Characterization of the physical, microbiological, and chemical properties of sonicated raw bovine milk

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ABSTRACT

Innovative processing technologies, such as ultrasonication, can change the properties of milk, allowing for the improvement or development of dairy foods. Yet taking bench-scale equipment to pilot plant scale has been challenging. Raw milk, standardized to 3% fat and warmed to inlet temperatures of 42 or 54°C, was exposed to continuous, high-intensity, low-frequency ultrasonication (16/20 kHz, 1.36 kW/pass) at flow rates of 0.15, 0.30, and 0.45 L/min that resulted in resident times within the reaction cell of 6, 3, and 2 min per pass, respectively. Multiple passes (3, 5, and 7, respectively) were required to obtain a total exposure time of 14 to 18 min. Evaluation of fat droplet sizes, enzyme coagulation properties, and microstructure of milk and milk gels, as well as determining compositional and lipid properties, were conducted to determine the potential of the ultrasound system to effectively modify milk. Laser scanning particle sizing and confocal microscopy showed that the largest droplets ($2.26 \pm 0.13 \mu\text{m}$) found in raw milk were selectively reduced in size with a concomitant increase in the number of submicron droplets ($0.37 \pm 0.06 \mu\text{m}$), which occurred sooner when exposed to shorter bursts of ultrasonication (0.45 L/min flow rates) and at an inlet temperature of 54°C. Ultrasound processing with milk entering at 42°C resulted in faster gelling times and firmer curds at 30 min; however, extended processing at inlet temperature of 54°C reduced curd firmness and lengthened coagulation time. This showed that ultrasonication altered protein-protein and protein-lipid interactions, thus the strength of the enzyme-set curds. Scanning electron microscopy revealed a denser curd matrix with less continuous and more irregular shaped and clustered strands, whereas transmission electron microscopy showed submicron lipid droplets embedded

within the protein strands of the curd matrix. Processing at inlet temperature of 54°C with flow rates of 0.30 and 0.45 L/min also reduced the total aerobic bacterial count by more than 1 log cfu/mL, and the number of psychrophiles below the limit of detection (10 cfu/mL) for this study. Ultrasonication exposures of 14 to 18 min had minimal effect on the milk composition, fatty acid profiles, and lipid heat capacity and enthalpy. The findings show that this continuous ultrasound system, which is conducive to commercial scale-up, modifies the physical and functional properties of milk under the parameters used in this study and has potential use in dairy processing.

Key words: raw milk, ultra-sonication, fat droplet size, coagulation, homogenization

INTRODUCTION

Innovative nonthermal processing technologies, such as ultrasonication (US), alter the properties of milk, allowing for the production of novel-textured, high-moisture dairy foods. Application of US in dairy processing is a relatively young field of study. Processing at high frequencies (>1,000 kHz), a nondestructive range of US, has been shown to separate milk fat into fractions containing different-sized droplets (Leong et al., 2014, 2016) and improve the creaming properties of recombined milk (Juliano et al., 2011). Studies using low frequencies (20 to 40 kHz) have reported that US improved the degassing of skim milk (Villamiel et al., 2000), the heat stability of whey proteins (Ashokkumar et al., 2010), the solubility of concentrated milk (Zisu et al., 2013; Yanjun et al., 2014), and ice cream production and quality (Mortazavi and Tabatabaie, 2008). Ultrasonication was also reported to be effective in reducing bacterial populations (Feng et al., 2008; Drakopoulou et al., 2009), including pathogens and spoilage bacteria in milk (Villamiel and De Jong, 2000b; Cameron et al., 2009; Chouliara et al., 2010).

The majority of food processing applications have been conducted between frequencies of 20 to 1,000 kHz, a range where micro bubbles form, pulse, expand (usually in the <100 μm range), and collapse within

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microseconds. The strongest acoustic cavitation effects are created at low frequencies (20 to 40 kHz) and high intensity (10 to 1,000 W) and result in microenvironments of high temperatures and pressures, turbulence, and shear throughout a liquid sample that permanently alters the physical and chemical properties of the food (Ashokkumar et al., 2010; Gallego-Juárez and Graff, 2015; Zisu and Chandrapala, 2015). As frequencies increase, the bubbles collapse sooner and with smaller radii so the cavitation effect is less.

The homogenizing property of US is well established, although comparisons among the array of different systems and processing conditions can be difficult. With sufficient power (usually 20 to 24 kHz and 50 W to 1 kW) and exposure time (10 to 120 min), US can reduce the wide range of lipid droplet sizes found in raw milk to a narrow band of submicron-sized droplets (Wu et al., 2001; Shanmugam et al., 2012). Thermosonication, which is US conducted at higher temperatures (usually above 55°C for dairy processing), improved the efficiency of the US treatments by shortening the time for these changes to occur (Ertugay et al., 2004; Riener et al., 2009, 2010). Ultrasonication of milk also improved its coagulation properties by decreasing the time to onset of gelation and increasing the curd firmness in whole (Marchesini et al., 2012) and skim milk (Nguyen and Anema, 2010; Liu et al., 2014). These findings demonstrate that US affected both the lipids and proteins found in milk. Ultrasonication has been reported to improve the water-holding capacity, increase curd firmness, and decrease syneresis in yogurt (Wu et al., 2001; Riener et al., 2009, 2010; Zisu et al., 2011).

The majority of studies using US technology to homogenize milk and milk products have been conducted at the bench-scale using US transducers submerged in small volumes of liquid. Larger, continuous US systems operating at 20 or 35 kHz, 150 W to 4 kW, and flow rates 0.01 to 6 L/min have reduced lipid droplets to submicron sizes while successfully inactivating milk enzymes (Villamiel and De Jong, 2000a), decreased the viscosity of whey protein and calcium caseinate solutions (Zisu et al., 2010), and reduced bacterial counts (Crudo et al., 2014). More research is needed on large-scale, continuous US systems as new concepts are incorporated into their design.

In this study, a continuous US system with dual-frequency transducers in a parallel plate configuration was employed. The system is an indirect system that does not allow direct contact between the milk and the US transducers, efficiently chills the reaction chamber to prevent heat exposure, and results in shorter processing times to achieve the desired US exposure treatments. The objectives of this study are to characterize the ef-

fects of continuous US technology, in combination with various inlet temperatures, flow rates, and total exposure times, on the chemical, physical, and chymosin-coagulation properties of raw milk and determine its suitability for altering the properties of milk.

MATERIALS AND METHODS

Processing

Milk. Raw milk (75 kg/shipment) was obtained from a local dairy farm. A portion of the farm milk was skimmed (F15 Disc Bowl centrifuge; Armfield Inc., Clarksburg, NJ) and used to dilute the farm milk to 3.0% fat. The standardized milk was stored in 5-L cans at 1.8°C until undergoing US processing. To optimize personnel and equipment usage, US was conducted on the 2nd and 3rd day; 2 US treatments were conducted per milk shipment. Before processing, a 1-L sample was removed from the can and used as a nonprocessed control.

Ultrasound Processing. The assembled system (Figure 1) consisted of a HTST/UHT heat exchanger/pump (FT74XTS, Armfield Limited, Ringwood, UK) that was used to heat and control the flow rate of milk to the US system. This was followed by a continuous US unit (DRC-8-DPP-FGS, Advanced Sonic Processing Systems, Oxford, CT) with a 2.4 kW dual-frequency reactor cell (parallel diaphragm plates) with 2 low-frequency (16 and 20 kHz, nominal 1,200 W each) transducers housed within a cooling jacket. The transducers were configured to eliminate standing waves, set at a maximum acoustic intensity of 100 W/cm³, and delivered 1.36 kW per pass. The acoustic cavity or reactor was cylindrical in shape and contained the 2 diaphragm stainless steel plates (diameter of 20.3 cm), separated by a 3.2-cm spacer. The reactor volume given by the manufacturer is 0.865 L. Milk entered the reaction cell through 20.3 cm of a 0.75" (1.9 cm) sanitary stainless steel pipe in the chilled transducer section, then entered the reactor through the side of the bottom of the 20-kHz transducer plate, flowed through the space between the plates, out the top of the second plate, and then through the 16-kHz chilled section, through a pipe with the same dimensions as the inlet pipe, and exited the system (flowing directly into the small-mouth collection container). Circulating water at 10°C was used to cool the transducers via the cooling jacket. Milk was warmed using the Armfield unit. After preliminary trials, the inlet temperatures of 42 or 54°C were chosen, a range in which milk entered the US chamber just above the melting point of milk fat (>40°C) or just below the temperature generally considered the start for thermo-

sonication ($>55^{\circ}\text{C}$). Although temperatures increased within the reaction cell during US, the cooling jacket reduced samples to exit temperatures ranging from 20 to 30°C . Milk was pumped into the US system at 3 different flow rates; the slowest (**s**) was at 0.15 L/min, the medium (**m**) was at 0.30 L/min; and the fastest (**f**) was at 0.45 L/min. The residence time (**t**) for a single pass through the reactor is given by $t = \text{volume of the reactor}/\text{flow rate}$. For the 3 flow rates above, the values of **t** are 6, 3, and 2 min, respectively. Several passes were used to obtain total residence times within the US reaction cell of around 15 min. The **s** flow rate required 3 passes and resulted in **t** of 6, 12, and 18 min. The **m** flow rate needed 5 passes for **t** of 3, 6, 9, 12, and 15 min. The **f** flow rate required 7 passes to obtain **t** of 2, 4, 6, 8, 10, 12, and 14 min. Following each pass through the US system, milk was collected and stored on ice before reheating to the desired inlet temperature. Samples for analyses were collected from each pass, exit temperatures were recorded, and samples to be assayed were immediately refrigerated at 1.8°C . Individual samples were identified according to inlet temperature ($^{\circ}\text{C}$), flow rate (**s**, **m**, or **f**), and total US residence time (min). For example, milk that entered the US chamber at 42°C at the slowest flow rate, and underwent 2 passes would be identified as 42s12 ($^{\circ}\text{C}$, flow rate, min).

Physical Properties

Fat Droplet Size. Particle size was measured in triplicate for all milk samples 24 h after US processing using a laser scanning particle size distribution analyzer (Partica LA-950V2, Horiba Instruments, Kyoto, Japan). The volume-weighted ($d[4,3]$) and area-weighted ($d[3,2]$) droplet sizes were calculated using a refractive index of 1.38.

Milk Imaging. Confocal microscopy was conducted as described by Tunick et al. (2016). Briefly, fresh samples (0.18 mL) were fixed with 0.02 mL of 25% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA), stained with 0.01% Fast Green FCF (protein) and 0.01% Nile Red (lipids), and examined using a confocal laser scanning microscope (TCS SPE, Leica Microsystems, Buffalo Grove, IL) at $100\times$ magnification.

Coagulation Assays. The viscoelastic properties of coagulating milk were tracked using a small amplitude oscillatory shear analyzer (Kinexus Lab Plus Rheometer, Malvern Instruments, Westborough, MA) fitted with a cup-and-bob attachment (PC25C019AL and C25 SW1399 SS, respectively). A strain sweep was conducted on 17.0 mL of milk at 25°C to determine the linear range. Enzyme coagulation properties were determined as described by Bucci et al. (2018). An ali-

Figure 1. Diagram of ultrasonication system.

quot (17.0 mL) of sample was warmed to 35°C in the cup before the addition of a 0.395-mL chymosin solution (2.00 g of Chy-Max in 100-mL water; Chr. Hansen, Milwaukee, WI). The bob was lowered into place, and time sweeps were conducted in duplicate at 35°C , 10 Hz, and 0.015% strain. Coagulation time (when elastic modulus, G' , began to rise) and curd firmness (G' at 30 min after enzyme addition) were recorded.

Milk Gel Imaging. Milk gel imaging was performed within 12 h of coagulation assays. Milk samples (10 mL) were warmed to 35°C before addition of the chymosin solution (0.1 mL); swirled gently and capped with parafilm; and incubated at 35°C for 30 min. A portion of the gel was immediately submerged in 2.5% glutaraldehyde for 30 min, cut into 3- to 5-mm³ pieces, and stored at 4°C until processed for scanning electron microscopy and transmission electron microscopy (TEM). Samples were washed in 0.01 M imidazole (Electron Microscopy Sciences), dehydrated through series of washes with increasing ethanol concentrations, critical point dried (Denton Vacuum, Cherry Hill, NJ), and mounted on stubs and gold-coated (150R ES, Electron Microscopy Sciences) for scanning electron microscopy (FEI Quanta 200 F SEM, Hillsboro, OR) imaging. Samples for TEM were washed in imidazole, stained with 1% osmium tetroxide (Electron Microscopy Sciences), dehydrated in ethanol washes, rinsed with propylene oxide (Electron Microscopy Sciences), infiltrated with an increasing series of resin (LX-112, Ladd Research, Williston, VT), and cured overnight in a vacuum oven at 60°C . Thin sections were prepared using a diamond knife (Diatome, Hatfield, PA) and then stained in uranyl acetate, then lead citrate (Electron Microscopy Sciences) for TEM (CM 12, Philips, Amsterdam, the Netherlands) imaging.

Microbiological and Chemical Properties

Microbiology. Milk samples were collected aseptically into sterile 15-mL tubes at the US outlet port and stored at 4°C for up to 3 h before plating. Serial dilutions were prepared using 0.1% peptone water, with 0.1 mL of each dilution dispersed in duplicate on plate count agar (Oxoid Limited, Basingstoke, UK). Total aerobic counts were determined following incubation at 32°C for 48 h, and psychrophilic counts were determined after incubation at 4 to 6°C for 10 d. Bacterial counts were reported as the mean log₁₀ colony-forming units per milliliter ($\pm$ standard deviation) from a minimum of 2 independent processing runs.

Milk Composition. The moisture, fat, protein, and lactose contents of milk were measured in triplicate at the time of US processing, using a mid-range infrared milk analyzer (Milkoscan Minor 6, FOSS North America Ltd., Chicago, IL; method 972.16; AOAC International, 2012). All samples were tested in duplicate for alkaline phosphatase activity (PAS-Lite, Charm Sci. Inc., Lawrence, MA).

Fat Properties. Fresh, non-processed control milk and milk US treated at the maximum 18-min exposure were centrifuged (Advanti J-301, Beckman Coulter, Indianapolis, IN) at 4°C for 30 min at $10,000 \times g$ and the top fat layer was collected. Fatty acid profiles were obtained using a modified FAME method based on Tunick and Van Hekken (2017). Briefly, triglycerides from approximately 23 mg of milk fat were dissolved in hexanes (Fisher Scientific, Fair Lawn, NJ) and converted to methyl esters using sodium methoxide (Sigma Aldrich, St. Louis, MO); water was removed using CaCl₂ (Fisher Scientific). Particles were removed by centrifugation ($700 \times g$ for 3 min at 22°C), and triplicate 1- μ L samples in hexane were analyzed by a gas chromatograph (Trace 1300, Thermo Fisher Scientific, Waltham, MA) fitted with a CP-Sil 88 column (100 m $\times$ 0.25; Agilent Technologies, Wilmington, DE) and a flame ionization detector set at 270°C. The Chromeleon Chromatography Data System (Dionex, Thermo Fisher Scientific) was used for instrument operation and data analysis. Separation began at 80°C and increased by 4°C/min. Following a 5 min hold at 220°C, the temperature was further increased by 4°C/min to 240°C, and held for 10 min. Peaks calculated as percentage of fatty acids were identified using references for standard carbon 4 to 24 methyl esters and conjugated methyl linoleate (448 and 678; Nu-Chek Prep, Elysian, MN).

Differential Scanning Calorimetry. Melt properties of US milk fat were evaluated in triplicate using the methodology described by Tunick and Malin (1997). Briefly, 12-mg samples of thawed milk fat were

warmed to 90°C for 2 h and stored in a desiccator at 22°C. Melt profiles of each sample were collected using a differential scanning calorimetry (DSC) unit (DSC Q100, TA Instruments, New Castle, DE) that tempered the sample at 50°C for 5 min, then lowered it by 5°C/min to -50°C to equilibrate for 5 min, and finally raised the temperature by 5°C/min to 50°C. Instrument software (Universal Analysis 2000, version 4.7A; TA Instruments) was used to integrate profiles and calculate temperature and enthalpy of melt.

Statistics

Processing runs were executed over a 10-wk period with 1 or 2 random processing treatments conducted each week; all treatments were performed in triplicate. Data analysis for particle size, coagulation time, curd firmness, composition, fatty acid profiles, and melt properties were conducted using ANOVA; a PROC MIXED statement (Version 9.4 SAS-STAT, SAS Institute Inc., Cary, NC). The inlet temperature and accumulated exposure time were the fixed variables and replication of the treatments (rep) was the random factor. The Bonferroni least significant difference test was used to determine significant differences ($P < 0.05$).

RESULTS AND DISCUSSION

Processing

Ultrasonication processing details are summarized in Table 1. Flow rates were reduced to maximize US exposure times per pass. Although processing runs lasted about 2 h to obtain 14 to 18 min of actual US exposure; for most of that 2-h period, the samples were stored on ice waiting for the next pass. Actual processing time can be reduced, and yield increased by placing multiple continuous US units in series but was out of the scope of this study. Another benefit to a continuous system is that the transducers are never in direct contact with the milk, thus improving food safety and the ability to clean in place.

Because the transducers are surrounded by cooling medium, the surface of the diaphragm plates remained at or near the temperature of the medium. This was measured indirectly by determining the increase in temperature of the cooling water that exited the jacket with or without fluid in the reaction cell. A nominal increase to the exiting cooling water temperature of no more than 2°C was observed without fluid in the reaction cell, with no additional increase detected when milk flowed through the system at any of the 3 flow rates tested. However, a significant net cooling effect on

Table 1. Processing details for ultrasonication of raw milk including circulation information and temperatures (means and SD) entering (inlet) and exiting after the last pass (outlet) the ultrasonication system¹

		Flow rate		
Processing property		0.15 L/min	0.30 L/min	0.45 L/min
Circulation details				
Exposure time per pass (min)		6	3	2
Passes conducted		3	5	7
Passage through system (min)		9.4 ± 1.6	5.6 ± 0.5	4.1 ± 0.3
Total processing (min)		133 ± 27.4	129 ± 7.5	124 ± 20.9
		Inlet	Outlet	
Temperature (°C)		42	19.5 ± 2.3	21.9 ± 2.0
		54	24.1 ± 0.8	28.3 ± 0.8
				24.8 ± 1.3
				31.4 ± 0.4

¹Treatments were conducted at 2 inlet temperatures and 3 flow rates.

the milk passing through the reaction cell was observed, decreasing the temperature by 16.4 to 29.1°C linearly with increasing flow rate. Samples exiting the system were below 40°C, a temperature where most fats in milk are not in the liquid state and not as susceptible to US penetration (Earnshaw et al., 1995).

Although US is considered a nonthermal process, Nguyen and Anema (2010) reported that without any control, temperature increased from 20 to >80°C within 5 min for 18 mL of skim milk when ultrasonicated at 22.5 kHz and 50 W, well above pasteurization conditions and into the range of whey protein denaturation. Although thermosonication has a synergistic effect, temperature control is essential to manage the desired US outcome. The use of water at 10°C to cool the US reaction cell demonstrated the efficiency of the system to mitigate any heat generated within the reaction cell, and adjustments to the temperature of this cooling water should establish any US conditions required.

Information on continuous US systems is still very limited. Villamiel and De Jong (2000a) reported that a continuous system centered on a transducer in direct contact with the sample operating at 20 kHz and 150 W with flow rates of 0.011 to 0.028 L/min inactivated milk enzymes after 102 s exposure at 75°C. Zisu et al. (2010) reported that a continuous system using a horn operating at 20 kHz and 1 to 4 kW with flow rates of 0.2 to 6.0 L/min decreased the viscosity of whey and casein ingredient solutions and improved the coagulation traits. Crudo et al. (2014) reported that a continuous system operating at 35 kHz and 370 W (1194 W/L) with a flow rate of 0.17 L/min reduced bacterial counts by 95% after a 10 min exposure at 37°C. Our continuous system with a 2.4 kW reactor cell, delivering 1.36 kW per pass, and a parallel plate configuration of 2 transducers (16/20 kHz), compares well with these systems although further optimization of flow rates, ex-

posure times, and temperatures are required to obtain a homogeneous submicron emulsion.

Physical Properties

Fat Droplet Properties. As expected, the typical wide distribution of fat droplet sizes in raw nonhomogenized 3% milk was altered when exposed to US treatments at different temperatures and increasing exposure times. The d[4,3] profiles (Figure 2), which were volume-weighted and more sensitive to larger particles, showed a single, broad-based peak with a mode diameter of $2.26 \pm 0.13 \mu\text{m}$ for the raw milk controls. Ultrasonication processing of milk with inlet temperatures at 42°C (Figure 2a, 2c, 2e) resulted in a narrower peak, with decreased distribution, as the averaged diameter shifted slightly to 2.10, 2.08, and 1.91 μm for the slowest, medium, and fastest flow rates, respectively. A second narrow peak with a mode diameter of $0.37 \pm 0.06 \mu\text{m}$ appeared after total US exposures of 18, 12, and 8 min for the slowest, medium, and fastest flow rates, respectively. Overall, results showed that shorter exposure times with more passes led to greater reductions in particle size. When milk with the inlet temperature of 54°C was ultrasonicated (Figure 2b, 2d, 2f), mode diameters decreased by 0.28, 0.54, and 0.64 μm after exposures of 18, 15, and 14 min at the slowest, medium, and fastest flow rates, respectively, with only slight decreases in distribution. The submicron peak also appeared earlier, after 12, 9, and 6 min at the slowest, medium, and fastest flow rates, respectively.

The d[3,2] profiles (Figure 3), which were area (surface)-weighted and more sensitive to smaller particles, reflected changes in the distribution of the smaller fat droplets. Only slight differences ($<0.02 \mu\text{m}$) in the main peak diameters were noted when milk at 42°C inlet temperature was ultra-sonicated (Figure 3a, 3c,

Figure 2. Comparison of volume-weighted particle sizes ($d[4,3]$) of fat droplets in raw milk controls (con; blue) and milk ultrasonicated at different inlet temperatures and flow rates. Columns, inlet temperatures of milk: left column (a, c, e), 42°C; and right column (b, d, f), 54°C. Rows, flow rates: top row (a, b), 0.15 L/min; middle row (c, d), 0.30 L/min; and bottom row (e, f), 0.45 L/min. To obtain a total ultrasonication exposure of 18, 15, or 14 min, samples underwent multiple passes; 3, 5, or 7 for flow rates of 0.15, 0.30, or 0.45 L/min, respectively; passes 1, orange; 2, gray; 3, yellow; 4, dark blue; 5, green; 6, purple; and 7, red.

Figure 3. Comparison of area-weighted particle sizes ($d[3,2]$) of fat droplets in raw milk controls (con; blue) and milk ultrasonicated at different inlet temperatures and flow rates. Columns, inlet temperatures of milk: left column (a, c, e), 42°C; and right column (b, d, f), 54°C. Rows, flow rates: top row (a, b), 0.15 L/min; middle row (c, d), 0.30 L/min; and bottom row (e, f), 0.45 L/min. To obtain a total ultrasonication exposure of 18, 15, or 14 min, samples underwent multiple passes; 3, 5, or 7 for flow rates of 0.15, 0.30, or 0.45 L/min, respectively; passes 1, orange; 2, gray; 3, yellow; 4, dark blue; 5, green; 6, purple; and 7, red.

and 3e) up to exposures of 12, 9, and 6 min for the slowest, medium, and fastest flow rates, respectively, and at 54°C (Figure 3b, 3d, and 3f) up to exposures of 6, 6, and 4 min, respectively. Once the submicron peak appeared, the distribution of the larger peaks decreased from 15 to 18% down to 5 to 8%, with an exception of 42m12 (inlet temperature of 42°C, medium flow rate, and total US exposure time of 12 min). The larger peak did not disappear completely (remained around 5%) but the diameter continued to decrease. When milk at 42°C inlet temperature was processed, the submicron peak was not present after 12 min at the slowest flow rate but was present by 8 min at the fastest flow rate. At 54°C, the submicron peak appeared between 4 and 6 min at the fastest flow rate.

The reduction of the larger peak with a concomitant formation of a narrower submicron peak was similar to that reported in other US studies. Wu et al. (2001) obtained this dual peak stage in a 150-mL sample at 15°C after ultrasonication for 10 min at 20 kHz and 90 W using a 13-mm US horn. Zisu and Chandrapala (2015) required 30 min of ultrasonication at 20 kHz and 50 W/min to reach this point. Our continuous system accomplished similar results in 14 to 18 min using 16/20 kHz and 1.36 kW per pass.

Confocal images visually supported the particle size changes noted above (Figure 4 illustrates the changes in fat droplet sizes in milk with an inlet temperature at 42°C). Control milk (Figure 4a, 4e, and 4i) was dominated by large fat droplets (red or yellow) with

Figure 4. Confocal images of raw milk controls and milk at inlet temperature of 54°C ultrasonicated at different flow rates and exposure times. Rows, flow rates: top row (a, b, c, d), 0.15 L/min; middle row (e, f, g, h), 0.30 L/min; and bottom row (i, j, k, l), 0.45 L/min. Column, exposure time: first column (a, e, i), controls; second column (b, f, j), pass before submicron peak appears; third column (c, g, k), pass when submicron peak appears; and fourth column (d, h, l), last pass with maximum exposure time. Scale bar represents 10 μ m. Lipids are stained red and yellow, and protein is stained green.

a background of protein (stained green). During US processing, the large fat droplets became less frequent as they were fractured into smaller droplets. In the pass before the appearance of the submicron peak, milk samples still contained many large or clustered fat droplets (Figure 4b, 4f, and 4j). After the submicron peak appeared, smaller droplets were plentiful among the medium-sized droplets with larger droplets appearing to be clusters of medium-sized droplets (Figure 4c, 4g, and 4k). After 14 min of US exposure (Figure 4d, 4h, and 4l), the submicron fat droplets dominated, although medium and smaller particles were still present. Confocal images of US milk with abundant submicron-sized fat droplets (Figure 4c, 4d, 4g, 4h, 4k, and 4l) were similar to those of 3% fat milk homogenized using a 2-stage homogenizer reported by Bucci et al. (2018).

Milk inlet temperature influenced the effectiveness of the US processing. Samples entering the sonication chamber at 42°C had larger reductions in diameters and the submicron peak appeared earlier than in samples at inlet temperatures of 54°C. A similar trend was also noted for flow rate as samples with the fastest flows (shorter bursts of US exposures) had greater reductions in particle diameters. Several studies have shown thermosonication was more effective in homogenizing fat droplets than US alone at lower temperatures (Ertugay et al., 2004; Riener et al., 2009, 2010). The efficient cooling within the US unit lowered the temperature of the milk to below the relatively small range of temperatures near 40°C, where the milk fat triglycerides transition between liquid and crystalline states. The heat generated within the reaction cell should have raised temperatures somewhat, but it is unknown if it ever exceeded 40°C. Samples that took 6 min to pass through the reaction cell exited the US unit 5 to 7°C cooler than samples taking 2 min per pass (Table 1).

Findings from this study suggested that shorter bursts and multiple passes were a more efficient process. The d[4.3] data also indicated that a minimum US exposure was needed before submicron droplets accumulated enough to be detected. Conditions used in this study had not reduced the fat droplet size below 0.3 μm , which may reflect a size stability for fat droplets. Although a homogeneous submicron particle size was not reached with the US conditions we tested, the selective nature of acting on the largest of the droplets gives insight into the potential finer control of the microstructure of the cheese matrix in future research.

Unlike microfluidization, another nonthermal processing technique, which creates homogeneous submicron fat droplets in the very first pass through the unit (Bucci et al., 2018), US causes gradual reduction in particle size. Many studies have obtained this homo-

geneous submicron emulsion but did so using batch conditions with a transducer tip submerged in the milk for long periods of time. Further testing will determine optimum processing conditions for generating that result in custom-made sized fat droplets for novelty dairy products.

Coagulation Properties. Rennet coagulation of 3% raw milk was altered by US processing (Figure 5). Ultrasonicated milk that had inlet temperatures of 42°C gelled faster and formed firmer curds than the raw milk control or milk that had inlet temperatures of 54°C. The 42°C inlet temperature US-treated milk showed decreased ($P < 0.05$) coagulation times (Figure 5a) and increased curd firmness (Figure 5b) within their first pass, which did not change after repeated US exposure. The samples that had inlet temperatures of 54°C and the medium or fastest flow rates also decreased in coagulation time and increased in curd firmness during their first pass, but not to the extent observed for milk that had inlet temperatures of 42°C. After the second pass, curd firmness decreased, and coagulation times lengthened, appearing similar to non-processed control milk after 14 min of exposure time. Milk entering the US chamber at 54°C at the slowest flow rate, increased ($P < 0.05$) in coagulation time, and decreased in curd firmness ($P < 0.05$) as exposure time progressed from 0 to 18 min. All samples showed improved coagulation time at the onset of US, well before a reduction in lipid droplet size was measured. Chymosin-set gels depend on the hydrolysis of the bond between Phe 105 and Met 106 in κ -casein and a slight decrease in pH to destabilize the casein micelles before they begin to coalesce (Lucey, 2004). Examination of the control curds microstructure using scanning electron microscopy (Figure 6a and 6e) illustrated the typical matrix made of long continuous protein strands that entrapped large lipid droplets (now void spaces). After 15 min of US exposure, the protein strands are closer together and less continuous. As found with the particle size data, milk receiving 6-min US exposures (Figure 6b and 6f) was most similar to the controls, whereas samples exposed to 2- and 3-min bursts of US had denser curd matrices (Figure 6d and 6h). Examination of protein strand microstructure using scanning electron microscopy (Figure 7) showed the differences between the raw milk controls (Figure 7a and 7e) and samples after 15 min of US exposure (Figure 7b, 7c, 7d, 7f, 7g, and 7h). Ultrasonicated milk gels showed that smaller lipid droplets acquired a distinct protein layer at their surfaces (arrows) and were embedded within the protein strands of the curd matrix. The degree of lipid insertion within the protein strands gives insight into the change in curd firmness.

Reduction of curd firmness at higher temperature, with increased US exposure, did not agree with previous studies that reported improved gelation for US-treated milk with homogeneous submicron emulsion stages. Shanmugam et al. (2012) reported that US (20 kHz, 20 and 41 W up to 60 min, 16°C) processing of pasteurized homogenized skim milk resulted in slight micelle size reduction, possibly because of the removal of the hairy layers, denaturation of whey proteins, and formation of whey-casein aggregates. Nguyen and Anema (2010) reported that US at 22.5 kHz and 50 W without temperature control reached >85°C within 5 min and 95°C by 15 min and showed increased micelle size initially, solubilized κ -CN from micelles, denatured whey proteins, and whey-casein aggregates. If tempera-

tures were controlled, preventing the denaturation of whey proteins, US had minimal effect on making acid gels. Chandrapala et al. (2012a) ultrasonicated skim milk, micellar CN, and CN powder at 20 kHz and found that after 60 min, there were no changes in micelle size, levels of free CN, or soluble calcium. They concluded that casein was stable when ultrasonicated. Riener et al. (2009) reported that whole milk ultrasonicated for 6 min at 24 kHz for yogurt making resulted in submicron-sized lipid droplets and had higher curd firmness, water holding capacity, and gelation pH over normal yogurt-set heated to 90°C for 10 min.

Homogenized milk is typically not used to make cheese as the smaller-sized fat droplets result in a short, noncontinuous protein matrix within the curd and poor syneresis properties (Scott, 1986). Queso Fresco is one of the few high-moisture cheeses that use homogenize milk to ensure a crumbly, high-moisture curd that does not melt (Van Hekken et al., 2012). Queso Fresco does have microbial food safety issues (Gould et al., 2014) and wheying off during storage (Van Hekken et al., 2012) that US may be able to address.

Microbiology. Raw milk samples contained 3.81 ± 0.70 log cfu/mL of total aerobic mesophilic bacteria, and 2.29 ± 0.87 log cfu/mL of psychrophilic bacteria (Figure 8a, inlet temperature of 42°C and 8b, inlet temperature of 54°C). After standardizing the milk fat concentration, samples were stored for up to 48 h at 4°C before ultrasound processing. During storage, the bacterial counts increased with mesophilic counts between 3.36 and 4.88 log cfu/mL (Figure 8, black bars), and psychrophilic counts between 2.59 and 4.35 log cfu/mL (Figure 8, white bars). The bacterial load was not affected in most samples by continuous flow ultrasound for milk with inlet temperatures at 42°C. The exception was a 0.5 log reduction in psychrophilic bacteria at the fastest flow rate (Figure 8a, dotted bars). Milk with inlet temperature of 54°C was shown to improve the antimicrobial activity of ultrasound processing, with mesophilic counts reduced by an average of 1.3 and 1.4 log when medium and fastest flow rates were used, respectively (Figure 8, gray bars).

The lack of greater microbial reductions was attributed to US at lower temperatures. These findings were in agreement with previous studies that showed ultrasound processing at temperatures above 50°C was effective in eliminating *Pseudomonas fluorescens* and *Streptococcus thermophilus* in milk (Villamiel and De Jong, 2000b). In our study, milk with an inlet temperature of 54°C was more effective than the 42°C inlet temperature in reducing the number of psychrophilic bacteria within raw milk, with counts dropping below the limit of detection (1 log cfu/mL) for all 3 flow rates

Figure 5. Coagulation properties [(a) coagulation time and (b) curd firmness 30 min after enzyme addition] of raw milk ultrasonicated for up to 18 min at different processing treatments. Treatments included inlet temperatures of 42°C (circles) and 54°C (squares) and flow rates of 0.15 (blue), 0.30 (yellow), and 0.45 (green) L/min.

Figure 6. Transmission electron microscopy images of control milk and milk ultrasonicated for the longest exposure times (14 to 18 min) at different inlet temperatures and flow rates. Rows, inlet temperature: top row (a, b, c, d), 42°C; and bottom row (e, f, g, h), 54°C. Columns, flow rates: first column (a, e), control; second column (b, f), 0.15 L/min; third column (c, g), 0.30 L/min; and fourth column (d, h), 0.45 L/min. Scale bar represents 0.5 μ m. Arrows point to fat droplets surrounded by dense protein layer.

tested (Figure 8b, dotted bars). Psychrophilic bacteria were expected to be less tolerant of the higher temperatures when compared with mesophilic bacteria, which may explain why the ultrasound treatment was effective against these bacteria, even at the slowest flow rate, which showed approximately 2 log reductions in

bacteria after 6 min of US processing. These results support previous findings that ultrasound may be an effective antimicrobial application for reducing the number of spoilage microbes in dairy products, thereby extending shelf life (Cameron et al., 2009; Chouliara et al., 2010).

Figure 7. Scanning electron microscopy images of control milk and milk ultrasonicated for the longest exposure times (14 to 18 min) at different inlet temperatures and flow rates. Rows, inlet temperature: top row (a, b, c, d), 42°C; and bottom row (e, f, g, h), 54°C. Columns, flow rates: first column (a, e), control; second column (b, f), 0.15 L/min; third column (c, g), 0.30 L/min; and fourth column (d, h), 0.45 L/min. Scale bar represents 5 μ m.

Chemical Properties

Composition. Milk contained $2.91 \pm 0.06\%$ fat, $3.12 \pm 0.07\%$ protein, $4.70 \pm 0.11\%$ lactose, and $11.51 \pm 0.21\%$ TS. The slight differences noted after US treatments were not significant. Alkaline phosphatase remained active in all samples after the last pass, indicating that samples were not exposed to pasteurization conditions during the processing run. A faint metallic-like-aroma was present in the US milk that increased in intensity as exposure time accumulated. Current research is underway to identify the undesirable volatile compounds present in our samples. A slight frosting of the metal wall within the reaction cell closest to the transducers suggested cavitation erosion caused by running at 100% power. Further research at lower power settings may eliminate the aroma. In a review of transducer erosion studies, Mawson et al. (2014) reported that measurable amounts of micron-sized metallic particles were formed by transducer sonotrodes operated at 18 and 20 kHz for 7.5 h. Although amounts were still below acceptable limits for drinking water, further research on sonotrode design or development of food-safe erosion resistance of stainless steel is needed. Other US studies that have reported off-odors that were products of the high heat of cavitation (Riener et al., 2009, 2010) and oxidation of PUFA hydroperoxides (Riener et al., 2009, 2010; Chouliara et al., 2010; Pingret et al., 2013).

Fatty Acid Properties. The fatty acid profiles for the control milk samples were within the accepted ranges reported for bovine milk (Kaylegian and Lindsay, 1995; Jensen, 2002). Although slight differences were present in the individual fatty acid concentrations among the nonprocessed control samples (0 s), the majority of fatty acid levels before and after US processing were similar ($P > 0.05$; Table 2). With the exception of C18:0, very small changes were detected for C4:0 to C20:0 in milk with inlet temperatures of 42°C and for C18:0, C14:1, and C18:2 *trans,trans* in milk with inlet temperatures at 54°C. Of the C18 PUFA currently believed to be important in human health, the n-3 linolenic, C18:3, and n-6 linoleic acid, C18:2 *cis-9,cis-12* were not altered by US processing. A slight increase occurred in the CLA C18:2 *trans,trans* following 14 to 18 min of US treatment. Previous studies for longer US periods reported increased levels of pentanal, hexanal, and heptanal, volatiles that indicated oxidation of PUFA (Riener et al., 2009, 2010; Chouliara et al., 2010; Pingret et al., 2013). Results suggested that the 14 to 18 min treatment in our system was not intense enough to induce significant chemical alterations in the fatty acid profile. Further research is suggested.

Although the DSC profiles showed similar ($P > 0.05$) maximum temperature ($62.04 \pm 1.45^\circ\text{C}$) and enthalpy ($12.57 \pm 0.27 \text{ J/g}$) among the raw milk controls and US samples, the melt profiles showed a subtle change. Controls (Figure 9a) had the typical milk fat profile with 3 regions, low, medium, and high melt fats separated by

Figure 8. Comparison of the total cell count of indigenous mesophilic and psychrophilic bacteria in raw milk at inlet temperatures of (a) 42°C or (b) 54°C; before (no sonication) and after ultrasonication at different flow rates (0.15, 0.30, and 0.45 L/min) for the longest exposure times (14 to 18 min). Total mesophilic (control, black bars; and 14 to 18 min of exposure, gray bars) and psychrophilic (control, white bars; and 14 to 18 min of exposure, dotted bars; ND = not detected) bacteria were determined by serial dilution and colony counts on plate count agar. Results are the average of a minimum of 2 independent experiments with SD error bars.

Table 2. Fatty acid profile of control milk and milk entering the ultrasonication unit at 42 or 54°C and sonicated for the longest exposure time of 14 to 18 min, depending on the milk flow rate

Fatty acid	42°C		54°C		SE
	0 s	14–18 min	0 s	14–18 min	
Saturated					
C4:0	1.910 ^a	1.864 ^b	1.794 ^a	2.080 ^a	0.121
C6:0	1.913 ^a	1.751 ^b	1.526 ^c	1.550 ^c	0.041
C8:0	1.307 ^a	1.220 ^b	1.083 ^c	1.100 ^c	0.022
C10:0	3.152 ^a	3.018 ^b	2.727 ^c	2.763 ^c	0.043
C12:0	3.604 ^a	3.516 ^a	3.267 ^c	3.292 ^c	0.356
C13:0	0.189 ^a	0.187 ^a	0.173 ^b	0.174 ^b	0.002
C14:0	11.93 ^a	11.88 ^a	11.38 ^b	11.54 ^b	0.073
C15:0	1.130 ^a	1.215 ^a	1.213 ^a	1.222 ^a	0.007
C16:0	32.87 ^b	33.28 ^a	32.10 ^c	32.04 ^c	0.145
C17:0	0.726 ^b	0.721 ^b	0.774 ^a	0.765 ^a	0.005
C18:0	11.92 ^c	11.83 ^c	12.79 ^a	12.53 ^b	0.095
C20:0	0.172 ^b	0.168 ^b	0.180 ^a	0.179 ^a	0.002
Monounsaturated					
C14:1 <i>trans</i>	0.220 ^b	0.222 ^b	0.228 ^a	0.229 ^a	0.001
C14:1	0.833 ^b	0.850 ^a	0.776 ^d	0.798 ^c	0.007
C16:1 <i>trans</i>	0.426 ^b	0.424 ^b	0.447 ^a	0.451 ^a	0.002
C16:1	1.731 ^a	1.733 ^a	1.753 ^a	1.757 ^a	0.012
C17:1	0.196 ^b	0.200 ^b	0.242 ^a	0.245 ^a	0.007
C18:1 <i>cis</i> -9	18.94 ^b	19.24 ^b	20.38 ^a	20.40 ^a	0.187
C18:1	0.519 ^b	0.533 ^b	0.586 ^a	0.599 ^a	0.008
C20:1	0.177 ^b	0.171 ^c	0.191 ^a	0.188 ^a	0.002
Polyunsaturated					
C18:2 <i>trans,trans</i>	0.249 ^c	0.253 ^{bc}	0.257 ^b	0.265 ^a	0.002
C18:2 <i>cis</i> -9,12	2.120 ^a	2.104 ^a	2.153 ^a	2.151 ^a	0.026
C18:3 α	0.348 ^b	0.344 ^b	0.365 ^a	0.367 ^a	0.004
C20:4	0.131 ^a	0.127 ^a	0.129 ^a	0.127 ^a	0.002

^{a–d}Means that do not share the same letter are significantly different ($P < 0.05$).

inflection points (arrows). The inflection point between medium and high melt fats is minimal, however. As the US exposure increased, this inflection point became deeper (Figure 9b). Although sample preparation for DSC eliminated the structure of the fat droplets, it appeared that the higher melt triglycerides may be easier to separate in low-frequency US-treated milk. It has already been established that high-frequency (>1 MHz) US is capable of fractionating milk fats by the size of the lipid droplets (Leong et al., 2014, 2016).

Although low-frequency US is excellent at making fine emulsions and homogenizing milk fat droplets to a uniform submicron size, this study examined the early effects of US, where submicron particles begin to accumulate and changes to coagulation properties are noted. Further optimization of the system is required to identify conditions for desired modification of milk.

CONCLUSIONS

Use of a continuous US unit, delivering 1.36 kW/pass within a 2.4-kW reactor cell with parallel diaphragm plates and 2 transducers (16/20 kHz) external to the

reactor for milk entering the unit at 42 or 54°C, resulted in the formation of significant amounts of submicron-sized fat droplets and altered the coagulation properties of the milk. Microscopic imaging illustrated the changes in fat droplet sizes in milk and the curd matrix microstructure, which gives insight into the altered protein-lipid and protein-protein interactions within the modified milk. The system also reduced microbial counts at temperatures below pasteurization conditions, although specific activity against bacterial pathogens is needed to validate US as an alternative to thermal processing. Further optimization of the system, including processing milk at temperatures above the melting point of milk fat yet below pasteurization temperature (63°C), is required; however, the results from this study suggest continuous US may be a suitable tool for dairy food processing.

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Figure 9. Differential scanning calorimeter profile of (a) typical control and (b) 18-min ultrasonicated milk samples. Arrows indicate the inflection points separating the ranges for low, medium, and high temperature melting fats.

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