Functionalised dairy streams: Tailoring protein functionality using sonication and heating

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Highlights

- Casein and whey protein functionality was modified by ultrasound and heat
- Heating of concentrated casein alone or in the presence of whey proteins, resulted in impaired gel formation
- Heating of whey protein in isolation prior to combining with casein, did not impair gelation
- Ultrasound could partially reverse the effects of heating less concentrated whey protein solutions

Abstract

Ultrasound can be used to modify the functional interactions between casein and whey proteins in dairy systems. This study reports on ongoing developments in understanding the effect of ultrasound and heating on milk proteins in systems with modified casein-whey protein ratios (97:3, 80:20 and 50:50), prepared from milk protein concentrates that were fractionated by microfiltration, based on protein size. Heating of concentrated casein streams (9% w/w) at 80 0 °C for up to 9 minutes resulted in reduced gelation functionality and increased viscosity, even in the absence of added whey proteins. 20 kHz ultrasonication at 20.8 W calorimetric power for 1 min was able to break protein aggregates formed during heating, resulting in improved gelation and reduced viscosity. Interestingly, when heated whey protein was recombined with unheated casein the gelation properties were similar to unheated controls. In contrast, when heat treated casein streams were recombined with unheated whey protein, the gel forming functionality was reduced. This study therefore shows that using specific combinations of heat and/or ultrasound, fractionated dairy streams can be tailored for specific functional outcomes.

1. Introduction

Bovine milk consists of water, fat, proteins, lactose, and a range of minerals [1]. The ability to selectively separate and fractionate these components from one another, enables more complete usage of the milk and the exploitation of the specific functional properties of the individual components [2]. For example, cross flow microfiltration can alter the protein composition of milk without affecting the native protein characteristics [3]. Microfiltration membranes with a pore size of ~ 0.1 μm, can separate casein micelles (CM) from the whey proteins (WP) in milk.

The use of ultrasound is of interest for a number of dairy applications, due to its capability to induce a range of useful effects. These include improving the heat stability of dairy proteins [4], emulsifying and homogenizing fats into dairy streams [5, 6], accelerating the solubilisation of milk protein concentrate powders [7], and improving shelf life by modulating enzyme and microbe activity [5, 8]. The primary mechanism of low-frequency high-power ultrasound (20-100 kHz) in dairy processing is an effect known as acoustic cavitation, which is the formation and collapse of bubble nuclei in fluids. The collapse of bubbles are high energy events that produce local temperature hotspots up to ~5000 °C and pressure shockwaves of several hundred atmospheres. The intense shear and temperature conditions on a localised scale produced by acoustic cavitation can partially unfold milk proteins, altering their functionality such as foaming capability [9] and heat stability [10].

Whey proteins denature at ≥ ~65°C, which can lead to protein aggregation through hydrophobic interactions and the formation of intermolecular disulfide bonds [11, 12]. This increases the viscosity or even cause gelation, which is generally undesirable during processing. Denatured whey protein can also attach to the surface of casein micelles [13], forming complexes that affect the functional properties of casein (CN) such as gelation [14, 15].

Ashokkumar et al. [4, 16] and Zisu et al.[17] outlined an approach to overcome heat instability of whey protein concentrates. The application of ultrasound for a very short duration after a pre-heating step, breaks down any formed aggregates, and prevents their reformation and any associated viscosity increase on subsequent heating. The authors attributed the observed viscosity reduction primarily to the physical forces generated during acoustic cavitation. Chandrapala et al. [10] found that the surface charge and reactive thiol groups remained unchanged by sonication applied in between a preheating and post-heating step. However, the surface hydrophobicity of these aggregates was decreased markedly. It was speculated that sonication broke down the protein aggregate networks through physical shear caused by acoustic cavitation, leading to the formation of smaller aggregates with lower surface hydrophobicity. These smaller aggregates are resistant to further aggregation during post-heating, thereby improving heat stability. Similarly, Kresic et al. [18] investigated the rheological and thermophysical properties of whey protein concentrate and isolate solutions subjected to sonication. Their results showed that ultrasound changed the flow behaviour, which was attributed to changes in the protein structure.

Although casein micelles are considered relatively heat stable entities, their composition and size respond to alterations in pH, temperature and milk protein concentration [19-21]. It is possible that the localized high temperatures and shear forces created by sonication can physically alter the casein micelles or their interactions with other milk components. Madadlou et al. [22] found that the average size of re-assembled casein micelles could be reduced by

exposure to 35 kHz ultrasound, (2 - 6.6 W for 6 h), between pH 6.35 and pH 11.4 while size reduction was significantly high when the pH was >8.0. It should be noted that the re-assembled casein particles were considerably larger (275 nm) and structurally and functionally different to native casein micelles [23]. In a study involving native casein micelles a decrease in particle size resulting from sonication was observed. This particle size decrease was attributed to a reduction in the casein micelle size, although this was not substantiated [14]. A recent study by Chandrapala et al. [24] showed that although sonication did not affect casein micelle size, its composition or the mineral balance in skim milk, it did reduce the size of the remaining fat globules. Further sonication helped break down the whey protein aggregates and non-micellar casein present in reconstituted casein powder systems. Zisu et al. [25] showed that ultrasound could reduce the viscosity of medium-heat treated skim milk concentrates containing 50-60% solids by approximately 10% for fresh material, and by >17% for highly viscous age-thickened material. Similarly, Yanjun et al. [26] found that ultrasound reduced the viscosity of reconstituted milk protein concentrates through the reduction in the size of the protein aggregates. These results show that controlled application of ultrasonic energy can help break up large casein and whey protein aggregates thereby influencing macroscopic properties such as viscosity, without inducing changes to the casein micelles or mineral balance.

Sonication has also been found to influence the gelation properties of milk streams. Recently, Liu et al. [27] reported accelerated rennet gelation in milk sonicated at pH 8.0 and re-adjusted back to pH 6.7 compared to milk sonicated at pH 6.7. Firmer gels were observed from milk sonicated at pH 6.7 compared to those made from non-sonicated control milk. Acid gel firmness (*G'*) was also altered when skim milk was ultrasonicated prior to acidification, although the effect was attributed largely to denaturation of whey protein caused simply by temperature increases (up to about 95 °C) resulting from sonication performed without temperature control [14]. Wu et al. [28] reported that high-intensity ultrasound (216, 540 and 1080 J/g electrical energy input, 20 kHz) not only effectively homogenized the fat present in milk but also significantly increased the resulting yogurt viscosity and water holding capacity and reduced the syneresis. These effects relate to the yoghurt structure, which is based on networks of interconnected casein micelles and denatured serum proteins that entrap serum and fat globules. Further, ultrasound can alter the fat globule membrane, increasing the interactions between the fat globules and casein micelles and the strength of the gel.

There is potential to manipulate the functionality of dairy streams using microfiltration to fractionate native casein and whey proteins, and then treating these streams by ultrasonication and heating to modify their properties. Previous studies have largely considered ultrasonication of whey proteins or casein in isolation, or in milk that naturally contains a fixed casein-whey protein ratio [7, 10, 24, 29-31]. Milk concentrate streams (defined here as a stream with >1.5X the natural protein concentration of milk ~ 3.5 % w/v) are economically important, often produced prior to spray drying [32] to reduce overall volumes to be processed, with potential to be used as a means to reduce transport costs [33] and/or to enhance the productivity of cheese making vats [34, 35]. Processing of concentrated milk streams also has potential to reduce the energy consumption and to produce more effective treatment outcomes [36]. This is particularly important for ultrasound processing, for which the effectiveness is localised near the emitters, and which therefore must be confined to relatively small volumes and geometries.

While studies have considered the effects of ultrasound on milk concentrates and reconstituted powders [10, 25, 26, 37], this study aims to expand upon existing knowledge by isolating the

effects on casein and whey proteins. In particular, the study aims to establish for the first time the specific effects of heating and ultrasonication on membrane filtered casein and whey protein concentrates (not subjected to pH adjustment or prolonged heat treatment) either in isolation or when combined together at different ratios, as a means to tailor the functionality of the resultant milk concentrate stream. To investigate this, concentrated milk streams with varied CN:WP ratios were subjected to various treatment conditions and assessed for key physical attributes, namely particle size, viscosity and rennet gelation capability.

2. Methods and materials

2.1 Milk protein solutions

All casein and whey protein solutions were made from reconstituted powders that were produced by microfiltration as previously described [38]. Whey solutions were prepared by dissolving 10% w/w of powder, which had a total protein content of 76.3% w/w (98.5% of which was whey protein), in Milli Q water at room temperature (~23 ^{0}C). This gave a whey protein concentration of 7.54 % w/w. Casein solutions were prepared by dissolving 9.% w/w of casein powder, which had a total protein content of 84.9% w/w (97.0% of which was casein protein), in Milli Q water. This gave a casein protein concentration of 7.40% w/w. To improve solubilisation of the casein powder, the solution was dissolved in Milli-Q water heated to 50 °C. Casein and whey protein solutions were stirred for at least 2 hours at 50 °C and room temperature respectively with a magnetic stirrer (~500 rpm), followed by overnight equilibration at 4 °C. To prevent microbial growth, sodium azide was added to the milk concentrates at a concentration of 0.04 wt %.

Unless otherwise specified, calcium chloride ($CaCl_2$) was added to all batches of reconstituted milks to achieve a total soluble calcium concentration of 7 mM. This value was based on a previous study by Pouliot, Boulet and Paquin that corresponds to the average soluble calcium content of the milks tested [39].

The prepared casein and whey protein solutions were used to formulate milk systems with CN:WP ratios of 97:3 (high purity casein), 80:20 (similar to skim milk) and 50:50, while keeping the total protein concentration constant at 7.5% w/w. The pH of all milk systems were measured to be between 6.6 and 6.8 post mixing.

2.2 Ultrasonication

A 20 kHz ultrasound unit (Branson 450-D digital sonifier) with an 11 mm horn was used for all sonication procedures. 80 mL samples of reconstituted milk were sonicated for 1, 2 or 3 minutes at 30% amplitude (which corresponded to 20.8 W calorimetric power delivery) in a water jacketed cell. Water equilibrated at room temperature was used for cooling, which enabled temperature during sonication to be maintained below 30 °C.

2.3 Heat treatment

Samples of reconstituted milk (20 mL) were placed in 50 mL centrifuge tubes, and immersed in a water bath (80 °C) for durations of 3, 6 and 9 minutes. These heating times resulted in a final milk temperature of 47 ± 1, 67 ± 1 and 73 ± 1 °C, respectively. The lowest temperature is below the denaturation temperature of whey protein. The higher temperatures correspond approximately to the denaturation temperature of some whey proteins (e.g. α-lactalbumin, BSA), and the temperature of milk pasteurisation at which the more heat resistant whey

proteins (e.g. β-lactoglobulin) also begin to denature. After heating, the samples were immediately removed and placed in a water bath at room temperature (22 ± 1 °C) for 7 minutes to cool.

Some samples were treated with a combined sequential heating/ultrasound protocol. For these samples, milk was either heated for 6 minutes followed by sonication for 1 minute, or sonicated for 1 minute then heated for 6 minutes. The shorthand notation used to describe the treatments are as follows: UN – untreated, S – Sonication, H – heating, SH – sonication 1 min followed by heating 6 min, HS – heating 6 min followed by sonication 1 min. Numbers following letters denote duration in minutes (i.e., S1 – sonication 1 minute).

2.4 Protein analysis

Protein analysis of samples was performed based on the Dumas Combustion method using a LECO Trumac NCS analyser (LECO Corporation, Michigan, USA) following a method that complies with the standard ISO 1489. Samples of milk (~ 2 g) or powder (~ 0.2 g) were weighed onto a nickel-lined ceramic boat and dried in an oven overnight (104 °C). The boats were then loaded into the LECO analyser and combusted at a temperature of 1100 °C within the furnace. Protein values were determined from the measured nitrogen percentage by multiplying by a conversion factor of 6.38 [40].

Total nitrogen, non-casein nitrogen and non-protein nitrogen were all assessed using this combustion method, to ascertain the proportional protein content of whey and casein in the milk streams. Briefly, the non-casein portions were obtained by acidifying milk samples to a pH of 4.6-4.7 at 40 °C, then centrifuging at 840 g for 10 minutes (Thermofisher Hereus Megafuge 8). Supernatants were collected and filtered through a 0.45 μm filter. Non-protein nitrogen samples were treated with an equivalent volume of 30 % trichloroacetic acid (TCA), mixed, then centrifuged at 840 g for 10 minutes. Supernatants were collected then filtered through a 0.45 μm filter.

2.5 Particle size measurement

Particle size was measured using a ZetaSizer Nano ZS (Malvern Instruments). Samples were diluted approximately 5000 fold using Milli Q water. A refractive index of 1.45 was used and the measurements were carried out at 25 ^{0}C. .

2.6 Rennet gelation kinetics

20 mL samples of milk were preheated to 31 °C prior to rennet addition (Chymax Plus, Cheeselinks) to reach a concentration of 0.035 IMCU/mL. Samples were mixed for 1 minute then loaded immediately into a cup and bob geometry (part no: 996284) of an AR-G2 Rheometer (TA Instruments, Newcastle, USA). The gelation was monitored using the rheometer for 1 hour at 31 ^{0}C using an oscillation time sweep procedure, with a constant frequency of 1 Hz and shear strain percentage of 2.5 %. The gelation kinetics were monitored from the storage, G', and loss, G'', modulus. The onset of gelation was arbitrarily defined as the time at which the storage modulus exceeded 0.2 Pa [27].

2.7 Viscosity measurement

0.1 mL samples were loaded into a cone and plate geometry (part no. 988134) of an AR-G2 rheometer (TA Instruments, Newcastle, USA). Samples were assessed using a flow procedure

where the shear rate was ramped from 0.1 to 200 s^{-1}. The temperature was kept constant at 31 °C.

2.8 Gel microstructure

Gels were prepared for microstructural analysis by warming 10 mL samples of treated milk to 31 °C, and adding rennet at a final concentration of 0.035 IMCU/mL and 2 drops each of Fast Green (2 mg/mL in water) (Sigma Aldrich) and Nile red (1 mg/ml in ethanol) (Sigma Aldrich) dyes. Samples were mixed for 1 minute then loaded into a concave well in a microscope slide (BRAND®, 76x26 mm, concavity 15-18 mm and depth of 0.6 – 0.8 mm) and covered with a cover slip. The microscope slides were incubated at 31 °C for 1 hour then removed and stored at room temperature until analysis. A confocal microscope (Leica SP5) was used to image the gels with the laser lines set at 486 and 633 nm for excitation. The fluorescence emission was captured through a 60X lens using 2 photo multiplier tubes set at 500-600 nm (Nile Red emission) and 655-755 nm (Fast Green emission). The separate images were overlayed to produce the gel cross-section images.

2.9 Calcium determination

Soluble and total calcium concentrations were analysed using an inductively coupled plasma (ICP) optical emission spectrometer (Varian 720-ES with an SPS 3 autosampler). Samples for soluble calcium measurement were first prepared by ultracentrifuging milk systems at 100 000 g using a Beckman Coulter Ultracentrifuge (Optima L-100 XP with type 70 Ti rotor) for 1 hour at 4 °C. The supernatant was collected and filtered using a 0.45 μm syringe filter. For both total and soluble calcium measurements, samples were acid precipitated with 100 % w/v TCA (1.2 mL TCA per 0.5 mL sample). Samples were then diluted to 10 mL with Milli Q water and centrifuged at 840 g for 5 minutes. The supernatant was collected and filtered through a 0.45 μm filter. 0.3 mL of this filtrate was diluted to 5.0 mL using Milli Q water. The diluted samples were analysed in the ICP at the following conditions; wavelength 315.887 nm, power 1 kW, plasma flow 15 L/min, auxiliary flow 1.5 L/min, nebulizer flow 0.75 L/min, pump rate 15 rpm.

2.10 Statistical analysis

All measurements were performed in triplicate unless otherwise stated. Where appropriate, the statistical significance of results was assessed with Minitab 18 using a Tukey t-test comparison (confidence interval of 95 %).

3.0 Results and discussion

3.1 Protein aggregate size

The effect of heating and sonication on protein aggregation in milk with different CN:WP ratios was investigated using blends of reconstituted casein micelle and whey protein powders obtained by MF at a total protein concentration of 7.5% w/v. In the untreated (UN) samples, there were typically two or three peaks that could be distinguished (Figure 1, blue distributions). The large particles (>1 μm) represent fat globules, the mid-range particles (100-300 nm) are casein micelles, and the smaller particles (<100 nm) are whey protein (aggregates). Note that there is natural variation in the size distribution of particles of the untreated samples across the different formulations being casein, 80:20 and 50:50 casein:whey ratio, due to the differing proportions of casein and whey in the samples.

Sonication had a relatively minor effect on the particle size distribution of high-purity casein (Figure 1A). With sonication, there was a decrease in the amount of signal from the fat globules and a corresponding increase in the casein micelle peak, with no other changes. This is consistent with a previous study by Chandrapala et al. [24] that showed sonication caused minimal changes to actual casein micelles, but reduced the size of the fat droplets. Heating of the high-purity casein, and application of ultrasound before or after heating, resulted in observable shifts of the casein micelle peaks to larger sized particles (Figure 1 B and C). The distributions of those samples subject to both heating and sonication (Figure 1 C) also became somewhat broader compared to the samples only subjected to sonication (Figure 1 A), presumably resulting from some degree of aggregation. As casein proteins are heat stable, the likely cause of any aggregate formation is the presence of whey protein, which can denature upon heating and bridge casein micelles [13]. As there was only a small amount of whey protein in the high-purity casein system, the changes observed with heating were relatively minor.

With a greater amount of whey protein (80:20 and 50:50 solutions), there was a slight shift towards smaller particles following sonication. In contrast, heat treatment increased the particle size due to the formation of aggregates formed via the denaturation of whey protein. The combined sonication/heat treatments were performed to ascertain if sonication could control the undesirable formation of protein aggregates. With a casein:whey ratio of 80:20, both sonication prior to and post heating, appeared to be effective in reducing/preventing formation of larger aggregates (Figure 1 F). When the whey fraction was increased to a ratio of 50:50, the order in which ultrasound was applied became important (Figure 1 H). When ultrasound was applied post-heating, large aggregates that were formed by the heating step appeared to be broken down to a similar size range to that in the original sample. However, when sonication was used prior to heating, there was a considerable increase in aggregates size upon heating (Figure 1 I), similar to when heating was performed without prior sonication (Figure 1H). This is an interesting result, that builds on the findings of a previous study on whey proteins (in the absence of casein micelles) that showed sonication of heat-treated whey proteins improved the stability upon subsequent additional heat treatment [4].

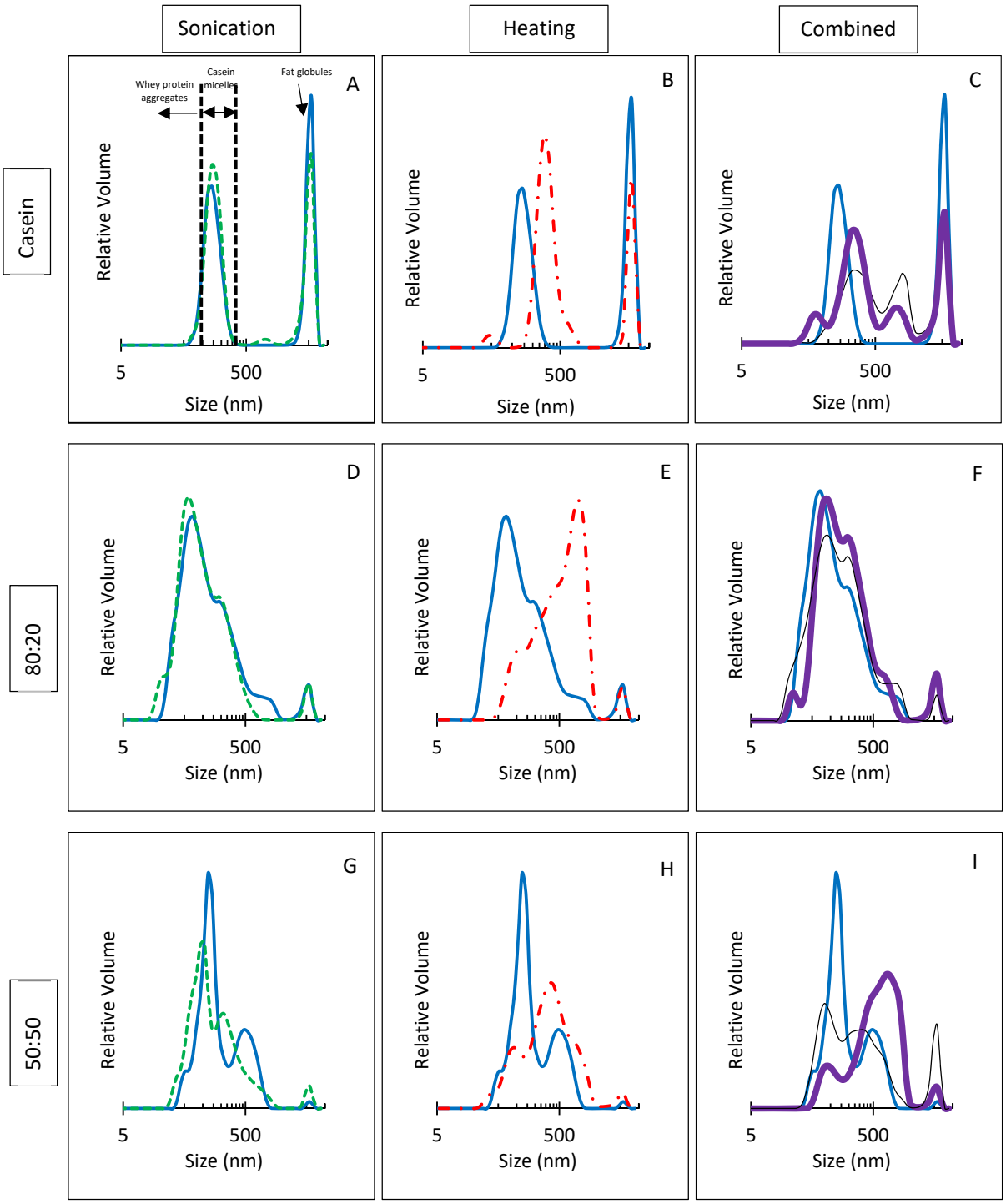
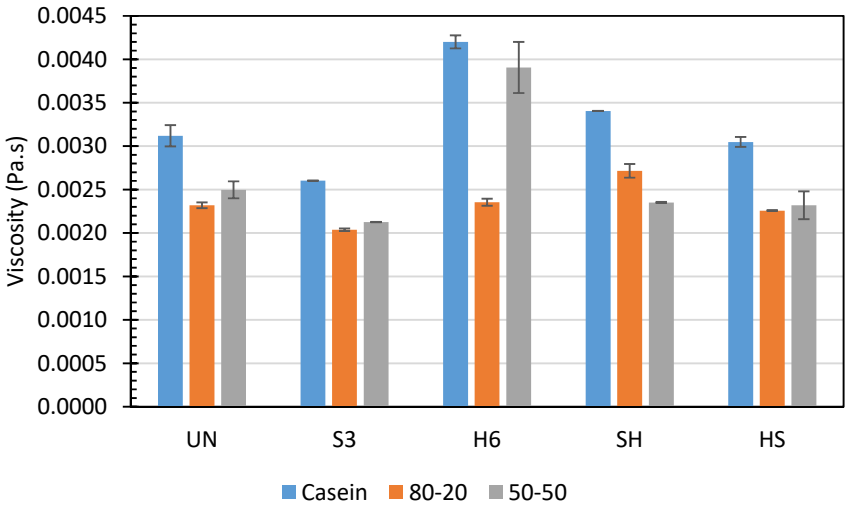


Figure 1: Size distribution (volume weighted average) of the milk concentrate streams subjected to selected treatments. The formulations 80:20 and 50:50 represent the casein:whey ratio. The colored lines represent: blue solid – untreated (UN), green dashed – sonicated for 3 min (S3), red dotted and dashed – heat treated for 9 min (H9), purple thick – sonicated for 1 min then heated treated for 6 min (SH), black thin – heat treated for 6 min then sonicated for 1 min (HS).

3.2 Viscosity

The viscosity of the different milk systems following ultrasonic and heat treatments was measured (Figure 2 A and B). Due to the high protein content, these milk streams were shear-thinning (Supplementary Information Figure S1). The apparent viscosity is reported here at a shear rate of ~50 s^{-1}, which provides a good indication of perceived thickness in foods [41], and at a temperature of 31 °C. For all sample types, the use of sonication alone reduced the viscosity relative to the untreated samples. This is largely consistent with the observed declines in the particle size distributions, and can be explained by the sonication reducing the amount of casein-casein, casein-whey and whey-whey protein aggregates present. The breakage of these aggregates reduces the strength of the interactions in the milk, thereby lowering the viscosity, as has been previously reported [17].

Contrarily, the use of heating, reported here for a fixed duration of 6 or 9 minutes (Figure 2 A and B), generally increased the viscosity. Streams heated for 9 minutes, resulted in orders of magnitude increase in viscosity relative to the untreated controls and trended with increasing proportion of whey protein as expected. Interestingly after 6 minutes of heat treatment, the high-purity casein and 50:50 streams, increased the viscosity more relative to the controls than heat treatment of the 80:20 stream. The 80:20 and 50:50 streams are expected to be less heat stable due to increasing presence of whey proteins that are heat sensitive. It is unclear why the high-purity casein stream had a higher viscosity after 6 min heating than the 80:20 stream. It may be due to the high concentration of casein resulting in a higher probability of casein-casein interactions leading to heat-induced thickening. As noted above, heating of the casein stream did result in an increase to the particle size distribution (Figure 1 B). Visually, the high concentration of casein present in the higher purity streams were ‘thicker’ after heating. It should also be noted that the viscosity of the pure casein streams was higher relative to the 80:20 and 50:50 streams prior to any treatment, due to casein micelles having a higher voluminosity than native whey proteins.



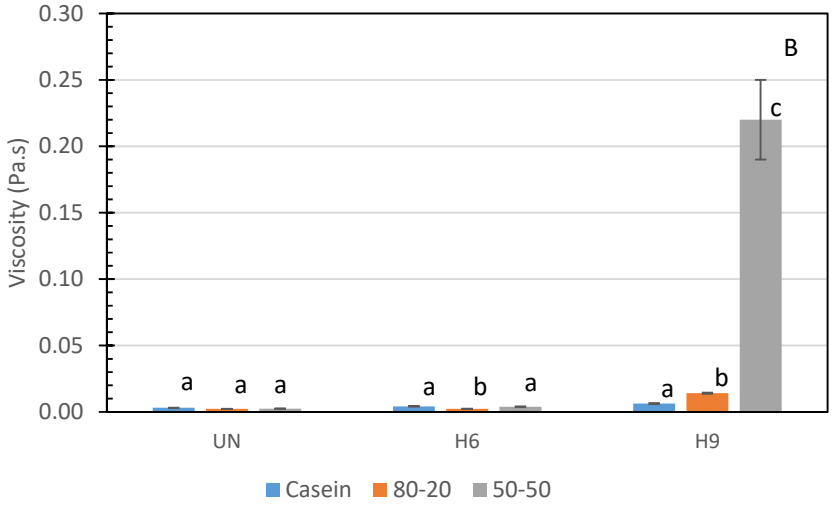


Figure 2: Viscosity of milk concentrate streams subject to selected treatments. For clarity, (A) compares all conditions except H9 and (B) compares H9 to UN and H6. The apparent viscosity is reported here for a shear rate of 50 s^{-1} and a temperature of 31 °C. Statistically significantly different results ($p<0.05$) for different milk systems within each treatment condition are denoted by different letters a, b and c. UN = untreated, S3 = sonication 3 min, H6 = heating 6 min, H9 = heating 9 min, SH = sonication 1 min followed by heating 6 min, HS = heating 6 min followed by sonication 1 min.

The use of combined heat and sonication treatments resulted in generally expected results. Firstly, the use of sonication post-heating tended to produce streams with a lower viscosity than those to which sonication was applied pre-heating. This is consistent with the particle size distribution obtained for the 50:50 streams, whereby aggregates formed upon heating were broken up by ultrasound. The breakage of heat-formed aggregates will tend to reduce the viscosity. Apart from the 80:20 stream, the combined treatments resulted in a lower viscosity than heating alone. For the 80:20 case, the application of sonication prior to heating resulted in a higher viscosity relative than heat alone. This is inconsistent with the other results, but as observed in the size distributions, sonication prior to heating has the potential to cause the formation of larger aggregates, which may be the reason for this apparent anomaly.

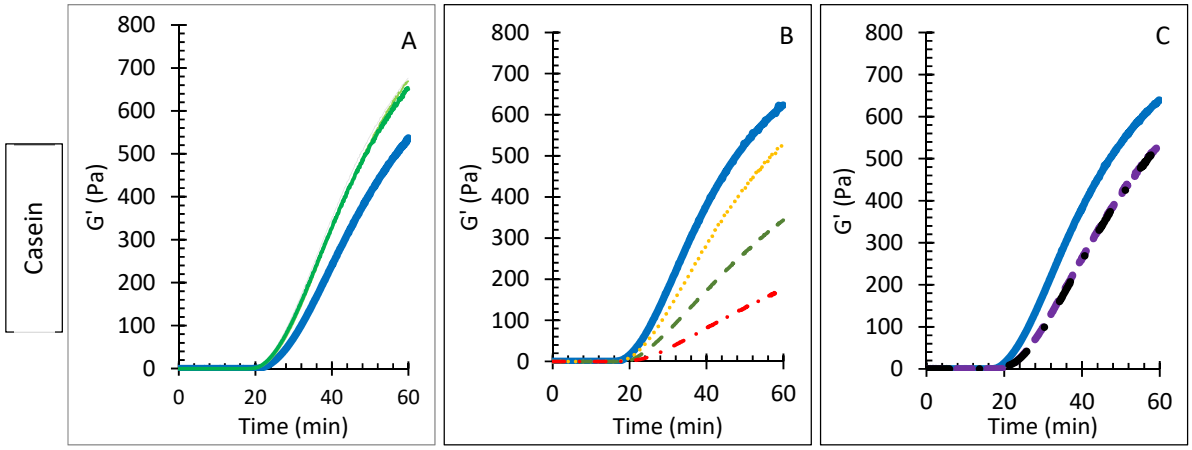
3.3 Gelation

One motivation for using membrane filtration to increase the protein and casein content of milk, is to accelerate rennet-induced gelation during cheese production, thereby improving productivity. The chymosin enzyme in rennet destabilizes the casein micelle by cleaving its hydrophilic κ-casein 'hairy' layer, producing a gel. Increasing the casein concentration makes the rennet gelation process more rapid [42, 43], while increasing the whey protein concentration has an inhibitory effect [38].

While it has been shown that sonication can improve the rate of gelation kinetics under selected conditions [27, 44], the effect of sonication is shown here to be highly dependent on the proportion of whey protein present in the milk stream. In the case of the high purity casein and 80:20 streams, sonication for up to 3 minutes increased the rate of gelation (Table 1) and gel firmness at 60 minutes (Figure 3 A, D).

Table 1: Rate of gelation of milk streams subject to different treatments. The rate is estimated from the slope of the storage modulus as a function of time, after the onset of gelation has occurred.

	Rate of gelation (Pa/s)		
	Casein	**80:20**	**50:50**
UN (Sonicated samples)	0.26	0.14	0.05
S1	0.35	0.16	0.06
S2	0.34	0.19	0.06
S3	0.33	0.17	0.06
UN (Heated samples)	0.34	0.18	0.05
H3	0.25	0.14	0.04
H6	0.15	0.11	0.02
H9	0.07	0.017	0.004
UN (Combined samples)	0.34	0.18	0.05
SH	0.26	0.08	0.01
HS	0.26	0.10	0.01



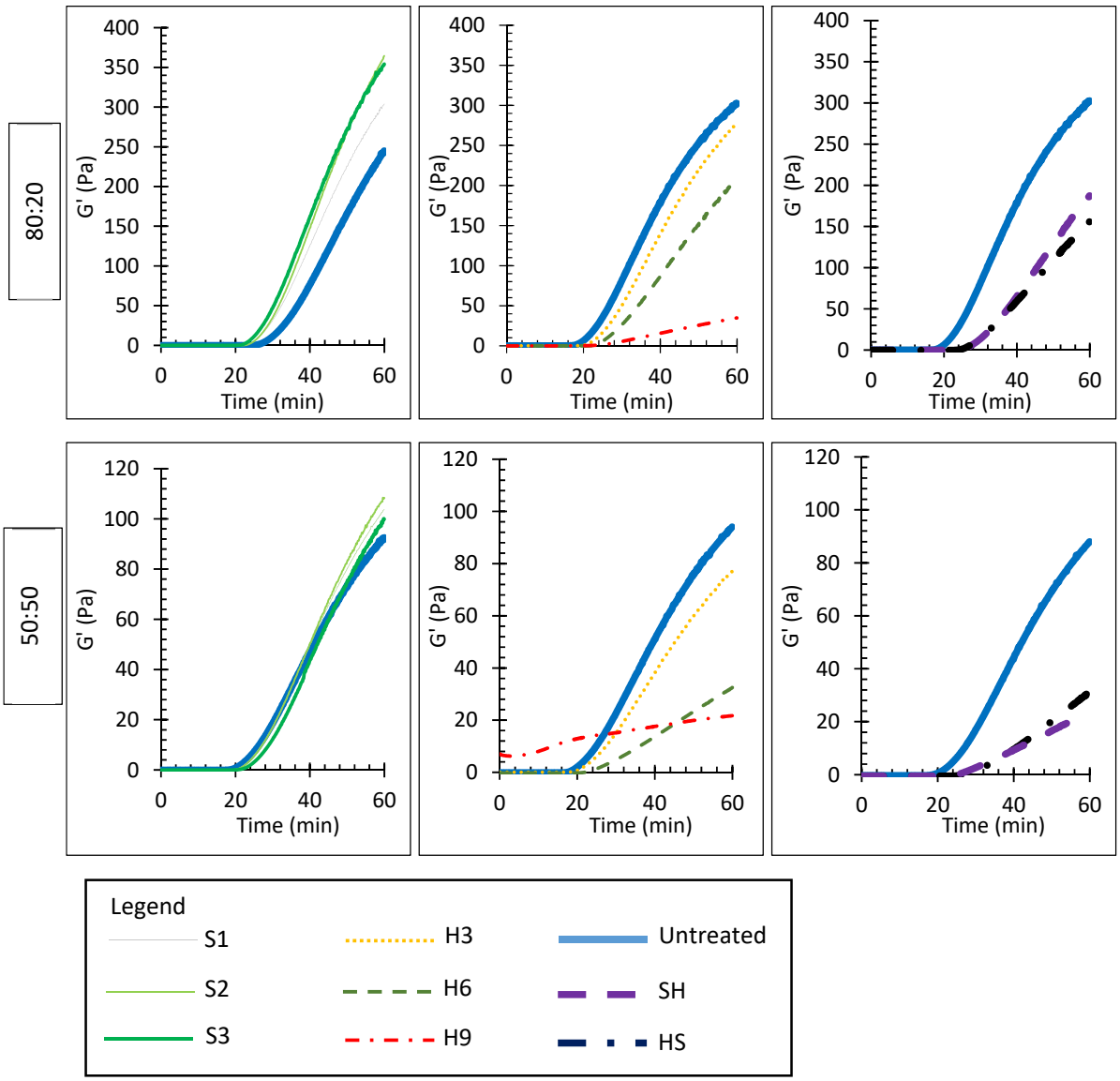
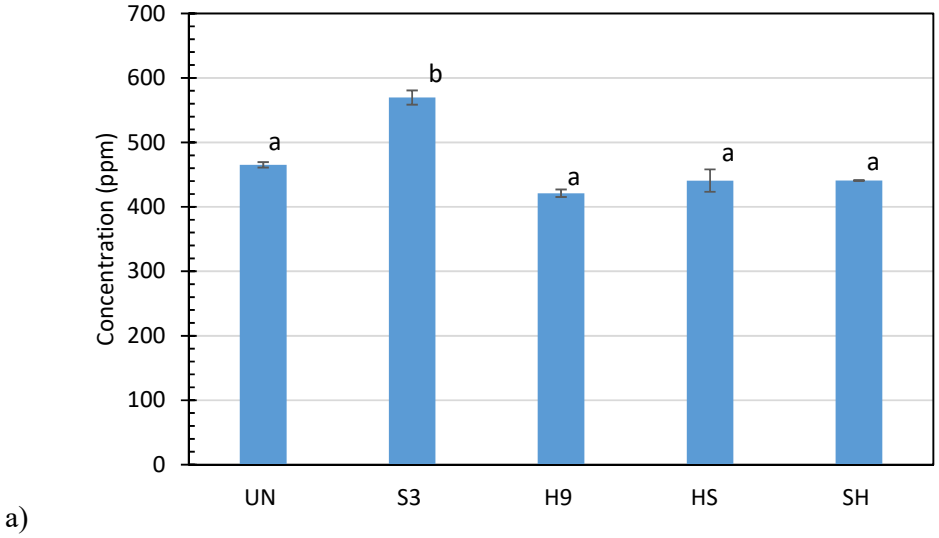
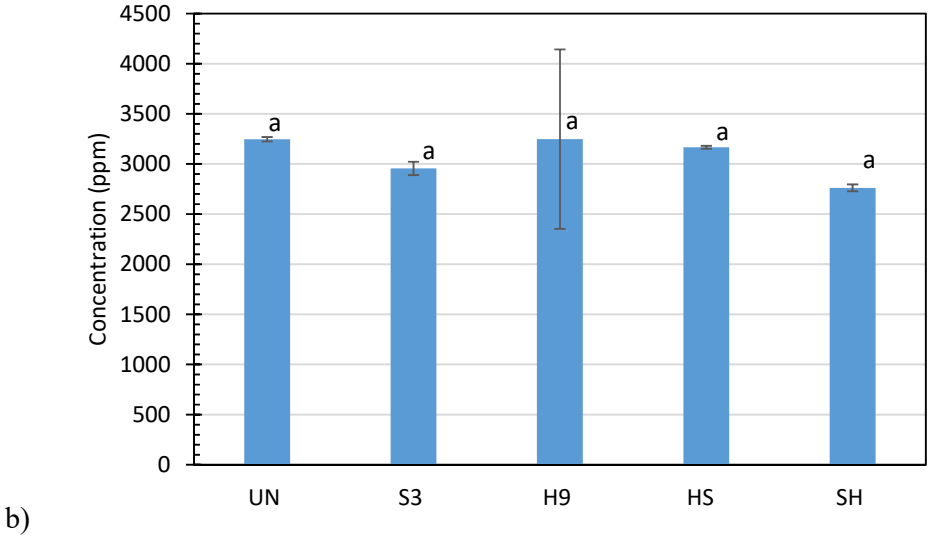


Figure 3: Rennet gelation kinetics in terms of storage module (G') as a function of time, for different milk concentrate streams subject to sonication, heat and combined treatments.

In all cases, heating delayed and slowed down the rennet-induced gel formation (Figure 3 B, E, H). In the presence of whey proteins, heat induced association of whey proteins onto the surface of casein micelles [13, 45] impedes the action of chymosin. The low concentration of whey protein in the high-purity casein stream suggests that there should be another explanation for the observed reduction in gelation rate following heating. One possible explanation could be heat induced changes to the mineral balance of the milk system, in particular a reduction in the soluble calcium [46] that is critical for rapid gelation. However, the soluble and total calcium of the pure casein streams were not significantly different after heating (Figure 4).



a)



b)

Figure 4: Concentration of a) soluble calcium and b) total calcium of the treated high-purity casein samples. Different letters represent significantly different results.

Another possible explanation for the reduced gelation rate after heating is a possible heat-induced thickening of the high-purity casein stream, resultant from the formation of casein-casein aggregates. Such an increase in viscosity could impair the action of chymosin and/or aggregation of the renneted casein micelles. As shown in Figure 2, the viscosity of the high-purity casein stream was indeed increased upon heating. The combined treatments of sonication and heating reduced the viscosity of the high-purity casein stream relative to the heated sample and this is consistent with enabling an increase of the gelation kinetics. There appears to be no specificity to the order of the combined treatments to enhance the gelation rate of the high-purity casein stream. This shows that sonication can partially reverse heat-induced increases in the viscosity of high-purity casein streams, both before and after heating, thereby increasing the rennet gelation rate.

In the 50:50 streams, the large amount of whey protein appeared to form a gel upon extended heating (e.g. 9 min treatment), as indicated by the measurable storage modulus at the initiation of the rennet gelation (Figure 3 H). This changed the kinetics of the rennet gelation and appeared to prevent it from going to completion. With whey present (80:20, 50:50) the

combined treatments were not able to restore the gelation kinetics back to those of the unheated samples. This suggests that the association of heat-denatured whey proteins onto the casein micelles could not be reversed by the shear forces resulting from ultrasound. Interestingly, sonication prior to heating slightly impaired the gelation of the 50:50 stream (Figure 3 I), which is consistent with the observed increase in viscosity (Figure 2) and particle size (Figure 1 I). This could be explained by sonication breaking apart larger casein-casein and whey-whey aggregates that enable increased whey-casein interactions upon subsequent heating.

Confocal scanning microscopy images of the rennet gels (Figure 5) were taken to further investigate the observed trends. The gels formed from the high-purity casein streams were much denser than those from the 50:50 streams due to the higher proportion of casein. The sonicated casein stream produced a gel that is visibly denser than the untreated casein sample, consistent with the observed increase in the rennet gelation kinetics. Visually, there appears to have been minimal effect of sonication on the microstructure of the 50:50 gel.

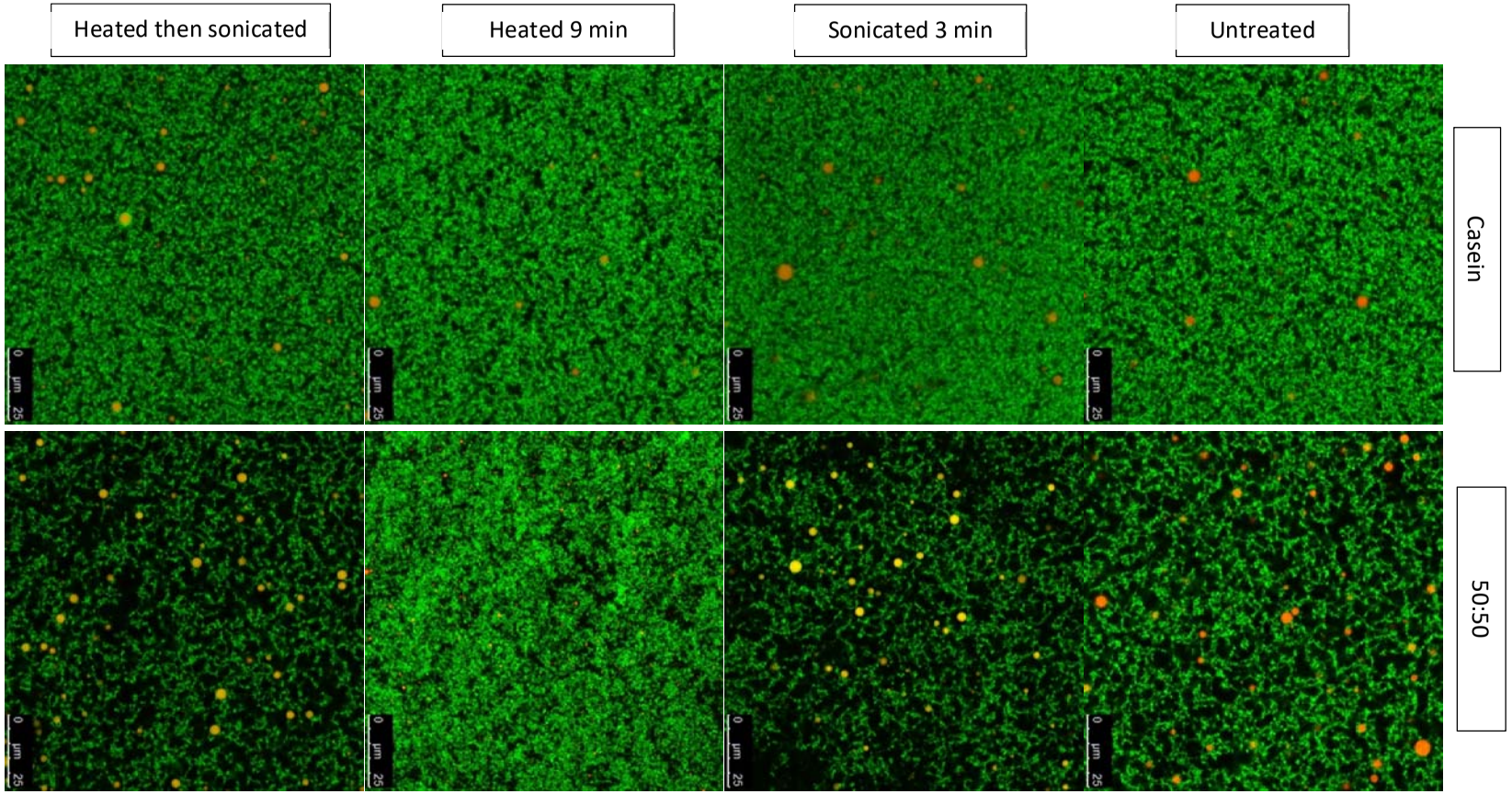
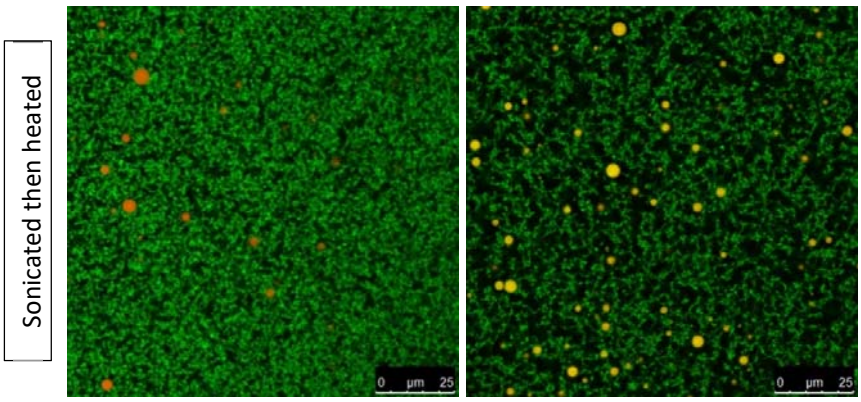


Figure 5: Confocal scanning micrographs for casein and 50:50 milk concentrate streams. The orange/red coloured circles within the matrix correspond to fat droplets dyed with Nile red.



Heating of the high-purity casein stream appears to have had minimal effect (despite reduced rate of gelation) on the gel structure. However, heating of the 50:50 stream resulted in gel with a denser network. This network is likely formed by heat-induced whey protein denaturation, which is consistent with the observed increase in viscosity upon heating. The combined heat and sonication treatments of high-purity casein produced gels with visually similar microstructures to the controls. For the 50:50 system, the combined treatments resulted in gels that were less dense. This was presumably due to inhibited rennet action resulting from the attachment of denatured whey protein onto the casein micelles. Sonication of these streams was unable to return the system to the same state as that of the untreated samples.

3.4 Separate heat and ultrasonic treatment of casein or whey protein

The above results provide insight into how to improve processing of concentrated milk streams, for example, by making them less prone to the heat-induced deterioration of rennet gelation capability. It is clear that the presence of whey proteins in milk leads to reduction in functionality of rennet gelation if subjected to heat treatment in the presence of casein. As microfiltration enables splitting of the casein and whey protein fractions into separate streams, it is possible to heat-treat and/or sonicate these proteins separately to potentially preserve key functionality. Here, the gelation of combined casein and whey proteins streams (blended at a 50:50 protein mass ratio), one of which had been separately treated by heat and/or ultrasound, were compared.

From Figure 6, it is clear that the heat treatment of casein prior to combination with untreated whey protein reduced the gelation functionality much more than the heat treatment of whey protein combined with untreated casein. The microstructure of gels produced from these concentrate streams (Figure 7) were consistent with the gelation behaviour. These results indicate that the creation of casein aggregates upon heating leads to an impairment of the rennet gelation process. These casein aggregates can be partially disrupted with sonication. The application of sonication to the heated casein stream in the absence of whey, partially recovered the gelation rate (Table 2), but it was still much slower than the untreated sample (Figure 6 and Table 2). This behaviour is distinct from that shown in Figure 3, in which sonication is unable to recover the rennet ability of sample in which whey proteins have been attached onto the casein during heat treatment. In these experiments sonication was applied for 3 mins and it may be possible that extended sonication would be able to recover this gelation ability further.

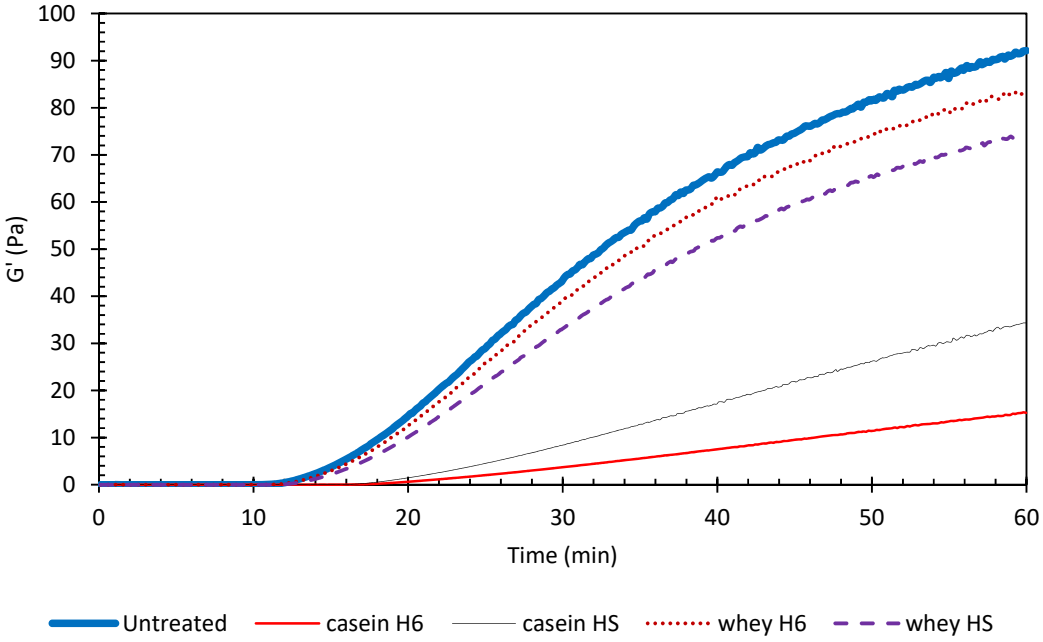


Figure 6: Storage modulus as a function of time during rennet gelation of separately treated casein and whey protein streams, recombined at a 50:50 ratio after one of the streams had been subjected to heat and/or ultrasonic treatment. Untreated = untreated whey protein and untreated casein. Casein H6 = casein heated for 6 min and untreated whey protein. Casein HS = casein heated for 6 min then sonicated for 3 min and untreated whey protein. Whey H6 = whey protein heated for 6 min and untreated casein. Whey HS = whey protein heated for 6 min then sonicated for 3 min and untreated casein.

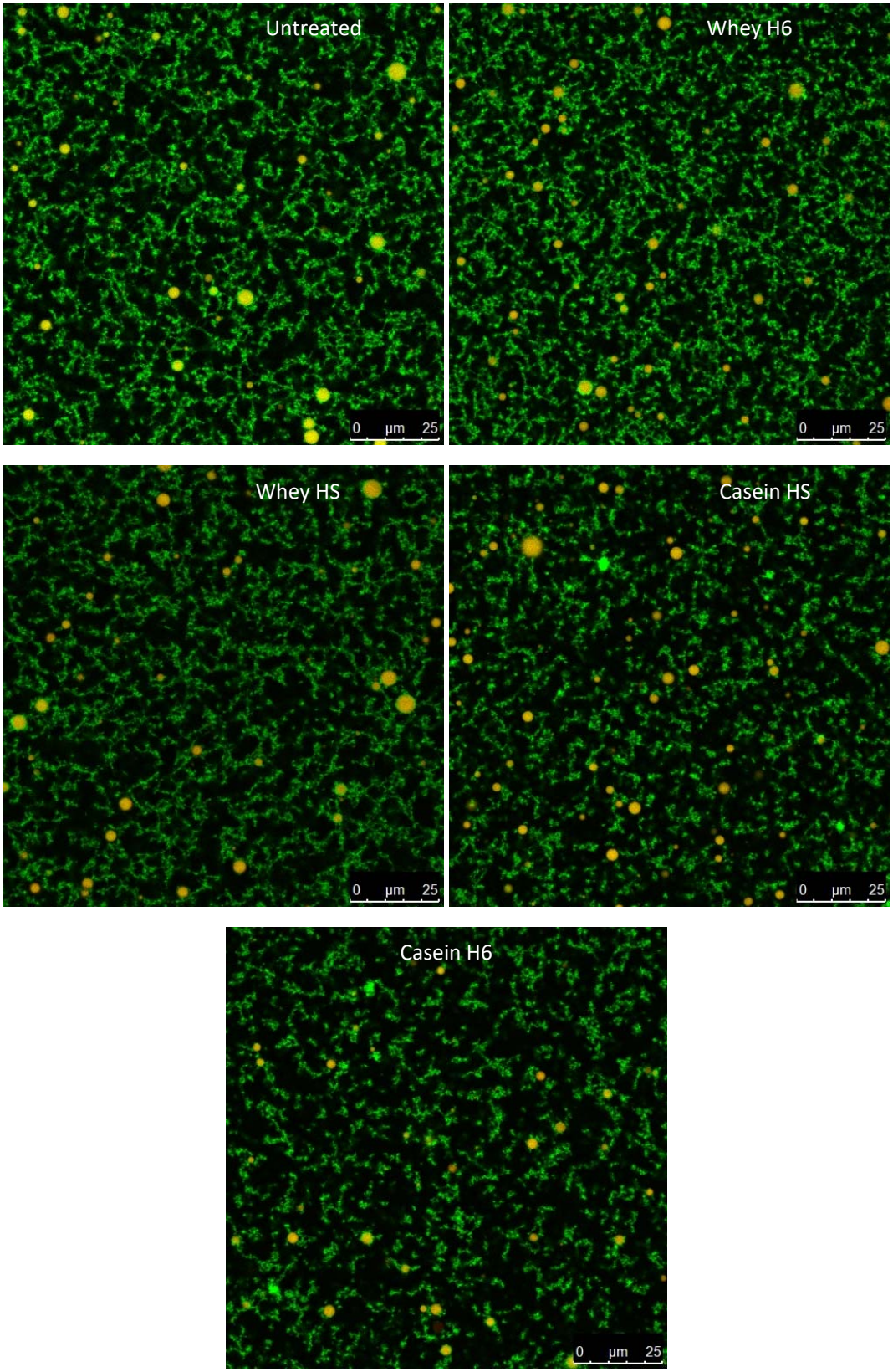


Figure 7: Confocal scanning micrographs of gels formed from milk streams subject to selected treatments. Whey H6 refers to whey protein heat treated for 6 minutes separately to the casein. Whey HS refers to whey protein heat treated for 6 minutes followed by 1 minute of sonication separately to casein. Casein HS refers to casein protein heat treated for 6 minutes followed by 1 minute of sonication separately to whey proteins. Casein H6 refers to casein protein heat

treated for 6 minutes separately to whey protein. The treated streams were recombined with untreated whey protein or casein respectively to form a 50:50 casein:whey ratio mixture prior to rennet gelation. The orange/red coloured circles within the matrix correspond to fat droplets dyed with Nile red.

Table 2: Rate of gelation of milk stream subject to separate treatment of the casein and whey protein streams by heat and/or ultrasonic treatment, prior to recombination to a 50:50 ratio. The rate is estimated from the slope of the storage modulus as a function of time after gel initiation has occurred.

	Rate of gelation (Pa/s)
Untreated	0.03
Casein H6	0.007
Casein HS	0.02
Whey H6	0.03
Whey HS	0.03

Interestingly, when the whey protein was heat treated separately from the casein, the rennet ability of the combined milk stream was very similar to the control that had undergone no treatment. When the heated whey protein was also subjected to sonication (after heating and prior to combination with casein), there was however a reduction in the rennet ability. This result suggests that the size of the whey-whey aggregates formed by heating play an important role in the rennet-induced gel formation. As reported by Gamlath et al. [38], native whey proteins have an inhibitory effect on rennet gelation. The larger whey-whey aggregates formed by heating are presumably less able to block access of the rennet to the κ-casein cleavage sites, thereby impairing rennet gelation less than whey-whey aggregates that have been reduced in size by ultrasound. There may be potential to further modify the rennet ability of separate whey/casein streams using more intensive heat treatment and subsequent US processing.

Conclusion

Sonication has been used to modify and manipulate the functional interactions between casein and whey proteins in concentrated dairy streams. Heating of milk results in a potentially undesirable decline in the gelation rate and an increased viscosity that have implications to productivity. Sonication alone can significantly improve the rennet gelation of casein rich streams while combined heat and sonication treatments can reduce the size of heat induced aggregates and thereby the increase in viscosity of both casein and whey protein rich streams. Further, heat treating whey protein alone before mixing with casein can mitigate the impaired rennet gelation that results when both proteins are heated together. Therefore using select strategies to avoid heating concentrated milk streams containing both casein and whey proteins together, it is possible to mitigate some of these changes, or even promote them to produce streams that have unique functional characteristics.

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