Effect of heat treatment of milk between 85 and 100 °C on the properties of acid milk gels

Master thesis by
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Preface

The present master thesis is a result of the work done in a project made at the section of Dairy Technology at Department of Food Science, Faculty of Science, University of Copenhagen (KU) in collaboration with Arla Strategic Innovation Center, Brabrand (ASIC). The work has been performed in the period from January 2012 to November 2012 and corresponds to 45 ECTS. The experimental part of the project was performed at Frederiksberg Campus (LIFE) at Department of Food Science, section of Dairy Technology and section of Quality & Technology, and at ASIC. This report is the final part towards obtaining a Master of Science in Food Science and Technology, with a specialization in Dairy Science.

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Last, but certainly not least, I would like to thank my family and friends, especially my husband Palle Lindkvist and my kids Silas and Lucas, for patience, support and love. Without you this thesis would not have been possible.

Date: 2/11 – 2012

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Maria Lindkvist, FSK09009
Abstract

Heat treatment of milk prior to yoghurt production significantly improves the textural qualities of the yoghurt, making the gel firmer and reducing syneresis. This improvement is due to denaturation and aggregation of the whey proteins (Donato & Guyomarc'h 2009). However, although the effect of heat treatment on the properties of acid milk gels have been extensively studied, little attention has been addressed to the effect of varying the intensity of the heat treatment on the properties of acid milk gels.

The effect of heat treating the milk between 85 °C and 100 °C on the acidification of the milk and the properties of stirred yoghurt was examined. The content of native whey proteins and the content of protein aggregates were investigated by RP-HPLC and SE-HPLC, respectively. The acidification of the milk was followed rheologically and by 1H LF-NMR. The stirred yoghurt was investigated rheologically, by 1H LF-NMR, by degree of syneresis, by the posthumus funnel, and by CLSM.

Increasing the duration of the heat treatment, from 2 to 60 seconds, or increasing the temperature of the heat treatment, from 85 °C to 100 °C, led to a significant decrease in the content of native whey protein in the milk samples, and a significant increase in the amount of protein aggregates in the milk samples.

When acidification of the milk was followed by 1H LF-NMR, a plateau in the acidification curve was obtained when the pH of the milk was about 4.8. The value of T2 in this plateau, T2-plateau, significantly decreased when the temperature or both the duration and the temperature of the heat treatment was increased. The coagulation time, Tgel, measured when acidification of the milk was followed rheologically, significantly decreased when the temperature or the duration of the heat treatment was increased. Furthermore, a correlation appeared to exist between T2-plateau and Tgel.

For both the content of native whey proteins, the amount of protein aggregates, T2-plateau and Tgel there was a shift in the effect of the applied heat treatment, when heating for at least 92.5 °C for at least 31 seconds, because heat treating the milk at a lower intensity only led to minor changes in these properties compared to heat treating the milk for at least 92.5 °C for at least 31 seconds. There thus seems to be a ‘minimum necessary’ heat treatment to cause significant changes to the properties of the milk proteins and the acidified milk gels.

For the stirred yoghurt there was much unexplained variation in all of the properties measured, making it difficult to make conclusions on the basis of these experiments. However, the results indicated that the ‘minimum necessary’ heat treatment to cause significant changes to the properties of the stirred yoghurt was 100 °C for 60 seconds, i.e. a much more intense heat treatment than was found during the acidification experiments. Furthermore, a correlation appeared to exist between T2, water binding, and the rheological properties of the stirred yoghurt. However, further studies in a more stable yoghurt system are necessary to confirm these conclusions.
Resume


Effekten af at varmebehandle melken mellem 85 °C og 100 °C på syrningen af melken og egenskaberne af røreyoghurt blev undersøgt. Indholdet af native valle proteiner og protein aggregater blev undersøgt vha. henholdsvis RP-HPLC og SE-HPLC. Syrningen af melken blev fulgt rheologisk og med 1H LF-NMR. Røreyoghurten blev undersøgt rheologisk, med 1H LF-NMR, via graden af synerese, med posthumus funnel og med CLSM.

Når varmebehandlingstiden blev forøget, fra 2 til 60 sekunder, eller varmebehandlingstemperaturen blev forøget, fra 85 °C til 100 °C, førte det til et signifikant fald i indholdet af native valle proteiner, og en signifikant forøgelse i indholdet af protein aggregater.


For indholdet af native valle proteiner, indholdet af protein aggregater, T2-plateau og Tgel sås en ændring i effekten af varmebehandling, når den var mindst 92,5 °C i mindst 31 sekunder. Varmbehandling af melken ved lavere intensiteter førte kun til mindre ændringer i disse parametre sammenlignet med varmebehandling ved mindst 92,5 °C i mindst 31 sekunder. Derved ser der ud til at eksistere en 'minimum nødvendig' varmebehandling for at give signifikante ændringer af egenskaberne af mælkeproteiner og syrnedede mælkegeler.

For røreyoghurten var der meget uforklarlig variation i alle de parametre, der blev målt, hvilket gjorde det vanskeligt at konkludere noget på basis af disse eksperimenter. Dog indikerede resultaterne at den 'minimum nødvendige' varmebehandling for at give signifikante ændringer af røreyoghurtens egenskaber var 100 °C i 60 sekunder, altså en noget højere varmebehandling end fundet under syrningsforsøgene. Derudover så der ud til at være en korrelation mellem T2, vandbinding, og de rheologiske egenskaber af røreyoghurten. Dog er det nødvendigt med yderligere studier i mere stabile yoghurt systemer for at bekræfte disse konklusioner.
## Abbreviations and symbols

<table>
<thead>
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<th>Description</th>
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<tr>
<td>$^1$H LF-NMR</td>
<td>Proton Low Field Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
</tr>
<tr>
<td>ASIC</td>
<td>Arla Strategic Innovation Center, Brabrand</td>
</tr>
<tr>
<td>CCP</td>
<td>Colloidal Calcium Phosphate</td>
</tr>
<tr>
<td>CLSM</td>
<td>Confocal Laser Scanning Microscopy</td>
</tr>
<tr>
<td>CPMG</td>
<td>Carr-Purcell-Meiboom-Gill</td>
</tr>
<tr>
<td>cryo-TEM</td>
<td>cryo-Transmission Electron Microscopy</td>
</tr>
<tr>
<td>DVS</td>
<td>Direct Vat Set</td>
</tr>
<tr>
<td>FID</td>
<td>Free Induction Decay</td>
</tr>
<tr>
<td>GDL</td>
<td>Glucono-δ-Lactone</td>
</tr>
<tr>
<td>HF-NMR</td>
<td>High Field Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>HSD</td>
<td>Honestly Significant Difference</td>
</tr>
<tr>
<td>HTST</td>
<td>High Temperature Short Time</td>
</tr>
<tr>
<td>LIFE</td>
<td>Frederiksberg Campus, Department of Food Science, Faculty of Science, University of Copenhagen</td>
</tr>
<tr>
<td>Min</td>
<td>Minute(s)</td>
</tr>
<tr>
<td>PC</td>
<td>Principal Component</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal Component Analysis</td>
</tr>
<tr>
<td>RF</td>
<td>Radio Frequency</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reverse Phase High Pressure Liquid Chromatography</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>sec (or s)</td>
<td>Second(s)</td>
</tr>
<tr>
<td>SEC</td>
<td>Size Exclusion Chromatography</td>
</tr>
<tr>
<td>SE-HPLC</td>
<td>Size Exclusion High Pressure Liquid Chromatography</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic Acid</td>
</tr>
<tr>
<td>UF</td>
<td>Ultra Filtration</td>
</tr>
<tr>
<td>UHT</td>
<td>Ultra High Temperature</td>
</tr>
<tr>
<td>B</td>
<td>Magnetic Field Strength</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celcius</td>
</tr>
<tr>
<td>CCP</td>
<td>Colloidal Calcium Phosphate</td>
</tr>
<tr>
<td>G'</td>
<td>Elastic Modulus (Storage Modulus)</td>
</tr>
<tr>
<td>G''</td>
<td>Viscous Modulus (Loss Modulus)</td>
</tr>
<tr>
<td>I</td>
<td>Nuclear Spin Quantum Number</td>
</tr>
<tr>
<td>M</td>
<td>Nuclear Magnetization Vector</td>
</tr>
<tr>
<td>M_x</td>
<td>The x-axis Component of the Nuclear Magnetization Vector</td>
</tr>
<tr>
<td>M_y</td>
<td>The y-axis Component of the Nuclear Magnetization Vector</td>
</tr>
<tr>
<td>n</td>
<td>Number of replicates</td>
</tr>
<tr>
<td>T</td>
<td>Temperature</td>
</tr>
<tr>
<td>$T_1$</td>
<td>Longitudinal Relaxation Time Constant</td>
</tr>
<tr>
<td>$T_2$</td>
<td>Transverse Relaxation Time Constant</td>
</tr>
<tr>
<td>$T_{2n}$</td>
<td>Transverse Relaxation Time Constant for the n$^{th}$ water population</td>
</tr>
</tbody>
</table>
\( T_{2\text{-plateau}} \) The value of \( T_2 \) in the plateau of the acidification curve
\( T_{gel} \) Coagulation time
\( t \) Time

\( \gamma \) Gyromagnetic Ratio (Section 2.5)
\( \gamma \) Shear Strain (Section 2.6)
\( \dot{\gamma} \) Shear Rate
\( \delta \) Phase Angle
\( \eta \) Viscosity
\( \tau \) Pulse Spacing (CPMG)
\( \sigma \) Shear Stress
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1 Introduction

It has long been recognized that heat treatment of milk prior to production of set and stirred yoghurts leads to improved textural qualities of the yoghurt, in the form of firmer gels and reduced syneresis (Kalab et al. 1976; Rønkilde Poulsen et al. 1982).

Heat treatment of milk leads to the denaturation of the whey proteins. Upon denaturation, the whey protein form aggregates with κ-casein, which are attached to the casein micelle or solubilized in the serum of the milk. The nature of these aggregates and in particular their influence on the properties of acid milk gels have been extensively studied, see e.g. recent review by Donato and Guyomarc'h (2009). These studies have in general applied heat treatments between 70 and 100 °C between 1 minute and 30 minutes, and often a heat treatment which is known to cause almost complete denaturation of the whey proteins is applied, e.g. 90 °C for 10 minutes. Only few studies have applied heat treatment for less than one minute (Rønkilde Poulsen et al. 1982; Dannenberg & Kessler 1988a; b), and to the knowledge of this author none of these studies have been performed recently.

Acidification of milk is typically followed rheologically (Lucey et al. 1998c; Guyomarc'h et al. 2003b; Laligant et al. 2003b), but other methods have also been applied, like proton low field nuclear magnetic resonance (1H LF-NMR) (Laligant et al. 2003b; Hinrichs et al. 2007). 1H LF-NMR is not sensitive to coagulation of milk, but when acidification of milk is followed by 1H LF-NMR, the proton mobility increases as pH decreases, reflecting the decreasing hydration of the casein structure due to solubilization of colloidal calcium phosphate (CCP) (Mariette et al. 1993; Famelart et al. 1997; Laligant et al. 2003b)

An in depth investigation of the effect of heat treatment of milk between 85 °C and 100 °C on the acidification of the milk and on the properties of stirred acid milk gels have not been performed recently. Furthermore, to the knowledge of this author, 1H LF-NMR have not been used to follow the acidification of milk heat treated at varying temperatures and durations, nor has there been made a direct coupling between following acidification rheologically and by 1H LF-NMR. The aim of this project was therefore to investigate the effect of heat treatment of milk between 85 °C and 100 °C on the acidification of milk, and on the properties of stirred yoghurt. The acidification of milk was followed rheologically and by 1H LF-NMR. The properties of the stirred yoghurt were investigated rheologically, by 1H LF-NMR, by degree of syneresis, by the posthumus funnel, and by CLSM.

This thesis consists of a theoretical part and an experimental part. The theoretical part is a literature study, where mainly scientific articles, but also book chapters are used. The experimental part provides a description of how the experiments were conducted, and the results of the experiments. These results are discussed and related to the relevant sections in the theoretical part as well literature references. Final conclusions are followed by perspective, with suggestions for further investigations.
2 Theory

2.1 Production of yoghurt

Yoghurt is a cultured milk product prepared by fermentation of milk by the starter cultures *S. thermophiles* and *L. delbrueckii subsp. bulgaricus* (Tamime & Robinson 2007). Yoghurt exists in several forms. The main processing steps for the production of set and stirred yoghurt can be seen in Figure 1. The main difference between set and stirred yoghurt is that when producing set yoghurt, the milk is acidified in the retail container, while when producing stirred yoghurt the milk is fermented in a fermentation tank, then stirred, cooled and filled into retail containers (Lucey 2004; Tamime & Robinson 2007). Stirred yoghurt is the type of yoghurt studied in this project. However, following the acidification of milk in the $^1$H LF-NMR spectrometer or in the rheometer corresponds to producing set yoghurt in small scale. Thus both types of yoghurt will be discussed in this report.

Prior to acidification of milk, the protein content of the milk is standardized, as increasing the protein content leads to improved texture of the yoghurt (Bylund 2003; Tamime & Robinson 2007). The fat content is standardized to achieve the required fat content in the finished product, which varies from 0.1 to 10% depending on product and the yoghurt type (Tamime & Robinson 2007). After standardization the milk is homogenized. The homogenization is performed to prevent creaming during the incubation period and to assure uniform distribution of the milk fat (Bylund 2003). During homogenization, casein and some whey protein absorb to the surface of the fat globules interface and this effectively increases the number of

![Figure 1: The main processing steps in the manufacture of set and stirred yoghurt (Adapted from Lucey (2004))](image-url)
structure-building components, leading to an increased firmness and viscosity of the yoghurt (Lucey 2004). Homogenization also reduces whey separation and increases the whiteness of the product (Tamime & Robinson 2007).

Milk for consumption is normally pasteurized at 72 °C for 15 seconds, or an equivalent heat treatment, to destroy pathogenic microorganisms, while milk for yoghurt production is given a much more intense heat treatment (Bylund 2003). The range of heat treatments applied to milk for yoghurt production, Figure 1, destroys pathogenic microorganisms and most of the milk enzymes, and improves the properties of the milk as a substrate for the starter culture, by lowering the oxygen level (Tamime & Robinson 2007). However, the main reason for subjecting milk for yoghurt production for such an intense heat treatment, as e.g. 95 °C for 5 min is to improve the texture properties of the yoghurt. The heat treatment typically applied to milk prior to yoghurt production, Figure 1, denatures most of the whey proteins in the milk, in particular β-lactoglobulin, Section 2.3, leading to the formation of soluble and micelle bound whey protein aggregates, Section 2.3.1. These aggregates interact with the casein micelles during acidification of milk, which leads to an increased pH at gelation. The increased pH at gelation leads to a decreased gelation time. Furthermore, yoghurt made from heat treated milk has increased gel strength and a decreasing syneresis level compared with yoghurt made from unheated milk, Section 2.4.1. Most of the experiments regarding the importance of heat treatment on the properties of yoghurt and acid milk gels have been investigating set style yoghurt, but examination of stirred yoghurt have led to similar conclusions (Rønkilde Poulsen et al. 1982; Ipsen 2003). It has also been shown that for firm gels changes in the gel properties induced by heat treatment is conserved after stirring the gel (Cayot et al. 2003).

After the heat treatment the milk is cooled to the acidification temperature and inoculated with the starter culture. The acidification temperature is typically 40-45 °C for the starter culture to have optimal growth conditions (Tamime & Robinson 2007). For model systems acidification with glucono-δ-lactone (GDL) is often used instead of using starter cultures (Vasbinder et al. 2001). GDL is a cyclic ester, which hydrolyses to gluconic acid when added to milk (de Kruif 1997). This occurs rapidly, especially at high temperatures, inducing a significant decrease in pH. The final pH of a gel acidified with GDL depends on the concentration of GDL and the buffer capacity of the milk (de Kruif 1997). Although GDL is used to mimic acidification with starter cultures, gels produced with GDL differs from gels produced with starter cultures. Gels made with GDL have higher storage modulus (G'), yield stress, and whey separation, than gels made with starter cultures (Lucey et al. 1998b). During acidification of milk colloidal calcium phosphate (CCP) dissolves from the micelles and the “hairy layer” of the micelles collapse, decreasing the steric stabilization of the micelles, leading to the formation of the gel, Section 2.4.

After acidification is completed, the yoghurt, made with starter cultures, needs to be cooled to stop the acidification, as it otherwise would continue until a pH about 4. As acidification continues after cooling, although slowly, the yoghurt is normally cooled at a pH a bit higher than the target pH in the product (Tamime & Robinson 2007). In set yoghurt, the yoghurt is blast cooled in the retail container after acidification and stored, while in stirred yoghurts, the yoghurt is stirred and then cooled, often in a plate system. The stirring and cooling have great impact on the properties of the yoghurt. By stirring, the gel is broken down into more or less stable microgel particles suspended in the whey. The properties of the stirred yoghurt is related to the properties of the initial gel (Cayot et al. 2003; Lee & Lucey 2006; Renan et al. 2009). If the stirring and cooling is optimally performed, the gel can regain most of its original viscosity.
This regain of structure is called rebodying, and it has been explained with over-acidification, when using bacterial cultures, and cooling of the stirred gel (Bylund 2003; Tamime & Robinson 2007), but rebodying has also been shown to take place when the over-acidification is prevented and when the gel is cooled to 4 °C prior to stirring (Renan et al. 2009). However, in particular for weak gels, minor differences in the cooling and stirring leads to major differences in the final product (Rønkilde Poulsen et al. 1982), and the highest increase in viscosity and elastic modulus is seen in gels, which has the highest viscosity and elastic modulus before and immediately after stirring (Renan et al. 2009). Therefore, when investigating stirred yoghurt, it is important to perform the cooling and stirring as consistent as possible, to minimize the variations in the final product due to inconsistent cooling and stirring.

2.2 Milk proteins

Commercial milk contain about 3.5% protein, although the protein concentration of the milk from individual cows change significantly during lactation (Fox & McSweeney 1998; Fox & Kelly 2004). The milk proteins are divided into caseins and serum proteins. The caseins are defined as the proteins, which precipitate from milk at pH 4.6. They constitute approximately 80% of the protein content in milk, and the whey proteins constitute the remaining 20% of the proteins in milk (Fox & McSweeney 1998; Walstra 2006a). About 3% of the total nitrogen in milk is soluble in 12% trichloroacetic acid and is referred to as non-protein nitrogen. The principal constituent of the non-protein nitrogen is urea (Fox & Kelly 2004).

2.2.1 Serum proteins

The serum or whey proteins of milk are dissolved in the serum of the milk. The definition of whey proteins also include peptides split off from κ-casein by rennet, but serum protein and whey protein are often used interchangeably when discussing the proteins dissolved in the milk (Walstra 2006a). The serum proteins consist mainly of β-lactoglobulin, α-lactalbumin and blood serum albumin, representing approximately 50, 20 and 10% of the total serum protein content in milk.

Apart from these 3 proteins, there are a number of other minor protein fractions in the milk serum, such as immunoglobulins (Fox & McSweeney 1998; Walstra 2006a). β-lactoglobulin and α-lactalbumin are milk specific, as they are synthesized in the mammary gland, as opposed to most of the other serum proteins in milk, which originate from blood or mammary tissue (Larson & Rolleri 1955; Fox & McSweeney 1998). In this section β-lactoglobulin and α-lactalbumin will be described, as they from aggregates with κ-casein upon heating, Section 2.3.1.

2.2.1.1 β-lactoglobulin

β-lactoglobulin is the main serum protein in milk, as it constitutes 50% of the serum protein and approximately 12% of the total protein in milk (Fox & McSweeney 1998). A number of genetic variants of the protein has been identified. The most common genetic variants of β-lactoglobulin is labeled A and B (Aschaffenburg & Drewry 1955; Fox & McSweeney 1998; Bikker et al. 2000; Edwards et al. 2008). β-lactoglobulin has a high biological value, and is rich in sulphur amino acids, as it contains 2 cystines and one cysteine per monomer of 18 kDa. β-lactoglobulin is able to bind several hydrophobic molecules, suggesting a role in their transport. However, the biological function of β-lactoglobulin has not been clearly established. It is proposed that β-lactoglobulin is primarily an important source of amino acids for the offspring (Fox & McSweeney 1998; Kontopidis et al. 2004). β-lactoglobulin is a globular protein, with a compact globular structure and a diameter of about 3.6 nm, Figure 2. In the native molecule the free cysteine is buried in the interior of the molecule, but upon denaturation the molecule unfolds and the
cysteine becomes reactive (Fox & McSweeney 1998). The quaternary structure of β-lactoglobulin is very pH dependent. Below pH 3.5 β-lactoglobulin exists as a monomer, between pH 3.5 and 5.5 it is an octamer, between pH 5.5 and 7.5 it is a dimer, and above pH 7.5 β-lactoglobulin undergoes a conformational change, dissociates to a monomer, and the thiol group become exposed (Fox & McSweeney 1998).

![Figure 2 Tertiary structure of β-lactoglobulin. The yellow bonds represent the disulfide bonds, C106:119 and C66:160, and the yellow dots represent the free cysteine Cys 121 (Chatterton 2011)](image)

2.2.1.2 α-lactalbumin

α-lactalbumin represents about 20% of the serum protein and approximately 3.5% of the total protein in milk. Milk of western cattle contains only one genetic variant (Fox & McSweeney 1998). It is a small, compact ellipsoid protein, 14 kDa, with dimensions of 2.5 x 3.7 x 3.2 nm, Figure 3 (Fox & McSweeney 1998; Walstra 2006a). About 10% of the α-lactalbumin molecules are glycosylated (Edwards et al. 2008). It binds calcium, and the binding of calcium stabilizes the protein conformation. Removal of calcium causes partial unfolding of the molecule into a molten globule state, where irreversible heat denaturation occurs at relatively low temperature. In the presence of calcium native α-lactalbumin shows complete renaturation after heat treatment, if no other proteins were present during heating (Walstra 2006a; Edwards et al. 2008). α-lactalbumin has a key biological role in the synthesis of lactose, as it acts as a ‘specifier protein’, controlling the rate of formation of lactose, as the enzyme catalyzing the synthesis of lactose becomes highly specific in the presence of α-lactalbumin (Fox & McSweeney 1998; Edwards et al. 2008).

![Figure 3 Tertiary structure of α-lactalbumin. The peptide chain is rainbow colored, beginning at the N-terminal in blue and progressing to the C-terminal in red. The binding of the Ca²⁺-ion is shown in the center of the molecule. The four disulfide bonds are shown in ball-and-stick representation (although one in the helical domain is obscured) (Edwards et al. 2008)](image)
2 Theory

2.2.2 The caseins

The caseins represent approximately 80% of the protein in bovine milk. Due to their commercial importance, they have been extensively characterized and are probably the best characterized food protein system (Fox & Brodkorb 2008). According to Fox and Brodkorb (2008) research on casein dates back to the beginning of the 1800 century. Due to the vast amount of literature available about the casein and the casein micelle recent review articles as well as book chapters will form the basis for this section, and other relevant literature will be cited where appropriate. Casein consists of \( \alpha_s1\)-, \( \alpha_s2\)-, \( \beta\)- and \( \kappa\)-casein in the approximate relative amount 4:1:3:5:1.5, respectively (Dalgleish & Corredig 2012). The caseins are highly phosphorylated, and \( \alpha_s1\), \( \alpha_s2\), \( \beta\) and \( \kappa\)-casein contain 8(9), 10-13, 5(4) and 1(2,3) moles phosphate per mole. The phosphorous is covalently bound to the protein and can only be removed by very severe heat treatment, high pH, or some phosphatases. The phosphate is mainly esterified to serine and the phosphorylation occurs in the Golgi membranes of the mammary cell (Fox & McSweeney 1998).

The caseins have very high proline content, resulting in a very low content of \( \alpha\)-helixes and \( \beta\)-sheets. They thus have little secondary or tertiary structure, making the caseins very susceptible to proteolysis without prior denaturation, thus making them easily digestible (Fox & McSweeney 1998). The lack of secondary and tertiary structure of the caseins makes them flexible, and probably explains their stability to denaturing agents, including heat. They have been described as “rheomorphic” or “naturally disordered” implying that they may adapt their structure to suit their conditions (Dalgleish 2011).

\( \alpha_s1\) - and \( \beta\)-casein contain no cysteine or cystine residues, thus increasing their flexibility. The two minor caseins \( \alpha_s2\)- and \( \kappa\)-casein have two cysteine residues pr. molecule. Under non-reducing conditions \( \alpha_s2\)-casein exists as a disulphide-linked dimer, while \( \kappa\)-casein exists as a series of disulphide-linked molecules ranging from dimers to decamers. The cysteine in \( \kappa\)-casein is quite important for the effect of heating on the milk proteins, Section 2.3.1. The degree of hydrophobicity varies among the caseins: \( \beta\)-casein is the most hydrophobic, \( \alpha_s2\)-casein the most hydrophilic. The C-terminal region of \( \kappa\)-casein is strongly hydrophilic, while the N-terminal region is strongly hydrophobic giving \( \kappa\)-casein a detergent-like structure (Fox & McSweeney 1998).

In calcium containing solutions \( \alpha_s1\), \( \alpha_s2\) and \( \beta\)-caseins are insoluble forming a coarse precipitate with calcium at the range of concentrations of protein and calcium found in most milks. \( \kappa\)-casein is not only soluble in calcium, but also interacts with and stabilizes the calcium insoluble caseins enabling the formation of the stable colloidal calcium protein complex in which the caseins exists in milk (Waugh & von Hippel 1956; Fox & McSweeney 1998; Qi 2007)

2.2.2.1 The casein micelle

The casein micelle is a complex aggregate between the caseins and the mineral calcium phosphate. The main purpose of the casein micelle is to solubilize calcium and phosphate and to fluidize the casein molecules, as to lower the viscosity of milk compared with if casein was not in a micelle (Fox & McSweeney 1998; Qi 2007; Horne 2008). Although many properties of the casein micelles have been clearly established, the actual structure has not. The models for casein assembly and structure fall into 4 categories, the sub-micelle model, the nano-cluster model, the dual-binding model, and the “sponge” model (Fox & McSweeney 1998; Qi 2007; Horne 2008; Dalgleish & Corredig 2012). The properties of the casein micelles,

\[1\] Number in bracket refer to the less frequently occurring number of moles phosphate per mole
which are generally accepted, will be outlines in the following, as they form the basis for the different casein models (Anema 2008). Firstly it should be noted, as pointed out by Dalgleish and Corredig (2012), that many studies on the casein micelle have been performed on non-native casein, and although casein micelles from milk powder or milk protein concentrate behave similar to native casein, it remains to be fully demonstrated that it is identical to native casein micelles. Furthermore, it has been reported that small amounts of whey proteins denature and become attached to the casein micelles even during the production of low heat skim milk powder (Martin et al. 2007).

Most of the casein in milk exists in the micelle, but the amount is temperature dependent, and as the temperature is lowered caseins, in particular β-casein, dissociates from the micelle. On a dry matter basis approximately 94% of the micelle is casein and approximately 6% is colloidal calcium phosphate, CCP, consisting of calcium, magnesium, phosphate and citrate. The micelles are highly hydrated, with the interior of the micelle containing approximately 2-3 kg water per kg protein, depending on the measuring method. Thus, although the weight of the caseins is only about 2.5% of the total weight of milk, the micelles occupy approximately 10% of the volume (Fox & McSweeney 1998; Horne 2008; Dalgleish & Corredig 2012). The casein micelle is roughly spherical in shape, although it does not have a smooth surface, with diameters ranging from 80 to 500 nm (average 150-200 nm), and molecular weight ranging from $10^6$ Da to >$10^9$ Da (average $10^8$ Da). There are many small micelles, but these represent only a small proportion of the total mass of the micelles. There are $10^{14}$-$10^{16}$ micelles/ml milk, and they are quite tightly packed with an average distance of about 240 nm, i.e. approximately twice the size of the average casein micelle (Fox & McSweeney 1998; Walstra 1999; Horne 2008; Dalgleish & Corredig 2012).

κ-casein is a critical feature of micelle structure and stability (Waugh & von Hippel 1956; Fox & McSweeney 1998; Fox & Brodkorb 2008). Mutation experiments on mice have demonstrated that κ-casein also is essential for micelle assembly and for lactation to occur in vivo (Shekar et al. 2006), at least in mice, but it is most likely also the case for other mammals. As described in Section 2.2.2 the calcium insensitive κ-casein stabilizes the calcium sensitive caseins. κ-casein can stabilize up to 10 times its mass of the calcium sensitive caseins (Fox & Kelly 2004). The relative proportion of κ-casein in the casein micelle is inversely related to the size of the micelles. The relative proportion of β-casein in the micelle increases with the size of the casein micelle, while the relative proportion of the CCP and the αs-caseins appear to be independent of the size of the micelle. This imply that κ-casein is predominantly, if not completely, on the surface of the micelle, β-casein is mainly present in the interior of the micelle, and the colloidal calcium phosphate and the αs-caseins are found throughout the micelle (Fox & McSweeney 1998; Marchin et al. 2007; Qi 2007; Dalgleish & Corredig 2012).

κ-casein is readily hydrolysed by chymosin (~35kDa) and reacts via sulphhydryl-disulphide interactions with β-lactoglobulin and whey protein complexes, when milk is heated, Section 2.3.1. Both of these reactions further support that κ-casein is positioned at the surface of the micelles, or that the micelles are very porous so that large molecules can diffuse readily through them (Fox & Brodkorb 2008; Dalgleish 2011). It has been demonstrated that κ-casein is not very exposed on the micellar surface, and that the surface of the micelle is not covered exclusively by κ-casein (Fox & Brodkorb 2008). It is generally accepted that most if not all of the C-terminal part of κ-casein protrudes from the surface of the casein micelle in the form of a “hairy layer”, which by steric and electrostatic repulsion prevents further aggregation of the micelles (Walstra 1999; Dalgleish 2007; Dalgleish & Corredig 2012). However, the previous concept of the casein
micelle as a fairly smooth hairy sphere seems to be misleading, as the surface of the casein micelle seems to contain both clefts and protruding protein tubules, Figure 4. With the position of κ-casein for optimal stability being on the end of the tubules, possibly in form of several κ-caseins linked by disulphide bonds on each end (Dalgleish et al. 2004; Dalgleish 2011; Dalgleish & Corredig 2012).

2.2.2.1.1 The sub-micelle model
The sub-micelle model, Figure 5, is the oldest of the casein models, which has not yet been fully rejected, and it has been revised several times (Walstra 1999; Fox & Kelly 2004; Qi 2007; Dalgleish 2011; Dalgleish & Corredig 2012). The sub-micelle model hypothesizes that micelles are formed from smaller aggregates (sub-micelles) linked together by small domains of calcium phosphate (Walstra 1999; Dalgleish & Corredig 2012). The bonds between the molecules in a sub-micelle are believed to be both hydrophobic and electrostatic (Walstra 1990). The model derived from the observation that the proteins in sodium caseinate form small aggregates via non-covalent interactions when dispersed in aqueous media, i.e. in the absence of calcium (Dalgleish & Corredig 2012). With the knowledge that κ-casein is mainly found on the surface of the micelles, it is implied that at least two types of sub-micelles must exist, a κ-casein rich and a κ-casein poor (Walstra 1990; 1999; Dalgleish & Corredig 2012). It has not been clearly demonstrated that such different particles exists neither in sodium caseinate nor in the casein micelles. However, it is known that κ-casein tends to self-aggregate via the formation of intermolecular disulphide bonds, that these aggregates are present in caseinate, and that they could possibly represent the κ-casein rich type of the sub-micelles (Fox & McSweeney 1998; Dalgleish & Corredig 2012). The existence of sub-micelles is supported by the raspberry-like appearance of the casein micelles when examined via electron microscopy, although it might be an artifact from sample preparation (Walstra 1999; Dalgleish & Corredig 2012). The synthesis of the casein micelle has also been claimed to support the sub-micelle model, as the caseins, during the synthesis...
of the casein micelle, appear to be spherical complexes of about 10 nm in diameter, before being phosphorylated and binding calcium in the process of forming the micelles (Qi 2007). However, although it seems to be clearly demonstrated that casein exists as small spherical complexes during synthesis (Qi 2007), it is not clearly demonstrated that these complexes remain intact in the casein micelle (Dalgleish et al. 2004; Dalgleish & Corredig 2012). One of the main evidences for the sub-micellar model, the raspberry like structure of the casein when examined via electron microscopy, does not seem to exist in newer research, where improved methods have been applied (Dalgleish et al. 2004; Fox & Kelly 2004; Marchin et al. 2007). Apart from the lacking proof of existence of the two types of sub-micelles, the sub-micelle model also suffers from the problem that the location of the calcium phosphate in the micelle was never clearly established (Walstra 1990; 1999; Fox & Kelly 2004; Dalgleish 2011). Furthermore, the location of calcium phosphate was altered by Walstra (1999), without giving an explanation for this change.

![Sub-micelle model](image)

**Figure 5 Sub-micelle model: Cross section through a casein micelle, highly schematic (Walstra 1990)**

### 2.2.2.1.2 The nano-cluster model

The nano-cluster model, Figure 6, was originally proposed by Holt (1992), who pictured the casein micelle as a tangled three-dimensional web of casein molecules cross-linked by randomly distributed nano-clusters of CCP, with the C-terminal region of the κ-casein extruding from the surface of the micelle as a “hairy layer” (Fox & McSweeney 1998). Holt et al. (1996) proposed that one of the biological functions of the casein micelle was to enable high concentrations of calcium and phosphate to be in the milk without precipitation, which could potentially cause painful calcification of the mammary gland. The basis of the formation of the nano-cluster model was the discovery that small domains of calcium phosphate precipitating from a supersaturated solution could be surrounded and prevented from precipitation by the presence of β-casein phosphopetides, forming small nano-clusters of calcium phosphate with a mass of 61 kDa and a radius of 2.4 nm surrounded by a shell of about 50 peptides. The tail of the peptides is assumed to associate via weak interactions to form a protein matrix. Weak interactions being hydrophobic interactions, hydrogen bonding, ion bonding, weak electrostatic Van der Waals attraction and other factors (Holt et al. 1996; Holt et al. 1998; Dalgleish 2011; de Kruijf et al. 2012). The presence of nano-clusters evenly distributed throughout the casein micelle has been supported by cryo transmission electron microscopy, cryo-TEM, studies, although the authors are not quite agreeing on the size of the clusters (Marchin et al. 2007; McMahon & Oommen 2008; Trejo et al. 2011). However, the proposed structure of the nano-cluster model has also been criticized for being incapable of explaining the changes of the casein micelle during processing (Horne 2006; Horne 2008).
2 Theory

Figure 6 Representation of the nano-cluster model, displaying a tangled web of casein molecules, red, held together by calcium phosphate nano-clusters, black dots (de Kruif et al. 2012)

2.2.2.1.3 The dual bonding model
In the dual bonding model by Horne (1998), Figure 7, investigations of the individual caseins have been employed in predicting their ability to cross-link through hydrophobic interactions. The model suggests that micellar assembly and growth take place by a polymerization process involving two distinct types of bonding: Cross-linking through hydrophobic regions of the caseins and by linkage of hydrophilic regions containing phosphoserine clusters to calcium phosphate nano-clusters. The bond formation is facilitated by a local excess of hydrophobic attraction over electrostatic repulsion (Horne 2008). κ-casein can link to the casein chains by its hydrophobic N-terminal, but it has a hydrophilic C-terminal and no phosphoserine cluster. κ-casein is thus considered a chain terminator, explaining the surface location of κ-casein (Horne 1998; Horne 2008). According to Dalgleish (2011) the dual bonding model describes the types of interactions by which the micelle can be assembled, but it fails to give the details of the actual interior structure of the micelle. de Kruif et al. (2012) also criticizes the dual bonding model, stating that the structure of the model, with interaction of only a pair of molecules, is thermodynamically unfavorable.

Figure 7 Schematic illustration of the dual bonding model, showing the bonding through hydrophobic interactions between casein, linkage through CCP, and the surface location of κ-casein (Horne 1998)

2.2.2.1.4 The "sponge" model
Arguing that none of the current models take into account the porous water filled structure of the casein micelle, as evident from the interior of the micelle containing 2-3 kg water per kg protein, Dalgleish (2011) recently proposed a new model for the structure of the casein micelle, Figure 8, and went on to explain the usefulness of the model during processing (Dalgleish & Corredig 2012).
In the different models of the structure of the casein micelle, the hydrophobic interactions between the micelles are believed to have a major influence on the stability of the casein micelle. However, this does not fit well with the presence of vast amounts of water in the interior of the micelle, unless the water is not evenly distributed. Leading to the conclusion that water channels must exist inside the micelle (Dalgleish 2011). In the “sponge” model the internal structure of the casein micelle is pictures as a bicontinuous system of water channels and strands formed of calcium phosphate/casein nano-clusters. The idea is that CCP, αs-casein and some of the β-casein aggregates together in strands, held together by short range interactions like calcium bridging, hydrogen bond formation and van der Waals interactions. These strands are then partially stabilized by the surfactant properties of mobile β-casein molecules, hydrophobically binding to other caseins, which also explains how up to 60% of β-casein can leave the micelle upon cooling. The exterior of the micelle is stabilized by κ-casein (Dalgleish 2011; Dalgleish & Corredig 2012). This model for the internal structure of the casein micelle is supported by the analysis of cryo-TEM and atomic force microscopy, AFM, images of the casein micelle (McMahon & Oommen 2008; Trejo et al. 2011; Ouanezar et al. 2012).

An interesting consequence of the “sponge” model is that for ‘smaller’ molecules, enzymes included, the idea of the surface of the micelle is challenged, as these molecules will be able to penetrate deep into the interior of the micelle via the water channels. However, for interactions between casein micelles, the idea of the casein micelle with a stabilizing “hairy layer” is unchanged by this model (Dalgleish & Corredig 2012).

![Figure 8 Schematic section through a casein micelle, showing regions of water within the structure (white area). The αs-casein and β-casein (orange) are connected via calcium phosphate nano-clusters (grey spheres). Some β-casein (blue) is hydrophobically bound to other caseins. Para-κ-casein (green) and caseinomacropeptide chains (black) are on the outermost parts of the surface. Not drawn to scale and the sizes of the water channels are exaggerated for clarity (Dalgleish & Corredig 2012).](image-url)

During the development of a deeper understanding of the structure of the casein micelle and the mechanism of casein micelle interaction and changes during processing several models for the structure of the casein micelle have been proposed as outlined in this section. At the time of their creation these models tried to incorporate current knowledge and ideas, and it seems likely that, as the knowledge about
the caseins, the CCP, and their interactions in the micelle, is expanded, or importance of current knowledge is realized, the current models will be revised or new models will occur.

2.3 The effect of heat treatment of milk on the milk proteins

The caseins are very stable to high temperatures, coagulating only after being heated at 140 °C for 15-20 min (Fox & McSweeney 1998). Unlike the caseins the serum proteins are heat labile, retaining their native conformation only within relatively limited temperature ranges (Anema 2008; Donato & Guyomarc'h 2009). It should here be noted that the denaturation of a protein involves the (reversible) unfolding of the protein often followed by irreversible aggregation reactions. As it is the irreversible aggregation that largely determine the functional properties of dairy products, it is common practice to define serum protein denaturation as the formation of irreversibly denatured and aggregated serum proteins (Anema 2008). In general denaturation of the major serum proteins, α-lactalbumin and β-lactoglobulin, occurs only at temperatures above ≈70 °C (Anema 2008). Even before it was realized that the proteins in the milk serum consisted of several different proteins, it was discovered that denaturation of the serum proteins was a kinetic phenomenon, thus depending on both the temperature and the duration of the heat treatment (Rowland 1933; Harland & Ashworth 1945). After the isolation of the individual serum proteins, it was possible to investigate the heat stability of the individual proteins (Larson & Rolleri 1955). According to Anema (2008) the investigation of the kinetics of the denaturation of the major serum proteins in milk, α-lactalbumin and β-lactoglobulin, over a large temperature range began with the study of Lyster (1970). The study indicated that the irreversible denaturation reaction was not a simple process, as a change in temperature dependence was observed at about 80-90 °C for both proteins (Lyster 1970). There is indications that the change in temperature dependence is due to a change from unfolding of the whey proteins being the rate determining step at lower temperatures, below 90 °C for β-lactoglobulin and below 80 °C for α-lactalbumin, to aggregation reactions being the rate determining step at higher temperatures (Dannenberg & Kessler 1988c; Kessler 2002; Anema 2008).

2.3.1 Aggregation of milk proteins upon heating

It has long been known that in model mixtures β-lactoglobulin and κ-casein interacts upon heating via disulphide aggregation (Zittle et al. 1962; Sawyer et al. 1963). When heating milk, whey protein denature and form aggregates with κ-casein, which are either soluble or micelle bound. However, the formation of these aggregates is a much slower process than the denaturation of whey proteins (Anema & Li 2003a; b; Guyomarc'h et al. 2003a; Vasbinder et al. 2004; Donato et al. 2007; Donato & Guyomarc'h 2009; Morand et al. 2011). The pathway for formation of these aggregates still remain to be fully established, for recent review see Donato and Guyomarc'h (2009). These aggregates have been shown to consist primarily of α-lactalbumin, β-lactoglobulin, and κ-casein held together by disulphide bonds (Guyomarc'h et al. 2003a; Jean et al. 2006), and to contain still reactive thiol groups (Vasbinder et al. 2003b). According to Corredig and Dalglish (1996a), when heated in a water bath, α-lactalbumin and β-lactoglobulin have similar kinetics of interaction with casein micelles, but when milk is heated in HTST and UHT systems α-lactalbumin reacted more slowly than β-lactoglobulin, indicating that the extent of formation of aggregates depends not only on the intensity of the heat treatment, but also on the type of heat treatment applied. Aggregation between whey protein and various concentrations of κ-casein, upon heating for 24 hours at 80 °C, to ensure maximum complex formation, have shown that an increase in κ-casein concentration leads to a decrease in the size of the aggregates, indicating that κ-casein acts as an inhibitor for the propagation of the whey protein complexes (Morand et al. 2011)
The mechanism of formation of aggregates between κ-casein and whey proteins changes with pH. A decrease in pH leads to an increase of the attachment of whey proteins to the casein micelle, while an increase in pH increases the amount of soluble aggregates, possibly because of increasing solubilization of κ-casein. At normal pH of milk about 30% of the whey protein aggregates bind to the micellar surface (Anema & Li 2003b; Donato & Dalgleish 2006).

When whey proteins form complexes with κ-casein on the surface of the casein micelles, the size of the micelles increase. Based on the increase in the size of the micelle and the number of whey proteins available per κ-casein, it seems that not all of the κ-casein participates in the formation of whey protein κ-casein complexes (Anema & Li 2003a). The extent of protein attachment to the casein micelles, have been shown to increase when the temperature of the heat treatment is increased, and most of the change in the casein micelle size is seen after more than ~80% of the whey protein had denatured (Anema & Li 2003a). However, there seems to be a limit to the extent in which whey proteins can bind to casein micelles (Corredig & Dalgleish 1996b; Guyomarc’h et al. 2003a).

When heating mixtures of whey proteins and casein micelles it has been shown that increasing the whey protein content increases the amount and the size of the soluble whey protein/κ-casein aggregates (Guyomarc’h et al. 2003a; Donato et al. 2007), but adding additional κ-casein has only minor effect on the amount and the size of these aggregates (Donato et al. 2007). However, when heating mixtures of whey proteins and κ-casein, aggregates are known to be formed (Jean et al. 2006; Donato et al. 2007; Morand et al. 2011). According to Donato et al. (2007) their results indicates that, when heated in a water bath at 90 °C, the preferential reaction of whey proteins with κ-casein is on the surface of the casein micelles, and the soluble complexes dissociates from the micelle into the serum, rather than being formed in the serum.

2.4 Acidification of milk

During the reduction in pH calcium and phosphate gradually dissociates from the micelle, due to increased aqueous solubility. The method of acidification and separation of soluble calcium and phosphate varies between studies, but in general only little change in the amount in soluble calcium and phosphate is seen above pH 6. Below pH 6, the amount of soluble calcium and phosphate increases, and reaches a plateau at 5.2-5.0 for phosphate and about 4.6 for calcium (Dalgleish & Law 1989; Mariette et al. 1993; Famelart et al. 1996; Gastaldi et al. 1996; Famelart et al. 1997; Le Graët & Gaucheron 1999). When using defined amounts of GDL to obtain the decreasing pH, it has been calculated that the ratio between calcium and phosphate in the micelles remains constant down to pH 5.5 (Dalgleish & Law 1989; Gastaldi et al. 1996). In spite of the loss of CCP, which is an essential structural feature, dissociation of casein from the micelles at decreasing pH is only seen at temperatures below 25 °C (Dalgleish & Law 1988). This stability has been coupled with the decreasing negative charge of the individual caseins, so that although the bonds to CCP are lost, the charges of the individual caseins become insufficient to force them apart. Thus the casein micelle is dominated by hydrophobic interactions, which increase in strength, when the temperature is increased, explaining the temperature dependence of the dissociation of caseins from the micelle upon acidification (Horne 2008; Dalgleish & Corredig 2012). The reduction in pH also decreases the charge of the stabilizing “hairy layer” of κ-casein on the micellar surface causing a collapse of the κ-casein layer, because intra- and interchain interactions are no longer sufficient to keep the chains extended (Dalgleish & Corredig 2012). The collapse of the “hairy layer” decreases the steric stabilization of the casein micelles, enabling them to diffuse closer to each other, and as short range attractive forces take over, a tree-dimensional network is
formed (Lucey & Singh 1998). In unheated milk, the aggregation occurs very close to the isoelectric point of the caseins, at a pH of approximately 4.8 (Vasbinder et al. 2003a; Rodriguez del Angel & Dalgleish 2006).

Recent investigation of the structure of the casein micelle upon acidification by cryo-TEM, multangle 3D light and x-ray scattering, and AFM has revealed that casein micelles lose their surface heterogeneity, shrink, and become more compact upon acidification, resulting in small smooth casein particles (Marchin et al. 2007; Moitzi et al. 2011; Ouanezar et al. 2012). According to Ouanezar et al. (2012) these results support the theory of the casein micelle having a sponge-like structure, Section 2.2.2.1.4, as internal gaps in the micellar structure would close upon the rearrangement and packaging procedure induced by the loss of CCP and the increase in net attraction between the proteins.

2.4.1 Effect of heat treatment on the properties of acid milk gels

It has long been known that when milk is heated prior to yoghurt making, the pH at gelation increases, the strength of the gel increases, and the syneresis decreases (Kalab et al. 1976; Rønkilde Poulsen et al. 1982), although excessive heat treatment was found to have a negative influence on the gel properties (Rønkilde Poulsen et al. 1982; Dannenberg & Kessler 1988b; Lucey et al. 1997). Studies of the effect of heat induced whey protein denaturation on the properties of the resultant gel, have shown a strong correlation between denaturation of whey proteins, in particular β-lactoglobulin, and an increase in gelation pH (Lucey et al. 1997; Lucey et al. 1999; Vasbinder et al. 2001; Ipsen 2003; Vasbinder et al. 2003a), an increase in gel strength (Dannenberg & Kessler 1988b; Lucey et al. 1997; Lucey et al. 1999; Cayot et al. 2003; Ipsen 2003), and a decreasing level of syneresis (Dannenberg & Kessler 1988a; Cayot et al. 2003). These results lead the conclusion that aggregation of denatured whey proteins with casein particles during the acidification of heated milk explains the effects of heating on the properties of acid milk gels (Lucey et al. 1997).

Heat denaturation of whey proteins lead to the formation of soluble and micelle bound whey protein aggregates Section 2.3.1. These aggregates cause the pH at gelation to increase from ~4.9 to ~5.4, and the strength of the gel to increases (Kalab et al. 1976; Lucey et al. 1998c; Guyomarc’h et al. 2003b; Vasbinder et al. 2004; Donato & Guyomarc’h 2009). These changes occurs irrespective of whether the denatured whey proteins are present as soluble aggregates, or bound to the micelle or as both soluble aggregates and aggregates bound to the micelle. However, both the gelation pH and the strength of the resultant gel is more affected by soluble complexes than micelle bound complexes (Guyomarc’h et al. 2003b; Vasbinder et al. 2004). It has been hypothesized that this reflects the ability of the soluble whey protein aggregates to rearrange and fill the voids between micelles, resulting in a larger number of linkages in the gel network than when the whey proteins are already attached to the surface of the protein particles before gelation (Dalgleish & Corredig 2012).

Confocal laser scanning microscopy (CLSM) on acidified milk systems of separately labeled casein micelles and whey protein complexes have shown that both soluble and micelle bound whey protein aggregates physically take part in the network formation in acid milk gels, as they appear to be co-located with the micelles (Vasbinder et al. 2004). Similar studies performing CLSM during the acidification indicates that heat induced serum complexes interacts with casein micelles at least at the point of gelation (Dubert-Ferrandon et al. 2006; Guyomarc’h et al. 2009). These studies also showed that in gels made from unheated milk, the casein micelle fraction had a homogeneous structure with small pores, and the whey

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2 Corresponding to heat treatment much more intense than needed for complete denaturation of β-lactoglobulin
proteins fraction appeared to be unstructured, while in gels made from heated milk the casein micelle fraction was more inhomogeneous, with larger pores, and the whey protein fraction was more structured. Visual inspection of the pore size, coupled an increasing pore size with an increase in the strength of the gel (Vasbinder et al. 2004). Examination of the gel strength of gels made in the presence or absence of N-ethylnmaleimide, a chemical compound preventing the formation of disulfide bonds, indicate that disulphide bonds play an important role during gel formation in heated milk, in particular in the rearrangements of the gel occurring after gelation. The presence of N-ethylnmaleimide did not affect the gel strength of gels made from unheated milk (Vasbinder et al. 2003b; Vasbinder et al. 2004).

The exact mechanism for the changes seen in the acid gelation of heated milk still remains to be fully established (Donato & Guyomarc'h 2009). Although, experiments in model milk systems, with protein aggregates with altered pl and surface hydrophobicity, have shown that increasing the pl of the protein aggregates, from 3.5 to 5.5, increases pH at gelation, without having an effect on the value of the elastic modulus of the gel, while increasing the surface hydrophobicity of the protein aggregates, increases both pH at gelation and value of the elastic modulus of the gel. However, excessive surface hydrophobicity of the whey protein aggregates resulted in gels with increased pore size and whey expulsion (Morand et al. 2012a; Morand et al. 2012b).

When increasing the gelation pH to above about 5.2, either by heat treatment of the milk (Guyomarc'h et al. 2003b; Dubert-Ferrandon et al. 2006), by increasing the acidification rate both in heated and unheated milk (Horne 2003; Anema 2009), by increasing the content of CCP in the casein micelles (Anema 2009), or by inducing coagulation with a combination of acid (GDL) and rennet (Lucey et al. 2000), the gel will exhibit a ‘maximum loss tangent’, \( \tan \delta_{\text{max}} \), and a ‘shoulder’ in the elastic modulus after gelation. This unusual rheological behavior has been attributed to the presence of CCP in the casein micelles at gelation. The continuing decrease in pH after gelation will dissolve CCP from micelles already partitioning in the gel network, causing an internal weakening in the gel particles. As the pH is further deceased, so is the charge of the proteins in the gel network, allowing reformation of internal bonds, inducing an increase in the gel stiffness (Horne 2003; Anema 2009).

In unheated milk at low acidification rates, the gelation occurs at a low pH, about 4.8, thus CCP is already dissolved before a network is formed, and the gel does not undergo an internal weakening or exhibit a \( \tan \delta_{\text{max}} \) (Lucey 2004). In heated milk with varying CCP content in the micelles, the \( \tan \delta_{\text{max}} \) is increased and shifted to a higher pH as the concentration of CCP is decreased, probably because the CCP is solubilized at a higher pH, when the CCP level is decreased (Anema 2009).

2.5 \(^1\text{H LF-NMR}\)

Nuclear magnetic resonance, NMR, is a non-invasive, non-destructive method of extracting physical and chemical information about a sample. It has the advantage that most systems are transparent to the method as nuclei and electrons that are magnetic occur naturally in all matter, but NMR is less sensitive than other spectroscopic methods (Eads & Davis 1994; Viereck et al. 2005).

Nuclei spin like a top, and thus have a spin angular momentum, the magnitude of which depends on the spin quantum number, \( I \). In NMR spectroscopy the sample is positioned in a strong external magnetic field,
and this allows for studies of specific nuclei in the sample with non-zero spin quantum numbers\textsuperscript{3} (Viereck \textit{et al.} 2005). The proton ($^1\text{H}, I = \frac{1}{2}$) is the most commonly used NMR nucleus, and as proton low field nuclear magnetic resonance, $^1\text{H}$ LF-NMR, is used to investigate the samples in this report, protons will be used as model nucleus in the remainder of this description of the theory of NMR.

In this report the focus is NMR relaxometry, which can be described by the Bloch equation (Bloch 1946):

\begin{equation}
\frac{d\mathbf{M}}{dt} = \gamma \mathbf{M} \times \mathbf{B}
\end{equation}

Where $\mathbf{M}$ is the nuclear magnetization vector, which is defined as the sum of all the spins, $\mathbf{B}$ is the magnetic field strength, and $\gamma$ is the gyromagnetic ratio, which is constant for a given nucleus. To facilitate the description of the spin manipulation giving rise to the NMR signal, an NMR instrument is normally ascribed axes: The $z$-axis is normally ascribed the direction of the external magnetic field, and the nuclear magnetization vector at equilibrium, the $x$- and the $y$-axis will normally be equipped with coils for applying radio frequency (RF) pulses and for detection. To flip the magnetization of the sample to the $xy$-plane, where the signal of the sample is to be detected, an RF pulse is applied. An RF pulse is a short pulse, typically few $\mu$s, in the radio frequency which result in a broad band excitation, exciting all resonances simultaneously, giving the strongest possible signal (Eads & Davis 1994). The angle of the flip of the nuclear magnetization vector depends on the duration of the RF pulse. An RF pulse with duration precisely long enough to flip the net magnetization vector into the $x$-$y$ plane is denoted a $90^\circ$ pulse or reading pulse, while a pulse with a duration long enough to flip the nuclear magnetization vector along the negative $z$-axis is donated a $180^\circ$ pulse. When the nuclear magnetization vector is in the $xy$-plane, it will continue to precess about the external magnetic field, inducing an oscillating signal in the detectors along the $x$- and $y$-axis (Viereck \textit{et al.} 2005).

After perturbation by a $90^\circ$ pulse, the obtained signal will decrease exponentially due to nuclear relaxation processes. The magnetization has a longitudinal component (parallel to the static magnetic field, the $z$-axis) and a transverse component (in the plane perpendicular to the static field, the $xy$-axis) (Eads & Davis 1994). The signal is measured in the $xy$-plane, so the decay of the signal reflects the decay of the transverse component, which decays as the spin system gradually loses coherence in the $xy$-plane. The loss of coherence is due to energy exchange between spins. The process is known as spin-spin or transverse relaxation, and is described by a time constant called $T_2$. Simultaneously with the loss of coherence in the $xy$-plane the protons will seek to regain equilibrium along the $z$-axis due to the influence of the external magnetic field. The time it takes for the protons to regain equilibrium depends on the probability of energy exchanges occurring between the spins and their environment (the lattice). This relaxation mechanism is referred to as longitudinal or spin-lattice relaxation and is described by a time constant called $T_1$. Interaction with less mobile species enhances $T_1$ relaxation, providing an indication of the degree of water mobility in the vicinity of macromolecules and surfaces. A long $T_1$ indicates greater water mobility (Viereck \textit{et al.} 2005; Hinrichs \textit{et al.} 2007).

\textsuperscript{3} Nuclei with even number of protons and neutrons are not visible by NMR spectroscopy, as $I = 0$
By the Bloch equations it is possible to derive the following equations:

Equation 2

\[
\frac{dM_x}{dt} = -\frac{1}{T_2} M_z
\]

Equation 3

\[
\frac{dM_y}{dt} = -\frac{1}{T_2} M_y
\]

Where \(T_2\) is the transverse relaxation, and \(M_x\) and \(M_y\) is the x- and y-magnetization, respectively. These equations can be solved as:

Equation 4

\[
M_x = M_{x0} \cdot \exp\left(\frac{1}{T_2} t\right)
\]

and likewise for the y-magnetization. Thus describing the exponential decrease of the magnetization in the xy-plane due to spin-spin relaxation.

2.5.1 Pulse experiments

A number of pulse experiments for the selection of specific signals give NMR applications an enormous diversity, here the focus will be on the two pulse experiments performed in this report, FID and CPMG.

2.5.1.1 FID (Free induction decay)

The FID experiment is the simplest possible experiment in NMR, as it is the application of a short unselective 90° RF pulse and measurement of the relaxation in the xy-plane following the pulse, Figure 9A. If several consecutive measurements are performed, a recycle delay is inserted between the measurements, to allow the system to regain equilibrium along the z-axis. As described above the relaxation after the 90° pulse is due to spin-spin interactions, and the time constant describing the FID curve is called \(T_{2*}\), to distinguish it from the true nuclear relaxation time \(T_2\) (Eads & Davis 1994).

The short unselective pulse applied in the FID experiment excites all resonances simultaneously, making it possible to view all nuclei of a given type in the sample, as the amplitude at time zero is proportional to the number of resonant nuclei (Eads & Davis 1994; Viereck et al. 2005). The decay rate gives physical information about a sample. In solid like phases, where molecular motion is restricted and slow, the signal will decay rapidly, whereas in liquid like phases, where molecular motion is unrestricted and fast, the signal will decay slowly (Eads & Davis 1994).

2.5.1.2 CPMG (Carr-Purcell-Meiboom-Gill)

The real \(T_2\) is measured by the CPMG experiment (Carr & Purcell 1954; Meiboom & Gill 1958), which consists of a 90° pulse followed by a number of 180° pulses, in the following sequence 90°-\(\tau\)-180°-2\(\tau\)-180°-2\(\tau\)-180°-..., with echoes at times 2\(\tau\), 4\(\tau\), 6\(\tau\) etc., Figure 9B. If several consecutive measurements are performed, a recycle delay is inserted between the measurements, to allow the system to regain equilibrium along the z-axis. The CPMG sequence creates a spin echo in the xy-plane, where effects due to inhomogenic spin-spin interactions are refocused, which means that the effect of the main part of the spin-spin interactions are averaged to zero at the time point 2\(\tau\), 4\(\tau\) etc.
2 Theory

Fitting the top point of the decaying echoes exponentially gives the $T_2$ value (Viereck et al. 2005). A disadvantage of the CPMG experiment is that the time from the perturbation of the system to the data acquisition is much longer than for the FID experiment, making it difficult to obtain a signal from the very fast relaxing components, representing solid phases of the sample, as they have relaxed before the data acquisition starts.

Figure 9 Illustration of the pulse experiments applied in this report. A: The FID experiment. B: The CPMG experiment (Larsen 2008).

2.5.1.3 Extracting $T_2$ values

In a complex food system protons typically exist in many forms measurable by NMR. These forms have different $T_2$ values: Free water, which relax slowly, has a $T_2$ of 100-2500 ms, bound water, which relax faster, has a $T_2$ of 50-100 ms, and solid fat, which relax fast, has a $T_2$ of 0.5-100 ms (Larsen 2008). In fact time domain NMR is quite sensitive in detecting differences in molecular mobility (van Duynhoven et al. 2010). The decay rate measured by FID and CPMG will comprise the $T_2$ value of all of the components in the sample, and if multiple components exist in a sample the relaxation decay curve will be a sum of multiple exponential terms (Hansen et al. 2010). To extract the different $T_2$ values of the individual components in the sample, the relaxation curves were fitted by a sum of exponential decays according to the following equation, note the similarity between this equation and Equation 4:

Equation 5

$$M(t) = \sum_{n=1}^{N} M_{2n} \cdot \exp \left(-\frac{t}{T_{2n}}\right) + e$$

Where $M(t)$ is the reduced magnetization at time $t$, $N$ is the number of components in the sample, $M_{2n}$ is the relative abundance of the $n^{th}$ component, $T_{2n}$ is the corresponding relaxation time constant, and $e$ is the residual error (Pedersen et al. 2002; Hansen et al. 2010).

2.5.2 Applications of $^1$H LF-NMR

NMR has many applications for food products and different types of dairy products (Karoui & De Baerdemaeker 2007; van Duynhoven et al. 2010). In this section the focus will be on NMR measurements during the acidification of milk and casein solutions.

In milk the proton mobility expressed as the proton relaxation rate has been described as the sum of three fractions of protons: (1) “free” water protons, the mobility of which does not depend on the motion of macromolecules; (2) hydration water protons, the mobility of which depends on the motion of
macromolecules, e.g. by interactions with proteins; and (3) water protons in exchange with exchangeable protein protons, such as the side chain protons in -OH, -SH and -NH (Mariette et al. 1993; Le Dean et al. 2004).

NMR studies of the changes in proton mobility during acid gelation of skim milk and caseinate solutions shows that as the pH decreases the proton mobility is increased (Mariette et al. 1993; Famelart et al. 1997; Laligant et al. 2003b; Hinrichs et al. 2007; Mok et al. 2008). In general little change is seen in the proton mobility down to pH 6, then the proton mobility increases until a pH below 5, after pH 5 the proton mobility stabilizes.

There is a high correlation between $^{17}$O relaxation rate and $^1$H relaxation rate, of the most abundant proton population, measured during static acidification of skim milk. As the $^{17}$O relaxation rate does not reflect molecular motions of water molecules in exchange with ex-changeable protein protons, this indicates that the changes in $^1$H relaxation rates during acidification of skim milk mainly reflects changes in the molecular motions of "free" and hydration water molecules, i.e. fraction 1 and 2, from the above description. This indicates an increase in the molecular motion of water molecules during acidification of skim milk (Mariette et al. 1993; Moller et al. 2011).

Mariette et al. (1993), performing a static investigation of the acidification of reconstituted skim milk with GDL, coupled the increase in water mobility during acidification as seen by a decrease in the $^{17}$O and $^1$H relaxation rates with the solubilization of CCP from the micelles, detected by $^{31}$P NMR. This correlation was also found by Famelart et al. (1997), who also performed a static experiment on reconstituted milk. Laligant et al. (2003b) performed continuous NMR measurements on heat treated reconstituted skim milk acidified with lactic acid bacteria at two acidification temperatures and found that the acidification temperature did not seem to affect the changes in the proton mobility during acidification. This result corresponds with the solubilization of colloidal calcium phosphate during acidification of milk being independent of the acidification temperature (Dalgleish & Law 1989; Laligant et al. 2003a). According to Laligant et al. (2003b) $T_2$ is not sensitive to gelation, but the time for the minimum value of $dT_2/dpH$ corresponded to the maximum of tan($\delta$), further supporting that the increase in water mobility measured during acidification relates to solubilization of CCP from the micelles, as the maximum in tan($\delta$) has been correlated with solubilization of CCP from the micelles (Horne 2003; Anema 2009).

Hinrichs et al. (2007) used a combined FID and CPMG experiment to describe the acidification of casein solutions with up to four water populations. An immobile and a weakly mobile water phase were found to vary very little with the decrease in pH. The $T_2$ value of the mobile water phase increased as the pH decreased until about pH 5.8-5.4, then a very mobile phase appeared which $T_2$ value increased until about pH 5. However, the authors without further explanation, and no supporting results, states that the water mobility of the gel is changed as soon as pH reaches the isoelectric point of casein. They suggest a correlation between the increase in the proton mobility during acidification and the disintegration of the casein micelles due to solubilization of colloidal calcium phosphate. The presence of a very mobile water phase in this study, which was not seen in the studies performed on reconstituted milk mentioned above might be because this study was done on casein solutions, as whey proteins have been shown to decrease the water mobility in solutions of native phosphor caseinate (Le Dean et al. 2004).
Summing up, the primary change in proton mobility during milk acidification is seen in the water protons, observed as mobile protons in NMR experiments. The measured proton mobility of the water protons represents an average of the water protons in the milk (Larsen 2012, personal communication). Casein micelles represent approximately 10% of the volume in milk, and their porous structure holds large amounts of water, Section 2.2.2.1. Therefore the mobility of the major water population in milk is affected by casein. Presumably the mobility of the water molecules inside the casein micelle is lower than the mobility of water molecules in the vicinity of the casein micelles. As the pH is lowered CCP leaves the micelles, and the casein micelles is increasingly held together by hydrophobic bonding, Section 2.4. Thus the interior of the micelle become more hydrophobic, expelling water from the micelles. As water is expelled from the micelles the average proton mobility increases, as the contribution of less mobile water populations from the interior of the micelles to the overall proton mobility decreases. The increasing proton mobility during acidification of milk thus reflects the decrease in hydration of the casein micelles (Laligant et al. 2003b; Ouanezar et al. 2012). When all of the CCP is expelled from the micelles, the change in proton mobility stops and presumably the proton mobility measured after most of the CCP has left the micelles gives a measure of how tight the protein network is in the gel, as the larger the distance from the water molecule to the casein, the higher the proton mobility. During acidification of reconstituted skim milk, an increase in the concentration of skim milk powder from 15 to 25% has been shown to decrease the proton mobility (Moller et al. 2011).

To the knowledge of this author, the effect of varying the intensity of the heat treatment of milk on the acidification of milk as measured by NMR has not been examined previously.

2.6 Rheology

Rheology is the science of the deformation and flow of matter: It is the study of the manner in which materials respond to applied stress or strain (Steffe 1996).

Shear stress, $\sigma$, is defined as the applied force per unit area on a material, shear strain, $\gamma$, is the deformation on the material length due to the applied force, shear rate, $\dot{\gamma}$, is the rate of deformation, and for an ideal Newtonian liquid the viscosity, $\eta$, is defined as (Barnes 2000b):

\[ \eta = \frac{\sigma}{\dot{\gamma}} \]

Thus the viscosity is independent of the applied shear rate. The viscosity of yoghurts is typically dependent on the shear rate, and it has often classified as a pseudoplastic viscoelastic fluid, sometimes with a yield stress (Skriver 1995). A pseudoplastic is a fluid where an increase in shear rate gives a less than proportional increase in the shear stress. A yield stress was originally perceived as a material having solid like behavior, or resistance to flow, at very small stresses. With the development of rheometers operating at very small stresses and measuring high viscosities, this concept has changed, as it was revealed that what was previously conceived as a resistance to flow, in fact was an abrupt increase in viscosity over a limited range of increasing shear stress (Barnes 1999). Although the concept of the yield stress has changed, it still remains an important process parameter.

In dynamic rheometry an oscillatory sinusoidal strain (or stress) is applied to a linear viscoelastic sample giving a sinusoidal stress (or strain) torque response (Barnes 2000a), which can be used to evaluate gel
strength and observe coagulation in milk. The oscillatory testing enables the determination of both the viscous and elastic properties of the material measured. The ratio between the viscous modulus, $G''$, and the elastic modulus, $G'$, is the loss tangent:

$$\tan(\delta) = \frac{G''}{G'}$$

In a gel the elastic properties dominate, and $\tan(\delta)$ can be applied directly to determine the gelation point, as the point where the phase angle ($\delta$) stays consistently below 45° (Ipsen 2003; Anema 2009).
3 Materials and methods

3.1 Overview of the experiments

Table 1 Overview of the heat treatments performed, the methods applied for analysis of the heat treated milk and yoghurt, and reference to the relevant section(s), where the data is presented and discussed, with minor sections in brackets. Note that apart from the yoghurt experiments all of the experiments have been performed on the milk or during acidification.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Milk</th>
<th>Heat treatment</th>
<th>Methods</th>
<th>Section</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preliminary</td>
<td>Skim milk powder</td>
<td>85 °C for 5, 10 and 30 min</td>
<td>pH during acidification</td>
<td>(4.1.1)</td>
</tr>
<tr>
<td>experiments</td>
<td></td>
<td>90 and 95 °C for 5 min</td>
<td>RP-HPLC</td>
<td>(4.1.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 °C for 5, 10, 15, 20, 25</td>
<td>^1H LF-NMR</td>
<td>(4.1.1-4.1.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>and 30 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preliminary</td>
<td>Pasteurised skim milk</td>
<td>unheated</td>
<td>pH during acidification</td>
<td>(4.2.2)</td>
</tr>
<tr>
<td>experiments</td>
<td></td>
<td>ASIC 1: 85, 92.5 and 100 °C</td>
<td>MilkoScan (only ASIC 1)</td>
<td>(4.2.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>for 2, 31 and 60 sec</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>LIFE: 92.5 and 100 °C</td>
<td>RPLC</td>
<td>(4.2.3.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>for 30, 60 and 270 min</td>
<td></td>
<td>(4.2.3.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ASIC 2: 85 °C for 2 and 60 sec</td>
<td>^1H LF-NMR</td>
<td>(4.2.4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>92.5 °C for 31 sec A and B</td>
<td>Rheology</td>
<td>(4.2.6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 °C for 2 and 60 sec</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yoghurt</td>
<td>Pasteurised skim milk</td>
<td>85 °C for 2 and 60 sec</td>
<td>pH during acidification</td>
<td>(4.3.1)</td>
</tr>
<tr>
<td>experiments</td>
<td></td>
<td>92.5 °C for 31 sec A and B</td>
<td>^1H LF-NMR</td>
<td>(4.3.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 °C for 2 and 60 sec</td>
<td>Rheology</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>SYNERESIS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Posthumus funnel</td>
<td></td>
<td>(4.3.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CLSM</td>
<td></td>
<td>(4.3.4)</td>
</tr>
</tbody>
</table>

3.2 Preliminary experiments

Preliminary experiments were made with low heat milk powder (Milex 240 LH, Arla foods ingredients, Viby J, Denmark) with specifications as seen in Table 2.

Table 2 Manufactures specifications for Milex 240 LH, the low heat skim milk powder used for the preliminary experiments

<table>
<thead>
<tr>
<th>Milk protein</th>
<th>36 % average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>51 % average</td>
</tr>
<tr>
<td>Ash</td>
<td>8% average</td>
</tr>
<tr>
<td>Moisture</td>
<td>4 % max</td>
</tr>
<tr>
<td>Milk fat</td>
<td>1.25 % max</td>
</tr>
</tbody>
</table>

For preparation of the milk samples 9 g powder was dissolved in 75 g water, stirred shortly, and then stored in the refrigerator overnight to ensure full hydration of the milk protein.
3.2.1 GDL acidification

To determine the amount of GDL (Merck, Germany) needed to obtain a pH of 4.6 after acidification, four batches of milk were made. The batches of milk were heated at 95 °C for 6 min in a water bath, cooled down to 44 °C and acidified with GDL. The four batches of milk were acidified with 1.2, 1.3, 1.4 and 1.5 g GDL per 100 g milk, as literature indicated that a GDL concentration within this range of concentrations could be optimal for achieving a pH of 4.6 after acidification in a skim milk prepared with 12 g milk powder per 100 g water (de Kruif 1997; Gastaldi et al. 1997; Lucey et al. 1998a; Lucey et al. 1998b). The pH was measured at irregular intervals during the acidification at 44 °C for 3.5 hours and the following day, after being stored overnight in the refrigerator. Based on the results (results not shown), it was decided to use 1.25 g GDL per 100 g milk for the preliminary experiments.

3.2.2 Heating of the milk

Preliminary experiments were used to determine the effect of heating at different times and temperatures, see Table 3. After preparing the milk samples as described, Section 3.2, the milk was heated in a water bath. To ensure a more uniform heat treatment, the samples were shaken every 10 seconds for the first minute of the heat treatment and then after 1½ min and after 2 min. The experiments were set up to evaluate the effect of going from a relatively mild heat treatment – 85 °C for 5 min – to a very extreme heat treatment – 100 °C for 30 min. The experiments were performed in a randomized order.

Table 3 Heating temperature, heating time and number of replicates made for the preliminary heat treatment experiments

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time (min)</th>
<th>Replicate measurements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unheated</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>85</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>85</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>85</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>90</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>90</td>
<td>5</td>
<td>1</td>
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<tr>
<td>90</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>100</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>100</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>100</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>100</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>100</td>
<td>30</td>
<td>2</td>
</tr>
</tbody>
</table>

After heat treatment 30 ml aliquots of the milk were taken for protein analysis and frozen, the rest of the milk sample was either stored in the refrigerator or frozen before NMR analysis.

3.2.3 Content of native whey protein by RP-HPLC

The protein analyses were performed at Arla Strategic Innovation Center, Brabrand (ASIC).

To separate the native whey proteins from the caseins, the whey protein aggregates, and the casein-whey protein aggregates, 20 ml of milk was acidified to pH 4.6 with 1 M HCl (Dannenberg & Kessler 1988c). This

---

4 The unheated sample was low heat skim milk powder, for specifications see Section 3.2.
was done by first acidifying the milk to pH 4.65, then stirring it for 10 min on a magnetic stirrer to allow for the protein equilibrium to settle, and then the pH was adjusted to 4.6. The samples were centrifuged at 17,100 g for 10 min at 4 °C, where after the supernatant was removed and analyzed by HPLC. The HPLC analyses were performed by Lene Buhelt Johansen from ASIC.

For the HPLC analysis 20 µl aliquots with a temperature of 5 °C were injected into the column. Separation of the individual milk proteins was obtained using a gradient flow with the 2 solvents 0.05% TFA in MilliQ water and 0.1% TFA in Acetonitrile. The gradient flow and the specifications for the method can be seen in Appendix 1. The column was maintained at 60 °C, the flow rate was 0.6 ml/min and the eluted peaks were detected by UV absorbance at 214 nm.

Single determinations were performed for the preliminary experiments.

As the analysis of the content of native whey proteins in the milk samples showed, results not shown, that even though the milk powder was low heat, more than one third of the β-lactoglobulin eluting the column had already been lactosylated even in the unheated sample, it was decided to use pasteurized skim milk instead of skim milk powder for the main heat treatment experiments.

3.2.4 $^1$H LF-NMR

The acidification of the milk samples at a temperature of 44 °C was followed continuously for 6 hours by time domain $^1$H LF-NMR.

The analyses were conducted using a benchtop 23.2 MHz Maran pulsed $^1$H-NMR spectrometer (Resonance Instruments Inc., Witney, UK). The software RINMR 4 (Resonance Instruments Inc., Witney, UK) was used to obtain the data. FID and CPMG measurements, Section 2.5.1, were used to determine the relaxation behavior during acidification. Prior to the measurements, the frequency of the instrument was adjusted on a 10 mM CuSO$_4$ standard sample. Approximately 10 g milk was mixed with GDL, 1.25 g GDL per 100 g milk, and immediately after mixing added to an $^1$H LF-NMR glass tube with an inner diameter of 18 mm. A stopper was placed on the tube to prevent evaporation during the measurements.

For the FID experiment the relaxation following the perturbation of the 90° pulse was measured with 8000 points for optimal resolution. For the CPMG experiment the top point of 4096 echoes were acquired, with a 90°-180° pulse spacing value (τ) of 100 µs, in order to properly describe the relaxation decay. For both experiments the recycle delay between measurements was set to 7 seconds, 16 consecutive scans were used for noise reduction and the receiver gain was set to 0.7.

For extraction of the transverse relaxation time constants T$_2^*$ and T$_2$, and the signal intensities amplitude M$_2^*$ and M$_2$, the FID and CPMG relaxation curves were fitted by exponential analysis as described in Section 2.5.1.3. The discrete exponential fitting of the FID and CPMG relaxation curves were performed in MatLab version 7.12.0 (The MathWorks Inc., Natick, MA) using in-house MatLab routines (Pedersen et al. 2002; Engelsen & Bro 2003).

Double determinations were done for the preliminary experiments.
3.3 Main experiments

3.3.1 GDL acidification
As it was decided to use pasteurized skim milk instead of skim milk powder, another experiment was performed to determine the concentration of GDL, which would give a pH of 4.6 after 6 hours of acidification. As the final pH of the milk depends on the concentration of both the milk protein and the GDL (de Kruif 1997; Gastaldi et al. 1997) and the concentration of milk protein in the pasteurized milk was lower than the concentration of milk proteins in the samples prepared from milk powder, the GDL concentrations tested in the experiment were between 0.60 and 1.40 g GDL per 100 g milk with an interval of 0.10 g/100 g giving a total of 9 samples.

For the experiment 9 batches of approximately 2 kg pasteurized skim milk was weighed and heated to 44 °C in a water bath. The amount of GDL to give a final concentration of between 0.60 and 1.40 g GDL per 100 g milk was added to the milk batches, when a temperature of at least 43.5 °C was obtained. After addition of GDL, the sample was stirred for about 15 seconds. The short stirring enabled the GDL to be added at 30 seconds intervals, thus minimizing the time difference between the measurements of pH in the samples, as previous experiments had shown that the pH measurement took about 30 seconds to perform per sample. After addition of GDL the pH was measured at irregular intervals for 6 hours.

Based on the results of this experiment, Section 4.2.2, it was decided to use 1.00 g GDL per 100 g milk for the acidification experiments.

3.3.2 Heat treatment of milk
After the preliminary experiments attempts were made to heat treat the milk in the plate heat exchanger at LIFE. This was, however, abandoned as too much heat was lost in the system, as approximately 4-5 °C was lost in the tubes from the heat exchanger to the holding cell and approximately 4-5 °C was lost in the holding cell, giving a heat loss from the plate heat exchanger to the end of the holding cell of about 10 °C. It was then decided to heat treat the milk in the plate heat exchanger at ASIC and one batch of milk was heat treated and analyzed.

**ACIS 1**: The milk was heated at 85, 92.5 and 100 °C for 2, 31 and 60 seconds. Giving a total of 10 samples, 9 heat treated samples and 1 unheated sample, which had been pasteurized at 72 °C for 15 seconds, Table 4.

After production aliquots were taken for protein analysis, and the remainder of the samples were frozen. Before analysis, the samples were defrosted in the refrigerator.

This milk was analyzed by following the acidification for 6 hours by $^1$H LF-NMR and by oscillatory rheometry. The protein content was analyzed by HPLC and SE-HPLC. The composition of the milk was analyzed by MilkoScan™ and the pH of the milk was registered.

Based on the results of the analysis of this first batch of milk, samples were chosen to be repeated and made in a larger scale to make it possible to make small scale yoghurt experiments. However, shortly before the scheduled date of the experiments, the heating element of the plate heat exchanger at ASIC broke.
3 Materials and methods

The plate heat exchanger at LIFE had been improved, as new tubes had been installed and the holding cell had become more isolated, so it was decided to attempt to heat treat the milk at LIFE once more. The holding cell at LIFE also had the advantage, that it was larger than the one at ASIC, making it possible to heat treat the milk for up to 270 seconds.

**LIFE:** The milk was heated at 92.5 °C and 100 °C for 30, 60 and 270 seconds. Giving a total of 7 samples, 6 heat treated samples and 1 unheated sample, which had been pasteurized at 72 °C for 15 seconds, Table 4.

Unless otherwise specified the samples had been frozen immediately after production. Before analysis, the samples were defrosted in the refrigerator.

This milk was analyzed by following the acidification for 6 hours by $^1$H LF-NMR and by oscillatory rheometry, the composition of the milk was analyzed by MilkoScan™ and the pH of the milk was registered.

As will be described, Section 4.2.4, 4.2.5, and 4.2.6, the analyses performed on the milk during acidification showed that the heat treatment in the plate heat exchanger at LIFE was incomparable with the heat treatment in the plate heat exchanger at ASIC. The results indicated that the holding time was longer than expected.

Fortunately, the plate heat exchanger at ASIC was repaired just in time to make a second heat treatment, making it possible to make the small scaled yoghurt experiments planned.

**ASIC 2:** The milk was heated at 85 °C for 2 and 60 seconds, at 100 °C for 2 and 60 seconds and at 92.5 °C for 31 seconds, as 92.5 °C for 31 seconds is the center point in the original experimental design, this heat treatment were performed twice to give an indication of the repeatability of the heat treatment. Thus a total of 7 samples were produced, 6 heat treated samples and 1 unheated sample, which had been pasteurized at 72 °C for 15 seconds, Table 4.

After production the samples were frozen. Before analysis the samples were defrosted in the refrigerator.

This milk was analyzed by following the acidification for 6 hours by $^1$H LF-NMR and by oscillatory rheometry, the composition of the milk was analyzed by MilkoScan™ and the pH of the milk was registered.

The yoghurt made from this milk was investigated rheologically, by $^1$H LF-NMR, by degree of syneresis, by the posthumus funnel, and by confocal laser scanning microscopy, CLSM.
Table 4 Overview of the heat treatment experiments performed at ASIC, the first (1) and second (2) batch, and LIFE. The table is set up with similar heat treatments in the same row, making it easier to get an overview of the number of times a particular heat treatment had been performed.

<table>
<thead>
<tr>
<th></th>
<th>ASIC 1</th>
<th>LIFE</th>
<th>ASIC 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>Time (s)</td>
<td>Temperature (°C)</td>
<td>Time (s)</td>
</tr>
<tr>
<td>unheated$^5$</td>
<td>unheated$^6$</td>
<td>unheated$^6$</td>
<td></td>
</tr>
<tr>
<td>85</td>
<td>2</td>
<td>85</td>
<td>2</td>
</tr>
<tr>
<td>85</td>
<td>31</td>
<td>85</td>
<td>60</td>
</tr>
<tr>
<td>92.5</td>
<td>2</td>
<td>92.5</td>
<td>30</td>
</tr>
<tr>
<td>92.5</td>
<td>31</td>
<td>92.5</td>
<td>31 A</td>
</tr>
<tr>
<td>92.5</td>
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<td>100</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td></td>
<td>270</td>
</tr>
</tbody>
</table>

3.3.3 Protein analysis

The protein analyses were performed at Arla Strategic Innovation Center, Brabrand (ASIC). The milk samples from the first heat treatment at ASIC, see Table 4, were analysed for the content of the individual whey proteins by HPLC and for the content of protein aggregates by SEC.

3.3.3.1 Content of native whey protein by RP-HPLC

The content of native whey proteins by RP-HPLC was determined as described in Section 3.2.3. Triplicate determinations were performed.

3.3.3.2 Content of protein aggregates by SE-HPLC

In SEC the chromatographic column is packed with small particles of a porous material. The space between the particles and pores are filled with a mobile phase. The samples are diluted in the same eluent as is used as the mobile phase and injected into a series of columns that are continuously flowed with the mobile phase. In the column the molecules permeate into the pores of the porous material, the column is packed with. Molecules are excluded from the pores with effective size smaller than the size of the molecules. Thus smaller molecules can permeate deeper into pores and can permeate into smaller pores. Consequently, the larger molecules elute the columns first, followed by molecules with decreasing molecular size. This principle is called steric exclusion and it is the main separation mechanism in SEC (Podzimek 2011).

For the SE-HPLC analysis 200 µl milk was transferred to an eppendorf tube and frozen until the time of the analysis. The SE-HPLC analyses were performed by Lene Buhelt Johansen from ASIC.

The mobile phase used was 0.1 M sodium-phosphate at a pH of 6.3. Before the SE-HPLC analysis 200 µl of milk were added to 1 ml of the mobile phase and shaken gently for 15 min. 2 µl aliquots with a temperature

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$^5$ The unheated sample had previously been pasteurised at 72 °C for 15 seconds
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of 5 °C were injected into the column, a column optimized for the analysis of proteins and their aggregates with molecular weights ranging from 10,000 to 450,000 Daltons (Waters 2012). The flow through the column was 0.3 ml/min. The compounds eluting the column were detected with a UV-detector, with a signal wavelength of 214 nm. The specifications for the method can be seen in Appendix 1.

2-4 determinations were performed.

3.3.4 Composition of the milk
The composition of the milk was analyzed with a MilkoScan™ FT2 (Foss, Hillerød, Denmark). The MilkoScan™ uses infra-red spectrometry in combination with chemometric analysis of the data to quickly and easily analyze the composition of the milk.

Each sample was measured 3 times. For each measurement the instrument provides a result which is an average of 2 consecutive measurements.

3.3.5 ¹H LF-NMR
The acidification of the milk samples at a temperature of 44 °C was followed continuously for 6 hours by time domain ¹H LF-NMR. Approximately 10 g milk was mixed with GDL, 1.00 g GDL per 100 g milk, and immediately after mixing added to an ¹H LF-NMR glass tube with an inner diameter of 18 mm. A stopper was placed on the tube to prevent evaporation during the measurement. The FID and CPMG experiments were performed as described in Section 3.2.4.

Double and triple determinations were done for the main experiments.

3.3.6 Rheology
The acidification of the milk samples at a temperature of 44 °C were followed by oscillatory rheometry on a StressTech rheometer (Reologica Instruments AB, Lund, Sweden). The software RheoExplorer (Reologica Instruments AB, Lund, Sweden) was used to obtain the data. The measurement, a dynamic oscillatory time sweep at a stress of 0.1 Pa, was performed in a bob-cup system. At least 15 g milk was mixed with GDL, 1.00 g GDL per 100 g milk, and immediately after mixing transferred to the cup. To prevent evaporation during the measurement the sample was covered with a thin layer of rapeseed oil.

Triplicate determinations were performed.

Based on the rheological measurements, the coagulation time, T<sub>gel</sub>, of the milk was determined as the time point where the phase angle dropped consistently below 45°, Section 2.6.

3.4 Production of yoghurt and experiments performed on the yoghurt
As described in Section 3.3.2 a small scale production of yoghurt was made from the second batch of milk made at Arla Strategic Innovation Center, Brabrand (ASIC2, Table 4). The experiment was made twice in two consecutive days, day 1 and day 2, to get an idea of the experimental variation. As the examination of 14 yoghurt samples is quite time consuming, the same type of experiment was completed on all of the yoghurt samples before another experiment was started. However, this meant that the age of the yoghurt varied at the time the experiments were performed, Table 5.
Table 5 The experiments performed on the yoghurt and the timespan of the age of the yoghurt when the experiment was performed.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Age of the yoghurt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confocal microscopy</td>
<td>7-9 days</td>
</tr>
<tr>
<td>Posthumus funnel</td>
<td>9-10 days</td>
</tr>
<tr>
<td>Rheology</td>
<td>13-18 days</td>
</tr>
<tr>
<td>Syneresis by centrifugation</td>
<td>17-18 days</td>
</tr>
<tr>
<td>pH</td>
<td>9 and 20-21 days</td>
</tr>
<tr>
<td>NMR</td>
<td>22-23 days</td>
</tr>
</tbody>
</table>

3.4.1 Acidification of the milk

For the production of yoghurt approximately 4 kg of each batch of milk was weighed and heated to 44 °C in a water bath. 1.00 g GDL per 100 g milk was weighed accurately and added to the milk batches when a temperature of at least 43.5 °C was obtained. After addition of GDL, the sample was stirred for about 15 seconds. The short stirring enabled the GDL to be added at 30 seconds intervals, thus minimizing the time difference between the measurements of pH in the samples, as the pH measurement took about 30 seconds to perform per sample. After addition of GDL the pH was measured at irregular intervals for 6 hours. The pH was measured almost constantly in the beginning of the experiment, and then gradually less frequently, as the rate of acidification decreased.

The acidification curve was fitted using the Curve Fitting Tool of Matlab version 7.12.0 (The MathWorks Inc., Natick, MA), fitting a line in the form \( y = ax + b \) to the data, with \( y: \) pH and \( x: \) ln(time), and \( y: \) ln(pH) and \( x: \) ln(time), as both plots appeared to be linear. Comparison of the lines with the data revealed that the function \( pH = a \cdot \ln(time) + b \) gave the best fit.

After the acidification, the yoghurt was treated in a yoghurt post-treatment device (Scandinox A/S, Galten, Denmark). The equipment is designed to cool and smooth the yoghurt after acidification. After being poured into the device the yoghurt is firstly cooled using a plate heat exchanger and thereafter it is smoothed by passing a tubular pipe with a constant counter pressure. All of the samples were cooled to 20 °C prior to being smoothed with a backpressure of 1 bar. This backpressure was chosen to smooth the yoghurt as gentle as possible. When being tapped from the post-treatment device, the first approximately 700 ml was discarded to ensure that there was no mixing of products, the rest was tapped into 6-7 500 ml beakers and stored at 5 °C. The product tapped from the post-treatment device appeared to vary in viscosity throughout the tapping of a single sample. The yoghurts expelled a lot of whey during production, and this could have made it difficult for the post-treatment device to adequately and consistently mix the gel and the whey. To mimic the variation in the results caused by this, the 3rd beaker tapped was used for all of the analysis, except for the posthumus funnel. However, that approach, might not give an accurate view on the sample, and in retrospect the 6-7 beakers tapped per sample should have been mixed after tapping, to mimic the variation in the sample originating from inconsistent mixing of gel and whey in the post-treatment device.

As these yoghurts are produced from skim milk, using a fast acidification, GDL, at a high temperature, 44 °C, Section 3.4.1, and had a pH above 4.6 after storage, Section 4.3.1.1, it could be expected that these yoghurts had a weak gel, which was excessively wheying-off (Lucey 2004), as was indeed the case for these yoghurts. Therefore great care was taken to gently mix the whey with the gel prior to all of the measurements.
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3.4.2 $^1$H LF-NMR
The samples were measured at 20 °C, as this was the lowest possible temperature setting of the LF-NMR.

Approximately 30 minutes before placing the sample in the $^1$H LF-NMR glass tube the sample was stirred gently with a spoon to mix the whey with the gel. Immediately before transferring the sample to the tube, the gel was stirred again and gently transferred to an $^1$H LF-NMR glass tube with an inner diameter of 18 mm. The sample was placed in the NMR spectrometer approximately 15 minutes before measurement for the temperature of the sample to equilibrate.

The FID and CPMG experiments were performed as described in Section 3.2.4. To ensure that the samples had reached a temperature of 20 °C when being measured the instrument was set up to make 5 consecutive measurements of FID and CPMG relaxation curves, each measurement took approximately 5 min. A slight increase in the value of $T_2$ was seen from the first to the fourth measurement, while the difference between the values of the fourth and the fifth measurement was very small. It was therefore decided to base the value of $T_2$ on an average of the value of $T_2$ measured in the fourth and the fifth measurement. This was considered a convenient way to check that the temperature of the sample had equilibrated before the measurement.

Double determinations were performed.

3.4.3 Rheological analyses
The rheological measurements were performed using the rheometer described in Section 3.3.6, in a bob-cup system. The measurements were performed at 13 °C, a temperature chosen to mimic the temperature of cold yoghurt, which is put in a warm mouth. Approximately 45 min before measurement the yoghurt was gently stirred with a spoon until the gel and the whey was mixed. Immediately before being placed in the rheometer, the gel were gently stirred again and transferred to the cup of the rheometer. After the yoghurt had been transferred to the cup of the rheometer, a 5 min interval before measurement was inserted for the yoghurt to regain structure and the temperature of the yoghurt to equilibrate. 5 min resting periods was also placed between the different measurements on the sample to ensure that the yoghurt had regained the structure that the applied stress had broken.

After 5 min of ‘resting’ the gel was measured. A stress sweep from 0.002 to 50 Pa at a frequency of 1 Hz was performed to determine the linear viscoelastic area. Following the stress sweep a frequency sweep was performed going from 0.01 to 50 Hz in 25 steps. The frequency sweeps was performed at a constant stress of 0.05 Pa, apart from the unheated day 2 sample and the second measurement of the day 2 sample heat treated at 85 °C for 2 sec, where the frequency sweep was performed at 0.01 Pa. From the frequency sweep the elastic modulus of the sample was determined at a frequency of 1 Hz. Flow curves were obtained by varying the shear rate logarithmically from $1 \cdot 10^{-4}$ s$^{-1}$ to 1000 s$^{-1}$ in 24 steps. A double logarithmic plot of the apparent viscosity versus shear stress was used to determine the apparent yield stress. The apparent yield stress was defined as the stress which resulted in an abrupt decrease in the apparent viscosity (Barnes 1999).6

Graphs of the stress sweeps, the frequency sweeps, and the flow curves can be seen in Appendix 4.
3.4.4 Syneresis by centrifugation
The examination of the degree of syneresis of the yoghurt by centrifugation was adapted from Torres (2012). Prior to the measurement the yoghurt was stirred gently to mix the whey with the gel. A 20 g sample of yoghurt was transferred to a 50 ml centrifugal tube and centrifuged at 1200 g for 10 min at 4 °C. After centrifugation the whey was drained of. The water holding capacity was calculated as the percentage weight of the whey separated from the gel in relation to the initial weight of the yoghurt.

Duplicate measurements were performed.

3.4.5 Posthumus funnel
The Posthumus funnel is an empirical rheological method. The apparatus consists of a cylindrical upper part and a conical lower part ending in a defined orifice with a size of 4 mm. A metal pin is placed in the boundary between the upper and the lower part of the funnel.

Prior to the measurement the yoghurt, which had a temperature of about 5 °C, was stirred gently to mix the whey with the gel. The measurement was conducted by filling the funnel with the yoghurt, approximately 200 ml from the 2nd or 4th beaker, while covering the orifice with a finger. The finger was removed simultaneously with starting a stopwatch and the time from the removal of the finger until the metal pin was visible was noted.

Triplicate determinations were performed on the yoghurt and on tab-water, which was measured for comparison.

3.4.6 Confocal laser scanning microscopy
Unlike traditional light microscopes, which are limited to very thin samples, the confocal laser scanning microscopy (CSLM) can image a single focal plane in a sample of arbitrary thickness (van de Velde et al. 2003). In CSLM the fluorescent light from the sample is collected by the objective lens and focused into a small pinhole to eliminate the out-of-focus light. Because of this pinhole, the confocal microscopy provides excellent resolution within the focal plane. An optical section of the sample (2D image) is obtained by a point by point scanning of the sample in the x- and y-direction within the focal plane. Three-dimensional (3D) images of the sample can be obtained by a stepwise movement of the focal plane of the instrument through the depth of the sample. In CSLM, contrast is obtained by differences in fluorescence, either by auto-fluorescence of the material or by the addition of specific fluorescent dyes (van de Velde et al. 2003).

For the confocal laser scanning microscopy examinations the proteins in the yoghurt gel was stained with nile red (Sigma-Aldrich, Denmark). Nile red is normally used for fat staining, but it has been shown to bind to β-lactoglobulin and κ-casein (Sackett & Wolff 1987). Prior to the experiment the yoghurt was stirred gently to mix the whey with the gel. To stain the proteins 1 ml of yoghurt was gently mixed with 12.5 µl nile red solution\(^7\). After mixing the samples were allowed to rest for at least one hour, where after 100 µl of stained yoghurt was transferred to a microscope slide with a conical depression. The samples were examined using a 63x water immersion objective on a Leica SP5 CSLM system (Leica Microsystems, Heidelberg, Germany) fitted with an inverted microscope and an argon/krypton laser. Nile red was excited at 514 nm and the emitted signal was collected from 580-650 nm. One slide was prepared per sample and at least 10 images of 1024x1024 pixels were recorded per slide.

\(^7\) The solution consisted of nile red dissolved in acetone at a concentration of 0.4 g/l.
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The images were analyzed by the ImageJ software (Abràmoff et al. 2004) with the macro Lines8 created by Landini (2012). First the image was converted to a binary image, and then Lines8 was run. The Lines8 macro skeletonizes the image, and for each particle it registers how many particles (including the particle itself) that exist in its 3x3 neighborhood. This is used to find the number of free ends, inside-line points, and the number of bifurcations, which is represented by 1, 2 and 3 neighborhood particles. The number of clusters is also registered, edge clusters was excluded. To minimize the “noise” from very small particles, the minimum cluster size was set to 100 pixels.

3.5 Statistics and Chemometrics

Analysis of variance was performed in JMP® version 9.0.2 (SAS Institute Inc.). When performing analysis of variance the unheated samples were set to 0 °C for 0 min for ease of comparison, as the unheated sample is the “zero” sample.

Comparison of means was also performed in JMP®, with the Tukey Kramer HSD, honestly significant difference, which takes the increased probability of finding a statistical difference between two means when doing multiple comparisons into account (Massart et al. 1997). On the basis of this comparison letters are assigned to the samples to distinguish samples which are significantly different (p<0.05). These letters are ordered, so the sample with the largest value is assigned a, the sample with the significantly different second-largest value is assigned b, and so forth.

Principal component analysis (PCA) was performed using the PLS-Toolbox in MatLab version 7.12.0 (The MathWorks Inc., Natick, MA). PCA is a mathematical method of constructing common latent factors (or principal components - PCs) from underlying latent structures in the original data from many samples. This can be described mathematically as

\[
X = TP^T + E
\]

where \(X\) is the data matrix (I × J), \(P\) contains the underlying profiles (J ×N; loadings), and \(T\) is the contributing amplitudes (I ×N; scores). The scalar \(N\) is the number of factors resolved and \(E\) (I ×J) holds residual unexplained variation. The scores can be seen as new variables, which summarize the old variables. The loadings inform how the variables are linearly combined to form the scores. The loadings unravel the magnitude (large or small correlation) and the manner (positive or negative correlation) in which the measured variables contributes to the scores. Chemometric data analysis has proven to perform well especially in quantitative calibration problems. The disadvantage of using chemometric models is that it requires data from many samples to be analyzed simultaneously (Eriksson et al. 2006).
4 Results and discussion

4.1 Preliminary experiments

4.1.1 Differences between reconstituted skim milk and pasteurized skim milk

As described in Section 3.2.4 the acidification of milk was followed for 6 hours by $^1$H LF-NMR. Both FID and CPMG experiments were performed, but while the $T_2$ value measured by the CPMG experiment appeared to change during the acidification of the milk, the $T_2$ value measured by the FID experiment did not seem to change during the acidification of the milk. However, in the FID experiment a difference between the samples prepared from skim milk powder and the samples prepared from pasteurized skim milk was noted. Most of the samples prepared from skim milk powder had two water populations throughout the acidification, one relaxing slowly, high $T_2^*$, with a relative abundance between 85-90%, and another water population relaxing faster, low $T_2^*$, with a relative abundance between 10-15%, see example in Figure 10.

Figure 10 A typical example of the acidification of milk prepared from skim milk powder, monitored by the FID experiment. The graph to the left shows the evolution of $T_2$ during acidification, while the graph to the right shows the corresponding relative abundances of the two water components.

None of the samples prepared from pasteurized skim milk had more than one water population, results not shown. The samples had a slowly relaxing water population having a $T_2^*$ value similar to the value of the slowly relaxing component of the samples prepared from skim milk powder, Figure 10. On this basis it could be speculated that the difference was not solely due to the use of powder, but might also be a consequence of the applied heat treatment, i.e. the appearance of two water populations in the samples from the preliminary experiments could be because they were heat treated in a water bath, as opposed to being heated in a plate heat exchanger. However, during the preliminary experiments a few samples prepared from skim milk powder, were heat treated in the plate heat exchanger at LIFE, and these samples also had two water populations throughout the acidification. An examination of why the fast relaxing water component was only seen in samples prepared from skim milk powder is beyond the scope of this report. A difference between the samples prepared from skim milk powder and the samples prepared from pasteurized milk is also seen in the analysis of the content of native whey proteins: As described in Section 3.2.3 more than one third of the β-lactoglobulin in the samples prepared from milk powder was lactosylated. In the samples prepared from pasteurized skim milk none of the β-lactoglobulin was lactosylated, results not shown. This difference along with other alterations of the proteins, in particular
the whey proteins, induced during the spray drying of milk, could be a part of an explanation of why a fast relaxation water component is seen during the acidification of samples prepared form skim milk powder.

4.1.2 $^1$H LF-NMR measurements

In Figure 11 the acidification curves for the samples heat treated at 85 °C and the unheated sample is shown. These curves are representative for the curves of all of the samples. For all of the samples the $T_2$ value at time 0 was $170.4 \pm 4.5$ ms, and all of the samples had a single water population throughout the acidification. The general shape of the curves, with an initial increase in $T_2$ value followed by a plateau after about 150 min, was seen in all of the curves measured. The increase in proton mobility, as seen by the increase in $T_2$, has been coupled with solubilization of CCP from the micelles, Section 2.5.2. As seen from Figure 11, the standard deviation of the points of the curve was quite large. Furthermore, the duplicate determinations, 85 °C for 5 min A and B, were not very similar. The standard deviation of the points on the curves of the other samples was in the same magnitude, results not shown. The variation in the standard deviation and between the duplicate determinations could indicate that the heat treatment performed in the preliminary experiments was not as uniform as one could have hoped. That the standard deviation of the unheated sample was smaller than the standard deviation of the other samples supports this theory.

![Figure 11](image.png)

The development of $T_2$, measured by CPMG, during acidification of milk prepared from skim milk powder and heat treated at 85 °C at various durations. Note that the y-axis starts at 150 ms. For clarity the standard deviation is only shown in a few points. (n=2)

The time for the onset of the plateau, about 150 min, seems to be similar for all of the samples, while the value of $T_2$ in the plateau of the acidification curve, $T_{2\text{-plateau}}$, depends on the duration of the heat treatment for the samples heat treated at 85 °C, Figure 11, as $T_{2\text{-plateau}}$ decreases as the duration of the heat treatment increases. Based on the general shape of all the curves, results not shown, $T_{2\text{-plateau}}$ seems to correlate with the effect of altering the intensity of heat treatment on the acidification of milk as measured by an $^1$H LF-NMR CPMG experiment. Therefore $T_{2\text{-plateau}}$ is calculated, as an average over the last 5 measuring points, representing approximately 25 minutes of measurement. The calculated $T_{2\text{-plateau}}$ of the samples is compared, Figure 12. The effect of increasing the heating time when heating at 85 °C is clearly seen, as it also is in Figure 11. For the samples heat treated between 85 °C and 100 °C for 5 min a slight decrease in $T_{2\text{-plateau}}$ is seen, although they are not significantly different. For the samples heat treated at 100 °C for 10

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*The remainder of the acidification curves can be seen in Appendix 2*
min, no further effect is seen when the duration of the heat treatment is increased. An analysis of variance of the effect of the duration and the temperature of the heat treatment on $T_2$-plateau showed that both the duration and the temperature of the heat treatment had a significant ($p<0.001$) negative effect on $T_2$-plateau. Thus when the temperature of the heat treatment or the duration of the heat treatment was increased $T_2$-plateau decreased. A decreasing $T_2$ indicates that the water relaxes faster, indicating a tighter protein network in the gel, Section 2.5.2, which could indicate that the gel is more firm and has a higher degree of water binding.

![Graph showing $T_2$-plateau of the acidification curve measured by CPMG. Note the y-axis starts at 300 ms. $T_2$-plateau is calculated as an average of the last 5 measurements of each sample. Standard deviation is shown as errorbars. Samples not sharing the same letter are significantly different ($P<0.05$). (n=2)](image)

As mentioned increasing the temperature of the heat treatment from 85 °C to 100 °C when heating for 5 minute only gave a slight decrease in $T_2$-plateau. Therefore the necessary decrease in the duration of the heat treatment due to limitations in the size of the holding cell of the plate heat exchanger at ASIC, is seen as optimal for the possibility of clearly seeing an effect of varying the intensity of the heat treatment on the acidification of milk measured by $^1$H LF-NMR spectrometry.
4 Results and discussion

4.2 Main experiments

4.2.1 MilkoScan™ and pH
The average pH of all the samples was 6.75±0.05. For all of the samples from the 3 batches, Table 4, the MilkoScan™ results were very similar, Table 6. The composition and pH of the milk were as expected for skimmed milk (Walstra 2006b). The casein content showed the greatest variation, Table 6, and appeared to slightly increase with the intensity of the heat treatment, results not shown. This increase might be caused by the attachment of whey proteins to the κ-casein on the exterior of the casein micelle, 2.3.1, making the casein micelles bigger (Anema & Li 2003a) and thus increasing the apparent casein content as measured by the MilkoScan™. However, the variation in the measured casein content was great even in measurements on the same sample, and the change in casein content between samples was not significant.

Table 6 The average content of fat, protein, casein, lactose and dry matter of the samples measured by MilkoScan™.

<table>
<thead>
<tr>
<th></th>
<th>Fat</th>
<th>Protein</th>
<th>Casein</th>
<th>Lactose</th>
<th>Dry matter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.13±0.02 %</td>
<td>3.60±0.02 %</td>
<td>2.82±0.07 %</td>
<td>4.89±0.03 %</td>
<td>9.60±0.03 %</td>
</tr>
</tbody>
</table>

4.2.2 Concentration of GDL
The acidification curves for the test to find the concentration of GDL to give a pH of 4.6 after 6 hours can be seen in Figure 13 A. As seen from the curves the rate of pH decrease is high immediately after addition of GDL, but then gradually the rate decreases and approaches zero. The lower the concentration of GDL, the faster the rate of pH decrease approaches zero.

As expected the final pH decreases, when the concentration of GDL increases, Section 3.3.1, and there seems to be a linear correlation between the concentration of GDL and the pH after acidification, Figure 13 B. It can be seen from Figure 13 that a concentration of GDL of 1.00 g GDL/100 g milk gives a final pH of 4.6, so this concentration of GDL was used for the acidification experiments.

![Figure 13 A](image1.png)  ![Figure 13 B](image2.png)

Figure 13 A The acidification curves from the test to find the concentration of GDL to give a pH of 4.6 after 6 hours of acidification. B The concentration of GDL vs. the pH after 6 hours of acidification.
4.2.3  Protein analysis

4.2.3.1  Content of native whey proteins

The percentage decrease in the content of native whey proteins for the ASIC 1 samples can be seen in Figure 14. The percentage decrease is calculated in relation to the content of native whey proteins in the unheated milk. Pasteurization at 72 °C for 15 seconds only denatures a few minor whey proteins, and generally has a negligible effect on the content of the major whey proteins (Dannenberg & Kessler 1988c; Kessler 2002; Patel et al. 2006). The plot clearly shows that the content of native whey proteins in the milk decreased as the intensity of the heat treatment increased. An analysis of variance on the effect of the duration and the temperature of the heat treatment on the percentage decrease in the content of native α-lactalbumin, and native β-lactoglobulin A and B supports this observation, as both duration, temperature and the combined effect of duration and temperature had a significant (p<0.001) positive effect of the percentage decrease of the content of native α-lactalbumin, and native β-lactoglobulin A and B. Thus when the temperature of the heat treatment, the duration of the heat treatment, or both the temperature and the duration of the heat treatment was increased the content of native α-lactalbumin, and native β-lactoglobulin A and B was decreased.

That the content of native whey proteins decrease upon heating, was expected, as it is a well-known fact, that whey proteins denature when heated at temperatures above 70 °C, Section 2.3 (Dannenberg & Kessler 1988c; Anema 2008). From Figure 14 it can be seen that β-lactoglobulin B appears to be more heat labile than β-lactoglobulin A, which is a trend generally seen, when examining the heat stability of the whey proteins (Gough & Jenness 1962; Dannenberg & Kessler 1988c; Allmere et al. 1998). However, compared with the extensive work of Dannenberg and Kessler (1988c) on the denaturation of whey proteins in skim milk, the degree of denaturation of the whey proteins, as represented by the percentage decrease, was much higher in these samples. In particular the denaturation of α-lactalbumin was much higher than expected. It is especially surprising that the denaturation of α-lactalbumin was almost 40 % in the sample.
heat treated at 85 °C for 2 seconds. When examining the effect of increasing the intensity of the heat treatment, it was noticed that a shift occurred in the content of native β-lactoglobulin, when the samples were heated for at least 92.5 °C for at least 31 seconds, as the percentage decrease in the content of native whey proteins was much higher for these samples than the samples which were subjected to a less intense heat treatment. When increasing the heating temperature or the duration of the heat treatment the content of native β-lactoglobulin A and B decreased much more than the content of native α-lactalbumin. So although the initial high decrease in the content of α-lactalbumin is unexpected, the general pattern of β-lactoglobulin being more sensitive than α-lactalbumin to an increase in the intensity of the heat treatment persist (Dannenberg & Kessler 1988c).

The sample heat treated at 85 °C for 60 seconds had a surprisingly high content of native whey proteins, in particular β-lactoglobulin, when compared with the other samples. As will be described in the following sections, Section 4.2.4 and 4.2.5, the acidification curves of this sample measured by oscillatory rheometry and by 1H LF-NMR were not as expected, and differed from the ASIC 2 sample heat treated at 85 °C for 60 seconds. This could suggest that this sample was not heat treated as expected. During the heat treatment the heating temperature was generally quite fixed to the target value, but for the ASIC 1 heated at 85 °C for 60 seconds the heating temperature fluctuated a bit, ±0.5 °C, which could be one of the reasons why this sample did not give the expected results.

When combining the denaturation degree of all of the whey proteins, the order of intensity of the heat treatment, expressed as the highest degree of overall denaturation of whey proteins, was 100 °C 60 sec > 100 °C 31 sec > 92.5 °C 60 sec > 92.5 °C 31 sec >> 85 °C 60 sec ≈ 85 °C 31 sec ≈ 100 °C 2 sec > 92.5 °C 2 sec > 85 °C 2 sec.

### 4.2.3.2 Content of protein aggregates

The influence of the heat treatment on the aggregation of the milk protein was measured by SE-HPLC. The nature of the proteins and protein aggregates which compose the different peaks were not examined, but it is known that the larger the molecule, the faster the elution time is, Section 3.3.3.2. It is thus assumed that the first peak, eluting at approximately 5-7 minutes, represents protein aggregates, while the second peak, eluting at approximately 8.5-11 minutes, represents native whey proteins, Figure 15. Under this assumption it is noticed that the size of the first peak is increased and shifted to the left, as the intensity of the heat treatment is increased, indicating that the content and size of the protein aggregates was increased as the intensity of the heat treatment is increased. It is also noticed that the second peak appears to consist of two peaks, where the size of the peak to the right, decreases when the intensity of the heat treatment is increased and the peak to the left does not appear to be altered by changes in the intensity of the heat treatment. As native α-lactalbumin and β-lactoglobulin are similar in size, and α-lactalbumin is an oval protein and β-lactoglobulin is more globular in structure, Section 2.2.1, it is possible that the left part of the peak represents primarily native α-lactalbumin, which, with its oval structure, might elute immediately prior to native β-lactoglobulin. The change in the size of the peaks with the intensity of the heat treatment corresponds with this theory, as β-lactoglobulin was found to be much more sensitive to increasing intensity of the heat treatment than α-lactalbumin, Section 4.2.3.1.
Figure 15 The signal intensity of the eluate from the SE-HPLC analysis of the proteins in the milk. The first part of the graph, where no proteins eluted, have been removed for clarity. Average of 2-4 measurements.

To further examine the elution profile, the SE-HPLC data was examined chemometrically, Figure 16 A and B. As expected based on the elution profile, the SE-HPLC data can separate the samples on the basis of the intensity of the heat treatment. In the plot in Figure 16 A the first component of the PCA model clearly separates the samples on the basis of the intensity of the heat treatment, as the intensity of the heat treatment the samples were subjected to is increased when going from the right part of the plot to the left part. The loadings of the first and second principal component, explaining 82% and 10% of the variation in the data, respectively, are seen in Figure 16 B. It is seen that the loadings have a negative influence on the model until at 9 minutes retention time, which is at the center of the second peak in Figure 15, between the two parts of the peaks. This corresponds with the observation that there is a shift in the response to the increased intensity of the heat treatment in that point, as the content of the compounds eluting before and after 9 minutes were increased and decreased, respectively, when the intensity of the heat treatment was increased. The observed shift in the first peak can also be observed in the loadings in Figure 16 B, where the peak now consists of two joined peaks, with the left part of the peak being larger than the right part. The loadings of the second principal component, explaining 10% of the variation in the data, differs from the loadings of the first principal component in particular in the first peak, where the first part is positive and the second part is negative. Based on the loadings it appears that the first principal component primarily explains the variation in the data due to denaturation of whey proteins and formation of aggregates, while the second principal component primarily explains the variation in the data due increasing size of the aggregates, Figure 15 and Figure 16 B.

It appears that increasing the heating temperature, when heating the samples for only 2 seconds had little effect on the aggregation of the proteins measured by SE-HPLC. For the samples heated at least 92.5 °C for at least 31 sec, there is a clear trend in the intensity of the heat treatment increasing when going from the bottom-center to the upper-left part of the graph, indicating that the denaturation of the whey proteins, the formation of aggregates, and the size of the aggregates increase, when the intensity of the heat treatment is increased.
Results and discussion

Figure 16 A Scores for the first vs. the second principal components and B Loadings for the first and the second principal component of the PCA model made on the SE-HPLC data. The data was mean centered prior to building the model. The Scores are colored according to the duration of the heat treatment. Some labels have been removed for clarity.

An analysis of variance on the effect of the duration of the heat treatment and temperature of the heat treatment was also performed on the area under the curve of the first peak, approximately 5-7 minutes retention time, assumed to represent protein aggregates, and the area under the curve of the second peak, which was split in two: Approximately 8.5-9 minutes retention time, the part of the peak which was unaffected by changes in the intensity of the heat treatment, assumed to represent native whey proteins, perhaps in particular α-lactalbumin, and approximately 9-10 min, the part of the peak which was highly affected by changes in the intensity of the heat treatment, assumed to represent native whey proteins, perhaps in particular β-lactoglobulin, Figure 17. This analysis of variance showed that both duration, temperature, and the combined effect of duration and temperature had a significant (p<0.05) positive effect on the area under the curve of the first peak, assumed to represent protein aggregates, and a significant (p<0.01) negative effect on the area under the curve of the second part of the second peak, assumed to represent native whey proteins, perhaps in particular β-lactoglobulin. Thus when increasing the temperature, the duration, or both the temperature and the duration of the heat treatment, the area under the curve of the first peak increased and area under the curve of the second part of the second peak was decreased.

It should also be noted that for the area under the curve for these two peaks almost all of the samples are significantly different, Figure 17, thus emphasizing the ability of SE-HPLC to distinguish the samples based on the intensity of the heat treatment they have been subjected to. Neither the heating time, nor the duration of the heat treatment, nor the combined effect of time and temperature had a significant effect of the first part of the second peak, assumed to represent native whey proteins, perhaps in particular α-lactalbumin.
Based on the scores of the first and second principal component in the PCA model, Figure 16 A, and the development in the size of the first peak and the second part of the second peak, Figure 17, the order of intensity of the heat treatment is 100 °C 60 sec > 100 °C 31 sec > 92.5 °C 60 sec > 92.5 °C 31 sec > 85 °C 60 sec ≈ 100 °C 2 sec > 85 °C 31 sec > 92.5 °C 2 sec > 85 °C 2 sec > unheated.

4.2.4 $^1$H LF-NMR

The acidification curve as measured by $^1$H LF-NMR for the heat treated pasteurized milk is similar to the acidification curve for the heat treated milk prepared from milk powder, Figure 11 and Figure 18 A. As for the preliminary experiments, all of the samples had the same starting point, approximately 188.5±4.5 ms, which was a bit higher than the starting point for the samples prepared from skim milk powder, where the starting point was 170.4±4.5 ms. This difference is most likely due to a higher dry matter content in the samples prepared from skim milk powder, as it was made with 12 g powder/100 g water, Section 3.2. In samples made from reconstituted skim milk increasing the dry matter content has been shown to decrease the proton mobility (Moller et al. 2011). The increase in proton mobility during acidification has been related to the solubilization of CCP from the micelles, Section 2.5.2. The onset of the plateau, at about 120 min, does not appear to vary with the intensity of the heat treatment. As for the preliminary experiments, it appears that $T_2$-plateau is related to the intensity of the heat treatment, Figure 20. Increasing the heating temperature or the duration of the heat treatment decreases $T_2$-plateau. It is noted that the ASIC 2 sample heated at 92.5 °C for 31 sec B, has an unusual low $T_2$-plateau and a high standard deviation. The acidification was only followed twice by $^1$H LF-NMR. The first measurement was made on the same day as the ASIC 2 sample heated at 100 °C for 60 sec was measured, and it is possible that the sample was taken from the wrong bottle. The ASIC 1 sample, which was heat treated at 85 °C for 60 sec, and had unexpectedly low values of whey protein denaturation, Section 4.2.3.1, is seen to have a higher $T_2$-plateau than the ASIC 2 sample, which was given the same heat treatment. This ASIC 1 85 °C 60 sec sample also had one of the highest standard deviations.

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9 The remainder of the acidification curves can be seen in Appendix 2
deviations all of the samples, Figure 18 and Figure 20. This supports the theory in Section 4.2.3, that this sample was not given the expected heat treatment. In particular as the other heat treatments, which were made at both ASIC 1 and ASIC 2 appear to have almost identical acidification curves and \( T_{2,\text{plateau}} \) Figure 17 and Figure 20.

To show how the acidification curve changes with the decrease in pH, the pH of the samples, calculated based on Equation 9 in Section 4.3.1, is plotted as the top axis in Figure 18 A. As the relationship between the time and the pH is not linear, neither is the top axis in Figure 18 A. The appearance of the plot, when plotted against a linearly decreasing pH, can be seen in Figure 18 B. Comparison of the two curves in Figure 18, shows that the influence of the intensity of the heat treatment on the value of \( T_2 \) in the plateau of the curve is visible in both curves. However, it is less clearly seen in Figure 18 B, as the plateau of the curve is much more narrow, because the change in pH is quite small in that part of the curve.

![Figure 18](image)

Figure 18 The development of \( T_2 \), measured by CPMG, during acidification of pasteurized skim milk heat treated for 60 seconds at various temperatures. The pH of the samples was calculated using Equation 9 in Section 4.3.1. Note that the y-axis starts at 150 ms. For clarity the standard deviation is only shown in a few points. A Displaying time linearly, with pH in the top x-axis. B Displaying pH linearly. (n=3 for ASIC 1 and n=2 for ASIC 2)

To further explore the relationship between the intensity of the heat treatment and the acidification curve as measured by \(^1\)H LF-NMR, a PCA model was made. Initially the 3 batches, ASIC 1, LIFE, and ASIC 2, Table 4, were compared to examine if a systematic difference existed between them, Figure 19 A. It is seen that there is no systematic difference between the acidification curves of the ASIC 1 and the ASIC 2 batches, while the acidification curves of the LIFE batch stands out. The acidification curves of the LIFE batch generally had a higher \( T_{2,\text{plateau}} \) than samples from ASIC 1 and 2 given the same heat treatment. The difference does not appear to stem from a difference in the pasteurized skim milk, as the acidification curves of the unheated samples from the 3 batches were almost identical, results not shown\(^{10}\). In Figure 19 A the pasteurized milk is the seven samples in the right part of the graph.

\(^{10}\) A plot of the acidification curves of the unheated samples from the 3 batches can be seen in Appendix 2
The difference between the acidification curves of the milk heated at LIFE and at ASIC was also seen when performing an analysis of variance on the effect of the duration and the temperature of the heat treatment on $T_2$-plateau, with the batch as a blocking factor, as the samples heated at LIFE were significantly different from the samples heated at ASIC. As will be described in Section 4.2.6, this difference is most likely due to the heat treatment at LIFE having a longer duration than expected. The samples heated at LIFE were therefore removed prior to a further examination of the data.

As the analysis of variance showed that there was no significant difference between the two batches made at ASIC, another analysis of variance was performed on $T_2$-plateau without the batch as a blocking factor. It showed that both the temperature of the heat treatment and the combined effect of duration and the temperature of the heat treatment had a significant ($p<0.001$) negative effect on $T_2$-plateau. Thus increasing the temperature of the heat treatment, or both the temperature and the duration of the heat treatment decreased $T_2$-plateau. However, changing only the duration of the heat treatment did not have a significant effect on $T_2$-plateau. Decreasing $T_2$-plateau means that the water relaxes faster, indicating a tighter protein
network after acidification, Section 2.5.2. This could mean that the gel is more firm and has a higher degree of water binding. Based on the $^1$H LF-NMR measurements during acidification, within the range of heating times and temperatures examined in this experiment, it would seem that raising the temperature of the heat treatment has a larger effect on the properties of the gel, than increasing the duration of the heat treatment.

A further examination of the data by PCA after removal of the LIFE batch was also performed, Figure 21 and Figure 22. The distribution of the samples on the PCA plot is similar to the distribution of the samples seen on the PCA plot of the analysis of protein aggregates by SE-HPLC, Figure 16 A. There is a clear trend in the graph, of the intensity of the applied heat treatment increasing when going from the right to the left of the graph. The trend seen in both protein analyses, Section 4.2.3.1 and 4.2.3.2, that a change in the effect of the heat treatment is seen when the heat treatment was for at least 92.5 °C for at least 31 seconds, is also seen in Figure 21, although it is not as clear as it was for the protein analyses. The loadings of the PCA model support the assumption that the main difference between the samples is seen in the plateau of the curve. As the first principal component, which explains 96% of the data, has the highest value in the plateau of the curve. The second principal component, which explains only 2.6% of the data, has the highest value at the end of the curve and just before the plateau of the curve. The latter variation probably stems from minor unsystematic differences in the pH during acidification, Section 4.3.1, inducing minor unsystematic differences in the solubilization of CCP from the casein micelles.

![Figure 21](image_url)

Figure 21 Scores for the first vs. the second principal components of the PCA model made on the 1H LF-NMR measurements during the acidification of milk, after removal of the LIFE batch. The data was mean centered prior to building the model. The scores are colored according to the heat treatment applied to the milk. The numbers 1-29 and 49-64 refer to the ASIC 1 and ASIC 2 batch, respectively.
On the basis of the PCA analysis the order of the intensity of the heat treatment as measured on the effect on the changes in $T_2$ during acidification of milk measured by an $^1$H LF-NMR CPMG experiment is $100 \, ^\circ C \, 60 \, sec > 100 \, ^\circ C \, 31 \, sec > 92.5 \, ^\circ C \, 60 \, sec > 92.5 \, ^\circ C \, 31 \, sec > 85 \, ^\circ C \, 60 \, sec$ ASIC $2 > 100 \, ^\circ C \, 2 \, sec > 85 \, ^\circ C \, 60 \, sec$ ASIC $1 \approx 85 \, ^\circ C \, 31 \, sec \approx 92.5 \, ^\circ C \, 2 \, sec$ ASIC $1 > 85 \, ^\circ C \, 2 \, sec >$ unheated.

4.2.5 Rheology

As was seen for the $^1$H LF-NMR measurements during acidification, Section 4.2.4, the rheological measurements during acidification showed that the samples heat treated at LIFE differed from the samples heat treated at ASIC. The coagulation time, $T_{gel}$, of the samples heat treated at LIFE, Figure 25, was significantly lower than $T_{gel}$ of the samples heat treated at ASIC, Figure 23 A. A principal component analysis of the development in the elastic modulus during acidification also showed that the samples heat treated at LIFE differed from the samples heat treated at ASIC. This principal component analysis had a similar appearance to the principal component analysis of the development of $T_2$ during acidification seen in Figure 19, results not shown. The samples heated at LIFE were therefore removed prior to further examination of the data.\textsuperscript{11}

The standard deviation of $T_{gel}$ was much larger for the ASIC 1 samples, than for the ASIC 2 samples, Figure 23 A, samples and the LIFE samples, Figure 25 A. This difference is due to a change in the way the experiment was set up, as described below.

Due to the low dry matter content and the fast acidification rate, the gels were very prone to contract and expel whey (Lucey 2004). For the ASIC 1 samples the milk was placed in the rheometer in a volume just covering the bob. As the gel developed in the rheometer and contracted, this induced cracks in the gel, where the oil covering the gel could sink down and blend with the gel. This happened in a varying degree for the ASIC 1 samples, in particular for the gels with a $T_{gel}$ of more than 50 min, Figure 23 A, thus inducing large variations in the results. After analyzing the ASIC 1 samples, it was realized that this might be a problem. It was therefore attempted to increase the volume of milk added to the rheometer, to the maximum possible, while still leaving space for the oil covering the sample. This change of practice greatly decreased the variation in the measurements of the LIFE samples. Thus this practice was continued when

\textsuperscript{11} Graphs of the elastic modulus and the phase angle for the samples can be seen in Appendix 3
analyzing the ASIC 2 samples. Because of this change of practice, it should be noted, when comparing $T_{gel}$ of the ASIC 1 samples with $T_{gel}$ of the ASIC 2 samples, that the data from the ASIC 1 samples is less reliable than the data from the ASIC 2 samples.

Figure 23 A $T_{gel}$ for the samples made at ASIC. Samples not sharing the same letter are significantly different ($p<0.05$), above the bars all samples, below the bars only ASIC 2. B The calculated pH at gelation, based on Equation 9 in Section 4.3.1, for the samples made at ASIC. Note that the y-axis in B starts at pH 4.5. ($n=3$)

$T_{gel}$ decreased when the intensity of the heat treatment increased. $T_{gel}$ decreased from about 75 min in the unheated ASIC 2 sample, to about 25 min in the samples heat treated at 100 °C for 60 sec, Figure 23 A. The decrease in coagulation time is caused by an increase in coagulation pH, from 5.0 to 5.4, Figure 23 B, which is caused by an increasing degree of whey protein denaturation, Section 4.2.3.1, leading to increasing formation of whey protein aggregates, Section 4.2.3.2, which are known to interact with casein micelles upon acidification, decreasing the pH at gelation, Section 2.4.1.

Due to the less reliable nature of the ASIC 1 samples, the analysis of variance of the effect of the duration and the temperature of the heat treatment on $T_{gel}$ was only performed on the ASIC 2 samples. The analysis of variance on the ASIC 2 samples showed that both the duration of the heat treatment and the temperature of the heat treatment had a significant ($p<0.001$) negative effect on the coagulation time. Thus increasing the duration or the temperature of the heat treatment significantly decreased $T_{gel}$. However, increasing both the duration and the temperature of the heat treatment did not have a significant effect on $T_{gel}$. A comparison of means of all of the samples from ASIC 1 and ASIC 2, showed that, only samples having a very different $T_{gel}$ were significantly different, and even the two samples heat treated at 85 °C for 60 sec was not significantly different, Figure 23 A. When the comparison of means was only performed on the ASIC 2 samples, it was found that most of the samples had significantly different $T_{gel}$, Figure 23 A.

In Figure 24 the phase angle and the elastic modulus of the ASIC 2 samples measured during acidification is plotted as a function of the calculated pH, Equation 9 in Section 4.3.1. The increase in gelation pH with increasing intensity of the heat treatment is clearly seen. It can be seen that, as expected, the unheated sample does not exhibit a ‘maximum loss tangent’, and as the intensity of the heat treatment increases, the ‘maximum loss tangent’ gets more pronounced, and the pH of the ‘maximum loss tangent’ slightly increases. From Figure 24 B it can be seen that increasing the intensity of the heat treatment greatly
increases the gel strength of the sample, as seen by an increase in the elastic modulus measured throughout the acidification. The sample heat treated at 100 °C for 60, which is the sample with the highest pH at gelation, has a ‘shoulder’ in the elastic modulus, indicating that CCP is still present in the micelle at gelation (Anema 2009), Section 2.4.1.

![Image](image-url)

**Figure 24 A** The phase angle and **B** The elastic modulus measured during acidification of the ASIC 2 samples as a function of the calculated pH, Equation 9 in Section 4.3.1. To indicate the magnitude of the standard deviation, it is shown in a few points on the graphs. (n=3)

Based on the $T_{gel}$ and the gel strength, as measured by the elastic modulus, the order of the intensity of the heat treatment of the ASIC 2 samples is $100 \, ^\circ C \, 60 \, sec > 92.5 \, ^\circ C \, 31 \, sec \, A \approx 92.5 \, ^\circ C \, 31 \, sec \, B = 85 \, ^\circ C \, 60 \, sec > 100 \, ^\circ C \, 2 \, sec > 85 \, ^\circ C \, 2 \, sec >$ unheated.

### 4.2.6 $^1$H LF-NMR and rheology for samples heat treated at LIFE

As mentioned in Section 3.3.2 attempts were made to heat treat the milk at LIFE, and a discussion of the differences between the samples made at LIFE and the samples made at ASIC will be outlined in the following.

$T_{gel}$ and $T_{2\text{-plateau}}$ of the LIFE samples can be seen in Figure 25 A and B. It should be noted that the sample heat treated at 92.5 °C for 60 seconds appears to give strange results, the reason for this is not known, but a likely scenario is that the intensity of the heat treatment was lower than expected. When comparing $T_{gel}$ for the samples heated at LIFE with the samples heat treated at ASIC, Figure 23 A, it is clearly seen that $T_{gel}$ is much lower for the samples treated at LIFE than the samples heat treated at ASIC. In particular the $T_{gel}$ of 14.8±2.1 min for the sample heated at 92.5 °C for 270 seconds is a quite low $T_{gel}$. The pH curve of the acidification of milk heated to above 100 °C for 60 seconds has not been measured. However, if the pH curves for these samples are similar to the pH curves measured for the samples heated for shorter durations, Figure 28, then the pH would have been approximately 5.5 at the time of gelation in the sample heated at 92.5 °C for 270 sec, which is a quite high pH at gelation even for heat treated milk. Thus about 15 min might be approaching the fastest possible $T_{gel}$ when acidifying skim milk with 1.00 g GDL per 100 g milk at 44 °C. As increasing the intensity of the heat treatment increases the pH at which gelation occurs, thus decreasing $T_{gel}$, the consistently lower values of the gelation time of the LIFE samples indicates, that the heat treatment has been more severe at LIFE than at ASIC. This is supported by $T_{2\text{-plateau}}$, which is also consistently lower for the samples heat treated at LIFE, than for the samples heat treated at ASIC.
Results and discussion

Figure 25 A $T_{gel}$ and B $T_2$-plateau measured by $^1$H LF-NMR, on the samples heat treated at LIFE. All of the samples has been frozen and defrosted before the measurement apart from the 100 °C 60 sec – unfrozen sample. Standard deviation is shown as errorbars. Samples from the same measurement not sharing the same letter are significantly different ($p<0.05$). (n=3)

On the basis of the above described differences between the samples heated at ASIC and the samples heated at LIFE, it is concluded that the heat treatment in the plate heat exchanger at LIFE did not correspond to the heat treatment at the plate heat exchanger at ASIC. The design of the two plate heat exchangers are quite different in particular in relation to the holding cell, where the system is much more compact at ASIC than at LIFE, Figure 26 A and B. In particular the tubes between the plate heat exchanger and the holding cell at LIFE are quite large, Figure 26 B. It is considered to be probable, that the heat treatment at LIFE appeared more severe than the heat treatment at ACIS, because the samples were given a longer heating time than anticipated. This longer heating time might be due to the length of the tubes from the plate heat exchanger to the holding cell, not being fully accounted for in the expected holding time.

Figure 26 Pictures of the plate heat exchangers at A: ASIC and B: LIFE. Note in particular the difference between the lengths of the tubes going from the plate heat exchanger to the holding cell in the two pictures.
4.2.7 Summary and conclusions on the main experiments

When comparing the order of the intensity of the heat treatment, as measured by the decrease in the content of native whey proteins, the increase in the content of protein aggregates, the decrease in T\textsubscript{2\_plateau}, and the increase in the elastic modulus measured during acidification, it is seen that for all of these measurements the samples heated for at least 92.5 °C for at least 31 seconds are the samples, which are clearly given the most intense heat treatment. The order of the intensity of the heat treatment for those samples was 100 °C 60 sec > 100 °C 31 sec > 92.5 °C 60 sec > 92.5 °C 31 sec for all of the measurements, and there was always a clear difference between the different heat treatments. For the samples given a lower heat treatment, the measured difference between the samples were smaller, in particular little difference was seen between these samples in T\textsubscript{2\_plateau}, indicating that the heat treatment applied to these samples was insufficient to change the properties of the gel made from this milk. The conclusion about the samples heat treated at 85 °C for 60 seconds was hampered by the great difference between the ASIC 1 and ASIC 2 samples. Based on the ASIC 2 sample it appears that this samples is grouped with the samples given a heat treatment above 92.5 °C for 31 seconds, where the intensity of the heat treatment applied to the ASIC 2 85 °C 60 sec sample is a bit less than the intensity of the heat treatment applied to the 92.5 °C 31 sec samples.

When comparing T\textsubscript{2\_plateau} measured by \textsuperscript{1}H LF-NMR with T\textsubscript{gel}, it was noticed that they appeared to respond in a similar manner to the changes in the intensity of the heat treatment, as both T\textsubscript{2\_plateau} and T\textsubscript{gel} decreased when the intensity of the heat treatment was increased. When plotting T\textsubscript{gel} against T\textsubscript{2\_plateau}, Figure 27, it is clearly seen that changes in the intensity of the heat treatment leads to similar changes in T\textsubscript{gel} and T\textsubscript{2\_plateau}. However, the ASIC 1 samples with a T\textsubscript{gel} of above 60 minutes and the LIFE sample heated at 92.5 °C for 60 sec disturbs the picture a bit. It can also be seen from this plot, that, although the duration of the heat treatment at LIFE is uncertain, Section 4.2.6, the relationship between T\textsubscript{gel} and T\textsubscript{2\_plateau} for the samples made at LIFE and ASIC are similar.

![Figure 27 T\textsubscript{gel} vs. T\textsubscript{2\_plateau}](image)

Note the x-axis starts at 370 ms. The standard deviation of T\textsubscript{gel} is shown as errorbars. (n=3)
4.3 Yoghurt experiments

When analyzing the data from the experiments performed on the yoghurt, Section 4.3.2-4.3.4, it became clear that the variations in the results were not only explained by the varying intensity of the heat treatment, nor by the varying pH at stirring, Section 4.3.1.1, which is known to influence the viscoelastic properties of stirred yoghurt (Renan et al. 2009). Attempts to perform analysis of variance brought more confusion than clarification, and indicated that the variation in the data was caused by more factors than was measured, results not shown. One factor might be related to the tapping of the yoghurt from the post-treatment device, Section 3.4.1. When producing yoghurt in the pilot plant at LIFE, many factors influence the properties of the produced yoghurt (Ipsen 2012, personal communication). Furthermore, it is known that even minor differences in the mechanical treatment applied to the yoghurt during stirring and cooling will affect the viscosity and syneresis of the gel, and the weaker the coagulum is before the mechanical treatment is applied, the greater this effect is (Rønkilde Poulsen et al. 1982). With this knowledge it was decided to merely present the results from the experiments performed on the yoghurt and describe clear tendencies. The actual evaluation of the effect of the varying intensity of the heat treatment of the yoghurt on the properties measured was performed by a principal component analysis, Section 4.3.5.

4.3.1 pH measurements during acidification

The acidification curves from the production of yoghurt seemed to be similar for all of the samples. All of the samples had an initial high rate of pH decrease, where after the rate of decrease fell, Figure 28. This was similar to the curves seen when testing different GDL concentrations, Section 4.2.2. As the rate of pH decrease started to fall the variation in the pH between the samples increased. However, the variation in pH did not seem to vary with the intensity of the heat treatment. It was hypothesized that the acidification curve was independent of the applied heat treatment. This hypothesis was tested by performing a comparison of the means of pH from the two production dates for each sample. As the pH was measured in random intervals, the samples from the two production dates had been measured at very different time points. However, it was possible to select 10 points where the difference between the measuring times at the two production dates were less than 2 minutes, which was considered a sufficiently small difference for comparison of the pH of the samples. The comparison of means showed that no pH mean was significantly different, and the hypothesis that the acidification curve was independent of the applied heat treatment was not rejected. Thus systematic variation in pH will not be considered, when discussing the measurements performed during acidification, Section 4.2.4 and 4.2.5. To be able to relate the measurements performed during acidification with the pH of the sample, a curve was fitted to the acidification curves of the samples as described in Section 3.4.1, Figure 28. The curve had the function:

\[ \text{Equation 9} \quad pH(t) = 6.45 - 0.334 \cdot \ln(t), R^2 = 0.978 \]

With t being time in minutes.
4.3.1.1 pH at stirring and after storage

The pH at stirring varied much more for the samples made the first production day, than for the samples made the second production day, Figure 29 A and B. During storage the pH increased for all of the samples. An analysis of variance showed that an increase in the pH at stirring led to a significant (p<0.001) increase in pH after 9 and 20-21 days of storage. Thus the pH after 9 and 20-21 days of storage showed much more variation for the samples made the first production day, than for the samples made the second production day. Furthermore, the pH of the samples made on the second production day was significantly higher than the pH of the samples made the first production day.

Figure 29 pH at stirring (n=1) and pH after 9 and 20-21 days of storage (n=3) for the two production dates, A day 1, and B day 2. Samples from the same measuring day not sharing the same letter are significantly different (p<0.05). Note the y-axis starts at pH 4.3
4.3.2 $^1$H LF-NMR, rheology, and syneresis

As described, Section 4.3, the results from the experiments performed on the yoghurt were very varying and were clearly affected by more than the applied heat treatment and the pH at stirring. The results of the $^1$H LF-NMR measurements, the rheological measurements and the measurement of the degree of syneresis of the yoghurts, Figure 30, were clearly very varying. Furthermore, they appeared to be correlated, as samples which had a high $T_2$ had a high percentage drainage, a low apparent yield stress, and a low elastic modulus at 1 Hz. This correlation was especially seen when the results from replicate measurements were compared. In particular, it was noticed that for the unheated sample and the sample heated at 85 °C for 2 seconds, the samples produced the second day had a higher $T_2$, than the samples produced the first day. However, for all of the other samples, $T_2$ was higher for the samples produced the first day, than for the samples produced the second day. A similar shift between these two samples and the other samples was also seen for the percentage drainage, for the apparent yield stress, and the elastic modulus at 1 Hz, Figure 30.

Figure 30 A $T_{21}$ and $T_{22}$ for the yoghurts measured by CPMG. B Percentage drainage for the yoghurts measured by centrifugation. Note the y-axis starts at 55%. C Apparent yield stress for the yoghurts. D Elastic modulus at 1 Hz for the yoghurts. Standard deviation is shown as errorbars. Bars in a plot not sharing the same letter are significantly different (p<0.05). (n=2)
It is difficult to say something conclusive about the effect of varying the intensity of the heat treatment on the basis of this data. However, the sample heated at 100 °C for 60 seconds have a lower $T_2$, a lower percentage drainage, a higher yield stress, and a higher elastic modulus than the other samples. This difference could be caused by the relatively more intense heat treatment applied to this sample, although the difference was not significant.

From the results of the $^1$H LF-NMR CPMG measurements on the yoghurts, another difference between the unheated sample, the sample heated at 85 °C for 2 seconds and the other samples is noticed, Figure 30 A. These two samples were the only samples, which had two water populations. They had a slowly relaxing water population, $T_{22}$, with a $T_2$ which was similar to the $T_2$ values of the other samples, and a fast relaxing water population, $T_{21}$, with a relative abundance of less than 5%. A possible explanation for the occurrence of the two water populations in these samples could be the occurrence of water cavities of varying sizes, which influences the dynamics of water. Thereby the fast relaxing water population can be ascribed to water bound to the surface of the proteins or in very small water cavities, while the slowly relaxing water population can be ascribed to water in larger cavities (Larsen 2012, personal communication).

This fast relaxing water population was not seen in any of the samples acidified in the NMR spectrometer, indicating that the occurrence of the fast relaxing water population could be related to the stirring, cooling or rebodying of the yoghurt. During stirring the gel structure is broken down into microgel particles which are suspended in the whey, and during storage the gel will regain structure (Renan et al. 2009). As it is known that the whey proteins affect the acid gelation of milk differently when they are denatured, Section 2.4.1, it is also possible that whey proteins affect the properties of the microgel particles and the rebodying of the gel differently, when they are denatured, as it is known that changes in the rheological properties of firm acid gels induced by heating is conserved after stirring (Cayot et al. 2003).

### 4.3.3 Posthumus funnel

![Figure 31](image_url)

*Figure 31 The time to elute the posthumus funnel for the yoghurts. Note the y-axis starts at 15 sec. Standard deviation is shown as errorbars. Bars not sharing the same letter are significantly different (p<0.05). (n=3)*
4 Results and discussion

As described, Section 3.4.1, the yoghurt for the posthumus funnel analysis was not from the same beaker, as the yoghurt for all of the other analysis, this was because approximately 200 ml of yoghurt was used per repetition. The result of the posthumus funnel analysis showed a rather large standard deviation for many of the samples, Figure 31, further supporting that there was variation in the viscosity of the product tapped from the post-treatment device. Most of the samples did not have a significantly different eluting time, Figure 31, and it is noteworthy that only the samples heated at 100 °C for 60 seconds had an eluting time, which is significantly different from the eluting time of water.

4.3.4 Confocal laser scanning microscopy

An example of two confocal images and their skeletonization can be seen in Figure 32. This skeletonization was used to describe the images, Section 3.4.6. The result is presented in Figure 33. Although it was attempted to take representative images of each sample, there was great variation in the images. In particular the amount of ‘structure’ varied a lot throughout a sample. This variation is also seen in the results of the skeletonization, where there in general was more variation within a sample than between samples. However, for the number of clusters, the samples heated at 100 °C for 2 and 60 seconds stands out as having more clusters than the other samples. It should also be noted that the samples from the first production date had a significantly (p<0.05) lower number of clusters, number of endpoints per cluster, and number of bifurcations per cluster than the samples from the second production date. The number of inside line points did not differ significantly between the two production dates.

![Figure 32 An examples of original images, left, and the corresponding binary image, center, and skeletonized image, right. A day 1 heat treated at 85 °C for 2 sec. B day 1 heat treated at 100 °C for 60 sec.](image)

12 Examples of the confocal images and skeletonized images of the remainder of the samples can be seen in Appendix 5.
4.3.5 Principal component analysis

The principal component analysis of the experiments performed on the yoghurt is seen in Figure 34 and Figure 35. As anticipated from the visual inspection of the data, Section 4.3.2-4.3.4, it is not possible to separate most of the samples based on the intensity of the heat treatment. However, the sample heat treated at 100 °C for 60 seconds is separated from the other samples on the first PC, which explains 41% of the data.
4 Results and discussion

Figure 34 Scores for the first vs. the second principal components of the PCA model made on the experiments performed on the yoghurt. The data was autoscaled prior to building the model. The scores are colored according to the applied heat treatment. Some labels have been removed for clarity.

Figure 35 Loadings for the first vs. the second principal components of the PCA model made on the experiments performed on the yoghurt. The data was autoscaled prior to building the model. Note that the loading for the number of end points is placed right next to the loading for the bifurcations, and therefore it can not be seen.

An inspection of the loadings reveal, that apart from the number of end points, inside line points and bifurcations per cluster, all of the properties measured were correlated with the separation of the sample heated at 100 °C for 60 seconds from the other samples. That this heat treatment resulted in an either lower or higher value for the results of these experiments was described in the previous sections, apart from the pH measurement where no clear connection between the intensity of the heat treatment and the pH was observed. The two samples heat treated at 100 °C for 60 seconds are quite separated on the second PC, which explains 21% of the data. This separation is correlated with the loadings for the number of end points, inside line points and bifurcations per cluster.

In the main experiments the effect of varying the intensity of the heat treatment on the properties of the acid gelation of milk had been investigated by acidification experiments in the $^1$H LF-NMR spectrometer and
in the rheometer, Section 4.2.4 and 4.2.5. These experiments were upscaled from 10-15 ml samples in the main experiments to 4 kg batches in the yoghurt experiments, without any adjustments of the experimental setup. Therefore it could be anticipated that the effect of varying the intensity of the heat treatment would be less clear. That the samples heat treated at 100 °C for 60 seconds can be separated from the other heat treatments, despite the lack of adjustment in the experimental setup and the huge variations in the results, makes this separation even more powerful.
5 Conclusion

In this study, the effect of varying the intensity of the heat treatment of milk applied prior to acidification on the properties of acid milk gels have been investigated.

Increasing the duration of the heat treatment, from 2 to 60 seconds, or increasing the temperature of the heat treatment, from 85 °C to 100 °C, led to a significant decrease in the content of native whey protein in the milk samples, as measured by RP-HPLC, and a significant increase in the amount of aggregates in the milk samples, as measured by SEC. However, the composition of these aggregates was not examined.

When acidification of the milk was followed by 1H LF-NMR, a plateau in the acidification curve was obtained after the milk had acidified for about 120 min, corresponding to a pH in the milk of about 4.8. The value of $T_2$ in this plateau, $T_{2\text{-plateau}}$, is believed to represent the ‘tightness’ of the protein network in the gel. When heat treating the milk between 85 °C and 100 °C for 2 to 60 seconds, $T_{2\text{-plateau}}$ significantly decreased when the temperature of the heat treatment or both the temperature and the duration of the heat treatment was increased. However, increasing only the duration of the heat treatment did not have a significant effect on $T_{2\text{-plateau}}$.

The coagulation time, $T_{gel}$, of the ASIC 2 samples, measured as the time, when the phase angle dropped below 45°, significantly decreased when the duration of the heat treatment, or the temperature of the heat treatment was increased. Increasing both the duration and the temperature of the heat treatment did not have a significant effect on $T_{gel}$.

$T_{2\text{-plateau}}$ was found to be correlated with $T_{gel}$. This correlation was seen for all of the heat treatments performed in plate heat exchangers. When the milk was heat treated between 85 °C for 2 seconds and 100 °C for 60 seconds, $T_{2\text{-plateau}}$ and $T_{gel}$ gradually decreased. However, increasing the duration of the heat treatment to above 60 seconds, when heating at 92.5 °C and 100 °C, only marginally decreased $T_{2\text{-plateau}}$ and $T_{gel}$, compared with heating for 60 seconds at 92.5 °C and 100 °C, respectively.

For both the content of native whey proteins, the amount of aggregates, $T_{2\text{-plateau}}$, and $T_{gel}$ there was a shift in the effect of the applied heat treatment when heating for at least 92.5 °C for at least 31 seconds, as heat treating the milk at a lower intensity only led to minor changes in these properties compared to heat treating the milk for at least 92.5 °C for at least 31 seconds or more. Thus there seems to be a ‘minimum necessary’ heat treatment to cause significant changes to the properties of the milk proteins and the acid milk gel.

For the stirred yoghurt, the conclusions from the acidification experiments could not be directly applied to predict the properties of the gel. For the small scale yoghurt experiments, there was much unexplained variation in all of the properties measured, making it difficult to make conclusions on the basis of these experiments. However, the analyses indicated, and a principal component analysis confirmed, that the samples heat treated at 100 °C for 60 seconds was different from the other samples. Thus the results indicated that heat treating the milk at 100 °C for 60 seconds prior to acidification could be sufficient to induce positive changes in the properties of the yoghurt, such as increased firmness and decreased separation of whey. However, the results should be confirmed in an experimental setup adjusted to reduce the unexplained variation in the results.
A correlation appeared to exist between $T_2$, water binding, and the rheological properties of the stirred yoghurt. However, this correlation also needs to be confirmed or rejected by further studies with an improved experimental setup.

Compared with the typical heat treatment applied to milk prior to yoghurt production, 90-95 °C for 5 min (Bylund 2003), heating at 100 °C for 60 seconds might be energetically more favorable, as although the temperature is increased, the holding time is substantially decreased. However, as described above, these results need to be confirmed in a more stable yoghurt system.
6 Perspective

A number of conclusions could be made based on the results in this report, but in several areas questions arose, which could only be answered by investigations which were outside the scope of this report.

The $^1$H LF-NMR FID experiment performed during the acidification of the milk revealed a fast relaxing component in the preliminary experiments, prepared skim milk powder, which was not present in the experiments prepared from pasteurized skim milk. This fast relaxing component thus appear to be related to properties of the skim milk powder, perhaps representing an incomplete dissolution of the skim milk powder, even after hydrating overnight. The hydration of the milk proteins could be investigated by HF-NMR, where it could be measured as the increase in protein content over a specified time period. It would also be possible to investigate the effect of different mixing procedures on the hydration of the milk proteins.

The acidification experiments showed that, within the range of temperatures investigated, a certain heat treatment, at least 92.5 °C for 31 seconds, induced more changes in the properties measured during acidification, than heating at lower durations or temperatures. When making the small scale stirred yoghurt experiment, the minimum heat treatment to induce changes in the properties of the yoghurt was 100 °C for 60 seconds. Although this result needs to be confirmed in a more stable yoghurt system, it could be interesting to investigate this 'minimum necessary' heat treatment more, and perhaps establish it for heat treatments at lower temperatures and increased duration of the heat treatment.

The yoghurt system could be made more stable by increasing the dry matter content, by lowering the acidification temperature to decrease the acidification rate, by increasing the concentration of GDL, to decrease the pH at stirring, or by using bacterial cultures instead of GDL. Given the fragile nature of the yoghurts produced, a combination of the suggestions is probably necessary to achieve a more stable yoghurt system.

There were also indications in the acidification experiments, that increasing the duration of the heat treatment to above approximately 60 seconds, when heating at 92.5 °C and 100 °C, only gave minor changes in the properties measured during acidification. The effect of this increased duration of the heat treatment on the properties of the stirred yoghurt was not investigated. Finding the boundary, where increasing the heat treatment only leads to minor changes in the properties of the yoghurt could be interesting, as overheating the milk could then be avoided. The effect of overheating the milk could also be established. The study of Rønkilde Poulsen et al. (1982) showed that excessive heating of the milk prior to yoghurt production had detrimental effects on the properties of the yoghurt. This could be investigated further, and perhaps the nature of the changes in the properties of the milk resulting in yoghurt of decreased quality could be discovered.

Further investigations of the degree of denaturation of the whey proteins and the amount and type of aggregates, which gives the most optimal yoghurt structure, could also be of commercial interest in relation to the production of skim milk powder used to enhance the protein content in yoghurt.
7 References


Appendix


Appendix

Ipsen, R. 2012, personal communication): RE: Personal communication with Richard Ipsen, section of Dairy Technology, Department of Food Science, Faculty of Science, University of Copenhagen, Denmark. E-mail: ri@life.ku.dk.


Larsen, F. H. (2008): NMR relaxometry slides. From the course Quantitative Food Spectroscopy. Quality & Technology, Department of Food Science, Faculty of Life Sciences, University of Copenhagen.

Larsen, F. H. 2012, personal communication): RE: Personal communication with Flemming Hofmann Larsen, section of Quality & Technology, Department of Food Science, Faculty of Science, University of Copenhagen, Denmark. E-mail: fhl@life.ku.dk.


Appendix


Appendix 1: Protein analysis

RP-HPLC

Device list
1290-ALS
1290-Bin Pump
1290-Column
VWD-SL+

Method name 120305 Symmetry WP.m

Inj.vol (m needle wash) 20 µl
Needle wash: Wash needle in flush port for 2.0 sec.

Sample temp. 5°C

Pump
Flow 0.600 ml/min
Low Pressure Limit 0.00 bar
High Pressure Limit 400.00 bar

Mobilfase A: 0,05% TFA i MilliQvand,
Mobilfase B: 0,1% TFA i Acetonitril

Gradient:

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Column temp 60°C +/- 0.8 °C

Column: Waters Symmetry300™ C18 5 µm 2,1x150mm
## Appendix II

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SE-HPLC

Mobile phase recommended by Waters for size exclusion chromatography of proteins. 0.1 M Na-fosfat pH 6.3; 2 L: 24.777 g NaH₂PO₄·H₂O, 12.521 g Na₂HPO₄·7 H₂O is dissolved in 2 L milliQ water. Measure pH to ensure that it is 6.3.

Device list
- 1290-ALS
- 1290-Bin Pump
- 1290-Column
- VWD-SL+

Method name **110927 SEC.m**
Inj.vol. 2 µl
Needle wash Wash needle in flush port for 10 sec
Sample temp. 5°C

Gradient
- Flow (ml/min): 0.3
- Pressure Max: 350 (ml/min)
- Solvent A: XX
- Solvent Ratio A: 100
- Solvent B: 20 % methanol
- Solvent Ratio B: 0

Column temp 40°C

Waters ACCQUITY UPLC® BEH200 SEC 1.7 µm 4.6x 150 mm with guard column Waters ACCQUITY UPLC® BEH200 SEC 1.7 µm 4.6x 50 mm.

Detector UV
- Pre-Run Balance Yes
- Post-Run Balance No
- Margin for -ve absorbance 100
- Peak Width GT 0.10 min (2.0s)
- Wavelength 214
- Output Zero Offset (%) 5 Output Attenuation 1000
- UV Lamp Yes
- Polarity 0

From 190 To 400

200 µl of milk is added to 1 ml mobile phase and gently shaken for 15 minutes before analysis.
Appendix 2: NMR graphs from the preliminary and the main experiments

Preliminary experiments

Figure 36: The development of T2, measured by CPMG, during acidification of milk prepared from skim milk powder and heat treated: A: at 85 °C at various durations, B: for 5 min at various temperatures, C: at 100 °C at various durations. Note that the y-axis starts at 150 ms. For clarity the standard deviation is only shown in a few points. (n=2)
Main experiments

Figure 37 The development of T2, measured by CPMG, during acidification of heat treated pasteurized skim milk. The pH of the samples was calculated using Equation 9 in Section 4.3.1. Note that the y-axis starts at 150 ms. For clarity the standard deviation is only shown in a few points. (n=3 for ASIC 1 and n=2 for ASIC 2)

Figure 38 The development of T2, measured by CPMG, during acidification of pasteurized skim milk heat treated at LIFE. The pH of the samples was calculated using Equation 9 in Section 4.3.1. Note that the y-axis starts at 150 ms. For clarity the standard deviation is only shown in a few points. (n=3)
Appendix 3: Rheology graphs from the main experiments

Elastic modulus

Figure 39 The elastic modulus measured during acidification of A: The ASIC 1 samples, B: The ASIC 2 samples, and C: The LIFE samples as a function of the calculated pH, Equation 9 in Section 4.3.1. To indicate the magnitude of the standard deviation, it is shown in a few points on the graphs. (n=3)
Phase angle

Figure 40 The phase angle measured during acidification of A: The ASIC 1 samples, B: The ASIC 2 samples, and C: The LIFE samples as a function of the calculated pH, Equation 9 in Section 4.3.1. To indicate the magnitude of the standard deviation, it is shown in a few points on the graphs. (n=3)
Appendix 4: Rheology graphs from the yoghurt experiments

Flow curves

Frequency sweep

Stress Sweep

Figure 41 Data from the rheological measurements of the yoghurt samples. Left column: Day 1. Right column: Day 2. To indicate the magnitude of the standard deviation, it is shown in a few points on the graphs. (n=2)
## Appendix 5: Confocal microscopy - Images and Connectivity

Table 7 Examples of the results from the CLSM analysis of the yoghurts made the first production date.

<table>
<thead>
<tr>
<th>Heat treatment</th>
<th>Image</th>
<th>Connectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unheated</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td>85 °C 2 sec</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td>100 °C 2 sec</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td>Heat treatment</td>
<td>Image</td>
<td>Connectivity</td>
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<tr>
<td>---------------</td>
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</tr>
<tr>
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<td><img src="image1" alt="Image" /></td>
<td><img src="connectivity1" alt="Connectivity" /></td>
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<tr>
<td>85 °C 2 sec</td>
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<tr>
<td>92.5 °C 31 sec - A</td>
<td><img src="image4" alt="Image" /></td>
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</tr>
</tbody>
</table>

Table 8: Examples of the results from the CLSM analysis of the yoghurts made the second production date.
Appendix

92.5 °C 31 sec – B

85 °C 60 sec

100 °C 60 sec