Master's thesis

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"Smart Yoghurt" – Combining innovative technologies towards more efficient yoghurt manufacturing

The potential of non-thermal combination technology with CO_2, high-pressure processing and ultrasound to improve the efficiency and sustainability of yoghurt manufacturing while maintaining product quality and storage stability of yoghurt produced with heat treatment

Master's thesis in Biotechnology
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Abstract

Heat treatment (>80 °C, up to 30 min) can comprise the nutritional and organoleptic properties of dairy products. To this end, non-thermal technologies have gained attention e.g. high-pressure processing (HPP), ultrasound (US) and carbon dioxide (CO_2), implemented alone or in combination as an eco-friendly alternative to heat treatment. In this study, reconstituted skim milk was subjected to either heat treatment (85 C° for 20 min, followed by inoculation with starter culture) as control or to a sequential process of CO_2-HPP-US (combination technology) according to the 2^3 full factorial design as follow: the milk sample was packaged with CO_2 in a sous-vide pouch and immediately subjected to HPP (400 or 600 MPa, for 5 or 15 min) prior to the inoculation and US treatment (68 kHz, 300 W, for 5 or 15 min at 42 °C). This led to eight combinations of HPP pressure, HPP time and US time. All samples were fermented at 42 °C until the pH reached ~4.6 and the resulting yoghurt gel was stored at 4°C overnight.

The combination technology, especially with the US treatment for 15 min reduced the total fermentation time compared to the traditional heat treatment. The yoghurt gel produced with HPP 400 MPa for 15 min exhibited the viscoelastic properties comparable to those of the control sample, but with a larger standard deviation indicating a variability in the gelation process. The lower yield strain and stress of the yoghurt gel indicated that the use of the combination technology in general resulted in more brittle gel with a weaker interconnectivity within the gel network compared to the control sample. Based on the total fermentation time and the viscoelastic and textural properties of the yoghurt gel, the combination technology 400 MPa, 15 min, US 15 min, was selected for further characterisation as compared to the heat treatment, including the microbial dynamics during the fermentation and native- and SDS-PAGE analysis of the milk proteins. In addition, the storage stability of the sample was assessed after 1, 7, 14, 21, 28 and 42 days of storage at 4°C with the vial count of the starter culture, titratable acidity and whey separation. The microbial dynamics during the fermentation were similar for the samples subjected to the heat treatment and the combination technology 400 MPa, 15 min, US 15 min, indicating the processing conditions did not influence the growth and metabolic activities of the starter culture. During storage, the yoghurt gel produced with the heat treatment or the combination technology showed similar viable count of the starter culture, postacidification and titratable acidity, but different amount of whey separation.

Sammendrag

Tradisjonell varmebehandling (>80 °C, inntil 30 min) kan påvirke de ernæringsmessige og organoleptiske egenskapene til meieriprodukter negativt, og påvirker også industriens øko-fotavtrykk. Teknologier som ikke tar i bruk varme har fått oppmerksomhet, f.eks. høytrykksprosessering (HPP), ultralyd (US) og bruk av karbondioksid (CO_2), enten alene eller kombinert, som et miljøvennlig alternativ til varmebehandling i meieriindustrien. Rekonstituert skummetmelk ble enten utsatt for varmebehandling (85 °C i 20 min, etterfulgt av inokulering med starter kultur) som en kontroll eller en sekvensiell behandling av CO_2-HPP-US (kombinasjonsteknologi) i et 2^3 fullt faktorialt forsøk som følger: melkeprøven ble pakket med CO_2 i en sous-vide pose og umiddelbart behandlet med HPP (400 eller 600 MPa, 5 eller 15 min), etterfulgt av inokulering og US behandling (68 kHz, 300 W, 42 °C, 5 eller 15 min). Dette førte til åtte kombinasjoner av HPP trykk, HPP tid og US tid. Alle prøvene ble fermentert ved 42 °C til pH 4.6 og yoghurten ble så lagret ved 4 °C.

Kombinasjonsteknologi, spesielt med US behandling i 15 minutt reduserte den totale fermenteringstiden sammenlignet med varmebehandlet kontroll. Yoghurtgel produsert ved HPP 400 i 15 minutter hadde viskoelastiske egenskaper sammenlignbare med kontrollen, men med større standardavvik som indikerer variasjon i geleringsprosessen. Lavere plastisk deformasjon og deformasjonskraft av yoghurten indikerte at bruk av kombinasjonsteknologi generelt resulterte i en skjørere gel med svakere bindinger innad i gelnettverket sammenlignet med varmebehandlet kontroll. Basert på total fermenteringstid, viskoelastiske egenskapene og teksturegenskaper av yoghurtgelen, ble kombinasjonsteknologien 400 MPa, 15 min, US 15 min valgt for videre karakterisering, blant annet mikrobiell vekst av startkulturen under fermentering, native- og SDS-PAGE analyse av melkeprotein og lagringsstabilitet etter 1, 7, 14, 21, 28 og 42 dagers lagring ved 4 °C. Den mikrobielle veksten under fermentering av lignende i melk behandlet med kombinasjonsteknologi og varme, noe som indikerer at behandlingsforholdene ikke påvirket veksten og den metabolske aktiviteten av starterkulturen. Under lagring hadde yoghurt produsert ved bruk av kombinasjonsteknologi og varmebehandling lignende antall kolonidannende enheter av starterkulturen, ettersyrning og titrerbar syre.

Preface

This master thesis is written as a part of my master's degree in biotechnology (MBIOT5) at the Department of Biotechnology and Food Science at NTNU, in collaboration with Nofima in Stavanger from September 2019 to May 2020.

I want to thank my supervisors and co-supervisors at NTNU and Nofima, Kari Helgetun Langfoss and Turid Rustad, Izumi Sone, Estefanía Noriega Fernàndez and Bjørn Tore Rotabakk, for all their feedback and support during this whole process. I would especially like to thank Izumi for all the support, patience and the countless hours you help me with this master thesis. I could not have done it without you. I would also like to thank Mette Risa Vaka for her help in the laboratory and all the others in Nofima who helped with their expertise.

Finally, I want to thank my family and friends for all their support throughout my master's degree.

Varhaug, May 2020

Mari Varhaug

List of abbreviations

Abbreviation	Full form
BSA	Bovine serum albumin
CCP	Colloidal calcium phosphate
CFU	Colony-forming unit
CO_2	Carbon dioxide
CN	Casein
EPS	Exopolysaccharide
GDL	glucono-δ-lactone
HPP	High-pressure processing
LA	Lactalbumin
LAB	Lactic acid bacteria
LB	*Lactobacillus delbrueckii ssp. bulgaricus*
LG	Lactoglobulin
MFG	Milk fat globule
MFGM	Milk fat globule membrane
MRS	Man, Rogosa & Sharpe
PAGE	Polyacrylamide gel electrophoresis
sCO_2	Supercritical carbon dioxide
SD	Standard deviation
SDS	Sodium dodecyl sulphate
ST	*Streptococcus thermophilus*
US	Ultrasound
WHC	Water holding capacity

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1 Introduction

Originally, yoghurt were produced to conserve the nutrient in milk, but it was soon discovered that yoghurt with different texture, flavours and consistency could be produced (McKinley, 2005). Yoghurt is a nutrient-dense food, and is a good source of proteins, calcium, phosphorous as well as vitamins and minerals (McKinley, 2005; Walstra, Wouters, & Geurts, 2006). The consumption of yoghurt is increasing. In 2015 the worldwide production of yoghurt were 35 529 000 MT, an 20% increase from 2010 ("The World Yoghurt Market Report 2000-2025," 2016). Yoghurt consumption is also increasing in Norway, from 6.8 kg yoghurt per person in 2000 to 10 kg per person in 2017 (*Utviklingen i norsk kosthold*, 2018). Today, milk from cows are most commonly used in the dairy industry, but milk from goat, sheep and buffaloes are also used ("Tetra Pak Dairy processing handbook," 2015).

Thermal treatment is extensively used in the dairy industry for preservation/processing purposes (e.g. thermalization, pasteurization or sterilization) despite its environmental footprint and undesirable effects on food nutritional (e.g. vitamin/mineral loss) and sensory (e.g. texture, colour, taste, flavour) attributes (Pardo & Zufía, 2012). Non-thermal processing technologies have received significant attention in the last decade in respond to the increasing consumer demand for safe, minimally processed and value-added products, with improved nutritional and sensory quality (e.g. fresh-like, healthy, long shelf-life) (Langelaan et al., 2013). For instance, High Pressure Processing (HPP) and Ultrasound (US) are promising non-thermal technologies for the dairy industry, typically combined with CO_2 addition. Non-thermal processing technologies are also beneficial for the manufacturing process through e.g. faster production rates, sustainable use of natural resources, energy and water savings and reduced food waste and green-house-gas emissions leading to reduced production costs and thus representing an environmentally friendly alternative to traditional heat treatment (Kourkoutas, Chorianopoulos, Nisiotou, Valdramidis, & Karatzas, 2016; Zhang, Wang, Zeng, Han, & Brennan, 2019).

This introduction will first address the main proteins in milk, which is important for the gelation of milk during yoghurt production. Furthermore, it will provide an overview of the different steps in yoghurt production, before a section about the use the non-thermal processing technologies CO_2, HPP and US in dairy processing reviewed. Lastly, the objectives of this master thesis are presented.

1.1 Milk proteins

1.1.1 Casein

Casein is the most abundant protein in bovine milk constituting approximately 80% of the total protein content. The main casein types include α_{s1}-, α_{s2}-, β- and κ-casein (Dalgleish & Corredig, 2012; Lucey, 2017). α_{s1}- and α_{s2}-casein make up 40% and 10% of the total casein in bovine milk and have a molecular weight of 23.6 kDa and 25.2 kDa after phosphorylation, respectively. α_{s1}-casein contains two centres of phosphorylation, while α_{s2}-casein contains three. β-casein accounts for 35% of the total casein in bovine milk, has a molecular mass of 24.0 kDa following phosphorylation, and has one centre of phosphorylation. κ-casein, which is the smallest casein with molecular weight of 19 kDa prior to post-translational modifications, accounts for 15% of the total casein in bovine milk, with no centres of phosphorylation (McSweeney & Fox, 2013).

Most of the casein proteins in bovine milk are incorporated in particles called casein micelles. In addition to casein, casein micelles also contain colloidal calcium phosphate (CCP). CCP are small granules of 2-3 nm, often called nanoclusters, composed of calcium and phosphate along with small amounts of magnesium, citrate and other compounds. The micelle is on average 150 – 200 nm and is highly hydrated. Although it only constitutes 2.5% of the total milk weight, it represents 10% of the milk volume (Dalgleish & Corredig, 2012; Lucey, 2017).

The micelle structure is important for understanding the properties and behaviour of milk. There have been several models suggesting how the micelle is built up, but its structure still has not been fully understood. Available hypothesis on the micelle structure include the submicelle model (Walstra, 1999), the nanocluster model (Holt, 1992), the dual-bonding model (Horne, 1998), and the most recent model suggested by Dalgleish (2011). In this master thesis, theoretical assumptions for the interpretation and discussion of results will be based on the model suggested by Dalgleish (2011), as described below and illustrated in Figure 1.

Casein interacts with CCP nanoclusters through phosphorylation centres. α_{s1}- and α_{s2}-casein contains multiple centres of phosphorylation and are therefore able to interact with several CCP nanoclusters. β-casein with one centre of phosphorylation will bind only to one CCP nanocluster. Interaction between α_s- and β-casein with CCP allows multiple CCP nanoclusters to be linked to each other, and casein proteins and the nanoclusters can grow into a micelle. κ-casein does not contain any phosphorylation centre and cannot interact with CCP

nanoclusters. Although it can associate with other types of casein through non-covalent interactions, the growth of the micelle will be restricted since it only contains one hydrophobic segment to interact through. It is well established that κ-casein is located on the surface of micelles. Parts of the κ-casein, the caseinomacropeptide (C-terminal region), projects from the surface of the micelles as a 5-10 nm thick hairy layer around the micelle and prevents micelles from aggregation with each other through steric repulsion. This hairy layer is not too dense, since β-casein is able to dissociate and re-associate during cooling and heating, and also due to the interactions of the micelle with whey proteins. It has been suggested that micelles contain water channels stabilised by β-casein. This could explain why the micelles are highly hydrated (Dalgleish & Corredig, 2012). The integrity of the micelle is maintained through hydrophobic interactions and CCP internally, and through steric repulsion of the κ-casein hairy layer on the surface (Anema, 2014; Dalgleish & Corredig, 2012; Lucey, 2017).

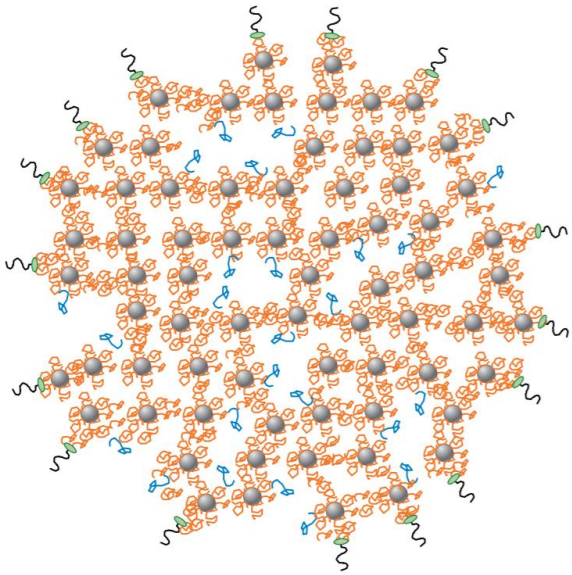

Figure 1: Structure of casein micelle according to the Dalgleish model. α_{s1}-, α_{s2}- and β-casein (orange) are linked to CCP (grey). Some β-casein (blue) stabilise the water channels in the micelle. κ-casein on the outer surface of the casein micelle consisting of two parts; para-κ-casein (green) and caseinomacropeptide chains (black). Picture adapted from Dalgleish and Corredig (2012).

1.1.2 Whey proteins

20% of the proteins in bovine milk are whey proteins, which remain soluble at pH 4.6 (McSweeney & Fox, 2013). Whey proteins are acid-soluble, highly structured, and heat-sensitive, the latter resulting in protein unfolding above certain temperatures (McSweeney & O'Mahony, 2016). There are many different whey proteins, with the main ones being β-lactoglobulin (β-LG), α-lactalbumin (α-LA), bovine serum albumin (BSA) and

immunoglobulins (Ig). Other minor whey proteins include lactoperoxidase, lactoferrin and serum transferrin (McSweeney & Fox, 2013).

β-LG represents 50% of whey proteins and 12% of total proteins in bovine milk. The protein is highly structured, compact and globular, with the isoelectric point at pH ~ 5.2. β-LG monomers are 18.3 kDa, with 162 residues per monomer. Each monomer has two intramolecular disulphide bridges and one mol cysteine (McSweeney & Fox, 2013). β-LG normally exist as a dimer at normal milk pH (6.7) (Olsen & Orlien, 2016). Under denaturing conditions, such as heat treatment above 75 – 80 °C, the dimer dissociate into monomers, and the cysteine-residues are exposed and can react with other molecules, e.g. κ- (Considine, Patel, Anema, Singh, & Creamer, 2007).

α-LA makes up 20% of whey proteins and 3.5% of total proteins in bovine milk. The protein is compact and globular, with ~14.2 kDa, and 123 residues per monomer. Its isoelectric point falls between 4.2 and 4.5. Each monomer has four intramolecular disulphide bridges. (McSweeney & Fox, 2013).

BSA is ~ 66 kDa, consists of 583 amino acids with 17 disulphides and 1 sulfhydryl. It has the ability to interact with α-LA and β-LG upon heat treatment, but due to its low level in bovine milk (0.1 – 0.4 g/L) is has little effect on the physicochemical properties of milk. Ig is a group of complex proteins, which also has little effect on physicochemical properties of milk due to low concentrations (0.6 – 1 g/L) (McSweeney & Fox, 2013).

1.2 Acidification of casein and gel formation

At normal milk pH (pH 6.6 – 6.8) (Walstra et al., 2006) the milk casein micelles are stabilised by negative charges and steric repulsions. When casein micelles acidify, CCP dissolves, leading to changes in the internal structure of the casein micelle. At the isoelectric point of casein (~pH 4.6), protein aggregation occurs (Dalgleish & Corredig, 2012; Lucey, 2014).

Lucey (2014) describes three pH-regions in the acidification of casein micelles. The first region range between pH 6.0 and 6.7, illustrated in Figure 2 (a). As the pH decreases there is less net negative charge on the casein micelle leading to reduced electrostatic repulsion and solubilisation of CCP. However, in this pH-range the amount of CCP being solubilised is still relatively small and does not affect the internal structure of the casein micelle much. The next pH-region falls between pH 5.0 and 6.0, illustrated in Figure 2 (b). As the pH decreases even more, this leads to a reduction in electrostatic repulsion. Eventually the stabilising hairy layer

of κ-casein collapses, so that its steric stabilising effect disappears. This allows casein micelles to diffuse closer to each other, eventually leading to gel formation. At pH 5.0 all CCP is dissolved from the casein micelle. The third pH region refers to pH ≤ 5.0, illustrated in Figure 2 (c). The net negative charge of the casein micelle decreases even more and there is an increase in +/- charge interactions, van der Waals forces and hydrophobic interactions. In presence of denatured whey proteins, complexes consisting of κ-casein and whey proteins are formed.

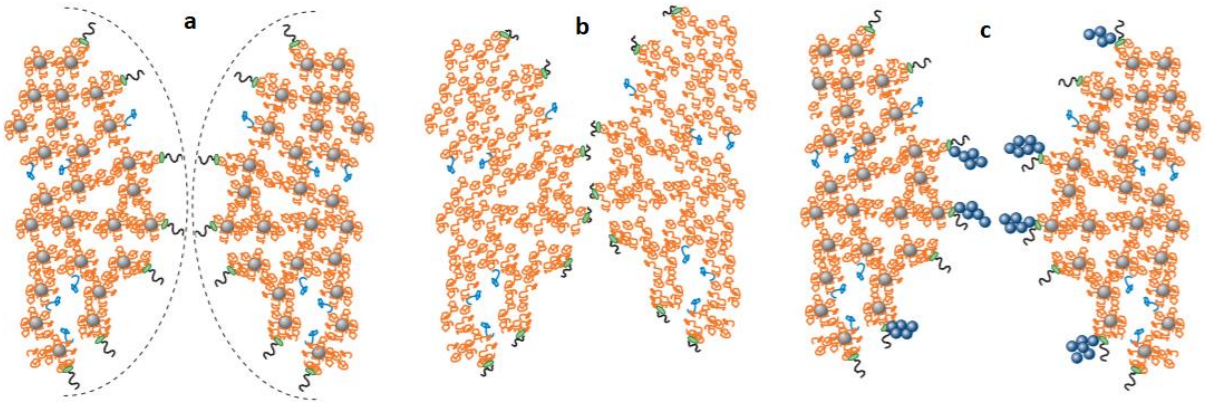

Figure 2: Interacting parts of casein micelles. (a) Native micelles steric stabilised by κ-casein hairy layer. The dashed line represents the zone in which the steric stabilising effect is acting. (b) Acidified micelles, CCP has solubilised and κ-casein hairy layer has collapsed. (c) Micelle in heated milk with κ-casein/whey protein complexes (dark blue spheres). These complexes provide interaction points between micelles during acid gelation. Para-κ-casein is green, the caseinomacropeptide chains are black, α- and β-caseins are orange, and calcium phosphate nanoclusters are represented by grey spheres. Some β-casein (blue) stabilise the water channels in the micelle. Picture adapted from Dalgleish and Corredig (2012).

1.3 Yoghurt production

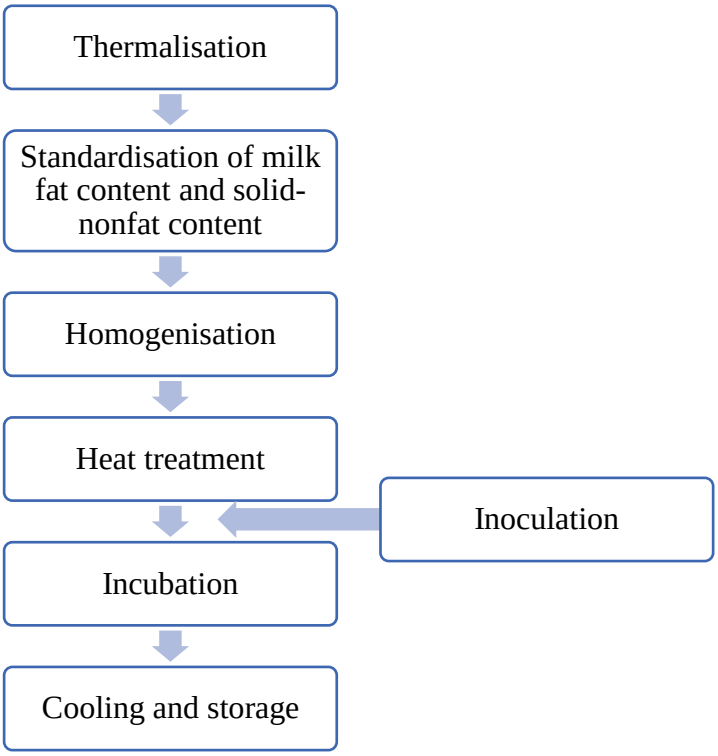

Figure 3: Flow chart for yoghurt production.

Codex Standard for fermented milks (243-2003) defines yoghurt as a fermented milk product obtained by the lactic fermentation through the action of *Streptococcus thermophilus* (ST) and *Lactobacillus delbrueckii* ssp. *bulgaricus* (LB). The microorganisms in the final product must be abundant and viable, and the total protein content in the yoghurt must be minimum 2.7% (m/m). The building blocks in the acid milk gel are casein micelles and denatured whey proteins (Lucey, 2014; Peng, Horne, & Lucey, 2009). There are different types of yoghurt, including set, stirred, drinking and frozen type yoghurts (Karam, Gaiani, Hosri, Burgain, & Scher, 2013). Set type yoghurt is incubated and cooled in the final package (Kim & Oh, 2013), and will be the type of yoghurt used in this master thesis. Figure 3 gives an overview of the different steps in yoghurt production.

Prior to homogenisation and heat treatment, milk is often thermalized and standardised. Thermalization is typically performed at temperatures from 60 to 69 °C for 20 to 30 s, and its goal is to inactivate vegetative cells and enzymes. Standardisation includes changes in the fat content of the milk and its solid-nonfat content. Normally this adjustment will involve a reduction of the fat content of the milk and an increase in the content of lactose, proteins,

mineral and vitamins. Standardisation is an important process for the quality of yoghurt, as the content of fat and solid-nonfat will affect the textural properties of the yoghurt gel (Chandan & O'Rell, 2013; Karam et al., 2013; Lucey, 2004; Sfakianakis & Tzia, 2014).

1.3.1 Homogenisation

Milk is homogenised to prevent a layer of fat to be formed on the surface of the milk. During homogenisation, the milk fat globule (MFG) is exposed to conditions which ruptures the MFG-membrane (MFGM), and when it is rebuilt, proteins from the milk serum will be incorporated into the MFGM, enhancing emulsion stability of the milk (Sfakianakis & Tzia, 2014). Commonly, homogenisation is applied at temperature between 55 and 80 °C and at pressures between 10 and 20 MPa, and the diameter of the MFG is reduced from 2-10 µm to 0.1-1µm. (Chandan & O'Rell, 2013; Sfakianakis & Tzia, 2014). Homogenisation reduce creaming and wheying off during storage, in addition to improves the consistency of yoghurt (Chandan & O'Rell, 2013).

1.3.2 Heat treatment

Heat treatment can be implemented via different methods, with the most typical one at industrial settings being through plate heat exchangers according to (Chandan & O'Rell, 2013). Time and temperature of the heat treatment can vary greatly depending on the goal of the heating (Anema, 2014). Heat treatment of milk has several important functions. Inactivating undesirable microorganisms in milk, including pathogenic and spoilage bacteria, and most milk enzymes is important for the safety of yoghurt as well as creating noncompeting conditions for the starter culture. Expulsion of oxygen, creating a more beneficial growth environment for lactic acid bacteria (LAB), and production of protein-cleaved nitrogenous compounds, leading to more available nutrients for the growth of LAB. Heat treatment also lead to physical changes in proteins, leading to interaction between denatured whey proteins and casein micelle. This has beneficial effects on the texture and quality of the yoghurt gel formed, including reduced whey separation and higher viscosity (Chandan & O'Rell, 2013).

Heat-induced changes in milk proteins

When whey proteins are subjected to heat above 70 °C they both denature and aggregate. Denaturation leads to dissociation into monomers if the native protein exists in an oligomeric state, followed by the unfolding of the monomer. Unfolding expose reactive groups in the protein, which can then react with other molecules. Disulphide bonds are covalent linkages of two cysteine residues in proteins. Unlike peptide bonds, disulphide bonds are reversible in nature allowing cleaved bonds to reform. α-LA only contains disulphide bonds,

while β-LG contains disulphide bonds as well as a free cysteine residue with a reactive thiol group. When β-LG denatures the reactive thiol group is exposed and can form disulphide bonds with other reactive thiol groups (Figure 4 A and B) and through thiol group-disulphide bond exchange reactions (Figure 4 C). In milk, which contains different proteins, the denaturation process is not reversible due to thiol group disulphide bond exchange reactions leading to formation of aggregates (Anema, 2014; Cho, Singh, & Creamer, 2003).

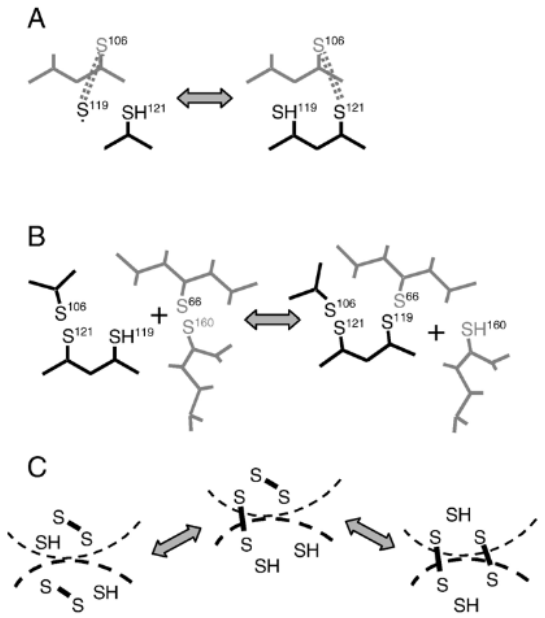

Figure 4: Thiol-disulphide interchange in β-lactoglobulin (β-LG). A and B: possible intermolecular interchanges. C: disulphide-bond interchange reaction between two β-LG leading to formation of dimer. Picture borrowed from Considine et al. (2007)

Casein has a random coil structure and is not susceptible to denaturation. When milk is heated, denatured β-LG will interact with κ-casein, which is present at the surface of the casein micelle, through thiol group-disulphide bond exchange reaction leading to disulphide bonds. Since α-LA does not contain reactive thiol groups it will not interact with the casein micelle directly, but through β-LG. Heat treatment of milk leads to a complex mixture of native whey proteins, whey protein aggregates and casein micelles coated with whey proteins. Variables including duration of heat treatment, temperature (see Figure 5), pH of milk, milk composition, protein concentration and salt concentration in milk will affect the denaturation and the interaction between whey proteins and casein micelles (Anema, 2014; Cho et al., 2003).

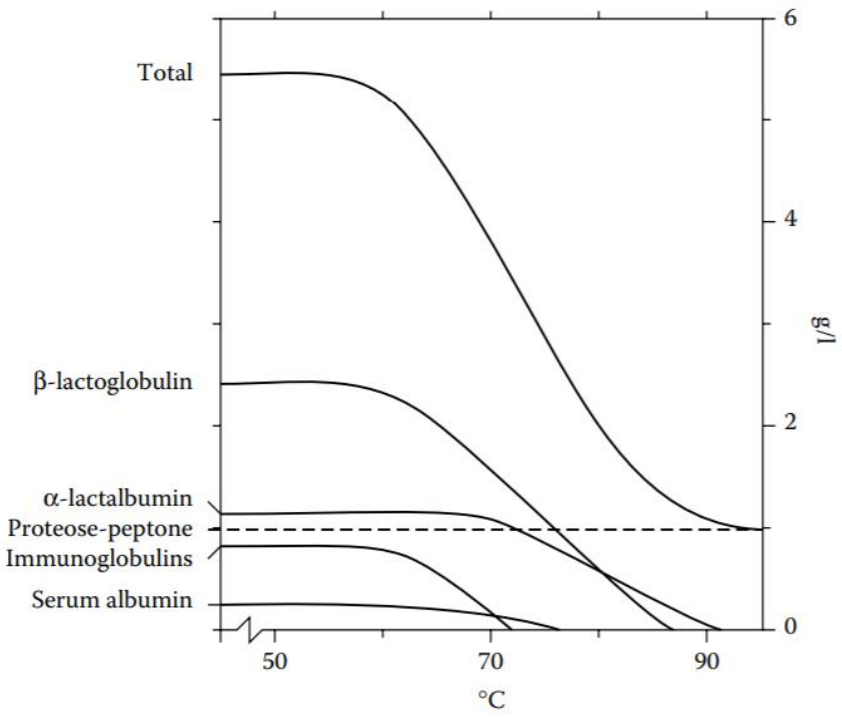

Figure 5: Amount of whey protein that remains dissolved after cooling and acidification to pH 4.6 when milk is heated at different temperatures for 30 min. Borrowed from Walstra et al. (2006)

Unheated milk forms a weak gel around pH 4.8. Heat treated milk, where denatured whey proteins have interacted with casein micelles, leads to reduced stability of the κ-casein hairy layer. This leads to a shift of the gelation point to a higher pH, since the isoelectric point of β-LG is 5.2. Thus, heat treatment of milk affects its gelling properties and leads to a stronger gel than in unheated milk (Dalgleish & Corredig, 2012; Lucey, 2014).

1.3.3 Fermentation

After milk processing, either thermal or non-thermal, milk should have a temperature around the optimum growth temperature of the starter culture prior to inoculation, in order to minimise the impact on bacterial metabolism. Inoculation with the starter culture is followed by incubation around the optimum growth temperature until the desired pH is reached. Commonly between pH 4.5 and 4.6. Typical incubation temperatures fall within the range of 31 to 45 °C, but most manufacturers recommend 41 to 42 °C (Chandan & O'Rell, 2013; Lucey, 2014; "Tetra Pak Dairy processing handbook," 2015). Incubations temperatures affect the rate of acidification, thus affecting rheological properties and whey separation of acid milk gels (Anema, 2008b), with lower temperatures having lower rate of acidification due to decrease metabolic activity of LAB, decreasing the rate of lactic acid production (Medeiros, Souza, & Hoskin, 2015).

Starter culture

Commonly used starter cultures are a mixture of two lactic acid LAB; ST and LB (Johnson & Steele, 2007). Sometimes the starter culture mixture also contains probiotic cultures. ST and LB produce lactic acid and reduce the pH of milk, leading to formation of a yoghurt gel (Baglio, 2014).

There are different forms of commercial starter cultures; fresh bulk starters, deep-frozen concentrated cultured, freeze-dried cultures and highly concentrated cultures (Direct Vat Set or Direct Vac Inoculation) ("Tetra Pak Dairy processing handbook," 2015). Inoculum level of the starter culture will depend on the manufacturer's recommendations. The starter culture strain chosen as well as their ratio will affect flavour, acidification rate, texture and rheological properties of yoghurt (*Development and Manufacture of Yoghurt and Other Functional Dairy Products*, 2010). Some strains produce exopolysaccharides, which affects the texture and rheological properties of yoghurt, including firmer body, higher viscosity and low syneresis (Mende, Rohm, & Jaros, 2016).

ST is a Gram positive, facultative anaerobic, nonmotile bacteria existing as spherical/ovoid cells (Baglio, 2014). Its optimal growth temperature is 37 °C (Chandan & O'Rell, 2013). ST produces lactase in substantial levels, which can break down lactose to glucose and galactose and, through subsequent steps, glucose further into lactic acid (Johnson & Steele, 2007). During the first stages of the fermentation, ST meets its need for nitrogen from free amino acids in the milk and during later stages from free amino acids produced by LB through endogenous peptidases (Johnson & Steele, 2007). Lactic acid concentrations of 1% inhibits the growth of ST (Chandan & O'Rell, 2013).

LB is also a Gram positive, nonmotile bacteria existing as slender rods with rounded ends. It is an aerotolerant anaerobe homofermentative and produces lactic acid, similarly to ST, as well as hydrogen peroxide (Baglio, 2014). Its optimal growth temperature is 45 °C. As compared to ST, LB tolerates lower pH and higher lactic acid concentration, e.g. up to 1.8% (Chandan & O'Rell, 2013). LB has cell wall-bound proteases, which are able to hydrolyse caseins into peptides (Johnson & Steele, 2007). However, it is dependent on ST to break down the peptides into free amino acids using peptidase, due to its low peptidase activity (Chandan & O'Rell, 2013).

Thus, LB and ST have a mutually beneficial symbiotic relationship, illustrated in Figure 6. Although ST and LB can survive and produce yoghurt as a single culture, they benefit from

each other's metabolism leading to faster acid production and pH drop (Chandan & O'Rell, 2013). For instance, ST's urease activity results in CO_2 generation, which stimulates the growth of LB. During the first stages of the fermentation (until pH 5.0), the maximum specific growth rate of ST is much higher than that for LB and it contributes to most of the lactic acid production. Later in the co-culture fermentation, LB growth dominates, as ST is affected by the increasing lactic acid levels in the media, and becomes the major contributor to the lactic acid production (Chandan & O'Rell, 2013; Walstra et al., 2006).

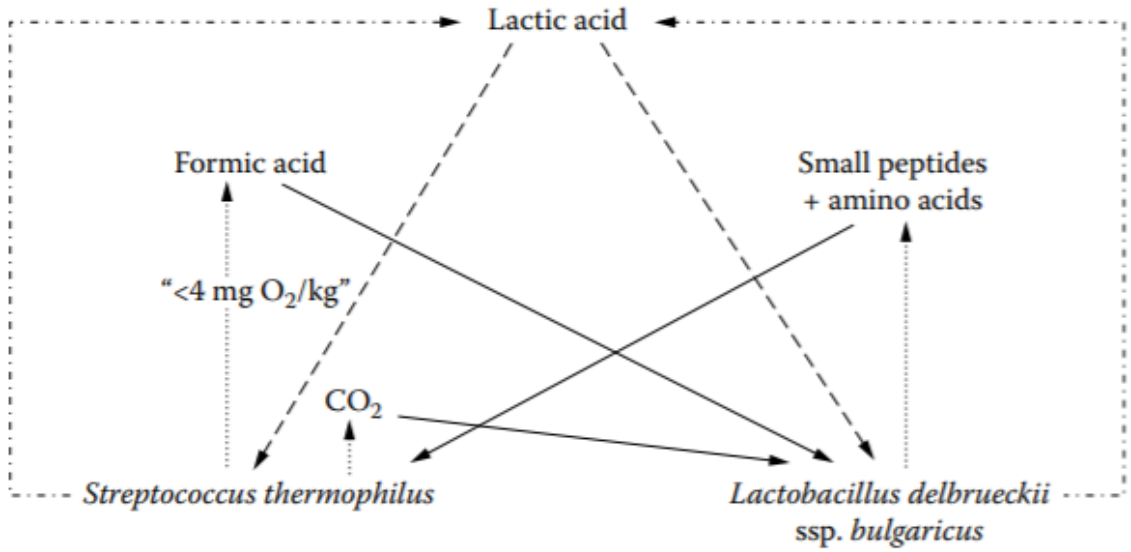

Figure 6: Overview of the symbiotic relationship between Streptococcus thermophilus and Lactobacillus delbrueckii ssp. bulgaricus. Picture adapted from Walstra et al. (2006)

1.3.4 Cooling and storage

When the desirable pH value is reached (pH 4.5), the cooling process starts ("Tetra Pak Dairy processing handbook," 2015). Cooling at this stage is important to stop the growth of the starter culture and further acidification. Set yoghurt is cooled in the container, either by transferring to cold storage, or by first blast cooling it in the fermentation chamber or cooling tunnels (Lucey, 2004). Cooling leads to increased firmness and viscosity of the yoghurt gel (Lucey, 2004, 2014). Yoghurt is stored at 4 to 5 °C to ensure the shelf life according to regulation since refrigeration temperatures slow down physical, chemical and microbial activity (Chandan & O'Rell, 2013; Lucey, 2004). Codex Standard for fermented milks (243-2003) require that titratable acidity, expressed as% lactic acid, is minimum 0.6%, and that the sum of viable microorganisms constituting the starter culture is minimum 10^7 CFU/g. These requirements have "to be verified through analytical testing of the product through to "the date of minimum durability" after the product has been stored under the storage conditions specified

in the labelling". In Norway, a storage time between 35 and 40 days is normal ("Naturell yoghurt,").

1.4 Use of non-thermal processing technologies in dairy products

Thermal treatment is extensively used in the dairy industry for preservation/processing purposes (e.g. thermalization, pasteurization or sterilization) despite its environmental footprint and undesirable effects on food nutritional (e.g. vitamin/mineral loss) and sensory (e.g. texture, colour, taste, flavour) attributes (Pardo & Zufía, 2012). Non-thermal processing technologies have received significant attention in the last decade in respond to the increasing consumer demand for safe, minimally processed and value-added products, with improved nutritional and sensory quality (e.g. fresh-like, healthy, long shelf-life) (Langelaan et al., 2013). For instance, High Pressure Processing (HPP) and Ultrasound (US) are promising non-thermal technologies for the dairy industry, typically combined with CO_2 addition within the frame of the hurdle technology. Hurdle technology, i.e. multi-target and mild combination of synergistic preserving factors ("hurdles"), is typically applied in the food industry to control foodborne pathogenic and spoilage microorganisms, thus improving food safety, whilst maintaining nutritional and sensory attributes and extending the product shelf life (Leistner, 2000). Typical hurdles include increased acidity, reduced water activity (a_w) and (high/low) temperature, but also modified atmosphere and addition of preservatives, with emerging contribution of innovative non-thermal processing (Singh & Shalini, 2016). Non-thermal processing technologies are also beneficial for the manufacturing process through e.g. faster production rates, sustainable use of natural resources, energy and water savings and reduced food waste and green-house-gas emissions leading to reduced production costs and thus representing an environmentally friendly alternative to traditional heat treatment (Kourkoutas et al., 2016; Zhang et al., 2019).

1.4.1 Carbon dioxide (CO_2)

Addition of CO_2 to milk leads to a decrease in pH since CO_2 reacts with water, resulting in formation of carbonic acid (Hotchkiss, Werner, & Lee, 2006), see Equation 1. CO_2, HCO_3^- and CO_3^{2-} are in an equilibrium, and their relative amounts will depend on the pH (Pedersen, Colmer, & Sand-Jensen, 2013), see Figure 7. Lactic acid bacteria used in yoghurt production are rather tolerant to CO_2, and CO_2 produced by ST has been found to stimulate the growth of LB (Driessen, Kingma, & Stadhouders, 1982). Louaileche, Bracquart, Saulnier, Desmazeaud, and Linden (1993) found that ST has an absolute CO_2 metabolic requirement for cell growth.

Equation 1

$$CO_2(dissolved) + H_2O \leftrightarrow H_2CO_3$$

$$H_2CO_3 \leftrightarrow HCO_3^- + H^+$$

$$HCO_3^- \leftrightarrow CO_3^{2-} + H^+$$

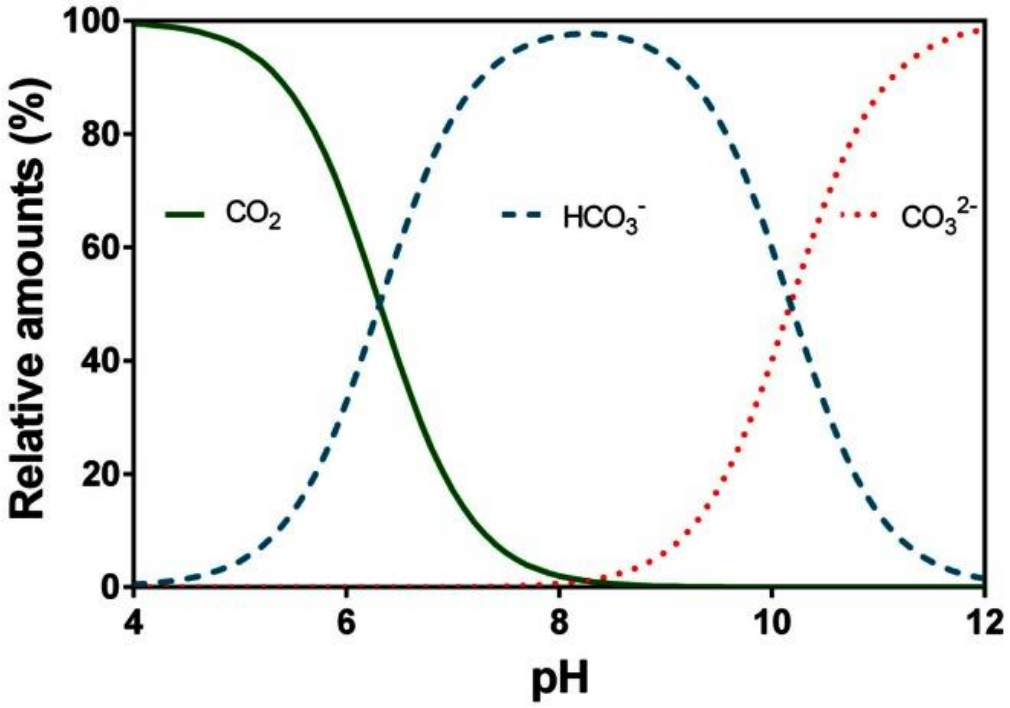

Figure 7: Relative amounts (%) of carbon dioxide (CO_2), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) in water as a function of pH. Picture borrowed from Pedersen et al. (2013).

Supercritical CO_2 (sCO_2) is CO_2 in a liquid state. This liquid state is reached when CO_2 is held above a critical temperature of 31.1 °C and a critical pressure of 7.4 MPa (Cheung, 1999), Figure 8. Benefits by using sCO_2 is that low viscosity and absence of surface tension allows for high diffusivity and solubility into both aqueous and fat phases of complex food materials (Amaral et al., 2017). Ceni et al. (2016) investigated the use of sCO_2 for inactivation of the enzyme alkaline phosphatase and *Escherichia coli* in milk, and found that at a CO_2: milk ratio of 0.05, 70 °C, 80 bar and a holding time of 30 min, *E.coli* and 94.5% of the enzyme were inactivated.

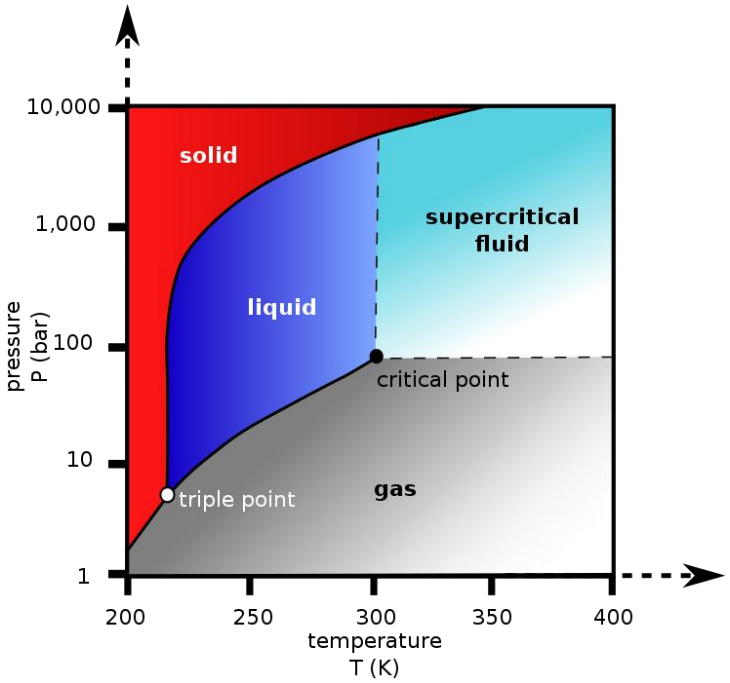

Figure 8: Phase diagram for pressure and temperature of carbon dioxide (CO_2). Picture borrowed from Ni, Song, Wang, and Shen (2016).

Calvo, Montilla, and Cobos (1999) found that skim milk acidified (with CO_2 bubbling) to pH 6.2 and 6.0 prior to yoghurt production (inoculated with LB and ST, incubated at 42 °C for 4 h) had significant higher ($p<0.05$) lactic acid production than control (skim milk without CO_2) after seven days storage. The concentration of L-lactic acid and D-lactic acid in pH 6.2, 6.0 and control were 5.5 and 0.85 g/kg, 6.0 and 1.0 g/kg and 5.1 and 0. 69 g/kg, respectively.

Another study conducted by Vinderola, Gueimonde, Delgado, Reinheimer, and Reyes-Gavilán (2000) found that the fermentation time (incubation at 42 °C until pH 5 was reached) was significantly shortened in CO_2-treated milk (carbonated with food-grade CO_2 to pH 6.3) compared to non-acidified control using two different starter combinations; (1) a combination of ST and *Lactobacillus acidophilus* and (2) a combination of ST, *L. acidophilus*, and *Bifidobacterium bifidium*. Fermentation time were reduced from 275.00 min in untreated milk to 217.50 min in CO_2-treated milk and from 235.00 to 177.50 min, for starter combination (1) and (2), respectively. The reduction in fermentation time was attributed to a lower initial pH (pH 6.3) as a result of addition of CO_2 and enhanced growth and metabolic activity of the starter cultures.

Peng et al. (2009) studied how preacidification of the milk (using glucono-δ-lactone (GDL), not CO_2) prior to fermentation would modify the amount of solubilised CCP and thus affect the textural properties of yoghurt. The pH of the milk was controlled using various

amounts of GDL to pH values of 6.55, 6.42, 6.10, 5.78, and 5.65. Preacidification of the milk prior to the fermentation (40 °C until pH 4.6 was reached), showed a linear increase in solubilisation of CCP with decreasing preacidification pH. Solubilised CCP decreased the number of cross-links between CCP and casein in the casein micelle, thus the number of CCP cross-linking during the gelation process leading to weaker gel. Comparing yoghurt made from preacidified milk, showed lower G', 139 and 183 Pa, lower yield stress, 16 and 23 Pa, and higher whey separation, 5.54 and 5.05%, at pH 6.1 compared to 6.55, respectively.

1.4.2 High pressure processing

High pressure processing (HPP) can be implemented either in batch or as a semi-continuous process. This master thesis focus on the batch process. In batch process food material is placed in a vessel that is filled with liquid and a pump or piston pressurises the vessel. When the target pressure is reached, the pressure is held for a certain holding time, before depressurisation and the food material can be removed. The liquid surrounding the food material act as a pressure-transmitting medium and could be water or another relevant media. Normal pressure treatment ranges between 50 and 1000 MPa (Hogan, Kelly, & Sun, 2005). Example of current implementation of HPP in the yoghurt industry is HPP treatment after packaging of the final yoghurt product, having the benefits of inactivation of yeast and mold for up to three months as well as reducing the number of LB, preventing postacidification ("Dairy,").

HPP can be implemented at room temperature or lower temperatures without affecting covalent bonds, which causes a minimal impact on flavour compounds and vitamins, as compared to thermal treatment, thus leading to better preservation of the nutritional value and sensory attributes (Hogan et al., 2005; López-Fandiño, 2006; Olsen & Orlien, 2016). A slight temperature increase of 3 °C per 100 MPa occurs during HPP treatment, due to adiabatic heating. Temperature will return to original temperature as soon as the pressure is released ("Everything you ever wanted to know about HPP concepts," 2020). Pressure assisted thermal sterilisation is a new technique combining heat and pressure to sterilise liquid food. Its goal is to reduce the sterilisation temperature needed to inactivate bacterial spore, thus minimising the thermal effect on nutrients (Wimalaratne & Farid, 2008).

The changes induced by HPP in milk proteins depend on several factors including protein structure and concentration, pressure level, duration of pressure treatment, temperature, pH, ionic strength and solvent composition (Kelly, Kothari, Voigt, & Huppertz, 2009; López-Fandiño, 2006). As previously mentioned, β-LG is a compact, globular protein that exists as a

dimer at milk's native pH. β-LG is pressure-sensitive and will denature at pressures > 100 MPa forming aggregates, as it contains reactive thiol groups and disulphide bonds, similarly to heat-treated milk. α-LA is more resistant towards pressure as it contains more intramolecular disulphide bonds and no free thiol groups, but will denature at pressures > 400 MPa (Huppertz, Fox, & Kelly, 2004; Olsen & Orlien, 2016).

Casein micelles dissociate at pressures > 250 MPa (Huppertz et al., 2004). Hydrophobic interactions are disrupted, CCP dissolved and calcium and phosphorous are released into the serum phase, and as a result the micelle dissociate into smaller micelles. When the pressure is released, dissolved calcium, phosphorous, casein and submicelles will re-associate into new micelle structures, but these micelles will have different size and structure than the original micelle (Dalgleish & Corredig, 2012; Olsen & Orlien, 2016).

Harte, Amonte, Luedecke, Swanson, and Barbosa-Cánovas (2002) studied the yield stress and microstructure of set yoghurt made from heat treated milk (85 °C, 35 min), HPP processed milk (193 or 676 MPa, for 5 or 30 min) or untreated milk. Milk treated at 193 MPa or untreated milk did not show any disruptive effect on the casein micelles or any denaturation of the whey proteins. Milk subjected to 676 MPa presented smaller casein micelles than heat treated milk, while heat treatment had no significant effect on the size of the casein micelles. Both 676 MPa and heat treatment denatured whey proteins, which interacted with κ-casein. Yoghurt made from milk treated at 676 MPa for 30 min had similar yield stress (49.3 Pa) as yoghurt made from heat treated milk (63.5 Pa), while yoghurt made from milk treated at 193 MPa and untreated milk had lower yield stress (<22.9 Pa). Treatment at 676 MPa for 30 min gave gels with similar characteristics to heat treated, while 676 MPa for 5 min gave a weaker gel. Yoghurt gels made from milk treated either with 676 MPa for 5 or 30 min or 85 °C for 35 min showed an average decrease of 0.7% in water holding capacity (WHC) after 20 days storage.

Anema (2010) adjusted the pH of skim milk to between 6.4 and 7.3 before HPP treatment (200-600 MPa, 30 min, 20 °C), and acidified the milk to pH 4.5 (30 °C, 3 h) using GDL (between 2.0 and 2.8% depending on pH of the milk). Acid milk gels made from milk with lower pH had lower final G' and yield stress than acid milk gels made from milk with higher pH. The levels of denatured β-LG increased with the magnitude of the pressure treatment and with the initial pH of the milk. They proposed that pressure treatment and pH affected the interaction between β-LG and casein, therefor affecting the properties of the acid milk gel.

Tsevdou, Tsevdou, Eleftheriou, and Taoukis (2013) found that HPP treated milk (600 MPa, 10 min at 55 °C) showed an increase in the firmness by texture analysis of the yoghurt gel (incubated at 42 °C until pH 4.75 was reached) compared to thermally treated milk (85 °C, 30 min). Fermentation time in HPP treated milk was also significantly decreased (by 30 to 40 min) compared to thermally treated milk. Both HPP and heat treatment lead to changes in milk serum, affecting the activity of enzymes involved in lactose hydrolysis, hence affecting the rate of acidification. These findings indicate that these changes are larger after HPP treatment than after heat treatment.

1.4.3 Low frequency ultrasound

The term ultrasound refers to soundwaves with a frequency above 16 kHz, which is above the normal human hearing frequency. According to Ojha, O'donnell, Kerry, and Tiwari (2016) there are three categories of ultrasound; low frequency ultrasound between 20 kHz and 100 kHz, high frequency ultrasound between 20 kHz and 2 MHz, and diagnostic ultrasound which is above 1 MHz, see Figure 9. Low and high frequency ultrasound can be used for food applications. High frequency ultrasound is used as a non-destructive analytical method, while low frequency ultrasound is used to change chemical and physical properties of various biological products as well as to inactivate microorganisms. Ultrasonic transducers convert electrical energy into vibrational sound energy, which is transmitted to the material either directly or indirectly through a probe or ultrasonic bath, respectively, see Figure 10. Cavitation refers to the creation, expansion and implosion of microbubbles in the liquid media when exposed to ultrasound. Low frequency ultrasound leads to a high rate of cavitation since the ultrasonic waves produce high acoustic pressure, while high frequency ultrasound has lower acoustic pressure which leads to a lower rate of cavitation (Ojha et al., 2016).

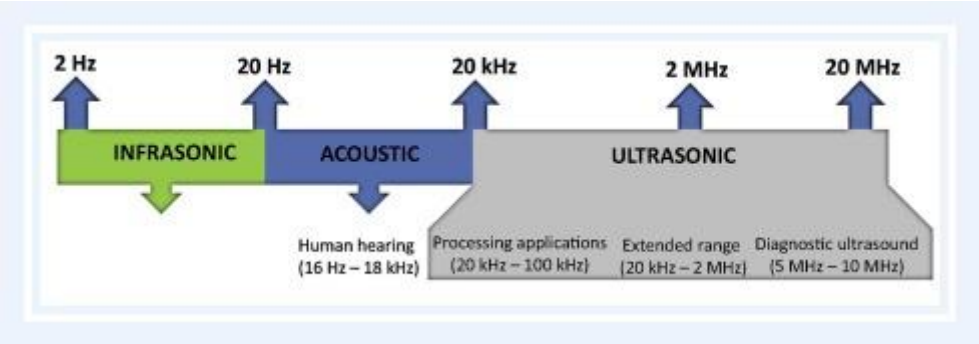

Figure 9: Overview of ultrasound categories. Picture borrowed from Ojha, Mason, O'donnell, Kerry, and Tiwari (2017).

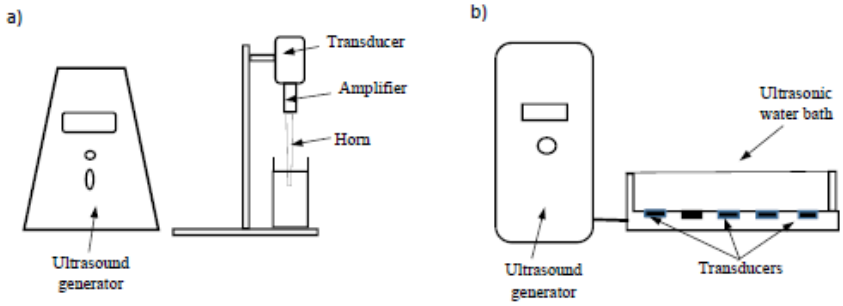

Figure 10: Ultrasonic setup: (a) ultrasound probe; (b) ultrasound bath. Picture borrowed from Abesinghe et al. (2019).

Low frequency ultrasound has been used in milk to inactivate undesired pathogenic and spoilage microorganisms, usually in combination with mild temperature, thus improving the product safety. Other applications include homogenisation, reduction in fermentation time and improved rheological properties of yoghurt gel (Marchesini et al., 2015).

Gregersen, Wiking, and Hammershøj (2019) studies effects of US treatment (flow cell) on the final G' of gel formed from milk (3.5% fat) acidified by GDL (540 mg GDL for 20 mL milk). Milk samples were treated in US flow cell, first heated to desired temperature (27, 50 or 70 °C) before US treatment (20 kHz, 10, 30 or 50 W) with a total treatment time of 30 min. The control samples received the same heat treatment and circulated in the US flow cell without receiving US treatment. They found that US treatment of milk lead to increased G' compared to control samples with the effect being dependent on both the power level and temperature. The final G' after 50 W treatment were significantly lower for 27 °C compared to 50 and 70 °C.

Wu, Hulbert, and Mount (2000) found that ultrasound had a homogenisation effect on the fat globules in milk, leading to reduced size of the fat globules. 90 W (20 kHz) for 10 min had similar homogenisation effect as conventional homogenisation (12 MPa at 60 °C). Higher power levels produced smaller fat globules and more uniform dispersion, thus having better homogenisation effect.Wu et al. (2000) found that ultrasound treatment lead to better WHC due to the reduced size of fat globules, and thus increased available area for interactions with casein of the fat globule membrane.

Wu et al. (2000) reported faster acidification (incubated at 43 °C) of inoculated (with mixture of ST, LB, *Bifidobacterium* and *Lactobacillus acidophilus*), sonicated milk (20 kHz, 15 °C, 180, 270 or 450 W, 8 min), which was attributed to modifications in the membrane permeability of the starter cultures, due to cavitation. Sonoporation lead to increased transport

of nutrients into and removal of waste products out of bacterial cells, including release of intracellular β-galactosidase. Normally lactose is transported into the bacterial cell where β-galactosidase hydrolyses it into lactic acid, but as an effect of sonoporation lactose can be hydrolysed in the extracellular environment, resulting in faster acidification of the media (Abesinghe et al., 2019).

Nguyen and Anema (2010) studied the effect of US treatment (22.5 kHz, 50 W, 30 min) on the acid gel formation using skimmed milk as compared to heat treatment (80 °C, 30 min), with and without temperature control. Samples under the temperature control were held at 20, 40, 60 or 70 °C under US, while the sample without temperature control reached 95 °C after 15 min. US treatment without temperature control led to whey protein denaturation, aggregation of casein micelles (indicated by increased particle size). With temperature control where the US temperature was held at 20 or 40 °C (below the denaturation temperature of whey proteins), no whey protein denaturation was observed, indicating that denaturation of whey proteins was attributed to the heat generated and not an effect of US. The resulting acid gels showed low final G' at these temperatures (20 and 40 °C), with a slight increase in G' (up to 50 Pa) with the prolonged US treatment of 30 min. With US temperature control held at 60 °C, acid gels would reach final G' as high as 260 Pa. Nguyen and Anema (2010) concluded that the only effect of US on the formation of the acid milk gel was an slight increase in firmness, especially at a prolonged US treatment, but that most of the effect were due to heat generated as a result of US treatment. Riener, Noci, Cronin, Morgan, and Lyng (2009) found 2-fold higher WHC and 25% higher final G' in yoghurt (incubated at 40 °C) made from US treated milk (24 kHz, 400 W, 45 °C, 10 min) compared to yoghurt made from heat treated milk (90 °C, 10 min).

1.4.4 Combination technologies

To the knowledge of the authors, very limited literature is available on the potential of combined non-thermal processing technologies in dairy products. Studies have been done on the combination of heat, HPP and/or US in dairy products. Anema (2008c) found that the combination of heat (65-100 °C, 30 min) and pressure (100-800 MPa, 30 min, 20 or 70 °C) gave higher levels of whey protein denaturation than heat or pressure treatment alone. Riener et al. (2009) found that combining heat treatment (45 °C, 10 min) with US treatment (24 kHz, 400 W, 10 min) led to yoghurt with higher WHC, greater viscosity and higher gelation pH compared to yoghurt made from heating at 90 °C for 10 min. Nonetheless, several studies have demonstrated the synergistic effects of combining CO_2, HPP and/or US in other foods. Marchesini et al. (2012) reported that the addition of CO_2 before US treatment (400W, 24 kHz)

of raw milk reduced the development of burnt off-flavours and the formation of oxidation products during storage at 4 °C. Abid et al. (2014) studied the effect of US (25 kHz, 70% amplitude, 20 °C, 60 min) and HPP (250 MPa, 350 MPa or 450 MPa, 10 min, room temperature) on the enzymes (polyphenolase, peroxidase and pectinmethylesterase), microorganisms (total viable plate counts, yeasts and molds) and phenolic compounds (total phenols, flavonoids and flavanols) of apple juice. Such a combination technology inactivated enzymatic and microbial activity, with the best results obtained at 450 MPa, demonstrating that HPP and US could have a potential hurdle effect on the safety of apple juice.

1.5 Objectives

The main objective of this thesis was to investigate the potential of non-thermal, combination technology with CO_2, HPP and US to improve the efficiency and the sustainability of the yoghurt manufacturing while maintaining the product quality and storage stability of the yoghurt produced with the conventional heat treatment (85 C° for 20 min). The secondary objectives are:

1. Investigate the effect of HPP pressure, HPP time and US time on the total fermentation time and the viscoelastic and texture properties of the yoghurt gel. Choosing an optimal combination of HPP pressure, HPP time and US time for further characterisation based on these results
2. Investigate the effect of combination technology on microbial dynamics during fermentation
3. Investigate the effect of heat, CO_2, HPP and/or US on protein denaturation
4. Investigate the storage stability during 42 days storage at 4 °C

2 Materials and methods

2.1 Materials

Non-fat dried milk powder was obtained from PanReac AppliChem ITW Reagents (Darmstadt, Germany) and stored under cool, dry conditions. The milk powder contained 35.0 ± 1.0% (w/w) protein, ~1.0% (w/w) fat, 52.0 ± 1.0% (w/w) lactose and 8.5 ± 0.5% (w/w) ash, according to the manufacturer. A yoghurt starter culture (YC-350) containing *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* (50 U) was kindly provided by Chr. Hansen (Hoersholm, Denmark) and stored at -40 °C upon reception. Vacuum pouches were supplied by Lietpak UAB (Vilnius, Lithuania). Solid CO_2 pellets were acquired from Seal Weld Pro AS, Norway. Rubber injection ports (A Peel&Stick™ Adhesive Rubber Injection Port) were supplied by Shroom Supply, Florida, USA.

MRS agar (De Man, Rogosa, & Sharpe, 1960) and M 17 agar acc. To TERZAGHI from Merck (New Jersey, US) were used for microbiological analysis of yoghurt samples, along with peptone water (CM0009) from Oxoid (Hampshire, UK).

2.2 Preparation of reconstituted skim milk

Reconstituted skim milk was prepared by mixing non-fat dried milk powder with sterile distilled water to a final concentration of 120 g/L. The milk powder was dissolved with gentle stirring (350 rpm) at room temperature overnight inside the laminar flow cabinet to ensure sterile conditions, avoiding the use of carcinogenic sodium azide (NaN_3), a bacteriostatic agent typically used to preserve protein-rich samples such as milk (Upadhyay, Goyal, Kumar, Ghai, & Singh, 2014). The total solid content of the reconstituted milk was measured after 18 h at 105 °C to 10.8 ± 0.0248% (N=6). The total solid content of the reconstituted milk was measured after 18 h at 105 °C. The temperature of the milk was standardised at 27 °C in a thermostatic water bath (Grant Instruments, Cambridge, UK) prior to processing to ensure the supercritical state of CO_2 during the HPP.

2.3 Milk processing

Milk was processed either by traditional heat treatment (85 °C for 20 min) or by a sequential combination of non-thermal processing technologies (smart processing), including CO_2, HPP and low frequency US treatment (hurdle technology). Figure 11 presents an overview of the milk processing steps following both smart and traditional schemes.

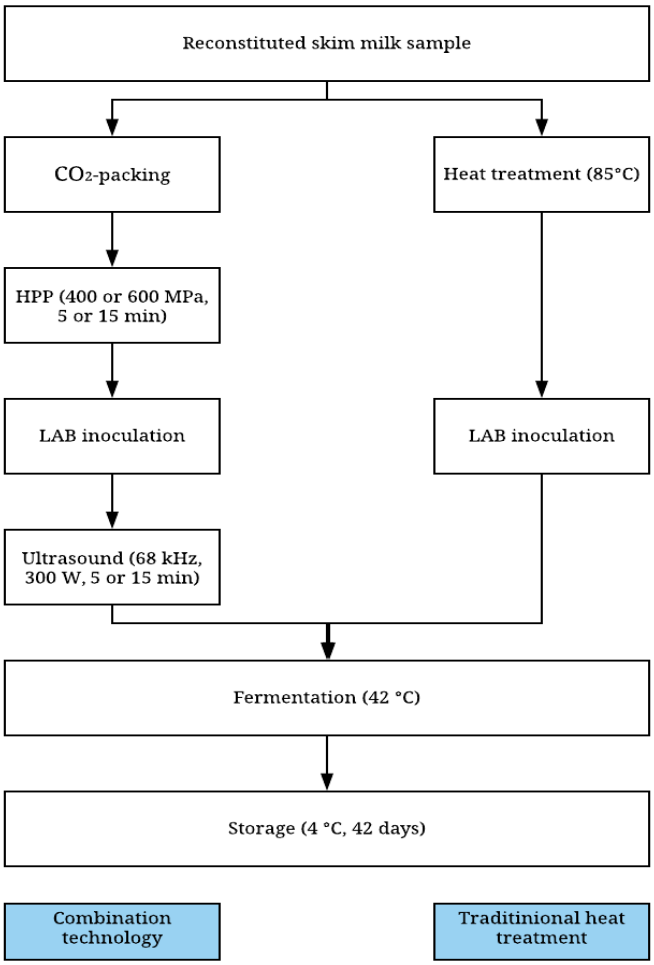

Figure 11: Overview of the milk processing steps according to non-thermal combination technology or traditional heat treatment.

2.3.1 Traditional heat treatment

500 mL reconstituted milk was heat-treated in a vacuum pouch at 85 °C for 20 min using a water bath. After the heat treatment, the milk was cooled down to 42 °C in ice water.

2.3.2 Inoculation of starter culture

Prior to inoculation into the processed milk, the freeze-dried starter culture, stored at -40 °C upon reception, was reactivated by transferring half the content of a pouch (25 U) to 500 mL reconstituted milk at 42 °C under sterile conditions. The freeze-dried starter culture was allowed to dissolve for 20 min on a magnetic stirrer plate (350 rpm, 42 °C). Then, 10 mL of the cell suspension was transferred for convenience into a 15 mL sterile Falcon tube under sterile conditions. 2 mL was sampled into a 3 mL sterile syringe with a sterile needle and aseptically injected into the headspace of the pouch (Figure 12) containing the processed milk, through a

sanitised rubber injection port. The final inoculum size in the milk was adjusted to 0.2 U/L, according to the manufacturer instructions. The inoculated samples were gently shaken to ensure the even distribution of the starter culture.

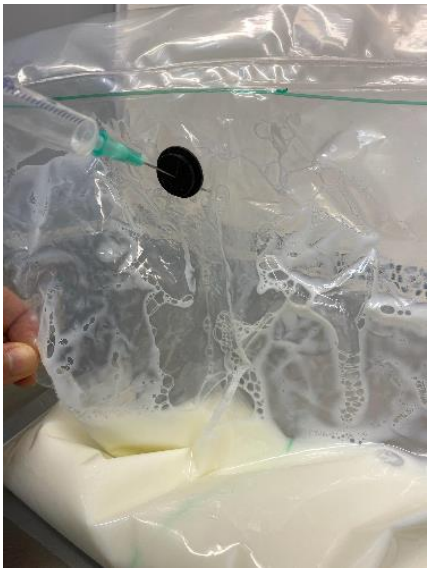

Figure 12: The placement of the rubber injection port at the headspace of the bag.

2.3.3 Non-thermal combination technology

Two level-full factorial experimental design

A 2^3 full factorial design was conducted to investigate the influence of HPP pressure (400 MPa or 600 MPa, at 27 °C), HPP holding time (5 or 15 min) and US treatment time (5 or 15 min, at 68 kHz, 300W, 42 °C) on the subsequent fermentation until pH 4.6 (Section 3.2) and textural and viscoelastic properties of the resulting set yoghurt (Section 3.3). This led to eight different combinations of the factors mentioned above, besides the traditional heat treatment (85 °C, 20 min) as shown in Table 1. The results from those experiments were evaluated to select the optimal set of combination technology for further analysis, including microbial dynamics during fermentation (Section 3.6), storage trial (Section 3.7) and native- and sodium dodecyl sulphate (SDS)- polyacrylamide gel electrophoresis (PAGE) analysis (Section 3.5) in comparison with the traditional heat treatment (Section 3.4).

Table 1: 2 level-full factorial design of the screening experiments to select the optimal combination.

Exp no.	**Pressure**	**HPP time**	**US time**
1	- (400 MPa)	- (5 min)	- (5 min)
2	+ (600 MPa)	- (5 min)	- (5 min)
3	- (400 MPa)	+ (15 min)	- (5 min)

4	+ (600 MPa)	+ (15 min)	- (5 min)
5	- (400 MPa)	- (5 min)	+ (15 min)
6	+ (600 MPa)	- (5 min)	+ (15 min)
7	- (400 MPa)	+ (15 min)	+ (15 min)
8	+ (600 MPa)	+ (15 min)	+ (15 min)
9	Traditional, 85 °C, 20 min		

- is the lowest level for each factor, + is the highest level for each factor.

Addition of CO_2

1.0 g dry ice was weighed out and immediately transferred to the pouch containing 500 mL reconstituted milk. This amount was selected because it represents a 1:1 ratio between the gas and the milk. The pouch with the sample was vacuum-packed (Supermax C, Webomatic, Germany) as shown in Figure 13, so that the O_2 and CO_2 content in the headspace after CO_2 dissolution was 1.9 ± 0.8 % and 89.8 ± 3.3 %, respectively (Section 2.6.2), while avoiding sample boiling during vacuum. The pouch with the milk was placed on a laboratory rocker (Rocker 25, Labnet international, New Jersey, US) at 80 rpm for 1 min to standardise the condition and the time for sublimation of the solid CO_2, and promote the dissolution of CO_2 into the milk. The gas composition and gas volume in the pouch were measured as described in Sections 2.6.2 and 2.6.3 respectively.

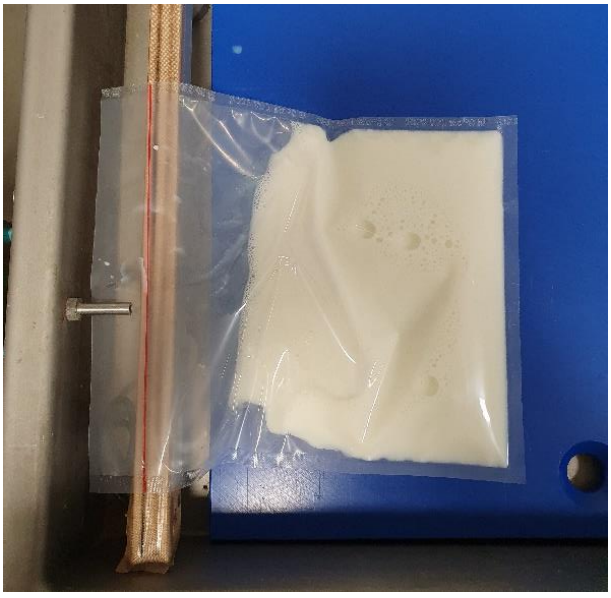

Figure 13: The standard placement of the vacuum bag containing milk and dry ice in the vacuum machine. The red line on the vacuum pouch is 3.5 cm from the top of the pouch and is placed over the black line in the vacuum machine. A 2 cm thick board was placed at the bottom of the vacuum machine to achieve 6 cm between the top of the pouch and the bottom of the pouch where the milk is.

High pressure processing (HPP)

High-pressure processing (HPP) of the milk treated with CO_2 was performed at 400 or 600 MPa for 5 or 15 min, temperature ranging between 11 and 38 °C, using a high hydrostatic pressure machine QFL 2L-700 (Avure Technologies Inc., Columbus, USA). The pressure levels were pre-programmed at 410 or 610 MPa to ensure actual values above 400 and 600 MPa, respectively, throughout the holding time. After the addition of CO_2, the pouch containing the milk was double-bagged before it was placed in the HPP treatment chamber filled with distilled water. The pressure and temperature changes in the chamber were recorded using a software (RSView runtime 1500 Ver. 06.02.20.00).

Inoculation

Following HPP, the surface of the sample pouch was disinfected with 70% v/v ethanol. The inoculation procedure followed that for the traditional heat treatment as described in Section 2.3.2).

Low frequency ultrasound (US)

After the inoculation, the sample was placed in an ultrasonic bath, a BT 130H bench top system (UPCORP, Illinois, USA) at a standardised position (see Figure 14) and treated at 68 kHz and 300W for 5 or 15 min. The temperature of the water in the tank ranged between 43.5 and 46.5 °C and was adjusted for each combination so that the temperature of the milk after the US treatment reached above 42 °C but not exceeding 45 °C. The temperature in the US bath remained stable after the treatment.

Figure 14: Detail of the sample pouch in the US bath. The pouch is placed approximately in the middle of the US bath (13.5 cm from short sides and 6.5 cm from the long sides) inside a metal cage.

2.4 Fermentation

The milk, either heat treated or using combination technology, was distributed into either 100 mL sterile cups (Sarstedt, Germany), approximately 60 mL in each, or 15 mL Falcon tubes, approximately 10 mL in each, while maintaining the temperature of the milk above 42 °C using a water bath at 43 °C. The milk in the cups and tubes was fermented at 42 °C in an incubator (B9000, Termaks, Bergen, Norway) with aluminium foil loosely over the top, as well as a Scotch bottle with water and lid off inside the incubator, to minimise evaporation. During the fermentation, a Falcon tube was sampled every 30 min and afterwards discarded, in order to monitor the evolution of pH and thus determine the total fermentation time, (Section 2.6.1). Total fermentation time was calculated by applying linear regression on the measured values from three hours of fermentation on, so that the time at which the pH reached 4.6 was interpolated. Small strain rheological measurements were performed during the fermentation to monitor the development of the viscoelastic properties of the sample (Section 2.6.6). Viable plate counts for both bacterial species in the starter culture were determined every 30 min during fermentation (Section 2.6.4), for the traditional treatment and the optimal combination technology. The cups with the yoghurt were transferred to an ice bath at the end of the fermentation i.e. when the pH in the Falcon tubes reached 4.6 and cooled down to 4 °C by using a water bath with ice.

2.5 Storage

The samples in the cups were stored overnight at 4 °C with lids on and afterwards subjected to texture measurements (Section 2.6.7).

2.5.1 42 days storage trial

Milk samples processed via traditional heat treatment and the optimal combination technology (HPP: 400 MPa for 15 min; US: 68 kHz, 300 W for 15 min), based on the results from the full factorial experimental design, were subjected to a storage trial of up to 42 days (Section 3.4). The milk treated accordingly was distributed into either 15 mL Falcon tubes or 25 mL conical flasks and fermented at 42 °C as described in Section 2.4. Besides microbial dynamics during fermentation, viable plate counts LB and ST (Section 2.6.4), titratable acidity (Section 2.6.8) and pH (Section 2.6.1) were analysed after 1, 7, 14, 21, 28 and 42 days of storage at 4 °C. The whey separation (Section 2.6.9) was measured after 1, 7 and 42 days.

2.6 Process and product characterisation

All processing steps, including fermentation, as well as the final yoghurt product were characterised, as shown in Figure 15. Analytical measurements to characterise milk processing included pH and temperature, gas volume and composition, and native- and SDS-PAGE. pH, fermentation time and microbial dynamics were used to characterise the fermentation process. Texture analysis and rheological measurements were conducted on the final yoghurt product, and viable plate counts for both LB and ST, titratable acidity and whey separation were analysed during 42 days of storage at 4 °C.

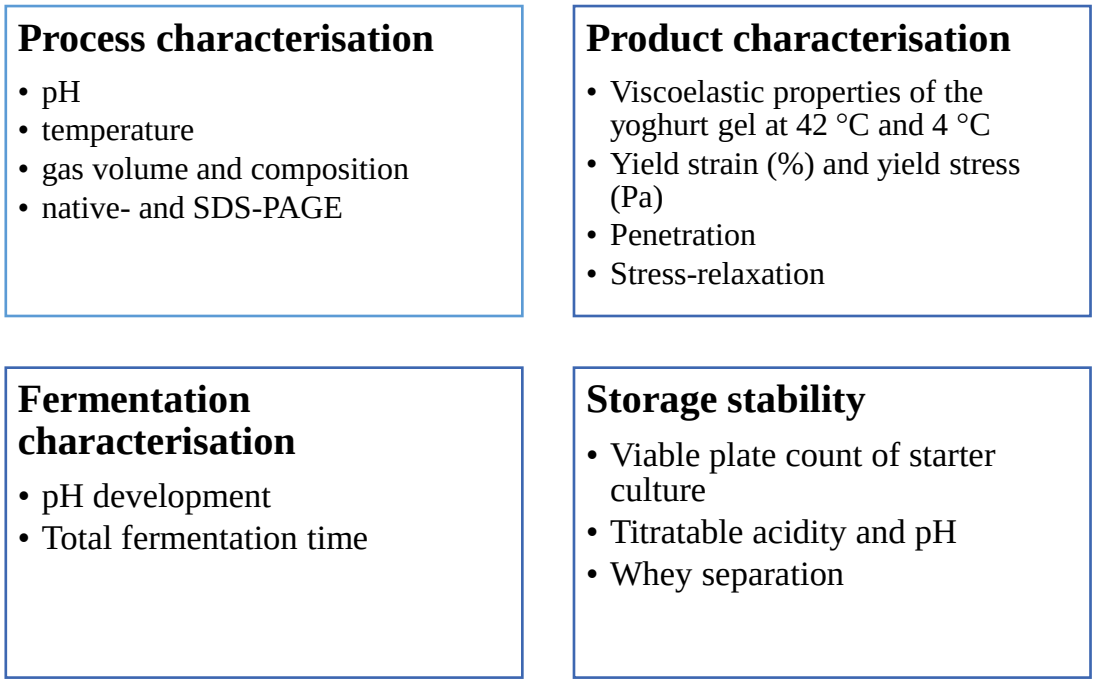

Figure 15: Overview of different analytical methods for product and process characterisation during yoghurt production.

2.6.1 pH and temperature measurement

A benchtop pH meter (EasyFive FP20, Mettler Toledo, Oslo, Norway) with an integrated temperature sensor (LE438) was used to measure the pH of the milk/set gel. The sample was mixed well with a disposal pipet before the measurement to minimise possible pH gradients along the z axis (sample depth), attributed to varying oxygen availability and thus, bacterial predominance and microbial metabolism. Temperature was measured using a handheld thermometer (104-IR, Testo SE & Co. KGaA, Titisee-Neustadt, Germany) throughout the experiments.

2.6.2 Gas composition

The gas composition in the headspace of the pouch was measured using a Checkmate9900 analyser (PBI-Dansensor, Ringsted, Denmark). The gas was collected from the headspace of the pouch by inserting a syringe through a foam rubber septum (Nordic Supply, Skodje, Norway). The foam rubber septum was used to avoid diffusion of false atmosphere into the gas analyser. The measurement was repeated for five pouches (N=5).

2.6.3 Gas volume

The gas volume in the headspace of the pouch was measured according to Rotabakk, Lekang, and Sivertsvik (2007) with modifications, using a TA.XT Plus Texture Analyser (Stable Micro Systems, Godalming, UK) equipped with a self-built probe (Figure 16). The gas volume was measured in a pouch containing only CO_2, or CO_2 with 500 mL reconstituted milk or 540 g distilled water, the latter based on the density of 60 g milk powder dissolved in 500 mL distilled water. The pouch was placed under the probe and submerged in a basin filled with water at 2 mm/s for 15 s before it was held for 30 s. The selected speed and holding time were chosen to minimise water movement in the beaker and to let the water stabilize before the measurements. The Buoyance force was measured after 26 s, 28 s and 30 s. The volume of the probe, measured at the start of the experiment using the same method as the gas volume measurement, was subtracted from the average of these three measurements to obtain the average volume of the headspace in the pouch. The measurement was repeated for five pouches for each set of analysis (N=5).

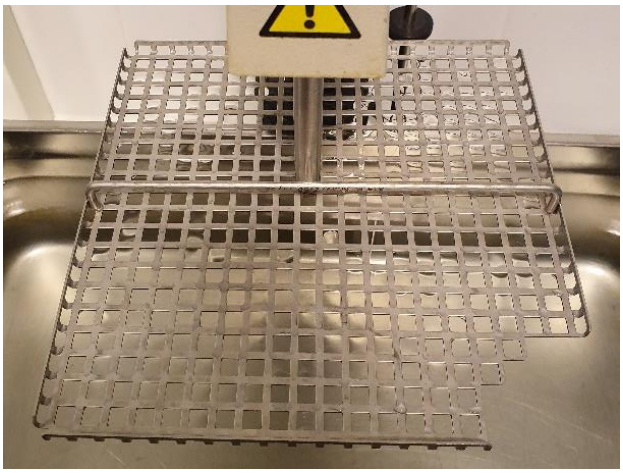

Figure 16: The homemade probe attached to the texture analyser used to measure the gas volume in the headspace of the vacuum pouch containing milk and CO_2.

2.6.4 Microbial dynamics during fermentation and storage

During fermentation (every 30 min) and storage at 4 °C (1, 7, 14, 21, 28 and 42 days), 1 mL aliquots of samples subjected to either traditional heat treatment or the optimal combination technology were transferred to 9 mL peptone water 0.1 % (w/v) and vortexed thoroughly until a homogeneous suspension was achieved. Serial decimal dilutions were prepared in peptone water and 100 µL of appropriate dilutions spread onto M17 and MRS agar plates for determination of ST and LB, respectively. Prior to enumeration of viable plate counts, M17 plates were incubated at 37 °C for 48 h under aerobic conditions. Anaerobic conditions were generated for LB by pouring three extra layers of MRS agar tempered at 45-48 °C over the dried, inoculated agar surface, followed by incubation at 37 °C for 72 h. Two technical and two biological replicates were analysed during fermentation, while three independent triplicates were sampled during the storage trial.

2.6.5 Native- and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Native- and SDS-PAGE of the milk was performed as described by Anema and McKenna (1996) and Anema and Klostermeyer (1997) with modifications. The milk subjected to Native- and SDS-PAGE analyses were (1) untreated reconstituted milk, (2) milk heat-treated at 85 °C for 20 min, (3) milk treated with HPP 400 MPa for 15 min, (4) milk treated with HPP 400 MPa for 15 min and US for 15 min, (5) milk with CO_2 treated with HPP 400 MPa for 15 min, and (6) milk with CO_2 treated with 400 MPa for 15 min and US for 15 min. The analyses were repeated twice using milk from two independent sample preparation (N=2).

Approximately 10 g of the sample was ultra-centrifuged at 100 000 g for 1 h at 20 °C (Optima XPN-100 Ultracentrifuge, Beckman Coulter, USA) in a centrifuge tube (Centrifuge Tubes Polycarbonate Thick Wall, Beckman Coulter, USA) and the associated Beckman Ti-70 rotor (Beckman Coulter, Ireland). Soluble caseins and whey proteins were defined as those that did not sediment from the milk during the ultracentrifugation. 400 mL of the resulting supernatant was transferred to a 1.5 mL Eppendorf tube and the pellets were discarded. Two replicates were made for each supernatant. The semi quantitative measurement of protein concentration of the supernatant was performed using a NanoPhotometer (Implen GmbH, Germany) based on the Warburg-Christian method (A280/ A260 method) (Warburg & Christian, 1941). 4 µL of sample was used for measurements with background corrections, and distilled water was used as blank. The protein concentration of the sample was adjusted to 3.0 mg/mL with distilled water.

The samples were run on SDS-PAGE and native-PAGE under reducing and non-reducing conditions, respectively. Dissociating and reducing conditions were obtained by mixing 20 µL sample with 20 µL SDS-sample buffer containing19 µL 2x Laemmli sample buffer (65.8 mM Tris-HCl, 26.3% (w/v) SDS and 0.01% Bromophenol Blue) and 1 µL β-mercaptoethanol and heated at 100 °C for 5 min. Non-denaturing conditions was obtained by mixing 20 µL sample with 20 µL Native Sample Buffer containing 62.5 mM Tris-HCl, 40% glycerol and 0.01% Bromophenol Blue (Bio-Rad, California, US). 10 µL sample (final protein concentration 1.5 mg/mL) was loaded in each well of a 12-well Mini-PROTEAN TGX Stain-Free Precast Gel (Bio-Rad) with 10 µL standard (Precision Plus Protein Unstained Standards, Bio-Rad). The gels were placed in a Mini-Protean Tetra Vertical Electrophorese Cell (Bio-Rad) with a 1:10 diluted running buffer (10x Tris/Glycerine/SDS buffer, Bio-Rad) and run at 300 V for 15 min. The gels were activated and imaged using ChemiDoc XRS+ (Bio-Rad) and analysed using Image Lab software (version 6.0.1).

The content of serum phase proteins was determined by comparing the band intensities of the treated reconstituted milk samples against those of the untreated reconstituted milk samples on native- and SDS-PAGE gels.

2.6.6 Rheological measurements

Rheological measurements of the gelifying milk during fermentation and on the final set gels were performed using a hybrid rheometer (Discovery HR-2,TA Instruments, Newcastle, UK) and a cone (40 mm, 2°) and plate geometry as described by Anema (2008a), with modifications. The geometry and the surface of the Peltier plate were disinfected with 70% v/v ethanol before 750 µL of the treated milk was added to the rheometer. The cone geometry was lowered into position and a solvent trap was placed around the sample to minimize evaporation. Time course measurements of storage modulus (G') and loss modulus (G'') were conducted at a frequency of 0.1 Hz and at a strain of 1.0% at 42 °C during the fermentation (Section 2.4). The frequency and strain chosen were within the linear viscoelastic range of acid milk gels (Marle & Zoon, 1995). Measurements were taken every 5 min. When the pH of the samples in the incubator reached 4.6, the temperature of the sample was reduced to 4 °C and stabilised for 15 min before the final G' and G'' values of the set gel at 4 °C were determined. The set gel was then subjected to a strain sweep (see Figure 17 for typical strain sweep curve) where the strain was increased from 0.5 to 300% at a constant frequency of 0.1 Hz. The yield strain and the yield stress of the set gel were determined by using the package "Segmented" (Muggeo, 2008) in the R program (The R Foundation for Statistical Computing, Vienna).

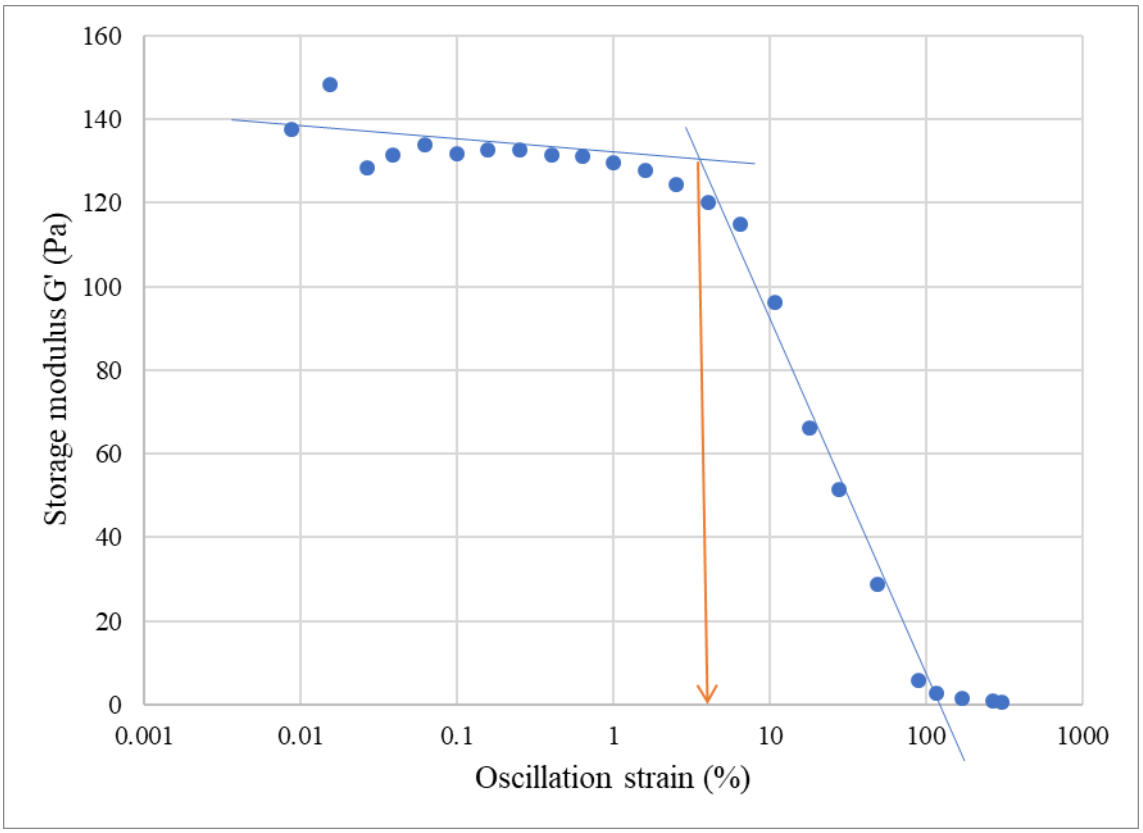

Figure 17: Typical strain sweep curve.

2.6.7 Texture analysis of set gel

Penetration

The penetration test was performed according to Vercet, Oria, Marquina, Crelier, and Lopez-Buesa (2002) with modifications. The measurement was performed using a TA.XT plus texture analyser with a 1/2'' Dia Cylinder Delrin Radiused probe (Stable Micro Systems Ltd, Godalming, UK). The sample temperature was 4 °C and only one measurement was performed in the middle of each cup containing approximately 60 mL set gel. The probe was placed less than 7 mm from the surface of the samples to ensure 15 mm of penetration depth. The test speed was set to 0.5 mm/s over a length of 22 mm. A typical penetration test curve is shown in Figure 18. Breaking force (g), force 15 mm (g), and area (g*s) were calculated. Breaking force shows how much force is required to break down the gel and force at 15 mm gives an indication about the firmness of the sample. The area under the penetration start from the start point until 15 mm down into the sample demonstrate how much work is required to get 15 mm down in the sample (N=3-6).

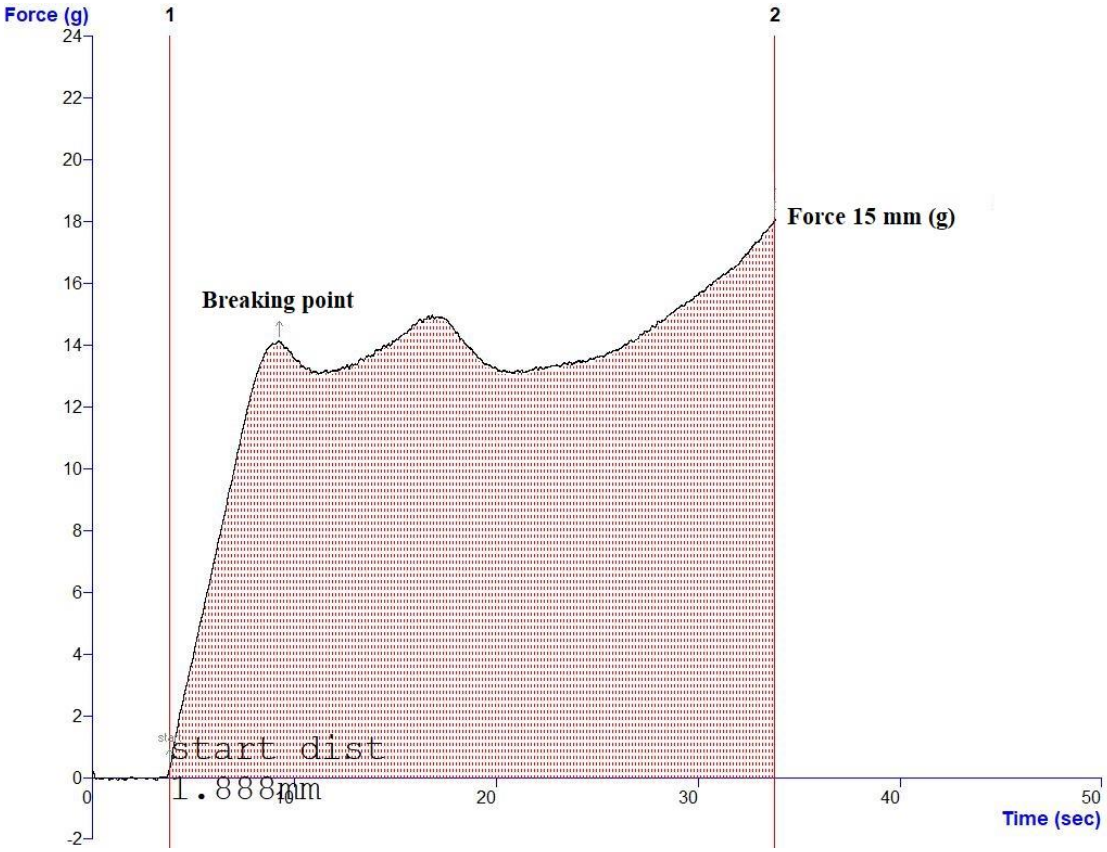

Figure 18: Typical curve from penetration test, with breaking point and force 15 mm (g), as well as area under the curve (marked in red).

Stress-relaxation

The stress-relaxation test was performed as described by Vercet et al. (2002) with modifications using a TA.XT plus texture analyser with a 1/2'' Dia Cylinder Delrin Radiused probe (Stable Micro Systems Ltd, Godalming, UK). The stress-relaxation test was performed on three replicates immediately after they were taken out of cold storage (4 °C). Stress-relaxation test was performed by maintaining a 2.5 mm deformation into the sample (at 1.0 mm/sec) for 120 s. A typical stress-relaxation test curve is shown in Figure 19. The following data were obtained from the stress-relaxation test: max force (g), minimum residual force (g), relaxation force (g), and% residual force. Max force is the maximum force at the surface of the set gel, while minimum residual force is the force that has not recovered at the end of the test (2 min). Relaxation force is the force that has recovered at the end of the test. Relaxation force and minimum residual force added together is the max force. Residual force (%) is the% of max force that is left after two mins holding time (N=3-7).

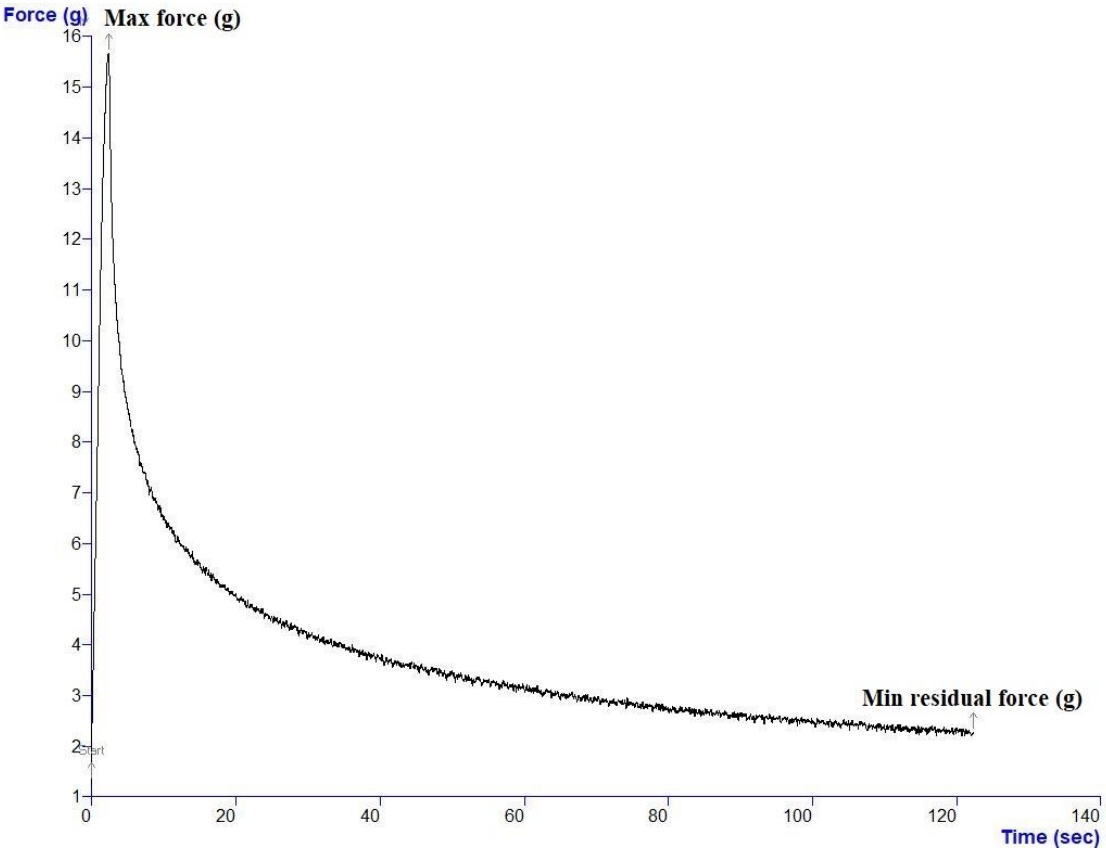

Figure 19: Typical curve from the stress-relaxation test, with max force (g) and min residual force (g). Residual force (%) is the quotient of min residual force over max force.

2.6.8 Titratable acidity

The content of lactic acid in yoghurt (% v/v) was determined by titration as described by Ghasempour, Moghaddas Kia, Golbandi, and Ehsani (2019), with modifications. Approximately 5 g yoghurt was mixed with 5 g distilled water and titrated with 0.1 M NaOH until the pH reached 8.30 ± 0.01 and stabilised at this pH for at least 30 s. Stable pH for 30 s was chosen since the yoghurt reacted, making the pH drop slowly, but continuously. Lactic acid has a molecular weight of 90.08 g/mol. Assuming that lactic acid is the predominant acid in the yoghurt sample, the percentage of lactic acid was determined using Equation 2.

Equation 2

$$Lactic\ acid\ (\%) = \frac{V * 0.009}{m} * 100\%$$

V (mL) = volume of 0.1 M NaOH

m (g) = mass of yoghurt

0.009 = titration acid expressed in units of lactic acid

2.6.9 Whey separation

Whey separation was determined using the volumetric flask method described by Lucey, Munro, and Singh (1998) and Peng et al. (2009) with modifications, after 1, 7 and 42 days of storage at 4 °C. Prior to conducting the experiment, the volumetric flasks were weighed and marked. Immediately after milk treatment, approximately 20 mL was transferred into 25 mL volumetric flasks and fermented at 42 °C in the incubator until the pH reached 4.6, cooled down to 4 °C in an ice bath and stored at 4 °C. Any liquid (whey) on the surface of a milk gel was gently poured out and the volumetric flasks were weighed again. The measurements were done in triplicate. The degree of whey separation was expressed as a percentage of the total weight of the set gel.

2.7 Replicates and statistical analysis

All experiments were conducted at least in duplicates. Error bars on graphs represent standard deviation (SD) of replicates. Data was analysed using SPSS (26.0, IBM, New York, US) where relevant using General linear model Univariate procedure and one-way ANOVA with Tukey as post hoc analysis. Results with $p \leq 0.05$ were reported as significant.

3 Results and discussion

3.1 Process characterisation

3.1.1 pH change during milk processing

The pH was measured at different stages of milk processing prior to the fermentation. The initial pH (Figure 20) of the milk was 6.6 ± 0.04 and decreased to 6.2 ± 0.03 after the addition of CO2. This decrease in pH was similar to Martin (2002) findings where the pH of milk decreased from 6.8 to 6.1 after CO_2 were bubbled into the milk. The average pH of the HPP-treated milk was 6.4 ± 0.04 and 6.8 ± 0.007 with and without the addition of CO2 respectively. After the inoculation and the following US treatment (68 kHz, 300 W, 5 min or 15 min), the pH of the treated milk decreased to pH 6.2 ± 0.05 in the presence of CO2. The pH of the milk increased by approximately 0.2 (from 6.6 ± 0.04 to 6.8 ± 0.007 without CO_2 and from 6.2 ± 0.03 to 6.4 ± 0.04 with CO_2) as a result of the HPP treatment. After US treatment, the pH of the milk decreased to a similar value of that prior to the HPP treatment, indicating that HPP affected the casein micelle. At pressures above 400 MPa, α-LA and β-LG denature, and the casein micelle dissociates along with the dissociation of CCP from the casein micelle. The release of CCP during pressure treatment could be a possible explanation for the temporary increase in pH. As the pressure is released, the casein micelle will reassociate, although changes in the size of the casein micelle gave been observed (Olsen & Orlien, 2016). The pH of the milk decreases again after US treatment, suggesting that US treatment might also alter the physiochemical properties of milk proteins. However, a study done by Chandrapala, Martin, Zisu, Kentish, and Ashokkumar (2012) on the effect of ultrasound (20 kHz, 450 W, 50% amplitude, within 10 °C of room temperature, up to 60 min) on the casein micelle integrity in skimmed milk did not find any significant changes on the concentration of soluble caseins no measurable changes in the concertation of soluble calcium. They did however find a small decrease in pH, probably as a result of formation of nitric acid through reaction between oxygen and nitrogen (Supeno & Kruus, 2000). The pH of the heat treated sample (85 °C, 20 min) was 6.5 ± 0.03 prior to the fermentation.

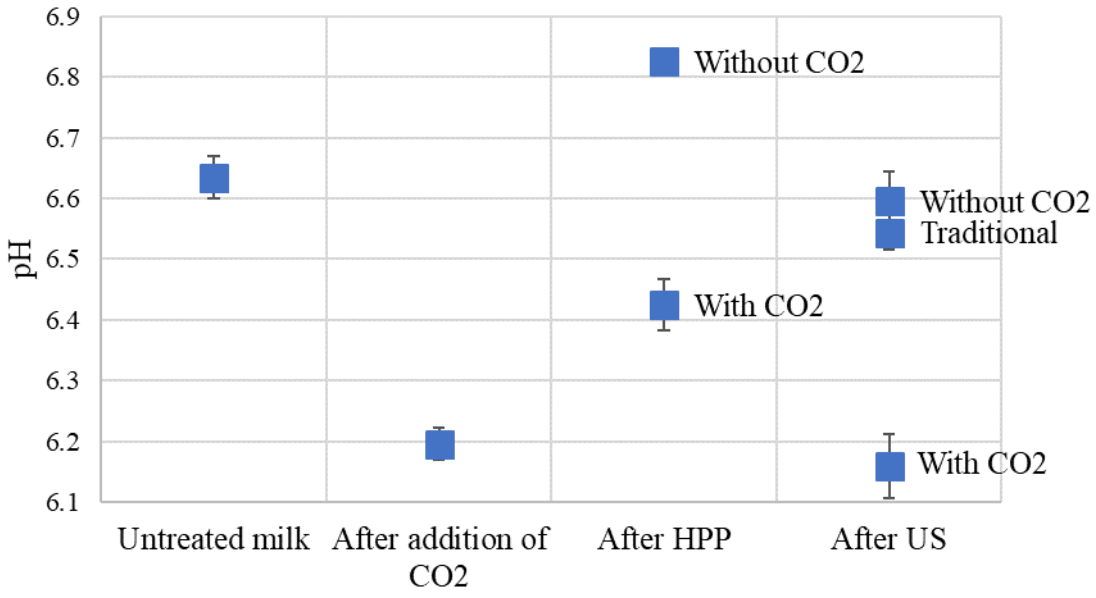

Figure 20: pH measured at the different steps of milk processing. In untreated milk, after CO2 addition, after HPP with and without CO2, and at the start of fermentation for the sample subjected to the traditional heat treatment (85 °C, 20 min) and to the combination technology (CO_2, HPP and US). The pH is reported as mean ± SD.

3.1.2 Supercritical CO_2 and temperature during HPP

CO_2 is in a supercritical state when the pressure is above 7.4 MPa (T_p) and the temperature is above 31.1 °C (T_c) (Cheung, 1999). Figure 21 shows the time that the temperature and pressure is above the critical temperature and pressure for CO_2. The temperature indicate the temperature in the chamber, but it is estimated that the temperature in the chamber and the milk are similar due to the milk being liquid and in good contact with the water inside the chamber and the volume of the milk (500 mL) is relative small compared to the volume of the chamber (2000 mL). The time CO2 is in critical state indicated here is just an estimate. The pressure for the combination technologies were 400 or 600 MPa, well above 7.4 MPa. The temperature of the untreated milk was standardised to 27 °C using a water bath. The temperature during HPP did not exceed 31.1 °C for the whole duration of the pressure treatment. Both 400 MPa, 5 min and 400 MPa, 15 min had approximately 1 min and 40 s in sCO_2 state. 600 MPa, 5 min had approximately 4 min and 35 s, while 600 MPa, 15 min had approximately 5 min and 15 s. After HPP treatment the temperature of the sample ranged between 11.4 ± 0.5 °C and 17.9 ± 0.5 °C (Table 2), depending on the pressure and the duration of the treatment. This is due to adiabatic pressure release.

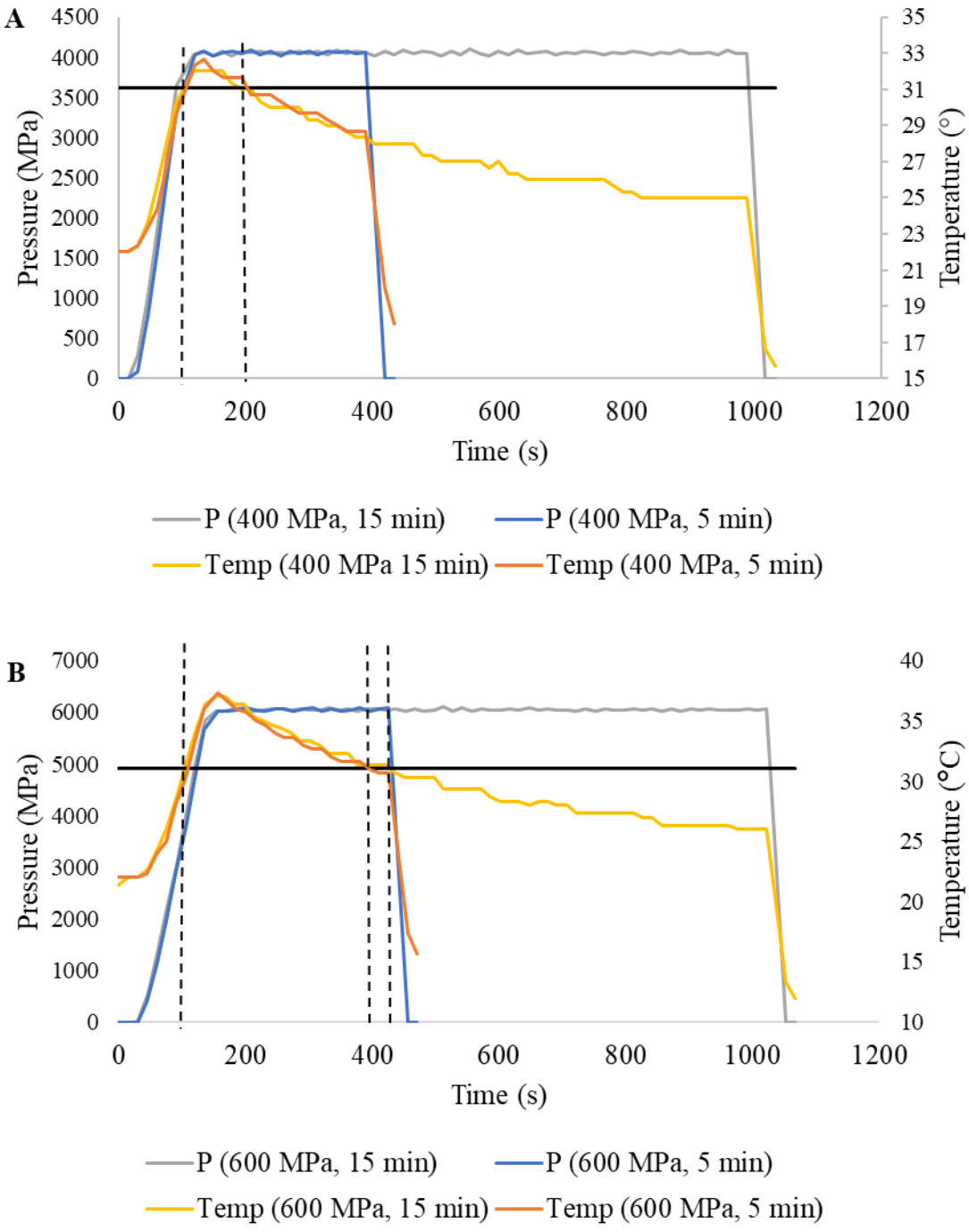

Figure 21: Development of pressure (P) and temperature within the pressure-chamber of the HPP apparatus for treatment at (A) 400 MPa, 5 and 15 min (B) 600 MPa, 5 and 15 min (N=4). The black line indicates the critical temperature, 31.1 °C, for supercritical CO_2, and the dashed lines indicates the time in supercritical state.

Table 2: Temperature in the milk after HPP treatment at either 400 MPa or 600 MPa for a duration of either 5 min or 15 min (mean ± SD, N=2-3).

HPP treatment	Temperature (°C)
400 MPa, 5 min	17.9 ± 0.5
400 MPa, 15 min	15.2 ± 0.3
600 MPa, 5 min	15.3 ± 0.2
600 MPa, 15 min	11.4 ± 0.5

3.1.3 Gas volume and composition

The gas volume and composition were measured as described in Section 2.6.2 and 2.6.3. The gas volume in 500 mL reconstituted milk with CO2 was 266.2 ± 23.5 mL. The CO_2 and O_2 content were 89.8 ± 3.3% and 1.9 ± 0.8%, respectively. There was some variability in the gas volume (8.8%) and gas composition (3.7% for CO_2 and 42.1% for O_2). This could be explained by the fast evaporation of dry ice as it came in contact with the milk at the time of the vacuum packing.

3.2 Fermentation

3.2.1 pH development

The pH of the sample was monitored every hour during the fermentation until the pH reached 4.6, as presented in Figure 22. pH at the start of the fermentation was higher for the heat treated milk (pH 6.5) compared to the samples subjected to the combination technologies (pH 6.2) (Figure 20). It was found that the pH at fermentation start was independent of the processing time (see Section 6.1).

The pH of the heat treated sample decreased continuously throughout the fermentation. For the sample subjected to the combination technology, there was a shoulder phase in the pH reduction during the first hour of fermentation, even with a slight increase in pH. Since no lag phase was apparently observed in the growth curves for both microbial species (Section 3.6), it is unlikely that the starter culture was negatively affected by the higher CO_2 concentration. Another explanation could be the evaporation of CO_2 during the first stage of fermentation as the sealed pouch with the sample was cut open after the processing. The CO_2 evaporation causing a slight pH increase may have concealed the decrease in pH by the starter culture.

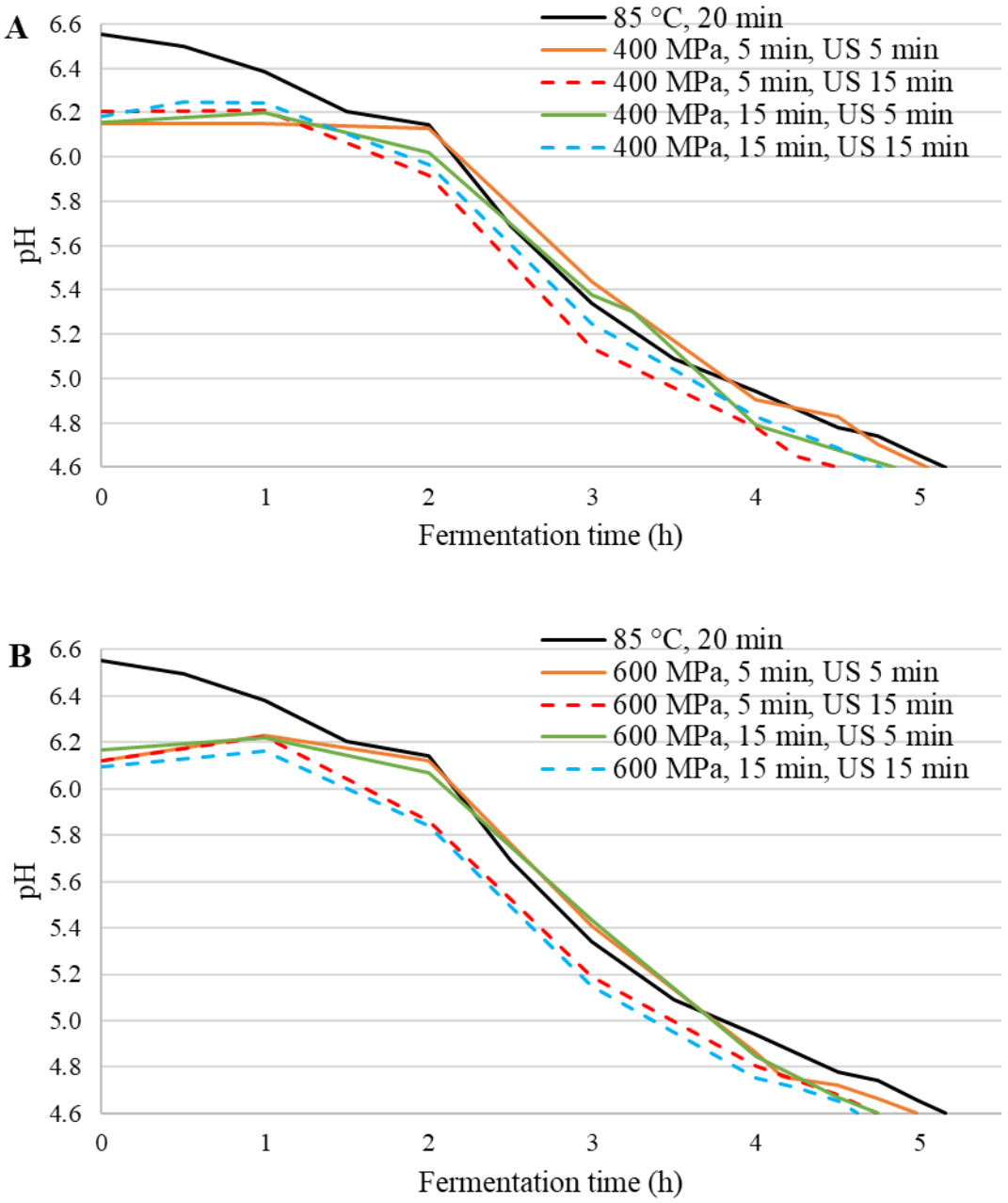

Figure 22: *pH development in the sample subjected to heat treatment (85 °C, 20 min) or combination technology at HPP 400 MPa (A) and 600 MPa (B) for 5 or 15 min, followed by US treatment at 68 kHz, 300 W, for 5 or 15 min. The samples subjected to US treatment for 15 min are shown as a dashed line, while the remaining samples are shown as a continuous line. Mean ± SD, N=2-4.*

3.2.2 Total fermentation time

The total fermentation time needed to reach pH 4.6 was calculated by linear regression based on the results presented in Section 3.2.1 (Table 3). The average fermentation time of all the samples subjected to the non-thermal processing technologies was shorter than that of the heat treated sample, suggesting that the use of CO_2, HPP and/or US promoted the fermentation. The samples treated with US (68 kHz, 300 W) for 15 min showed a faster reduction in pH

compared to the sample treated with US for 5 min, especially in combination with HPP treatment at 600 MPa for 5 or 15 min (Figure 22). The results are consistent with the previous findings. Wu et al. (2000) found that US treatment resulted in reduction of up to 30 min in the fermentation time mostly due to accelerated acidification in the last part of the process, while Vinderola et al. (2000) reported a reduced fermentation time by 57.5 min in fermented milk with the addition of CO_2. Vercet et al. (2002) found the exact opposite, with longer fermentation time due to retardation acidification in the last part of the process, after combined US and pressure treatment (20 kHz, 2 kg/cm^3 pressure, 40 °C, 12 s) compared to control. Ozcan, Horne, and Lucey (2015) found shorter fermentation time (approximately 25 min) in milk with initial pH of 6.2 (pH lowered with 0.5 N HCl) compared to initial pH of 6.7, and suggested that this could due to the lower initial pH reducing the acid-base buffering of the milk due to solubilisation of CCP, and thus making it easier to decrease the pH. A trend is seen towards 15 min US treatment having a faster fermentation time compared to 5 min US treatment, indicating that longer treatment time in the US is having a bigger promoting effect on the growth of the starter culture. US treatment leads to selective permeabilization (sonoporation) of the cell membrane of the starter culture, leading to increased transport of nutrients into and removal of waste products from the bacteria cell, possibly indicating that the sonoporation effect is greater after 15 min without being so large that the cells rupture.

The SD of the total fermentation times ranges between 4 and 25 min. A variability in the fermentation time will be expected, influenced by the concentration of the starter culture, balance and variation between the different strains in the culture, and fermentation temperature ("Tetra Pak Dairy processing handbook," 2015). Variation in fermentation times are also the case in the industry.

Table 3: Total fermentation time (h) required to reach pH 4.60. The fermentation time (h) is presented in ascending order from fastest to slowest (mean ± SD, N=2-4).

Yoghurt combination	**Fermentation time (h)**
400 MPa, 5 min, US 15 min	4.50 ± 0.16
600 MPa, 15 min, US 15 min	4.62 ± 0.07
600 MPa, 5 min, US 15 min	4.74 ± 0.18
600 MPa, 15 min, US 5 min	4.75 ± 0.36
400 MPa, 15 min, US 15 min	4.76 ± 0.20
400 MPa, 15 min, US 5 min	4.85 ± 0.42
600 MPa, 5 min, US 5 min	4.98 ± 0.32

400 MPa, 5 min, US 5 min	5.05 ± 0.11
85 °C, 20 min	5.16 ± 0.17

3.3 Product characterisation

3.3.1 Viscoelastic properties of the yoghurt gel at 42 °C and 4 °C

Milk samples were treated according to the respective processing conditions and their viscoelastic properties were followed during the fermentation at 42 °C and after 15 min of cooling (4 °C). Those subjected to the traditional heat treatment (85 °C, 20 min) and the combination technology with HPP 400 MPa (5 or 15 min, followed by US, 68 kHz, 300W, for 5 or 15 min) were repeated twice, while only one rheological measurement was performed for the remaining samples with HPP 600 MPa (5 or 15 min, followed by US 5 or 15 min), due to time constrains. The final elastic modulus (G') and loss modulus (G'') of the yoghurt gel at 42 °C at the end of fermentation (at pH 4.6) and at 4 °C are presented in Figure 23. G' describes the solid-state behaviour of a sample, while G'' describes the viscoelastic behaviour ("Basics of rheology,"). The SD in the final G' and G'' of the milk gel produced using 400 MPa for 5 or 15 min is large at both temperatures, indicating a large variability in the gelation process in the samples as compared to the traditional heat treatment (Figure 24).

The treatment with HPP 400 MPa for 15 min resulted in a yoghurt gel with the higher G' and G'' than with HPP 400 MPa for 5 min and which were comparable to those of the sample subjected to the heat treatment, regardless of the US time (5 or 15 min). These findings are in agreement with the findings by Harte et al. (2002) (although at different pressures), which found that milk treated at 676 MPa for 30 min gave gels with similar rheological curves as heated milk, while milk treated at 676 MPa for 5 min gave yoghurt with weaker gel structure. Our findings at 600 MPa did not give results in agreement with that of Harte et al. (2002). 600 MPa lead to weaker yoghurt gels for both 5 and 15 min holding time. Other studies agreed with our findings for 400 MPa 5 min and 600 MPa 5 and 15 min, which gave weaker G' profiles and final G' compared to milk with higher pH (which in our case is heat treated milk) (Anema, 2010; Ozcan et al., 2015).

Nguyen and Anema (2017) found that ultrasonification (22.5 kHz, 50 W) gave yoghurt gels with similar final G' as heat treated (80 °C, 30 min) milk (475 Pa), while longer treatment time gave decreasingly lower final G', with lowest G' (175 Pa) after 30 min. Similar trends

were seen in this experiment, with lower final G' for all treatments after US 15 min compared to US 5 min, except for HPP 600 MPa, 15 min.

The G' and G'' of the gel is determined from the number of connections between the constituents of the gel as well as the type of connections (Anema, 2008c). This indicates that the traditional yoghurt has more bonds inside the gel network than yoghurt made from non-thermal processing technologies, with exception of 400 MPa, 15 min. US treatment has a homogenising effect on the fat globules in milk, reducing their size (Wu et al., 2000). It is therefore expected that this will affect the number of bonds and their distribution in the yoghurt gel network formed, along with the effect HPP has on milk proteins. There is need for repetition of the rheological measurements, especially for HPP 600 MPa.

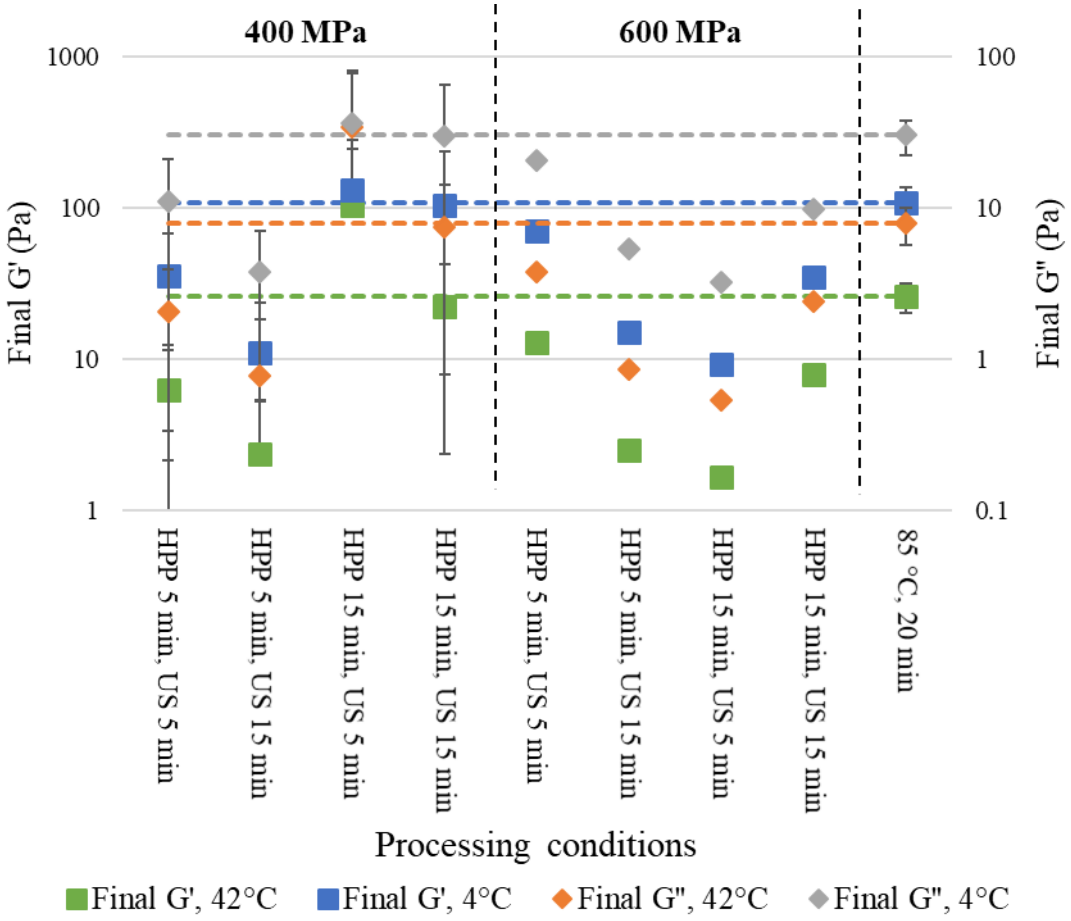

Figure 23: Final G' (■) and G'' (♦) for yoghurt gel formed from milk treated either traditionally or using non-thermal processing technologies at the end of the gelation process (42 °C) and after cooling (4 °C) (mean ± SD, N=1-5). Non-thermal processing conditions are CO_2, HPP (either 400 or 600 MPa for either 5 or 15 min), followed by US (68 kHz, 300 W for either 5 or 15 min). Traditionally is heat treatment at 85 °C for 20 min. The dashed horizontal lines are as comparison against traditional heat treatment.

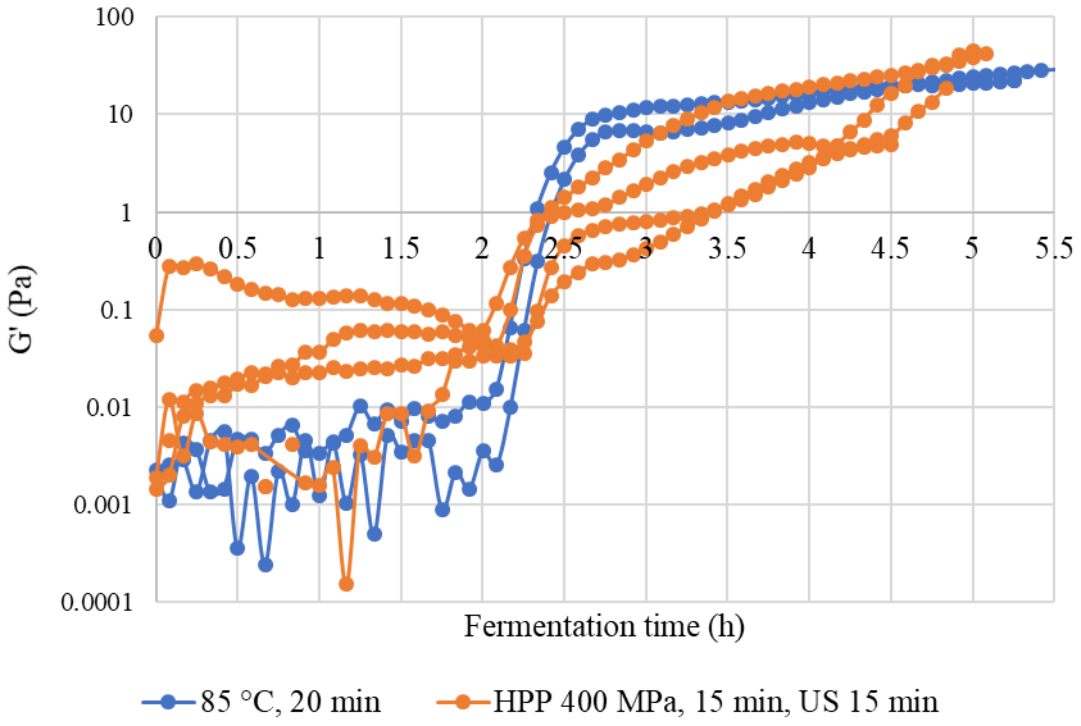

Figure 24: G' (Pa) during the gelation process of yoghurt produced using traditional heat treatment (85 °C, 20 min) and combination technology (400 MPa, 15 min, US 15 min).

3.3.2 Yield strain (%) and yield stress (Pa)

Strain sweep were performed on the final yoghurt gel after 15 min of cooling at 4 °C. Typical strain weep curves are shown in Figure 17. The onset point represented the point at which the gel structure yielded. The corresponding strain (%) and stress (Pa) were determined as the yield strain and stress of the yoghurt gel at 4 °C respectively. Figure 25 displays the yield strain and stress of the samples prepared as described in Section 2.6.6. As in the final G' and G'' values, the SD between the replicates was large, indicating the variability in the gel properties of the samples. Nonetheless, the yoghurt produced from the heat treated milk showed higher yield strain (%) and yield stress (Pa) than the samples subjected to the combination technology. Needs et al. (2000) also found lower yield stress in pressure treated milk (600 MPa, 15 min) compared to heat treated milk (85 °C, 20 min), while Anema (2008c) found the exact opposite. Yield strain depends on the strand curvature, meaning that a higher yield strain is observed for higher strand curvature as the strand first have to be straighten before it is stretched and yield (Anema, 2008c). Low yield strain indicates a brittle gel (Lucey, 2001). Yield stress depends on the number and type of bonds in the gel network, with more and stronger bonds, e.g. covalent bonds instead of non-covalent bonds, giving larger yield stress before yielding (Anema, 2008c). The higher yield stress and strain obtained for the traditional sample suggested that this gel contained more and/or stronger bonds and could resist more stress (rearrangements)

before the gel was yielded, compared to the gel made using the combination technology. 400 MPa for 15 min gave higher yield stress than 5 min, suggesting that 15 min lead to formation of more and stronger bonds. Little effect was seen from US time. There was little variation in the yield strain between the 400 MPa samples, indicating that 400 MPa affected the interconnectivity within the gel network, thus the gel properties, while US time did not. 600 MPa showed different trends but need more repetition.

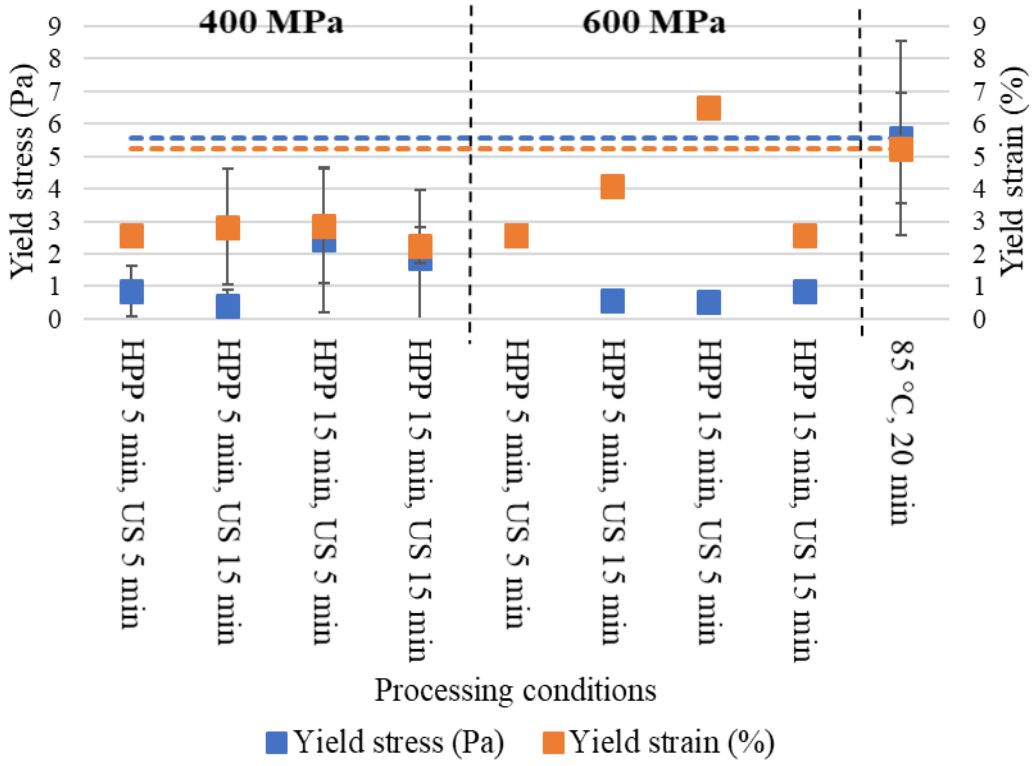

Figure 25: Yield stress (Pa) (orange) and yield strain (%) (blue) calculated based on G' for yoghurt gel formed from milk treated either traditionally or using non-thermal processing technologies (mean ± SD, N=1-5). Non-thermal processing conditions are CO_2, HPP (either 400 or 600 MPa for either 5 or 15 min), followed by US (68 kHz, 300 W for either 5 or 15 min). Traditionally is heat treatment at 85 °C for 20 min. The dashed horizontal lines are as comparison against traditional heat treatment.

3.3.3 Penetration

Breaking force (g) is the force required to break down the gel surface and is shown in Figure 26. There is no significant difference between the breaking point of the yoghurt made using combination technology and traditional heat treatment. Force 15 mm down into the sample gives an indication to the firmness, and area under the curve is the total work of penetration required to penetrate 15 mm down in the sample. Both force at 15 mm (g) and area (g·s) is shown in Figure 27. Area and force 15 mm seem to follow the same trend for all of the nine samples. Most of the yoghurt made using combination technology are not significantly different to yoghurt made using heat treatment, except for 400 MPa, 15 min, US 15 min, 600

MPa, 5 min, US 15 min and 600 MPa, 15 min, US 15 min which have higher values for force 15 mm (g) and area (g*s)., indicating that these three combinations have a firmer gel structure after one day storage at 4 °C. These three samples have 15 min US treatment in common, and US time showed a strong significant effect (p=0.001) on the area and force 15 mm, indicating that 15 min US treatment affects the firmness of the yoghurt gel formed. Our findings agree with Vercet et al. (2002) findings that samples submitted to a combination of heat (40 °C), US (20 kHz) and pressure (0.2 MPa) had a firmer structure and increased consistency and viscosity than heat treated samples. This was possibly due to denaturation of serum proteins by US. There were no significant interactions effects of the variables.

600 MPa, 15 min, US 15 min showed both higher breaking force and higher area and force 15 mm compared to the remaining samples. From these results it may be concluded that this combination has a higher gel firmness after one day storage at 4 °C compared to the others.

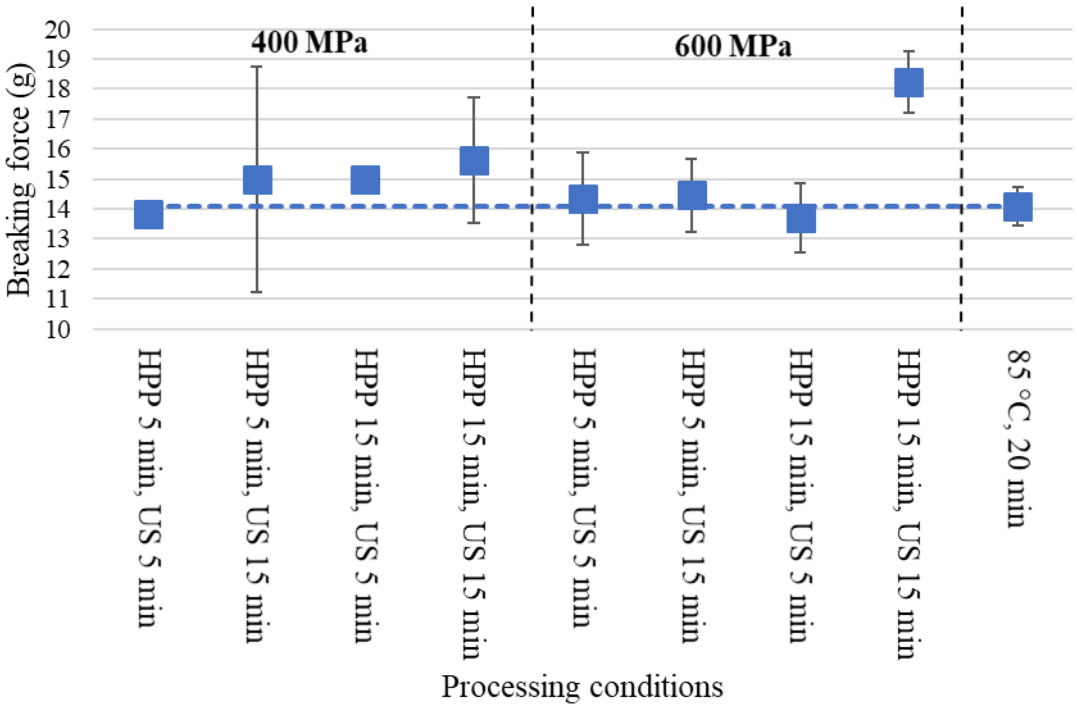

Figure 26: The force needed to break the surface of the gel is plotted for the different processing conditions (mean ± SD, N=3-6). Non-thermal processing conditions are CO_2, HPP (either 400 or 600 MPa for either 5 or 15 min), followed by US (68 kHz, 300 W for either 5 or 15 min). Traditionally is heat treatment at 85 °C for 20 min. The penetration test was performed after one day storage at 4 °C. The dashed horizontal lines are as comparison against traditional heat treatment.

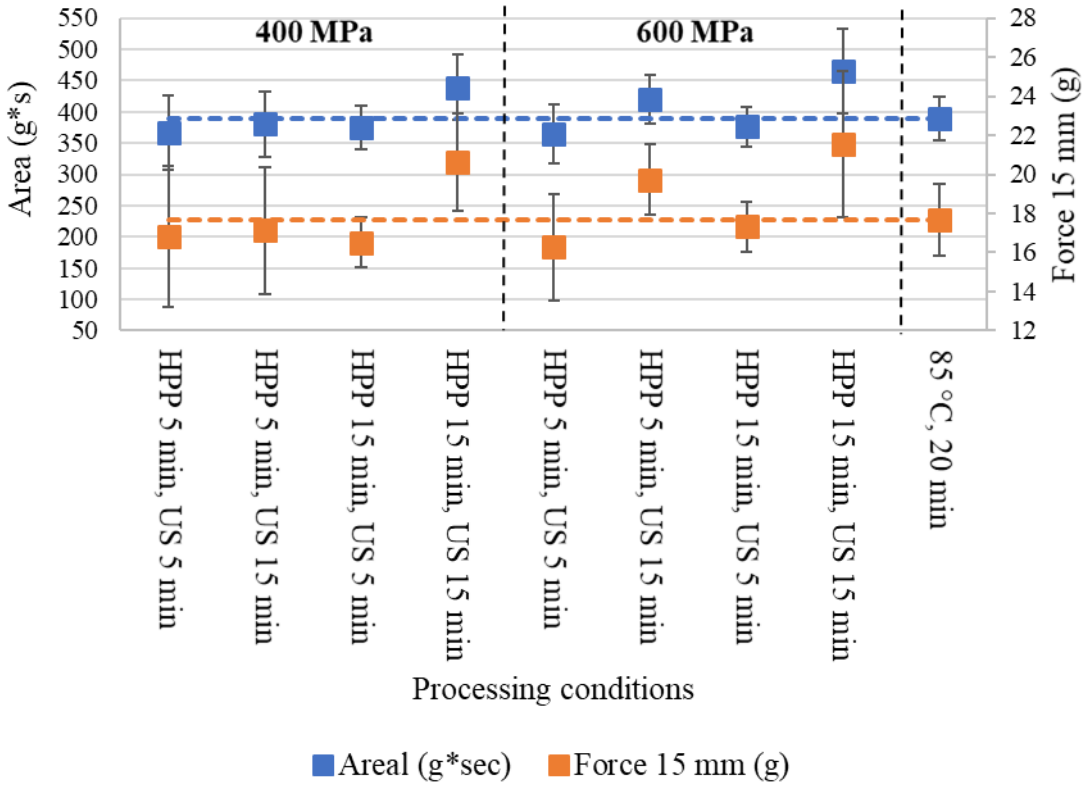

*Figure 27: Area (g*s) under the penetration curve and the force (g) after 15 mm compression plotted for the different processing conditions (mean ± SD, n=3-6). Non-thermal processing conditions are CO_2, HPP (either 400 or 600 MPa for either 5 or 15 min), followed by US (68 kHz, 300 W for either 5 or 15 min). Traditionally is heat treatment at 85 °C for 20 min. The penetration test was performed after one day storage at 4 °C. The dashed horizontal lines are as comparison against traditional heat treatment.*

3.3.4 Stress-relaxation

Maximum force (g) is the force required to break the surface of the gel and gives an indication on the hardness of the gel. Residual force (%) is the% of max force that is left after two mins holding time. Maximum force (g) and residual force (%) are presented in Figure 28. All samples, except for 600 MPa, 15 min, US 15 min, showed a lower maximum force than traditional and similar or higher residual force (%). Vercet et al. (2002) found that yoghurt made form milk subjected to heat (40 °C), pressure (0.2 MPa) and ultrasound (20 kHz) had slightly lower (18.2%) residual force than yoghurt made from traditionally heated milk (19.4%), which does not correspond to our findings. 600 MPa, 15 min, US 15 min on the other hand has higher maximum force (g) and lower residual force (%). This suggests that this gel structure is harder, but more brittle, since less % of the maximum force was left after 2 min. These findings correspond to the results from the penetration test, where HPP 600 MPa, 15 min, US 15 min also had the highest breaking force (g).

Statistical analysis found a significant interaction effect (p=0.01) between US and HPP time for residual force (%). Further post hoc analysis revealed that HPP 15 min, US 5 min had a significant higher residual force (p=0.012) than HPP 15 min, US 15 min. This suggest that when the sample was HPP-treated for 15 min, US 5 min produced a more brittle yoghurt gel than US treatment for 15 min. An interaction effect (p=0.034) was found between HPP pressure, HPP time and US time for maximum force (g). Post hoc analysis revealed that the maximum force of the yoghurt gel was significantly higher (indicating a harder gel) when the sample was subjected to the HPP treatment for 15 min than for 5 min at 600 MPa (p=0.023). The US treatment for 15 min resulted in a significantly higher maximum force of the yoghurt gel than for 5 min (p=0.012), and this significant effect of US time was not found when the HPP 600 MPa was limited to 5 min or the HPP pressure was at 400 MPa. The treatment with 600 MPa, 15 min, US 15 min resulted in a significantly higher maximum force of the gel than 400 MPa, 15 min, US 15 min (p=0.007). These finding indicate that 600 MPa, 15 min, US 15 min produced a harder gel than HPP at 400 MPa, for 5 min followed by US treatment for 5 min.

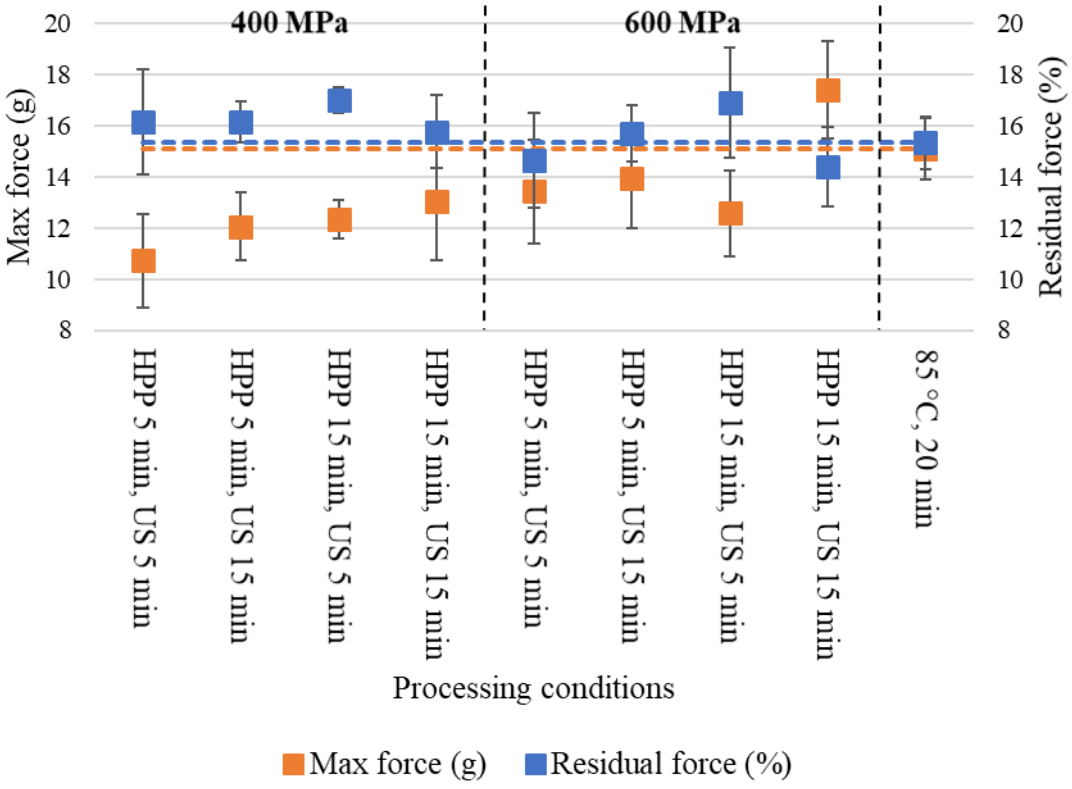

Figure 28: The force needed to break the gel surface (max force (g)) and the force that did not recuperate after two min (residual force (%)) are plotted for the different processing conditions (mean ± SD, N=3-7). Non-thermal processing conditions are CO_2, HPP (either 400 or 600 MPa for either 5 or 15 min), followed by US (68 kHz, 300 W for either 5 or 15 min). Traditionally is heat treatment at 85 °C for 20 min. Relaxation tests were performed at yoghurt gels stored at 4 °C after one day storage. The dashed horizontal lines are as comparison against traditional heat treatment.

3.4 Selection of processing conditions

For further characterisation (microbial analysis during fermentation, native- and SDS-PAGE) and for the storage trial up to 42 days, the treatment 400 MPa, 15 min, US 15 min was selected among the eight combinations of CO2, HPP and US (Table 1) based on the total fermentation time (Section 3.2.2), the rheological properties (Section 3.3.1 and 3.3.2) and texture analysis (Section 3.3.3 and 3.3.4). Based on the results on the total fermentation time, US treatment for 15 min showed a larger stimulatory effect on the starter culture than the US treatment for 5 min. 400 MPa, 15 min, US 15 min was chosen as it has similar results to heat treated milk in the textural analysis. In addition, the sample subjected to the respective treatment showed the Final G' and G'' at 42 °C and 4 °C comparable to those of the heat treated sample.

3.5 Native- and SDS-PAGE

The milk subjected to Native- and SDS-PAGE analyses were (1) untreated reconstituted milk, (2) milk heat-treated at 85 °C for 20 min, (3) milk treated with HPP 400 MPa for 15 mins, (4) milk treated with HPP 400 MPa for 15 min and US for 15 mins, (5) milk with CO_2 treated with HPP 400 MPa for 15 mins, and (6) milk with CO_2 treated with 400 MPa for 15 mins and US for 15 mins. Soluble caseins and whey proteins (serum phase proteins) were defined as those that did not sediment from the milk during the ultracentrifugation. The relative content of the serum phase proteins was determined by comparing the band intensities of the treated reconstituted milk sample against those of the untreated samples on native- and SDS-PAGE gels. See Figure 29 and Figure 30 for an example of native- and SDS-PAGE gels, respectively.

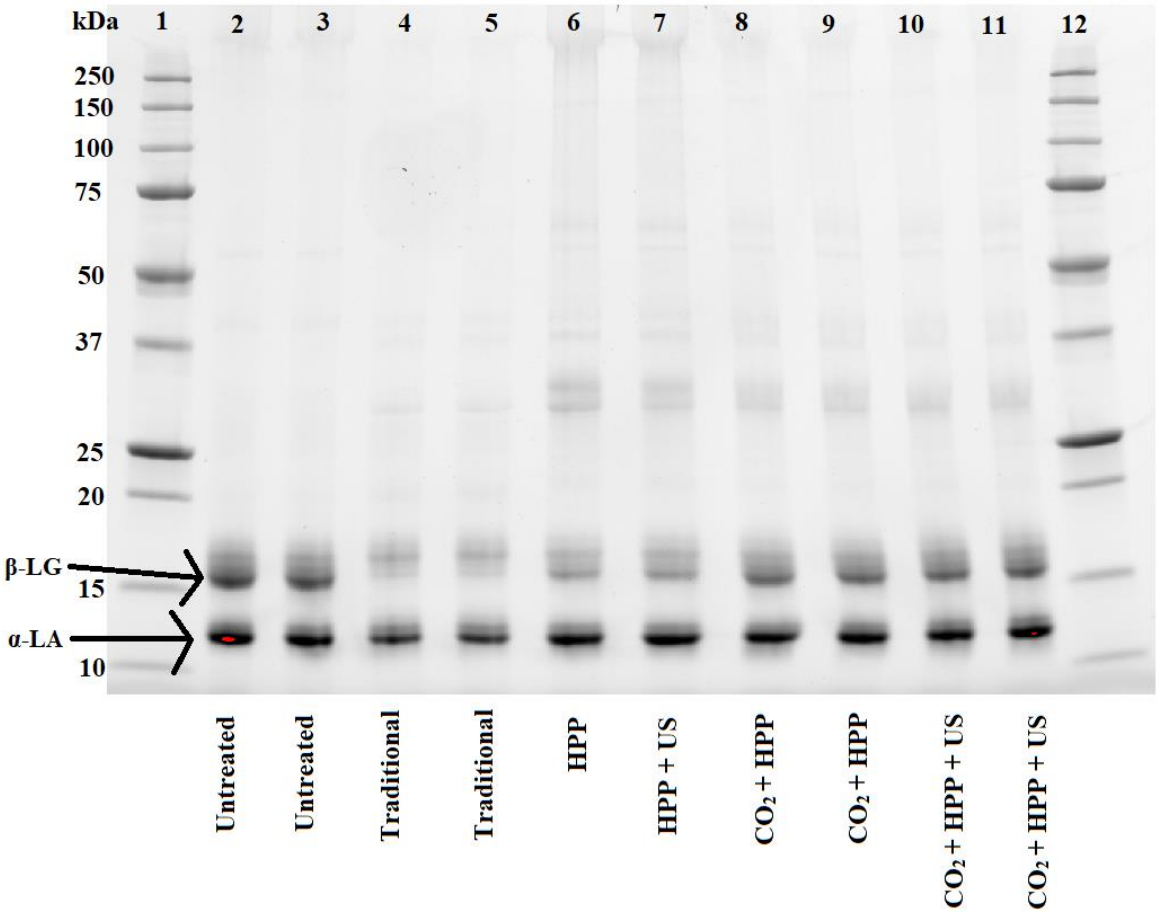

Figure 29: Native-PAGE of milk either untreated or subjected to different treatments; traditional (85 °C, 20 min), HPP (400 MPa, 15 min), HPP + US (400 MPa, 15 min and 68 kHz, 300 W, 15 min), CO_2 + HPP (1 g dry ice and 400 MPa, 15 min) and CO_2 + HPP + US (1 g dry ice, 400 MPa, 15 min and 68 kHz, 300 W, 15 min). The milk samples were centrifuged at 100 000 g for 1 h at 20 °C, the supernatant with a final concentration of 1.5 mg/mL was run on native-PAGE. Column 1 and 12 contains Precision Plus Protein Unstained Protein Standard. The bands of α-LA (14.2 kDa) and β-LG (18.3 kDa) are marked with arrows.

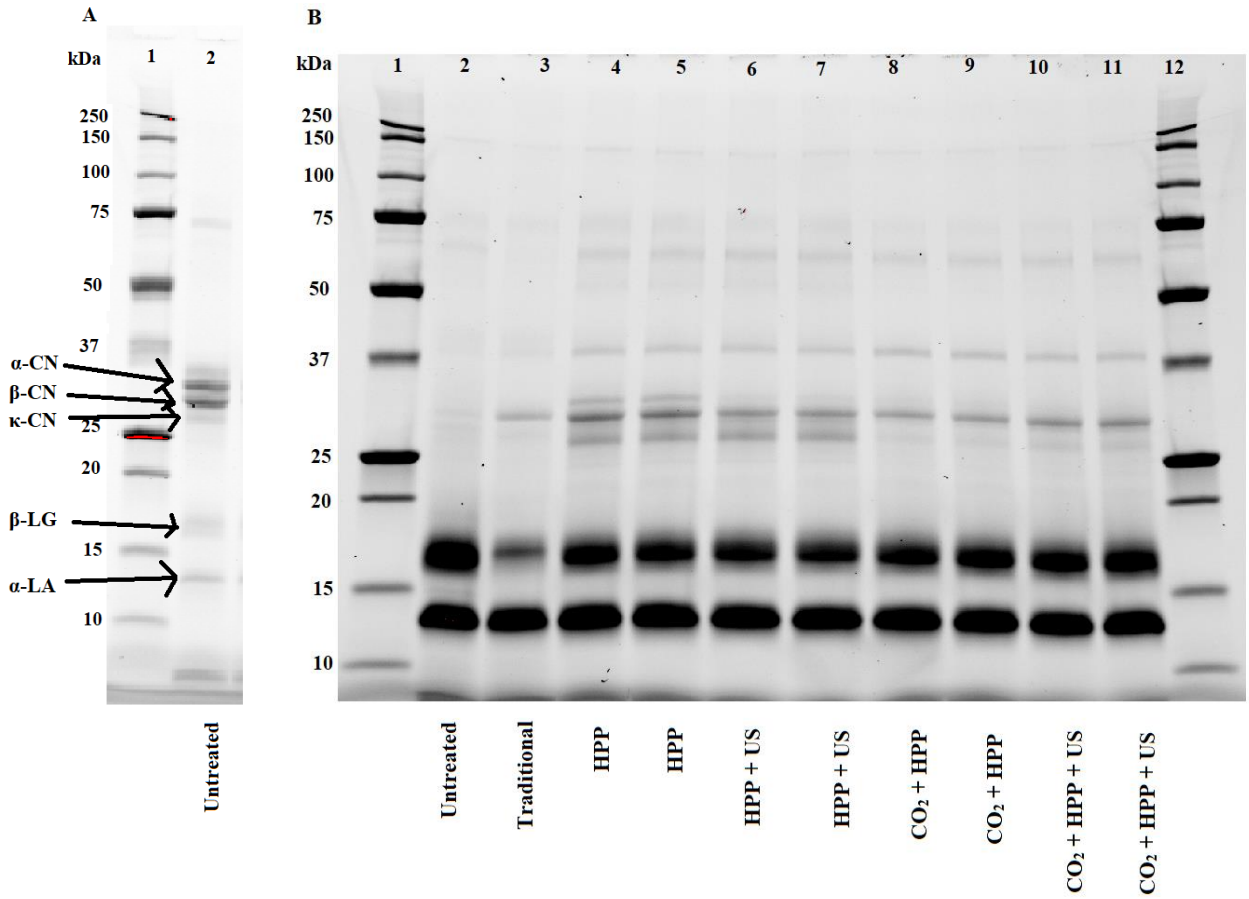

Figure 30: SDS-PAGE of (A) untreated milk with a final concentration of 1.5 mg/mL loaded on the gel. (B) either untreated milk or milk subjected to different treatments; traditional (85 °C, 20 min), HPP (400 MPa, 15 min), HPP + US (400 MPa, 15 min and 68 kHz, 300 W, 15 min), CO_2 + HPP (1 g dry ice and 400 MPa, 15 min) and CO_2 + HPP + US (1 g dry ice, 400 MPa, 15 min and 68 kHz, 300 W, 15 min). The milk samples were centrifuged at 100 000 g for 1 h at 20 °C, the supernatant with a final concentration of 1.5 mg/mL was run on the gel. Column 1 and 12 contains Precision Plus Protein Unstained Protein Standard. The bands of α-LA, β-LG, α-CN, β-CN and κ-CN are marked with arrows.

The effect of heat and different processing conditions of CO_2, HPP and/or US on the level of the serum phase whey fractions is presented in Figure 31 A and B. The whey proteins remaining serum phase of the milk in native-PAGE after ultracentrifugation is a mixture of native whey proteins, denatured whey proteins which has not associated with casein micelles and aggregates. Low levels of β-LG were observed in the serum phase of heat treated milk (approximately 10 and 30% in native- and SDS-PAGE, respectively), compared to pressure treated milks. Pressure treated milk without CO_2 had approximately 30 and 65% in native- and SDS-PAGE, respectively, thus indicating that a greater proportion of β-LG denatured during heat treatment and associated with casein proteins (in casein micelles), therefor being sedimented during ultracentrifugation. Pressure treated milk with CO_2, thus having a lower pH, had even higher levels of β-LG, approximately 70 and 80% for native- and SDS-PAGE,

respectively. These results indicate that more β-LG is denatured at milk native pH (6.6), than at lower pH. US treatment had little effect on the β-LG denaturation. The same trends were seen for the percentage of β-LG in the serum phase after different treatments for both native- and SDS-PAGE, but at different levels. This could be due to SDS-PAGE being run under reducing conditions, thus dissociating β-LG dimers into monomers and protein aggregates, giving higher levels of β-LG compared to native-PAGE where whey proteins remain in their native state.

α-LA levels in serum phase of the milk were higher than β-LG, with approximately 75% compared to untreated milk after heat treatment and approximately 100% after pressure treated. US and lowering of pH by CO_2 had little effect on the level of α-LA. Anema (2008c) observed more denaturation of α-LA and β-LG during heat treatment (80 °C, 30 min), 70 and 80% respectively, than during pressure treatment (400 MPa, 30 min), 0 and 70%, respectively. This is in agreement with our findings, where we observed a higher level of both α-LA and β-LG in the serum phase after pressure treatment compared to heat treatment. Anema (2008a) found 40 and 50% non-sedimentable whey proteins in heat treated milk (80 °C, 30 min) at pH 6.2 and 6.6, respectively, with the level increasing markedly with increasing pH, which is the exact opposite of our findings, with decreasing level of non-sedimentable whey proteins with increasing pH.

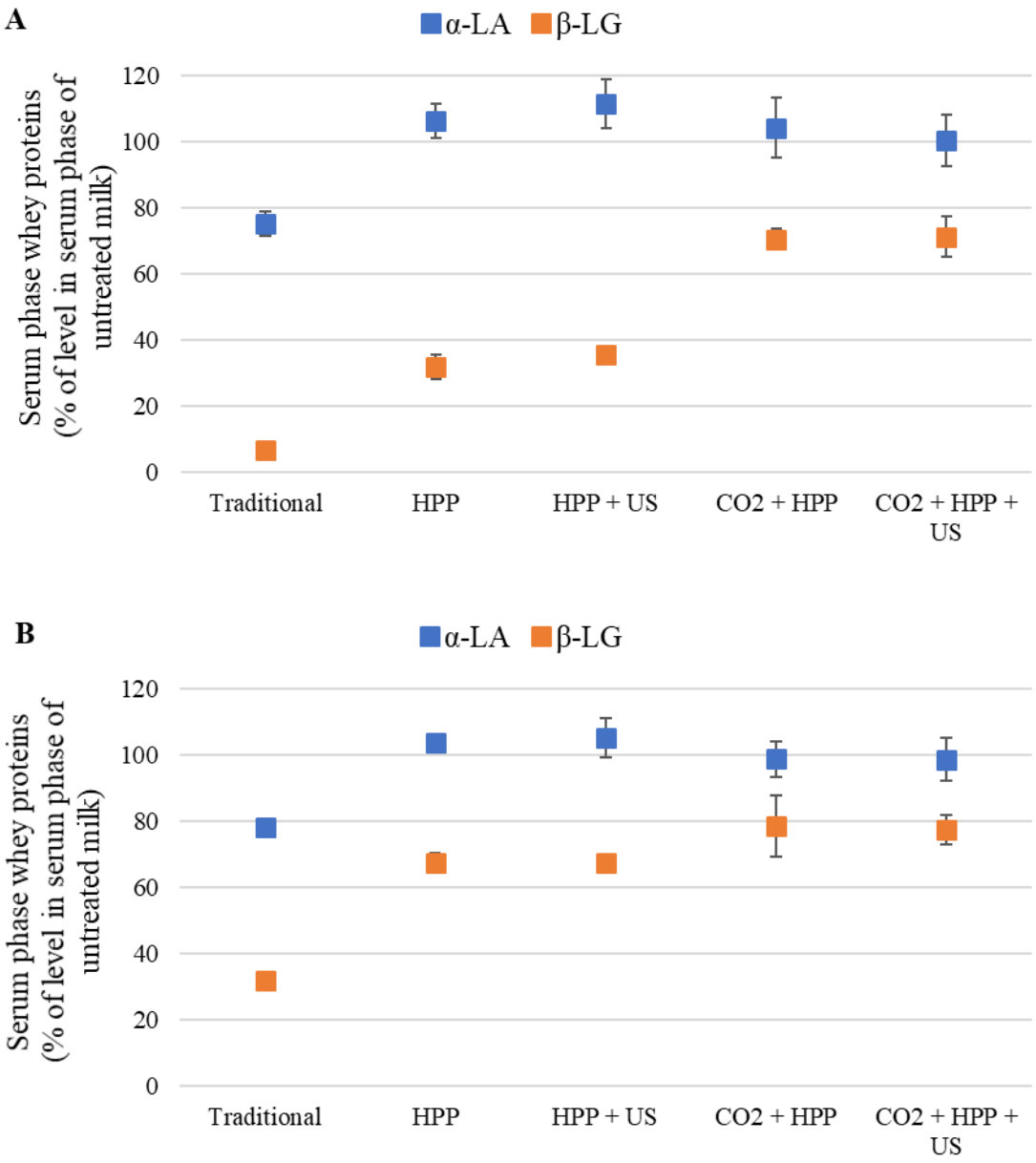

Figure 31: Effects of different processing conditions on the level of the serum phase whey proteins (α-LA and β-LG) when compared to the untreated milk on (A) native-PAGE gel and (B) SDS-PAGE: traditional heat treatment (85 °C, 20 min), HPP (400 MPa, 15 min), HPP (400 MPa, 15 min) + US (68 kHz, 300 W, 15 mins), CO_2 + HPP (400 MPa, 15 min) and CO_2 + HPP (400 MPa, 15 min) + US (68 kHz, 300 W, 15 min) (mean ± SD, N=2, a=2).

The effect of heat and different processing conditions of CO_2, HPP and/or US on the level of the serum phase casein fractions is presented in Figure 32. If the treatment of the milk leads to dissociation of casein from the casein micelle, these proteins will not sediment with the casein micelle during ultracentrifugation but be found in the serum phase. α-casein were only observed in the serum phase after HPP treatment (with and without CO_2), indicating that HPP led to a greater dissociation of the casein micelle than the heat treatment, but that the casein micelle reassociated after US treatment. β-casein was found in the serum phase for all the

samples at approximately 20% of the untreated milk, except for the sample after HPP treatment where a higher proportion, although not statistically significant, of the β-casein (40%) was observed when compared to the heat treatment, suggesting that HPP led to greater dissociation of β-casein from the casein micelle. However, when HPP was applied at lower pH (pH 6.2, with the addition of CO_2), β-casein was found to be 20% of the untreated milk, indicating that the dissociation of β-casein from the casein micelle is pH dependent. A higher proportion of the κ-casein dissociated from the micelle than the remaining casein fractions after heat treatment (50 %) and HPP (more than 70%) regardless of US treatment. However, this was not the case for the sample subjected to the HPP treatment (with or without US treatment) at lower pH, which showed a lower amount (20%) of k-casein in the serum phase than when HPP was applied without CO_2. Anema (2010) found that the amount of κ-casein in the serum phase increased with pH, from 35% at pH 6.4 to 60% at pH 6.8, supporting our findings. A decrease in β-casein after US treatment for HPP treated milk without CO_2 (from 40 to 20%) was observed. This could possibly due to acoustic cavitation caused by US leading to increased interaction between the β-casein molecules and reassociation of β-casein with the casein micelle (Shanmugam, Chandrapala, & Ashokkumar, 2012).

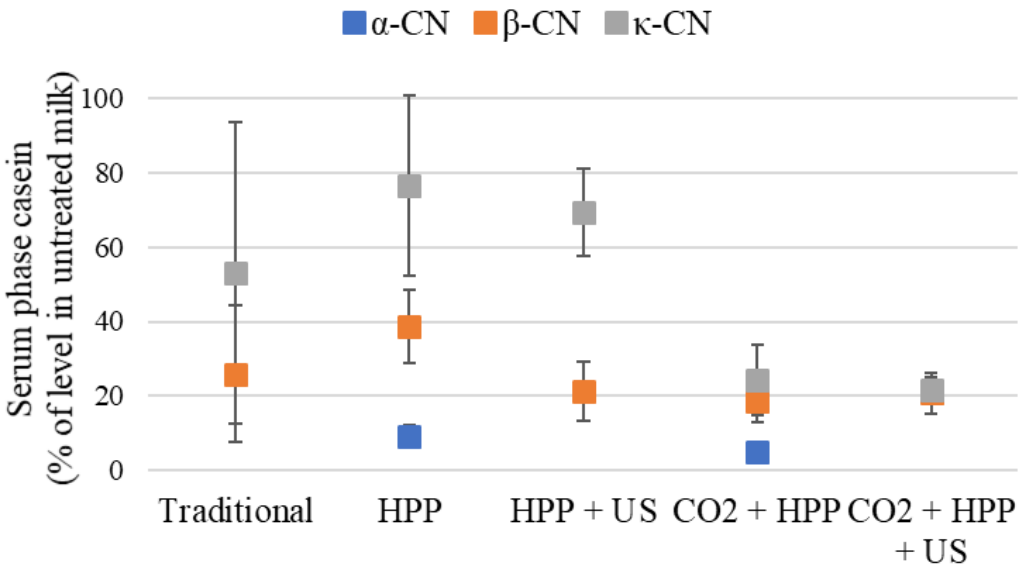

Figure 32: Effects of different processing conditions on the level of the serum phase casein (CN) (α-CN, β-CN and κ-CN) when compared to the untreated milk on SDS-PAGE*: traditional heat treatment (85 °C, 20 min), HPP (400 MPa, 15 min), HPP (400 MPa, 15 min) + US (68 kHz, 300 W, 15 mins), CO_2 + HPP (400 MPa, 15 min) and CO_2 + HPP (400 MPa, 15 min) + US (68 kHz, 300 W, 15 min) (mean ± SD, N=2, a=2).*

Combining the amount of whey proteins and casein in the serum phase after HPP treatment with CO_2, it could seem that at lower milk pH (6.2) there is less dissociation of the

casein micelle (especially less dissociation of κ-casein), thus leaving more β-LG in the serum phase since there is less k-casein available to interact with.

Denatured β-LG binds to κ-casein through disulphide bonds. These complexes can exist in the colloidal phase (casein micelle) or in the serum phase of milk. In the serum phase of milk, they are believed to form strand which are able to interconnect between casein micelles. Higher level of denatured β-LG/κ-casein complexes in the serum phase thus yoghurt gels with increased G' and higher yield stress. The pH affects the distribution of these complexes between the colloidal phase and the serum phase, with the level in the serum phase increasing with increasing pH upon heating (Anema, 2008a).

3.6 Microbial growth dynamics during fermentation

The growth curves of ST and LB during the fermentation of both types of yoghurt, i.e. from heat treated (85 °C, 20 min) milk and non-thermally treated milk (400 MPa, 15 min, US 15 min, 68 kHz, 300 W), are shown in Figure 33. During yoghurt fermentation, longer lag phase for LB as compared to ST, and double exponential phase for ST have typically been reported (Sieuwerts, 2016). However, absence of lag phase for both microorganisms and only one exponential phase for ST have been observed in the present work for both types of yoghurt, which could be attributed to the particular type of starter culture (direct vat set) and inoculum sizes (ratio) of both microorganisms.

No noticeable difference in the growth dynamics (i.e. slope of the exponential phase or maximum specific growth rate (μ_{max}), and maximum concentration at the stationary phase of growth (N_{max})) was observed for ST, independently of the yoghurt type. However, the growth curve of LB in heat treated milk presented a slightly less pronounced slope at least during the first 2 h of the exponential phase (μ_{max}), as compared to non-thermally treated milk. However, the maximum concentration of LB at the stationary phase of growth was similar for both types of yoghurt. This behaviour could be attributed to a positive effect of US treatment on the growth of LB (Abesinghe et al., 2019). On the other hand, the initial concentration of both LB and ST was the same for both types of milk, which confirms that the applied US conditions after the inoculation of the milk with the starter culture, did not cause cell viability loss, as compared to the non US treated milk (Abesinghe et al., 2019).

As above mentioned, the growth curves of both microorganisms in both heat treated and non-thermally treated milk did not present an evident lag phase, so that the presence of CO_2 in the milk seemed not to affect negatively the bacterial growth, given that ST is a facultative

anaerobe and LB, an aerotolerant anaerobe (Baglio, 2014) . Despite the difference in the initial concentration of ST and LB, the growth curves of both microorganisms in the non-thermally treated milk followed a similar pattern with regards to the slope of the exponential phase (μ_{max}). Undugoda and Nilmini (2019) also found a similar growth pattern for yoghurt fermented with a LB:ST ratio of 1:2. Although the total bacterial concentration in the yoghurt after fermentation was much above that the limits set by the Codex Standard 243-2033 (10^7 CFU/g), the individual concentrations of ST (10^9 CFU/mL) and LB (10^6 CFU/mL) were lower than the values reported in literature (Mani-López, Palou, & López-Malo, 2014) specially for LB, which again could be attributed to the type of starter culture and inoculum size/ratio.

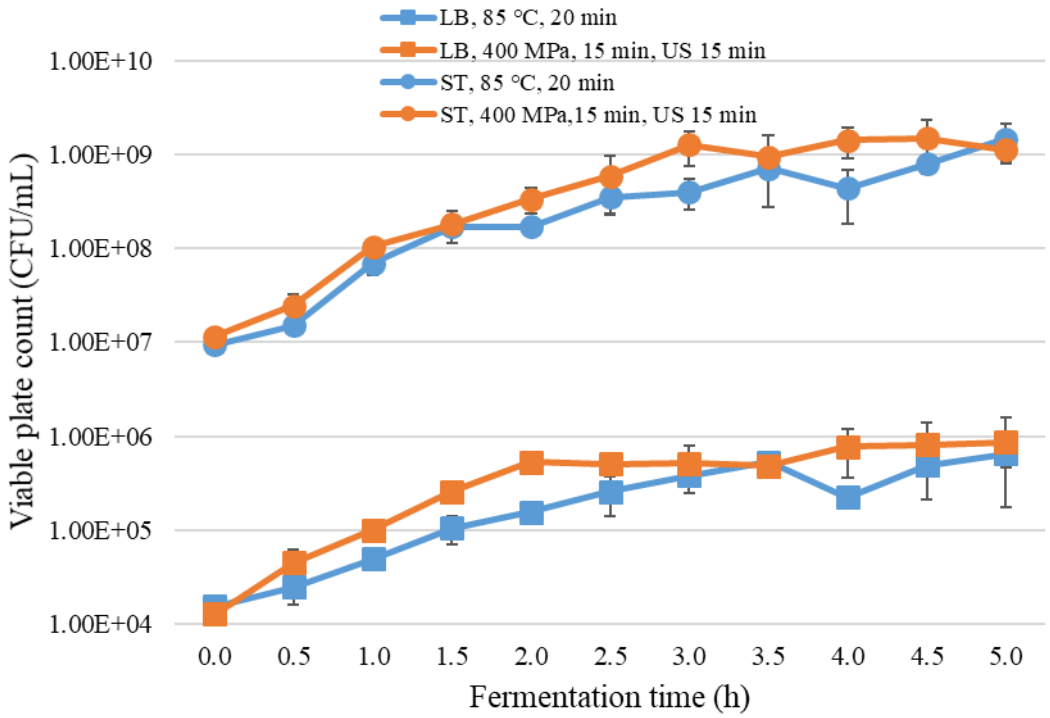

Figure 33: Microbial growth dynamics during the fermentation process. Streptococcus thermophilus (ST) (●) and Lactobacillus delbrueckii subsp. bulgaricus (LB) (■) (mean ± SD, N=2-3, a=2). Yoghurt made from traditionally heat treated (85 °C, 20 min) milk is represented in blue colour, yoghurt made from non-thermally treated (400 MPa, 15 min, US 15 min) milk is represented in orange colour.

3.7 Storage trial

Codex Standard 243-2003, an internationally recognised standard for fermented milk products, require that titratable acidity of yoghurt, expressed as% lactic acid is minimum 0.6%. The standard also states that the sum of starter culture microorganisms in the yoghurt needs to be minimum 10^7 CFU/g in total, and that this has "to be verified through analytical testing of the product through to "the date of minimum durability" after the product has been stored under the storage conditions specified in the labelling" ("Codex standard for fermented milks ", 2003).

Thus, the storage trial was performed over a period of 42 days at 4 °C on the yoghurt produced from the heat treated milk (85 °C, 20 min) and the milk subjected to the selected condition of the combination technology (400 MPa, 15 min, US 15 min). Viable plate counts for ST and LB, titratable acidity and whey separation of the corresponding yoghurt gels were studied to see that it fullfilled Codex Standard 243-2003 and to compare the storage stability of the yoghurt produced with thetraditional heat treatment and combination technology.

3.7.1 Microbial stability during storage

The concentration of ST and LB was monitored during 42 days of storage at 4 °C, as presented in Figure 34. Both ST and LB levels remained stable throughout the full storage period, as also reporter in other studies (Bozova, Kök Taş, & Guzel-Seydim, 2018), with concentrations of about 10^9 CFU/mL and 10^6 CFU/mL, respectively, for yoghurt made using both traditional heating and non-thermal processing. Thus, the total concentration of LAB in the yoghurt was well above the minimum requirement of 10^7 CFU/g set by the Codex Standard 243-2003.

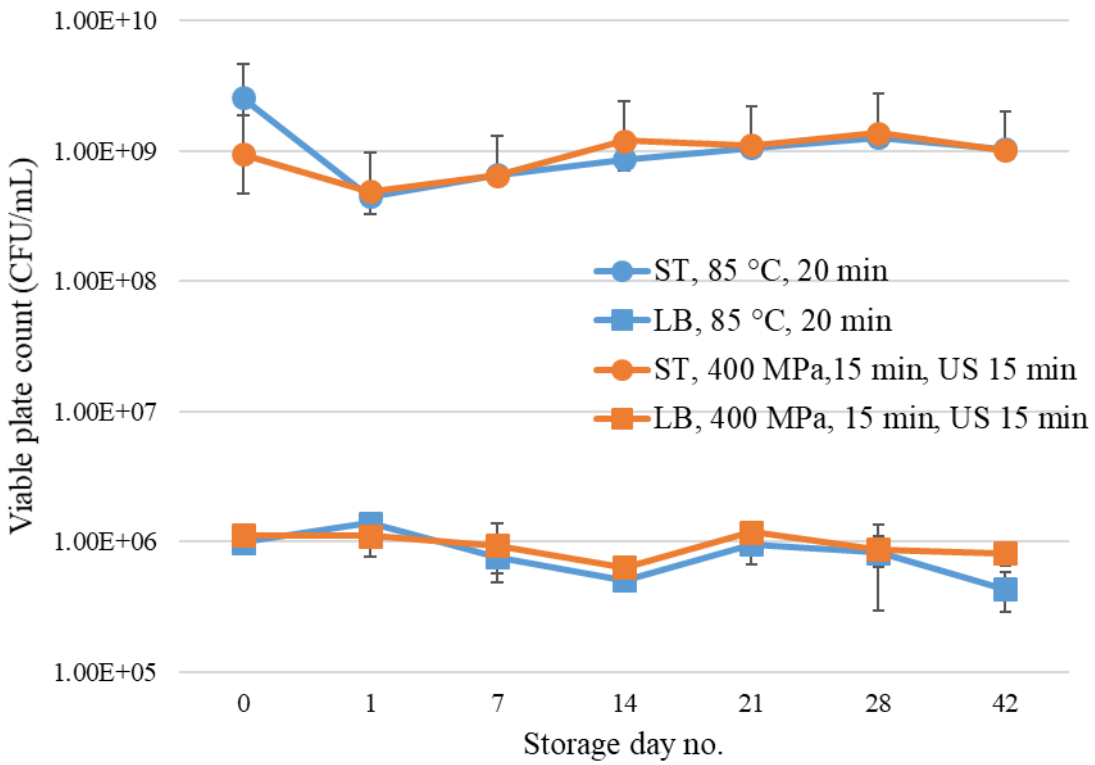

Figure 34: Viable plate counts of ST and LB during storage. Streptococcus thermophilus (ST)(●) and Lactobacillus delbrueckii subsp. bulgaricus (LB) (■) (mean ± SD, N=3). Yoghurt made from traditionally heat treated (85 °C, 20 min) milk is represented in blue colour, yoghurt made from non-thermally treated (400 MPa, 15 min, US 15 min) milk is represented in orange colour.

3.7.2 Titratable acidity and pH

Lactic acid (%) in the yoghurt was determined using the titratable acidity method described in section 2.6.8 on the assumption that lactic acid is the predominant acid in yoghurt. The lactic acid (%) over 42 days of storage is presented in Figure 35. There were not significant differences between the lactic acid concentration of both types of yoghurt at any day of the storage days. The lactic acid concentration was also above the Codex Standard 243-2003 requirement of at least 0.6% lactic acid. At day 1 of storage the concentration was well above 0.7% for both types of yoghurt, and the concentration increased during storage, ending at above 0.9% at storage day 42. Our findings are consistent with findings by others with an initial lactic acid concentration of approximately 0.7% and increasing lactic acid concentration during storage due to post acidification (Ghasempour et al., 2019; Mani-López et al., 2014). The pH values over 42 days of storage are presented in Figure 36. A significant decrease in pH was observed from day 1 to day 7 of storage ($p<0.0005$) and from day 7 to day 42 ($p<0.0005$) for both yoghurt types. Other studies also observed a decrease in pH during storage due to postacidification. Tomovska, Gjorgievski, and Makarijoski (2016) found that the pH decreased from 4.4 to 4.2 from day 1 to 15.

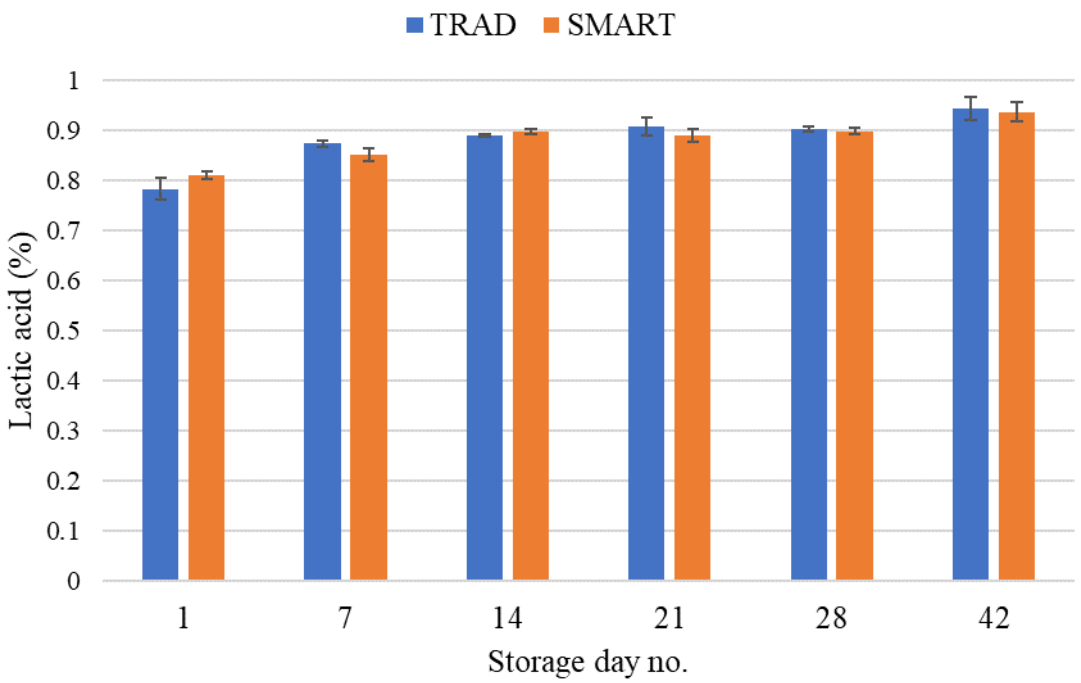

Figure 35: Lactic acid (%) during storage. Measurements of lactic acid concentration was performed weekly over a period of 42 days, with the data from day 35 missing due to lab closure as a consequence of covid-19 (mean ± SD, N=2-3). TRAD is the yoghurt made from traditionally treated (85 °C, 20 min) milk, SMART is the yoghurt made from non-thermally treated (400 MPa, 15 min, US 15 min) milk.

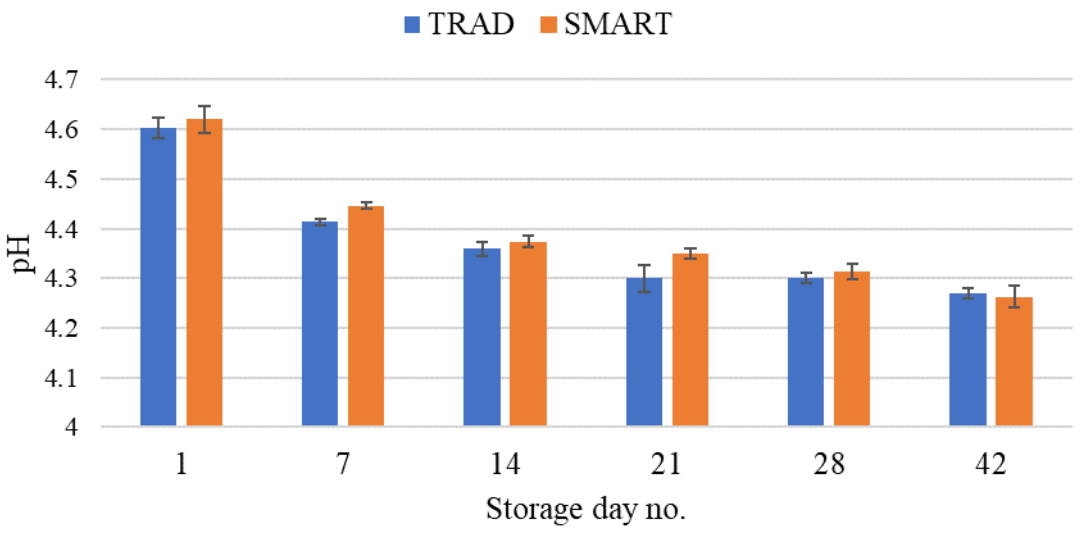

Figure 36: pH during storage. pH measurements were performed weekly over a period of 42 days, with the data from day 35 missing due to lab closure as a consequence of covid-19 (mean ± SD, n=3). TRAD is the yoghurt made from traditionally treated (85 °C, 20 min) milk, SMART is the yoghurt made from non-thermally treated (400 MPa, 15 min, US 15 min) milk.

3.7.3 Whey separation

Whey separation (%) was determined as described in section 2.6.9, at day 1, 7 and 42 of storage. The results are presented in Figure 37. The whey separation of traditionally produced yoghurt increased from 2.2 ± 0.6% to 14.8 ± 1.8% during, while the whey separation of yoghurt produced using non-thermal technologies decreased from 5.8 ± 0.4% to 2.2 ± 0.7% during storage. Lee and Lucey (2003) found that milk heated for 30 min at 82.5 °C had a whey separation of 1.07 ± 0.07% if incubated at 40 °C and 2.85 ± 0.24% if incubated at 45.7 °C until pH 4.6 was reached. Our samples were incubated at 42 °C, and for yoghurt made from heat treated milk the whey separation at day 1 agrees with their findings. Wu et al. (2000) found that ultrasound treatment lead to better WHC due to the reduced size of fat globules, and thus increased available area for interactions with casein of the fat globule membrane. Riener et al. (2009) also explains an observed 2-fold increase in WHC in yoghurt (incubated at 40 °C) made from US treated milk (24 kHz, 400 W, 45 °C, 10 min) compared to yoghurt made from heat treated milk (90 °C, 10 min), due to protein-protein and protein-lipid interactions enhancing the water binding within the three-dimensional network of the gel and thus increasing the WHC. This could possibly be the reasons why the whey separation of yoghurt produced from non-thermal technologies decrease during storage.

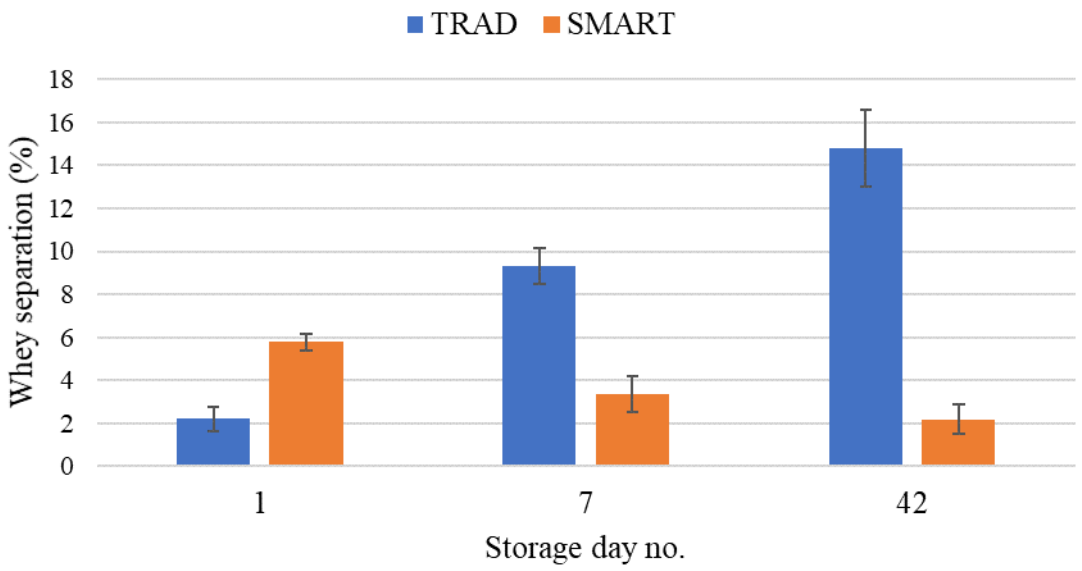

Figure 37: Whey separation during storage. Measurements were done at day 1, 7 and 42, (mean ± SD; N=2-3). TRAD is the yoghurt made from traditionally treated (85 °C, 20 min) milk, SMART is the yoghurt made from non-thermally treated (400 MPa, 15 min, US 15 min) milk.

4 Conclusions and future work

In conclusion, the use of the combination technology 400 MPa, 15 min, US 15 min for yoghurt production showed great potential as eco-friendly alternative to conventional heat treatment for yoghurt manufacture. 400 MPa, 15 min, US 15 min was selected as the optimal combination based on total fermentation time and viscoelastic properties of the yoghurt gel. It has shorter total fermentation time, similar microbial dynamic of the starter culture during fermentation, and similar storage stability as yoghurt made from heat treated milk. The gelation process is more unstable, less whey protein is denatured, and it gives lower yield strain and stress compared to heat treatment, indicating that the gel is more brittle with weaker interconnectivity within the gel network. Therefore, further experiments have to be conducted to optimise the combination technology further, but with shorter fermentation time and being eco-friendly it has great potential.

Possible future work includes colour and particle size distribution measurement of the milk sample treated with the combination technology in comparison to the heat treated milk. Particle size distribution could give insight into the homogenisation effect of US treatment and how HPP and US treatment in combination affects the casein micelle. It would be interesting to perform a sensory evaluation on the yoghurt gel to see how the combination technology affects the organoleptic properties compared to the heat treatment. Different HPP pressures, HPP time and US times could also be of interest to investigate, to see if there are other parameters to be optimised than the ones studied in this master thesis, e.g. in order to improve the yield stress and strain which were lower in the yoghurt gels subjected to the combination technology than the heat treatment. Investigating the use of combination technology on raw milk would also be interesting, to investigate the potential of the non-thermal combination technology towards microbial and enzymatic inactivation to improve the food safety. Further, it is relevant to assess the homogenising effect of HPP and US in combination and how the different fat content will affect the viscoelastic properties of the resulting yoghurt gel.

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6 Appendix

6.1 pH vs process time

The pH at the start of fermentation was plotted against the processing time used with non-thermal processing technologies (Figure 38). Processing time was the time from the milk treatment started until the milk samples was placed in the incubator. Milk processing using non-thermal processing technologies had different length due to different holding time in HPP and US (5 or 15 min). This plot shows that the pH at the start of the fermentation is not influenced by the length of the milk processing.

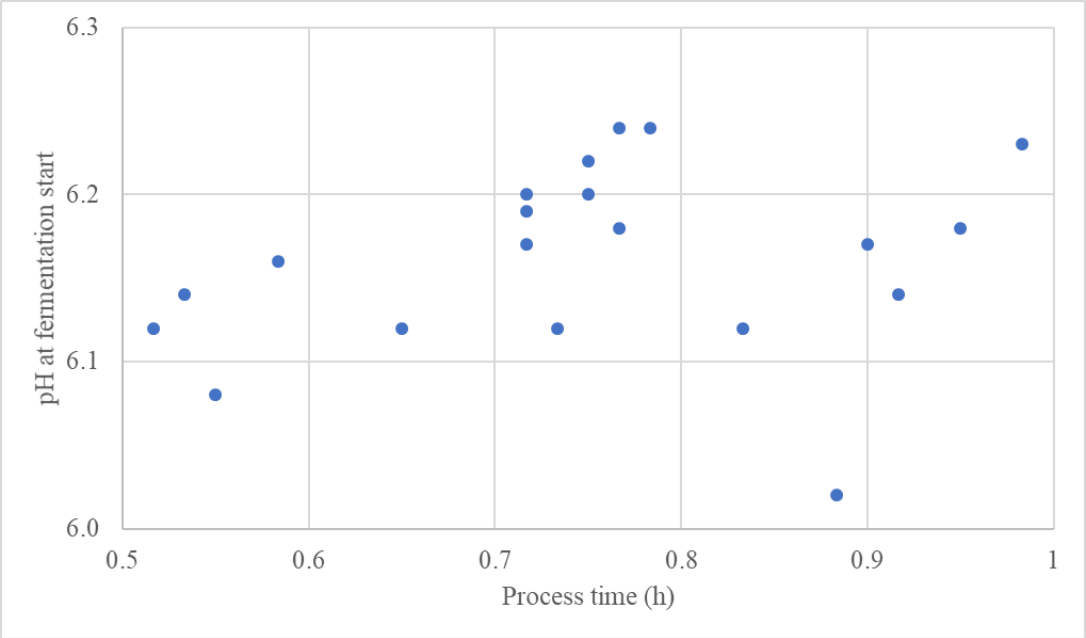


Figure 38: pH at fermentation start vs process time (h) for non-thermal processing of milk.

6.2 pH vs texture analysis

Since the texture analysation were performed on set style yoghurt, nothing could be inserted into the gel prior to analysation as this could affect the gel structure. Therefore, an assumption that the fermentation process was equal in each cup, was made so that the pH could be measured in other containers than the ones used for texture analysation. Unfortunately, a variation in the final pH of the different cups were observed, see Figure 39, which affected the result of the texture analysis. Due to this, the pH of each cup was measured after performing the texture analysis, and if the pH was ≥ 4.7, the result was discarded. The texture analysis was performed on three different cups each time and repeated on at least twice. Due to the great variability in pH and having to discard results with pH ≥ 4.7, this did unfortunately not give us 6 parallels. But the texture analysis was repeated so that at least three samples were obtained with a desired pH value. A possible explanation to the variability in pH is use of an alive starter culture. Each microorganism is independent and over a time period of up to five hours, which is the case for the fermentation process, there could be differences in their growth and acid production. If each cup does not contain the exact same number of bacteria this could also lead to a difference in acid production and decrease in pH.

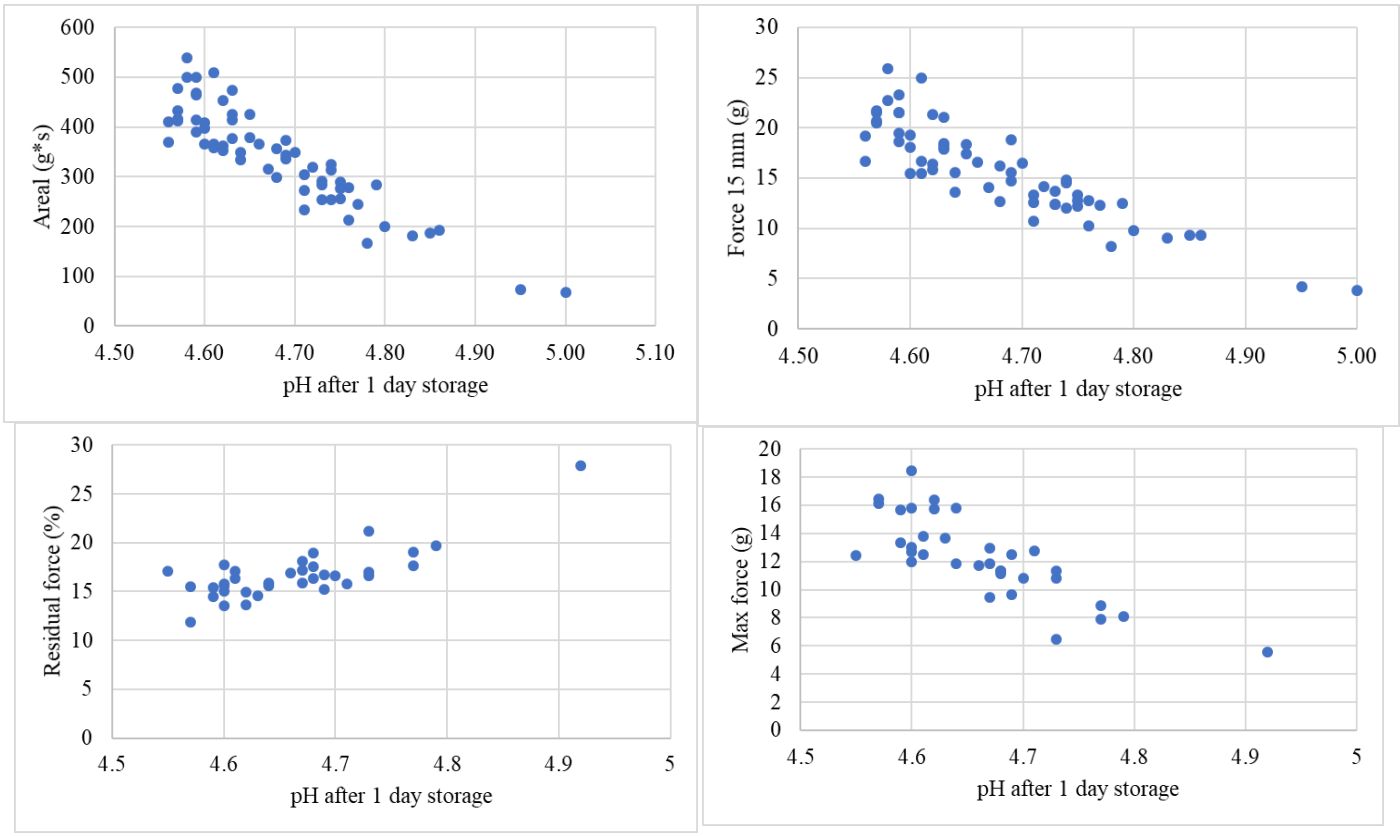


Figure 39: Results from penetration and stress-relaxation test vs. pH after one day storage.

6.3 Gelation process

The G' during the gelation process of heat treated milk (Figure 40), milk treated with 400 MPa, 5 or 15 min, US 5 or 15 min (Figure 41) and milk treated with 600 MPa, 5 or 15 min, US 5 or 15 min (Figure 42) is shown below.

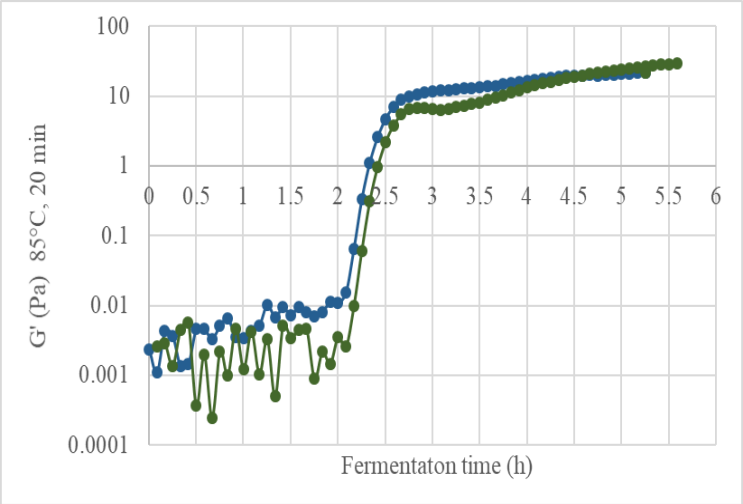


Figure 40: G' (Pa) during the gelation process of yoghurt produced using traditional heat treatment (85 °C, 20 min).

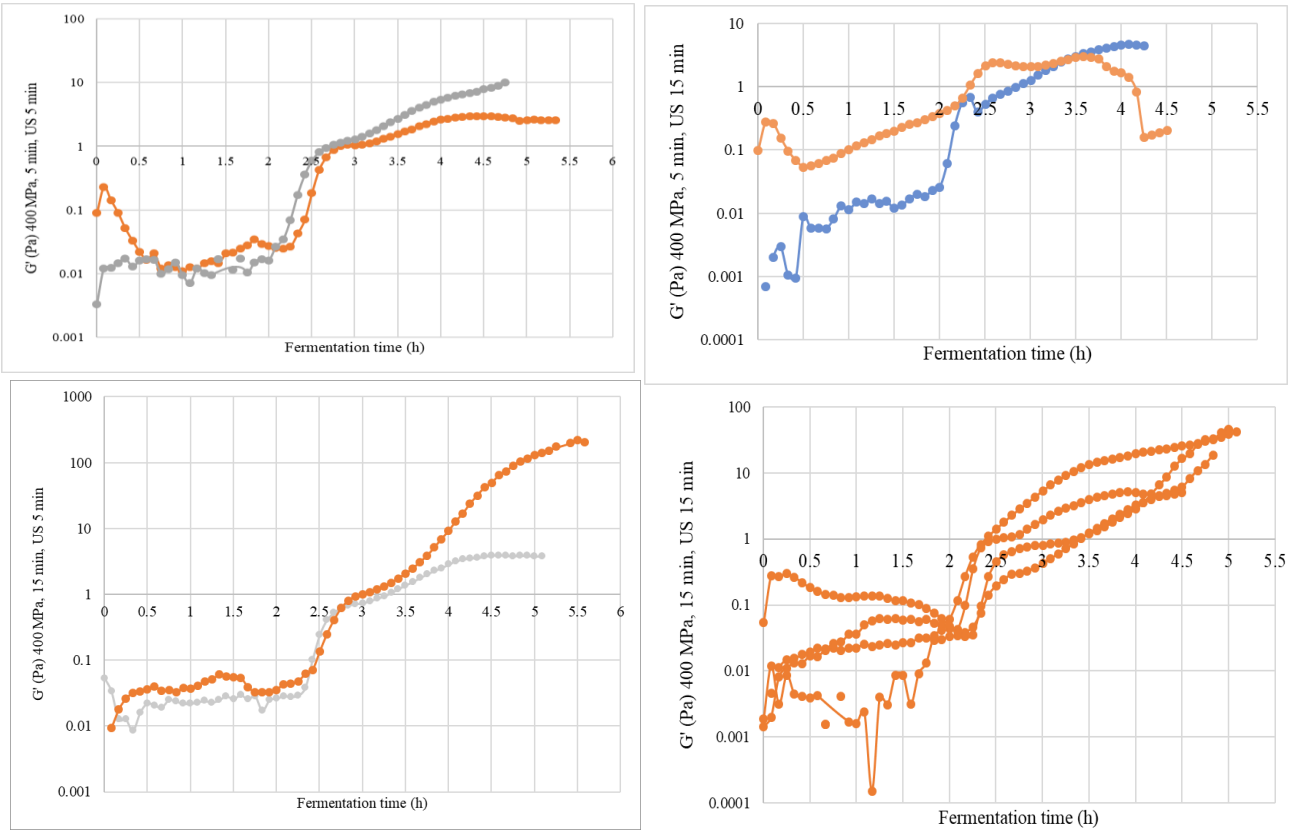


Figure 41: G' (Pa) during the gelation process of yoghurt produced using combination technology (400 MPa, 5 and 15 min, US 5 and 15 min).

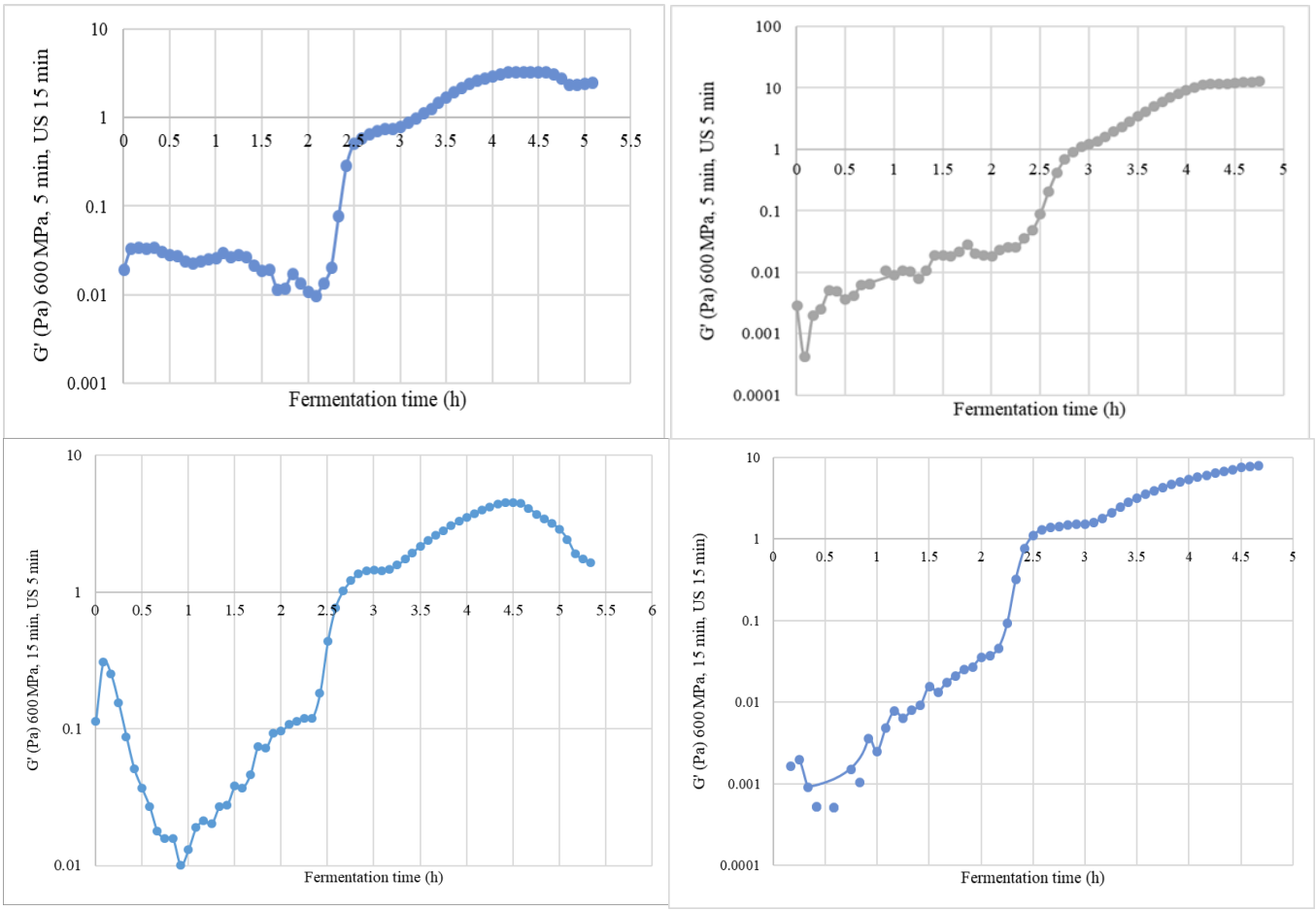


Figure 42: G' (Pa) during the gelation process of yoghurt produced using combination technology (600 MPa, 5 and 15 min, US 5 and 15 min).

6.4 Preliminary trials

The process for production of yoghurt using CO_2, HPP and US had to be established as this master thesis was the first time doing so. Preliminary trials were therefore performed to find the optimal process conditions.

Packing the milk with CO_2 was tested in different ways. One method tested was flushing CO_2 gas into the sous-vide pouch containing the milk simultaneous with vacuum packing. However, this led to great variability in the gas volume in the head space of the pouch (379 ± 45 mL). Some pouches (not included in the gas volume measurement) collapsed during packing with CO_2, thus barely containing any CO_2, while others were packed with so much CO_2 that they did not fit in the HPP chamber. Therefore, it was concluded that packing with CO_2 gas was too unreliable and decided to test packing the milk with dry ice. Although dry ice started to evaporate once it came in contact with the milk during vacuum packaging, this method was more reliable than packing with CO_2. The pouch did not collapse during packing and was never too big for the HPP chamber either.

After HPP, the temperature of the milk varied depending on the pressure and holding time due to adiabatic pressure release as explained in Section 3.1.2. Test had to be performed to find the optimal US bath temperature depending on the temperature of the milk after HPP treatment and the length of the US treatment (5 or 15 min), see Table 4.

Table 4: Optimal temperature of the US bath for each HPP and US treatment

HPP treatment	**Temperature (°C) of milk after HPP treatment**	**US time**	**Temperature (°C) of US bath**
400 MPa, 5 min	17.9	5 min	46
400 MPa, 5 min	17.9	15 min	43.5
400 MPa, 15 min	15.2	5 min	46.5
400 MPa, 15 min	15.2	15 min	43.5
600 MPa, 5 min	15.3	5 min	46.5
600 MPa, 5 min	15.3	15 min	43.5
600 MPa, 15 min	11.4	5 min	46.5
600 MPa, 15 min	11.4	15 min	43.5

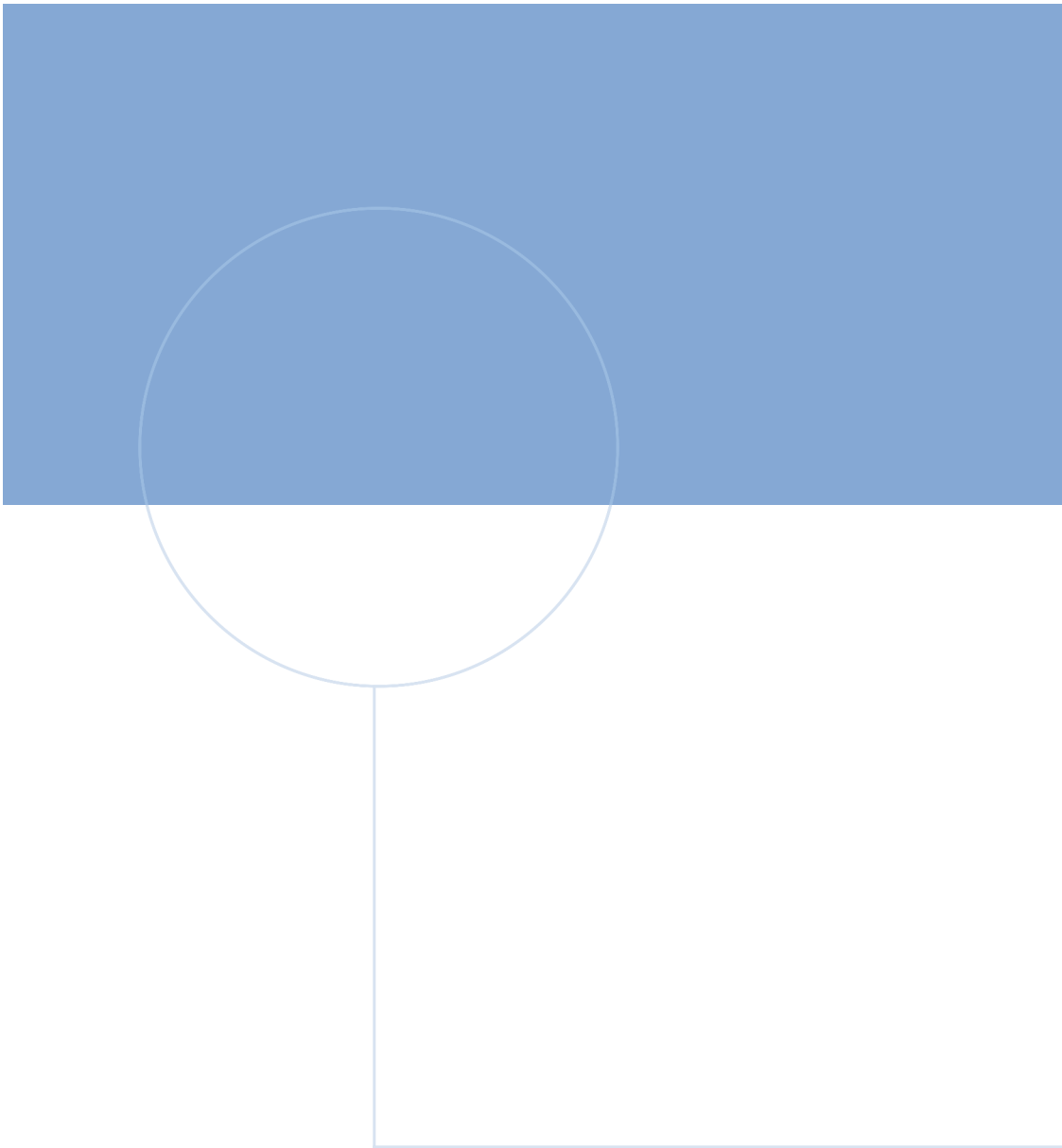
A small experiment was also performed with heat treatment followed by US treatment (5 min) before and after inoculation to see if US treatment prior to inoculation proved more

beneficial for shorter fermentation time than our experiment setup with US treatment after inoculation. However, the fermentation time of heat treated milk followed by US treatment before inoculation had 30 minute longer fermentation time than heat treated milk followed by US treatment after inoculation, and it was decided not to do more experiments with this and to stick with inoculation followed by US treatment.

The milk was 42 °C after US treatment and had to be kept at this temperature until placed in the incubator at 42 °C, to ensure optimal growth conditions for the starter culture. The US bath was in a different lab than the incubator, thus meaning that the temperature of the milk would drop in the time it took to move from one lab to another. The solution to this problem was to use a water bath set at 43 °C on a trolley, and to have the samples in a home-made rack inside the water bath.

Different fermentation temperatures (40 and 42 °C), different containers to incubate the milk in, and open or closed incubation was tested. The different containers tested was stand-up pouches, glass jars, 50 mL Eppendorf tubes and plastic cups. The stand-up pouch and 50 mL Eppendorf tube was not suitable for texture analysis, due to the plastic pouch moving during the texture analysis and the Eppendorf tube having too small diameter too avoid wall-interaction effects. The glass jars were recyclable, and it was too much work cleaning and sterilising them between each experiment. The plastic cups were found to be the most suitable as they were sterile and had large enough diameter to avoid wall-interaction effects. Closed incubation gave yellow bubbles inside the yoghurt gel, probably due to CO_2, and it was therefor decided to have open incubation (with alu foil loosely over the top to minimise evaporation).

Testing was done to find the best way to measure pH during the fermentation process. Manual pH measurements, where a sample was taken from the incubator every hour during fermentation and mixed well with a disposal pipet before measurement, was found to be the best alternative, to minimise possible pH gradients along the z axis (sample depth), attributed to varying oxygen availability and thus, bacterial predominance and microbial metabolism. With continuous pH measurement inside the incubator the measurement was in the same sample throughout the fermentation without any mixing. Different pH probes were also tested, with a probe that adjusted the temperature automatically being found to be the best option.



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