Comparison of micellar casein isolate and nonfat dry milk for use in the production of high-protein cultured milk products

D. J. Wilbanks,1* M. R. Lee,2 Y. S. Rahimi,3 and J. A. Lucey1,4
1Department of Food Science, University of Wisconsin, Madison 53706
2Department of Food and Nutrition, Daegu University, Gyeongsan 38453, South Korea
3Arla Foods, Viby J 8260, Denmark
4Center for Dairy Research, University of Wisconsin, Madison 53706

ABSTRACT

High protein levels in yogurt, as well as the presence of denatured whey proteins in the milk, lead to the development of firm gels that can make it difficult to formulate a fluid beverage. We wanted to prepare high-protein yogurts and explore the effects of using micellar casein isolate (MCI), which was significantly depleted in whey protein by microfiltration. Little is known about the use of whey protein-depleted milk protein powders for high-protein yogurt products. Microfiltration also depletes soluble ions, in addition to whey proteins, and so alterations to the ionic strength of rehydrated MCI dispersions were also explored, to understand their effects on a high-protein yogurt gel system. Yogurts were prepared at 8% protein (wt/wt) from MCI or nonfat dry milk (NDM). The NDM was dispersed in water, and MCI powders were dispersed in water (with either low levels of added lactose to allow fermentation to achieve the target pH, or a high level to match the lactose content of the NDM sample) or in ultrafiltered (UF) milk permeate to align its ionic strength with that of the NDM dispersion. Dispersions were then heated at 85°C for 30 min while stirring, cooled to 40°C in an ice bath, and fermented with yogurt cultures to a final pH of 4.3. The stiffness of set-style yogurt gels, as determined by the storage modulus, was lowest in whey protein-depleted milk (i.e., MCI) prepared with a high ionic strength (UF permeate). Confocal laser scanning microscopy and permeability measurements revealed no large differences in the gel microstructure of MCI samples prepared in various dispersants. Stirred yogurt made from MCI that was prepared with low ionic strength showed slow rates of elastic bond reformation after stirring, as well as slower increases in cluster particle size throughout the ambient storage period. Both the presence of denatured whey proteins and the ionic strength of milk dispersions significantly affected the properties of set and stirred-style yogurt gels. Results from this study showed that the ionic strength of the heated milk dispersion before fermentation had a large influence on the gelation pH and strength of acid milk gels, but only when prepared at high (8%) protein levels. Results also showed that depleting milk of whey proteins before fermentation led to the development of weak yogurt gels, which were slow to rebody and may be better suited for preparing cultured milk beverages where low viscosities are desirable.

Key words: micellar casein, high protein, yogurt gels, whey

INTRODUCTION

Yogurt is a popular fermented milk product enjoyed throughout the world. It is produced via fermentation of milk by lactic acid bacteria, specifically Lactobacillus delbrueckii ssp. bulgaricus and Streptococcus salivarius ssp. thermophilus. These bacteria convert lactose to lactic acid, thereby decreasing the pH of the system and lowering the net surface charge of milk proteins (Tamime and Robinson, 2007). At low pH values (pH ~4.6), the attractive forces dominate once enough of the electrostatic repulsive forces of the proteins have been sufficiently decreased, and a system-spanning network of protein particles forms a gel (Lucey and Singh, 2003). Higher protein levels thus lead to more crosslinking and interconnections between the protein particles in acidified milk. For this reason, protein fortification is commonly used to increase gel firmness, or viscosity, of cultured milk products. Some yogurt types, such as drinkable yogurt, require a weak body and a fluid consistency, so higher-protein formulations can be a significant obstacle for manufacturers to successfully overcome.

Casein proteins make up ~80% of milk proteins in bovine milk and form the primary structure in the particle gel that forms as a result of the acidification of milk (Damodaran, 2008). Caseins lack significant levels
of tertiary protein structures but instead form supramolecular protein aggregates commonly referred to as casein micelles. These micelles are made up of thousands of individual casein monomers held together by a combination of hydrophobic and electrostatic forces, with the latter at least partially involving phosphorylated protein residues associated with calcium. These calcium phosphate groups, or colloidal calcium phosphate (CCP), form bridges that crosslink the caseins in the micelle and are solubilized during acidification mainly at pH values 5.0 to 5.5 (Horne, 1998).

The solubilization of CCP has 3 main effects on acidified milk: (1) pH buffering (increase) due to the release of phosphate ions, (2) an increase in the ionic strength of the serum, and (3) loosening of micellar internal structure (Dalgleish and Law, 1988). The increase in ionic strength due to CCP solubilization also weakens electrostatic attractive forces as a result of charge shielding (Roefs et al., 1990); however, the effects of a weakened micellar structure on acid milk gels are more complex. The sequence (timing) of gel formation and complete CCP solubilization can have a major influence on the final gel structure. If an acid gel is formed before most of the CCP has been solubilized, then the loosening of the internal micellar structure already part of the network results in an increase in bond mobility as calcium-protein crosslinks are lost (Lucey and Horne, 2018). This increased internal structural flexibility is eventually overcome as the pH further declines below 5.0, after all the CCP is fully solubilized. The increased bond mobility created by CCP solubilization in gelled samples can lead to the formation of large pores and dense clusters, indicative of coarse gels that are weak (at the point of gel formation) and more prone to spontaneous syneresis (van Vliet et al., 1991; Lucey, 2001).

In contrast to caseins, whey proteins are globular and are soluble across the pH range encountered in yogurt fermentation. However, whey proteins are denatured with moderate heat >72°C (Dalgleish, 1990) and, in their denatured form, become insoluble near their isoelectric point (pI). These denatured whey proteins form aggregates with both whey and caseins that lead to acid gel formation at higher pH values than observed in unheated milk (Lucey et al., 2022). Denatured whey proteins also provide additional covalent (disulfide) linkages between protein aggregates and thus contribute to overall firmer gels (Krzeminski et al., 2011). Milk used in yogurt production is therefore typically heated to high temperatures before fermentation.

Milk fractionation via membrane filtration is rapidly growing in popularity as a tool to physically separate milk components without greatly altering their chemical properties (Saboya and Maubois, 2000). Microfiltration can separate casein from soluble milk components such as whey proteins, while maintaining the micellar structure of casein. Historical methods for casein isolation involve techniques such as acid precipitation of milk and then neutralization of the curd with base (alkali) to obtain caseinates. These caseinates do not retain the original micellar structure due to the depletion of CCP and so form very different yogurt gels than those made from native milk proteins (Peng et al., 2009). Micellar casein powder is the dried form obtained from the microfiltration retentate of milk, and considerable amounts of soluble ions are lost along with whey proteins during the extensive filtration and diafiltration processes needed for its manufacture. The ionic strength of rehydrated micellar casein dispersions can therefore differ greatly from NDM powder, which could also influence acid gel formation.

Although many studies have examined the effects of protein fortification of milk for use in yogurt production (Bong and Moraru, 2014; Meletharayil et al., 2015), micellar casein as the sole protein source for high-protein yogurt has been largely unexplored. The absence of denatured whey proteins in unheated milk has been shown to lead to weaker acid gels (Lucey et al., 1997a), which may be desirable for fluid yogurt beverages, especially those with higher protein contents. The objectives of this research were to investigate the effects of using different types of reconstituted milk powders with various levels of denatured whey proteins. We also explored the influence of the ionic strength of milk protein dispersions and its effects on yogurt gel properties.

**MATERIALS AND METHODS**

No animals were used in this study, and ethical approval for the use of animals was thus deemed unnecessary.

Protein dispersions (8%, wt/wt) were prepared from micellar casein isolate (MCI; Arla Foods) or low-heat NDM (Foremost Farms). Preparations were made in duplicate, as shown in Table 1, as separate batches and prepared on different days. Micellar casein dispersions were supplemented with lactose (laboratory grade; Fisher Scientific) and peptone (Becton, Dickinson, and Co.) to aid fermentation due to the very low levels of lactose and small peptides or nonprotein nitrogen found in MCI (depleted during the diafiltration process). For MCI dispersions in water, lactose was added at either 5.0 (designated as MCI-LL) or 11.4% (MCI-HL; wt/ wt); the former was determined by preliminary work to be the minimum lactose content required for the fermentation to reach pH 4.3, and the latter was chosen to align with the lactose content in NDM (when it...
was rehydrated to 8% protein, wt/wt). Micellar casein isolate was also dispersed in UF milk permeate powder (MCI-UF; Idaho Milk Products) at 13.3% (wt/wt), to align its lactose content with NDM and also to provide a soluble mineral environment similar to that of concentrated NDM milk. Peptone was added to MCI samples at various levels to try to minimize differences in the fermentation times needed to reach pH 4.6. Without peptone addition, the cultures took $\gg 10$ h to reach pH 4.6 for MCI-based dispersions. The NDM powder was dispersed in water with no added lactose or peptone. The conductivity of dispersions, which was used as an approximate indicator of ionic strength, was measured using a conductivity meter (Cond6+ Hand-Held Conductivity Meter; Oakton Instruments).

Dispersions were rehydrated at room temperature for 2 h using an overhead stirrer (model 2002; Fisher Scientific) at ~700 rpm, and then transferred to a refrigerator and stirred for 12 h at 4°C. After overnight rehydration, dispersions were heated at 85°C for 30 min while stirring and then rapidly cooled to 40°C for fermentation. The contribution of insoluble calcium phosphate to the buffering capacity of 3% (wt/wt) protein dispersions made with MCI or NDM dispersed in water were determined as described by Hassan et al. (2004). Higher-protein dispersions were difficult to analyze due to coagulation under acidic conditions and some foaming.

Stock cultures were prepared from aliquots of YC-380 mixed yogurt starter culture (Chr. Hansen) that had been inoculated into sterilized milk (121°C for 30 min) and fermented to pH 5.0 before immediate storage at ~80°C. On the day of yogurt fermentation, working cultures were prepared from thawed stock cultures that had been inoculated into sterilized milk and fermented for ~2 h at 40°C. Approximately 200 mL of MCI and NDM dispersions were inoculated with 2% (vol/vol) working culture, and fermentation took ~10.5 h at 40°C to reach pH 4.3. Sodium azide (0.02% wt/wt; Fisher Scientific) was then added to inactivate microbes and prevent post-acidification, and yogurts were sheared at low speed for 30 s in a blender (4 blades, 1.2-L container; Waring) to create stirred yogurt. The stirred yogurts were then poured into 120-mL transparent plastic containers (MVP Plastics) and disposable rheology cups (C-CC27; Anton Paar) for aging at ambient temperature for 4 wk. Stirred yogurt samples were left undisturbed for aging and not stirred again.

An Anton Paar MCR 301 rheometer was used to measure viscoelastic properties of yogurt gels. Small-amplitude oscillatory rheology (SAOR) was used to measure the elastic ($G'$) and viscous ($G''$) moduli of samples during fermentation. Samples were measured at a frequency of 0.1 Hz and a low amplitude (0.5% strain) to diminish disruption to the developing network (van Vliet et al., 1989; Mezger, 2006). Inoculated dispersions were placed in the rheometer at 40°C and fermented in situ while the pH was recorded in a separate sample via a pH meter (Fisher Scientific). All set-style yogurt measurements were made with a cup-and-bob measurement system (CC27, Anton Paar). In this study, a gel was experimentally determined to have formed when $G'$ values >1 Pa (Lucey et al., 1997b). The yield stress values of stirred yogurt samples were measured using a vane geometry (ST24-4V-30, Anton Paar) at a constant shear rate (0.01 s$^{-1}$). The yield stress was determined as the force required to break the gel, evidenced by a maximum in the shear stress value during the application of a constant low shear rate (Luyten et al., 1994). Yield stress testing was conducted on undisturbed stirred yogurt samples stored in disposable rheometer cups (C-CC27, Anton Paar) to minimize sample disruption when loading onto the rheometer. Measurements of SAOR and yield stress were made initially after the preparation of stirred yogurt and after 4 wk of storage at ambient temperature, to observe rebodying during storage. Ambient temperature was chosen for the storage condition because this would accelerate the

Table 1. Milk protein dispersions (8%, wt/wt) prepared from micellar casein isolate (MCI) or NDM (n = 2); peptone was added to some samples to accelerate acidification and more closely align fermentation times to pH 4.6

| Item                      | MCI in low-lactose water (MCI-LL) | MCI in high-lactose water (MCI-HL) | MCI in UF permeate (MCI-UF) | NDM in water (NDM) |
|---------------------------|-----------------------------------|------------------------------------|----------------------------|-------------------|
| Protein, %                | 8.0                               | 8.0                                | 8.0                        | 8.0               |
| Casein:whey               | 95.5                              | 95.5                               | 95.5                       | 82.18$^1$        |
| Lactose, %                | 5.0                               | 11.4                               | 11.4                       | 11.4              |
| Ash, %                    | 0.6                               | 0.6                                | 1.8                        | 1.9               |
| Peptone, %                | 1.0                               | 1.0                                | 0.5                        | —                 |
| TS, %                     | 15                                | 21                                 | 22                         | 22                |
| Conductivity (mS/cm)      | 1.7                               | 1.8                                | 6.7                        | 7.7               |
| pH of dispersion          | 6.8                               | 6.6                                | 6.5                        | 6.5               |
| Time to pH 4.6 (min)      | 430                               | 360                                | 330                        | 330               |

$^1$Estimated, approximate value based on true protein.
protein-protein interactions responsible for rebodying, and the sodium azide added to samples after fermentation prevented post-acidification.

Particle size was measured using a Malvern Mastersizer 2000 (Malvern Instruments Ltd.) to determine changes in the size of primary protein clusters throughout storage. The particle size of stirred yogurt was initially measured after stirring and after 4 wk of storage at ambient temperature by dispersing several drops of yogurt into water. Refractive indices of 1.33 and 1.57 were used for water and casein, respectively, as described by Ambrose Griffin and Griffin (1985).

To visualize the microstructure of set yogurt gels, a confocal scanning laser microscope (Nikon A1R-SI+) was used. Dispersions were inoculated with the working culture, stained with Fast Green FCF fluorescent dye (Lee and Lucey, 2003), and fermented in an incubator at 40°C for 10.5 h before imaging. Samples were fermented undisturbed in microscope slides with a cavity well and observed using 100× magnification with an oil immersion objective.

The permeability of set yogurt gels was determined using the method developed by van Dijk and Walstra (1986) and Roefs et al. (1990). The permeability coefficient ($B$) was calculated using the equation described by Lucey et al. (1998a):

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

where $B$ is the permeability coefficient ($m^2$); $h_\infty$ is the height of whey in the reference tube (m); $h_{t2}$ is the height of the whey in the gel tube (m) at time $t_2$ (s); $h_{t1}$ is the height of the whey in the gel tube (m) at $t_1$ (s); $\eta$ is the viscosity of the whey (Pa·s$^{-1}$); $H$ is the height of the gel in the gel tube (m); $\rho$ is the density of the whey (kg/m$^3$); and $g$ is acceleration due to gravity (9.8 m/s$^2$).

Acid whey for each dispersion was collected by filtration using cheesecloth, and viscosity was determined using a constant shear rate of 1 s$^{-1}$ using a cup-and-bob geometry at 40°C. The viscosity of acid whey was determined to be 0.941, 0.947, 0.905, and 1.23 mPa·s$^{-1}$ for yogurts prepared from MCI in water with added lactose (low and high levels), MCI in UF permeate, and NDM in water, respectively.

RESULTS

Micellar casein isolate and NDM were dispersed in water at 3% protein (wt/wt) to determine the contribution of CCP to the buffering capacity. High protein levels were not tested due to the length of time that would be required for performing this titration test and some coagulation and foaming issues at the high protein levels. The buffering capacity was determined as the area between acid and base buffering curves of milk dispersions in the pH range between 4.0 and 5.7. Micellar casein isolate showed a lower buffering capacity than NDM, with average values of $6.9 \times 10^{-4}$ and $8.2 \times 10^{-4}$, respectively. The compositions of 8% protein dispersions used to make yogurt samples, including the conductivity, pH, and acidification times to reach pH 4.6, are shown in Table 1. Peptone was added to help accelerate fermentation and replace some of the small peptides and amino acid sequences normally found in milk dispersions. Less peptone was needed for MCI-UF, and no peptone was added to the NDM samples. The protein dispersions (8%, wt/wt) were inoculated with starter culture, and gel properties were measured via SAOR during fermentation at 40°C (Figure 1).
roughly the same at pH 4.6 and were significantly higher than the other set-style yogurts (Table 2). The MCI dispersed in UF permeate formed the weakest gels at pH 4.6, and NDM formed set gels that were stiffer than MCI dispersed in UF permeate (Figure 1) but weaker than both MCI samples dispersed in water (MCI-LL and MCI-HL). Rheological properties for set-style yogurt gels are summarized in Table 2.

Gelation occurred at the highest pH values for 8% MCI dispersions in water, with either low or high lactose levels (Table 2). Generally, earlier gel formation was associated with stiffer gels at pH 4.6. The dispersions with high ionic strength, MCI-UF and NDM, formed gels near the pH values typically reported for unheated (4.9–5.0) and heated (5.2–5.4) milks, respectively (Vasbinder et al., 2004). The loss tangent (LT) value (G′′/G′) indicates the viscoelastic character of the system as either fluid (LT >1) or solid-like (LT <1). The LT values also indicated that the onset of gelation for each dispersion occurred at different pH values, which was dependent on both the presence of denatured whey proteins and the dispersant used. The LT values measured during acidification indicated early gel formation (LT <1) for MCI dispersions in water (MCI-LL and MCI-HL). For samples that formed gels before pH 5.0, an increase of the LT to a (secondary) maximum value around pH 5.1, referred to as the LTmax (Lucey et al., 1997a), was observed. The NDM yogurt samples exhibited the lowest LTmax values, and no LTmax was found for MCI-UF yogurt, which did not form a gel until pH ~4.9 (Table 2).

To further investigate the roles of ionic strength and denatured whey proteins in yogurt gel development, we also prepared 3% (wt/wt) protein dispersions of MCI in water, MCI in UF permeate, and NDM. These dispersions were prepared similarly, or proportional, to the 8% (wt/wt) protein milk dispersions listed in Table 1, but with reduced concentrations of components proportional to 3% protein (4.3% lactose for each dispersion, 8.3% TS, and so on). All dispersions had similar lactose contents, and 3% protein was chosen because this is approximately the protein level usually found in unfortified milk; therefore no low-lactose sample (MCI-LL) was prepared at the 3% protein level. Additionally, the ionic strength of 3% MCI dispersed in 5.6% (wt/wt) UF permeate and 3% NDM dispersed in water were approximately what would be expected in native (fresh) milk samples. The 3% (wt/wt) protein dispersions were microbially acidified similar to the previously described samples, and their SAOR profiles

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**Table 2.** Rheological properties of set-style yogurts prepared at 8 or 3% protein from micellar casein isolate (MCI) or NDM

| Item | MCI in low-lactose water (MCI-LL) | MCI in high-lactose water (MCI-HL) | MCI in UF permeate (MCI-UF) | NDM in water (NDM) |
|------|----------------------------------|-----------------------------------|----------------------------|-------------------|
| 8% protein | | | | |
| G′ at pH 4.6 (Pa) | 1.002±103 | 1.132±346 | 1.47±19 | 768±29 |
| LT at pH 4.6 | 0.342±0.012 | 0.312±0.054 | 0.359±0.013 | 0.260±0.003 |
| LTmax | 0.756±0.001 | 0.699±0.012 | NA | 0.426±0.001 |
| Gel pH | 5.70±0.21 | 6.03±0.28 | 4.90±0.04 | 5.27±0.08 |
| 3% protein | | | | |
| G′ at pH 4.6 (Pa) | — | 27.3±14.4 | 32.5±2.8 | 70.7±15.7 |
| LT at pH 4.6 | — | 0.469±0.086 | 0.412±0.019 | 0.360±0.025 |
| LTmax | — | 0.795±0.065 | NA | 0.516±0.004 |
| Gel pH | — | 4.93±0.13 | 4.95±0.04 | 5.48±0.08 |

* Different letters within a row indicate significant differences (P < 0.05).

1 Milk dispersions were heated at 85°C and inoculated at 40°C with yogurt starter culture (n = 2). Mean values ± 1 SD are shown.

2 G′ = storage modulus; LT = loss tangent; max = maximum; Gel pH = gelation pH; pH at which G′ value >1 Pa.

3 NA = not applicable, no LTmax observed.
are shown in Figure 2. The rheological properties of the set-style yogurt samples made with 3% protein (wt/wt) are summarized in Table 2. Similar to the results for 8% protein gels (Figure 1), the 3% (wt/wt) MCI dispersed in UF permeate exhibited lower gel stiffness ($G'$) during fermentation as well as a lower gelation pH compared with NDM. However, 3% MCI dispersed in water formed weaker gels than those made from NDM. Additionally, MCI dispersed in water formed a gel at a much lower pH value (~4.93) when prepared at 3% protein compared with 8% gels, and the gelation pH was similar (~4.95) to that for MCI-UF (Table 2).

To better understand the structure of high-protein acid milk gels, confocal scanning laser microscopy was used to obtain images of set-style yogurts made with 8% protein (wt/wt; Figure 3). All MCI samples had coarser gel networks with larger pores than the gels made with NDM. The NDM gels (Figure 3d) had smaller pores, finer clusters, and more uniform appearance. To obtain an estimate of the size of pores observed in the confocal microscopy images, the permeability levels of 8% protein set-style gels were measured (Figure 3e, inset). The permeability levels of all MCI-based yogurts were similar and were higher than those of gels made from NDM. Although MCI-UF samples exhibited much lower gelation pH values and no discernible $LT_{\text{max}}$ values compared with other MCI samples, the mean permeability coefficient was only slightly lower than that of MCI yogurts dispersed in water (MCI-LL and MCI-HL). Yogurt gels made from NDM, however, exhibited a very homogeneous, fine gel network (Figure 3d) that had lower permeability (Figure 3e, inset).

After shearing yogurts for 30 s, most of the initial gel structure was destroyed ($G'$ values <1 Pa), but the gel texture was partially restored over time. The reformation of bonds in yogurt gels over time, referred to as rebodying, is not completely understood (Renan et al., 2008) but is generally considered to be the result of new bonds forming within and between protein strands or clusters. The yield stress values of stirred yogurt samples were immediately measured after shearing, and the highest values observed were for NDM samples (Figure 4). After undisturbed storage for 4 wk, stirred yogurt samples stored in disposable rheology cups were placed onto the rheometer and measured again for yield stress after careful insertion of the vane geometry into the samples. After 4 wk of ambient storage, all stirred samples exhibited higher yield stress values compared with initial measurements, which was indicative of rebodying. The MCI-UF and NDM samples had the highest yield stress values and exhibited the largest increases in yield stress during storage.

Stirred yogurt samples were also collected from the plastic storage bottles after 4 wk of ambient storage and gently shaken by hand to break the gel. Samples were then poured into cups for immediate SAOR analysis using a cup-and-bob geometry with traditional rheology cups (CC27), and the dynamic moduli of the shaken samples were measured for 1 h at 22°C (Figure 5). The $G'$ values measured initially after stirring and after 4 weeks of ambient storage (and then shaking) showed large initial rates of increase in $G'$ with time after stirring for gels made from MCI in UF permeate and NDM (Figure 5A, C). However, ~30 min after stirring or shaking, the rate of increase of the $G'$ values, as shown by the $dG'/dt$ values, roughly approached zero (Figure 5B, D). The MCI dispersed in water exhibited very slow rebodying, regardless of lactose levels, and similarly had low yield stress values (Figure 4).

The particle size of stirred yogurt samples was initially measured after stirring and after 4 wk of storage at ambient temperature, and particle size distributions are shown in Figure 6. The particle size for each sample increased throughout storage and shifted from mainly monomodal distributions (peak at ~10 μm) initially after stirring to bimodal distributions at 4 wk with secondary clusters that had sizes between 100 and 1,000 μm. Mean particle size values (D1,3 volume-weighted mean diameter; μm) are shown in the inset for each graph. The mean particle size of stirred yogurt samples was initially highest in NDM yogurt and increased rapidly for samples with high ionic strength (MCI-UF and NDM in water) throughout storage. Syneresis levels for stirred yogurt samples (~90 mL of stirred yogurt) after 4 wk of aging in 120-mL plastic bottles are shown in

![Figure 2](66)
DISCUSSION

Set Yogurt

As described by Lucey et al. (1993), the increase in the buffering index observed during the acidification of milk near pH ~5 can be used to estimate the amount of insoluble CCP present. At similar protein levels, the MCI samples contain a higher overall amount of micellar casein compared with NDM (as NDM also contains more whey proteins and so less casein than MCI). Because CCP is a major buffer in milk (along with soluble salts; Lucey and Fox, 1993), a higher concentration of micellar casein (and therefore CCP) would be expected to have increased the buffering during milk acidification. However, a lower buffering capacity was observed for MCI, likely due to the loss of some CCP as well as most soluble salts during the extensive diafiltration used for micellar casein production (Alexander et al., 2011).

The fermentation times needed to reach pH 4.6 for MCI-LL and MCI-HL were slightly longer than those for MCI-UF or NDM (Table 1), possibly due to their lower concentrations of soluble milk components such as amino acids, which aid bacterial fermentation (Aryana and Olson, 2017). For MCI dispersions in water,
The inclusion of additional lactose (MCI-HL, 11.4% lactose) above the minimum amount required to reach pH 4.3 (MCI-LL, 5.0% lactose) led to faster acidification. Although very high concentrations of sugar can slow the acidification rates for yogurt starter cultures, osmotic tolerance is apparently strain-dependent, and some commercial strains have been shown to tolerate lactose levels up to 12% (wt/wt; Tamime and Robinson, 2007). The higher molar concentrations of solutes for the MCI-HL, MCI-UF, and NDM samples were apparently not high enough to limit bacterial growth by osmotic stress, and so acidification was rapid for these samples. This agreed with other studies that showed a generally robust resistance to osmotic stress for many lactic acid bacteria (van de Guchte et al., 2002).

Acid milk gels made from unheated milk (or caseinate) usually gel at pH values <5.0 (Lucey et al., 1997a) due to the absence of denatured whey proteins. Although the presence of denatured whey proteins in heated milk has been widely reported to contribute to an earlier onset of acid milk gelation (Lucey et al., 1998b; Andoyo et al., 2014), other studies (Fameheart et al., 1996; Auty et al., 2005) have reported that the ionic strength in chemically acidified micellar casein dispersions can also influence the gelation pH. The MCI samples in this study were significantly depleted of whey proteins (casein:whey ratio of 95:5) and so were expected to have low gelation pH values. Gelation pH values were instead very high (pH 5.8–6.0) in 8% protein gels made with MCI dispersed in water (Table 2). This study clearly showed that microbiologically acidified micellar casein dispersions formed gels across a wide pH range, highly dependent on the ionic composition, as well as the presence (or absence) of denatured whey proteins. Yogurts made with heated NDM had much lower gelation pH values (e.g., pH 5.3) than MCI dispersed in water, in agreement with previous studies on heated milk (Lee and Lucey, 2003; Anema, 2009).

The influence of the ionic environment on gel formation was highlighted by the fact that both the highest and lowest gelation pH values for yogurt made with 8% protein were obtained with MCI; the different gelation pH values were caused by differences in the ionic composition of the dispersants used. The presence of denatured whey proteins in NDM did lead to a higher gelation pH compared with whey protein-diminished milk dispersed in a similar ionic environment (MCI-UF). Although the mechanism for earlier gelation in yogurt with denatured whey proteins has been attributed to their higher pI, the mechanism for the earlier gelation observed in this study with 8% MCI dispersed in water is less understood (as MCI has only a small amount of residual whey proteins). A higher ionic strength should lead to increased charge shielding and a shorter Debye length, resulting in weaker charged protein interactions (Walstra and Jenness, 1984). Electrostatic (charged) interactions are important for the formation of acid gels (Lucey, 2002), and less charge shielding may facilitate earlier casein particle aggregation via electrostatic or hydrophobic interactions.

At high protein levels and low ionic strength, the MCI dispersed in water aggregated at higher pH values presumably due to the lack of charge screening (or due to high calcium ion activities at low ionic strength, or to both). The high protein content could have created a more crowded, or close-packed, environment that also likely promoted earlier aggregation. This could explain why the 3% protein MCI samples dispersed in water gelled at lower pH values than the 8% protein MCI sample, as the protein particles would be less tightly packed and thus less affected by short-range interactions. The lower ionic strength could also promote additional Ca bridging between protein particles (at pH values >5.5) due to a higher Ca$^{2+}$ ion activity, which has been observed in milk protein powders concentrated to very high levels (Crowley et al., 2014; Meletharayil et al., 2016). The high Ca$^{2+}$ ion activity may also contribute to casein insolubility during storage of high-protein milk powders (Gazi and Huppertz, 2015). The lower gelation pH values observed in samples prepared in an environment of high ionic strength (UF permeate) agrees with prior studies of acid gels made with

![Figure 4. Yield stress of stirred yogurts prepared from micellar casein isolate (MCI) or NDM measured initially after stirring and after undisturbed storage for 4 wk at ambient temperature. MCI was dispersed in water with 5% lactose (MCI-LL), water with 11% lactose (MCI-HL), or UF milk permeate (MCI-UF). The NDM was dispersed in water. Gels were fermented to pH 4.3 at 40°C, stirred at low speed with a blender for 30 s, cooled to ambient temperature, and measured for yield stress via a constant 0.01 s$^{-1}$ shear rate. The values shown are mean values (n = 2) with error bars representing 1 standard deviation of the mean.](image-url)
sodium caseinate (Lucey et al., 1997b; O’Kennedy et al., 2006), which reported delayed gelation at higher levels of added NaCl.

Gelation of tightly packed (concentrated) micellar casein dispersions has also been observed in liquid (not rehydrated) micellar casein fractions obtained by microfiltration and diafiltration once the dispersions are sufficiently concentrated and cooled to <~22°C (Amelia and Barbano, 2013; Lu et al., 2015). The cold gel that forms in these highly concentrated dispersions can occur near pH 7 and is different from acid milk gels in that it is thermally reversible upon warming. Lu et al. (2015) observed an increase in the possible temperature where these cold gels formed with increasing protein level, supporting the suggestion of the importance of close packing of protein particles. In a follow-up study, Lu et al. (2016) found that decreasing the pH of 12% protein micellar casein dispersed in recombined milk led to lower temperatures needed to form a cold gel. The close-packed cold gels that form at high pH values demonstrate the ability of casein particles to aggregate at pH values well above their pI under certain conditions; cold gelation has been attributed to the disruptions of steric hinderance due to the overlapping of packed casein particles (Lu et al., 2015). Our 8% protein MCI sample did not gel at 40°C, but presumably reducing the pH could have reduced their steric stability enough to allow aggregation.

The lack of charge screening and close packing of protein particles, which led to earlier acid gel formation, also likely explains the different G’ values observed at pH 4.6 for the 8% protein gels (Table 2). Large differences in the ionic strength of high-protein samples were demonstrated by the very low conductivity measurements of MCI dispersed in water (MCI-LL and MCI-HL) compared with MCI in UF permeate and NDM dispersed in water (Table 1). Similar to the trends observed for the gelation pH of high-protein gels, MCI showed extremes for gel stiffness (G’) at pH 4.6 that were highly dependent on the dispersant used. Heated milk is known to create stiffer yogurt gels due to the incorporation of denatured whey proteins into the gel matrix, so it was initially surprising that the stiffest yogurt gels were formed from whey protein-depleted milk. The earlier gel formation of MCI samples with low ionic strength (pH ~5.8–6.0) may have allowed for more time for protein-protein bonds to form, which may have influenced the final G’ value. The low ionic

Figure 5. Rebodying of stirred yogurt, as measured by storage modulus (G’) values, at ambient temperature initially after stirring (A and B) and after ambient temperature storage for 4 wk (C and D). Milks were prepared using either micellar casein isolate (MCI) or NDM, fermented at 40°C to pH 4.3, blended for 30 s, and cooled to ambient temperature for storage. Milk samples: ○ = MCI dispersed in low-lactose water (MCI-LL); ▽ = MCI dispersed in high-lactose water (MCI-HL); □ = MCI dispersed in UF permeate (MCI-UF); ◊ = NDM dispersed in water. The values shown are means of 2 replicates.
strength may also have contributed to additional protein-protein bonds, especially near the pI of casein (pH 4.6), as less protein charge shielding (by ions) would occur. The observation of lower dynamic moduli with higher levels of added salts was similar to what has been observed in unheated acid casein gels made with added NaCl (Roefs and van Vliet, 1990). Lucey et al. (1997b) also found a slowed rate of gel development for caseinate-based acid gels with added NaCl (120 mM).

Ionic strength was not the only parameter that affected set-style yogurt gel properties. Micellar casein isolate dispersed in UF permeate had a similar ionic composition to NDM in water, with the major difference being the presence of denatured whey proteins in NDM. Comparing the G’ values of our set-style yogurts at pH 4.6 for MCI dispersed in UF permeate and NDM in water, NDM formed considerably stiffer gels (Table 2)—likely a result of the presence of denatured whey proteins formed as a result of milk heat treatment (Lucey et al., 2022). The contribution of denatured whey proteins in NDM to the formation of a stiffer set-style yogurt gel was evident at both protein levels explored in this study.

An interesting feature was observed in the LT values for acidified milk gels made from heated milk (which form gels before the complete solubilization of CCP). This feature is the appearance of an increase in the LT value to a maximum peak around pH 5.1, referred to as the LTmax (Lucey and Horne, 2018). The LTmax observed in acid milk gels is attributed to earlier gelation due to the involvement of denatured whey proteins (pI around 5.3), but in our experiments an LTmax was also observed with whey protein-depleted milk dispersed in water (MCI-LL and MCI-HL; Figure 1). The MCI-UF yogurt samples did not exhibit an LTmax due to their later gel formation (pH 4.9), which occurred after CCP solubilization.

As was previously reported for unheated milk gels formed by a combination of rennet and acid (Lucey et al., 2000), any method that causes early onset of casein gelation before complete CCP solubilization during a fermentation process leads to an increase in the LT...

![Figure 6](image-url)
value. The LT value of the high-lactose MCI sample in water (MCI-HL), which formed a gel at pH ~6.0, exhibited a steady increase up to its maximum value at pH ~5.1 (Figure 1). The low-lactose MCI sample (MCI-LL), which formed a gel slightly later (pH ~5.7), exhibited a more rapid increase in the LT values to its maximum value. The different LTmax values observed between MCI-LL and MCI-HL (Table 2) could be a result of the higher osmotic pressure in the MCI samples with added lactose, or it could be a result of differences in acidification rates between samples.

The MCI-HL, MCI-UF, and NDM samples needed similar acidification times to reach pH 4.6 (Table 1), and therefore any differences in the LTmax for these samples were not due to differences in acidification rates. The lower LTmax values at both 3 and 8% protein for NDM yogurt, compared with MCI samples, indicated lower bond mobility in these gels when formed in the presence of denatured whey proteins. The LT values of all MCI samples, despite differences in the gelation pH and LTmax values, eventually approached approximately similar values by pH 4.6 (Figures 1 and 2). Throughout fermentation, NDM samples exhibited lower LT values, and thus a more elastic nature, compared with MCI samples. This more elastic gel nature observed in NDM yogurt is best explained by the presence of denatured whey proteins and the covalent, permanent bonds formed with casein.

Both confocal microscopy and permeability measurements (Figure 3) showed only small differences in the gel microstructures between MCI-based yogurts dispersed in either water or UF permeate. These small differences in their microstructures cannot explain the large differences observed in their rheological properties (Table 2). Rheological differences between the set-style yogurt gels made with MCI and prepared in different ionic environments were likely explained by altered local protein-protein interactions. Large pores in the microstructure observed in MCI samples allow for higher permeability and coarser gel networks (Roefs and van Vliet, 1990). The large pores observed in the microstructure for MCI yogurts (Figure 3a-c) are in agreement with their higher B-values (Figure 3e inset).

Stirred Yogurt

The yield stress values of stirred yogurt samples were measured with disposable rheology cups after undisturbed storage for up to 4 wk. These yield stress measurements were made directly from their storage containers by inserting a vane geometry—rather than traditional measurements, which involve the transfer of sample from a storage container to the rheometer cup or plate—and so provide an almost undisturbed view of the rheology of aged, stirred yogurt. Yield stress values (Figure 4) for stirred yogurt samples further suggested that ionic composition may also play an important role in the rebodying process. The G’ values of stirred samples (Figure 5) were measured using a more traditional cup-and-bob measurement system (wherein the stirred yogurt samples were shaken to fluidize the sample and allow for its transfer to a rheology cup). Both yield stress and G’ values showed differences in gel stiffness initially after stirring (1 d) as well as different rates of growth of the dynamic moduli during storage, which were influenced by the ionic environment. The MCI dispersed in water (MCI-LL and MCI-HL) had the lowest initial and stored (4-wk) yield stress and G’ values, indicating slower rearrangement of bonds in these samples made with lower concentrations of soluble salts. The yogurts made from MCI dispersed in UF permeate and NDM experienced much more rapid rebodying, likely promoted by the higher concentration of milk salts present.

The dG’/dt values (Figure 5b, d) also indicated faster rebodying in the yogurts made with MCI-UF and NDM, suggesting that much of the elastic character of
these gels that contributed to a resistance to flow (i.e., yield stress) likely reformed very soon after disruption of the gel network. It was not clear from this study whether differences in rebodying rates were a result of general ion interactions or perhaps specific ion interactions, such as those observed in the Hofmeister series or divalent ion (Ca\(^{2+}\)) bridging between protein aggregates. It is possible that Ca bridging occurs at yogurt pH values (~4.3–4.6), as so-called caseinate calcium remains bound to casein residues at pH values as low as pH ~3.5 (White and Davies, 1958; Le Graet and Brulé, 1993). It is possible that with higher levels of Ca, as with MCI-UF and NDM, more Ca could be involved in crosslinking or bridging neighboring casein strands together in stirred yogurt. The general ion interactions may also contribute to charge shielding and would be enhanced at higher salt levels, which would likely strengthen hydrophobic interactions. Regardless of whether Ca\(^{2+}\) bridging or hydrophobic interactions were responsible for the rebodying observed, all evaluated samples showed an increase in yield stress values over time. Samples with high ionic strength (MCI-UF and NDM) exhibited more rebodying (higher yield stress values), and the rebodying rate did not appear to be greatly affected by the amount of denatured whey proteins present in the system.

Because of the disruption to samples that can result from the stirring and dilution of yogurt that was necessary to load samples onto the particle size analyzer, particle size measurements of stirred yogurt should only be used to approximate the degree of aggregation of protein clusters in the gel, rather than try to represent the exact size of individual particles in the stirred, weak gel. Over time, an increase in protein aggregation was expected, as the low pH and ambient temperature continued to promote electrostatic and hydrophobic interactions between protein clusters. Unlike the large increase in yield stress values observed during storage, only a marginal increase in particle size occurred after aging for 4 wk for yogurts made from MCI dispersed in water, regardless of lactose levels (Figure 6a, b). Stirred yogurt prepared from NDM exhibited the largest initial D\([4,3]\) particle size, and the D\([4,3]\) values greatly increased at 4 wk, which highlighted the more dynamic protein aggregation process that was present after stirring for NDM samples. Much of the increase in particle size during storage involved clusters between 100 and 1,000 μm.

The greater propensity for rearrangements during storage for stirred yogurts made from NDM—as reflected in the large increases in particle size, yield stress, and G’ values—likely promoted the greater expulsion of serum (water) that would otherwise be trapped within the gel (Figure 7). This would agree with models for syneresis that suggest that endogenous syneresis pressure or rearrangements were partly responsible for the expulsion of whey from casein gels (van Vliet et al., 1997). As protein strands within the gel break and reform, the strands would assume a more energetically favorable conformation (i.e., shorter strands with decreased tensile stress), which causes the gel to contract, leading to an increase in endogenous pressure. This is noteworthy because confocal images and permeability measurements of set-style yogurt (Figure 3) indicated that NDM created the most crosslinked gels (i.e., low permeability and smooth or fine microstructure). However, once the initial gel microstructure was disrupted by stirring, the denatured whey proteins, which initially had helped to create a crosslinked and stable set-style gel, instead produced the least stable gel with high syneresis due to their strong propensity for bond reformation.

**CONCLUSIONS**

High-protein milk dispersions with low ionic strength (MCI-LL and MCI-HL) formed yogurt gels at high pH values (pH ~6.0) and were very stiff at pH 4.6. These whey protein-diminished gels exhibited much higher G’ values than those observed for heated NDM. Milk protein dispersions prepared at 3% protein were less impacted by alterations in the ionic environment, which suggested that charge shielding and close packing of protein particles were at least partly responsible for the different gel properties observed in high-protein acid milk gels rehydrated in various dispersants. Alteration of the ionic strength of MCI dispersions (or possibly other milk protein isolates that have been extensively dialyzed) could be used by manufacturers to manipulate the texture of high-protein yogurts. Stirred yogurts made with MCI and dispersed in water (low ionic strength) exhibited low yield stress and a slow rate of rebodying, both of which would be desirable for drinkable yogurt products.

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