca content increased with an increase in the fermentation time.

Rheological Properties

The rheological properties (G' and LT values) of yogurt gels made from different preacidification pH values and a constant fermentation time (375 min) are shown in Figure 4a. Similar gelation times were observed for samples with different preacidification pH values but similar fermentation times (375 min). At pH values >5.1 , the G' values of gels with different preacidification pH values were similar. At pH values ≤ 5.1 , the gelation profiles of yogurt gels made with a lower preacidification pH value had a slower increase in G' values, compared with gels made with the higher preacidification pH values. Storage modulus values at pH 4.6 significantly ($P < 0.001$) decreased with a decrease in preacidification pH value (Table 2). The effects of preacidification of milk on the rheological and physical properties of yogurt gels are summarized in Table 3. The G' values at pH 4.6 in yogurt gels made with preacidification pH values of 6.55, 6.10, and 5.65 were 183, 139, and 83 Pa, respectively. The gelation pH (~ 5.3 – 5.4) and pH at loss tangent maximum (LT_{max}) (~ 4.95) of yogurts made from different preacidification pH values were not significantly different. The LT_{max} values ranged from 0.43 to 0.45 for yogurt gels made from different preacidification pH values. The yogurt gels made from the lowest preacidification pH value of 5.65 had a significantly lower LT_{max} value than the other 2 preacidified samples (Table 3). The σ_{yield} values in yogurt gels made with preacidification pH values of 6.55, 6.10, and 5.65 were 23, 16, and 11 Pa, respectively. Preacidification of milk before fermentation resulted in a marked decrease in both the G' at pH 4.6 and the σ_{yield} of yogurt gels (Table 3).

The rheological properties of yogurt gels made with a constant preacidification pH value of 6.10 but with different fermentation times are shown in Figure 4b. The effects of fermentation time on the rheological and physical properties of yogurt gels are summarized in Table 4. The gelation pH and pH at LT_{max} shifted to a low pH value in gels with very long fermentation times (Figure 4b, Table 4). At pH values < 5.2 , a slower increase in G' values were observed with an increase in fermentation time. The G' at pH 4.6 was significantly negatively related to the fermentation time (Table 2). The G' values at pH 4.6 in yogurt gels made with fermentation times of 250, 375, and 500 min were 170, 139, and 99 Pa, respectively. Gelation time decreased with a reduction in the fermentation time. The σ_{yield} values in yogurt gels made with fermentation times of 250,

375, and 500 min were 22, 16, and 14 Pa, respectively. Increasing the fermentation time resulted in a decrease in LT_{max} and σ_{yield} (Table 4).

Whey Separation and Permeability

Fermentation time resulted in an increase in whey separation (Figure 5). Preacidification had a less pronounced effect compared with fermentation time on whey separation of yogurt gels, and fermentation time was a significant factor on whey separation (Table 2). Whey separation significantly increased in gels with long fermentation times, which is in agreement with previous work in yogurt gels made with a lower inoculation rate; that is, long fermentation time (Lee and Lucey, 2004).

The B value gives information about the inhomogeneities at the level of the gel network (Roefs and van Vliet, 1990; Lucey et al., 1997b). Preacidification pH and fermentation time had significant effects on the B of yogurt gels (Table 2). A higher B value was observed with a decrease in preacidification pH (Tables 2 and 3) and with an increase in fermentation time (Tables 2 and 4), indicating that larger pores were formed in yogurt gels made from milk with lower preacidification pH values and with longer fermentation times. Higher B values were previously observed in yogurt gels made at lower inoculation levels (Lee and Lucey, 2004).

Fluorescence Microstructure

The micrographs of yogurt gels made from heated milks with different preacidification pH values and different fermentation times are presented in Figure 6. The yogurt gels made with low preacidification pH values (e.g., Figure 6c) had larger clusters and fewer apparent interconnections in the strands making up the gel network, whereas the yogurt gels made with high preacidification pH values (e.g., Figure 6a) had a finer network structure with more branching and greater apparent interconnectivity. Large pores were present in the networks of yogurt gels made from low preacidification pH values compared with gels made from high preacidification pH. The yogurt gels made with long fermentation times (Figure 6e) had large strands and fewer apparent interconnections in the strands making up the gel network compared with yogurt gels made with short fermentation times (Figure 6d), which had a finer structure with more branching and apparent interconnectivity.

Response Surface Plots

The second-order polynomial models for each of the dependent variables are given in Table 2. The response

Figure 4. Storage modulus (solid symbols) and loss tangent (open symbols) of yogurt gels made with (a) various preacidification pH values and constant fermentation time (375 min), (b) with various fermentation times with a constant preacidification pH (6.10). (a) Yogurt gels were prepared with preacidification pH of 6.55 (■, □), 6.10 (▲, △), and 5.65 (●, ○) and incubated for 375 min. (b) Yogurt gels were prepared with the same preacidification pH of 6.10 and incubated for 250 (■, □), 375 (▲, △), and 500 min (●, ○). Results are the means of triplicates with error bars for standard deviation.

surface plot for the G' values at pH 4.6 and B as a function of preacidification pH and incubation temperature are shown in Figure 7. A good prediction model for G' at pH 4.6 ($R^2 = 0.76$) was obtained. Storage modulus at pH 4.6 decreased with a reduction with preacidification pH and with an increase in fermentation time (Figure 7a). The model for B was significant ($R^2 = 0.78$), and B was negatively affected by preacidification pH ($P < 0.001$) and positively affected by fermentation time ($P < 0.001$; Table 2). The B value increased with

a decrease in preacidification pH and an increase in fermentation time (Figure 7b). The prediction models for whey separation ($R^2 = 0.87$) and soluble Ca content at pH 5.0 ($R^2 = 0.78$) were significantly positively affected by fermentation time ($P < 0.001$; Table 2).

DISCUSSION

The loss of CCP is one of the major chemical changes in casein micelles during acidification. Significant amounts

Table 2. Second-order polynomial models obtained for dependent variables

Dependent variable ¹	Independent variable ²	Coefficient	R ² (adjusted) ³	P-value
G' at pH 4.6	Constant	129	0.76	<0.001
	pH***	25.403		
	T***	-16.968		
σ_{yield}	Constant	16.84	0.41	<0.01
	pH***	2.327		
	T**	-1.786		
LT _{max}	Constant	0.459	0.56	<0.05
	pH***	0.106		
	T*	-0.005		
	pH ² ***	-0.007		
pH at gelation	Constant	5.35	0.47	<0.01
	pH**	0.060		
	pH $\times$ T***	0.097		
pH at LT _{max}	Constant	4.93	0.40	<0.05
	pH**	0.038		
	T*	0.030		
	pH $\times$ T**	0.056		
Whey separation	Constant	5.38	0.87	<0.001
	T***	1.219		
	T ² ***	-0.446		
<i>B</i>	Constant	2.92	0.78	<0.001
	pH***	-0.130		
	T***	0.198		
Ca content at pH 5.0	Constant	89.9	0.78	<0.001
	T***	6.790		
	T ² ***	4.579		

¹G' = storage modulus (Pa); σ_{yield} = yield stress (Pa); LT_{max} = maximum loss tangent; *B* = permeability coefficient.

²pH = preacidification pH; T = fermentation time.

³R² values were adjusted for the degree of freedom.

P* < 0.05; *P* < 0.01; ****P* < 0.001.

of CCP were dissolved from within casein micelles with a reduction in pH (Figures 2 and 4). It is well known that CCP is solubilized as milk pH decreases (Dalglish and Law, 1989; Choi et al., 2007). Virtually all the CCP was dissolved at pH 4.6, which was the end of yogurt fermentation (Figure 3b); similar results were found in milks directly acidified with HCl (Dalglish and Law, 1989; Gastaldi et al., 1996). Gastaldi et al. (1996) also

found that practically all of the micellar insoluble P was solubilized during bacterial acidification of milk to pH 5.1 at 20°C.

The solubilization of CCP during fermentation is a slow and dynamic process and the point at which CCP is completely dissolved varies with the conditions of acidification (e.g., pH, time, rate of acidification and temperature). The results for the soluble calcium con-

Table 3. Effect of preacidification pH on rheological properties, whey separation, and permeability of yogurt gels made with a constant fermentation time (375 min)¹

Item	Preacidification pH		
	6.55	6.10	5.65
Insoluble Ca (mg/100 g)	101.8 $\pm$ 3.0	88.3 $\pm$ 2.4	69.8 $\pm$ 4.3
pH at gelation	5.40 $\pm$ 0.02 ^a	5.37 $\pm$ 0.05 ^a	5.38 $\pm$ 0.04 ^a
Maximum loss tangent (LT _{max})	0.457 $\pm$ 0.013 ^a	0.464 $\pm$ 0.007 ^a	0.438 $\pm$ 0.009 ^c
pH at LT _{max}	4.93 $\pm$ 0.02 ^a	4.95 $\pm$ 0.05 ^a	4.96 $\pm$ 0.10 ^a
Storage modulus, G' at pH 4.6 (Pa) ²	183 $\pm$ 13 ^a	139 $\pm$ 10 ^b	83 $\pm$ 10 ^c
Yield stress (Pa) ²	23 $\pm$ 2 ^a	16 $\pm$ 3 ^b	11 $\pm$ 1 ^c
Whey separation (%) ²	5.05 $\pm$ 0.05 ^a	5.54 $\pm$ 0.08 ^b	6.06 $\pm$ 0.07 ^c
Permeability coefficient (10 ⁻¹³ m ²) ²	2.63 $\pm$ 0.10 ^a	2.86 $\pm$ 0.07 ^b	3.17 $\pm$ 0.05 ^c

^{a-c}Values with different letters within the same row are significantly different (*P* < 0.05).

¹Values are means $\pm$ standard deviation.

²These properties were determined when the pH of yogurt gels reached 4.6.

Figure 5. Whely separation of yogurt gel made from milk with different preacidification pH and different fermentation time. Whely separation was measured when gels reached pH 4.6. Results are the means of duplicates with error bars for standard deviation. ^{a-d}Columns with different letters are significantly different ($P < 0.05$).

tent of gels at pH 5.0 indicated that fermentation time significantly affected the rate of solubilization of CCP. Long fermentation times (slow acidification) allowed more time for the solubilization of CCP (higher soluble Ca content in gels), whereas short fermentation times (fast acidification) allowed less time for this process to occur (lower soluble Ca content in gels). A lower pH value may have been required to completely dissolve CCP during a short fermentation time compared with a slow fermentation in which the CCP could have been completely dissolved at a slightly higher pH value. The effect of the loss of CCP on the properties of casein micelles can be viewed in the context of the dual-binding model for casein micelle (Horne, 1998). Solubilization of CCP reduces the number of CCP crosslinks and induces (temperature-dependent) dissociation of caseins from casein micelles (Walstra, 1990). The solubiliza-

tion of CCP should also modify the localized balance between hydrophobic interactions and electrostatic repulsion by exposing charged phosphoserine residues groups, thereby increasing the local electrostatic repulsion between caseins, although as pH decreases, the charge on the phosphate groups is also reduced (Lucey, 2002). The rate of solubilization of CCP was considered an important factor on whely separation and weak gel formation (Lee and Lucey, 2004). We hypothesized that modification of the internal CCP-casein interactions could affect the gelation properties of yogurt gels. The loss of CCP from inside micelles before, during, and after gelation may induce greater rearrangements of casein particles.

The turbidity of preacidified milk at 600 nm initially decreased with a reduction in pH as CCP was removed from casein particles during preacidification, and CCP

Table 4. Effect of fermentation time on rheological properties, whely separation, and permeability of yogurt gels made with the same preacidification pH (6.10)¹

Item	Fermentation time (min)		
	250	375	500
Insoluble Ca at pH 5.0 (mg/100 g)	42.8 ± 4.4 ^a	40.3 ± 1.7 ^a	21.0 ± 6.0 ^b
pH at gelation	5.38 ± 0.08 ^a	5.37 ± 0.05 ^a	5.22 ± 0.05 ^c
Maximum loss tangent (LT _{max})	0.474 ± 0.012 ^a	0.464 ± 0.007 ^b	0.447 ± 0.004 ^c
pH at LT _{max}	4.98 ± 0.02 ^a	4.92 ± 0.05 ^a	4.84 ± 0.01 ^c
Storage modulus, G' at pH 4.6 (Pa) ²	170 ± 13 ^a	139 ± 10 ^b	99 ± 11 ^c
Yield stress (Pa) ²	22 ± 2 ^a	16 ± 3 ^b	14 ± 4 ^b
Whely separation (%) ²	3.41 ± 0.06 ^a	5.54 ± 0.08 ^b	6.23 ± 0.16 ^c
Permeability coefficient (10 ⁻¹³ m ²) ²	2.52 ± 0.03 ^a	2.86 ± 0.07 ^b	3.17 ± 0.05 ^c

^{a-c}Values with different letters within the same row are significantly different ($P < 0.05$).

¹Values are means ± standard deviation.

²These properties were determined when the pH of yogurt gels reached 4.6.

Figure 6. Microstructure of yogurt gels prepared with the preacidification pH of (a) 6.55, (b) 6.10, and (c) 5.65 and incubated for 375 min. Microstructure of yogurt gels prepared with the same preacidification pH of 6.10 and incubated for (d) 250 min, (b) 375 min, and (e) 500 min. Scale bar = 50 μm . The protein matrix is white, whereas pores are dark.

contributes to the light scattering of micelles. A minimum in the turbidity profiles was observed between pH 5.9 and pH 6.1. Chardot et al. (2002) found a minimum in turbidity around pH 5.9, which corresponded to the first minimum of casein voluminosity that was related to collapse of the micellar hairy layer. Mizuno and Lucey (2005) reported a decrease in turbidity with the chelation of CCP inside micelles by emulsifying salts. A significant increase in turbidity was observed as the pH decreased from ~ 6.0 to 5.65, which indicated the formation of larger particles or clusters, which might be caused by some aggregation of particles that occurred before gel formation was detected by the rheometer (Figure 1).

We observed the appearance of an LT_{max} (Figure 4a and 4b), which was in agreement with previous studies of yogurts made from heated milk (Lee and Lucey, 2004). The solubilization of CCP weakened casein-casein interactions and probably contributes to the constant or slightly decreased G' values in this shoulder region and the initial increase in LT (i.e., before a maximum appeared; Lucey, 2002; Figures 4a and 4b). It was previously reported that the LT_{max} was caused by a loosening of the intermolecular forces in casein

particles that are already part of the gel network due to the solubilization of CCP (Lucey et al., 1998a; Lucey, 2002). Gels with lower preacidification pH values had slightly lower LT_{max} values because more CCP was dissolved at the lower pH used. Ozcan-Yilsay et al. (2007) reported that the solubilization of CCP was responsible for the LT_{max} in gels made from heated milk because the removal of CCP removed this LT_{max} peak.

Preacidification of milk affected the gelation properties of yogurt gels. Heat treatment has little effect on the pH-dependent release of Ca and phosphate from micelles during acidification (Law, 1996; Singh et al., 1996). Preacidification decreased the number of CCP crosslinks and weakened protein-protein interactions inside casein particles before fermentation, which may have decreased the rate of bond formation and the formation of cross-links between strands as indicated by the low G' and yield stress of gels (Table 3 and Figure 4a). Similar results were previously reported in rennet gels, in which there was a decrease in maximum G' values of rennet gels as the pH value of milk was reduced from 6.4 to 5.4 (Choi et al., 2007). With a lowering of the preacidification pH, the net negative charges on individual caseins and electrostatic repulsion decrease.

Figure 7. Response surface plots for the effect of preacidification pH value and fermentation time on (a) storage modulus values (G') at pH 4.6 and (b) permeability coefficient (B).

As a result, the steric stabilization of casein micelles diminishes and micelles exhibit attractive interactions, which might enhance the opportunities for casein particles to cluster as indicated by the increase in turbidity with a decrease in preacidification pH. Preacidification may promote more internal rearrangements as indicated by the formation of large clusters in the microstructure in the gel, high whey separation, and large B value. The larger casein clusters caused by preacidification did not “disperse” during gelation and therefore this dictated that gels from preacidified milks had larger clusters and pores. The overall result of preacidification was the formation of weaker gels that were more prone to rearrangement and whey separation. Similar trends were observed in gels made at high incubation temperature (low storage modulus and yield stress, high B and whey separation; Lee and Lucey, 2004). Fermentation time then controls the number and extent of rearrangements that can occur subsequently.

Fermentation time greatly affected yogurt gel formation, including the time to gelation, pH at gelation, time to $LT_{\max}$, pH at $LT_{\max}$, and time to reach pH 4.6. Fermentation time directly affected the rate of bond formation as indicated by the low G' value at pH 4.6 and low σ_{yield} values in yogurt gels made with a long fermentation time. When gelation occurred at a lower pH (i.e., long fermentation time), electrostatic repulsion and CCP crosslinks decreased, which could lead to greater loss of internal structure and modification of the type of bonds formed during fermentation. The rate of particle aggregation during acidification is affected by net charge on protein molecules, which in turn is influenced by the amount of bound calcium associated with casein at a given pH (Kim and Kinsella, 1989). With an increase in the fermentation time, there was an increase in the time allowed for rearrangements in the gel network. The increased (opportunity for) rearrangements allowed strands and clusters to aggregate further (coalesce or form denser clusters) and this resulted in the observed increase in whey separation and the formation of larger pores (Figure 6; higher B value). Similar results were observed in yogurts with low inoculation levels by Lee and Lucey (2004). Fermentation time affected the extent of CCP solubilization during the critical stage of gel formation (Figure 3c), which might favor increased rearrangements of the gel network (because there were fewer crosslinks). We have demonstrated that fermentation time affected the acidification rate, gelation kinetics, rate of aggregation, the type of interactions, as well as the time available for rearrangements of casein particles in gel network, which collectively contributed to the formation of weak and unstable gel networks.

CONCLUSIONS

Yogurt gels made with lower preacidification pH and longer fermentation time had lower storage modulus and yield stress values, higher whey separation and permeability, and formed coarse networks. Removing CCP by acidification before gelation decreased the concentration of CCP crosslinks and may have increased repulsion between the newly exposed phosphoserine residues, resulting in weaker gels. Increasing the fermentation time resulted in a lower rate of bond formation (as indicated by the low G' just after gelation and low σ_{yield}), weaker gels, and occurrence of large pores in the gels, probably due to greater loss of CCP crosslinks at the critical aggregation/gelation stage, and more time allowed more opportunity for greater casein rearrangements. Preacidification of milk before starter culture addition should be avoided as preacidification resulted in a weakening of the gel. The yogurt industry gener-

ally favors short fermentation times during processing and the results of this study suggest that reducing the fermentation time improved gel properties.

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