Studying stirred yogurt microstructure and its correlation to physical properties: a review.

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Abstract:

Microstructure is an important part of the understanding and the control of food properties as rheological properties, water holding and sensory properties. Stirred yogurt microstructure is being under study for decades. Observations at several length scales have been used to probe the structure. Some methods using optical techniques were recently introduced to provide a quick microstructure assessment of stirred yogurt. This review aims to provide a description of stirred yogurt microstructure and a short overview of the main techniques to characterize stirred yogurt microstructure allowing to highlight their complementarity. In general, stirred yogurt microstructure is described as a suspension of interconnected microgels into a continuous serum phase. While the relationship between yogurt microstructure and its physical and sensory properties has been discussed in numerous reviews, models or studies the impact of microgels sizes on rheological properties, water holding capacity, and creaminess, has not always been

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confirmed. Even if, other features such as microgels aggregation, shape, and compaction have shown to be involved in sensory or physical properties of stirred yogurt gel, a challenge remains for the characterization of microstructural characteristics of microgels without destructuring the network.

Keywords: stirred yogurt microstructure, microgels, rheological properties, microscopy, particle size

1. Introduction:

The processing of set and stirred fermented milk gels is a well-known and widely used food technology (Aryana & Olson, 2017; Tamime & Robinson, 2007b). From stirred yogurt to cream cheese, a large variety of processes and techniques are available to obtain a large variety of texture, taste and appearance. These attributes are assessed by consumers to evaluate the products as of a good quality or with defects. For example, serum separation from yogurt (syneresis) during storage is generally considered as a defect, while a firm and thick product is a sign of a good quality for stirred or concentrated yogurt but not in drinking yogurt. Many of yogurts' attributes can be controlled using process and formulation (Sodini, Remeuf, Haddad, & Corrieu, 2004).

The effect of dairy mix formulation on fermented dairy gels has been extensively studied and reviewed over the past years (Aryana et al., 2017; Karam et al., 2013; Lesme et al., 2020; Lucey, 2004; Sodini et al., 2004). In order to obtain viscous products with a good water retention capacity, it is advised to increase dry matter, protein and fat contents. The reduction of the casein to whey protein (CN:WP) ratio compared to the natural ratio in milk (4:1) is also commonly used for similar results. However, this ratio should not go under a value of 2 otherwise the

product is described as grainy and can display syneresis (Gilbert, Rioux, St-Gelais, & Turgeon, Submitted; Jørgensen et al., 2015; Krzeminski, Großhable, & Hinrichs, 2011; Lesme et al., 2020; Lucey, 2004). Other ingredient additions such as stabilizer (gelatin, starch, pectin,…) (Sodini et al., 2004) or particulated whey proteins (Lesme et al., 2020) are also reported to allow a better control of yogurt properties, specifically in low-fat or zero fat products (Ares et al., 2007; Fiszman, Lluch, & Salvador, 1999; Hess, Roberts, & Ziegler, 1997; Lesme et al., 2020).

Several processing steps are common to almost all fermented dairy products: milk standardization, homogenization, heat treatment, and fermentation (Chandan & O ' Rell, 2013; Lucey, 2004). Each of these steps having consequences on the final product properties. For example, heat treatments more intense than pasteurization (e.g. 95 °C / 5 min or 85 °C/30 min) or homogenization gives yogurts with higher viscosity and firmness while the syneresis is reduced (Sodini et al., 2004). Set-style yogurts are fermented directly into their packaging, cooled down, stored and then consumed as is. Stirred acid dairy gel (stirred yogurt, drinking yogurt) or concentrated product (Greek yogurt, labneh, cream cheese) are fermented in large vats before being pumped, filtered, smoothed, mixed with fruits or other flavouring agents and cooled (Chandan et al., 2013; Mokoonlall, Nöbel, & Hinrichs, 2016; Tamime et al., 2007b). The sequence, conditions and combinations of processing steps are specific to each product and manufacturer. It has been highlighted for stirred products (Afonso & Maia, 1999; Mokoonlall et al., 2016) that each processing step with an increasing level of shear intensity applied to the product is responsible for changes in rheological properties such as lower viscosity, elasticity and firmness (Mokoonlall et al., 2016). During storage, rheological properties are recovered partially due to a phenomenon called rebodying due to gel reorganization (Abu Jdayil & Hazim, 2002; Renan et al., 2008; Serra, Trujillo, Guamis, & Ferragut, 2009). More recently, it has been noticed that not only the shear intensity of the process, but the order and temperature of processing steps impact strongly stirred yogurt properties and their evolution during storage

(Guénard-Lampron et al., 2020a; Guénard-Lampron, St-Gelais, Villeneuve, & Turgeon, 2018; Guénard-Lampron, Villeneuve, St-Gelais, & Turgeon, 2020b; Leroux, 2018; Lussier, 2017). It has been shown that cooling before smoothing, using a plate heat exchanger rather than a tubular heat exchanger, or smoothing at low temperature led to yogurt with a reduced firmness measured using a compression test, and possibly reduced rebodying effects during storage.

The changes in acid dairy gels (set or stirred) properties that are observed when formulation or process are modified have often been correlated with a modification in gel microstructure (Gilbert, 2020; Harte et al., 2002; Laiho et al., 2017; Lucey, Munro, & Singh, 1998a; Torres et al., 2018; van Marle, van Den Ende, de Kruif, & Mellema, 1999). The main structural characteristics used to describe the gel (set or stirred) and known to impact, or correlate with, acid gel properties are the changes in network's crosslinking density, pore size, network heterogeneity or protein particle sizes (Guénard-Lampron et al., 2020a; Lee, W. J. & Lucey, 2004; Puvanenthiran, Williams, & Augustin, 2002). However, in literature, many different techniques are used to characterize and describe acid dairy gels, offering different length-scales of observations but also inducing various levels of sample destructuration (Gilbert, Rioux, St-Gelais, & Turgeon, 2020b; Lee, W. J. et al., 2004; Moussier et al., 2019a; van Marle, 1998). Theoretical representation of casein gels microstructure considers that structural features of each length-scale level are interconnected and each has a specific impact on physical characteristics (rheology, syneresis…) of the final products (Mellema, Walstra, van Opheusden, & van Vliet, 2002; van Marle et al., 1999).

The microstructure of stirred fermented dairy products is often described as a "suspension" of microgels (weakly interconnected) into serum (Gilbert et al., 2020b; Lucey, 2004; Moussier et al., 2019a; Moussier, Huc-Mathis, Michon, & Bosc, 2019b; van Marle et al., 1999; Zoon, 2003). Microgels are generally defined as individual or aggregated fragments of set gel that were not destroyed during shearing (Gilbert et al., 2020b; Mellema et al., 2002; Moussier et al., 2019b;

Rasmussen, Janhøj, & Ipsen, 2007; van Marle, 1998; Weidendorfer, Bienias, & Hinrichs, 2008). Size, shape and the tendency to aggregate of microgels were characterized (Gilbert et al., 2020b; Guénard-Lampron et al., 2020a; Rasmussen et al., 2007). In their review Mokoonlall et al. (2016) explain that microgel size is impacted by the post-fermentation processing steps depending on shear intensity or temperature as observed by Zhang, Folkenberg, Amigo, and Ipsen (2016), Gilbert et al. (2020b) and Guénard-Lampron et al. (2020a) using different techniques of microgels characterization. Milk formulation and pre-fermentation treatments (heat-treatment, homogenization) as well as fermentation conditions (temperature, time, starters…) have been reported to modulate microgels sizes and consequently functional properties (Cayot et al., 2008; Ciron, Gee, Kelly, & Auty, 2010, 2012; Körzendörfer et al., 2018; Krzeminski et al., 2011; Küçükçetin, Weidendorfer, & Hinrichs, 2008b; Laiho et al., 2017; Nöbel et al., 2016; Rasmussen et al., 2007).

The control of formulation and process aims at producing a fermented product appreciated by the consumer. Sensorial analyses are from far the best way to predict consumer hedonic response, but they are time-consuming and costly. Therefore, there is a need for methodological approaches to measure and predict the sensorial attributes. However, a challenge remains to understand the structural and physical properties of stirred yogurt involved into the sensorial feeling during consumption. If particle sizes seem to partly explain the smooth and creamy characteristics of stirred yoghurt gels (Cayot et al., 2008; Laiho et al., 2017; Sonne, Busch-Stockfisch, Weiss, & Hinrichs, 2014), these two characteristics also depend on the nature and rigidity of the particles in the stirred gel (Baniasadidehkordi & Joyner, 2019b, 2019c; Krzeminski et al., 2013). Physical characteristics usually measured using viscometry, viscoelastic characterization, and texturometry (Mortazavian, Rezaei, & Sohrabvandi, 2009) are still not sufficient to explain sensory results. Moreover, during consumption, physical properties and sensory perception are also influenced by the presence of saliva (Scholten, 2017; Sonne et

al., 2014; Vardhanabhuti et al., 2010). Recently, new techniques such as tribology have been introduced to model tongue-palate interaction in the mouth in the presence of saliva (Joyner, 2018; Scholten, 2017).

This review aims to report the different strategies used in the literature to understand stirred fermented gel microstructure and how the microstructures at different scales correlate with the physical properties of stirred fermented gel. A brief description of stirred fermented dairy gels microstructure formation is proposed based on the literature. Different techniques of measurement and observation are presented and compared. Finally, this review provides a discussion about the relationships and correlations found by different authors between microstructural features and stirred gel properties.

2. Stirred yogurt microstructure

Milk gelation is defined as the physical transition during which the milk changes from a low viscosity Newtonian fluid to a semi-solid or to a solid state (Foegeding, Vardhanabhuti, & Yang, 2011). In yoghurts and fermented milks, it is achieved through a slow acidification by lactic acid bacteria. Milk acidification induces major changes into the milk protein organization (Dalgleish & Corredig, 2012; Tamime, Hassan, Farnworth, & Toba, 2007a) resulting in the formation of a porous protein network in which the serum is entrapped (Lucey, 2004; Tamime et al., 2007a). Depending on the milk heat-treatment, denatured whey proteins contribute to increase the density of the protein network and it changes gel properties in both set gels (Gregersen et al., 2021; Puvanenthiran et al., 2002) and stirred gels (Gilbert et al., Submitted; Jørgensen et al., 2015; Laiho et al., 2017). Milk fat content has an important effect on gel structure especially if a homogenization step is applied to reduce fat globule size. The homogenized fat globule interface consequently includes caseins and may then participate actively to the gel network and

impact their properties (Ciron et al., 2010; Ciron, Gee, Kelly, & Auty, 2011; Gregersen et al., 2021; Lucey et al., 1998a). Finally, if stabilizers are added or exopolysaccharides (EPS) producing starters are used, the network is going to be modified in accordance with polymer characteristics through associative or segregative interactions with milk proteins (Corredig, Sharafbafi, & Kristo, 2011; Crispín-Isidro et al., 2015; Hassan, Ipsen, Janzen, & Qvist, 2003b; Sodini et al., 2004). Exopolysaccharides have different composition and structural features (capsular or free, flexibility, linear or ramified structure, neutral or charged, molecular weight) which will induce different contributions to the network structuration and their effect on gel structure and properties (Gentès, St-Gelais, & Turgeon, 2013; Gomand, 2019; Hess et al., 1997; van Marle, 1998; Zhang et al., 2016).

Stirred gel microstructure results from a controlled destructuration of the set gel (Figure 1) occurring during processing (stirring, pumping, smoothing, …). When shear treatments are applied, some intermolecular bonds responsible of the network structure and integrity may be broken and the gel is reorganized. Acidic gels are generally referred to as aggregated particle gels (Horne, 1999; Mellema et al., 2002; Puvanenthiran et al., 2002; van Marle et al., 1999) and several models were used to described them (Lucey, 2016). Repulsive electrostatic interactions between casein micelles in milk are neutralized during acidification allowing the formation on an aggregated protein network (Dalgleish et al., 2012). Mellema et al. (2002) used a fractal scaling model to study rearrangement in a casein gel. This model defines microstructural organization at four length scales: Sub-particle (< 0.2 µm); particle (0.2-1 µm), fractal cluster (1-40 µm) and macroscopic level (whole gel). At the sub-particle level are found the elementary building blocks that can rearrange, or aggregate. For fermented dairy product it would be protein, small protein aggregates, fat globules, and stabilizing polymers or EPS (Lucey, 2004). At the particle level are found the rigid segments composed of a tight rearrangement and linkage of several building blocks (Puvanenthiran et al., 2002). The fractal cluster level is made of

strands of particles which according to Mellema et al. (2002) are the stress carrying blocks of the whole structure. Finally, the whole gel is the macroscopic result of the complex assembly of fractal clusters altogether. During stirred yogurt process, the resistance of each of these levels of structure toward mechanical treatment in the set gel is determinant for stirred yogurt microstructure in the final product. According to van Marle (1998), during the shearing process of yogurt gel, weaker strands may rupture first, leading to the formation of unbroken gel fragments defining the future microgels (Figure 1). Then, during the process, more shear forces and friction will induce erosion and more fragmentation and reduction of their size until the end of the process (Figure 1) (Javanmard, Wong, Howes, & Stokes, 2018; Mokoonlall et al., 2016).

Microgels are discrete protein dense structures observed in the stirred yogurt at the end of the processing. Depending on experimental conditions microgels are reported to vary in size between few µm to few millimeters (Hahn et al., 2012a; Körzendörfer et al., 2018; Moussier et al., 2019a; van Marle et al., 1999). The size, shape, degree of compaction and heterogeneity of microgels depend on two main factors: (i) the network rigidity of the set gel before shearing (strength of the different bonds maintaining its structure together), and (ii) the intensity and sequence of operation during the shearing process after fermentation. The set gel network rigidity, is largely determined by three factors: dairy mix formulation, pre-treatment (homogenization, heat-treatment, …) and fermentation conditions (temperature, starters, inoculation rate, duration…) (Lesme et al., 2020; Lucey & Singh, 1998b; Sodini et al., 2004). The effect of each processing unit operation on yogurt is modulated by both the shear intensity and conditions during the processing. (Gilbert, 2020; Gilbert et al., 2020b; Guénard-Lampron et al., 2020a; Javanmard et al., 2018; Zhang et al., 2016). For example, recent works have shown that lower smoothing temperature produced yogurt with smaller microgels (Gilbert, 2020; Gilbert et al., Submitted; Guénard-Lampron et al., 2020a).

During storage and up to the consumption, microgels are able to re-aggregate, forming new structures, explaining the phenomenon called rebodying. Rebodying is the change of the rheological properties of the product presenting higher viscosity, elasticity (storage modulus measured by oscillatory rheology) during storage (Renan et al., 2008, 2009). The change in microstructure also modifies syneresis (Mellema et al., 2002; Mokoonlall et al., 2016). Post-processing changes in particle size has been studied at length-scales ranging from few µm to few mm using different analytical techniques (Gilbert et al., 2020b; Guénard-Lampron et al., 2020a; Rasmussen et al., 2007) and recent works performed at length scales larger than 10 µm highlighted aggregation during storage. In Gilbert et al. (Submitted), aggregation during storage, measured as microgels particle size growth, was observed only at lower CN:WP ratio (whey proteins addition into the formulation). Although the rebodying did occur in yogurt without whey protein addition, in this study, there was no significant difference in microgel sizes. Similarly Körzendörfer, Nöbel, and Hinrichs (2017) noticed that the presence of EPS could hinder microgel aggregation. These observations at larger length scales does not exclude that aggregation phenomenon may occur at a smaller scale (< 10 µm). Several process parameters such as, for instance, starters, machinery vibration during processing, temperature or holding time in vats may also influence aggregation of microgels (Gilbert, 2020; Hahn, Sramek, Nöbel, & Hinrichs, 2012b; Hahn et al., 2012c; Körzendörfer et al., 2017; Nöbel et al., 2016; Rasmussen et al., 2007).

Rebodying has also been attributed to the swelling of microgels during cooling (Lucey, 2004; Weidendorfer et al., 2008) based on the assumption that hydrophobic interactions get weaker at lower temperature leading to an increase in microgel voluminosity (Mokoonlall et al., 2016). Swelling with lowering the temperature has been observed for casein micelles at neutral pH (Nobel, Weidendorfer, & Hinrichs, 2012; Walstra, 1990) or in casein hydrogels crosslinked (transglutaminase) at pH 5.7 (Kruif et al., 2015). In yogurts, it would be consistent with the

results of Gilbert et al. (2020b) where higher smoothing temperature (42 °C *vs* 20 °C) led to larger microgels in stirred yogurt after storage at 4 °C. The microgel swelling during rebodying was also in accordance with the observations of Guénard-Lampron et al. (2020a) who have seen a reduction of pore areas in CLSM pictures of stirred yogurt between day 1 and day 22 after production. This corresponds to the fractal cluster scale defined by Mellema et al. (2002). Similarly, Javanmard et al. (2018) highlighted the presence of aggregation phenomenon as soon as the shear treatment stops or is reduced in intensity. An intense pre-shear of the acidic dairy gels (1000 s^{-1}, 600 s) followed by a 2 min period of relaxation time, and then a second shear period (shear rates: 0.001-100 s^{-1}) until steady state allowed to observe that, microgels were able to reorganize and aggregate under shear. Sizes were larger for lower shear rates. The microgel average size D(4,3) was 11 µm and it grew to values ranging from 61±17 to 223±25 µm after shear rates between 10 and 0,001 s^{-1} . Lower shear rate (≤0.1 s^{-1}) profiles were bimodal with a first population around 40 µm and the second population over 200 µm. This experiment nicely shows the evolutive behavior of microgel structuration in acidic dairy gels and that rebodying starts as soon as the shear treatment is interrupted.

The notion of length-scale, particle interaction, and molecular interaction are essential to describe the stirred gel microstructure. Each technique used to probe stirred fermented dairy gel microstructure is characterized by a specific scale of observation. The choice of a technique to characterize stirred yogurt microstructure should be based on a good understanding of strengths and limits of each approach and this will be discussed in the following section.

3. Techniques to observe stirred yogurt microstructure at different length-scales

3.1. Microstructure characterization and microgel size measurement

A large variety of techniques are available to characterize stirred gel microstructure giving access to different scales of observation. Scanning and transmission electronic microscopy allow to distinguish structural elements which are the building blocks and strands of the yogurt structure. From the wide variety of techniques used in the literature to characterize stirred yogurt microstructure (Table 1), the smallest sizes of microgels detected are between 5 to 10 µm (Moussier et al., 2019a), while the larger sizes were over 1 mm (Körzendörfer et al., 2017; Küçükçetin, A., 2008). The structure described as "microgels suspended into serum" can be observed at small scale (1 to 100 µm) (Gregersen et al., 2021; Laiho et al., 2017; Zhang et al., 2016) with well-defined individual microgels. At larger scale (10 µm to few mm) (Gilbert, Rioux, St-Gelais, & Turgeon, 2020a; Gilbert et al., 2020b) the network seems to be separated in three main structures: microgels, reorganized network, and serum pouches (Figure 2). It can be argued that the reorganized network observed, in this case, is simply a complex assembly of very small microgels (< 10 µm) that were too small to be differentiated by the analytical technique used (optical microscopy).

Techniques can be classified according to different criteria: sample preparation with or without dispersion into water or other aqueous dispersant, methods based on direct observation (microscopy), measurement of physical characteristics (particle size), and dynamic *vs* static techniques (if samples are measured under shearing or at rest) (Washington, 1992). Dispersion and agitation of samples are considered partially destructive and some structural information of the stirred gel may be lost (Gilbert et al., 2020b). Laser diffraction and sieving methods are statistically highly accurate giving access to a broad range of particle size and sample dispersion is needed. Observation of the structure and counting techniques using microscopy and image analysis have a lower statistical accuracy (Kippax, 2005; Washington, 1992) but provide high resolution details and a large variety of information about the structure (shape, compaction, …). Table 1 presents a non-exhaustive list of available techniques to characterize stirred yogurt gel

266 microstructure and the present section aims to give a brief description of the main techniques
267 found in literature.

268 *3.1.1 Laser diffraction spectrometry*

269 Laser diffraction is the granulometric technique the most widely used for particle size analysis
270 in many scientific field (Washington, 1992). Since it became available decades ago, it
271 progressively replaced the use of older techniques such as sieving or sedimentation (Bürkholz
272 & Polke, 1984; Washington, 1992). This method relies on the properties of suspended particles
273 to scatter a specific beam of light depending on their sizes, their refractive properties and the
274 dispersant refractive properties (Bürkholz et al., 1984; Washington, 1992). The main limitations
275 and source of error which should be taken into account for stirred yogurt gels characterization
276 are a possible overestimation of larger particles in the distribution as it is volume-weighed
277 (Washington, 1992) and the calculation models which has been developed for spherical particles
278 (surface-equivalent sphere, volume-equivalent sphere) while microgels and protein aggregates
279 are not always spherical (Gomand, 2019; Moussier et al., 2019a). For specification on other
280 important features that may impact accuracy of this technic (type of laser used, choice of
281 refractive index for the particle and the dispersant, theoretical model used, …) further reading
282 in the following review articles is suggested (Bürkholz et al., 1984; Kippax, 2005; Lee Black,
283 McQuay, & Bonin, 1996; Washington, 1992).

284 Dilution of sample can also mask some phenomenon as experienced by (Gilbert et al., 2020b)
285 while microgel aggregation during storage was observable with imaging techniques but not by
286 laser diffraction. Others attributed this difference to non-spherical particle orientation which
287 could influence imaging techniques but not laser diffraction measurement (Guénard-Lampron
288 et al., 2020a). Rasmussen et al. (2007) proposed a different approach to identify microgel
289 aggregation using laser diffraction. They specifically considered the particle size region on the

laser diffraction profile corresponding to sizes larger than their smoothing screen. They defined microgel aggregation as the proportion of microgels being larger than the screen size.

3.1.2 Direct observation of structures combined with image analysis

The use of microscopic techniques allow direct observation of the structures and can be used to probe the localization of different constituents as proteins, hydrocolloids and their interactions in the matrix (polysaccharides, protein aggregates, fat droplets, …) (Gaonkar & McPherson, 2016; Kaláb, Allan-Wojtas, & Miller, 1995). As for any biological or food sample care must be taken to adapt sample preparation and choose the observation technique to limit sample modification and artifact. After image acquisition, image analysis can be used to translate qualitative observation into numerical values to characterize the microgels by their size, shape, length, solidity (Gilbert et al., 2020b; Guénard-Lampron et al., 2020a; Hahn et al., 2012a; Moussier et al., 2019a). It can also be used to characterize gel roughness or gel heterogeneity (Gilbert et al., 2020b; Küçükçetin et al., 2008b), inter-pore distance or distance between different structures' centers of mass (Glover et al., 2019; Gregersen et al., 2021), approximate the fractal dimension of the network (Andoyo, Guyomarc'h, Burel, & Famelart, 2015; Moussier et al., 2019a) or the volume occupied by pores compared to the network in the image (Guénard-Lampron et al., 2020a).

Imaging techniques can be classified depending on their length-scale. To observe the building block particles and the fractal cluster level, transmission (TEM) or scanning electron microscopy (SEM) have be used (Kalab, Emmons, & Sargant, 1975; Remeuf, Mohammed, Sodini, & Tissier, 2003). But the most common techniques used to probe microstructure of stirred fermented dairy products is confocal laser scanning microscopy (CLSM). With a window of observation of approximately few hundred square microns (Gregersen et al., 2021; Moussier et al., 2019a; Zhang et al., 2016), it can differentiate structures as small as 0.5 µm and gives information on the three-dimensional organization of the network if images are taken at different

depths of the gel (Moussier et al., 2019a). The scale length of observation would be in-between the particle level and the fractal cluster level in the fractal scaling model. The structure of microgels has been observed, described and differentiated by many authors using this technique on undiluted samples (Gregersen et al., 2021; Zhang et al., 2016). The choice of a microscopic technique has to be done based on the information sought. TEM and SEM gives access to smaller structures, but the preparation procedure may induce artefacts and should be realized with great care. Sample preparation is very simple for CLSM, the gel may be gently placed in to the well of the microscopic slide before observation. CLSM visualization is based on fluorescence of component at specific wavelengths and the use of probes allows to distinguish two or more different macromolecules simultaneously by superposition of images taken at different wavelength. As for other food matrices, care must be taken that the probes do not induce changes in the gel. EPS in the network have been observed by Cryo-SEM or CLSM using lectin as an EPS probe (Tamime et al., 2007a). However, in CLSM micrograph, EPS may appear as thicker zones than what is observed in cryo-SEM due to the lower resolution of CLSM compared to Cryo-SEM.

Recently, Moussier et al. (2019a) combined CLSM techniques with a pre-dispersion of the sample to characterize microgels using image analysis. They compared this technique of microgel size analysis to laser diffraction and another technique called dynamic image analysis. In this last technique the sample is dispersed and stirred into water to be pumped into an analyzing cell. Micrographs of the dispersion are taken by a camera to determine microgels sizes. The range of sizes measured depends on the device and accessories used: Hahn et al. (2012a) reported sizes ranging from 0.4 µm to 2.5 mm, while for Moussier et al. (2019a) it was between 1 to 750 µm. When comparing the three techniques (laser diffraction, CLSM, dynamic size analysis), Moussier et al. (2019a) found similar results for size distributions, but dynamic image analysis tended to give fractal dimension values lower than laser diffraction or CLSM

image analysis. Fractal dimension allows to give a representation of microgel particles without the assumption of sphericity. In Hahn et al. (2012b), the use of microgel solidity and lengths data helped to detect microgel aggregation happening during cream cheese tempering in vat for longer at higher temperature (56 °C/60 min *vs* 38 °C/1 min).

In addition to microgel characteristics some authors have noticed using image analysis of microscopic images that the heterogeneity of the gel network in stirred yogurt can vary radically between samples (Gilbert et al., 2020a, 2020b; Gregersen et al., 2021; Körzendörfer et al., 2017; Küçükçetin, A., 2008). It is especially noticeable when using optical microscopy which has a window of observation of few mm^2 (Gilbert et al., 2020b; Tribst et al., 2020) allowing the differentiation of structures by image analysis ranging from 10 µm to few mm approximately. As the scale is larger than CLSM (Figure 2) it offers a more global view of the product and can overview the global organization of the structure (scale length would be in-between the fractal cluster and the macroscopic gel in the fractal scaling model).

Among the structures described using optical microscopy there are microgels and reorganized protein network. The reorganized network is probably constituted of small microgels tightly assembled, but the scale of observation is too large to differentiate them (Figure 2). When using CLSM the scale allows to clearly see the protein network with the serum pores, but it cannot be interpreted in terms of distinct microgel and reorganized network (Figure 2). This brings-up a question: if CLSM observations were performed on the same products, but once looking inside a microgel (as defined using optical microscopy) and once looking inside the reorganized protein network (as defined using optical microscopy), how different would be the structures?

Other imaging techniques are available to observe and characterize the product at a real scale size. It is the case of a light transmission technique that allows to count microgels larger than 300 µm and to observe the global apparent roughness (opposite of smoothness) of the stirred gel (Körzendörfer et al., 2017; Küçükçetin, Weidendorfer, & Hinrichs, 2009). An electronic

eye can also be used to measure light reflection at a specific angle on the surface of the stirred gel. Image analysis allows to quantify shininess and smoothness of the stirred gel and the results have been related to sensory analysis (Johansen et al., 2008; Møller, 2012). It has been proposed as a tool for a daily quality assessment in industry.

3.1.3 Sample preparation

Each technique requires a specific sample preparation as careful sampling and deposition of the gel into a microscopic well or sample dilution for laser diffraction measurements. The conditions in which the sample is dispersed (choice of dispersant, temperature, shearing intensity…) is a key factor to ensure accuracy (Beliciu & Moraru, 2009; McCrae & Lepoetre, 1996). The dispersant, for example, has to be chosen depending on the nature of the hydrocolloids (fat globule, protein aggregate,…) and the structures under study (Nollet, 2004). Generally for matrices containing casein micelles a dispersant with similar mineral composition and buffering capacities as the dairy matrix continuous phase is preferred (McCrae et al., 1996). Indeed, the dispersant composition can cause milk protein gels or microgel to swell or shrink (Shewan & Stokes, 2013). For instance, Kruif et al. (2015) showed that, casein hydrogels (of around 1 cm^3 obtained by rennet or by crosslinking) and casein micelles swell or shrink when immerged in different buffers with varying pH (from pH 6.9 to 5.1), salts composition ($CaCl_2$ from 10^{-2} to 10^{1} % or NaCl from 10^{-2} to 10^{2} %), or temperature (from 0 to 60 °C). It shows that hydrogels have a different swelling kinetics depending on the buffer used. In the literature, laser diffraction experiments of stirred fermented gels were performed using both acidified milk ultrafiltrate or distilled water. Comparison of distilled water and acidified milk permeate as dispersant to measure particle size of five different commercial yogurts (varying on fat and protein contents) using laser diffraction gave similar results (supplement A: Figure A.1). No noticeable differences were seen in the results obtained using one dispersant or the other. By extrapolation from the results of Kruif et al. (2015), the lack of difference in laser diffraction results between

those two dispersants may be related to the kinetics of microgel swelling or shrinking in those two different buffers.

Most of the time for stirred yogurt, imaging techniques involve minimal sample destructuration. If no coloring probe is necessary, when techniques such as optical microscopy, confocal laser microscopy, transmission imaging, or light reflection are used, the stirred yogurt is simply conditioned inside a spacer, a well or a sample holder and no further treatment is required (Table 1). Thin samples must be prepared to allow microscopic observations. Usually, yogurt would be carefully sampled, placed on a microscopy slide before being gently squashed under a lamella. In the case of fragile structures such as stirred yogurt gels, it would shear and distort the structures under study (Gaonkar et al., 2016). To solve this problem, some authors (Gilbert et al., 2020a, 2020b) chose to adapt a technique used for transmission images (Körzendörfer et al., 2017; Küçükçetin, Ahmet, 2008). The sample is gently spread inside a spacer with a specific depth adapted to observation technique (≈ 150 µm for optical microscopy, 0.6 to 1.2 mm for transmission images) and no lamella is applied in order to minimize sample destructuration. Some techniques (transmission or scanning electron microscopy for instance), or the need to add coloring probes in the sample can damage the structure during sample preparation and introduce artifacts in the micrographs (Kaláb et al., 1995; Lucey et al., 1998b; Tamime et al., 2007a), which is the case for most food matrices. For example, during sample preparation for Cryo-SEM if the sublimation time is too long after instant freezing, it may introduce artifacts due to the freeze-drying of the product (Tamime et al., 2007a).

4. Correlations between microstructure and stirred yogurt properties

4.1. Relationship between microstructure and rheological properties

Rheological and textural measurements are considered as the response at the macroscopic level of the properties at the microscopic level of food (Rao, M. A., 2007); and rheological properties of a yogurt gel network depend on interactions between the building blocks forming strands and between strands as well as on the concentration of strands forming a continuous network (Lucey, 2016; Rao, M. A., 2007). For stirred yogurt, three types of rheological experiments are generally used (Foegeding et al., 2011; Mortazavian et al., 2009): flow experiments using a rotational rheometer to characterize viscosity (apparent viscosity at a given shear rate, flow modelization, …) ; small deformation oscillatory experiments using a rheometer to characterize the visco-elastic properties of the stirred gel (complex viscosity, storage modulus, loss modulus, …), penetration or texture profile analysis (TPA) test using a texturometer to measure yogurt firmness. Recently, large amplitude oscillatory shear (LAOS) was used to probe gel network inner-interaction. In this type of experiments, viscoelastic properties can be studied in function of strain amplitude outside of the linear regime. The advantage of this method is to probe viscoelastic properties in a nearly non-destructive way at low strain (<2%), and to study the matrices behavior when slowly increasing the level of destructuration by increasing the strain toward 100 % and more. Low level of destructuration at small deformation allows to characterize the gel structure rigidity (G, G'',…). At larger destructuration, while both G' and G'' decrease with increasing strain, the strain at which G'=G" is used to characterize network strength at a scale ranging between strands and fractal clusters (Arshad, Paulsson, & Dejmek, 1993; Crispín-Isidro et al., 2015; Hess et al., 1997; Yazar, Caglar Duvarci, Yildirim Erturk, & Kokini, 2019). While there is an increasing interest for this technique, it is not always used at its full potential and the complexity of interpretation is a part of the limitations.

In stirred yogurt, the apparent viscosity can be considered as a function of microgels volume fraction, which depends on microgel size and shape, and serum viscosity (surrounding fluid) (Loewen, Nöbel, & Hinrichs, 2017; Walstra, Geurts, Walstra, & Wouters, 2005; Zoon, 2003).

Apparent viscosity increases with microgel volume fraction and serum viscosity (Loewen et al., 2017). According to Zoon (2003), an increase of only 1 mPa.s in serum viscosity could increase yogurt viscosity by 10 Pa.s (representing approximately an increase of 50% of the initial viscosity). This phenomenon has been reported when using EPS producing starters (Surber, Mende, Jaros, & Rohm, 2019; van Marle, 1998). However, to measure serum viscosity, it has to first be extracted by centrifugation (Ruas-Madiedo, Alting, & Zoon, 2005; Surber et al., 2019), and depending on the type of EPS (capsular, free, interacting with the network,…) EPS may stay in the pellet limiting observations to understand the links between EPS presence, serum viscosity et yogurt viscosity (Surber et al., 2019). However, depending on the state of shear destructuration, EPS in the serum may not be homogeneously distributed throughout the gel. The impact of EPS on yogurt viscosity, can differ with the type of EPS produced and EPS location (solubilized in the serum, entangled inside microgels, entrapped into microgels pores, …) influencing microgel properties and overall stirred gel viscosity (van Marle et al., 1999; Zhang et al., 2016). Furthermore stirring modifies EPS interactions with the protein network and their localization into the gel which may impact the rheological properties (Tamime et al., 2007a).

At increasing shear intensity, microgel sizes is decreasing while their sphericity increases, resulting in lower viscosity values (Walstra et al., 2005) (Figure 3). Both van Marle et al. (1999) and Javanmard et al. (2018) observed a reduction in microgel sizes with increasing shearing intensity. van Marle et al. (1999) used a micro-rheological model to describe yogurt viscosity based on yogurt microstructure: properties of microgels (elasticity, network rigidity, porosity, …), the ability of protein aggregates interactions to break and reform. While Javanmard et al. (2018) used both physical measurement (laser diffraction) and a rheological model to highlight the link between microgels size and aggregation with the rheological behavior of stirred yogurt including shear thinning and thixotropy. A large diversity of model

exists in literature to relate flow properties of stirred dairy gels and their internal structure depending on whether the model take account of the time dependency of the flow behavior, or the solid fraction of the dispersion (Rao, M. Anandha, 2014). van Marle et al. (1999) built a model in which stirred yogurt gels are an aggregating dispersion of protein particles considered as hard spheres. The model separates the shear stress during steady shear (σ) into two components: the stress due to the fractal structure (σ^{struct}), and the stress due to the hydrodynamic component (σ^{hydr}) (eq 1). This last parameter was calculated using the Krieger-Dougherty equation (relating the relative viscosity of a suspension solid part and particle volume fraction of hard spheres; eq.2) to consider the hydrodynamic volume of the suspension.

$$\sigma = \sigma^{struct} + \sigma^{hydr} \text{ (eq. 1)}$$

$$\eta_r = \left(1 - \frac{\phi_{agg}}{\phi_m}\right)^{-[\eta]\phi_m} \text{(eq.2)}$$

$\eta_r$is the relative viscosity of the suspension, ϕ_{agg} is the aggregates volume fraction, ϕ_m is the volume fraction of densely packed spheres, $[\eta]$ is the intrinsic viscosity of solids (=2.5 for rigid spheres). For a more complete explanation of the model, readers are invited to read van Marle et al. (1999). Javanmard et al. (2018), used a more macroscopic approach using the structural kinetic model (SKM). In this model, the concept of structural breakdowns and buildups is introduced as a kinetic parameter using a structural parameter, λ, into any other classical model for flow properties (Abu-Jdayil, 2003; Javanmard et al., 2018). During a flow shear experiment, this parameter depends on shearing intensity and duration, it can take values between 1 and 0, the former represents the state of fully structured matrix and the latter the fully unstructured matrix (Abu-Jdayil, 2003; Benezech & Maingonnat, 1994; Javanmard et al., 2018). For instance, Javanmard et al. (2018) used the Herschel-Buckley model multiplied by the structural parameter λ (eq.3). In eq.3, λ is considered to follow a second order kinetic model (eq.4), σ_0 is the yield

stress, $K_H$is the stirred yogurt consistency, $n_H$is the flow behavior index, $K_1$is a function of shear rate, λ is the structural parameter at particular shear rate and time and become equal to λ_e when the steady state is reached at a particular shear rate.

$$\sigma = \lambda(\sigma_0 + K_H\dot{\gamma}^{n_H}) \text{ (eq.3)}$$

$$\frac{d\lambda}{dt} = -K_1(\lambda - \lambda_e)^2, \lambda > \lambda_e \text{ (eq.4)}$$

4.1.1. Correlation between particles sizes and viscosity and firmness

The literature is abundant on the relationship between microstructural observations and physical properties of stirred fermented dairy gel. Table 2 presents a collection of experimental data coming from 19 different studies on stirred yogurt in which various factors (casein: WP ratio, smoothing temperature, homogenization, polysaccharide or exopolysaccharide presence, storage time, etc.) known to have an impact on their microstructure and physical properties were varied. Among the known factors usually correlated with physical properties, lets first explore the microgel size. Laser diffraction was generally used to determine microgel sizes in combination with another method to probe microstructure (optical microscopy, CLSM, SEM and light transmission). The changes on microgel sizes induced by modulation of a factor was compared to the impact on physical properties of stirred yoghurts for each study. In this set of observations (Table 2), 28 looked at both microgel sizes (all methods confounded: D43 or D32 with laser diffraction or image analysis, etc.) and viscosity (flow properties, apparent viscosity,…) or firmness (measured by the peak force in a penetration test). Larger particles are expected to produce more viscous and firmer yogurts. Accordingly, 20 observations showed an increase of viscosity or firmness when microgel size increased, and a decrease in viscosity or firmness when microgel size decreased. In the Gilbert (2020) study, the correlations between microgel sizes (all methods confounded) and viscosity or firmness vary between 0.5 and 0.98

depending on the variable studied (smoothing temperature, whey protein addition, storage time, EPS producing strains). However, correlation between sizes and rheological properties is not systematically reported among the studies. For instance, in the experimental plan of Krzeminski et al. (2013), when looking at the principal component analysis of particle size, rheological properties and sensory analysis of stirred yogurts the microgels sizes were not correlated behavior with other measurements.

Other microgel properties than size may be influential, as rigidity. In the work of Cayot et al. (2008) stirred yogurt gel were processed to obtain different microgel sizes using the shearing process (syringes with different dimensions and flow rate) and the milk heat treatment (varying intensity). Their results showed that the increase in microgel size due to the shearing process had no impact on viscosity, while the increase in microgel size due to the heating conditions had a significant impact on stirred yogurt viscosity. It could be hypothesized that the microgel had different rigidities. The one obtained with intense heat treatment being more rigid and resistant during viscosimetric measurement and thus resulting in higher apparent viscosity. Similar observations were found by Gilbert et al. (Submitted). Yogurts were produced with different levels of whey protein addition before heat treatment and were smoothed at 3 different temperatures. Viscosity and texture were measured and two techniques of microgel size analysis (digital microscopic image analysis, laser diffraction) were used. Viscosity and laser diffraction detected an increase in microgel size and viscosity due to the whey protein addition but did not detect differences between samples smoothed at different temperatures. However, both texturometry and image analysis techniques detected a simultaneous increase in microgel sizes and firmness due to both whey protein addition and smoothing temperature increase. It has been hypothesized that changes in structure due to different smoothing temperatures rely on weak interactions (eg. aggregated microgels) not detected by laser diffraction and having no impact on viscosimetry results, while less destructive techniques (texturometry and digital microscopic

image analyses) could detect these differences. Since aggregated microgels could be disrupted at shear rate as low as 10 s^{-1} (van Marle et al., 1999), it underlines the importance of using techniques with different degree of destructuration to give a better overview of the microstructure and its impact on physical properties of the gel.

Other parameters such as microgel shape and compactness or pore sizes may also have a significant role. A modification in both these parameters can be indicative of microgels aggregation and network compaction accompanied with an increase of firmness during storage for yogurt smoothed at different temperatures (Guénard-Lampron et al., 2020a). In another example, microgels made of polysaccharides with different shapes and sizes were directly introduced into a standard yogurt formulation (Rohart, Sieffermann, & Michon, 2015). The results showed that in addition to the concentration of added microgels, the main structural characteristics of microgel impacting the viscosity and texture of the product was: microgel size, shape (length/width ratio), and the entanglement of microgels between themselves. Increasing these parameters resulted in higher viscosity values. The inner properties of microgel (rigidity, porosity, …) are currently inaccessible and are often considered similar to those of the initial set gel. For a better understanding of the implication of microstructure into rheological behavior of stirred yogurt gel, it would be of a great interest to isolate and study individual microgel. For instance, in the work of Körzendörfer et al. (2018), large microgels (> 1mm) had a protein content three times higher than the yogurt, meaning that the inner properties of those microgels are different from the original set gel. This underlines a certain gap in the knowledge of stirred yogurt microstructure, while the formation of microgels has been established in the literature, questions remain on how different they are from the original gel, how they interact between themselves, and how the phenomena of swelling and aggregation affect stirred yogurt properties. The possible role of the surrounding media is not mentioned in any studies in Table 2 and has been explored in EPS producing media only.

4.2.2 Relationship with syneresis

Yogurt is a network in which water is physically entrapped and the spontaneous expulsion of serum due to contraction of the gel is called syneresis. Serum separation negatively affects consumer perception. The diversity of techniques to characterize syneresis in stirred fermented products (Sodini et al., 2004) can influence results (Hassan, 2008), and the complexity of phenomenon involved into syneresis (serum viscosity, gel porosity, shrinkage of the network, amount of closed pores, gel elasticity, gel heterogeneity,…) can complicate data interpretation resulting in contradictory conclusion (Hinrichs et al., 2004; Hinrichs, Götz, & Weisser, 2003; Lucey, 2001; Mokoonlall et al., 2016; Ruas-Madiedo & Zoon, 2003; Serra et al., 2009; Zhang et al., 2016). Spontaneous serum expulsion is a time-dependent phenomena and experimental methods were developed to accelerate the process using centrifugation or filtration to characterize the water holding capacity of the gel. In Table 2, only the results from induced syneresis methods are presented, because it is the most frequently used.

In set and stirred yogurt gels, syneresis is promoted by gel reorganization during storage. In the fractal scaling model, it corresponds to the reorganization of the sub-particular length-scales where fusion of particles can lead to micro-localized syneresis. The accumulation of these localized reorganization slowly leads to the formation of serum channels and growing serum pouches which will at a certain point be responsible for macro-syneresis (visible separation of serum on top of the gel) (Mizrahi, 2010; Rohart, Michon, Confiac, & Bosc, 2016; Silva & O'Mahony, 2018). The main microstructural characteristics of stirred fermented dairy gels that are often mentioned to correlate with syneresis are the microgel sizes, gel heterogeneity and "openness" of the network (presence of large pores, serum channels or pouches). Larger microgels are associated to higher syneresis values as they are related to heterogeneous network (Gilbert et al., 2020a, 2020b; Guénard-Lampron et al., 2020a; van Marle, 1998). Others have found opposite results (Gilbert, 2020; Zhang et al., 2016) and associated the presence of smaller

586 microgels with a highly broken network with less pores able to entrap serum leading to more
587 syneresis. It has been proposed by Hinrichs et al. (2003) using low frequency nuclear magnetic
588 resonance on proton (^{1}H-LF-NMR) to estimate the proportion of closed pores and gel
589 permeability in yogurt gels. In Gilbert (2020), a difference was measured between induced
590 syneresis (serum expulsion obtained by centrifugation at 238 *g*) and spontaneous syneresis
591 during storage as quantified by ^{1}H-LF-NMR. The two results were not necessarily identical and
592 a complex relationship between microstructure and syneresis was noticed. Based on the
593 structure described by optical microscopy (Figure 1) and water mobility measured by ^{1}H-LF-
594 NMR, the authors hypothesized that there would be an optimum proportion of the different
595 microstructure types (large microgels, reorganized microgels and serum pouches) to minimize
596 syneresis. The presence of serum pouches is a promotor of syneresis. To limit their formation
597 large microgels able to entrap serum efficiently and reorganized microgels that can slow down
598 the separation of the serum are necessary. Too much reorganized microgels would promote
599 serum pouches growth due to network shrinkage during post-acidification (Patrignani et al.,
600 2009), and very large microgel would induce a heterogeneous network in which flocculating
601 microgels may sediment during storage. Weidendorfer et al. (2008) mentioned another
602 phenomenon that could promote syneresis, if microgels do not interact with each other, it may
603 facilitate serum separation from the microgels. In addition, serum composition and viscosity
604 may also influence syneresis. For instance, the presence of EPS could create micro-phase
605 separation between the protein network and the serum, creating heterogeneity in the network
606 with large pores area. However, if the EPS has a high water binding capacity it can increase
607 serum viscosity and reduce syneresis (Ruas-Madiedo et al., 2003; Tamime et al., 2007a). To
608 sum up gels associated with low syneresis are those in which serum is homogenously distributed
609 and having no or low number of serum pouches. Serum pouches development can be limited by
610 both the presence of large microgels and reorganized network in adequate ratio, and by limiting
611 the capacity of microgels to aggregate (using EPS, keeping WP addition at low level, adding

612 milk fat in the formulation, etc.). Syneresis can also be controlled by increasing serum viscosity
613 or the water binding capacity of the gel network or addition of stabilizers.

614

615 *4.2.3 Microstructure and sensory analysis*

616 Consumption of yogurt is associated with a complex sensory perception referred to as
617 creaminess (Upadhyay, Aktar, & Chen, 2020). Many authors have tried to define and
618 decompose what makes a creamy product for the consumer as hedonic responses is strongly
619 positively correlated to creaminess (Frøst & Janhøj, 2007). The main physical characteristics
620 often mentioned to predict creaminess of a stirred dairy gel are viscosity (thickness of the
621 product), smoothness (not grainy or lumpy visually or in mouth, small particle size), absence of
622 syneresis, and shininess (visual glossiness, light reflectance) (Cayot et al., 2008; Johansen et al.,
623 2008; Krzeminski et al., 2013; Laiho et al., 2017; Rohart et al., 2015; Sonne et al., 2014). Once
624 again, the microgel size has been found to be extremely important for visual and in mouth
625 appreciation of the product. Microgel sizes limit above which the sample is perceived as grainy
626 (not smooth) by panelists has been determined by several authors. For example Cayot et al.
627 (2008) found a detection limit of 150 µm of diameter (based on a calculation to identify the
628 coarser particles as measured by laser diffraction) above which the panelist did not characterize
629 the product as creamy, while in Laiho et al. (2017) panelists started to detect an increasing
630 graininess (visual or in mouth) for yogurt with microgel sizes above 50 µm of diameter (D[4,3]
631 measured by laser diffraction). This difference can be explained first by the criteria used (coarser
632 particles sizes *vs* D[4,3]), but also by the factors under study (heating process and smoothing
633 intensity *vs* CN:WP ratio) which probably have created microgels with very different nature and
634 inner properties, the microgel resulting from CN:WP reduction being probably less brittle
635 (Gilbert et al., Submitted; Lesme et al., 2020). According to Rohart et al. (2015) in addition to
636 microgel shape and dimension their entanglement may increase perceived thickness of stirred

yogurts. Some authors used an electronic eye to predict the perception of creaminess, glossiness and grainy appearance from analysis of the yogurt surface structure (Johansen et al., 2008).

Human Textural perception is highly complex and recently, thin-film rheology known as tribology allowing to measure lubrication aspects important in oral processing gained interest (Scholten, 2017) to include a different perspective. This technique mimic the tongue-palate friction movement (Joyner, 2019). Structural information was able to predict the creamy characteristic of a product combining rheology, particle size and tribology data (Sonne et al., 2014). Use of tribology allows to explore the microstructural destructuration happening in the mouth with saliva and structural characterization of yogurt in saliva buffer which is closer to consumption conditions (Baniasadidehkordi et al., 2019c; Laguna et al., 2017; Laiho et al., 2017; Morell, Chen, & Fiszman, 2017). Tribology can also be used to better characterize the microgel suspension in conditions that does not necessary simulate human consumption (Joyner, 2019). Typically, when simulating consumption of oral processing different parameters have to be considered: the temperature, the sliding speed, the geometry and the properties of the probes (sliding surfaces porosity, softness, hydrophobicity), and the addition of saliva (Joyner, 2019; Scholten, 2017). To mimic mouth conditions, experiments are usually done at constant, or short range of sliding speed (in-between 0.01 to 100 $mm.s^{-1}$) at a temperature around 25 to 37 °C (Joyner, 2019). For yogurt some authors have chosen a range of slow sliding speed (0.1 to 10 $mm.s^{-1}$), because in those speed range, the frictional behavior corresponds to the boundary lubrication regime where friction does not depend on speed and would better correlate with sensory attributes such as creaminess (Morell et al., 2017).

The addition of saliva (artificial or human) is necessarily affecting the stirred yogurt microstructure during tribological test (Baniasadidehkordi et al., 2019c; Laiho et al., 2017). Firstly, because it dilutes the stirred yoghurt in a very different buffer, and secondly because of the proteins and enzymes contained in the saliva that can interact with the yogurt matrix. For

662 instance, solubilized starch can be hydrolyzed by amylase of saliva (Morell et al., 2017;
663 Scholten, 2017), and mucin, which is charged negatively at pH 7 (pH of saliva), can induce
664 repulsive interaction with other negatively charged proteins such as WP leading to depletion
665 phenomena (Scholten, 2017). The interaction between saliva and dairy food matrices is not
666 entirely understood, however, in some cases saliva has been found to act as lubricating agent
667 (Joyner, 2019) or being involved into the astringent feels (Vardhanabhuti et al., 2010). In full
668 fat stirred yogurt the addition of saliva has been found to increase protein network density and
669 cause fat coalescence (Baniasadidehkordi et al., 2019c).
670 Tribology results are strongly impacted by the characteristics of the particles inside the matrix
671 (size, shape, rigidity, deformability), and by the continuous phase lubricant properties. Large
672 dense particle aggregates, typically formed when increasing protein content, especially WP
673 content into yogurt formulation has been reported to increase friction coefficient
674 (Baniasadidehkordi et al., 2019c; Laiho et al., 2017; Morell et al., 2017; Scholten, 2017), while
675 the presence of deformable small particles has been associated to increase lubrication
676 (Baniasadidehkordi & Joyner, 2019a; Baniasadidehkordi et al., 2019c; Scholten, 2017). Fat
677 droplets tend to coalesce during the experiment, especially if saliva is present, the fat can form
678 interfacial films in between the two sliding probes that reduces friction during the experiment;
679 *i.e* increase lubrication (Baniasadidehkordi et al., 2019a, 2019c; Scholten, 2017).

680 Both Sonne et al. (2014) and Laiho et al. (2017) studied yogurt using sensory analyses, particle
681 size with laser diffraction, and tribology (same parameters). While Sonne et al. (2014) varied
682 the CN:WP ratio, the fat and the protein content of the yogurts, Laiho et al. (2017) only changed
683 the CN:WP ratio of a non-fat yogurt. In both cases particle size and tribological values correlated
684 well with sensorial creaminess in mouth (correlation absolute values from 0.58 to 0.98).
685 However, the descriptors grainy, lumpy, or smooth (determined visually and in mouth)
686 correlated well with particle sizes only (correlation absolute values from 0.81 to 0.99).

687 Considering that those two studies, the main difference reside in the formulation factors under
688 study, it shows that depending on which factor is studied, there is not a universal method able
689 to predict sensorial attributes of stirred yogurt. Actually, Sonne et al. (2014) theorized a model
690 linking each step of the mouth-processing (entry into the front oral cavity, tongue palate
691 frictions, swallowing) to a sensory attribute linked to a rheological or microstructural
692 measurement. Similarly, observation has been made by (Morell et al., 2017) that the addition
693 of modified starch increased lubrication of the yogurt during tribological test with saliva,
694 explaining well yogurt with a creamier feeling. However, tribological data could not explain
695 sensorial attributes relative to astringence such as gritty, grainy, or rough when adding whey
696 protein concentrate into yogurts. It seems that both tribological and microstructural observations
697 could complete each other to better predict sensorial properties of stirred yogurt.

698 **5. Conclusion**

699 The structure of stirred dairy gel can be described over different length scales which is
700 representative of the interrelations between the 3D organization of the yogurt network but also
701 introducing the challenge to understand how each level impact each other and creates spatial
702 heterogeneity into the stirred dairy gel. From a structure built in set gels, processing steps induce
703 a reorganization into a dynamic and complex structure with microgels interconnected into a
704 continuous serum phase. The size and shape of microgels will depend first on how brittle the set
705 gel is at the beginning, and then on the shearing process conditions (temperature, pH, shear
706 intensity…).

707 Many microstructural features are involved into stirred yogurt properties. Microgel sizes is often
708 correlated with viscosity, firmness, creaminess, or syneresis. It is the most accessible
709 microstructural feature to measure and is now part of most studies on yogurt. Other properties
710 such as microgels shape or compactness have been reported to impact stirred yogurt properties
711 these features are accessible only by imaging techniques. The use of techniques involving

different degree of sample destructuration can help to unravel the complexity of structural organization of microgels, their interactions and aggregation to explain phenomena as rebodying during storage or the effect of process and formulation on stirred yogurt properties. Additional techniques such as tribology, or water mobility measurements (^{1}H-LF-NMR) are helpful tools to assess the stirred yogurt microstructure, probing molecular interactions or water entrapment into the network. However, limited information is available on inner properties of microgels (network rigidity, porosity, entanglement with other microgels or EPS…) and how to control them to optimize stirred yogurt properties. Methodological approaches and adapted techniques are needed to unravel microgels role.

Acknowledgments

The authors want to acknowledge the support of Dr. Turgeon from NSERC-discovery grant program.

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Figure Legend

Figure 1: General pattern of stirred fermented dairy gel processes and structure organization (scales are not respected).

Figure 2: Differences in observation and interpretation of microstructure obtained from optical, digital, and confocal laser scanning microscopy (CLSM) of a non fat yogurt (4 % protein, ratio casein:whey protein = 2.8, total solids = 14 %). Red bar represents 250 µm. CLSM pictures were reproduced with permission of V. Guénard-Lampron (personal communication).

Figure 3: Schema of stirred yogurt gel viscosity and microstructure destructuration during a constant shear experiment at a constant temperature - φ is the volume fraction of microgels.

1093 Table 1: Different techniques available to observe and characterize stirred yogurt microstructure.

Technique of data acquisition	Sample preparation	scale of structures observation	data collection type	Example and references
Laser diffraction	dispersion under stirring into aqueous dispersant (milk serum, deionized water,…)	0.01 µm to 2-3 mm	Particule size distribution -microgel sizes -fractal dimension	Gilbert et al. (2020b) Moussier et al. (2019a) Zhang et al. (2016)
Diffused photon density wave spectroscopy of concentrated suspension	conditioned into a translucid sample holder	0.01 µm to 3 mm	Particle size, number, density	Tanguchi, Murata, and Okamura (2010)
Light scattering	dispersion into aqueous dispersant (milk serum, deionized water,…)	< 1 µm to 600 µm	Protein aggregate sizes distribution	van Marle (1998)
Diffuse reflectance spectroscopy	conditioned inside a sample holder	0.01 to ? µm	Particle sizes	Abildgaard et al. (2016)
Dynamic image analyses of microgel suspensions	dispersion under stirring into aqueous dispersant (milk serum, deionized water,…)	0.4 µm to 2.5 mm	Image analyses: microgel sizes distribution, shape, microgel compaction	Hahn et al. (2012a) Moussier et al. (2019a) Guénard-Lampron et al. (2020a)
Confocal laser scanning microscopy	conditioned into spacer/well equipped microscope slide	0.5 µm to ≈ 200 µm	Qualitative description, localisation of exopolysaccharides, fat, proteins,… Image analyses: pores sizes, heterogeneity of fat repartition into the protein network – fractal dimension	Torres, Amigo Rubio, and Ipsen (2012) Andoyo et al. (2015) Guénard-Lampron et al. (2020a) Zhang et al. (2016) Gregersen et al. (2021)
Confocal laser scanning microscopy	dispersion into water and conditioned into spacer/well equipped microscope slide	0.5 µm to 2-3 mm	Image analyses: microgel sizes distribution, shape, microgel compaction – fractal dimension	Moussier et al. (2019a) Guénard-Lampron et al. (2020a)
Optical microscopy, digital microscopy	Spread into spacer (≈ 150 µm deep) equipped microscope slide	10 µm to 1 mm	qualitative description Image analyses: microgel sizes distribution, shape, microgel compaction, network heterogeneity	Gilbert et al. (2020b) Gilbert et al. (2020a) Tribst et al. (2020)
Optical microscopy	dispersion into water and conditioned into spacer/wells equipped microscope slide or petri dish	> 0.5 mm	Image analyses: large microgel count	van Marle (1998) Remeuf et al. (2003)
Image acquisition from light transmission	Spread into spacer (0.6 to 1.2 mm deep) equipped glass slide	> 1 mm	Image analyses: large microgel count	Küçükçetin, Weidendorfer, and Hinrichs (2008a) Körzendörfer et al. (2017)
Particle size sieving	sample washed with yoghurt serum on agitating sieves	125 to > 800 µm	Image analyses: particule size distribution	van Marle (1998)
camera / electronic eye (angle measure technique; light reflection)	conditioned inside a sample holder	Macroscopic scale	Image analyses: glossiness, graininess, …	Møller (2012) Johansen et al. (2008)

Scanning electron microscopy	sample chemical fixation and dehydration	< 10 µm	Qualitative description, localization and attachment of exopolysaccharides, fat, proteins,…	(Kalab et al., 1975)
Cryo-Scanning electron microscopy	Sample fast freezing and sublimation	< 10 µm	Qualitative description, localization and attachment of exopolysaccharides, fat, proteins,…	(Hassan, Frank, & Elsoda, 2003a)
Transmission electron microscopy	sample chemical fixation and dehydration	< 10 µm	Qualitative description, localization and attachment of exopolysaccharides, fat, proteins,…	(Kalab et al., 1975)

1094

1095 Table 2: Relationship between microstructural features and stirred yogurt gel properties in literature

Evolutions of variables depending on the factors applied (results factor 2 – results factor 1)								
Ref.	Compared factors			Microgel (or aggregated protein cluster) sizes		NH[1] Optical microscopy	Rheological parameter	Syneresis (%)
	Compared factor	1	2	Laser diffraction	Optical microscopy			
Gilbert et al. (2020b)	ST[3] (°C)	20	42	D[4,3][2] ↗	↗	↗	visco.(K)[5] ↗	↗
Gilbert et al. (2020a)	Stabilizers	Polysac.[4]	Gelatin	NA[a]	↘	↘	NA	↘
Gilbert et al. (Submitted)	ST (°C)	15	25	D[4,3] =	↗	=	visco.(K) = firm.[5] ↗	=
	WP[7] addition [Day 1][6]	WP0[7]	WP1[7]	D[4,3] ↗	↗	↗	visco.(K) ↗ firm. ↗	↘
	Day	1	23	NA	↗ [WP1] = [WP0]	=	visco.(K) ↗ [WP1] firm.(K) ↗ [WP1] visco.(K) ↗[WP0] firm. = [WP0]	↘
Gilbert (2020) (Chapter 5)	ST (°C)	4	27	D[4,3] ↗	=	↗	visco.(K) ↗ firm. ↗	↘
	Day	1	12	D[4,3] =	=	=	visco.(K) ↗ firm. ↗	↘
Ref.	Compared factors			Microgel (or aggregated protein cluster) sizes		NH[1] CLSM	Rheological parameter	Syneresis (%)
	Compared factor	1	2	Laser diffraction	CLSM[7]			
Liu et al. (2016)	Ratio CN:WP[7]	2.3	1	D[4,3] ↗	NA	NA	visco.(η_{app}100)[5] ↗ firm. ↗	↘
	Ingredient	WPN[7]	WPM[7]	D[4,3] ↗	NA	NA	visco.(η_{app} 100) ↗ firm. ↗	↘
Krzeminski et al. (2011)	Ratio CN:WP [0 % Fat]	4	1.5	d50[2] ↗	NA	=	visco.(σ 50)[5] ↗	NA

	Fat content (% w/w) [CN:WP = 4]	0	12	d50 ↗	NA	(CLSM) ↘	visco. (σ 50) ↗	NA
Laiho et al. (2017)	Ratio CN:WP	4	1	D[4,3] ↗ D[3,2] [2] ↗ d90[2] ↗	↗	↗	visco.(η_{app} 50 ;100) [5] ↗	NA
Cayot et al. (2008)	HT[9]	NHT[9]	95 °C/5 min	cp[2] ↗	NA	NA	visco.(η_{app} 50) ↗	NA
	SP[10] intensity	high	low	cp ↗	NA	NA	visco.(η_{app} 100) =	NA
Zhang et al. (2016)	SP Back-pressure	0 bar	4 bar	pl[2] ↘	↘	↘	visco.(η_{app} 100) ↘	↗
Lee, W.-J. and Lucey (2006)	HT (°C/30 min)	75	85	NA	NA	↘	visco.(η_{app} 10;50;100) ↗	NA
	FT[2] (°C)	32	44	NA	NA	↗	visco.(η_{app} 10;50;100) ↘	MA
Guénard-Lampron et al. (2020a)	ST °C [Day 1]	22	35	(DIA[11]) =	NA	↗	visco.(η_{app} 10.5) = firm. ?	
	ST °C [Day 22]	22	35	(DIA) ↗	NA	↗	visco. (η_{app} 10.5) = firm. ?	↗
	Storage	Day 1	Day 2	(DIA) ↗ D[4,3] = D[3,2] =	NA	↘	visco. (η_{app} 10.5) = firm. ↗	↘
Ciron et al. (2010)	Homo. [non-fat milk]	CH[16]	MFZ[16]	D[4,3] = d90 =	NA	↗	firm. ↘	↗
	Homo. [low-fat milk]	CH	MFZ	D[4,3] ↗ d90 ↗	NA	↗	firm. =	=
Hassan et al. (2003b)	EPS	EPS --	EPS ++	NA	NA	↗	visco. (σ_H;K_H) [5] ↗	NA
Ref.	Compared factors			Microgel (or aggregated protein cluster) sizes		NH[1]	Rheological parameter	Syneresis

	Compared factor	1	2	Optical microscopy - diluted sample (grain > 1 mm/mL)	SEM	SEM		(%)
Remeuf et al. (2003)	Ingrédients + 2 % protein [HT = 90 °C/ 5 min]	SMP	WP	↗	NA	↘	visco.(η_{app} 10) ↗	↗
		SMP	NaCn	↗	NA	↗	visco.(η_{app} 10) =	↗
	HT [SMP +2 % protein]	90 °C/ 1 min	90 °C/ 5 min	=	NA	↘	visco.(η_{app} 10) ↗	↘
Chua, Deeth, Oh, and Bansal (2017)	Milk protein ingredient	+1 % SMP	+1 % WPI	NA	NA	↘	visco.(σ_H) ↗ firm. ↗	NA
Damin, Alcântara, Nunes, and Oliveira (2009)	Ingredient	+0.75 % SMP	+0.75 % SCN	NA	NA	↗	visco. (τ_0) ↗ firm. ↗	NA

Ref.	Compared factors			Microgel (or aggregated protein cluster) sizes		NH[1]		Syneresis (%)
	Compared factor	1	2	Laser diffraction	Light transmission images (grain > 1 mm/mL)	Light transmission images	Rheological parameter	
Küçükçetin, A. (2008)	Ratio CN:PS [HT: 95 °C/256 s]	4	1.5	NA	↗	↗	visco.(σ_0) ↗	↘
	HT [CN:PS = 3]	95 °C/256 s	130 °C/80 s	NA	↗	=	visco.(σ_0) ↘	=
Küçükçetin et al. (2009)	FT [EPS--]	37 °C	42°C	NA	↗	↗	visco.(σ_0) ↗	NA
	EPS [FT: 42 °C]	EPS--	EPS ++	NA	↘	↘	visco.(σ_0) ↘	NA
Körzendörfer et al. (2018)	Vibration during fermentation	none	intense	d90 ↘ D[3,2] [1] ↘	↗	↗	visco.(η_{app} 100) ↘	NA

[a] Not analyzed

[1] Network heterogeneity (Presence of large pores or area of loose network) – no units

[2] The reported distribution factor are D[4,3] = volume weight mean, D[3,2] = volume weight mean, d50 = quantile 0.5, d90 = quantile 0.9, dcp = coarse particle, pl (peak localization)

[3] ST =Smoothing temperature; FT = Fermentation temperature

[4] Polysaccharide

[5] Rheological parameters: texture (firm.) obtained using a penetration test or by viscosimetry; viscosity (visco.) obtained viscosimetry, the compared parameters are expressed in brackets: K= consistency (Pa.s) obtained using the power law model, σ_H,K_H = the yield stress (Pa) and consistency (Pa.s) obtained using the Hershel-Buckley model, η_{app} 10;50;100 = apparent viscosity (Pa.s) at 10, 50 , and 100 s^{-1}, σ50 =shear stress (Pa) at 50 s^{-1}, σ_0 = yield stress (Pa) obtain by an angular frequency sweep, τ_0= yield stress obtained using control stress ramp.

[6] Precision in squared brackets means that there is an interdependence with another factor

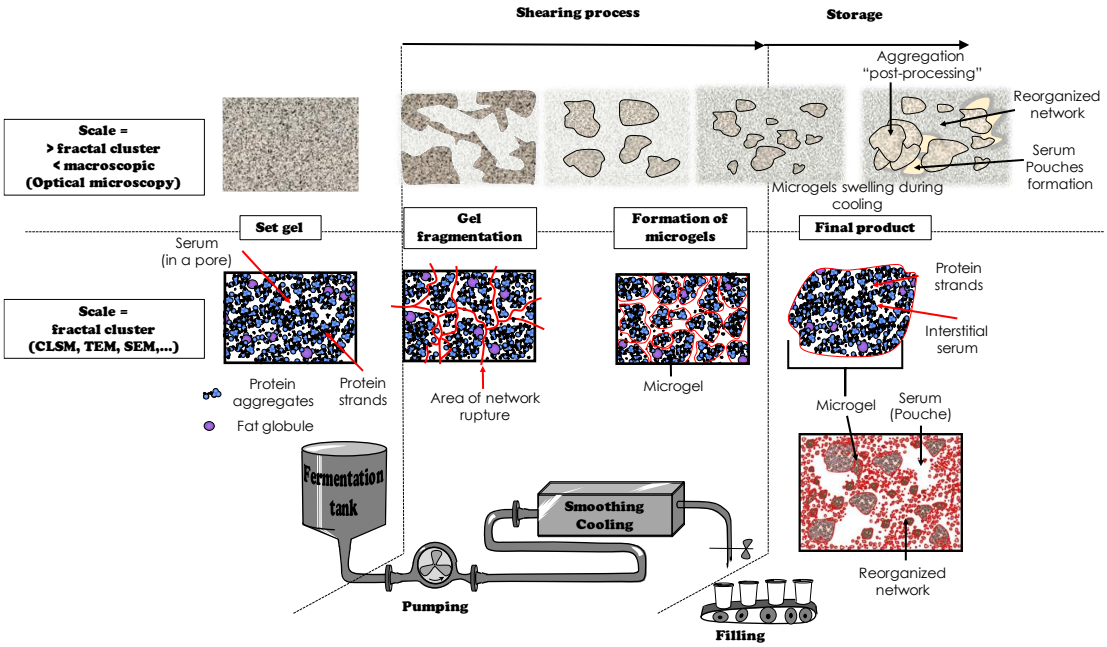
[7] WP = protein WP0 = No whey protein addition; WP1= 1% (w/w) of whey protein addition; ratio CN:WP = ratio casein:whey protein; WPM = microparticulated whey protein; WPN = nanoparticulated whey protein;

[8] Confocal laser scanning microscopy

[9] HT = Heat treatment; NHT = No heat treatment

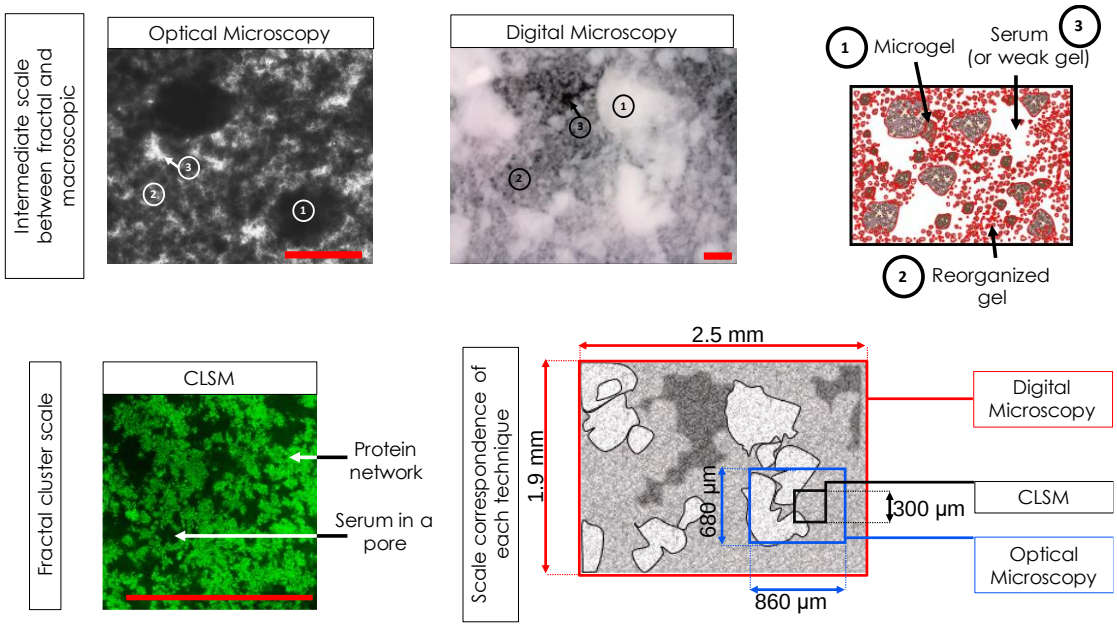
[10] Shearing process

[11] Dynamic image analyses instead of laser diffraction



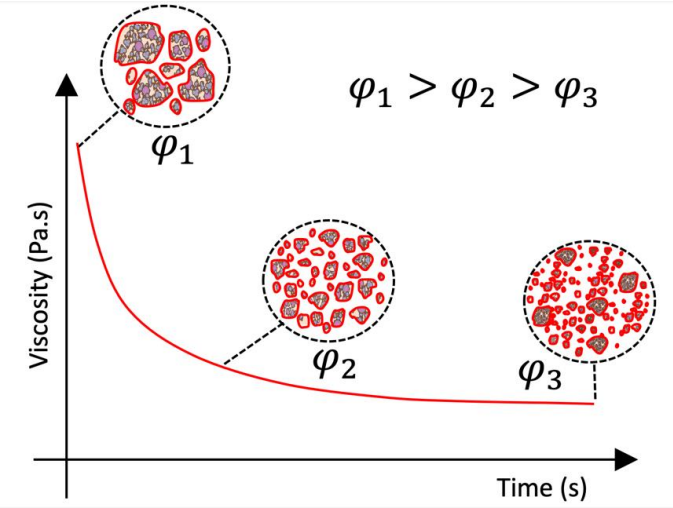
1113

1114 Figure 1



1115

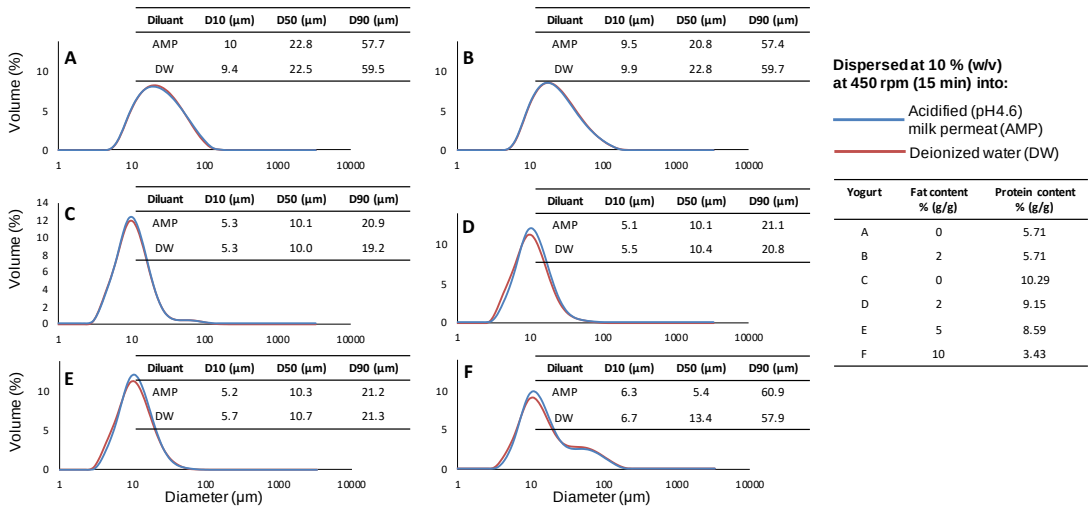
1116 Figure 2

1117 

1118 Figure 3

1119

ANNEXE A:



Yogurt	Fat content % (g/g)	Protein content % (g/g)
A	0	5.71
B	2	5.71
C	0	10.29
D	2	9.15
E	5	8.59
F	10	3.43

Figure A.1 : Particle size distribution of 6 different commercial yogurts (without stabilizer) obtained by laser diffraction according to the protocol of Gilbert et al. (2020b) using acidified milk permeate or deionized water. Size are reported using the D10, D50, and D90 quantiles obtained from a triplicate. Standard deviation is <0.9 µm for D10, <0.5 µm for D50, <8.4 µm for D90.