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Quantification by analysis of super-resolution microscopy images

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Microstructural changes in acid milk gels due to temperature controlled high-intensity ultrasound treatment: Quantification by analysis of super-resolution microscopy images

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Running Title: HIU milk gel super-res image analysis

Abstract

High Intensity Ultrasound (HIU) can be applied to food materials to create novel structures and textures, and as a method of processing. HIU treatment of milk modifies the gelation behaviour, but the microstructural impact is poorly understood. Milk samples were treated with HIU at three temperatures; 27°C, 50°C, and 70°C. Acid milk gels were formed from the milks and imaged using Super-resolution Stimulated Emission Depletion (STED) and confocal microscopy. Quantitative image analysis was applied and demonstrated that HIU treatment lead to measurable changes in both protein network morphology and degree of association of the fat droplets with the protein network.

Keywords

Super-resolution microscopy, Stimulated Emission Depletion (STED) microscopy, Quantitative image analysis, High intensity ultrasound, milk acid gel microstructure.

Introduction

Emerging alternatives to traditional processes are being explored within the food industry. High Intensity Ultrasound (HIU) treatments are an example of alternative processes that are capable of modifying the functionality and behaviour of materials (Watson *et al.* 2013), including foods (Awad *et al.* 2012; Povey 2017), which can produce altered functionalities conferring specific benefits to the manufacturer. High power ultrasound pre-treatment of milk has been explored in recent years and has been shown to lead to changes in the gel strength of acid milk gels (Gregersen Wiking and Hammershøj, 2019; Nguyen and Anema, 2010; 2017) and can improve yoghurt texture (Riener *et al.* 2009). An understanding of the effects of HIU treatment on gel microstructure at a spatial resolution related to physical and chemical properties is lacking.

Ultrasound is defined as sound waves with a frequency above the human audible range (20 kHz). There are two broad classes of ultrasound that may be utilised in food and dairy product structures, low intensity ultrasound (< 1 W/cm^2) can be utilised to measure and characterise materials as it is non-destructive, whereas HIU refers to an intensity range that will lead to cavitation and material modification (>1 W/cm^2) (Awad *et al*. 2012). HIU leads to material modification through several processes; fluctuations in pressure lead to the formation of bubbles, the size of which is related to the acoustic frequency. Bubbles move through the fluid due to the acoustic field and though the process of rectified diffusion grow until they reach a certain size at which point they collapse. Bubble collapse produces locally high temperatures and turbulence (Kentish and Fang 2014). The turbulence generated from HIU treatment has a homogenising effect in food and dairy systems, which can result in functional and structural changes and can be exploited by manufactures which has been reviewed recently (Carrillo-Lopez *et al.* 2021).

When considering structured dairy products the major macro components of interest are proteins and fat. Milk fat is present in the form of milk fat droplets, predominantly composed of triglycerides, with a surrounding phospholipid stabilising layer; the Milk Fat Globule Membrane (MFGM). The MFGM contains an array of complex proteinaceous and glycosylated components and provides stability to the milk fat globules (Huppertz and Kelly 2006). Milk proteins can be split into the caseins, which are insoluble at pH 4.6, with the remainder being the whey proteins (20%). Caseins are present in the form of casein micelles, which are a complex supramolecular assembly of four monomeric subunits held together through hydrogen bonding, hydrophobic interactions and ionic interactions mediated through calcium phosphate nanoclusters (De Kruif *et al.* 2012). Casein micelle aggregation can be induced through acidification to overcome the net negative surface charge present on the micelles or by chymosin cleavage of the electronegative region of κ-casein, present at the micelle surface and contributes to steric and electronegative stability to the micelles. The whey proteins are soluble, globular proteins and the dominant whey protein in bovine milk is β-lactoglobulin, which when denatured exposes a free reactive thiol group which can lead to protein-protein disulphide bond interactions (Kethireddipalli

Hill and Dalgleish 2011; Chatterton *et al.* 2006). Under shear homogenisation the milk fat droplets are broken apart into more, smaller droplets. Smaller fat droplets have an increased surface area in relation to the volume, hence, the native MFGM is not sufficient to cover this newly exposed surface. Casein and whey proteins associate with the fat globule surface as they are amphipathic and lower the total surface energy. Homogenising therefore effectively functionalise the surface of fat droplets with molecules that can participate in further protein-protein interactions and network formation (Cho Lucey and Singh 1999). Processes that can affect the degree of whey protein denaturation and fat droplet composition have potential to fine tune the functional behaviour of milk for different applications.

HIU treatment has been demonstrated to lead to microstructural changes in various protein gel systems. HIU has been shown to improve gel properties of porcine myosin by reducing aggregate size and increasing solubility (Sun *et al.* 2021) and to modify the properties of α-lactalbumin (Qayum *et al.* 2021) and surimi (Gao *et al.* 2021; Liang *et al.* 2020) leading to increased water holding capacity and altered gel morphology (Liang *et al.* 2020). The strength of rennet induced goat milk gels has also been shown to increase following HIU treatment, due to protein structure modifications and changes in gel morphology (Ragab *et al.* 2020). The combination of HIU and heat have been demonstrated affect gel strength and water holding in soy protein isolate (Zhao *et al.* 2021). In dairy products, HIU treatment can lead to whey protein denaturation below the denaturation temperature of whey protein (Gregersen *et al.* 2019; Shanmugam, Chandrapala and Ashokkumar 2012; Villamiel and de Jong 2000). HIU induced homogenisation of milk fat globules leading to changes in the MFGM protein composition (Gregersen *et al.* 2019) and has been shown to retain more of the MFGM proteins compared to traditional homogenisation (Liu *et al.* 2021). Furthermore, the intensity of HIU treatment leads to modification in protein-protein and protein-lipid interactions (Bui *et al.* 2021), which is likely to have an effect on how the protein and lipid components form structure.

Whilst HIU treatments have been demonstrated to alter the interaction between protein and fat in dairy samples, the subsequent impact upon gel microstructure has not been visualised or quantified to date. In food science, the ability to draw a causal link between a material's microstructure and its emergent physical and chemical properties remains elusive (Aguilera, Stanley and Baker 2000). Through the application of high-resolution microscopy and complementary image analysis methods, the effects of alternative processing methods can be quantified allowing comparison to known physical and chemical changes, providing a more complete map of interactions at different length scales.

Stimulated Emission Depletion (STED) microscopy is a super-resolution technique (Hell and Wichmann 1994; Hell 2007) that has recently been applied to the imaging of dairy product structures but remains under-exploited in the imaging of food systems (Glover and Holmes 2020; Glover *et al.* 2020). The advantage of STED in imaging colloidal structures is that the only perturbation required is the addition of a fluorescent dye. Samples do not have to be fixed or dried, minimising the potential for structural artefacts as well as reducing analysis time (Glover *et al.* 2019a).

Quantitative image analysis is required to extract the full level of detail contained within super-resolution images. Methods based on autocorrelation analysis have been used to quantify structures in various food materials (Ako *et al.* 2009; Urbonaite *et al.* 2016;

Ersch *et al.* 2016; Urbonaite *et al.* 2015; Ainis *et al.* 2019). Recent developments have allowed quantification of the microstructure of milk acid gels (Glover, *et al.* 2019a; Glover *et al.* 2019b; Gregersen *et al.* 2021). A combination of autocorrelation and cross-correlation analyses have been presented to quantify the protein network and the spatial relationship between protein and fat in two component Pickering stabilised emulsions and dairy gels (Araiza-Calahorra *et al.* 2020; Glover *et al.* 2019b). Quantification aids the understanding of how molecular changes during HIU treatment affect the structuring following treatment, and how that leads to changes in macroscopic properties. It has been shown recently that relevant parameters extracted from microstructural quantification relate to the macroscopic behaviour of a gel system for milk samples treated with hydrodynamic cavitation and pressure homogenisation (Gregersen *et al.* 2021).

The aim of this study was to be able to understand the microstructural changes that occur during temperature controlled high intensity ultrasound treatment. Two temperatures below denaturation temperature of whey protein were used, where whey protein denaturation has been shown to occur due to HIU (Gregersen *et al.* 2019). A temperature of 70 °C where both thermal and HIU effects can lead to whey protein denaturation and structural modification. Microstructural changes were quantified through image analysis of STED microscopy images. The output from the image analysis was compared to measurements of the casein micelle size and discussed in relation to previously acquired macroscopic data.

Materials and Methods

Milk samples were obtained and treated as per (Gregersen et al. 2019), a brief description follows.

Materials

Commercial full fat (3.5% fat), pasteurised (72 °C, 15 s), un-homogenised milk was obtained from a local store at maximum 2 days after processing at the dairy and stored at 5 °C. 1L packaged milk samples were pooled from which subsamples were taken. Samples were analysed for protein, fat and dry matter content by a Milkoscan unit (Foss, Hillerød, Denmark), which showed an average milk composition in g/L of; 35.4 ± 0.05, 35.2 ± 0.07 and126.2 ± 0.86 for protein, fat and dry matter, respectively.

Ultrasound treatment

Milk was subjected to ultrasound treatment in a 100 mL ultrasonic flow cell (Sonolab SL10 ultrasonic, Syrris Ltd, United Kingdom) operating at 20 kHz. Milk samples were heated to three treatment temperatures (27 °C, 50 °C and 70 °C) using a water bath, and ultrasonication was performed at 30 W with an estimated field energy intensity in the flow cell of 273W/L. These parameters were selected to be able to investigate the microstructure in samples where other physical and chemical parameters have been described (Gregersen *et al.* 2019). A total treatment volume of 800 mL milk was circulated in the flow cell system at 200 mL/min (Watson-Marlow 520DU peristaltic pump, United Kingdom) from a jacketed glass reactor (2000 mL) with circulating water for temperature control. During treatment, mixing was ensured with a magnetic stirrer at 200 rpm (IKA RCT basic IKAMAG®

magnetic stirrer, IKA®-Werke GmbH & Co. KG, Germany). The total treatment time was 30 min, given an average residence time in the flow cell of 4.13 min. Samples were prepared in duplicate.

Sample Preparation

Milk fat was stained for imaging with Nile Red (Sigma Aldrich, UK) to a concentration of 2.5 μM in the milk. Stained samples were left overnight at 5°C. Milk acid gels were prepared by adding 0.25 g of Glucono-δ-Lactone (GDL)(Sigma-Aldrich, USA) to 10 mL of the pre-stained milk giving a concentration of 2.5% GDL in the milk. The 10 mL of milk with GDL was inverted by hand for 1 min, and 600 μL of milk was sampled to which 3 μL of Atto 488 NHS-Ester (Atto-Tec GmbH, Siegen, Germany), dissolved in DMSO (99.9% pure, Sigma-Aldrich) was added to give a concentration of 510 μM in the milk. This volume was then transferred to a μ-Slide 8 Well chamber (ibidi, Germany), and incubated at 35 °C for 90 min. Five gels were produced per milk sample, giving 10 gels per treatment, as milk samples were prepared in duplicate.

Imaging Protocol

Imaging was performed using a Leica TSC SP8 STED microscope (Leica Gmbh, Mannheim, Germany). Images were taken with two sequential channels; first with excitation using an incident laser at 560 nm with detection between 570 and 700 nm, second with excitation at 480 nm and detection between 490 and 550 nm with STED depletion at 592 nm. The fat droplets were imaged with confocal resolution as the microscope was equipped for single channel STED imaging, the protein network was imaged with STED resolution. Both setups utilised a pulsed white light laser and gated hybrid detector (0.3 - 6 ns). The pixel size 34 nm using a zoom of 2.5X, acquiring images of 1384 x 1384 pixels equal to 46.5 μm^2 using a HCX PL AP 100X/1.40 OIL STED objective. A minimum of 10 images were taken per gel, i.e. a total of 100 images per sample type. Images were taken >5 μm above the glass interface to avoid boundary effects. The suitability of these sample preparation methods has been shown by Glover *et al.* (2019a).

Quantitative Image Analysis

Image analysis was conducted with MATLAB R2018b (Mathworks, U.S.A.) as described by Glover *et al.* (2019a; 2019b) using auto- and cross-correlation-based methods. The parameters extracted from the image analysis are: ξ_p (μm), the typical length scale of the protein domains in the protein channel image, λ (μm), the inter-pore distance in the protein channel image, D_f (-), the fractal dimension in the protein channel image, ξ_f (μm), the typical size of the fat droplets in the fat channel image and Λ (μm), the typical distance from the fat droplet interface to the centre of mass of the neighbouring protein domain determined between the fat and protein channels.

One-way Analysis of Variance (ANOVA) with treatment temperature as class variable and with post-hoc Tukey's honestly significant difference tests at minimum 95 %-level ($P \leq 0.05$) were conducted on the data extracted from the image analysis.

Casein Micelle Size

Skim milk was obtained by centrifugation at 2643 × g for 30 min at 4°C, and diluted 1:10 with simulated milk ultra-filtrate buffer (SMUF), prepared according to Jenness and Koops (1962). The average casein micelle size (Volume based) was determined by dynamic light scattering using a NANO-flex particle size analyzer (Microtrac Inc., Montgomeryville, PA, USA).

Results and Discussion

Fig1

Fig 2

Table 1

Figure 1 shows representative super-resolution STED images of the acid milk gel samples upon which quantitative analysis was performed. Figure 2 shows the outcome from the image analysis of the four gels samples, and Table 1 shows casein micelle size against the output parameters from the image analysis. It is optimal to compare changes in distributions of image analysis parameters, but the mean and standard deviations are included in Table 1 for statistical comparison.

The mean casein micelle size showed a trend to increase from the untreated control to the HIU treated samples, which was significantly highest at 70 °C, (Table 1). A temperature of 70°C will lead to denaturation of whey protein, and therefore higher temperatures during HIU treatments leads to increased denaturation of whey protein. HIU treatment below the denaturation temperature of the whey proteins does still lead to whey protein denaturation as previously shown (Gregersen et al. 2019) and therefore the treatment of 70 °C shows the combined effects of thermal and HIU treatment. When β-lactoglobulin denatures, a free thiol-group is exposed, which can covalently bind to both free thiol groups on the κ-casein molecules, situated on the surface of the casein micelles (Steffl *et al.* 1999; Lucey, Munro and Singh 1999).

The modification in casein micelle size with HIU treatment at high temperature was accompanied by a thinner gel structure, shown by the size of the typical length scale in the images, ξ_p, which corroborates with previous observations, where smaller ξ_p values also correlated to gels with a higher G' (Gregersen *et al.* 2021). Thus, a higher degree of denatured whey protein effectively increased micelle size, and modified the behaviour of the aggregating micelles and altered the gel structures, which was quantified here. It has previously been shown that higher levels of denatured whey protein relate to firmer gels being formed as there are more protein-protein interactions in the gel network (Anema and Li, 2000; 2003; Glover *et al.* 2020; Gregersen *et al.* 2021). It is a counter-intuitive observation that the size of the aggregating particles increases, but the typical size of the aggregated structures decreases. If the same quantity of protein is arranged to have thinner structures, there will be an increase in the protein network's surface area, which leads to increased moisture binding and increased gel strength (Glover *et al.* 2020). Increased moisture binding in protein gels following HIU treatment has been documented in several other materials such as surimi (Liang *et al.* 2020) and soy protein isolate (Zhao *et al.* 2021). STED microscopy and the image analysis applied here allows for the detection and quantification at a significant level, allowing gels produced from milks with different processing histories to be differentiated from each other. The combination of the interpore distance and fractal dimension can significantly differentiate between the four gel samples prepared, highlighting the fact that one single parameter is not sufficient to capture the subtle differences in gel structure and morphology. It can be seen in figure 2 that there is a trend in λ, the typical long order repeat distance in the images, which represents the interpore distance, where the control sample has the largest λ values. HIU treatment

leads to a reduction in the inter-pore distance which decreases with increasing temperature of treatment. When considered alongside ξ_p, the reduction in both structure size and interpore distance indicates a structure with more pores and a thinner continuous protein phase. The fractal dimension can be interpreted as showing how ragged the gel interface and to what extend the network completely fills a space (Glover *et al.* 2019). Figure 2c shows that the network in the control sample is the most space filling, supporting the interpretation that HIU treatment leads to morphological changes of thinner structures and increased porosity which are less space filling.

The fat droplet radius, ξ_f, decreased from the untreated control to HIU treatment at 50 °C and 70 °C, and also show a narrowing in the distribution with HIU treatment compared to the control sample. ξ_f is a measure of the average size of the fat droplets within the microscopy image and is not equivalent to a complete particle size distribution. The parameter, Λ, which relates to the typical distance from a fat droplet interface to the centre of mass of a neighbouring protein domain shows the largest difference between the control sample and the HIU treated samples. The fat droplets in the HIU treated samples are more closely embedded in the protein matrix. Following a size reduction, milk proteins will adsorb to the newly exposed fat droplet surface and HIU treatment is shown to provide a shift in protein associated with the MFGM (Gregersen *et al.* 2019). Hansen *et al.* (2020) showed that whey protein association to the milk fat globule membrane is not only mediated through di-sulphide bond formation and occurs through other intermolecular interactions such as hydrophobic interactions. Protein adsorption effectively functionalises the fat droplets to be able to become an active filler in the protein network increasing the gel strength (Van Vliet 1988), which has been observed in acid milk gels (Gregersen *et al.* 2021). The image analysis used here can measure the degree to which the fat droplets become more deeply incorporated into the protein network. The distances determined are below the diffraction limit of light and could therefore only be measured using a super-resolution technique.

The effects of whey protein denaturation on the structure, rheological properties and water binding capacities of conventionally processed dairy products are well studied (Cho *et al.* 1999; Van Vliet 1988). The results shown in this study highlight the fact that the structural changes are temperature dependent and therefore the degree of modification introduced by HIU treatment could be modulated through temperature control. When considering the fate of industrially produced dairy gels there are two broad possibilities. Firstly, where a high level of whey protein denaturation is beneficial in order to have more protein participate in the network, increase yield and water holding, improving the texture and minimising syneresis as typical with yoghurts and similar fermented dairy products (Lopez Camier and Gassi, 2007). Alternatively in gels produced in a cheese vat the level of denatured whey proteins needs to be controlled, partially because increased levels of whey protein confer bitter flavours to matured cheeses and also because whey proteins extracted from the liquid whey are much more valuable economically compared do being in the cheese matrix (Yadav *et al.* 2015). The results from the microstructural analysis suggested that it may be possible to use HIU treatments at lower temperatures for a homogenising effect, leading to a reduction in milk fat globule size and association of the fat droplets and protein network, without incurring higher levels of protein denaturation, supported by the less drastic change in micelle size measured here and corroborated by the study of Gregersen *et al.* (2019). Therefore, temperature control during treatment could allow a cheese maker to optimise the homogenisation process to maximise the entrapment of fat and boost yield,

whilst minimising whey protein losses, therefore maximising the potential of all the milk components. This study utilised pasteurised milk and therefore already contained some quantity of denatured whey protein and potential changes in the milk fat globules prior to treatment. HIU treatment of raw milk could potentially have even more pronounced effects in modifying structure, as the whey proteins will be denatured at the same time as the fat droplets are homogenised, thereby increasing the likelihood of protein-fat interactions, which is worth future consideration.

The results presented here demonstrate the effects that HIU pre-treatment has on the microstructure of dairy acid gels in terms of modification of the protein network, the homogenising effect HIU treatment has on the fat droplets and the subsequent interactions between the fat droplets and protein matrix. Whilst several studies have measured the various physical and chemical changes that occur during HIU treatment, this is the first demonstration with quantitative measures showing that the gel microstructure is significantly altered. The combined method of super-resolution imaging and quantitative image analysis have demonstrated value in being able to assess and differentiate samples prepared with emerging, alternative techniques. The use of quantitative imaging methodologies will be required to reach a stage where causal links can be drawn between microstructure and the emergent physical behaviours such as rheology, water holding or consumer acceptance.

Conclusions

A combination of super-resolution STED microscopy and correlation-based image analysis techniques quantified differences in the microstructure of acid gels from untreated and HIU treated milk. HIU treatments at 27 and 50 °C showed the effects of high intensity ultrasound on the sample, whereas the changes shown at 70 °C illustrate the combined effect of thermal denaturation and HIU treatment. Results demonstrate how HIU, at all temperatures tested, leads to formation a finer gel structures, shown by the size of the typical length scale in the images, ξ_p. HIU treatment also provide a closer association of the fat globules with the protein network, quantified in terms of a reduction in parameter, Λ, which relates to the typical distance from a fat droplet interface to the centre of mass of a neighbouring protein domain shows the largest difference between the control sample and the HIU treated samples. The quantified differences in structure are of importance for understanding HIU induced changes in the acid gel formation of milk.

Conflict of Interests

The authors have no competing interests.

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Data Availability Statement

Research data are not shared

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Figures

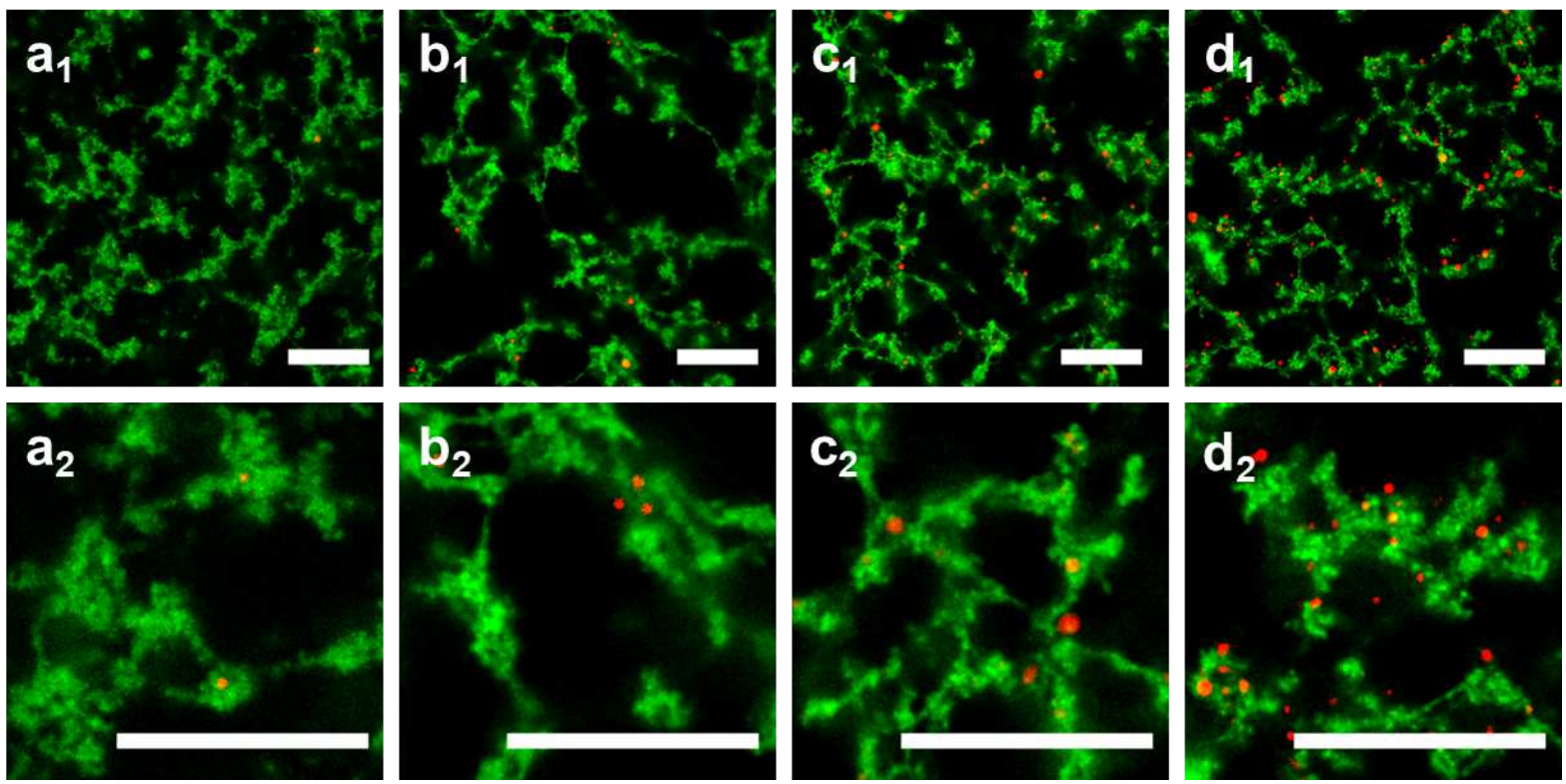

Figure 1: Typical two-colour Super-resolution Stimulated Emission Depletion (STED) and confocal microscopy images of acid milk gels with fat shown in the red channel (confocal) and the protein network shown in the green channel (STED). Acid gels were prepared from whole untreated milk control (a_1,a_2) and milk treated with high intensity ultrasound at 27°C (b_1, b_2), at 50°C (c_1, c_2) or at 70°C (d_1, d_2). Horizontal bar represents 10 µm.

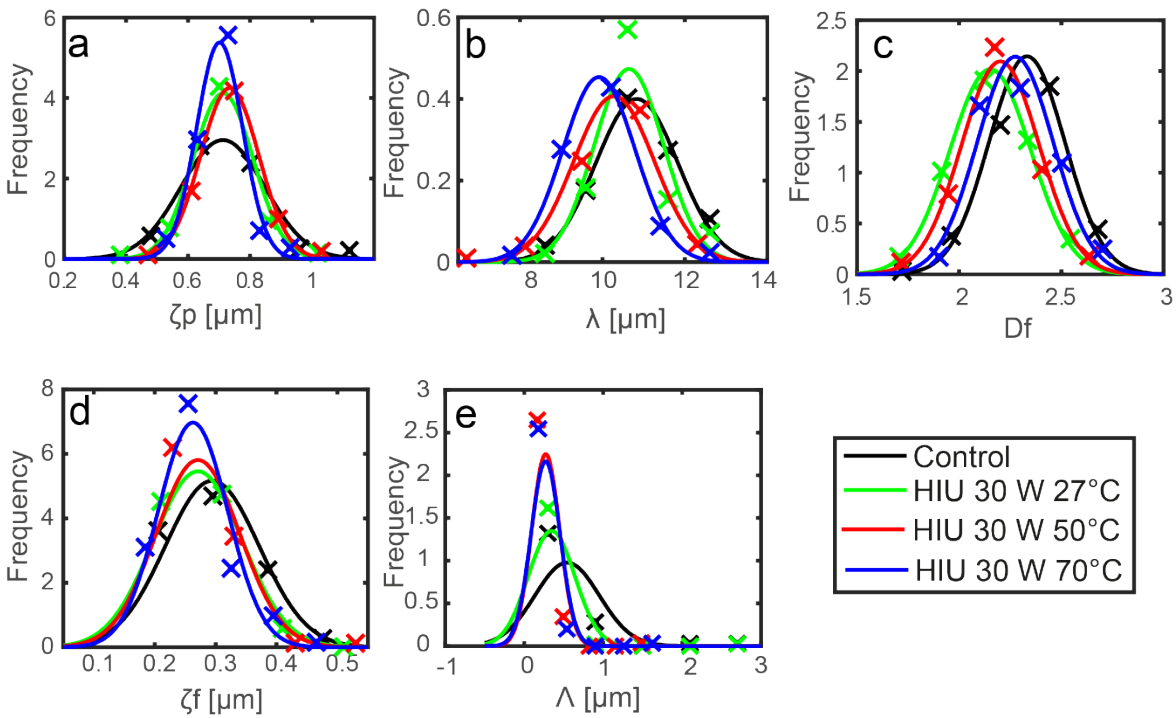

Figure 2: Image analysis parameters extracted from microscopy images of acid milk gels prepared from whole untreated milk (control) or milk treating milk with high intensity ultrasound (HIU) at 27 °C, 50 °C or 70 °C. Typical length of the protein domain extracted from autocorrelation analysis (a). Average inter-pore distance in protein network from autocorrelation analysis (b). Fractal dimension determined from Fourier space analysis (c). The typical length scale of the fat droplets determined with autocorrelation analysis (d). Typical distance from fat droplet interface to centre of mass of neighbouring protein domain determined from cross-correlation analysis (e).

Table

Table 1: Effect of high intensity ultrasound treatment (HIU) of milk at different temperature on the casein micelle size and characteristic of the microstructure, by image analysis of microscopy images, in terms of the typical length scale of the protein domains (ξ_p), the inter-pore distance (λ), the fractal dimension in the protein channel (Df), the typical size of the fat droplets (ξ_f) and the typical distance from the fat droplet interface to the centre of mass of the neighbouring protein domain (Λ). Lower case letters within each column denote significant differences between treatments ($P< 0.05$, n=10).

	Casein micelle size (µm)	ξ_p (µm)	Λ (µm)	D_f (-)	ξ_f (µm)	Λ (µm)
No treatment	196.5 ± 12.2 ^{a}	0.570 ± 0.11^{b}	11.10 ± 0.91^{b}	1.60 ± 0.26 ^{b}	2.24 ± 0.68 ^{b}	0.543 ± 0.48^{b}
HIU 27 °C	221.6 ± 18.0 $^{a, b}$	0.546 ± 0.10$^{a, b}$	10.77 ± 0.96$^{a, b}$	1.60 ± 0.37 $^{a, b}$	2.23 ± 0.51$^{a, b}$	0.346 ± 0.30^{a}
HIU 50 °C	238.2 ± 15.1 $^{a, b}$	0.537 ± 0.09$^{a, b}$	10.68 ± 1.15$^{a, b}$	1.50 ± 0.32 $^{a, b}$	2.10 ± 0.68^{a}	0.272 ± 0.18^{a}
HIU 70 °C	277.8 +16.5^{b}	0.477 ± 0.07^{a}	10.65 ± 1.00^{a}	1.49 ± 0.29^{a}	1.98 ±0.30^{a}	0.271 ± 0.18^{a}

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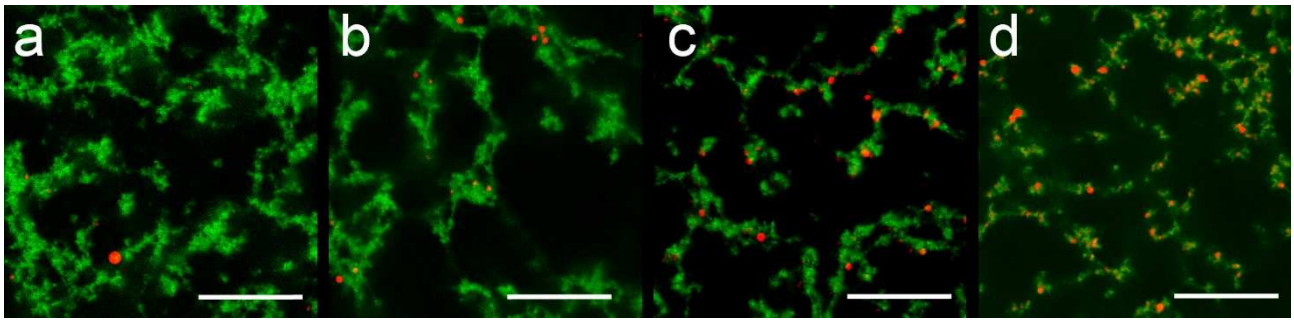

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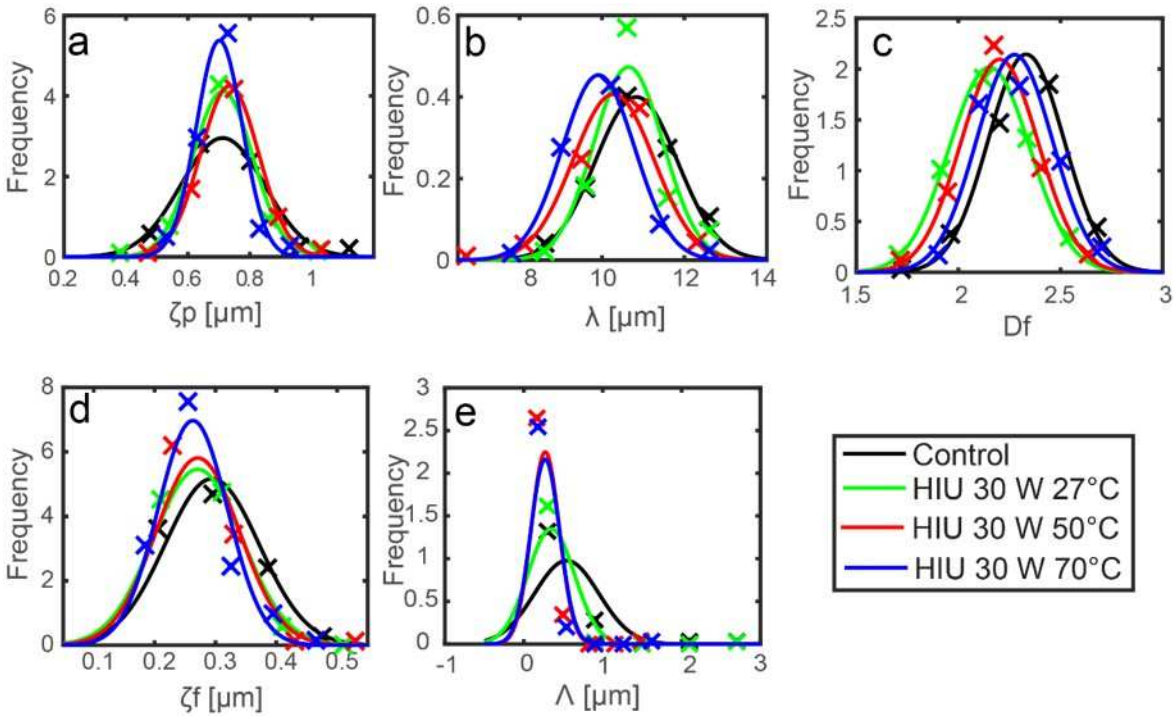

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