Smoothing temperature and ratio of casein to whey protein: Two tools to improve nonfat stirred yogurt properties

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ABSTRACT

Consumers are not always ready to compromise on the loss of texture and increased syneresis that nonfat stirred yogurts display compared with yogurts that contain fat. In this study, we investigated milk protein composition and smoothing temperature as a means to control nonfat yogurt microstructure, textural properties, and syneresis. Yogurts were prepared with different ratios of casein to whey protein (R1.5, R2.8, and R3.9). Yogurts were pumped through a smoothing pilot system comprising a plate heat exchanger set at 15, 20, or 25°C and then stored at 4°C until analysis (d 1, 9, and 23). Yogurt particle size and firmness were measured. Yogurt syneresis and water mobility were determined, respectively, by centrifugation and time domain low-frequency proton nuclear magnetic resonance (¹H-LF-NMR). Increasing the smoothing temperature increased gel firmness and microgel (dense protein aggregates) sizes independently of the whey protein content. Also, yogurt microgel sizes changed with storage time, but the evolution pattern depended on protein ratio. Yogurt R1.5 showed the largest particles, and their sizes increased with storage, whereas R2.8 and R3.9 had smaller microgels, and R3.9 did not show any increase in microgel size during storage. Micrographs showed a heterogeneous gel with the empty area occupied by serum for R1.5, whereas R2.8 and R3.9 showed fewer serum zones and a more disrupted gel embedding microgels. Induced syneresis reduced with greater whey protein content and time of storage. This is in agreement with ¹H-LF-NMR showing less bulk water mobility with increasing whey protein content during storage. However, ¹H-LF-RMN revealed higher values of spontaneous serum separation during storage for R1.5 and R3.9 yogurts, whereas these were lower and stable for R2.8 yogurt. Microgels play an important

structural role in yogurt textural attributes, and their characteristics are modulated by whey protein content and smoothing temperature. Optimization of these parameters may help improve nonfat stirred dairy gel.

Key words: stirred yogurt, microgel particle, syneresis, whey protein, time domain-nuclear magnetic resonance (TD-NMR)

INTRODUCTION

Yogurt is an acidic dairy gel obtained after the fermentation of milk by lactic bacteria (Codex Alimentarius, 2003; Aryana and Olson, 2017). Two main types exist: (1) firm (set) yogurt is obtained directly after fermentation; (2) stirred yogurt is obtained with additional post-fermentation processes, in which yogurt is stirred and smoothed (Mokoonlall et al., 2016).

In Canada, the yogurt market is dominated by stirred yogurt. Demand by consumers for low-fat products is increasing (Aryana and Olson, 2017), but low-fat yogurts tend to display major organoleptic defects such as non-creamy texture. The creaminess of stirred yogurt has often been linked to 4 physical variables: viscosity, firmness, syneresis (watery feeling), and granularity (Pereira et al., 2003; Lee and Lucey, 2006; Cayot et al., 2008; Tomaschunas et al., 2012; Krzeminski et al., 2013).

Syneresis occurs when the serum is expelled from the gel. It is controlled by the gel pore size and interconnections in the network (van Marle, 1998; Hinrichs et al., 2003; Zoon, 2003; Lee and Lucey, 2010). During storage, interactions in the casein network evolve, decreasing the number of pores and increasing their size, which facilitates water movement in the gel, leading to syneresis (van Marle, 1998; Lucey, 2001; Zoon, 2003; van Vliet et al., 2004; Lee and Lucey, 2006; Lee and Lucey, 2010). In a previous work (Gilbert et al., 2020a), low-frequency nuclear magnetic resonance (NMR) was used to study syneresis by characterization of bulk water mobility in the gel and quantification of spontaneous syneresis. Both were related to the yogurt microstructure, which

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itself was controlled by yogurt formulation and post-fermentation processes such as stirring.

Syneresis and texture defects can be reduced using several approaches, such as milk standardization, milk processing (heat treatment, homogenization), starter selection, fermentation settings (time, temperature), and post-fermentation processing conditions (e.g., stirring temperature, pumping pressure). For example, stabilizers (such as gelatin, gums, and starch) added during milk standardization can efficiently increase yogurt viscosity and reduce syneresis (Keogh and O'Kennedy, 1998; Fiszman et al., 1999; Sodini et al., 2004; Pang et al., 2016). However, consumers' concern about additives in food products is increasing (Asioli et al., 2017), and alternative solutions are needed. In their reviews, Sodini et al. (2004), Karam et al. (2013), and Lesme et al. (2020) showed that viscosity or firmness can be improved, while syneresis in yogurt decreased by increasing total solids or protein contents, or by lower ratio of casein (CN) to whey protein (WP) in yogurt.

The effects of the CN:WP ratio on set and stirred yogurt properties have been thoroughly investigated in interaction with milk heat treatment. All research performed on CN:WP ratios agree that yogurts with low CN:WP ratios (<2) had higher viscosity and firmness and lower syneresis but presented large protein aggregates (diameter $>100\text{ }\mu\text{m}$), inducing unwanted grainy perception (Puvanenthiran et al., 2002; Küçükçetin, 2008; Tomaschunas et al., 2012; Chua et al., 2017; Laiho et al., 2017). To our knowledge, no study has looked at the interaction between CN:WP ratio and post-fermentation processes (temperature changes, shear intensity) of stirred yogurts.

During the post-fermentation process, stirring and smoothing steps take place in a continuous circuit, where the gel undergoes different levels of structure modification through pipes, pump, cooling devices, and finally smoothing nozzle (Chandan and O'Rell, 2013; Mokoonlall et al., 2016). These processing treatments induce mechanical shearing that creates a new microstructure for stirred yogurt (Mokoonlall et al., 2016; Gilbert et al., 2020b). Microscopic observations (scale between $10\text{ }\mu\text{m}$ and $250\text{ }\mu\text{m}$, depending on the study) have revealed highly disrupted and reorganized protein network with intact microfragments (microgels) from the initial set gel (van Marle, 1998; Laiho et al., 2017; Gilbert et al., 2020b; Gregersen et al., 2021). Microgels can be defined as dense protein aggregates; their sizes and rigidity have been identified as important parameters for understanding the perceived granularity of stirred yogurt (Cayot et al., 2008; Laiho et al., 2017). A correlation between microgel size and syneresis has

often been mentioned for stirred yogurts, with some conflicting conclusions (van Marle, 1998; Lee and Lucey, 2006; Zhang et al., 2016; Gilbert et al., 2020b).

Few studies have focused on the influence of processes, such as passage through pipes (Schmitt et al., 1998; Abu-Jdayil et al., 2013) or cooling in a plate heat exchanger (Afonso et al., 2003; Fernandes et al., 2005; Fernandes et al., 2006), on yogurt microstructure and properties. The evolution of yogurt's fluid behavior during processing has been investigated, but the final product properties were not characterized in these studies. Zhang et al. (2016) used a technical scale unit (3-L production), where the effect of high- or low-pressure treatment during smoothing (impact shearing intensity) was studied. Those authors found that increasing pressure treatment from 1 to 4 bar during post-fermentation treatment reduced stirred gel viscosity, elasticity, and microgel sizes, whereas syneresis was increased. Guénard-Lampron et al. (2019) studied the influence of smoothing temperature using a technical scale unit (30 L) and showed that low smoothing temperature (10°C) reduced syneresis but also reduced viscosity and texture. However, yogurt microstructure was not investigated. In our previous work (Gilbert et al., 2020b), the effects of smoothing temperature in a temperature-controlled rheometer at laboratory scale ($<40\text{ mL}$) were linked to gel structures and syneresis in stirred yogurts. Higher smoothing temperatures (42 versus 20°C) were responsible for microgels with larger particle sizes and higher gel heterogeneity in the final products, leading to higher induced syneresis. To our knowledge, no study has yet investigated the effects of both smoothing temperature and milk standardization using a technical scale unit ($>3\text{ L}$) as reviewed in (Mokoonlall et al., 2016). Knowing that low CN:WP ratio and higher smoothing temperature are responsible for large microgels, it would be of interest to better understand how the association between smoothing temperature and CN:WP ratio determines microstructures of stirred yogurt, and whether those microstructures are linked with stirred yogurt properties (viscosity, syneresis, texture).

The aim of the present study was to understand how the combination of smoothing temperature and addition of whey protein affects nonfat stirred yogurt gel microstructure, texture, and syneresis, using a technical scale ($>3\text{ L}$). The methodological approach combined methods generally used to observe yogurt properties (texture analysis, rheology, laser diffraction, and induced syneresis) and non-destructive or less-destructive methods [microscopic image analysis, low-frequency proton NMR ($^1\text{H-LF-NMR}$)].

MATERIALS AND METHODS

Yogurt Production

Starter Culture. Milk was reconstituted at 12% (wt/wt) with skim milk powder (SMP; Agropur) and deionized water. It was then sterilized (121°C, 10 min) and inoculated at 0.1% with a commercial starter Yo-Dolce 1 (Biena) containing *Streptococcus thermophilus* (ST) and *Lactobacillus delbrueckii* ssp. *bulgaricus* (LB) in a 95:5 ratio. Fermentation took place at 41°C (Iso-temp incubator 200 series, model 230D, Fisher). The pH was regularly measured (SB20 Symphony, VWR) until pH 4.6 ± 0.05 was reached. Pre-culture was placed in a cold temperature-controlled room at 4°C and used the following day.

Yogurt Gel Manufacture. Yogurt milk (32 L) was prepared by using water, a low-heat SMP (Quadra Chemicals), whey protein concentrate (WPC; Agropur), whey protein isolates (Bipro, Davisco), and lactose (Quadra Chemicals). Standardization was realized to obtain 14% total solids, 3% caseins, and 3 different CN:WP ratios: 1.5:1 (R1.5), 2.8:1 (R2.8), and 3.9:1 (R3.9, the natural ratio of the SMP used). All ingredients were mixed for 10 min (21-inch Heavy-Duty Big Stik Immersion Blender, Waring Commercial) and stored at 4°C overnight.

Yogurt milks were heated at 50°C, homogenized in a 2-stage homogenizer (34.5 bar first stage and 137.9

bar second stage; Ariete NS2006H, GEA/NIRO), heat-treated (HT) at $95 \pm 1^\circ\text{C}$ for 5 min (Laboratory UHT/HTST Electra Series, MicroThermix), quickly cooled to $20 \pm 2^\circ\text{C}$ and stored at 4°C. The next day, 3 vats of 10 L of milk were inoculated with 200 mL of pre-culture each and incubated at $42 \pm 0.5^\circ\text{C}$ in a double-jacketed stainless-steel mini-vat (INRA/CFR 10 L, INRA). The pH was regularly measured, and fermentation was stopped when the pH reached 4.60 ± 0.05 (around 225 min).

The coagulum was mechanically stirred for 20 s using the blade agitator integrated in the mini-vat and transferred to a 10-L smoothing pilot system (Figure 1). The product was pumped (Varidrive Positive Pump, US Motors) and transported across the system using an assembly of stainless-steel pipe sections (4-cm diameter). The pump was set at 6 L/min when pumping water through the entire system, but it decreased to approximately 3 L/min when pumping yogurt, regardless of what temperature was used to smooth. A pre-smoothing stainless-steel semi-spherical nozzle (2-mm pore size) was used before the product reached the heat plate exchanger (MS3-SBL, Tetra Pack) to cool the yogurt to the 3 final targeted temperatures: 15, 20, and $25 \pm 1.5^\circ\text{C}$, based on the work of Guénard-Lampron et al. (2020). A stainless-steel smoothing conical nozzle (0.5-mm pore size) was placed at the end after the heat plate exchanger. Yogurt was then packaged and stored at 4°C until analyses.

Figure 1. Technical-scale scheme for stirring process.

Yogurt Milk Composition and Particle Size

Composition. Total solids and ash were measured in the heat-treated milk according to official methods (AOAC International, 2012). Total nitrogen, noncasein nitrogen, and NPN contents were measured by the Kjeldahl method (AOAC International, 2012) in unheated and heat-treated milk.

Similarly to Gilbert et al. (2020b), the ingredients used in this study already contained an initial amount of denatured WP (the amount of insoluble WP at pH 4.6 was approximately equal to 21, 32, and 43% in whey protein isolates, WPC, and SMP, respectively), resulting in reconstituted milk that already contained denatured WP. To determine the quantity of denatured WP already present in reconstituted milk from powders, the CN:WP ratio was evaluated using SDS-PAGE in reductive conditions as described previously (Gilbert et al., 2020b). The amount of denatured WP in untreated milk and in HT milk was approximated using a calculation based on Kjeldahl and SDS-PAGE results. Briefly, unheated milk samples (2 mg of protein/mL) were mixed at a 1:1 ratio with reductive sample buffer (2× Laemmli Sample Buffer, Biorad). Samples (10 µg) were loaded in duplicates in precast 4–20% polyacrylamide gels (Mini-Protean TGX Gels, Biorad). To control repeatability between gels, a milk protein standard containing α-casein, β-casein, κ-casein, α-lactalbumin, and β-lactoglobulin (Sigma-Aldrich) was also loaded in each gel. Protein band relative densities (PRD, in percentage of intensity) were determined (Quantity One 4.5.2, Biorad). The percentage of denatured whey protein calculation in milks before or after HT was adapted from Rioux and Turgeon (2012). Native whey proteins were presumed to be the difference between soluble nitrogen at pH 4.6 (NCN) and nitrogen soluble in trichloroacetic acid (NPN). The percentage of denatured whey protein was thus calculated as follows:

$$\%WP \text{ denaturation} = 100 - \left[\left(\frac{NCN_i - NPN_i}{TN_{HT} \times PRD_{\text{whey proteins}}} \right) \times 100 \right].$$

When the calculation is performed on reconstituted milk before HT, i = reconstituted milk before HT. When calculation is performed on reconstituted milk after HT, i = HT milk.

Aggregate Sizes in Milk. Particle size was measured in milk using light scattering (Zetasizer, Nano Series, Malvern Ltd.). Unheated and heat-treated milk were diluted 20 times in neutral milk permeate (Gilbert et al., 2020b). Milk permeate was produced as follows. Pasteurized skim milk was purchased in a local grocery store, and permeate was produced with

an ultrafiltration membrane (10 kDa; Desal membrane, PW2540F1074, GE Water and Process Technologies). An antibacterial agent, sodium azide (EMD), was added at 0.02% (wt/wt). Permeate was then divided into 2 batches: one left at neutral pH, the other acidified to pH 4.60 ± 0.05 using hydrochloric acid, 5 *N* and 1 *N*, for yogurt particle size analyses.

Yogurt Analyses

Bacterial Count in Yogurt. At d 0 after production, yogurt was diluted in sterilized (120°C; 15 min) peptone water (0.1%; Bacto Peptone, BD Bioscience). Lactobacilli and streptococci were enumerated by plate spreading on acidified de Man, Rogosa, and Sharpe (EMD Millipore) and M17 (Oxoid) agar media supplemented with lactose, respectively. Inoculated petri dishes were incubated under anaerobic conditions for 48 h at 37°C before enumeration.

pH of Yogurt. Yogurt pH was measured at d 1, 9, and 23 (SB20 Symphony, VWR) after production.

Laser Light Diffraction

At d 1 after production, yogurt samples were diluted 10 times into acidified milk permeate under magnetic stirring (15 min, 450 rpm), and a few drops were immediately used to measure yogurt particle size using laser light diffraction (1,000 rpm at 20°C; Mastersizer 3000, Malvern Ltd.). Particle size results were obtained from the mean of 5 consecutive measurements, and each yogurt sample was measured in triplicate. Because the particle size distributions were unimodal in all yogurts, the volume-weighted mean diameter $D[4,3]$ was used to express particle sizes.

Particle Analysis from Microscopic Image Analyses

Microscopic slides (75 × 25 mm) were prepared by fixing a microscopic cover glass (25 × 25 mm) on each side with clear nail polish, creating a volume of 0.109 cm³ in which yogurt was gently spread. Measurements were performed at d 1, 5, and 23 after production (Gilbert et al., 2020b). Ten random pictures (5 on 2 slides) were taken immediately. A digital microscope camera with the zoom set at 150 (AM4515ZT4 Dino-Lite Edge Microscope, Dino Canada) was positioned below the yogurt slide. Image analyses were performed using ImageJ2 software (<https://imagej.net/ImageJ>). Images were first scaled and then converted to a gray format for image analyses.

The surfaces of white masses found on the pictures, which are dense protein aggregates, also defined as microgels (Gilbert et al., 2020a,b), were measured by

bipolarizing images using the percentile-selective pattern of gray as threshold adjustment. Microgels were then further separated using the watershed program. All microgels detected on image edges were automatically eliminated from analyses, and the microgels' surfaces were defined as the mean obtained from 10 pictures. Gel heterogeneity was quantified by measuring the gray level variation within the picture (Gilbert et al., 2020a,b). Gray values on images vary between 0 (black) and 255 (white): pixels close to 255 indicate the presence of a dense protein network at this location; the lack of a protein network is represented by black pixels. The protein network density heterogeneity was thus quantified using the network heterogeneity index (**NHI**), which was equal to the standard deviation for the gray values on the image.

Flow Properties Measurement

At d 1, 9, and 23, viscosity was measured at 4°C using an ARES-G2 rheometer (TA Instruments) using a cup (height: 42 mm; diameter: 30 mm) and dyne (diameter: 28 mm) geometry. Approximately 24 mL of yogurts were gently introduced (using a 30-mL cut syringe) into the cup. The dyne geometry was slowly lowered into the cup. Flow curves were obtained by recording viscosity and shear-stress with increasing shear rate from 1 to 700 s⁻¹. Using the steady-state mode, 10 points per decade were recorded during the experiment. Stress curves were fitted to the power law model ($\eta = K\dot{\gamma}^{n-1}$) between shear rate of 5 to 631 s⁻¹ ($R^2 > 0.97$). In this model, η represents the apparent viscosity; K (Pa·s) is the intercept of the model curves, also called the consistency; n is an index representing the flow behavior of the semi-solid product (Foegeding et al., 2011). Yogurts usually have a shear-thinning behavior (pseudoplastic) with $n < 1$ (Abu-Jdayil et al., 2013).

Texture Measurement

At d 1, 9, and 23, firmness was measured using a TA-XT2 plus (Texture Technologies Corp.) equipped with 5-kg module and a cylindrical Teflon geometry (diameter: 3 cm; height: 3.5 cm). The compression test was performed at 4°C by lowering the geometry 2 cm deep into the gel at a speed of 1 mm/s. The maximum penetration force applied at 2 cm divided by the area of the probe was defined as the gel firmness (Guénard-Lampron et al., 2020).

Induced Syneresis

At d 1, 9, and 23, yogurt aliquots (20 mL) were weighted and stored in a chamber at 10°C for 3 h. They

were then centrifuged (Eppendorf centrifuge 5804R V3.3) using a swinging bucket rotor (Eppendorf Rotor A-4-44) at 238 g for 10 min at 10°C (Laneville and Turgeon, 2014). Syneresis was then calculated as the percentage of lost serum during centrifugation.

Water Mobility (¹H-LF-NMR Time Domain Analyses)

Water mobility was assessed at d 1 for milk and at d 1, 9, and 23 after production for yogurts using ¹H-LF-NMR. The NMR measurements were realized at 20 MHz in a Minispec Mq20 (Bruker Optik GmbH). The 10-mm probe was equipped with a temperature unit BVT 3000 (Bruker Optik GmbH) allowing all samples to be maintained at 4°C during measurement. The Carr-Purcell-Meiboom-Gill sequence was used to induce transversal relaxation signal decays on protons. The sequence was composed of 32 consecutive scans; the pulse separation (τ) was 0.5 ms, and the relaxation delay was 10 s. Laplace transformation was then performed on signal decays (Contin, Brucker). Data were fitted with 500 points from 0.1 to 2,000 ms. The transversal relaxation time constant [$T_2(i)$] and the relative intensity [$I_2(i)$] of each individual proton pool were obtained. The peak position on the relaxation time axis was defined as $T_2(i)$, and the area under the peak gave the relative intensity [$I_2(i)$] of each pool when divided by the total area under the spectrum (Han et al., 2009; Gilbert et al., 2020a). Each sample was measured in triplicate.

Experimental Design and Statistics

The yogurts were produced following a split-plot design with 3 repetitions for each experimental unit. The CN:WP ratio was the main plot, with smoothing temperatures as the subplot, and the effect of storage was introduced into the statistical model using the mixed procedure in SAS software (SAS v. 9.4, SAS Institute Inc.). The corrected Akaike's information criterion indicated the best correlation structure for repeated measurement. To satisfy ANOVA assumptions, some variables were transformed. Inhomogeneous variation or non-normal repartitions were corrected using data transformations according to the Box-Cox procedure. In addition, when the data set had a heteroskedastic issue, more than one standard error was inserted in the statistical model for a specific factor using the statement "group = specific_factor." Based on significant effects in the model, the protected Fisher's least significant difference method was used to obtain multiple comparisons. The Shapiro-Wilk statistic allowed us to check the normality assumption, and homogeneity of variances was verified by looking at the residual plots.

Results are reported as the means $\pm$ standard error. Correlations between variables were also estimated using Pearson correlation coefficients, and only correlation values higher than 0.5 and with $P < 0.01$ are discussed in the paper.

RESULTS AND DISCUSSION

Composition and Particle Size of Yogurt Milk with Different CN:WP Ratios

The composition of the different yogurt milks reached the targeted values (Table 1). No statistical difference was detectable for the total solid (14%), casein (3%), and ash (1%) contents depending on the ratios (Table 1). As expected, total and true protein contents were significantly influenced by the yogurt milk ratio ($P < 0.0001$). The R1.5 milk had the highest total and true protein contents. The total protein content was higher in R2.8 than in R3.9 milk, but we found no statistical difference for true protein, although CN:WP ratios in yogurt milks were statistically different.

The WP denaturation was similar for all yogurt milks (after HT), with values around 81% (Table 1). Higher WP content, especially β -lactoglobulin, has been reported to increase the WP denaturation rate in WP solutions and milk (Roefs and Kruif, 1994; Corredig and Dalgleish, 1996; Beaulieu et al., 1999). Thus, a higher denaturation level was expected for R1.5 having the highest WP content. As mentioned, milk was prepared from dairy powders, already containing denatured WP

proteins. When comparing denatured WP content in milk before heat treatment (Table 1), the amount of WP denaturation decreased with the level of WP added to the milk. Therefore, denaturation level changed from 26% before HT to 82% after HT in R1.5 milk compared with R3.9, for which denaturation values increased from 43% of WP before HT to 80% after HT.

Particle size of unheated reconstituted milk was equivalent in all yogurt milk (Table 1). Heat treatment resulted in larger particle sizes depending on CN:WP ratio in milk ($P < 0.01$; Table 1). Particle diameter in R1.5 heated milk was more than twice those of particles found in R2.8 and R3.9 milks, which was consistent with literature (Beaulieu et al., 1999). Beaulieu et al. (1999) compared protein aggregate sizes using photon correlation spectroscopy in milks with different CN:WP ratios before and after HT (95°C, 5min). Heat-treated milks with CN:WP ratios of 4, 1.5, and 0.7 had protein aggregate sizes of respectively 145, 210, and 340 nm. The values are different from aggregate sizes obtained in the present study; however, it should be noted that Beaulieu and colleagues used raw milk, whereas milks reconstituted from powders were used here. Lower values were also obtained in previous work (Gilbert et al., 2020b) for a formulation made from raw milk and adjusted with milk powders. The milk, which was equivalent to the R2.8 formulation, had protein aggregate sizes of approximately 190 and 205 nm for respectively untreated and HT (94°C, 5 min) milks. These values are lower than those obtained with reconstituted milks in the present paper.

Table 1. Milk composition and particle size¹

Item	Milk formulation (CN:WP ratio ²)			SEM
	R1.5	R2.8	R3.9	
Composition (g/100 g)				
Total solids	14.2 ^a	14.0 ^a	13.9 ^a	<0.1
Ash	1.1 ^a	1.0 ^a	0.9 ^a	<0.1
Total protein content	5.1 ^a	4.1 ^b	3.8 ^c	<0.1
True protein content	4.8 ^a	3.9 ^b	3.7 ^b	<0.1
CN content	3.0 ^a	2.9 ^a	2.9 ^a	<0.1
WP content	1.8 ^a	1.0 ^b	0.8 ^c	<0.1
CN:WP ratio	1.7 ^c	2.8 ^b	3.6 ^a	<0.2
WP denaturation before HT ³ (%)	26.0 ^c	36.2 ^b	42.5 ^a	<1.8
WP denaturation after HT (%)	82.3 ^a	80 ^a	80.4 ^a	<0.9
Particle size ⁴ (nm)				
Reconstituted unheated milk	226 $\pm$ 24 ^{a,B}	223 $\pm$ 2 ^{a,B}	222 $\pm$ 2 ^{a,A}	<25
HT milk	469 $\pm$ 24 ^{a,A}	230 $\pm$ 2 ^{b,A}	212 $\pm$ 2 ^{c,B}	<25

^{a-c}Means in a row with different superscripts are significantly different ($P < 0.05$).

^{A,B}Means in a column with different superscripts are significantly different ($P < 0.05$).

¹All values are expressed as mean $\pm$ SE (n = 3).

²WP = whey protein. Formulations include (CN:WP) R1.5 (1.5:1), R2.8 (2.8:1), and R3.9 (3.9:1).

³HT = heat treatment.

⁴Milk aggregate size obtained by light scattering.

Yogurt pH and Bacterial Concentrations

Post-acidification occurred during storage and was dependent on the CN:WP ratio ($P < 0.01$; Figure 2A). The pH decrease was smaller with lower CN:WP ratio. This could be attributable to the increasing protein content of lower CN:WP ratios in our study, which would result in a higher buffering capacity of the mix. Jørgensen et al. (2015) studied the effect of CN:WP (constant protein content and dry matter) and WP level of denaturation on stirred yogurt properties. They also observed post-acidification in their yogurts, but with opposite trends. Lower CN:WP ratio tended to decrease fermentation time and to increase post-acidification. In another work, Antunes et al. (2005) examined the effect of WPC addition, at a constant protein content, on properties of yogurts with or without probiotics. When no probiotics were used, no difference was found in post-acidification over storage between control yogurt and yogurt enriched with WPC.

In the present study, we found a difference in yogurt pH depending on smoothing temperature, independently of storage time ($P < 0.01$; Figure 2B). Yogurt smoothed at 25°C had a small but significantly lower pH (pH = 4.25) compared with yogurt smoothed at 15 and 20°C (pH = 4.29). During cooling and storage, bacteria are still fully active until 20°C, which probably explains the slightly more acidic pH in yogurts smoothed at 25°C because they spent a longer time over 20°C. Moreover, the enzymes responsible for lactic acid production in lactic acid bacteria are still active between 0 and 5°C, promoting post-acidification (Champagne et al., 1998; Antunes et al., 2005; Kailasapathy, 2006). When looking at bacterial counts, the ST enumeration and the ratio of ST to LB were not affected by the yogurt milk ratio or smoothing temperature (Appendix Figure A1). However, LB counts in yogurts were affected by the

smoothing temperature ($P < 0.05$; Appendix Figure A1). Yogurt smoothed at 25°C contained 10% more LB compared with yogurt smoothed at 15°C. This slight difference in LB population could be due to the temperature above 20°C, as observed for post-acidification during storage. Surprisingly, post-acidification in R3.9 yogurt was higher, although its LB population was not different from other yogurts. The results of Antunes et al. (2005) showed no relationship between WPC addition and survival of LB and ST, but they showed that specific probiotic strains of lactic acid bacteria had better survival when yogurts were enriched with WPC. This suggests that the influence of CN:WP ratio on bacteria population is strain-specific.

Particle Size Analyses and Gel Structures

Digital microscopic images of gels are presented in Figure 3. As seen in microscopic images, yogurt microstructure can be described according to 3 main elements (Gilbert et al., 2020a,b): (1) microgels (dense white particles in images); (2) reorganized protein gel around microgels; (3) serum-rich zones (protein-depleted black areas; Figure 3, top panel). Microscopic images showed that R1.5 was highly heterogeneous, with lower amount of reorganized protein gels, more serum separation, and large coarse particles (microgels) compared with R2.8 and R3.9 (Figure 3, bottom panel). Also, when smoothing temperature increased, microgel sizes tended to increase.

Quantification of the surface occupied by microgels and their size by image analysis confirmed that microgel size was influenced by the interaction between storage time and ratio ($P < 0.0001$; Figure 4A) and by the smoothing temperature ($P < 0.05$; Figure 4B). The R1.5 yogurt gels had the largest microgels, and their size increased during storage from 7,500 to 11,500 μm^2 .

Figure 2. pH of stirred yogurts smoothed at different temperatures during storage. (A) Effects of casein-to-whey protein ratio (R1.5, R2.8, R3.9) and storage time [d 1 (dark gray), 9 (mid gray), and 23 (light gray) after production] on pH. (B) Effects of smoothing temperature on pH. Results are reported as the means of the significant factor $\pm$ SE (A: $n = 9$; B: $n = 27$). Letters (a-c) represent significant differences ($P < 0.05$).

Figure 3. Description of stirred yogurt gel microstructure (1) and digital microscopy images of stirred yogurt gels at d 1 (2). Yogurts with a casein-to-whey protein ratio of 1.5 (A, D), 2.8 (B, E), or 3.9 (C, F) at 2 smoothing temperatures (15°C, A–C; or 25°C, D–F) are presented. Black scale bars represent 250 μm .

The R2.8 and R3.9 yogurts had similar microgel sizes during storage, averaging 3,500 μm^2 . A small increase was observed at d 23 for R2.8 microgels surface values, reaching 4,000 μm^2 . Gel NHI measured on the same image varied only depending on milk CN:WP ratio ($P < 0.0001$; data not shown). Higher NHI values were found in R1.5 samples (index = 44), and no differences were found between R2.8 and R3.9 (index = 15; data not shown). Higher NHI values indicate heterogeneous gel network, as seen in Figure 3.

Microgel sizes measured by laser diffraction (Figure 4C) were influenced by the CN:WP ratio ($P < 0.0001$). Because laser diffraction was measured at d 1 only,

no information was available on time-dependency for this variable. However, in a previous work (Gilbert et al., 2020b), stirred yogurt with CN:WP ratio of 2.8 prepared with the same starter and the same smoothing temperature showed an increase in microgel size over time, as observed with the image analysis method but not by laser diffraction. Microgel sizes measured by laser diffraction indicated that R1.5 stirred yogurt had microgels diameters 2.5 times larger than diameters at R2.8 and R3.9 microgels.

We detected good correlation (0.85; $P < 0.0001$) between microgel sizes obtained by laser diffraction and microgel sizes determined from image analysis at d 1,

Figure 4. Yogurt microgel size measured by image analysis of digital microscope slides (A, B) and by laser diffraction in milk permeate dilution (C). (A) Effects of casein-to-whey protein ratio (R1.5, R2.8, R3.9) and storage time on microgel sizes [d 1 (dark gray), 9 (mid gray), and 23 (light gray) after production]. (B) Effects of smoothing temperature (15°C, 20°C, 25°C) on microgel sizes. (C) Effect of the casein-to-whey protein ratio (R1.5, R2.8, R3.9) on microgel sizes (mean diameter, D[4,3]). Results are reported as the means of significant factor, and bars represent SE (A: n = 9; B: n = 27; C: n = 9). Letters (a–d) represent significant differences ($P < 0.05$).

indicating good consistency between those 2 methods (data not shown). Laser light diffraction technique implies breakdown of the gel structures due to dilution and shearing. The use of those 2 methods allowed us to differentiate 2 organization levels of yogurt microgels:

(1) primary microgels, measured by laser diffraction, and (2) a substructure of agglomerated (aggregated) microgels over storage. This type of aggregated structure has previously been mentioned by van Marle (1998) and Rasmussen et al. (2007).

Because the low CN:WP ratios in yogurt were responsible for large microgel sizes in both image analysis and laser light diffraction, it can be hypothesized that the size of the primary microgels is due to WP content. Several other studies have reported that stirred yogurt with CN:WP ratio lower than 2 presented coarse structures with large microgels and pores creating heterogeneous gel (Cayot et al., 2008; Küçükçetin, 2008; Krzeminski et al., 2011; Jørgensen et al., 2015; Chua et al., 2017; Laiho et al., 2017). In set-style yogurt, low CN:WP ratio led to 2 types of interaction: (1) disulfide bonds that conferred a high elastic modulus to the microgel, and (2) stronger hydrophobic interactions between WP aggregates that led to more cross-linking between protein aggregates inside the flock (Puvanenthiran et al., 2002; Andoyo et al., 2015). Indeed, Laiho et al. (2017) showed that microgels found in yogurts with low CN:WP ratio (<2) would be made of large protein aggregates obtained after heat treatment, which would be linked tightly by soluble WP aggregates through hydrophobic interaction, creating a secondary protein network.

Microgel sizes measured by image analysis were larger in yogurt smoothed at 25°C (Figure 4B). This has not been observed with the laser light diffraction method. Image analysis may be less disruptive and allow low-energy interaction to be maintained intact in weak gels. This allows us to highlight that higher smoothing temperatures favor microgel aggregation during smoothing and storage. Microgel aggregation due to storage or higher smoothing temperature can be explained by hydrophobic interactions of the surface of microgels (Cayot et al., 2003, 2008), which are probably intensified at low CN:WP and favored by higher temperature during smoothing. Working on acidic gelation at room temperature of pre-heated 2% (wt/wt) WP solutions, Alting et al. (2000) demonstrated that WP aggregates form disulfide bonds during acidification at room temperature. Although Weidendorfer et al. (2008) have dismissed the possibility of disulfide bond formation between WP aggregates during storage in stirred yogurt, some authors have suggested that they could be involved in rebodding (Krzeminski et al., 2011; Cheng et al., 2017). Küçükçetin (2008), using similar image analysis and laser diffraction techniques, did find microgels with larger particle sizes with low CN:WP ratio, but did not report aggregation of microgels over time. As discussed in Gilbert et al. (2020b), this may be explained by techniques not being able to detect microgel aggregations, with laser diffraction being too

Figure 5. Firmness (A, B), consistency (C), and flow behavior index of yogurts (D). (A, C, and D) Effects of the casein-to-whey protein ratio (R1.5, R2.8, R3.9) and storage time [d 1 (dark gray), 9 (mid gray), and 23 (light gray) after production]. (B) Effects of smoothing temperature carried out at 15, 20, and 25°C. Results are reported as the means of significant factor, and bars represent SE (A: n = 9; B: n = 27; C: n = 9; D: n = 9). Letters (a–g) represent significant differences ($P < 0.05$).

invasive and image analysis detecting only microgels over 1-mm perimeter.

Rheological Properties of Stirred Gels

Both firmness and viscosity (consistency, flow behavior index) results of yogurts varied along storage and according to the yogurt CN:WP ratio ($P < 0.01$; Figure 5). Smoothing temperature only influenced firmness ($P < 0.01$). Yogurt firmness globally decreased with the reduction of WP content (higher CN:WP ratio; Figure 5A) and increased with smoothing temperature (Figure 5B). Chua et al. (2017) have already reported similar results by testing the effects of both CN:WP ratio and protein content on stirred nonfat yogurt firmness. In their study, yogurt firmness increased with protein content, but, more importantly, yogurts with lower CN:WP ratios (enriched with WPC) were firmer than yogurts with high CN:WP ratios. It could be hypothesized that firmness increases in the present study may be related to WP content in addition to higher protein content. However, it is not possible to dissociate the effects of these factors. In our study, firmness increased over storage time, and this was more pronounced with

higher WP content. Firmness of R1.5 increased during storage by 18.4% between d 1 and 9 and an additional 7.8% after 23 d (Figure 6A). Firmness of R2.8 and R3.9 increased by only 7.1 and 10.1%, respectively, after 9 d, to reach a plateau, as no further increases were observed after 23 d.

The effects of CN:WP ratio on consistency (Figure 5C) had a behavior similar to firmness, which is expected, as this represents the value at zero shear. These 2 variables were significantly correlated ($r = 0.82$; $P < 0.0001$; Appendix Table A1). The R1.5 yogurt had the highest consistency, which increased between d 1 and 23, whereas R2.8 and R3.9 had smaller consistencies that were relatively constant over storage time. Laiho et al. (2017) reported similar results when testing different CN:WP ratios with a constant protein content. Those authors compared the viscosity of yogurts with different CN:WP ratio, decreasing from 4 to 1, and they found that yogurt viscosity significantly increased only when the CN:WP ratio was lowered to values below 1.5. Those results were expected, because addition of WP in yogurt milk before heat treatment has been associated with viscosity increases in yogurt, as reviewed by Sodini et al. (2004) and more recently by Lesme et

Figure 6. Induced syneresis (A, B), bulk water mobility measured by low-frequency proton NMR (^1H -LF-NMR; C, D), and spontaneous syneresis measured by ^1H -LF-NMR (E) of stirred yogurts. (A, C) Effects of the casein-to-whey protein ratio of 1.5 (R1.5), 2.8 (R2.8), or 3.9 (R3.9). (B, D) Effects of storage time (d 1, 9, and 23 after production). (E) Effects of the casein-to-whey protein ratio of 1.5 (R1.5), 2.8 (R2.8), or 3.9 (R3.9) and storage time [d 1 (dark gray), 9 (mid gray), and 23 (light gray) after production] on spontaneous syneresis. Results are reported as the means of significant factor, and bars represent SE (A: $n = 27$; B: $n = 27$; C: $n = 27$; D: $n = 27$; E: $n = 9$). Letters (a-c) represent significant differences ($P < 0.05$). $T_2(3)$ = relaxation time constant; $I_2(4)$ = relative intensity.

al. (2020). The addition of WP before heat treatment allows formation of WP aggregates around the casein micelles and in the serum (Donato and Guyomarc'h, 2009). According to Beaulieu et al. (1999), reducing the CN:WP ratio to 3 or less will saturate casein micelle surface sites where WP aggregates can form during heat treatment, favoring the formation of soluble WP aggregates in the serum phase. The presence of denatured WP attached to the casein micelles and the higher content in soluble WP aggregates increase gel network interconnectivity, which will increase strength,

elasticity, firmness, and viscosity of the gel (Lesme et al., 2020).

The flow behavior index (n) was similar for R2.8 and R3.9 yogurts ($n = 0.25$) and did not change due to storage (Figure 5D). However, R1.5 yogurts had a higher flow behavior index than the other ratios (Figure 5D). This value was lower after 9 d of storage, which reflects a faster decrease of viscosity with increasing shear rate. When n is higher, yogurt structure may have better resistance to breakdown when submitted to shear (Barnes, 1997; Abu-Jdayil et al., 2013). Our

R1.5 yogurts were firmer and their structure was more resistant to shear than R3.9 and R2.8 yogurts.

During storage, high correlation was found between microgel sizes measured by microscopic image analysis and both firmness ($r = 0.87$; $P < 0.01$) and consistency ($r = 0.74$; $P < 0.01$; Appendix Table A1). Because consistency and firmness were also highly correlated (Appendix Table A1), microgel size was probably a common structural factor influencing both consistency and firmness. Microgels with larger particle sizes would contribute to increase yogurt consistency and firmness. This agrees with Zoon (2003) and Walstra et al. (2005), who previously proposed a simplified model in which stirred yogurt flow properties are functions of stirred yogurt microgels properties (size, elasticity, porosity, volume fraction) and serum (or continuous phase) viscosity.

Increasing smoothing temperature increased firmness but had no effect on flow properties. Firmness increased with higher smoothing temperature (Figure 6B). When comparing flow properties and firmness measurements, these 2 methods do not induce the same level of shearing and destruction into the sample. Viscosity measurement is a more destructive method. Maybe, as observed for laser light diffraction and image analysis, some effects of increasing shear cannot be seen by viscosity measurements due to weaker interactions (weakly aggregated microgels) compared with texture measurements.

Syneresis and Bulk Water Mobility

The ability to force serum out of the gel (induced syneresis) depended on the CN:WP ratio ($P < 0.01$) and storage time ($P < 0.0001$; Figure 6A and B). Induced syneresis was reduced in enriched WP yogurts (Figure 6A), as previously reported (Sodini et al., 2004; Andoyo et al., 2015). Serra et al. (2009) attributed a decrease of induced syneresis during storage to post-acidification. According to those authors, post-acidification increases protein-protein interactions, which strengthen the gel and reduce the measured induced syneresis. A limit of the induced syneresis method is that it measures simultaneously spontaneous syneresis and serum forced out of the gel during centrifugation, and it is influenced by gel rigidity (Lucey, 2001). Other techniques, such as ^1H -LF-NMR, allow monitoring of water mobility and spontaneous syneresis within a gel.

When ^1H -LF-NMR was investigated previously in Gilbert et al. (2020a) to describe yogurt's water retention, 4 different proton populations were found in yogurt. Two of them have been identified as populations linked to water retention of yogurts. The third

proton population, with a relaxation time $T_2(3) > 100$ ms and a relative signal intensity $I_2(3) > 60\%$, represents the bulk water mobility in the yogurt. The fourth population is defined by a relaxation time $T_2(4) \geq 1,000$ ms and represents the water inside the serum that has been expelled by the gel (spontaneous syneresis). In other words, $T_2(3)$ has been identified as bulk water mobility inside the protein network; the lower the bulk water mobility is, the smaller $T_2(3)$ becomes, whereas $I_2(4)$ represents how much serum is separated from the gel (Gilbert et al., 2020a). Figure 6 present results for $T_2(3)$ and $I_2(4)$.

The $T_2(3)$ depended on the CN:WP ratio and storage time ($P < 0.0001$). Figure 6 (C, D) shows that $T_2(3)$ decreased with lower CN:WP ratios and storage time. Furthermore, $T_2(3)$ was correlated with induced syneresis ($r = 0.63$; $P < 0.0001$; Appendix Table A1). Bulk water mobility in the network [$T_2(3)$] can be limited by small gel pore sizes or high serum viscosity (Han et al., 1995; Hinrichs et al., 2003; Williams et al., 2011). During heat treatment, when the CN:WP ratio gets lower, soluble WP aggregates concentration in serum increases (Beaulieu et al., 1999; Vasbinder et al., 2003). A secondary protein network of WP aggregates may hinder CN interactions during acidic gelation and reduce network shrinking (Vasbinder et al., 2001; Andoyo et al., 2015; Laiho et al., 2017). However, the network becomes denser because of more interactions between WP aggregates, explaining lower induced syneresis and bulk water mobility when CN:WP ratio was low (Figure 6 A, C). It should be noted that the reduction of induced syneresis and $T_2(3)$ could also be related to the increase of protein content with lower CN:WP ratio (Sodini et al., 2004), making it difficult to differentiate the effects of protein concentration and WP content.

The relative signal intensity $I_2(4)$ measured by ^1H -L-NMR evolved with storage time and depends on CN:WP ratio in yogurt ($P < 0.01$). Both R1.5 and R3.9 had increasing $I_2(4)$ over time, whereas R2.8 showed low stable $I_2(4)$ values (Figure 6E). In R1.5 yogurts, the higher $I_2(4)$ corresponded with higher spontaneous syneresis. As seen in image analyses, R1.5 samples presented local phase separation due to heterogeneous gel and flocculated microgels, which may have led to sedimentation and thus increased spontaneous syneresis over time. Laiho et al. (2017) obtained similar results when characterizing the visual appearance of yogurt with different CN:WP ratios by sensorial analysis at d 1 after production. They found that lowering the CN:WP ratio to 1.5:1 and below increased the granular aspect of the product. A ratio of 1:1 significantly increased panelists' perception of the watery edge of the product. Lesme et al. (2020) mentioned other studies reporting

whelying off in yogurts formulated with higher WP content. Higher spontaneous syneresis [$I_2(4)$] found in R3.9 gels at d 23 was probably due to the gel shrinking during storage. Indeed, in R3.9 yogurt, the rebodding may have been affected by post-acidification (Figure 2). In R3.9 yogurts, the low WP content offers less interference to CN interactions, allowing gel contraction, and thus more serum is expelled during storage. The stability of R2.8 against serum separation is attributable to its homogeneous gel combined with a network of denatured WP aggregates, forming a secondary gel and hindering CN interaction.

To sum up, this study showed how formulation parameters such as the CN:WP ratio and smoothing temperature can be used to modulate yogurts properties. Those variables dictated yogurt gel microstructure. Similarly to Gilbert et al. (2020a), we found that it was possible to show the close relationship between microstructure, rheological properties, and syneresis using 2 methods (image analysis of microscopic pictures and ^1H -LF-NMR) that are less destructive than laser diffraction and induced syneresis. In the present study, using more experimental conditions with a factorial plan design, these methods proved their relevance to study complex systems such as stirred yogurt. This allowed better insight on stirred yogurt gels at different length scales and consideration of the effects of weak interactions inside the gel.

CONCLUSIONS

This study demonstrated that microgels seemed to be a structural determinant of textural attributes. High whey protein content led to larger and more solid microgels that aggregated over storage and were responsible for higher firmness and viscosity. Higher smoothing temperature also led to larger particles affecting only firmness measurements. Only less-destructive methods, such as image analysis and firmness, were able to detect that effect. Although increasing whey protein content caused less bulk water mobility in the gel, serum tended to separate less at an intermediate whey protein content level (R2.8), because microgel aggregation was moderated compared with R1.5, and the casein network would be less prone to shrinking during storage compared with R3.9. Both formulation and process controlled stirred yogurt microstructures. In the future, it would be of interest to examine interactions between smoothing temperature and other parameters of standardization, such as increased total solids, increased protein content, or, more interestingly, the use of exopolysaccharide-producing starters, as this aligns well with consumers' concern for clean labeling.

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APPENDIX

Figure A1. Enumeration of *Streptococcus thermophilus* (black bars), and *Lactobacillus delbrueckii* ssp. *bulgaricus* (gray bars) at d 0 of production after cooling at 4°C. Yogurts were smoothed at 15°C, 20°C, or 25°C. Error bars represent SE ($n = 27$). Letters (a, b, A) represent significant differences between smoothing temperatures ($P < 0.05$).

Table A1. Pearson's pairwise correlation coefficient between different instrumental measurements¹

Item	SDM	GH	T ₂ (3)	T ₂ (4)	I ₂ (4)	K	N	Firm	Syn	pH
SDM	1.00	0.68*	−0.79*	−0.47	0.42	0.74*	0.56*	0.87*	−0.21	0.16
GH		1.00	−0.80*	−0.46*	0.40	0.65*	0.72*	0.84*	−0.09	0.28
T ₂ (3)			1.00	0.55*	−0.41	−0.72*	−0.75*	−0.92*	0.63*	−0.15
T ₂ (4)				1.00	−0.50*	−0.44	−0.42	−0.50*	0.04	−0.05
I ₂ (4)					1.00	0.41	0.18	0.43*	0.20	−0.23
K						1.00	0.25	0.82*	−0.10	0.19
N							1.00	0.66*	−0.24	0.19
Firm								1.00	−0.30	0.14
Syn									1.00	0.09
pH										1.00

¹SDM = microgel surfaces measured by digital microscopy. GH = gel heterogeneity. T₂(i) = transversal relaxation time constant. I₂(i) = relative intensity of individual proton pool. K = consistency index of the power law model. N = shear behavior index of the power law model. Firm = firmness. Syn = syneresis.

*Correlation coefficients >0.5 and significant at $P < 0.01$.