Norwegian University of Life Sciences
Faculty of Chemistry, Biotechnology and Food Science

Philosophiae Doctor (PhD)
Thesis 2017:58

Protein fractionation by microfiltration in high-protein yogurt processing

Bruk av mikrofiltrering for proteinfraksjonering ved produksjon av proteinrik yoghurt

Camilla Elise Jørgensen

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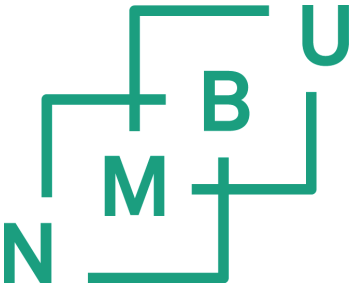

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Abstract

The main objective of the work presented in this thesis was to investigate the optimization of the microfiltration (MF) process in the production of high-protein yogurts (≥ 5.6% protein). High-protein yogurts have gained popularity during the last decade. However, research-based information about the impact of processing conditions on rheology, structure, and sensory properties of high-protein yogurt is limited.

The main proteins in milk, the caseins and whey proteins, can be fractionated into a casein-rich retentate and a permeate containing native whey proteins with the use of MF with membranes of 0.05–0.20 µm pore size. The native whey proteins can be further concentrated with ultrafiltration to a native whey protein concentrate (NWPC).

The effect of ceramic membrane pore size and filtration temperature on protein fractionation of skim milk by MF in a uniform transmembrane pressure system was investigated. An industrial MF application was modeled by performing MF with a constant permeate flux to a volume concentration factor of 2.5. Removal of native whey proteins increased with increasing pore size, giving the permeate from MF with the 0.20-µm membrane a significantly higher concentration of native whey proteins than the permeates from the 0.05- and 0.10-µm membranes (0.50, 0.24, and 0.39%, respectively). Significant amounts of small casein micelles (~130 nm) permeated the 0.20-µm membrane, resulting in a permeate with a white appearance, a casein content of 1.4%, and a casein distribution (α_{s2}-casein (CN): α_{s1}-CN: κ-CN: β-CN) similar to that of skim milk. The 0.10-µm membrane was found to be the most optimal for protein fractionation of skim milk into a casein concentrate and a permeate with native whey proteins and free from casein. Increasing the temperature of MF from 50 to 60°C when using the 0.10-µm membrane caused a reduction in native whey protein permeation and a steeper increase in transmembrane pressure during filtration. This was explained by potential interactions between whey proteins with casein micelles deposited on the membrane surface.

The permeate with native whey proteins from MF of skim milk was concentrated to a NWPC by ultrafiltration. Yogurt milk bases with ~8% protein were made by adding NWPC to casein concentrate in different whey protein:casein ratios (10:90–45:55). The degree of whey protein denaturation was then controlled by subjecting the yogurt milk base to varying

degrees of high temperatures. The denaturation status of the whey proteins and the ratio of whey protein to casein significantly influenced the microstructure, coagulum particle size, storage modulus (G'), firmness, and sensory properties of the resulting stirred, high-protein yogurts. The addition of NWPC in low or moderate levels (whey protein to casein ratio 25:75 or 35:65, respectively) in combination with heat treatment of the yogurt milk base at 75°C for 5 min yielded yogurts with significantly lower firmness, lower G', less coarse and granular appearances, and smoother consistencies, compared with corresponding yogurts produced from yogurt milk bases heat-treated at 95°C for 5 min or with control yogurts (no addition of NWPC). The addition of NWPC to the yogurt milk base after heat treatment or to the fermented yogurt before cooling gave stirred yogurts with unacceptable sensory appearances and consistencies. Thus, sensory acceptable high-protein yogurts, characterized as smooth and viscous, with considerable amounts of undenatured whey proteins (13-15 mg mL^{-1}) (approximately 50% of the available whey proteins in the yogurt) could be produced by adding NWPC to the yogurt milk base and by controlling the denaturation degree of the whey proteins by heat treatment.

MF with 0.20-µm membranes was used to fractionate skim milk with an average casein micelle size of ~174 nm into a retentate and a permeate containing "large" (~183 nm) and "small" (~129 nm) casein micelles, respectively. The permeate with small casein micelles was further concentrated with 0.10-µm membranes. Casein micelle size of yogurt milk bases significantly influenced the rheological properties of set type, high-protein yogurts (~5.6% protein). Yogurt milk base with small casein micelles yielded yogurts with higher storage modulus (G') and higher firmness than yogurt milk base with large casein micelles. Increased gelation capacity of small casein micelles can be explained by the increased amount of κ-CN.

The results obtained in this study revealed that MF of skim milk before fermentation can be utilized when producing high-protein yogurts. The choice of membrane pore size influences the protein composition of the resulting fractions, and thus the functional properties. High-protein yogurts with various properties regarding composition, structure, rheology, and sensory properties can be tailored with the use of retentates and permeates from protein fractionation by MF, and by controlling the degree of whey protein denaturation by heat treatment of the yogurt milk base.

Sammendrag

Hovedmålet til dette forskningsprosjektet var å undersøke hvordan mikrofiltrering (MF) kan optimaliseres og benyttes ved produksjon av proteinrik yoghurt (≥ 5,6 % protein). Gjennom det siste tiåret har yoghurt med høyt proteininnhold blitt stadig mer populært. Det er imidlertid begrenset forskningsbasert informasjon om innvirkningen av ulike prosessbetingelser på reologiske, strukturelle og sensoriske egenskaper til proteinrik yoghurt.

Melkas hovedproteiner, kaseiner og myseproteiner, kan ved bruk av MF og membraner med porestørrelse fra 0,05 til 0,20 µm fraksjoneres til et kaseinrikt retentat og et permeat som inneholder native myseproteiner. De native myseproteinene kan videre konsentreres til et nativt myseproteinkonsentrat ved bruk av ultrafiltrering.

Effekten av porestørrelsen til keramiske membraner og filtreringstemperatur ble undersøkt ved proteinfraksjonering av melk ved bruk av et MF-system med et uniformt transmembrantrykk. MF av melk ble utført ved en konstant permeatfluks til en volumkonsentrasjonsfaktor tilsvarende 2,5 for å etterligne industriell anvendelse av MF. Mengden native myseproteiner som ble fjernet fra melka økte med økende membranporestørrelse. Det var en signifikant høyere konsentrasjon av native myseproteiner i permeatet fra MF med 0,20 µm-membraner enn i permeatene fra MF med 0,05 µm- og 0,10 µm-membraner (henholdsvis 0,50, 0,24 og 0,39 %). Betydelige mengder små kaseinmiceller (~130 nm) passerte gjennom 0,20 µm-membranen og ga et permeat med 1,4 % kasein. Dette resulterte i et permeat med et hvitt utseende og en kaseinsammensetning (α_{s2}-kasein (KN): α_{s1}-KN: κ-KN: β-KN) lik den som er i melk. Forsøket viste at membranen med 0,10 µm porestørrelse var den best egnede til å fraksjonere melk til et kaseinkonsentrat og et kaseinfritt permeat inneholdende native myseproteiner. En økning av filtreringstemperaturen fra 50 til 60 °C ved bruk av 0,10 µm-membranen førte til en reduksjon i mengde native myseproteiner som passerte gjennom membranen, samt en kraftigere økning i transmembrantrykk under filtreringen. Dette funnet ble tilskrevet potensielle interaksjoner mellom myseproteiner og kaseinmiceller som avsettes på membranens overflate.

De native myseproteinene i permeatet fra MF av skummet melk ble konsentrert til et nativt myseproteinkonsentrat ved bruk av ultrafiltrering. Yoghurtmelk med ~8 % protein ble laget ved å tilsette nativt myseproteinkonsentrat til kaseinkonsentrat i ulike myseprotein:kasein-forhold (10:90–45:55). Denatureringsgraden til myseproteinene ble kontrollert ved å utsette yoghurtmelka for varierende grad av høye temperaturer. Denatureringsgrad og myseprotein:kasein-forhold hadde signifikant effekt på mikrostruktur, gelpartikkelstørrelse, elastiske egenskaper (G'), grad av fasthet og sensoriske egenskaper til proteinrik yoghurt. Tilsetning av nativt myseproteinkonsentrat i lave eller moderate mengder (henholdsvis myseprotein:kasein-forhold 25:75 eller 35:65) i kombinasjon med varmebehandling av yoghurtmelka ved 75 °C i 5 min, resulterte i yoghurt med signifikant lavere grad av fasthet og elastiske egenskaper (G'), mindre klumpete og fnokkete utseende og glattere konsistens, sammenliknet med tilsvarende yoghurt framstilt av yoghurtmelk varmebehandlet ved 95 °C i 5 min eller kontroll-yoghurten som ikke var tilsatt nativt myseproteinkonsentrat. Tilsetning av nativt myseproteinkonsentrat til yoghurtmelka etter varmebehandling, eller til den fermenterte yoghurten før avkjøling, ga en rørt yoghurt med sensorisk uakseptabelt utseende og konsistens. Viskøs og glatt proteinrik yoghurt med et betydelig innhold av native myseproteiner (13-15 mg mL^{-1}, omtrent 50% av de tilstedeværende myseproteinene) kunne framstilles ved å tilsette nativt myseproteinkonsentrat til yoghurtmelka og ved å kontrollere myseproteinenes denatureringsgrad under varmebehandlingen av yoghurtmelka.

MF med 0,20 µm-membraner ble benyttet til å fraksjonere skummet melk med en gjennomsnittlig kaseinmicellestørrelse tilnærmet 174 nm til et retentat og et permeat med henholdsvis «store» (~183 nm) og «små» (~129 nm) kaseinmiceller. Permeatet med de små kaseinmicellene ble videre konsentrert opp ved bruk av 0,10 µm-membraner. Yoghurtmelkas kaseinmicellestørrelse hadde signifikant innvirkning på de reologiske egenskapene til en set-type (urørt) proteinrik yoghurt (~5,6 % protein). Yoghurtmelka med små kaseinmiceller ga yoghurt som var fastere og hadde høyere grad av elastiske egenskaper (G') enn yoghurtmelka med store kaseinmiceller. De små kaseinmicellenes forbedrede evne til å danne gel ble koblet til det økte innholdet av κ-KN.

Resultatene fra dette arbeidet har vist at MF av skummet melk før fermentering kan benyttes til framstilling av proteinrik yoghurt. Membranenes porestørrelse påvirker proteinsammensetningen til fraksjonene, og derved deres funksjonelle egenskaper. Anvendelse av fraksjoner fra MF av melk, og kontroll av myseproteiners denatureringsgrad

under varmebehandling av yoghurtmelk, kan gi et bredt spekter av proteinrike yoghurtvarianter med ulik proteinsammensetning og sensoriske egenskaper (både struktur og konsistens).

Preface

The experimental part of this thesis was carried out at the Norwegian University of Life Sciences (Ås, Norway) and at the TINE R&D department (Oslo, Norway) during 2013-2017. The research work was financially supported by TINE SA and the Research Council of Norway (project number 222983) via the industrial PhD scheme.

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To my ever-inspiring and loving mom.
In loving memory of my beautiful dad.

Camilla Elise Jørgensen
Oslo, June 2017

Contents

List of publications

I Jørgensen, C. E., Abrahamsen, R. K., Rukke, E.-O., Johansen, A.-G., Schüller, R. B., & Skeie, S. B. (2016). Optimization of protein fractionation by skim milk microfiltration: Choice of ceramic membrane pore size and filtration temperature. *Journal of Dairy Science, 99*, 6164-6179. http://doi.org/10.3168/jds.2016-11090

II Jørgensen, C. E., Abrahamsen, R. K., Rukke, E.-O., Johansen, A.-G., Schüller, R. B., & Skeie, S. B. (2015). Improving the structure and rheology of high protein, low fat yoghurt with undenatured whey proteins. *International Dairy Journal, 47*, 6-18. http://doi.org/10.1016/j.idairyj.2015.02.002

III Jørgensen, C. E., Abrahamsen, R. K., Rukke, E.-O., Johansen, A.-G., & Skeie, S. B. (in press). Fractionation by microfiltration: Effect of casein micelle size on composition and rheology of high protein, low fat set yoghurt. *International Dairy Journal* (2016), http://dx.doi.org/10.1016/j.idairyj.2016.11.018

IV Jørgensen, C. E., Johansen, A.-G., Hoffmann, T. K., Skeie, S. B., Abrahamsen, R. K., & Rukke, E.-O. (2014). *Yoghurt with native whey proteins and processes for production thereof.* Patent application (priority number EP 2014/0199350) TINE SA, Oslo, Norway.

List of abbreviations

CCP	colloidal calcium phosphate
CN	casein
DF	diafiltration
GDL	glucono-δ-lactone
MCC	micellar casein concentrate
MF	microfiltration
MPC	milk protein concentrate
NWPC	native whey protein concentrate
RO	reverse osmosis
SMP	skim milk powder
TMP	transmembrane pressure
UF	ultrafiltration
UTP	uniform transmembrane pressure
VCF	volume concentration factor
WPC	whey protein concentrate
α-LA	α-lactalbumin
β-LG	β-lactoglobulin
κ-CN	κ-casein

Objectives and hypotheses

Consumer interest in high-protein (≥ 5.6%) yogurt has increased in the last decade. This development has been attributed to the increased consciousness about health benefits of dairy proteins, and consumers' preferences for thicker and creamier yogurts. However, the production of high-protein yogurts has challenged the dairy industry both with respect to environmental liability and sensory properties of the product. Concentration of the fermented yogurt to the desired protein content produces significant amounts of acid whey. Acid whey from yogurt production is, owing to its composition, a challenging by-product in downstream processing. Sensory defects such as graininess, bitterness, and too acidic flavor are other challenges faced by manufacturers of high-protein yogurts. Thus, novel methods of processing high-protein yogurts to overcome these challenges are important to the dairy industry.

The main proteins in milk, the caseins and whey proteins, have different molecular sizes and can be separated by the use of microfiltration (MF) with membrane pore sizes ranging from 0.05–0.20 μm. Milk fractions rich in caseins or native whey proteins could be good starting materials for tailoring and developing dairy products, such as high-protein yogurts. If the protein content of yogurt is increased prior to fermentation, production of acid whey is avoided.

MF of milk has been implemented industrially for casein standardization of cheese milk. The permeate fraction, containing the native whey proteins, can be concentrated to a native whey protein concentrate (NWPC) by ultrafiltration (UF), and utilized as an ingredient in the milk base for producing high-protein yogurts.

The limited amount of published research on the use of concentration processes prior to fermentation of high protein yogurt underpins the need to investigate this potential. The main objective of this thesis was to investigate how MF can be utilized when producing high-protein yogurts.

Protein fractionation by MF of skim milk is influenced by several factors, including filtration temperature and pore size of the membranes. Optimal fractionation of casein and whey proteins is of interest owing to their different functional properties. MF of skim milk

using ceramic membranes is typically carried out at temperatures ranging from 50 to 55°C. Operating at higher temperatures (e.g. 60°C) gives the potential benefit of reducing microbial growth and increasing flux, owing to reduced viscosity of milk at increased temperatures. The casein micelles in milk are polydisperse, varying in diameter from 50 to 500 nm with a mean diameter close to 200 nm. Ceramic membranes with different pore sizes are available on the market. It was hypothesized that both filtration temperature and ceramic membrane pore size would influence the protein fractionation of skim milk by MF (Publication I). Figure 1 illustrates the experimental approach used to test this hypothesis.

The proteins in the yogurt milk base are the key components in the gel network formed during yogurt fermentation. Protein composition and heat treatment of the yogurt milk base are known factors influencing the structure and rheology of traditional yogurts (< 5.6% protein).

An increased protein content in the yogurt milk base yields a yogurt with increased firmness and storage modulus, due to the increased amount of protein participating in the gel network. Excessive firmness of the yogurt gel could prevent the breaking of the yogurt gel into smaller coagulum particles during stirring, resulting in a grainy and coarse stirred yogurt. Yogurt milk bases with various whey protein to casein ratios (10:90–45:55) were obtained by adding NWPC to casein concentrate from MF of skim milk. It was hypothesized that the sensory properties of stirred high-protein yogurts (~8% protein) could benefit from the addition of native whey proteins to the yogurt milk base in combination with a reduced degree of denaturation of the whey proteins, compared to the denaturation degree normally obtained after high heat treatment of the yogurt milk base (Publications II and IV) (Figure 1).

It was observed that MF with 0.20-µm ceramic membranes could be used to obtain milk fractions with different casein micelle size distributions (Publication I). During renneting of milk, smaller casein micelles produce firmer rennet gels, explained by the increased total surface area of the para-casein micelles, allowing for more interaction points during

coagulation[*]. Based on this, it was hypothesized that the casein micelle size also could influence the rheology of high-protein yogurts (~5.6% protein), with smaller casein micelles giving a firmer yogurt gel (Publication III) (Figure 1).

The results of this thesis (Publications I-IV) are implemented and discussed in the following literature review on the challenges and possibilities of producing high-protein yogurts.

[*] Gustavsson, F., Glantz, M., Buitenhuis, A.J., Lindmark-Månsson, H., Stålhammar, H., Andrén, A., Paulsson, M.: Int. Dairy J. **39** (1) 201–208 (2014); Logan, A., Leis, A., Day, L., Øiseth, S.K., Puvanenthiran, A., Augustin, M.A.: Int. Dairy J. **46** 71–77 (2015); Walsh, C.D., Guinee, T.P., Reville, W.D., Harrington, D., Murphy, J.J., O'Kennedy, B.T., FitzGerald, R.J.: Int. Dairy J. **8** (8) 707–714 (1998).

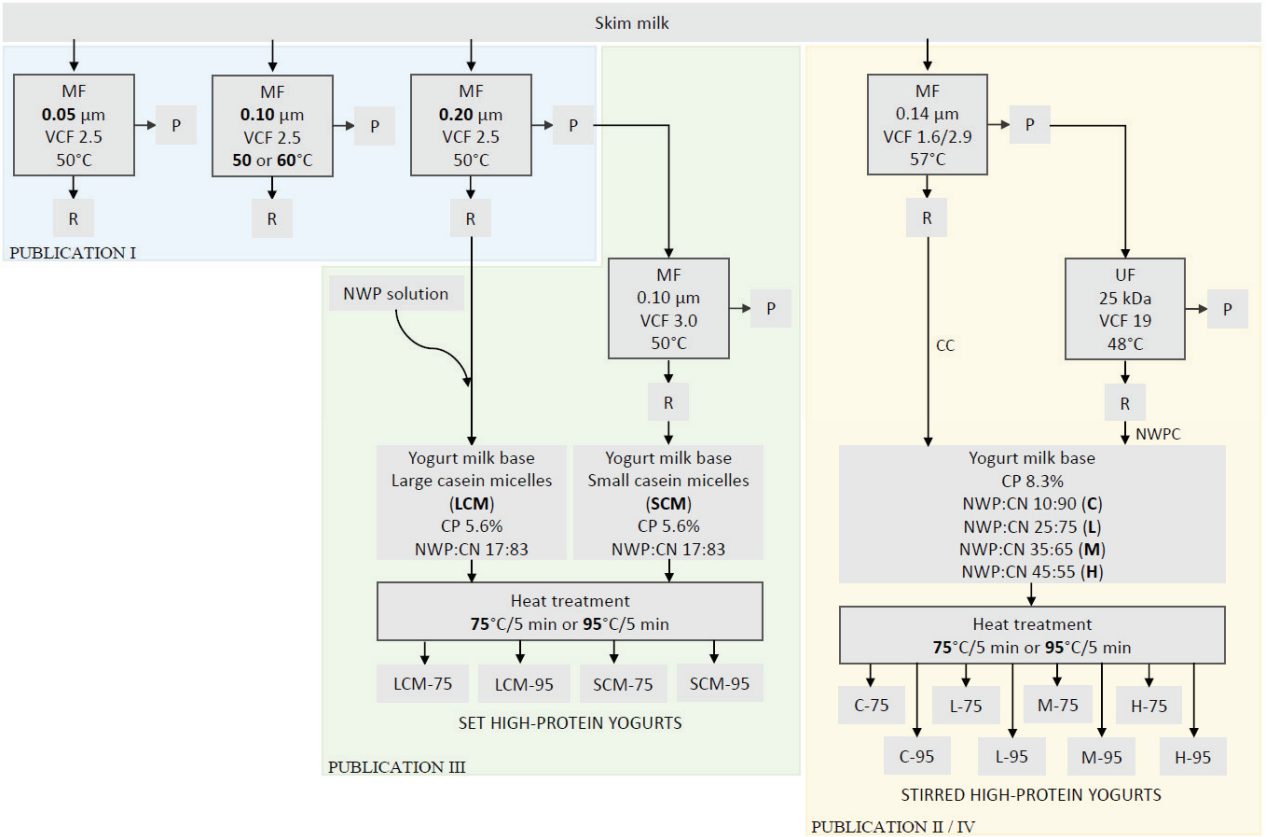

Figure 1: Process flow chart for publications I, II / IV, and III. Abbreviations are: MF, microfiltration; UF, ultrafiltration; R, retentate; P, permeate; VCF, volume concentration factor; NWP, native whey protein; CN, casein; CP, crude protein; LCM, large casein micelles; SCM, small casein micelles; CC, casein concentrate; NWPC, native whey protein concentrate. Denotations 75 and 95 refer to heat treatment of yogurt milk bases; 75°C 5 min; and 95°C 5 min, respectively. For publications II / IV: denotations C, L, M, and H refer to the added level of NWPC in terms of NWP:CN ratios in the yogurt milk bases; control (no addition); low; medium; high, respectively.

Literature review

This part of the thesis focuses on challenges and possibilities of processing technologies for high-protein yogurt.

Abstract

Background: High-protein yogurt has gained increased consumer interest over the recent years. Drivers behind the increased consumption of high-protein yogurts are, among others, product improvements in taste and texture, and increased amounts of scientific documentation have claimed health benefits of dairy proteins. Protein content of yogurt can be increased prior to fermentation by the addition of dairy powder, by evaporation, or by membrane filtration, or alternatively after fermentation with straining, mechanical separation, or membrane filtration. Concentration of the yogurt after fermentation produces large volumes of acid whey, which has been a major concern in the dairy industry. By concentrating prior to fermentation, production of acid whey is avoided. The different processing techniques influence the yogurt composition, structure, rheology, and sensory properties.

Scope and approach: Different challenges related to product and process can occur in the production of high-protein yogurts:

- Production of acid whey from concentrating fermented yogurt
- Sensory defects such as graininess, bitterness, too acidic flavor, and whey separation
- Technological challenges related to concentrating proteins prior to fermentation with the use of membrane filtration

This part of the thesis aims to overview the influence of the macro components in milk on the structure, rheology, and sensory properties of high-protein yogurt. The results obtained in Publications I–IV are included and discussed in conjunction with other published work. The thesis briefly touches on challenges with the existing classification system of high-protein yogurt, and a definition of such yogurt is suggested. Different processing techniques available for producing high-protein yogurts and their impacts on yogurt composition, structure, rheology, and sensory properties are discussed, along with their benefits and drawbacks for the dairy producer.

1 Introduction

High-protein yogurts and fermented milks have existed for a long time in many countries, and many names are applied to these products. Labneh (Eastern Mediterranean), Torba (Turkey), Stragisto (Greece), Chakka (India), and Ymer (Denmark) are all examples of concentrated or strained fermented milks with different geographical origins (Tamime, Hickey, & Muir, 2014). In the US, consumers were accustomed to thinner, more liquid-like yogurt before the introduction of high-protein yogurt, marketed as "Greek yogurt" or "Greek-style yogurt". Primarily, the texture benefits (thicker and creamier) of the increased protein content, but also the increased amount of scientific documentation claiming health benefits of dairy proteins (Fekete, Givens, & Lovegrove, 2013; Pasiakos, 2015; Phillips, Tang, & Moore, 2009), drove the "Greek yogurt" market in the US. In Europe, the growth of high-protein yogurt has been slower than in the US market; however, there has been an increased intake of high-protein yogurt in Northern Europe (Scandinavia, the Netherlands, Belgium, UK, and Germany) over recent years (Mellentin, 2013, 2014).

Tamime et al. (2014) surveyed the information contained on labels of 109 commercial concentrated fermented milks with different geographical origins. They classified the sample origins into four groups: group 1, fermented milks from the Eastern Mediterranean (Greece, Turkey, Lebanon, Jordan, Iran); group 2, the British Isles (UK, Ireland); group 3, Australasia (Australia, New Zealand); and group 4, North America (USA and Canada). The carbohydrate and fat contents of the fermented milk products were widely distributed and ranged from 1 to 12 g/100 g and 0 to 20 g/100 g, respectively. There were no distinct differences in carbohydrate or fat content among the groups. In contrast, the protein content among the groups was distinctly different. Group 1 comprised fermented milk such as Labneh, Süzme yogurt and strained yogurt, and had high-protein contents. The first quartile value (Q1)[1] was 8.0 g/100 g and the interquartile range was small (1.9 g/100 g). Groups 2, 3, and 4 had Q1 values of 4.5, 4.93, and 5.68 g protein/100 g, respectively. For group 2, 8 out of 21 samples had protein contents of 4.5 g/100 g or less. The most popular nomenclatures for products in groups 2–4 were "Greek" yogurt or "Greek-style" yogurt. The authors proposed that yogurts labeled "Greek" or "Greek-style" yogurt with a protein

[1] When observations are arranged in increasing order, the median is the midpoint. The first quartile (Q1) is the midpoint of the smallest number and the median of the observations. The third quartile (Q3) is the midpoint of the highest number and the median of the observations. Interquartile range equals Q3-Q1.

content of < 5 g/100 g were misnamed. Some countries had legal provisions covering the composition of the products. The provisions seemed, however, to be mostly concerned about the fat content of the products, and in some cases also the solids-not-fat or fat-in-dry matter content. Only in three of the investigated products was the content of protein defined; ≥ 5.6% protein for Süzme (Turkey) and Ymer (Denmark), and at least ≥ 8.4% protein depending on fat content for Chakka (India). It is worth discussing whether including protein content in legislative provisions could clarify the distinction between traditional and concentrated yogurts to guide both manufacturers and consumers.

According to the Codex standard for fermented milk (CODEX STAN 243-2003), yogurt is milk fermented with a symbiotic culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* containing a minimum of 2.7% milk protein and less than 15% fat (Codex Alimentarius, 2011). Concentrated fermented milk is a fermented milk where the protein has been increased prior to or after fermentation to a minimum of 5.6%. There is no legal standard to define "high-protein yogurt". However, the "concentrated fermented milk" term may comprise "high-protein yogurt". In that sense, "high-protein yogurt" is a yogurt containing a minimum of 5.6% protein (as determined by total Kjeldahl nitrogen multiplied by 6.38). Quarg is a product closely related to high-protein yogurt but usually the "fresh cheese" term comprises quarg. "Fresh cheese" is a product in which the whey protein to casein ratio does not exceed that of milk (i.e. 20:80). Fresh cheese can be obtained by coagulating milk through the action of rennet and/or other suitable coagulating agents, and by partially draining the whey resulting from the coagulation. The protein content of the fresh cheese is "distinctly higher" than the protein level of the blend from which the cheese was made; however, the protein level is not defined (CODEX STAN 283-1978) (Codex Alimentarius, 2011). In other words, the "fresh cheese" term or the "yogurt" term could comprise a fresh cheese like quarg if the following requirements are met: 1) coagulation is obtained at least by using a symbiotic culture of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, 2) no rennet is added, and 3) the whey protein:casein ratio does not exceed that of milk. The vagueness of the Codex standard may give dairy producers an option to choose whether this product (quarg) belongs to the "yogurt" category or the "fresh cheese" category. This raises a delicate issue with respect to the World Trade Organization's tariffs based on generic descriptions of products, as cheese has a tariff approximately 10 times higher than yogurt. One of the purposes with the Codex Alimentarius Commission is to ensure fair food trade practices. However, there seems to be

a lack of consistency in the Codex standards, which creates a loophole jeopardizing the global dairy trade. On the other hand, the vagueness of the Codex standard opens up opportunities for novel processing technologies for high-protein yogurts, fresh cheeses, and related products.

Based on the Codex standard definition of "concentrated fermented milk", it is hereby proposed that "high-protein yogurt" is a yogurt fermented with a symbiotic culture of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, containing a minimum of 5.6% protein, and less than 15% fat. The protein content can be obtained prior to fermentation by fortification with milk powder, evaporation, or membrane filtration, or after fermentation by straining (draining), mechanical separation, or membrane filtration. In this literature review, the term "high-protein yogurt" includes yogurt processed by increasing the protein content both prior to and after fermentation.

A plain yogurt with a high consumer acceptance should in general have a smooth, uniform and spoonable texture; be free from lumps, graininess, and visual whey separation (Lucey, 2004; Lucey & Singh, 1997); and should have a clean and typical yogurt flavor. Acetaldehyde, diacetyl, and lactic acid are the major aroma components of yogurt, but also other aromatic components, like acetone; acetoin; and acetic, formic, butanoic, and propanoic acids; contribute to yogurt flavor (Routray & Mishra, 2011). In a sensory evaluation of a wide range of commercially available plain yogurts, "Greek-style yogurts" (strained) with different fat levels were distinguished from the other yogurt samples (stirred or set-type) by having a thicker and firmer consistency (Brown & Chambers, 2015). Full-fat (8.8 or 20%) "Greek-style yogurts" differed from the low-fat (2%) and non-fat (< 0.05%) "Greek-style yogurts" by having a less chalky mouthfeel (dry, powdery sensation in the mouth). All "Greek-style yogurts" had a relatively high degree of smoothness irrespective of fat content. Desai, Shepard, and Drake (2013) reported that a full-fat strained yogurt received a higher overall impression score than low-fat and non-fat "Greek yogurts" in a consumer acceptance test. This yogurt was characterized by the descriptive panel to have high sensory intensities of milk fat flavor, viscosity, firmness, and denseness, and moderate amounts of sweet and sour taste. Although full-fat high-protein yogurts have preferable sensory properties, the largest dairy companies offer a wide range of non-fat and low-fat high-protein yogurts to meet consumer demands.

Sensory and texture attributes such as creaminess, viscosity, and smoothness are important drivers of liking of high-protein yogurts (Desai et al., 2013) and low-fat yogurts (Frøst & Janhøj, 2007). The sensory and physical properties of a high-protein yogurt are influenced and controlled by the composition of the yogurt milk base and by the conditions and parameters chosen during processing. Several reviews on the production of yogurt and its influence on physical and sensory properties have been published. During the last decade, the amount of research focusing on high-protein acid milk gels and yogurts has increased.

Different challenges related to product and process can occur in the production of high-protein yogurts:

- Production of acid whey from concentrating fermented yogurt
- Sensory defects such as graininess, bitterness, too acidic flavor, and whey separation
- Technological challenges related to concentrating proteins prior to fermentation with the use of membrane filtration.

This literature review aims to overview the influence of the milk macro components on the structure, rheology, and sensory properties of high-protein yogurt (section 2). Different processing techniques available for production of high-protein yogurts (section 3) and their impacts on yogurt composition, structure, rheology and sensory properties are discussed, along with their benefits and drawbacks for the dairy producer (sections 2 and 4).

2 Influence of milk macro components on high-protein yogurt

2.1 Protein

Protein is the crucial milk macro component in the formation of an acid milk gel such as yogurt. Several authors have reviewed the formation of acid milk gels in general (Dalgleish & Corredig, 2012; Livney, Corredig, & Dalgleish, 2003; Lucey, 2002; Lucey & Singh, 1997; van Vliet, Lakemond, & Visschers, 2004) and of yogurts in particular (Heertje, Visser, & Smits, 1985; Lee & Lucey, 2010; Lucey, 2004; Sodini, Remeuf, Haddad, & Corrieu, 2004).

Heat treatment of the yogurt milk base is regarded as a premise to obtain a good yogurt structure (Robinson, Lucey, & Tamime, 2006). Conventional heat treatment of the yogurt milk base, i.e. 95°C for 5 min or 80°C for 30 min, almost completely denatures β-

lactoglobulin (β-LG) (Anema, 2000; Dannenberg & Kessler, 1987) and denatures approximately 75% of α-lactalbumin (α-LA) (Anema, 2001). The aggregating whey proteins form disulfide-linked complexes with κ-casein (κ-CN) (Oldfield, Singh, Taylor, & Pearce, 1998), which are distributed between the micellar ("bound", associated with the casein micelles) and serum phase ("soluble") (Anema, Lee, Lowe, & Klostermeyer, 2004; Guyomarc'h, Law, & Dalgleish, 2003a; Kethireddipalli, Hill, & Dalgleish, 2010; Ozcan, Horne, & Lucey, 2015; Vasbinder, Alting, & de Kruif, 2003). Kethireddipalli et al. (2010) reported that about 30% of whey proteins are micelle-bound when heating recombined milk at 90°C for 10 min at the natural pH of milk (6.7). Similar amounts of micelle-bound aggregates (27%) were observed by Ozcan et al. (2015) when heating recombined skim milk at 85°C for 30 min at pH 6.7. Vasbinder et al. (2003) reported that 25% of both α-LA and β-LG were present as soluble aggregates, and 65% of β-LG and 50% of α-LA were present as micelle-bound aggregates after heating skim milk at 90°C for 10 min. The differences in the reported distributions of complexes between the micellar and serum phase (soluble) may be due to different sample preparations, and different methods for separation and quantification of the micelle-bound and soluble complexes.

The catabolism of lactose to lactic acid by the yogurt starter bacteria *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, leads to a gradual pH reduction and the formation of a gel network. When the pH is reduced, the buffering compounds of milk, like organic and inorganic phosphate, citrate, and phosphoserine residues of caseins, become protonated (Gaucheron, 2005; Lucey, Hauth, Gorry, & Fox, 1993; Salaün, Mietton, & Gaucheron, 2005). In response, colloidal calcium phosphate (CCP), which is attached to the α_s- and β-CN in the interior of the native casein micelle (Dalgleish & Corredig, 2012), is solubilized. At pH ~5.2–5.1, the solubilization of inorganic phosphate is complete, while approximately 10% of the calcium remains in the casein micelles and is not completely solubilized until about pH 4.8 (Heertje et al., 1985; Singh, Roberts, Munro, & Teo, 1996).

The onset of gelation occurs at about pH 5.5–5.4 in skim milk heated at 90°C for 10 min (Vasbinder et al., 2003), and is accompanied by a moderate increase in the elastic modulus[2] of the gel (Dalgleish & Corredig, 2012). The onset of gelation during acidification of heat-treated milk is related to the isoelectric pH of β-LG (~5.3) and the reduced electrostatic

[2] Elastic modulus/storage modulus (G') is assessed by rheological analyses and indicates solid-like properties, also referred to as "stiffness" by some authors.

repulsion between aggregated whey proteins at this pH (Horne, 1998; Lucey, Teo, Munro, & Singh, 1997). Just after the onset of gelation (pH 5.2–5.0), a weakening of the gel can be observed, measured as a maximum in the loss tangent (tan δ)[3]. This weakening could reflect the transition from an initial denatured whey protein-induced gel to a network dominated by casein-casein interactions at lower pH values (≤ 5.0) (Lucey, Tamehana, Singh, & Munro, 1998c). In addition, this weakening can be explained by the continued solubilization of CCP after the onset of gelation, resulting in increased electrostatic repulsions between the exposed phosphoserine residues in the interior of the micelles (Lucey, 2004; Lucey et al., 1998c).

As the pH is reduced further down to pH 4.6, charge neutralization occurs and the acid gel strengthens due to increased casein-casein interactions (Dalgleish & Corredig, 2012). According to Anema et al. (2004) and Guyomarc'h, Queguiner, Law, Horne, and Dalgleish (2003b), micelle-bound aggregates have a significant effect on the final storage modulus (G')[2] of an acid gel. However, soluble aggregates more dominantly contribute to an increase in the storage modulus (G') than the micelle-bound aggregates. Thus, in a yogurt milk base with both soluble and bound aggregates, there are many aggregating particles. Aggregation can occur between denatured whey proteins, between denatured whey proteins and the casein micelles, and between the casein micelles (Anema et al., 2004). On the other hand, Lucey et al. (1998c) observed that bound aggregates were important for increasing the G' of acid gels from heated milk, while soluble aggregates had relatively little effect on the rheological properties of acid gels. As suggested by Anema et al. (2004), the contradictory results of Lucey et al. (1998c) could be due to their preparation of soluble aggregates in the absence of casein micelles. In the absence of casein micelles, whey proteins may form large aggregates during heating that may not participate in the gel network during acidification (Anema et al., 2004). Figure 2 gives a schematic illustration of the heat-induced formation of micelle-bound and soluble complexes of κ-CN and whey proteins in a yogurt milk base, and the formation of a protein gel network during acidification.

[3] Loss tangent (tan δ) is the ratio of G'' to G', where G'' is the viscous/loss modulus. Indicates viscoelastic properties (liquid-like or solid-like), e.g. a high loss tangent indicates liquid-like behavior.

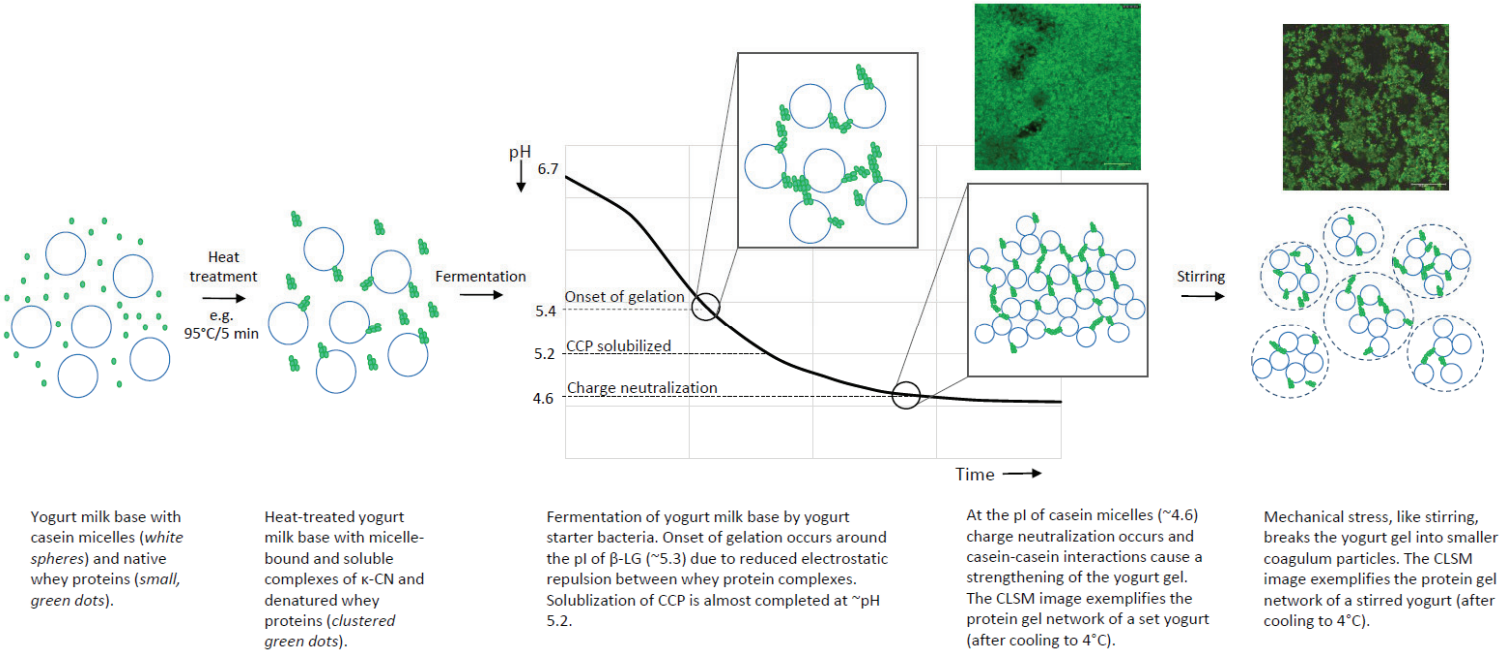

Figure 2: Schematic illustration of heat-induced formation of micelle-bound and soluble complexes of κ-CN and whey proteins in a yogurt milk base, and their participation in the yogurt gel network during acidification. Illustrations of protein networks of set and stirred yogurts are given. Explanations are given in the Figure. Abbreviations are: CN, casein; β-LG, β-lactoglobulin; CCP, colloidal calcium phosphate; CLSM, confocal laser scanning microscopy. Not drawn to scale.

Anema et al. (2004) and Guyomarc'h et al. (2003b) observed an increase in gelation pH and an increase in the final storage modulus (G') of acid gels with increasing amounts of soluble aggregates. Guyomarc'h et al. (2003b) proposed that aggregation of soluble complexes around the pI of β-LG (~5.3) could initiate destabilization of the casein micelles through hydrophobic interactions between soluble κ-CN whey protein aggregates and the partially neutralized casein micelles. In both studies (Anema et al., 2004; Guyomarc'h et al., 2003b), acid gels were formed with the use of glucono-δ-lactone (GDL). According to Lucey, Tamehana, Singh, and Munro (1998b), acid skim milk gels made with GDL or a bacterial culture have different rheological and physical properties. Acid gels made by acidification with GDL had much shorter gelation times and higher storage modulus (G') values than acid gels made by bacterial fermentation. The observed differences were explained by the very different mode and rate of acidification of the two gel systems, with GDL giving a rapid initial pH reduction. Ozcan et al. (2015) investigated the effect of soluble and bound aggregates on the rheological properties of acid gels made by bacterial fermentation. They observed that a milk sample with a mixture of soluble and bound aggregates (27% bound) yielded a stiffer yogurt gel (G' at pH 4.60) than milk samples where most of the aggregates were either bound (85%) or soluble (11% bound). The results of Ozcan et al. (2015) also support that a shift in aggregates from bound towards soluble increases the yogurt gel stiffness because milk with 11% bound aggregates yielded a yogurt gel with significantly higher storage modulus (G') than milk with 85% bound aggregates, which is in accordance with results from Anema et al. (2004) and Guyomarc'h et al. (2003b).

The variation in protein content among commercial yogurts and concentrated fermented milks (Tamime et al., 2014) leads to great variation in physical and sensory properties among yogurts on the market. In general, an increase in the protein content of a yogurt milk base yields a yogurt with increased firmness/viscosity[5] and storage modulus (G'), mainly due to the increased amount of protein that can participate in the gel network (Abrahamsen & Holmen, 1980; Biliaderis, Khan, & Blank, 1992; Mistry & Hassan, 1992; Schkoda, Hechler, & Hinrichs, 2001b). However, protein and total solids content are often dependent variables in experiments; thus, the effect of the protein content is often confounded with the total solids content (Sodini et al., 2004).

[5] Firmness is assessed by textural analyses (e.g. compression test), also referred to as gel strength or thickness. Viscosity can be determined by rheological analyses or a viscometer.

Jørgensen et al. (2015) (Publication II) observed that a high protein content (~8%) and a conventional heat treatment (i.e. 95°C for 5 min) of yogurt milk bases yielded stirred yogurts with a grainy and coarse appearance with large coagulum particle size. The high protein content (8%) yielded very firm yogurt gels, and the shearing applied during manual stirring was insufficient to break the gel network into smaller coagulum particles. Ozer, Bell, Grandison, and Robinson (1998), Ozer, Stenning, Grandison, and Robinson (1999a), and Ozer, Stenning, Grandison, and Robinson (1999b) investigated the effect of protein concentration and manufacturing techniques on the rheological and structural properties of concentrated yogurts (Labneh) with final total solids contents of ~23%. Yogurts produced with the traditional cloth bag method, or ultrafiltration (UF) of the yogurt milk base or the fermented yogurt yielded yogurts with higher protein content (~9%) than yogurts produced by reconstituted milk powder or reverse osmosis (RO) before or after fermentation (~6.5% protein). Production of concentrated yogurt with the traditional cloth bag method gave the stiffest yogurt (G'), followed by UF of the yogurt milk base or the fermented yogurt. Yogurts manufactured by RO prior to or after fermentation, or by reconstituted milk powder gave weaker final gels. The reduced stiffness of these yogurts was linked to the lower protein content of these yogurts (~6.5%), and to the detrimental mechanical effect of RO on the delicate gel structure. The yogurt produced with the traditional cloth bag method had a more compact microstructure than the yogurts concentrated with RO or UF, which had more discontinuous networks with thicker casein clusters. UF or RO of the yogurt milk base gave yogurts with fine, continuous microstructures (not stirred after fermentation).

Fortification of the yogurt milk base with milk powders prior to fermentation is a processing option in the manufacturing of high-protein yogurt. Available milk powders vary widely in their composition. Milk protein concentrate (MPC) is used in the commercial production of high-protein yogurts. Fortification of the yogurt milk base with higher-protein MPCs provides protein enhancement without adding significant amounts of lactose (Agarwal, Beausire, Patel, & Patel, 2015). Mistry and Hassan (1992) reported that sensory acceptable non-fat set yogurts fortified with MPC had protein contents less than 8% and lactose contents of at least 5%. Yogurts with a protein content above 8% gave a grainy texture. The desired level of protein could also be reached by fortifying the yogurt milk base with micellar casein concentrate (MCC). Bong and Moraru (2014) produced high-protein (~9.5% true protein), non-fat stirred yogurt by fortification of skim milk with MCC-88 (88% total protein in dry matter) or MCC-58 (58% total protein). Yogurts produced by MCC

fortification were compared to a lab-produced strained yogurt (cheesecloth), and a commercial "Greek-style yogurt", both containing the same amounts of protein. The amount of lactic acid (g/100 g) in the products was significantly higher in the MCC-fortified yogurts than in the strained yogurt, although the final pH values were similar (~4.3). Yogurt fortified with MCC-58 and the commercial yogurt had similar storage modulus (G') and flow behavior, suggesting similar textural properties. The strained yogurt had the highest water holding capacity, followed by the commercial "Greek-style yogurt" and the MCC-58 yogurt. The better water holding capacity of the strained yogurt was linked to the lower casein to serum protein ratio of this yogurt, and thus the increased cross-linking of the gel network and the high water-binding capacity of whey proteins. The MCC-58 yogurt had better water holding capacity than the MCC-88 yogurt, which was explained by the higher total solids content of the MCC-58 yogurt (~19% versus 15% total solids). Based on these findings, the authors proposed that MCC-58 could be a suitable protein source in the production of high-protein yogurt. However, the authors did not study the sensory properties of the yogurts. Jørgensen et al. (2015) (Publication II) observed that a high-protein (~8%), low-fat stirred yogurt produced from casein concentrate from microfiltration (MF) of skim milk had a coarse and granular appearance and a mealy consistency. This yogurt had a native whey protein to casein ratio (10:90) similar to that of the MCC-58 yogurt produced by Bong and Moraru (2014), indicating that the MCC-58 yogurt probably would have less acceptable sensory properties.

Several authors have investigated the effect of whey protein addition on the rheological properties of acid gels and yogurts. In general, increased amount of whey proteins has been reported to increase the final storage modulus (G'), maximum compression force (penetration test) and/or viscosity of acid milk gels (Chever, Guyomarc'h, Beaucher, & Famelart, 2014; Guyomarc'h et al., 2003b; Lucey, Munro, & Singh, 1999), yogurts with protein content < 5.6% (Krzeminski, Großhable, & Hinrichs, 2011; Kücükcetin, 2008a; Laiho, Williams, Poelman, Appelqvist, & Logan, 2017; Puvanenthiran, Williams, & Augustin, 2002; Remeuf, Mohammed, Sodini, & Tissier, 2003; Zhao, Wang, Tian, & Mao, 2016), and high-protein yogurts (> 5.6% protein) (Jørgensen et al., 2015) (Publication II). On the contrary, Guzmán-González, Morais, Ramos, and Amigo (1999) and Modler and Kalab (1983) reported that adding whey protein concentrates (WPC) decreased yogurt viscosity and firmness. In most of these studies, except for those by Guyomarc'h et al. (2003b) and Jørgensen et al. (2015), the whey protein source was a WPC or a whey protein

isolate, usually obtained from cheese whey. WPCs vary in their compositions (e.g. degree of β-LG lactosylation, mineral content, etc.) (Holt et al., 1999a; Holt et al., 1999b), and this could be the reason for the conflicting results of Guzmán-González et al. (1999), and Modler and Kalab (1983). This was also underpinned by the results of Modler and Kalab (1983), as yogurt prepared from fresh skim milk fortified with ultrafiltered WPC had higher gel strength (firmness) than yogurt prepared from milk fortified with WPC desalinated with electrodialysis or ion exchange.

Recently, Jørgensen et al. (2015) (Publication II) investigated the effect of adding native whey protein concentrate (NWPC) to casein concentrate from MF of skim milk on the rheological, structural, and sensory properties of stirred yogurt. Native whey is, opposed to cheese whey, free from somatic cells, lactic acid bacteria, bacteriophages, remnants of rennet (Maubois, 2002), cheese fines, and the glycomacropeptide from κ-CN, and has a neutral pH and taste. Dispersions of native whey protein powders made from MF of milk have been reported to give a significantly higher gel strength than dispersions of whey protein powders from cheese whey (Heino, Uusi-Rauva, Rantamäki, & Tossavainen, 2007). Heino et al. (2007) attributed the excellent gelling properties of native whey protein powders to the lack of glycomacropeptide and the high amount of native whey proteins. Jørgensen et al. (2015) (Publication II) observed that reducing heat treatment from 95°C/5 min to 75°C/5 min of yogurt milk bases with high whey protein to casein ratios (25:75–35:65) gave viscous, stirred high-protein yogurts (~8%) with rather small coagulum particle size, relatively smooth sensory consistency and shiny appearance. Thus, keeping considerable amounts of the whey proteins in their undenatured form (~40–50%) improved the sensory properties of these high-protein yogurts. However, the storage modulus (G') and the firmness of the yogurts were reduced compared to those of the yogurts from yogurt milk bases, where almost all the whey proteins were denatured (heat treatment at 95°C for 5 min). Chever et al. (2014) also observed reduced viscosity, firmness, and coagulum particle size of stirred, high-protein acid gels (9.2% protein) when an increasing amount of whey protein was kept in its undenatured form. Schmidt, Sistrunk, Richter, and Cornell (1980) reported that heat treatment of a yogurt milk base (6.4% protein) at 90°C for 30 min resulted in a grainy body of set yogurt, while a reduction in the heat treatment temperature to 80 or 85°C for 30 min yielded a smooth and firm-bodied yogurt. However, the improved sensory properties could likely not be explained by the presence of undenatured whey proteins,

because heat treatment at 80°C for 30 min is expected to completely denature whey proteins (Dannenberg & Kessler, 1987).

From a nutraceutical perspective, it could be interesting to produce high-protein yogurts with a considerable amount of undenatured whey proteins from native whey (Gryson et al., 2014; Hamarsland et al., 2017; Sousa et al., 2012; Walrand et al., 2016). Guggisberg, Eberhard, and Albrecht (2007) and Patocka, Cervenkova, Narine, and Jelen (2006) observed a reduction in the storage modulus (G') of yogurts when whey proteins were added to yogurt milk after heat treatment to retain the whey proteins in their undenatured state. Addition of whey proteins to the yogurt after fermentation resulted in two separate phases comprising fluid whey and coagulated protein mass (Patocka et al., 2006). The yogurts produced by these authors were not sensorially evaluated. Jørgensen et al. (2014) (Publication IV) investigated, however, the effect of adding NWPC to yogurt milk base (casein concentrate) before heat treatment, after heat treatment, or to the fermented yogurt before cooling, on the sensory properties of high-protein yogurt (~8% true protein, whey protein to casein ratio 30:70). They observed that adding NWPC to the yogurt milk base after heat treatment or to the yogurt after fermentation yielded yogurts with unacceptable sensory properties (mealy and granular). However, the addition of NWPC to the yogurt milk base followed by a reduced heat treatment (i.e. 75°C/5 min) compared to the conventional heat treatment (i.e. 95°C/5 min) gave a smooth and shiny yogurt.

Casein micelles are polydisperse and vary in diameter from 50 to 500 nm as measured with electron microscopy (Fox & Kelly, 2004). Skim milk with an average micelle diameter ranging from 149 to 222 nm (Devold, Brovold, Langsrud, & Vegarud, 2000), can be fractionated into a retentate containing "large" casein micelles (~186 nm) and a permeate containing "small" casein micelles (~130 nm) with the use of MF (Jørgensen et al., 2016) (Publication I). Jørgensen, Abrahamsen, Rukke, Johansen, and Skeie (in press) (Publication III) reported that a yogurt milk base with small casein micelles (~129 nm) gave high-protein set yogurts (5.6% crude protein) with higher storage modulus (G') and higher firmness (maximum compression force) than a yogurt milk base with large casein micelles (~183 nm). The authors proposed that this increased gelation capacity could be attributed to an increased amount of κ-CN in small casein micelles. Donato, Guyomarc'h, Amiot, and Dalgleish (2007) observed a higher content of soluble complexes of whey proteins and κ-CN in heated milk with a naturally high content of κ-CN. Jørgensen et al. (in press)

(Publication III) did not measure the distribution of bound and soluble complexes of whey proteins and κ-CN. However, they suggested that a possible higher content of soluble complexes in the yogurt milk base with small casein micelles could provide more points of attachment during acidification, as previously reported by Anema et al. (2004) and Guyomarc'h et al. (2003b). On the other hand, Horne (2003) observed no effect of casein micelle size on stiffness of gels made with GDL. However, these gels were made of non-heat-treated milk. Smaller casein micelles have previously been reported to produce firmer rennet gels (Gustavsson et al., 2014; Logan et al., 2015; Walsh et al., 1998).

Most of the work discussed in this section supports that firmness and storage modulus of acid gels and yogurts increase with increasing protein content and increasing amount of denatured whey proteins. In addition, research suggests that smaller casein micelles and a shift from bound towards soluble aggregates of whey protein κ-CN in the heat-treated milk could enhance the protein network of acid milk gels and yogurts. Furthermore, research suggests that yogurt milk bases with increased ratios of denatured whey protein to casein at constant total protein contents yield firmer yogurts with stronger protein networks (Jørgensen et al., 2015; Krzeminski et al., 2011; Kücükcetin, 2008a; Laiho et al., 2017). These latter studies covered ratios of denatured whey protein to casein from 10:90–60:40. Lucey et al. (1999) suggested that undenatured whey proteins act as inert fillers in the gel matrix, while denatured whey proteins associated with the casein micelles act as a bridging material by interacting with other denatured whey proteins. Increased amounts of protein particles participating in the gel network lead to increased branching, and consequently gels with higher G' (Lucey et al., 1997). Guyomarc'h et al. (2003a) observed that an increase in whey protein:casein ratio of a heat-treated (95°C, 10 min) milk-based dairy mixture (4.7% total protein, 5.3% lactose) increased the amount and average size of soluble aggregates of denatured whey proteins and κ-CN. An increase in the whey protein:casein ratio from ~15:85 to ~33:67 increased the soluble aggregate size from 3.5×10^6 to 5×10^6 Da. The ratio of whey proteins to κ-CN in the aggregates increased with increasing amount of whey protein in the mixture, while the proportion of κ-CN involved was rather consistent. The authors estimated that the soluble aggregates could be globular particles of more than 10 nm in diameter or hundreds of nanometers long linear particles. They suggested that the increased amount and size of soluble aggregates could explain the observed higher G' values of acid gels of milk-based dairy systems with increased whey protein:casein ratios (Guyomarc'h et al., 2003b). Furthermore, a firmer yogurt gel yields increased coagulum

particle size of the stirred yogurt with increased sensory roughness, coarseness, lumpiness, and graininess (Jørgensen et al., 2015; Krzeminski et al., 2011; Krzeminski et al., 2013; Kücükcetin, 2008a; Laiho et al., 2017; Tomaschunas, Hinrichs, Köhn, & Busch-Stockfisch, 2012).

The protein trends and the consumer interest for high-protein yogurt are expected to continue (Mellentin, 2016). Drivers behind the protein trend in the latest years are: consumer awareness of the health benefits of protein, inclusion of protein in more mainstream products such as yogurt, improvements in taste and texture of protein-rich products such as high-protein yogurt, the low carbohydrate trend, and the fact that high-protein yogurt and dairy products are regarded as "naturally functional". The protein trend is also connected to the weight wellness trend and consumer interests for products with limited additives ("clean label") (Mellentin, 2013). With the use of liquid dairy concentrates (casein concentrate, NWPC, milk retentate) and/or dairy protein powders (WPC, MCC, or MPC), high-protein yogurts with different whey protein to casein ratios can be produced. High-protein yogurts with a high content of whey proteins could be beneficial in infant-, elderly-, or sports nutrition due to the ability of whey proteins to increase plasma amino acids (Boirie et al., 1997; Hall, Millward, Long, & Morgan, 2003), and trigger muscle protein synthesis (Garlick, 2005; Tipton et al., 2007). Furthermore, high-protein yogurts could be beneficial in calorie-restricted diets, because the energy intake from protein seems to have a greater effect on satiety than intake of fat or carbohydrate (Benelam, 2009).

2.2 Fat

The fat content of yogurt varies from 0–10%, with most common values between 0.5 and 3.5% fat (Lucey & Singh, 1997). Traditional concentrated yogurts, such as Labneh from the Eastern Mediterranean region, typically have a fat content of 6-11% (Nsabimana, Jiang, & Kossah, 2005; Tamime & Robinson, 2007). Today, the largest dairy companies offer a wide range of fat-reduced, concentrated yogurts in the US and European markets, typically containing 0, 2, or 4% fat. According to the U.S. Food and Drug Administration (2016), these yogurts are described as non-fat (< 0.5% fat), low-fat (0.5–2.0%), and regular (≥ 3.25%), respectively.

The fat content of concentrated yogurts contributes to the sensory profile and to the textural and rheological properties of the product. In a consumer survey with female consumers (n=520, ≥ 18 y) who had consumed "Greek yogurt" at least once within the last three months, most respondents (54%) consumed low-fat yogurts, 26% consumed non-fat yogurts, and 20% consumed full-fat yogurts (Desai et al., 2013). Preference mapping of descriptive panel results and consumer acceptance testing (n=155) of ten "Greek yogurts" (from non-fat to full-fat with protein content from 5.8–10.6%) revealed that milk fat flavor was, among other attributes, an important driver of liking. The yogurt with the highest fat content had the highest sensory intensity of milk fat flavor, viscosity, and cohesiveness according to the descriptive panel results, and also received the highest overall impression score in consumer acceptance testing (Desai et al., 2013). Additionally, for yogurts lower in protein content (similar to that of raw milk), fat content is positively associated with sensory properties like creamy flavor, visual gel firmness, and mouthfeel (Folkenberg & Martens, 2003a). In a blind tasting (n=69) of the same yogurts, the participants preferred the high-fat yogurts to the low-fat yogurts (Folkenberg & Martens, 2003b).

Homogenization (150-200/50 bar) of a yogurt milk base increases the total surface area of the fat globules. The new surface layer of the fat globules is made up of casein micelles and fragments of casein micelles, whey proteins, and milk fat globule membrane material (Sharma, Singh, & Taylor, 1996). The new membrane material allows the fat globules to interact as pseudocasein particles in the protein gel network (Fox, Guinee, Cogan, & McSweeney, 2000), increasing the number of interacting particles of the yogurt gel. Thus, increasing the fat content of homogenized yogurt milk bases increases the storage modulus (G') of the yogurt (Lucey, Munro, & Singh, 1998a). If the milk is not homogenized or the homogenized fat is added after fermentation, the fat globules act as structure breakers and reduce the viscosity of the yogurt (Schkoda, Hechler, & Hinrichs, 2001a; van Vliet & Dentener-Kikkert, 1982).

Sensory and texture attributes like creaminess, viscosity, and smoothness are important drivers of liking of high-protein yogurts (Desai et al., 2013) and low-fat yogurts (Frøst & Janhøj, 2007). An increased fat content reduces the coagulum particle size of the yogurt and increases the viscosity and storage modulus (G') (Brauss, Linforth, Cayeux, Harvey, & Taylor, 1999; Krzeminski et al., 2011). A small coagulum particle size is correlated with perceived increased smoothness and reduced graininess (Cayot, Schenker, Houzé, Sulmont-

Rossé, & Colas, 2008; Jørgensen et al., 2015; Krzeminski et al., 2013). Sensory properties like smoothness, viscosity (relatively high, but not too high), fatty afterward mouthfeel, fat-related flavors (cream, butter), and also sweetness, are related to perceived creaminess of yogurt (Frøst & Janhøj, 2007). The positive effect of fat on sensory and physical properties of yogurts can, to a certain extent, be compensated by an increased protein content in reduced-fat yogurts (Tomaschunas et al., 2012). However, although increasing the protein content increases the fat-related attributes (creamy taste and texture, fatty mouth feel), very high intensities of these attributes can only be achieved with a high fat content (Tomaschunas et al., 2012).

With the bad reputation of milk fat in the 1980s and the introduction of dietary guidelines recommending lean dairy products, non-fat and low-fat yogurts entered the market (Mellentin, 2013). Since then, an increasing amount of scientific work reporting the neutral or beneficial effect of milk fat on health has been published (de Oliveira Otto et al., 2013; Mozaffarian, 2016; O'Sullivan, Hafekost, Mitrou, & Lawrence, 2013; Patterson, Larsson, Wolk, & Åkesson, 2013; Pimpin, Wu, Haskelberg, Del Gobbo, & Mozaffarian, 2016). A possible change in the image of dairy fat could reduce the demand for non-fat and low-fat yogurts within the next decades. However, such a change is expected to happen slowly because consumers have grown accustomed to low-fat and non-fat products (Mellentin, 2014), and changing dietary recommendations and dietary patterns occurs slowly. Nevertheless, changing the image of dairy fat could give the dairy industry increased opportunities for applying fat to develop and tailor sensory and rheological properties of high-protein yogurts.

2.3 Lactose

Lactose provides an energy source for the yogurt starter bacteria, and thus is essential for reducing pH through catabolism of the lactose to lactic acid and other yogurt flavor compounds (Tamime & Robinson, 2007). There seems to be little research on the direct influence of lactose on the rheological and structural properties of yogurt. However, lactose has been shown to influence the heat denaturation of whey proteins (Anema, 2000; Anema, Lee, & Klostermeyer, 2006), which in turn influences the rheology and structure of yogurt (Anema et al., 2004; Dannenberg & Kessler, 1987; Jørgensen et al., 2015; McKenna & Anema, 1993). Anema et al. (2006) studied the effect of lactose on heat denaturation of β-

LG and α-LA by recombining low-heat skim milk powder (SMP) in lactose solutions of 5, 10, and 15% to a protein content equal to the that of a 9.6% total solids skim milk. Skim milk samples were heated at temperatures between 75 and 100°C for 0–60 min in a thermostatically controlled oil bath. The irreversible denaturation of β-LG and α-LA decreased with increasing lactose concentration. Lactose increases the ordering of the water structure around protein molecules and thereby stabilizes the native protein conformation (Anema et al., 2006). However, for β-LG, the stabilizing effect of lactose diminished at higher heat treatment temperatures > 90°C (e.g. 95°C for 5 min) (Anema, 2000; Anema et al., 2006). That means that when heating skim milk with lactose content varying from approximately 5–20% at 95°C for 5 min, β-LG denaturation is extensive and varies from approximately 95% to 85%, respectively (Anema et al., 2006). Thus, the firmness of a yogurt made from yogurt milk bases subjected to the conventional heat treatment (i.e. 95°C for 5 min) would probably be mostly unaffected by varying the lactose content from 5–20%. If the heat treatment temperature was reduced (e.g. 80°C for 5 min), variation in the lactose content of the yogurt milk base is expected to have a greater influence on the thermal denaturation degree of β-LG (Anema et al., 2006), and thereby yogurt firmness (McKenna & Anema, 1993).

Meletharayil, Patel, Metzger, and Huppertz (2016b) investigated the effect of lactose level (no added lactose, 5.6% or 11. 2%) on acid gels (4% protein) of reconstituted MPCs heat-treated at 90°C for 10 min and acidified with GDL. Increasing the lactose content of the MPC dispersions to 5.6 or 11.2% increased the final G' and water holding capacity and decreased the microstructural porosity of the acid gels at pH 4.6. This observation was linked to increased levels of non-sedimentable (soluble) κ-CN and whey protein aggregates of the heat-treated MPC dispersions with increasing lactose concentration. Higher amounts of soluble aggregates of κ-CN and whey protein have previously been reported to increase the number and density of gelling protein particles, thereby increasing the storage modulus (G') of acid gels due to increased points of attachment during acidification with GDL (Anema et al., 2004; Guyomarc'h et al., 2003b) or starter culture (Ozcan et al., 2015). However, for bacterially fermented yogurt gels, a balance of both soluble and bound aggregates of κ-CN and whey proteins (shifted towards soluble) seem to contribute to stiffer gels (Ozcan et al., 2015). Meletharayil et al. (2016b) studied the effect of lactose on acid gels in a model system using GDL. Due to the reported different rheological and physical properties between acid gels made with GDL or bacterial fermentation (Lucey et al.,

1998b), the observed effect by Meletharayil et al. (2016b) of lactose on storage modulus (G') of acid gels should be investigated using bacterial cultures.

A high-protein yogurt can, for instance, be obtained by fortifying milk with dairy powders such as MPCs or MCCs to reach the desired protein level (Agarwal et al., 2015; Bong & Moraru, 2014; Meletharayil, Patel, & Huppertz, 2015). Protein fortification with low-protein MPC or MCC significantly increases the lactose content of the yogurt milk base. For instance, protein fortification of skim milk with MPC42 (42% protein, 46% lactose) to a protein content in the yogurt milk base of approximately 9% would concurrently increase the lactose content to approximately 11%. Vinderola, Costa, Regenhardt, and Reinheimer (2002) investigated the effect of lactose concentration (5, 15, or 20%) on the growth of some strains of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* cultured in MRS broth and Elliker broth, respectively. A lactose concentration of 15% inhibited some strains of these lactic acid bacteria. Thus, the lactose content of a yogurt milk base must be taken into consideration, because excessive lactose content may inhibit and decline the rate of acid production by the yogurt culture due to osmotic pressure (Vedamuthu, 2006).

The influence of lactose on rheological properties of high-protein yogurt seems to be a relevant focus for further research. Lactose has become a surplus milk component with the emerging use of membrane filtration technologies in the dairy industry. Application of lactose to manage texture and water holding capacity of yogurts could be an interesting option for reducing cost of yogurt production, however not for high-protein yogurts.

Trends in the food industry, such as high protein and weight wellness, have driven forward a carbohydrate reduction trend (Mellentin, 2014). Furthermore, free-from products, including lactose-free dairy products, are an integral part of the digestive wellness trend (Mellentin, 2016). The growing number of people following lactose-restricted diets, such as the low FODMAP diet (Gibson & Shepherd, 2010; Mellentin, 2016), has increased the demand for lactose-free yogurts and opened up market opportunities for reduced-lactose yogurts. Methods for producing lactose-free milk involving the use of membrane filtration and/or enzymatic hydrolysis with β-D-galactosidase have been patented (Lange, 2004; Tossavainen & Sahlstein, 2002; Wang, 2004). Elevating the protein content of a yogurt milk base with MF or UF in combination with diafiltration (DF) facilitates a yogurt milk base with a reduced lactose content and an increased protein to lactose ratio (Alvarez et al., 1998;

Biliaderis et al., 1992; Kosikowski, 1979; Mistry & Hassan, 1992). The lactose content must, however, be sufficient to ensure growth conditions for the yogurt culture (lactose ≥ 2%) (Alvarez et al., 1998) and to avoid flat flavor (Kosikowski, 1979). Furthermore, reducing the lactose content of the yogurt milk base with the use of MF DF or UF DF causes loss of minerals and compositional changes in the milk serum phase (Alexander, Nieh, Ferrer, & Corredig, 2011; Li & Corredig, 2014), which has implications for the production and properties of yogurt (Anema, 2009; Koutina, Knudsen, Andersen, & Skibsted, 2014; Ozcan-Yilsay, Lee, Horne, & Lucey, 2007; Ozcan, Horne, & Lucey, 2011; Ramasubramanian, Restuccia, & Deeth, 2008; Singh & Muthukumarappan, 2008).

3 Processing techniques for increasing protein content of yogurt

Protein content of yogurt can be increased prior to fermentation by adding dairy powder, by evaporation, or by membrane filtration, alternatively after fermentation with straining, mechanical separation, or membrane filtration. Concentrating the yogurt after fermentation produces large volumes of acid whey, which has been a major concern in the dairy industry. By concentrating prior to fermentation, acid whey production is avoided. Another possibility is to combine concentration prior to and after fermentation. The main focus in this section will be on methods for protein concentration prior to yogurt fermentation, because these methods eliminate the production of acid whey. However, the various manufacturing possibilities for concentrating fermented yogurt are also briefly mentioned.

3.1 Concentration after fermentation

The effect of different production methods on the rheology and microstructure of high-protein yogurt (i.e. Labneh) has been previously studied (Abu-Jdayil, Jumah, & Shaker, 2002; Ozer et al., 1998; Ozer et al., 1999a, 1999b; Tamime, Kalab, & Davies, 1991) and reviewed (Nsabimana et al., 2005; Özer, 2006).

Traditionally, strained yogurt can be made by using cloth bags to drain whey. Yogurt is poured into cloth bags and strained at cold temperatures (e.g. 4°C) to the desired total solids content. Depending on the pressure applied, the drainage time can be shortened to 6–18 hours. Today, cloth bags are mostly replaced by nozzle or quarg separators in industrial productions. Briefly, fermented milk is vigorously stirred and optionally passed through a

metal strainer or filter to break up any large clumps. Optionally, the fermented yogurt is thermized at 50–60°C to release some whey. The yogurt is concentrated at 35–40°C, cooled to ~15°C, cream is optionally added, and the product is packaged. Fat standardization can also be performed prior to fermentation if specially designed separators are used, such as that available from GEA Westfalia Separator Group GmbH (Nsabimana et al., 2005; Tamime et al., 2014). Membrane technologies, mainly UF, are other options for yogurt concentration. The fermented, warm (~40°C) yogurt is gently stirred and concentrated by a two- to four-stage UF plant with 5–6 bar inlet pressure. The concentrated yogurt is cooled to ~10–20°C and packaged. A thermization step can be added prior to concentration to improve the release of whey, inactivate most of the lactic acid microflora in the product, reduce the extent of proteolysis, and improve the keeping quality (Zakrzewski, Stepaniak, Abrahamsen, & Sørhaug, 1991; Özer & Tamime, 2013). The thermization step may be undesirable if a content claim is made on the product label, referring to the presence of a specific microorganism (Codex Alimentarius, 2011).

Manufacturing technique influences the rheological and structural properties of the concentrated yogurt, as investigated by Ozer et al. (1998), and Ozer et al. (1999a, 1999b), and as mentioned in section 2.1. Concentrated yogurt (~9% protein) produced with the traditional cloth bag method has a more compact microstructure than yogurt concentrated with UF after fermentation (tubular system, 25 kDa cut-off, 42°C), which had a more discontinuous network with thicker casein clusters (Ozer et al., 1999b). Tamime et al. (1991) reported that the firmness of Labneh (~8% protein) increased with increased temperature at UF of the warm yogurt, from 35–55°C. UF Labneh concentrated at 55°C was firmer and had more complex micellar chains than Labneh concentrated at lower temperatures, explained by agglomeration of casein particles caused by the higher temperature. UF at 50 or 55°C yielded Labneh with a similar firmness to Labneh concentrated using the cloth bag method (~9% protein); however, transmission electron microscopy revealed that the traditional Labneh had simpler and less complex protein chains. The lower firmness of the UF Labneh concentrated at 35°C than the traditional Labneh was attributed to the different processing conditions during concentration, i.e. pressure-driven concentration vs. gravitational concentration. Abu-Jdayil et al. (2002) observed a greater loss of apparent viscosity measured at a shear rate of 106 s^{-1} as a function of shearing time for commercial Labneh produced by the traditional method compared to

commercial Labneh produced by centrifugation. They suggested that the different production methods produced products with different space occupancies in the structure.

The use of centrifugation in the production of high-protein yogurt has been adapted from fresh cheese manufacturing processes (e.g. quarg). The resemblance between processing technologies for high-protein yogurts and fresh cheeses, like quarg, might underpin the need for a clearer distinction between products in the "concentrated fermented milk"-category and the "fresh cheese"-category, to close loopholes in the Codex standard, as discussed in section 1.

3.2 Concentration prior to fermentation

With the use of liquid dairy protein concentrates (e.g. casein concentrate, NWPC, milk retentate) and/or dairy protein powders (e.g. MCC, WPC, MPC, or their isolates), high-protein yogurts with different whey protein to casein ratios can be produced.

3.2.1 Concentration by membrane filtration

MF and UF are pressure-driven processes using semi-permeable membranes. UF of milk gives a protein-rich milk fraction, designated as milk retentate, and a protein-free fraction, designated milk permeate (CODEX STAN 207-1999, Codex Alimentarius (2011)). It is common industrial practice to concentrate milk with UF to increase the protein content of the yogurt milk base before fermentation into traditional yogurt (< 5.6% protein) (Rattray & Jelen, 1996). Evaporation or addition of SMP are other common industrial methods. However, these two methods also significantly increase the lactose content of the yogurt milk base, which influences the nutritional quality of the product, and its textural characteristics (Abrahamsen & Holmen, 1980). In the production of high-protein yogurt (> 5.6% protein), it is beneficial to use a technology that renders it possible to mainly increase the protein content, while minimally elevating lactose content. This can be obtained by UF.

UF membranes are offered in a variety of module configurations, including hollow fibers, tubular, plate and frame, and spiral wound (Pouliot, 2008). For producing milk retentates, the spiral wound configuration is typically used (Gésan-Guiziou, 2013). Milk UF can be performed at around 50°C or around 10°C. Permeation fluxes are higher at 50°C, but the

process duration must be reduced due to mineral precipitation of calcium phosphate in the membrane pores and due to possible bacterial growth in the retentate. UF at around 10°C results in little bacterial growth, and the process duration can be doubled depending on process parameters (Gésan-Guiziou, 2013). However, UF at 10°C and with a membrane cutoff greater than ~20 kDa potentially increases the β-CN permeation, as β-CN leaks out from casein micelles during low temperature treatment (Farrell et al., 2004; Liu, Weeks, Dunstan, & Martin, 2013; Rose, 1968; Schmitt, Saulnier, Malhautier, & Linden, 1993; van Hekken & Holsinger, 2000). Milk retentate produced by UF of milk with a membrane cutoff of ~10 kDa (or smaller) has a whey protein to casein ratio, which is unchanged from that of the original milk (~20:80).

MF of milk with membranes with pore sizes in the range from 0.05–0.20 µm produces a casein rich retentate ("casein concentrate") and a permeate with native whey proteins, commonly referred to as native whey, ideal whey, virgin whey, or serum proteins. The content of whey proteins in the MF permeate cannot exceed the content of whey proteins in the feed (milk). However, with the use of UF (e.g. cutoff ~10 kDa), the whey proteins in the MF permeate can be concentrated into a NWPC (Maubois, 2002). The casein concentrate has increased contents of casein and CCP compared to the original milk (Brandsma & Rizvi, 1999; Jørgensen et al., 2016; Neocleous, Barbano, & Rudan, 2002). The casein concentrate can industrially be used in the production of cheese, especially hard cheese varieties (Daufin et al., 2001; Kumar et al., 2013), due to improved rennet coagulation of cheese milk with a moderate increase in casein content (e.g. ~30–40 g kg^{-1}) (Heino, Uusi-Rauva, & Outinen, 2009, 2010; Maubois, 2002). However, in yogurt production, the presence of whey proteins in the yogurt milk base is essential. Addition of NWPC (Jørgensen et al., 2015) (Publication II) or whey powders, like WPC or whey protein isolate, makes the casein concentrate suitable as a milk base for yogurt manufacture.

The major concern in protein fractionation of skim milk by MF is to minimize and control fouling. Fouling means the deposition of milk components, such as proteins and calcium phosphate, on the membrane surface or in the pores of the membrane (Koh, Ashokkumar, & Kentish, 2013; Saxena, Tripathi, Kumar, & Shahi, 2009). Fouling appears as a flux decline with filtration time at a constant transmembrane pressure (TMP), or as a TMP increase at a constant flux. TMP is the force that drives fluid through the membrane and is the difference in pressure between the retentate and permeate sides of the membrane. Flux is the amount of

permeate (mass or volume) removed from the feed stream per unit of membrane area and time (Hausmann, Duke, & Demmer, 2013). The term "critical flux" describes the flux at which fouling begins to occur (Field, Wu, Howell, & Gupta, 1995; Howell, 1995). Below the critical flux, there is a linear relationship between flux and TMP, where the selectivity of the MF process is controlled by the membrane (Bacchin, Aimar, & Field, 2006) (Figure 3). Operation in this region is termed subcritical (Howell, 1995) and is advised for optimal separation of casein and whey proteins (Brans, Schroën, van der Sman, & Boom, 2004). However, the flux in this region is low and a larger membrane area is required to process a given amount of product within a certain time, thus, affecting the economics of the filtration operation (Smith, 2013b). Therefore, MF for protein fractionation is often operated above, but close to the critical flux, where the relationship between flux and TMP is no longer linear (Brans et al., 2004). Different hydraulic concepts are available to ensure a stable MF process in this region. This is further discussed in the next paragraph. The critical flux is reached when fouling occurs locally on the membrane (Bacchin, 2004). Above the critical flux, the deposit layer (fouling) acts as a secondary membrane, which leads to an alteration of the selectivity of the MF process and a decrease in whey protein permeation (Koh et al., 2013). The term "limiting flux" describes the highest flux that can be achieved by increasing TMP at specific hydrodynamic conditions (Bacchin et al., 2006). The limiting flux is reached when the whole membrane surface is controlled by the deposit layer (Bacchin, 2004). Further increases in TMP cause compaction of the deposited layer, and ultimately flux decline (Brans et al., 2004). Bacchin et al. (2006) introduced the term "sustainable flux", meaning the flux that the system can operate at for extended periods of time (acceptable fouling rate between cleaning cycles). The sustainable flux refers to operational and economic sustainability of the MF process, and is somewhere between the critical and limiting flux, where the fouling rate is low. Critical, limiting, and sustainable fluxes must be determined for each filtration process.

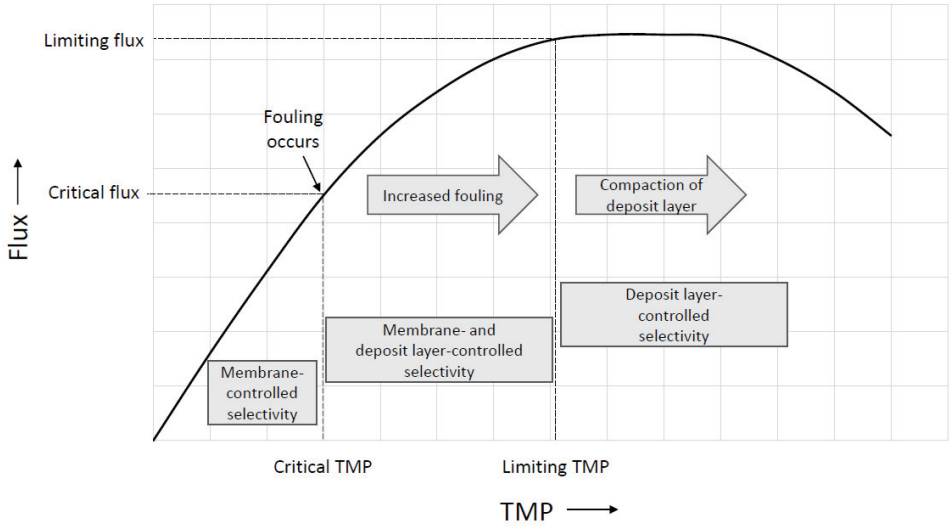

Figure 3: Effect of fouling on flux as a function of transmembrane pressure (TMP) during microfiltration (MF) of skim milk. Below the critical flux, in the sub-critical flux regime, there is no fouling and there is a linear relationship between flux and TMP. Protein fractionation in this region is controlled by the membrane. At the critical flux, fouling begins to occur, and increases with increasing TMP or flux. Fouling alters the selectivity of the MF process, and protein fractionation is controlled by the membrane and the deposit layer. Hydraulic concepts (uniform transmembrane pressure or inhomogeneous membranes) ensure stable MF in this region. At a certain elevated TMP, the flux cannot longer increase, and a limiting flux is reached. MF in this region is time dependent and controlled by the deposit layer. Continued MF in this region leads to compaction of the deposit layer, and ultimately flux decline. Figure based on information from Bacchin et al. (2006); Brans et al. (2004); Field et al. (1995); Howell (1995); Piry (2010).

MF of milk became industrially feasible with the hydraulic concept proposed by Sandblom (1974). The pressure-driven cross-flow of milk through the filter channels, tangential to the filter area, creates a pressure drop along the module. The pressure drop is relatively large because of the high cross-flow velocity required to obtain high permeation flux and accurate membrane selectivity (Saboya & Maubois, 2000; Smith, 2013b). The pressure drop on the retentate side causes heterogeneous fouling (Saboya & Maubois, 2000). To obtain a constant TMP over the length of the module, a permeate pump is installed, causing the permeate to recirculate co-currently with the feed/retentate stream in a separate loop, creating a pressure drop on the permeate side similar to the pressure drop on the feed/retentate side (Sandblom, 1974). The uniform transmembrane pressure (UTP) results in better control of the protein deposit (fouling) and consequently in acceptable MF performance (Gésan-Guiziou, 2013).

The membranes used can be formed by combining metals such as aluminum, titanium, or zirconium with support materials, and are commonly referred to as ceramic membranes. Ceramic membranes can tolerate a wide range of pH values (0 to 14) and temperatures, high pressures (up to ~300 bar), and high TMPs (> 170 bar). Some drawbacks of ceramic systems include high capital costs, sensitivity of the membranes to fast temperature changes, and labor-intensive membrane replacement (Smith, 2013a). The increased operational costs caused by the energy demand of the permeate pump in the UTP system can be reduced by filling the permeate compartment with plastic beads (Saboya & Maubois, 2000).

Another hydraulic concept that ensures a stable MF regime along the membrane is inhomogeneous ceramic membranes, with a higher hydraulic resistance at the membrane inlet where the TMP is high and a lower resistance at the membrane outlet (Gésan-Guiziou, 2013). The longitudinal permeability gradient can be built into the support structure (Membralox, Pall Corporation), often referred to as ceramic graded permeability membranes (Garcera & Toujas, 1997), or can be obtained by modifying the thickness of the separating layer (Isoflux, TAMI Industries) (Skrzypek & Burger, 2010). Both these commercial concepts avoid the need for a permeate pump, thus reducing the investment and running costs compared to the UTP system. Skrzypek and Burger (2010) reported that industrial plants using 0.14-µm Isoflux membranes were established in Poland and the Czech Republic in 2007 for casein standardization of skim milk for quarg production. MF of skim milk to a casein concentration factor of 1.6–2.0 allowed for a 40–60% reduction in the amount of acid whey.

Protein fractionation of milk can also be performed with polymeric spiral wound membranes. However, polymeric membranes have, in general, a wider pore size distribution than ceramic membranes, and a shorter membrane life (Brans et al., 2004; Pouliot, 2008). Zulewska, Newbold, and Barbano (2009) reported that ceramic membranes (0.1-µm pore size) in a UTP system gave significantly better whey protein removal from skim milk than ceramic graded permeability membranes (0.1 µm) and polymeric spiral-wound membranes (0.3 µm). The permeate from MF with ceramic graded permeability membranes had the highest proportion of casein. Under the operational conditions used in their study, the highest flux value was observed for the ceramic graded permeability membrane, followed by MF with the ceramic UTP system. Protein fractionation with the polymeric spiral wound membrane was, however, only effective after the formation of a boundary layer of milk

proteins on the surface of the membrane. At the beginning of the filtration experiment, before the formation of the boundary layer, significant amounts of caseins were detected in the permeate. Lawrence, Kentish, O'Connor, Barber, and Stevens (2008) also observed that the effect of MF with polymeric spiral wound membranes (0.3- and 0.5-µm pore size) was dictated by a protein layer that rapidly formed on the membrane surface. Formation of a protein layer increased the rejection of caseins; however, rejection of β-LG also increased. Today, a significant portion of dairy plants installed polymeric spiral wound systems for milk protein fractionation (~80%) (Tetra Pak Filtration Solutions, personal communication). Despite the better protein separation ability and flux values of ceramic systems, the lower investment, operation, and replacement costs of polymeric spiral wound systems seem to be decisive for a major part of the dairy industry. Additionally, long-standing technical and operational knowledge of polymeric spiral wound systems from processing of cheese whey might be an underlying factor. In an ongoing research project (AiF 18553 N) at the Technical University of Munich, polymeric spiral wound membranes are investigated and developed, aiming for an increased separation efficiency of caseins and whey proteins. Development of polymeric spiral wound membranes with high separation ability could, to a higher degree, challenge ceramic systems in the future.

To date, protein fractionation with ceramic membranes is a strategic choice for optimal separation of caseins and whey proteins. However, membrane designs and systems are not the only factors influencing protein fractionation. Other factors influencing the composition of retentates and permeates from protein fractionation of skim milk by MF are: composition of the skim milk, pretreatment of the skim milk (Brandsma & Rizvi, 1999; Svanborg, Johansen, Abrahamsen, & Skeie, 2014), membrane pore size (Jørgensen et al., 2016; Punidadas & Rizvi, 1998), membrane channel diameter (Hurt, Adams, & Barbano, 2015b, 2015c), membrane length (Piry et al., 2008), filtration temperature (Hurt, Adams, & Barbano, 2015a; Jørgensen et al., 2016; Kersten, 2001), volume concentration factor (VCF) (Kersten, 2001; Punidadas & Rizvi, 1998), ratio of permeation flux to wall shear stress (Gésan-Guiziou, Boyaval, & Daufin, 1999; Le Berre & Daufin, 1996), and fouling (Gésan-Guiziou et al., 1999; Jimenez-Lopez et al., 2008). Table 1 gives an extracted overview of some experimental approaches and reported effects from some of the referred works.

Table 1: Extracted results from work investigating the effect of various factors on composition of permeates and retentates from protein fractionation by ceramic MF. Abbreviations are: MF, microfiltration; UTP, uniform transmembrane pressure; TMP, transmembrane pressure; VCF, volume concentration factor; VRR, volume reduction ratio; GDL, glucono-δ-lactone; CN, casein; β-LG, β-lactoglobulin; α-LA, α-lactalbumin.

Factor and experimental approach	Results	Reference
Pretreatment of skim milk		
Ceramic UTP MF, 0.2 µm (Jiuwu); feed: unpasteurized or pasteurized (73°C/15 s) skim milk; average filtration temperature 56.3°C; average VCF 2.47.	Permeate from MF of unpasteurized skim milk contained higher amounts of calcium, phosphorus, and native whey proteins, and fewer casein fragments than permeate from MF of pasteurized skim milk.	Svanborg et al. (2014)
Ceramic UTP MF, 0.2 µm (Membralox P19-40, US Filter Corp.); feed: pasteurized skim milk; filtration temperature 50°C; cross-flow velocity 7.5 m s^{-1}; VCF 8-9 (to ~20% total protein in retentate); GDL added to retentate during filtration to reduce pH from 6.6 to 6.3 and 6.0.	A reduction in retentate pH from 6.6 to 6.0 decreased retentate calcium content by 20.1%, increased whey protein retention by 12.6%, and reduced permeate flux.	Brandsma and Rizvi (1999)
Membrane pore size		
Ceramic MF with asymmetric membranes (Ceramem), 0.05- or 0.20-µm pore size; feed: pasteurized skim milk; filtration temperature 50°C; cross-flow velocity 5.4 m s^{-1}; mean pressure on retentate side 138 kPa; concentration factor 2.5.	MF using a membrane with 0.05-µm pore size retained all caseins and allowed whey proteins to permeate. Permeate from MF using a membrane with 0.2-µm pore size contained a significant amount of casein. Higher cross-flow velocity gave a higher flux for the membrane with 0.05-µm pore size.	Punidadas and Rizvi (1998)
Ceramic UTP MF, 0.05- and 0.10-µm (Orelis), 0.20-µm (Atech) pore sizes; feed: pasteurized skim milk; filtration temperature 50°C; cross-flow velocity 6.9 m s^{-1}; constant flux 44 L m^{-2} h^{-1} for 0.05 µm, ~58 L m^{-2} h^{-1} for 0.10 and 0.20 µm; VCF 2.5.	MF using membranes with 0.10- and 0.05-µm pore size retained all caseins. Transmission of native whey proteins increased with increasing pore size. Significant amounts of small casein micelles permeated the membrane with 0.20-µm pore size and resulted in a permeate with 1.4% casein and a casein distribution (α_{s2}-CN: α_{s1}-CN: κ-CN: β-CN) similar to that of skim milk.	Jørgensen et al. (2016) (Publ. I)
Membrane length		
Ceramic MF, 0.1-µm pore size (Atech); feed: pasteurized skim milk; filtration temperature 55°C; wall shear stress 115 Pa; cross-flow velocity ~6 m s^{-1}; permeate and retentate were recirculated (no concentration). A special module design consisting of 1.2-m long membrane with four sections. Average TMPs in section 1 to 4: 82 kPa to 17 kPa.	Flux was independent of TMP in the first three sections, filtration was deposit layer-controlled. Section 4 had pressure-dependent flux. Permeation of β-LG increased from 38% in section 1 to 87% in section 4. The deposit layer was responsible for the retention of whey proteins in the first sections. An optimum exists for the relationship of protein permeation and flux.	Piry et al. (2008)

Table 1: (*Continued*)

Filtration temperature		
Ceramic UTP MF, 0.10-µm pore size (Orelis); feed: pasteurized skim milk; filtration temperature 50 or 60°C; cross-flow velocity 6.9 m s^{-1}; constant flux ~59 L m^{-2} h^{-1}, VCF 2.5, no recirculation.	Permeate from MF at 50°C contained significantly more native whey proteins and calcium than MF at 60°C. Retentate from MF at 60°C had less caseins, probably due to deposition of caseins on the membrane surface. MF at 60°C had a more rapid increase in TMP.	Jørgensen et al. (2016) (Publ. I)
Ceramic UTP MF, 0.10-µm pore size (Membralox, Pall Corp.); feed: pasteurized skim milk; VCF 3.0; total recirculation mode; filtration temperature sequentially increased from 50 to 55 to 60 to 65°C, MF operated for 1 h at each temperature.	Content of calcium and native whey proteins in permeate decreased as the filtration temperature was increased to 65°C, and at the same time casein contamination in the permeate decreased. Increasing the MF temperature from 50 to 65°C decreased the TMP required to maintain a flux of 54 kg m^{-2} h^{-1}.	Hurt et al. (2015a)
Volume concentration factor		
Ceramic UTP MF, 0.10-µm pore size (Société des Céramiques Techniques); feed: pasteurized skim milk; filtration temperature 55°C; wall shear stress 150 Pa; constant difference in pressure loss on the retentate side to the permeate side 40 kPa; concentration factor from 1 to 5, expressed as the ratio of casein content in retentate to skim milk, which is close to VCF during 0.1 µm MF.	Flux decreased with increasing casein concentration factor. Permeation of β-LG and α-LA increased with increasing concentration factor. The specific mass flux of β-LG increased with increasing concentration factor and reached a maximum at concentration factor ~2.5. A further increase in concentration factor to 5 decreased the specific mass flux. The protein fractionation was optimal at concentration factor ~2.5.	Kersten (2001)
Wall shear stress		
Ceramic UTP MF, 0.10-µm pore size (Membralox, Société des Céramiques Techniques); feed: skim milk heated at 63°C/15 s; filtration temperature 50°C; VRR 2.0; wall shear stress from 40 to 110 Pa; various constant flux from 30 to 110 L m^{-2} h^{-1}.	Micellar casein retention was > 0.99 in all MF runs. MF at flux 90 L m^{-2} h^{-1} and wall shear stress 110 Pa gave steady MF for 400 min with 70-80% whey protein transmission and almost total casein rejection. Higher flux and/or lower wall shear stress gave divergent runs with a sudden increase in fouling and sharp decrease of transmission. A critical ratio of flux to effective wall shear stress (convection towards the membrane/erosion) was found: ~1.0 L m^{-2} h^{-1} Pa^{-1}. MF below this value gave slow increase of fouling resistance and high whey protein transmission with slow decrease.	Le Berre and Daufin (1996)

Table 1: (*Continued*)

Fouling		
Ceramic UTP MF, 0.1-µm pore size (Orelis); feed: skim milk heated at 63°C/15 s; filtration temperature 50°C; VRR 2.0; wall shear stress 100 Pa; retentate pressure 400 kPa; gradual increase in TMP from 0 kPa to 100 kPa.	TMP < 10 kPa: linear relationship of flux as function of TMP, permeate turbidity low, transmission of whey proteins high, thickness of deposit low. TMP ~10–30 kPa: increased deposit thickness, decreased transmission of whey proteins, increased permeate turbidity. TMP ~30–100 kPa: flux stabilized at a limiting permeation flux (~75 L m^{-2} h^{-1}), continued decrease in whey protein transmission, deposit thickness remained constant.	Gésan-Guiziou et al. (1999)
Ceramic UTP MF, 0.1 µm pore size (Kerasep, Novasep); different feed solutions: skimmed milks, suspensions of native casein micelle powder, and aqueous phases of milk; filtration temperature 48°C; constant flux 50 L m^{-2} h^{-1}; VRR 2.0; wall shear stress decreased step-by-step from 120 Pa to the critical wall shear stress (55 Pa), after 1 h wall shear stress was increased step-by-step to 120 Pa.	MF of thermized skim milk: before the formation of deposit layer (wall shear stress 120 to 80 Pa), TMP was stable (~12 kPa), soluble protein (whey) transmission was constant (~65%). Decrease in wall shear stress to 55 Pa gave quick and sharp increase in TMP and soluble protein transmission was decreased down to 25%. Increase in wall shear stress to 120 Pa yielded 67% increase in TMP, soluble protein transmission was 55%. Aqueous phases of milk (no casein): Decrease in wall shear stress to 55 Pa gave no sharp increase in TMP; however, TMP increased at the end of the filtration. Soluble protein transmission (~80%) was higher than for thermized skim milk. A critical ratio of flux to wall shear stress was found for MF of milk: ~0.91 L m^{-2} h^{-1} Pa^{-1}. Change in transmission of soluble proteins at critical wall shear stress was due to fouling. Casein micelles were the major milk constituent responsible for fouling. Minerals enabled easier set-up of the fouling layer (membrane-micelle interaction and micelle-micelle interaction). Soluble proteins had no effect on the set-up of the fouling layer, but their presence in milk increased irreversible fouling by 20%. Soluble proteins could be rejected or entrapped (e.g. electrostatic interactions) in the micelle deposit, limiting their permeation.	Jimenez-Lopez et al. (2008)

To obtain maximal separation of caseins and whey proteins by MF of skim milk, the skim milk should preferably be unpasteurized (Svanborg et al., 2014) (Table 1). Prior to protein fractionation by MF, the microbial load of the unpasteurized skim milk can be reduced by MF with pore sizes in the range 0.8 to 1.4 µm (Maubois, 2002). The pore size of the membranes for protein fractionation should be chosen carefully to ensure rejection of casein micelles and optimal permeation of whey proteins (Jørgensen et al., 2016; Punidadas & Rizvi, 1998). Casein micelles are polydisperse (Fox & Kelly, 2004), and research supports that the mean diameter of casein micelles varies between individual cows (de Kruif & Huppertz, 2012; Devold et al., 2000), feeding regimens (Devold et al., 2000), and seasons (Glantz et al., 2010). Thus, retention of casein micelles with a specific membrane pore size depends on the casein micelle size distribution of the original milk. In fact, there is no standard to define the separation ability of ceramic membranes, thus the given pore size of a membrane should be considered as an indication of its separating ability, not as a precise definition. The VCF during MF affects the flux and permeation of whey proteins, and a moderate VCF is advantageous for high specific mass flux of whey proteins (Kersten, 2001) (Table 1). The filtration temperature should preferably be high enough to limit microbial growth and promote high flux values ($\geq 50°C$), but low enough to avoid heat denaturation of whey proteins and to limit possible interactions between whey proteins and caseins deposited on the membrane surface ($< 60°C$) (Hurt et al., 2015a; Jimenez-Lopez et al., 2008; Jørgensen et al., 2016; Kersten, 2001). A critical value of the ratio of flux to wall shear stress has been reported (~1.0 L m^{-2} h^{-1} Pa^{-1}), and parameters should be chosen to ensure MF operation below this value, thereby limiting fouling while maintaining high whey protein transmission (Jimenez-Lopez et al., 2008; Le Berre & Daufin, 1996).

The various available hydraulic concepts, the wide range of possible operation parameters, and the interacting hydrodynamic conditions influencing protein fractionation, makes protein fractionation by MF a complex unit operation. Future research should aim for high industrial relevance by modeling industrial applications and procedures, contributing to easier and reliable implementation of MF for optimal protein fractionation in the dairy industry.

Other membranes, like microsieves and dynamic filtration devices, have been developed to exceed performances of conventional MF membranes (Jaffrin, 2008; Saxena et al., 2009). Microsieves have well-defined, uniform pores and low flow resistance due to a very thin

selective silicon-nitride layer. The main advantage of microsieves is the high permeate flux obtainable (Saxena et al., 2009). In 2008, Tetra Pak and FluXXion BV signed a partnership agreement to develop microsieves for removing bacteria from milk. However, the partnership dissolved (Tetra Pak Filtration solutions, personal communication) due to unknown reasons. So far, research on applications of microsieves in milk processing seems to be limited to bacteria removal (Brito-de la Fuente, Torrestiana-Sánchez, Martínez-González, & Mainou-Sierra, 2010; Verwijst, Baggerman, Liebermann, & van Rijn, 2015). In dynamic or shear-enhanced filtration systems, high shear rates are created to limit deposit formation, resulting in higher permeate fluxes and increased membrane selectivity (Jaffrin, 2008). Dynamic filtration systems for processing milk have been investigated for protein concentration by UF (Akoum, Jaffrin, & Ding, 2005; Ding, Zhang, Ould-Dris, Jaffrin, & Tang, 2016; Meyer, Mayer, & Kulozik, 2015) and protein fractionation by MF (Al-Akoum, Ding, Chotard-Ghodsnia, Jaffrin, & Gésan-Guiziou, 2002; Espina, Jaffrin, Frappart, & Ding, 2008). Interestingly, Meyer et al. (2015) and Meyer, Hartinger, Sigler, and Kulozik (2017) suggested that dynamic filtration systems can be operated in cascade mode as a supplement to conventional cross-flow filtration processes to reach higher volume reduction ratios during UF of skim milk (e.g. 30% protein in the final retentate). The investment costs per membrane area of microsieves and dynamic filtration systems are relatively high compared to conventional cross-flow systems with ceramic membranes or polymeric spiral wound membranes (Jaffrin, 2008; Meyer et al., 2015; Saxena et al., 2009; Verwijst et al., 2015). Jaffrin (2008) concluded that the sales of industrial dynamic systems seemed to be limited in volume, but predicted that further development of dynamic systems could make these filtration devices more popular in the future.

3.2.2 Addition of membrane-manufactured powders

Traditionally, SMP has been used to enrich protein and total solids of yogurt milk bases (< 5.6% protein). Tamime et al. (2014) reported from their survey on commercial strained fermented milks, that the carbohydrate content of the products ranged from 1 to 12 g/100 g. They proposed that products with higher carbohydrate contents probably were made by fortifying the yogurt milk base with SMP or whey powder to enhance the yield of strained yogurt, or the product was made by fortification without removing acid whey. Yogurts produced by the addition of SMP or whey powders had higher lactose content and lower protein content, and thus, lower firmness than yogurts produced by concentration after

fermentation, or from UF retentate. The use of membrane-manufactured powders containing less lactose and more protein than in SMP, could enhance the composition and textural characteristics of high-protein yogurts produced by fortification.

Because there are no regulations defining membrane-manufactured powders, a great diversity of terms is used in trade and literature. The dry form of milk retentate from UF of milk is often termed “milk protein concentrate” (MPC), but is also referred to as “retentate powder”, “native milk protein concentrate”, “milk powder from ultrafiltered skim milk”, “skim milk retentate powder”, and “high-protein lactose-free milk powder”. The dry form of retentate from protein fractionation of skim milk by MF is often referred to as “micellar casein concentrate” (MCC). Other names are “native phosphocaseinate”, “micellar casein powder”, and “micellar casein isolate” (Carr & Golding, 2016).

MPC powders are manufactured to contain protein on a dry basis from 42% (MPC42) to 85% (MPC85). The content of lactose of these powders is typically in the range from 46% (MPC42) to 4% (MPC85). Fat and ash contents are around 1.5 and 7%, respectively. Because there is no standard of identity for MPCs, the compositions of these powders can be modified and adapted to their specific applications (Agarwal et al., 2015). When producing MPCs with protein contents above 70%, DF is applied to “wash out” more of the components that are small enough to pass the UF membrane (lactose and ash). The retentate from UF and DF is evaporated to remove more water, and finally spray dried (Singh, 2007).

The composition of MCC powders with respect to ratio of whey protein to casein and content of protein, lactose, and ash depends on the membrane processing (Hurt & Barbano, 2015; Hurt, Zulewska, Newbold, & Barbano, 2010). MPCs provide casein and whey protein in the same ratio as milk, while MCCs in general have an increased casein to whey protein ratio or can be almost devoid of whey proteins. MPCs are therefore used to fortify yogurt (Agarwal et al., 2015), although the addition of WPCs or NWPCs to MCCs (or MPCs) could provide a yogurt milk base with beneficial protein composition.

Solubility, flavor, gelation, water binding, and viscosity are important functional attributes of MPCs used to fortify yogurt milk bases for manufacturing high-protein yogurts. Because the dissolution of an MPC powder is necessary for the expression of other functional

attributes, solubility is regarded as a critical functional property of MPC (Agarwal et al., 2015). MPCs with high protein contents (e.g. MPC 80) have poor solubility upon reconstitution in water at 20°C. However, the solubility is increased at elevated reconstitution temperatures (e.g. 37°C) and if the reconstituted solution is homogenized (e.g. 138 bar) (Sikand, Tong, Vink, & Walker, 2012). Additionally, the solubility of MPCs is higher when reconstituted in milk permeate (Sikand et al., 2012) or in milk (Udabage, Puvanenthiran, Yoo, Versteeg, & Augustin, 2012), than in water. Sikand, Tong, Roy, Rodriguez-Saona, and Murray (2011) observed that the solubility of commercial MPC80s and milk protein isolate (~90% protein) depended on their mineral composition. Solubility was correlated with increased sodium content and reduced calcium, magnesium, and phosphorus content. Mao, Tong, Gualco, and Vink (2012) reported that the solubility of MPC80 could be enhanced by adding NaCl during the DF stage. Gazi and Huppertz (2015) observed that MPC35–MPC90 powders were fully soluble immediately after production. However, the solubility of MPCs with protein contents ≥ 60% changed during storage, and depended on the storage temperature. Solubility of MPC80–MPC90 remained high upon storage at 20°C for 60 d, however it decreased strongly to ~50% solubility upon storage at 37°C for 60 d. The reduced solubility was due to the reduced solubility of caseins, and it was suggested that the insoluble caseins primarily were in the micellar form. According to Udabage et al. (2012), high pressure treatment (e.g. 200 MPa at 40°C) applied to the concentrate before spray drying improved MPC solubility. This increased solubility was linked to the increased concentration of non-micellar casein. Reducing colloidal calcium content by carbon dioxide treatment of the milk before and during UF (Marella, Salunke, Biswas, Kommineni, & Metzger, 2015; Meletharayil, Metzger, & Patel, 2016a), or calcium removal by ion exchange (Bhaskar, Singh, & Blazey, 1999), are other options to tailor the solubility of MPCs for their application in high-protein yogurts. Further research seems to be needed to determine the possible effect of increased content of non-micellar casein in a yogurt milk base prepared from MPC on the various properties of high-protein yogurt.

MPC with a high protein content is, due to its lower lactose content, favorable for fortifying high-protein yogurts. One criterion for MPCs is the solubility of the powder, which can be improved by different processing techniques and controlled by storage temperature (e.g. 20°C) and time. It seems, however, that more research is needed to determine further criteria for optimal MPC application in high-protein yogurts. Further research focusing on the effect

of processing conditions and powder composition on MPC functionality, particularly when applied to high-protein yogurts, could ease the selection of MPC for yogurt manufacturers.

4 Challenges and possibilities in producing high-protein yogurt

4.1 By-products

Concentrating fermented yogurt produces large volumes of acid whey, which became a major concern to the dairy industry with the increased production of high-protein yogurts. Nishanthi, Vasiljevic, and Chandrapala (2017) reported that the composition of acid whey from commercial production of "Greek-style yogurt" differed from sweet whey obtained from commercial hard rennet cheese production. The acid whey contained ~3.2% lactose and, as expected, it had a low pH (4.55) due to relatively high amounts of lactic acid (0.55%). Furthermore, it contained low amounts of total protein (0.24%) and high amounts of calcium (0.13%) and total phosphate (0.18%) compared to the sweet whey (1.04%, 0.06%, and 0.07%, respectively). Only limited amounts of whey proteins are lost to the whey stream during yogurt concentration because most whey proteins are denatured and retained in the product.

According to Wijayasinghe, Vasiljevic, and Chandrapala (2015), the presence of lactic acid hindered the removal of water during evaporation of acid whey and limited lactose crystallization in freeze-dried powders. Additionally, the presence of calcium was reported by Chandrapala and Vasiljevic (2017) to impair lactose crystallization in spray dried lactose powders at certain ratios of lactic acid to calcium (e.g. 1% and 0.12%, respectively). The composition of acid whey makes lactose crystallization challenging. For many manufacturers of high-protein yogurt, there remain two options; distribution of acid whey as animal feed, or disposal of acid whey, both options creating additional costs. Thus, a reduction in the volume of acid whey from production of high-protein yogurts could be beneficial for the dairy industry.

Concentration of the yogurt milk base prior to fermentation remains as a good option to reduce or avoid acid whey production. However, the production of membrane-manufactured liquid concentrates or powders produces milk permeate as a by-product. Milk permeate is, as opposed to acid whey, free from lactic acid, galactose from lactose catabolism, and

fermentation metabolites from yogurt production, and has a neutral pH. The relatively simple composition of milk permeate makes it more suitable for downstream processing than acid whey. Milk permeate could for instance be used for "down-standardization" of SMP to a minimum protein content of 34% (Codex Alimentarius, 2011; Rattray & Jelen, 1996; Williams, D'Ath, & Zisu, 2008). Concentration of the yogurt milk base prior to fermentation can also be combined with subsequent concentration of the yogurt after fermentation. Figure 4 gives an example of the volume distribution of by-products from the production of a high-protein yogurt (8% protein) as influenced by the sequence of the concentration step in the production line. Concentration of the yogurt after fermentation produces ~1.3 kg of acid whey per kg yogurt. Concentration of the yogurt milk base to 5% crude protein by i.e. UF, and subsequent concentration of the fermented yogurt by e.g. nozzle separator approximately halves the volume of acid whey to ~0.6 kg per kg yogurt. By concentrating the yogurt milk base prior to fermentation, production of acid whey is avoided. In the latter examples, the volume reduction of acid whey is balanced by a corresponding volume increase of milk permeate.

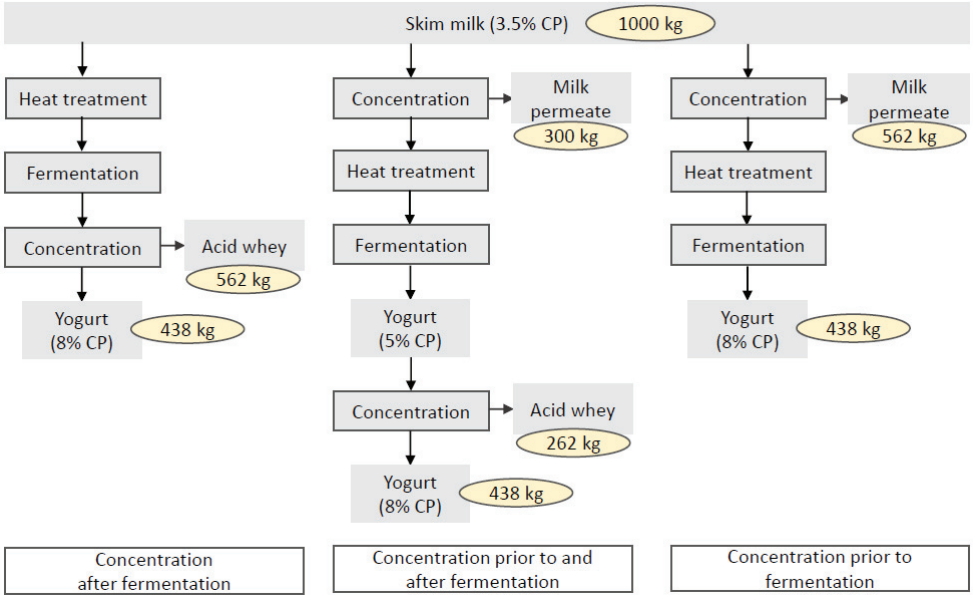

Figure 4: Illustration of volume distribution of by-products from production of high-protein yogurt as influenced by the sequence of the concentration step; concentration after fermentation (e.g. nozzle separator); combination of concentration prior to and after fermentation (e.g. ultrafiltration and nozzle separator); concentration prior to fermentation (i.e. ultrafiltration). Yellow ellipses indicate volumes. Abbreviation: CP, crude protein.

4.2 Yogurt structure and rheology

When manufacturing traditional plain yogurts (< 5.6% protein), textural defects such as a weak body and wheying-off can be caused by low protein levels in the yogurt milk base, insufficient whey protein denaturation during heat treatment, excessive mechanical shearing during pumping and stirring, and/or physical mishandling during distribution (Lucey, 2004). When producing high-protein yogurts (≥ 5.6% protein), an increased protein content of the yogurt milk base gives a yogurt with increased firmness/viscosity and storage modulus (G'), mainly due to the increased amount of protein that can participate in the gel network (Abrahamsen & Holmen, 1980; Biliaderis et al., 1992; Mistry & Hassan, 1992; Schkoda et al., 2001b). Set yogurts fortified with MPC to a protein content above 8% have been reported to possess a grainy texture (Mistry & Hassan, 1992). Stirred yogurts made from yogurt milk bases of liquid protein concentrates with 8% protein and heat-treated at 95°C for 5 min were perceived as grainy and coarse (Jørgensen et al., 2015) (Publication II). The firmness and storage modulus (G') of these yogurts tended to increase with increasing ratio of whey protein:casein. Sensory roughness, coarseness, and graininess of stirred yogurts correlates well with the coagulum particle size of the yogurt (Jørgensen et al., 2015; Krzeminski et al., 2011; Krzeminski et al., 2013; Kücükcetin, 2008a; Laiho et al., 2017; Tomaschunas et al., 2012). Thus, very firm gels with high interconnectivity in the gel network and/or insufficient mechanical shearing of the yogurt gel, makes it more difficult to break the yogurt gel into smaller coagulum particles during stirring.

If the protein content of the yogurt is increased after fermentation, the concentration step will both increase the protein content and provide mechanical shearing. Tamime et al. (1991) observed that pressure driven concentration of Labneh with UF at 35°C decreased the firmness compared to Labneh concentrated by gravitation (cloth bag). Underpinning these results, Ozer et al. (1999a) observed that the viscosity and gel strength of Labneh was halved when using UF at 42°C compared to the traditional cloth bag method. Furthermore, Tamime et al. (1991) observed that an elevated UF temperature (50-55°C) reduced the loss of firmness during pressure-driven concentration with UF. Thus, the forces applied to the yogurt during concentration and the parameters chosen during processing (i.e. temperature) influence the physical properties of the end product.

The coagulum particles of stirred high-protein yogurt should be small enough to ensure a smooth texture (Cayot et al., 2008; Jørgensen et al., 2015; Krzeminski et al., 2011; Krzeminski et al., 2013; Kücükcetin, 2008a; Laiho et al., 2017; Tomaschunas et al., 2012). Cayot et al. (2008) reported that it was impossible to perceive creaminess of stirred yogurts when the coagulum particles were larger than 150 µm. For stirred yogurts with particle sizes below 100 µm, the perception of creaminess was related with the visual and oral consistency of the product, and creaminess increased with increased sensory thickness. Thus, small coagulum particles contribute to smoothness and creaminess, which both are important drivers of liking of high-protein and low-fat yogurts (Desai et al., 2013; Frøst & Janhøj, 2007). However, in the production of high-protein yogurts, processing conditions ensuring small coagulum particles would, to some degree, sacrifice firmness and high viscosity. Because thickness is important for the perceived creaminess of yogurts, a challenge would be to establish processing conditions that yield optimal coagulum particle size, without extensive losses in firmness and viscosity.

Adjustment of the heat treatment load to the yogurt milk base, or implementation of shearing devices in the process line after fermentation seem to be useful unit operations for tailoring structural, rheological, and sensory properties of high-protein yogurts. As reported by Jørgensen et al. (2015) (Publication II), leaving a considerable amount of the whey proteins in their undenatured state (~40-50%) significantly reduced the coagulum particle size and significantly increased the smoothness and shininess of high-protein yogurts (8% protein) containing whey protein:casein ratios of 25:75–35:65. Reducing the heat treatment of the yogurt milk base from 95°C/5 min to 75°C/5 min also reduced the firmness and storage modulus (G') of the stirred yogurts. However, this was necessary to obtain a viscous, smooth, and shiny yogurt.

A reduced heat treatment temperature could possibly result in remaining active bacteriophages in the yogurt milk base, which can cause slow or incomplete fermentation (Quiberoni, Moineau, Rousseau, Reinheimer, & Ackermann, 2010). Ebrecht, Guglielmotti, Tremmel, Reinheimer, and Suárez (2010) isolated bacteriophages of *L. delbrueckii* from yogurt samples of industrial batches with fermentation problems. They reported that heat treatment at 72°C for 2 min could inactivate 99% of bacteriophages, however, heat treatment at 82°C or 90°C inactivated all bacteriophages in less than 2 min. Binetti and Reinheimer (2000) isolated a bacteriophage of *S. thermophilus* from yogurt. Heat treatment

at 72°C for ~10 min gave 99% inactivation, while heat treatment at 90°C for 5 min gave total inactivation. Furthermore, Quiberoni et al. (2003) observed that a bacteriophage of *L. delbrueckii* subsp. *bulgaricus* isolated from yogurt only was totally inactivated at 90°C for 15 min. Thermal resistance is phage-dependent, however, conventional heat treatment (95°C for 5 min) yields a higher log reduction of bacteriophages than 75°C for 5 min. Protective measures against bacteriophages are, in addition to sufficient heat treatment of the yogurt milk base, rigid sanitation in the dairy plant, aseptic handling of yogurt cultures, and culture rotation. Interestingly, it seems that also MF could be a protective measure. Phage retention by membrane filtration has been reported to be dependent on phage morphology (e.g. length) and deposit layer formed during filtration (Gautier, Rouault, Méjean, Fauquant, & Maubois, 1994; Samtlebe et al., 2017a; Samtlebe et al., 2015, 2017b). A 3.4 log lactococcal phage retention was achieved during MF of cheese whey with a 0.1-µm pore size membrane (Samtlebe et al., 2017a). Similarly, a 99.6-99.86% retention was observed for lactococcal phages during MF of milk with a 0.1-µm pore size membrane (Gautier et al., 1994). Thus, the research supports that the NWPC from MF of skim milk with 0.1-µm membranes would be almost phage-free. Yogurt milk bases prepared by blending milk retentate or casein concentrate with NWPC (Jørgensen et al., 2015) (Publication II) could possibly be made phage-free without extensive denaturation of the whey proteins in the NWPC, by separate heat treatments of the concentrates. However, a sufficient amount of whey protein should preferably be present during heat treatment (e.g. 95°C for 5 min) of the casein concentrate to obtain a yogurt milk base with a mixture of micelle-bound and soluble aggregates of whey proteins and κ-CN, thus providing large amounts of aggregating particles (Anema et al., 2004; Guyomarc'h et al., 2003b; Ozcan et al., 2015). Subsequent addition of pasteurized (e.g. 72°C for 15 s) NWPC to the heat-treated yogurt milk base, could provide a good basis for further fermentation into a smooth and viscous high-protein yogurt.

Hahn et al. (2012) investigated the effect of post-processing fresh cheese (8.4% protein) with a rotor/stator-device (Ytron Process Technology GmbH & Co KG) at rotational speeds 300, 1500, and 3000 min^{-1}. Increasing the rotational speed at post-processing reduced the coagulum particle size and storage modulus of the fresh cheese, and at the same time, the product was perceived as more smooth. Similarly, Weidendorfer, Bienias, and Hinrichs (2008), reported that the storage modulus (G') of yogurt (3.9% protein) was reduced upon increased mean peripheral velocities during post-processing of yogurt with a colloidal mill. Below a certain storage modulus (e.g. 170 Pa), syneresis was observed on the yogurt surface

after 3 weeks of storage. This observation underpins the importance of determining the effect of processing conditions on the amount of surface-whey, which is considered as a quality defect in yogurt (Lucey, 2001). However, it is conceivable to think that high-protein yogurts could have a better resistance against whey separation caused by post-processing than traditional yogurts (< 5.6% protein), as high-protein yogurts have higher storage modulus and higher firmness caused by a more dense coagulum. Meletharayil et al. (2016a) studied the effect of hydrodynamic cavitation (SPX Flow Technology) at different rotor speeds on rheological and structural properties and water-holding capacity of "Greek yogurt" (~9% protein). Increasing the extent of cavitation (rotor speed) increased the structural breakdown, and thereby reduced the firmness and number of grains (perimeter > 1 mm) in the yogurt. Interestingly, cavitated yogurt had better water-holding capacity than a non-cavitated yogurt and a commercial "Greek yogurt". Their results suggested that cavitation led to the incorporation of moisture as finely dispersed molecules into the protein matrix. Application of post-processing unit operations, like rotor/stator devices or shock wave reactors (e.g. hydrodynamic cavitation), seems promising in terms of reducing coagulum particle sizes of high-protein yogurts. The effect of these treatments on the yogurt bacteria due to elevated temperatures caused by cavitation (Milly, Toledo, Kerr, & Armstead, 2008; Moholkar & Pandit, 2001) might require investigation. Additionally, concentration prior to fermentation could potentially yield a very firm yogurt gel, challenging the pumping capacity and downstream processing, for instance agitation of fermented yogurt and emptying of fermentation tanks.

The structure of high-protein yogurt could also be improved by adding fat to the yogurt milk base. An increased fat content reduces the coagulum particle size of the yogurt and increases the viscosity and storage modulus (G') (Brauss et al., 1999; Krzeminski et al., 2011). Cream can be added to the yogurt milk base or to the fermented yogurt. The addition of cream to the fermented yogurt gives the opportunity to produce yogurts from the same yogurt milk base with various fat and protein compositions, which increases the flexibility of yogurt production. However, as observed by Schkoda et al. (2001a), the added fat only contributed to the gel network as pseudocasein particles and increased the viscosity of the fermented milk when the cream was added to the milk prior to fermentation and homogenized (150 bar at 62°C) together with the other milk constituents. When the homogenized cream (100/20 bar at 70°C) was added to the fermented milk after fermentation, a slight increase in serum-

binding capacity was observed; however, the viscosity of the fermented milk decreased as the amount of added cream increased.

For yogurts with reduced fat content, microparticulated whey proteins with a high ratio of native-to-denatured whey proteins can be added to the yogurt milk base to increase the elastic modulus (G') and creaminess of the yogurt (4.25 or 5% protein, 0.5% fat) (Torres, Janhøj, Mikkelsen, & Ipsen, 2011). Yazici and Akgun (2004) reported that microparticulated whey proteins added to the yogurt milk base (0.5% fat) could improve the sensory properties of strained yogurt (~2% fat, ~12% protein), depending on amount of microparticulated whey proteins added and storage time of yogurt. However, creaminess is, besides smoothness and thickness/viscosity, correlated with fatty afterward mouth-feel and creamy/milk fat flavor. Thus, high-protein yogurt with a high intensity of creaminess is only obtainable if milk fat is also present (Desai et al., 2013; Folkenberg & Martens, 2003a; Frøst & Janhøj, 2007; Tomaschunas et al., 2012).

Yogurt milk bases with high protein contents have high buffering properties, resulting in increased fermentation times to obtain a predetermined pH-value (Salaün et al., 2005). Peng, Horne, and Lucey (2009) studied the effect of fermentation time by varying the amount of yogurt culture added to yogurt milk bases of recombined SMP. Longer fermentation time yielded yogurts with lower storage modulus (G' at pH 4.60), increased whey separation, and microstructures with large strands with fewer apparent interconnections in the strands and larger pores. Jørgensen et al. (2015) (Publication II) observed that stirred high-protein yogurt (~8% protein) produced from casein concentrate from MF of skim milk, had microstructures with large protein clusters and large pores, and the yogurt was perceived as granular and coarse by sensory evaluation. The increased fermentation time of this yogurt, compared to yogurts with increased whey protein to casein ratios, was linked to the increased amount of buffering compounds such as caseins and CCP. Peng et al. (2009) explained that increased fermentation time increased the time allowed for rearrangements, allowing strands and protein clusters to aggregate further, resulting in denser clusters and larger pores. Shorter fermentation time gave yogurts with finer structures and more branching. Thus, it is possible that reducing the CCP of liquid or dried concentrates from membrane filtration could improve structural and rheological properties (e.g. finer microstructures, smoother texture) of high-protein yogurts produced by concentration prior to fermentation. The calcium and ash content of casein concentrates

(MF) or milk retentates (UF) can be reduced by pre-acidification of the milk prior to filtration (Brandsma & Rizvi, 1999; Marella et al., 2015). Meletharayil et al. (2016a) observed that a high-protein yogurt (~9% protein) produced from MPC with reduced CCP had shorter fermentation time than a yogurt produced from MPC where no CCP had been removed. The use of MPC with reduced CCP in combination with hydrodynamic cavitation of the fermented yogurt yielded a high-protein yogurt with similar physical characteristics (titratable acidity, rheological properties, and microstructure) as a commercial strained high-protein yogurt. On the other hand, Peng et al. (2009) reported that lower pre-acidification pH of recombined SMP (added GDL before heat treatment and fermentation), gave yogurt gels (~4% protein) with lower storage modulus (G') and higher whey separation. However, it is conceivable to think that high-protein yogurts would respond differently than yogurts with lower protein content (e.g. 4%) to yogurt milk bases where the CCP has been reduced prior to fermentation. As discussed previously, sensory properties of high-protein yogurts can benefit from treatments that reduce storage modulus (G') and firmness of the yogurt gel to a certain degree.

4.3 Flavor

Some reported flavor defects in high-protein yogurts are burnt/beefy flavor, too acidic flavor, bitter flavor, and astringent mouthfeel.

Desai et al. (2013) reported that burnt/beefy flavor, i.e. aromatics associated with sulfurous compounds or beef broth, was a consistent driver of dislike of commercial "Greek yogurts". A burnt/beefy flavor was detected in some fortified "Greek yogurts" containing WPC or MPC, but not in the strained yogurts. Sulfurous compounds may originate from the WPC powder (Carunchia Whetstine, Croissant, & Drake, 2005; Carunchia Whetstine, Parker, Drake, & Larick, 2003; Lee, Laye, Kim, & Morr, 1996; Wright, Zevchak, Wright, & Drake, 2009) or the MPC powder used for fortification (Drake, Miracle, & Wright, 2009; Smith, Campbell, Jo, & Drake, 2016), or may be produced during heat treatment of the yogurt milk base (White, Fox, Jervis, & Drake, 2013). According to Drake et al. (2009), rehydrated NWPC (34% protein) has a superior flavor to WPC from Cheddar whey (34% protein), due to its bland taste and low flavor intensity. Evans, Zulewska, Newbold, Drake, and Barbano (2009) reported that sulfur-containing aroma compounds were more prevalent in spray-dried

than in freeze-dried WPC and NWPC (serum protein concentrate). This was explained by the higher denaturation degree of whey proteins during spray drying, and consequently higher amount of degradation products (e.g. dimethyl disulfide) from sulfur-containing amino acids. The aroma intensity of dimethyl disulfide measured by gas chromatography-olfactometry was higher in WPC than in NWPC regardless of drying technique, yet the overall aroma intensities of these powders were low as evaluated by a sensory panel. Evans et al. (2009) also compared WPC and NWPC produced in their study to six commercial WPCs with similar moisture and protein contents. The commercial WPCs generally had higher sensory intensities of the attributes cardboard, diacetyl, and astringent, and higher concentrations of volatile compounds related to lipid oxidation (hexanal, heptanal, pentanal), fermentation (diacetyl), and degradation of sulfur-containing amino acids (dimethyl disulfide). These differences indicated the significant influence of factors such as milk source, processing conditions, and storage on whey protein powders. In the study by Desai et al. (2013), one of the best-liked yogurts, after the full fat yogurt, was a fat-free protein-fortified yogurt (fortification not defined). Thus, careful selection of dairy protein powders for fortification of yogurt milk bases in the production of high-protein yogurts seems important.

Sensory analysis of high-protein yogurts produced from yogurt milk bases of liquid NWPC and casein concentrates showed no off-flavors or bitter flavor (Jørgensen et al., 2015) (Publication II). The use of liquid dairy protein concentrates in the production of high-protein yogurts may exclude potential undesirable flavor compounds produced during powder manufacture and storage. However, further research is needed to evaluate the influence of the origin (source, processing, storage) of whey protein and casein on the physical and sensory properties of high-protein yogurts.

According to Desai et al. (2013), consumers of “Greek yogurt” differed in respect to liking of sour taste, defined as the basic taste associated with acid. Consumer cluster analysis revealed that one group of consumers liked yogurts with high sour taste, while for another group of consumers, sour taste was disliked. Fortified yogurts were scored as “too sour” by 50% or more of the consumers (n=155). The rate of pH reduction during fermentation is controlled by the buffering properties of the yogurt milk base. Yogurt milk bases with increased protein contents will have high buffering capacities due to the elevated presence of buffering compounds, such as CCP and caseins (Salaün et al., 2005). Jørgensen et al.

(2015) (Publication II) reported that the rate of pH reduction during fermentation of yogurt milk bases with true protein contents of ~8% to pH 4.60 significantly decreased with decreasing whey protein to casein ratios. The yogurts with the longest fermentation times (highest casein, calcium, and phosphorus content), also had the highest content of lactic acid in the final yogurts. Similar observations were reported by Amatayakul, Halmos, Sherkat, and Shah (2006) for yogurts with ~3% total protein. Jørgensen et al. (2015) (Publication II) found, however, no differences in perceived intensities of acid taste of high-protein yogurts with different pH reduction rates. Less lactic acid is produced if the fermentation of the yogurt milk base is stopped at a higher pH. A higher final fermentation pH (e.g. 4.8) may positively influence the characteristics of the yogurt, in terms of less acidic taste and smoother yogurt structure (Kücükcetin, 2008b; Martin, Skokanova, Latrille, Beal, & Corrieu, 1999). A higher final fermentation pH has, however, been reported to give higher degree of syneresis, lower storage modulus (G'), and lower apparent viscosity of final yogurts with protein content less than 5.6% (Kücükcetin, 2008b; Martin et al., 1999). Stopping the fermentation of high-protein yogurts at a higher pH could be an interesting approach to reduce challenges related to excessively firm yogurt gel, graininess, and acidic taste. Further research is needed to evaluate the effect of a higher final fermentation pH on sensory and physical properties of high-protein yogurt produced by concentration prior to fermentation.

Another important aspect of producing high-protein yogurts with the use of liquid or dried protein concentrates is the possible presence of plasmin. Because plasmin is concentrated with the caseins during MF (Aaltonen & Ollikainen, 2011), the use of casein concentrates could give a yogurt milk base with increased plasmin activity. Plasmin and plasminogen-derived activity has also been observed in commercial MPCs and micellar casein isolates (Gazi, Vilalva, & Huppertz, 2014). The optimum activity of plasmin is at pH 7.5 and 37°C (Bastian & Brown, 1996; Ismail & Nielsen, 2010), however, proteolysis by plasmin during fermentation (42°C) and storage (7°C) of yogurt (pH ~4.25) has been reported (Gassem & Frank, 1991). Plasmin can cause hydrolysis of caseins in yogurt, leading to the formation of bitter peptides (Lemieux & Simard, 1991, 1992). Bitter taste in yogurt has been reported to positively correlate with astringent mouthfeel, described as a puckering or tingling sensation on oral tissues (Brown & Chambers, 2015). Astringency in milk products can be caused by different compounds (Lemieux & Simard, 1994), including γ-CN from plasmin-induced degradation of β-CN (Harwalkar, Cholette, McKellar, & Emmons, 1993). Desai et al.

(2013) reported that fortified commercial "Greek yogurts" in general, and among other descriptors, were described as astringent, while strained yogurts were not. Mistry and Hassan (1992) suggested that the bitterness of high-protein yogurt produced by MPC fortification could be linked to proteolytic activity of the yogurt starter bacteria in the absence of lactose.

Whether astringency and bitterness of high-protein yogurts can be linked to possible casein degradation by plasmin or proteolytic activity of the yogurt bacteria, or presence of other compounds, like calcium salts (Lemieux & Simard, 1991; Tordoff, 1996; Yang & Lawless, 2005), remains to be investigated. The plasmin system in milk is a complex system influenced by the presence of activators and inactivators and processing conditions such as heat treatment (Ismail & Nielsen, 2010). Further research could reveal the effect of yogurt milk base composition (i.e. whey protein to casein ratio, calcium salts) and heat treatment on plasmin activity and potential development of bitter taste and astringency, especially in high-protein yogurts produced by concentration prior to fermentation.

The use of membrane-manufactured powders or liquid concentrates for preparation of yogurt milk bases changes the milk substrate and affects the growth of the yogurt starter culture. Özer and Robinson (1999) investigated the behavior of a yogurt culture with a 1:1 ratio of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* in concentrated yogurts produced by concentration prior to or after fermentation. In the yogurt milk bases with 160 g kg^{-1} total solids content, *S. thermophilus* had an exponential growth phase ending at around 180 min of incubation (pH 5.2–5.6). After this, *S. thermophilus* entered a stationary phase, while *L. delbrueckii* subsp. *bulgaricus* grew more rapidly until the end of fermentation at pH 4.3. Fermented yogurts were concentrated to ~23% total solids content and 7.5% protein by UF or by straining (cloth bag). The counts of *S. thermophilus* continued to increase during and after concentration (storage), although the temperature during UF was 50°C. For the yogurt milk base concentrated to ~22% total solids and 7.8% protein by UF prior to fermentation, *S. thermophilus* increased rapidly up to 180 min and then growth ceased due to high lactic acid content (1.1%). The growth of *L. delbrueckii* subsp. *bulgaricus* started at an earlier point (120 min) than in the unconcentrated yogurt milk bases, and continued to increase during fermentation and storage at 4°C. Yogurt concentrated by UF after fermentation had significantly more acetaldehyde and had lower acidity than the yogurt produced from UF milk, reflecting the favorable growth pattern of

yogurt bacteria in the yogurt concentrated after fermentation. Interestingly, this difference was not noted when the sensory panel evaluated aroma/flavor intensity. The results of Özer and Robinson (1999) support that manufacturers should take into consideration the production method when selecting yogurt bacteria. Yogurt starters that allow the development of yogurt aroma (e.g. acetaldehyde) with restricted post-acidification and post-proteolytic activity are favorable.

5 Conclusions and future perspectives

The reviewed research supports that firmness and storage modulus (G') of acid milk gels and yogurts increases with increasing protein content and increasing amount of denatured whey proteins. Furthermore, research suggests that firmness of yogurts with constant protein contents increase with increasing proportions of denatured whey proteins in the yogurt milk base (reviewed studies covered whey protein to casein ratios from 10:90–60:40). Additionally, the firmness and storage modulus of high-protein yogurts have shown to increase with the use of yogurt milk bases with smaller casein micelle size distributions.

Very firm yogurt gels with high interconnectivity in the gel network and/or insufficient mechanical shearing of the yogurt gel possess increased coagulum particle size, correlated with increased sensory roughness, coarseness, lumpiness, and graininess of the stirred high-protein yogurt. Because consumer liking of high-protein yogurts is driven by smoothness, high viscosity, thickness, and creaminess, manufacturers should strive to obtain high-protein yogurts with small coagulum particles without excessive losses of firmness and viscosity.

Research supports that high-protein yogurts with high sensory qualities can be obtained by concentrating the yogurt milk base prior to fermentation, and thereby without production of acid whey. High-protein, non-fat yogurts (8% protein) can be obtained by adding NWPC to casein concentrate from MF of milk. A reduction of the heat treatment load to the yogurt milk base is necessary to reduce whey protein denaturation, and thus reduce the firmness of the yogurt gel and the coagulum particle size of the stirred yogurt. The combination of a high protein content and remaining undenatured whey proteins in the heat-treated yogurt milk base (13-15 mg mL^{-1}, approximately 50% of the available whey proteins in the yogurt) ensures a smooth and viscous stirred high-protein yogurt.

High-protein yogurts can also be produced by fortifying the yogurt milk base with dairy protein powders, such as MPC. Selection of a dairy protein powder with a bland flavor could provide a high-protein, non-fat yogurt with good sensory properties. The use of CCP-reduced MPC in combination with post-processing treatment with shearing devices (e.g. rotor/stator or shock wave reactors) could reduce the coagulum particle size and provide a smooth high-protein yogurt.

Research-based knowledge about the impact of processing conditions on rheology, structure, and particularly sensory properties, of high-protein yogurt, is still limited. Further research is needed on the following areas:

- A fundamental understanding of how the composition (e.g. whey protein:casein ratio, casein micelle size, CCP, lactose) and heat treatment of the yogurt milk base influence the formation of micelle-bound and soluble aggregates of whey proteins and κ-CN, and furthermore how this affect the mechanisms of yogurt gel formation, and the structure, rheology, and sensory properties of high-protein yogurt. Micelle-bound and soluble aggregates of whey proteins and κ-CN provide points of attachment during yogurt gel formation. The balance of bound and soluble aggregates, and the size of the soluble aggregates, could influence the firmness and structure of the yogurt gel, and thus the sensory properties of the yogurt.
- Investigation of the impact of a higher final fermentation pH, or a reduced CCP content of the MPC or the casein concentrate, for instance by pre-acidification of the milk prior to filtration. A shorter fermentation time could provide a finer yogurt structure with more branching, avoiding rearrangements of the gel into dense protein clusters. A higher final fermentation pH could ameliorate challenges in the production of high-protein yogurt related to too-firm yogurt gel, graininess, and too acidic flavor.
- Studies investigating the reasons for development of bitterness and astringent mouthfeel of high-protein yogurts, with an emphasis on the effect of plasmin present in the MPC or casein concentrate, proteolytic activity of the yogurt bacteria, and/or development of calcium salts during fermentation.
- Evaluation of the effect of the origin of whey protein and casein ingredients on the physical and sensory properties of high-protein yogurt. Sulfurous compounds causing off-flavor of high-protein yogurt may originate from the dried protein powder (e.g. MPC or WPC) used for fortification. The flavor of high-protein yogurts

may benefit from the bland flavor of liquid protein concentrates (e.g. casein concentrates, NWPC, or milk retentate).

- Studies on the effect of concentration prior to fermentation by using liquid or dried protein concentrates, in combination with concentration after fermentation on the composition, physical, and sensory properties of high-protein yogurts. The forces applied to the yogurt during concentration (mechanical or pressure-driven), and the parameters chosen during post-processing (i.e. temperature) influence the physical properties of the end product.

Furthermore, there seems to be a lack of consistency in the Codex standards with respect to distinguishing high-protein yogurts and fresh cheeses like quargs. Closing these loopholes could ensure fair practices in the trade of fermented dairy products; however, efforts should be made to ensure that the Codex standards allow for innovative ways of processing high-protein yogurt. Legislative provisions covering the composition of fermented dairy products could clarify the distinction between traditional and high-protein yogurts.

There is no simple and straight forward answer to what is the best approach to produce high-protein yogurts. However, the dairy industry should strive for optimal utilization of the macro components of the milk. MF is a technology with a high potential for optimal utilization of the milk proteins, and provides superior ingredients for further processing into high-protein yogurts with high sensory qualities.

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Optimization of protein fractionation by skim milk microfiltration: Choice of ceramic membrane pore size and filtration temperature

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ABSTRACT

The objective of this study was to investigate how ceramic membrane pore size and filtration temperature influence the protein fractionation of skim milk by cross flow microfiltration (MF). Microfiltration was performed at a uniform transmembrane pressure with constant permeate flux to a volume concentration factor of 2.5. Three different membrane pore sizes, 0.05, 0.10, and 0.20 μm, were used at a filtration temperature of 50°C. Furthermore, at pore size 0.10 μm, 2 different filtration temperatures were investigated: 50 and 60°C. The transmission of proteins increased with increasing pore size, giving the permeate from MF with the 0.20-μm membrane a significantly higher concentration of native whey proteins compared with the permeates from the 0.05- and 0.10-μm membranes (0.50, 0.24, and 0.39%, respectively). Significant amounts of caseins permeated the 0.20-μm membrane (1.4%), giving a permeate with a whitish appearance and a casein distribution (α_{S2}-CN: α_{S1}-CN: κ-CN: β-CN) similar to that of skim milk. The 0.05- and 0.10-μm membranes were able to retain all caseins (only negligible amounts were detected). A permeate free from casein is beneficial in the production of native whey protein concentrates and in applications where transparency is an important functional characteristic. Microfiltration of skim milk at 50°C with the 0.10-μm membrane resulted in a permeate containing significantly more native whey proteins than the permeate from MF at 60°C. The more rapid increase in transmembrane pressure and the significantly lower concentration of caseins in the retentate at 60°C indicated that a higher concentration of caseins deposited on the membrane, and consequently reduced the native whey protein transmission. Optimal protein fractionation of skim milk into a casein-rich retentate and a permeate with native whey proteins were obtained by 0.10-μm MF at 50°C.

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Key words: protein fractionation, ceramic membrane pore size, filtration temperature, uniform transmembrane pressure, constant flux

INTRODUCTION

The main proteins in milk, the caseins and whey proteins, differ in their functional and nutritional characteristics, and it is of interest to the dairy industry to separate these proteins. The caseins can be used to produce cheese, and high protein beverages and fermented milks. Whey proteins derived from microfiltration (**MF**) of milk are commonly referred to as native whey, ideal whey, or virgin whey. Native whey, as opposed to cheese whey, is free from somatic cells, lactic acid bacteria, bacteriophages, remnants of rennet (Maubois, 2002), cheese fines, and the glycomacropeptide from κ-CN (Brans et al., 2004). The neutral taste and pH, native protein conformation, and nutritional quality of whey proteins make native whey an excellent end product or ingredient in products addressed to infant, elderly, or sports nutrition.

Casein micelles and whey proteins can be separated by the use of MF with membranes with pore sizes in the range of 0.05 to 0.20 μm (Brans et al., 2004). The MF membrane material (i.e., ceramic, polymeric) and the membrane design and system [i.e., ceramic gradient, ceramic uniform transmembrane pressure (**UTP**), polymeric spiral-wound] influence the efficiency of whey protein removal, but also overall costs and cleaning procedures. Ceramic membranes in a UTP system give significantly better whey protein removal than ceramic graded permeability membranes and polymeric spiral-wound membranes (Zulewska et al., 2009). Optimal separation of caseins and whey proteins is of interest to the dairy industry. Therefore, the focus of this paper is on ceramic membranes in a UTP system. The composition of retentates and permeates from MF of skim milk is also influenced by several other factors: the composition of the skim milk, the pretreatment of the skim milk (Brandsma and Rizvi, 1999; Hernández and Harte, 2009; Svanborg et al., 2014), membrane

pore size (Punidadas and Rizvi, 1999), channel geometry (Adams et al., 2015a), filtration temperature (Van Hekken and Holsinger, 2000; Hurt et al., 2015; Seibel et al., 2015), concentration factor (Punidadas and Rizvi, 1999; Kersten, 2001), wall shear stress (Le Berre and Daufin, 1996), and fouling (Le Berre and Daufin, 1996; Gésan-Guiziou et al., 1999, 2000; Jimenez-Lopez et al., 2008; Lawrence et al., 2008; Adams et al., 2015b).

Ceramic MF of skim milk to separate caseins and whey proteins is usually carried out at temperatures ranging from 50 to 55°C. Operating at higher filtration temperatures (>50°C) gives the potential benefit of reducing microbial growth (Walstra et al., 2006) and increasing flux as reported by Kersten (2001). However, Kersten (2001) observed a flux decline at temperatures above 55°C, explained by the precipitation of calcium phosphate. Hurt et al. (2015) reported, as opposed to Kersten (2001), that calcium phosphate precipitation did not cause membrane fouling when increasing the MF temperature from 50 to 65°C. They observed, however, a decrease in whey protein transmission with increasing filtration temperature, partly explained by the possible denaturation of whey proteins. Thus, the possible denaturation of whey proteins may be another disadvantage of MF of skim milk at higher temperatures. Significant denaturation of α-LA and β-LG occurs on heating milk above about 70°C (Anema, 2009), although conformational changes of β-LG has been reported to take place already at temperatures of 40°C (Qi et al., 1995, 1997). The separation of milk into cream and skim milk is usually carried out at around 57°C (range 55–65°C). In a continuous milk treatment process with ceramic MF at 60°C, the skim milk can be fed directly to the filtration process without temperature adjustment. The pumping energy and the friction forces arising from the flow of feed through the MF channels contribute to a temperature increase of the feed, and the temperature is likely to rise from 57 to 60°C. Thus, ceramic MF at 60°C might be relevant for the dairy industry.

Ceramic membranes with different pore sizes are available on the market. The effect of membrane pore size on the composition of MF retentate and permeate could possibly influence the choice of membrane pore size in an industrial MF application. Optimal fractionation of caseins and whey proteins is of interest to the dairy industry due to their different functional properties. Optimization of native whey protein removal and utilization of native whey proteins in value-added products may be a key to increase profitability of a ceramic MF process. For instance, relatively small differences in whey protein concentration in the MF permeate could have a major effect on the economic feasibility of an MF process with the goal to produce a native whey protein concentrate. Punidadas and Rizvi (1999) investigated the effect of gradient membranes with pore sizes of 0.05 and 0.20 μm on the composition of retentates and permeates from MF of skim milk. They reported that a portion of casein passed through the 0.20-μm membrane, whereas almost all the caseins were retained by the 0.05-μm membrane at the same time that whey proteins permeated the membrane. According to Zulewska et al. (2009), the permeate from MF of skim milk with gradient membranes has a higher casein proportion compared with permeate from MF of skim milk with ceramic membranes operated at a UTP. Information concerning the effect of different pore size on the protein fractionation of skim milk in a UTP MF system seems to be lacking and should be further investigated.

Milk proteins are the most valuable constituent of milk, and quantitative determination is important. Electrophoresis and column liquid chromatography are the main techniques used to separate and quantify milk proteins (Dupont et al., 2013). Protein compositions as found with capillary electrophoresis and reversed-phase HPLC are often given as relative values (Miralles et al., 2003; Heck et al., 2008; Svanborg et al., 2014) because of the difficulties in using standard curves for quantification of protein concentrations due to the impurity of the protein standards. Important information may get lost with the interpretation of relative values. A useful procedure to calculate real protein concentration values based on capillary electrophoresis is therefore presented in the present study.

Kersten (2001) and Hurt et al. (2015) studied the effect of temperature on protein fractionation using an MF system run in recycle mode at a constant transmembrane pressure (**TMP**) resulting in a minimal change in flux with filtration time. Investigation of the effect of MF temperature in recycle mode cannot preclude potential effects of the recycle time on the protein fractionation. Industrially, it is common to run MF processes at flux values above the critical flux to maximize the utilization of the membrane area, although operation above the critical flux causes fouling and can reduce operating time (Gésan-Guiziou et al., 2000). In an industrial MF process, the flux is kept constant because downstream unit operations in a continuous process are depending on a constant flow.

The objective of this study was to investigate the effect of membrane pore size and filtration temperature on protein fractionation of skim milk by MF to optimize fractionation of caseins and whey proteins. Ceramic MF in a UTP system was performed with a volume concentration factor of 2.5 and with a constant permeate flux to model an industrial MF application.

MATERIALS AND METHODS

Experimental Design

Three pore sizes, 0.05, 0.10, and 0.20 μm, were investigated at filtration temperature of 50°C. At a pore size of 0.10 μm, 2 filtration temperatures were investigated: 50 and 60°C. The scope of the experiments rendered it impossible to investigate the effect of all factor levels on the same milk; therefore, the experiments were performed with 7 different milk deliveries. Two MF experiments were performed on milk from each milk delivery (the day of delivery and the following day); thus, a total of 14 MF experiments were conducted.

Microfiltration Feed Preparation

Milk was obtained from the university farm and separated (Westfalia Separator AG, MSD50–01–076, Oelde, Germany) at 63°C to an average fat content of 0.06 ± 0.01%. The skim milk was pasteurized in a plate heat exchanger (M6-MFMC, Alfa-Laval, Lund, Sweden) at 73°C for 15 s and cooled to 4°C. The pasteurized skim milk was split into 2 parts. One part was microfiltered the same day, and one part was stored at 4°C and microfiltered the following day. Before MF, the skim milk was gently heated under continuously stirring to 45°C (Δ4°C–45°C = 27 to 34 min) in a steam connected double-jacketed 300-L tank. The skim milk was kept at 42 to 45°C for 15 min to partially reverse potential β-CN leakage from the casein micelle (Rose, 1968; Liu et al., 2013) and solubilization of minerals (Schmitt et al., 1993) caused by cold storage of the milk.

Microfiltration

Microfiltration was performed on a pilot-scale MF system (Membranteknikk AS, MTCVV 3–25, Flekkefjord, Norway) equipped with a permeate pump (UTP system) and a cold permeate system according to US Patent No. 13/635,335 (NO Pat. No. 330,181; Hoffmann, 2011). The MF system is schematically presented in Figure 1 and the specifications of the ceramic membranes used are given in Table 1. All ceramic membranes were 1.178 m in length and had round retentate flow channels.

Before MF of milk, the system was equilibrated with water. The centrifugal pump was adjusted to reach a crossflow velocity of 6.7 m/s with water, leading to a target crossflow velocity of 6.9 m/s with pasteurized skim milk (Table 1). Table 2 gives an overview of the operational values during MF of pasteurized skim milk. The MF was performed with a volume concentration

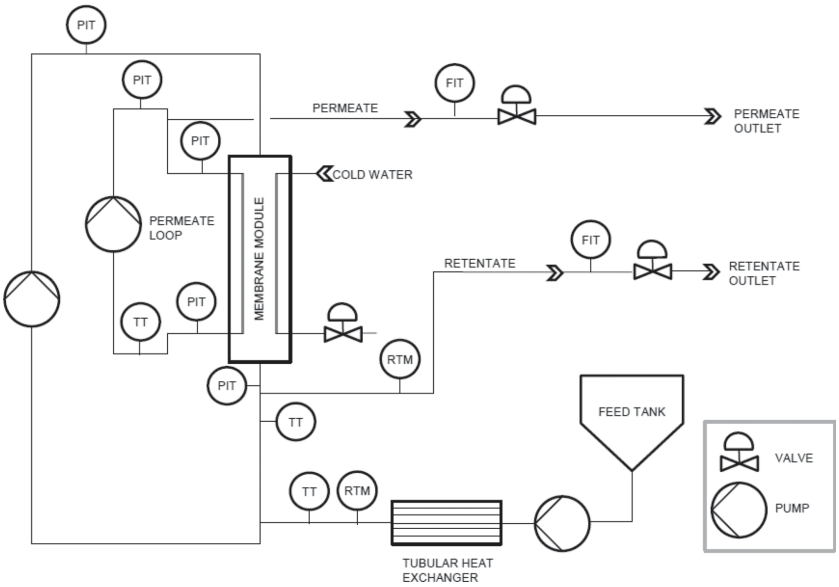

Figure 1. Schematic diagram of the microfiltration membrane system including a permeate loop with cold permeate system. FIT; flow indicator transmitter, PIT; pressure indicator transmitter, RTM; relative turbidity monitor, TT; temperature transmitter.

Table 1. Specification of ceramic membranes used for microfiltration of pasteurized skim milk

Item	Membrane pore size (μm)		
	0.05	0.10	0.20
Producer[1]	Orelis	Orelis	Atech
Hydraulic diameter (mm)	2.9	2.9	3.3
Number of membranes used for microfiltration	2	2	3
Membrane area/membrane (m^2)	0.34	0.34	0.24
Number of channels/membrane	31	31	19
Membrane material	TiO_2/ZrO_2	TiO_2/ZrO_2	α-$Al_2O_3/TiO_2/ZrO_2$
Δ Pressure (kPa) giving crossflow velocity of 6.9 m/s	290[2]	290[2]	260[3]

[1]Orelis Environnement SAS, Salindres, France. Atech Innovations GmbH, Gladbeck, Germany.

[2]Information given by producer.

[3]Calculated value.

factor of 2.5 at constant flux. Due to the increased TMP with decreasing pore sizes, the constant flux at MF with the 0.05 μm membrane was set to 44 L/m^2 h compared with a constant flux of 58.6 L/m^2 h for membranes with 0.10 and 0.20 μm pore sizes. Minimal variation in the skim milk composition made it possible to use the same volume concentration factor in all replicate blocks.

The skim milk temperature at the inlet of the feed tank was 45°C. The filtration temperature of the skim milk was measured at the outlet of the module and was adjusted by controlling the tubular heat exchanger and the cold permeate system (Figure 1). In addition, the pumping energy and the friction forces arising from the flow of feed through the membrane channel contributed to a temperature increase of the feed. The lower pump capacity required to obtain the set crossflow velocity during MF with the 0.20-μm membrane (larger hydraulic diameter) resulted in a lower temperature increase than for the 0.05- and 0.10-μm membranes. For this reason, the feed temperature adjusted by the tubular heat exchanger was increased to a set temperature of 50°C for the 0.20-μm membrane (Table 2). The higher increase in temperature caused by friction in the membranes with smaller hydraulic diameters (0.05 and 0.10 μm) was adjusted by reducing the permeate temperature (Table 2). A filtration temperature of 60°C during MF with the 0.10-μm membrane was reached by increasing both the feed temperature and the permeate temperature.

A MilkoScan FT1 (Foss, Hillerød, Denmark) with Fourier transform infrared analytical technology was

Table 2. Operational values (mean ± SD, n = 3 or 4) during microfiltration of pasteurized skim milk at 50°C with ceramic membranes with pore sizes of 0.05, 0.10, and 0.20 μm, and at 60°C with a 0.10-μm membrane

Item	Filtration temperature 50°C			Filtration temperature 60°C
	0.05 μm	0.10 μm	0.20 μm	0.10 μm
Pressure retentate inlet (kPa)	487 ± 0	487 ± 1	459 ± 1	487 ± 1
Pressure permeate inlet (kPa)	398 ± 7	407 ± 9	416 ± 0	413 ± 5
Pressure retentate outlet (kPa)	196 ± 0	195 ± 1	196 ± 0	195 ± 1
Pressure permeate outlet (kPa)	106 ± 7	114 ± 8	153 ± 0	120 ± 5
Mean TMP[1] (kPa)	89 ± 7	81 ± 8	43 ± 1	75 ± 5
τ_w[2] (Pa)	179	180	180	184
Feed temperature (°C)	44.0 ± 0.2	45.4 ± 1.6	50.3 ± 0.3	52.7 ± 0.6
Filtration temperature (°C)	50.1 ± 0.1	50.1 ± 0.2	50.0 ± 0.2	59.9 ± 0.1
Permeate temperature (°C)	44.4 ± 0.1	43.8 ± 0.1	48.6 ± 0.3	55.6 ± 0.2
Flow retentate (L/h)	20 ± 0	27 ± 0	28 ± 0	27 ± 0
Flow permeate (L/h)	30 ± 0	40 ± 0	42 ± 0	40 ± 0
Flux (L/m^2 h)	44.1 ± 0.0	58.9 ± 0.1	58.3 ± 0.0	58.9 ± 0.1
Volume concentration factor[3]	2.5 ± 0.0	2.5 ± 0.1	2.5 ± 0.0	2.5 ± 0.1

[1]TMP = transmembrane pressure = $\{[(Rp_i - Pp_i) + (Rp_o - Pp_o)]/2\}$, where Rp_i = retentate pressure inlet, Rp_o = retentate pressure outlet, Pp_i = permeate pressure inlet, Pp_o = permeate pressure outlet.

[2]τ_w = wall shear stress = $\{\Delta P \times [d_{hydraulic}/(4 \times L)]\}$, where $\Delta P = (Rp_i - Rp_o)$, $d_{hydraulic}$ is the hydraulic diameter, L is the membrane length.

[3]Volume concentration factor = [(flow retentate + flow permeate)/flow retentate].

used to measure the macro composition of skim milk, retentates, and permeates during MF. When a protein content of 7.35 ± 0.05% in the retentate from MF at pore size 0.05 and 0.10 μm was reached, the collection of retentate and permeate in separate cooling tanks started (time 0). The accumulation of retentate and permeate continued for 100 min. The time from the pasteurized skim milk was fed into the system and until the collection of fractions started was 90 to 95 min for the 0.10-μm membrane, and 120 min for the 0.05-μm membrane. Due to loss of proteins to the permeate during MF at 0.20 μm, the protein content of the retentate did not reach the target value of 7.35 ± 0.05% at the given volume concentration factor. Therefore, the collection of retentate and permeate from MF at 0.20 μm started at a protein concentration of the retentate of 5.95 ± 0.05% (after 120 min). During MF, samples of retentate and permeate were directly sampled from the outlet every 15th min and measured by MilkoScan FT1. Representative samples for chemical and physical analyses were sampled from the tanks containing accumulated retentate and permeate, respectively.

Cleaning Procedure

After displacing milk by water, the system was cleaned with 3.5% (vol/vol) alkaline detergent (Ultrasil 25, Ecolab Deutschland GmbH, Monheim am Rhein, Germany) at 80°C for 20 min followed by a permeate flush for 3 min. The alkaline cleaning procedure was repeated with 1.5% (vol/vol) alkaline detergent at 80°C for 20 min followed by a permeate flush for 6 min. The system was finally cleaned with 1.5% (vol/vol) acidic detergent (nitric acid 65%, VWR International, Fontenay-sous-Bois, France) at 50°C for 20 min followed by a permeate flush for 6 min. Before every MF run, the system was conditioned with 1.5% (vol/vol) alkaline detergent at 80°C for 20 min, followed by a permeate flush for 6 min. The system was always thoroughly rinsed with water before feeding the system with milk.

Chemical Analyses

Samples for chemical analyses were freshly frozen and thawed the day of analysis. The content of total solids was determined according to oven drying at 102 ± 2°C for 24 h (IDF, 2010a). Fat content was determined according to the Röse Gottlieb method (IDF, 2010b). Minerals (Ca, P, K, Na, Mg, Cu) were quantified by the method described by Jørgensen et al. (2015). For quantification of minerals, ERM-BD150 and ERM-BD151 (Institute for Reference Materials and Measurements, Geel, Belgium) were used as reference materials. Total nitrogen (**TN**), CP, NPN, and noncasein nitrogen (**NCN**) were determined using the Kjeldahl method [IDF, 2014 (TN and CP), IDF, 2001, and IDF, 2004, respectively]. True protein (**TP**) was calculated by subtracting NPN from TN. Casein was calculated by subtracting NCN from TN. Native whey protein (**NWP**) was calculated by subtracting NPN from NCN. A multiplying factor of 6.38 was used to calculate the amount of the various protein components. Lactose was quantified by HPLC as described by Moe et al. (2013). The pH was measured with a pH meter equipped with a temperature probe (Radiometer Copenhagen, Nerliens Kemisk Tekniske AS, Oslo, Norway).

Transmission of Proteins. Transmission rate of proteins (T) as measured with MilkoScan FT1 was calculated by using the relation T (%) = (Cp/Cr) × 100, where Cp and Cr are the protein concentration in permeate and retentate, respectively (Morin et al., 2004).

Capillary Electrophoresis. Run buffer (pH 3.0 ± 0.1) was made according to Heck et al. (2008) and filtered through a 0.20-μm filter (no. 83.1826.001, Sarstedt, Nümbrecht, Germany). Sample buffer (pH 8.6 ± 0.1) was prepared according to Recio et al. (1997). Sample buffer (900 μL) and milk sample (600 μL) were mixed, vortexed, and left on a benchtop shaker for 1 h at room temperature and finally filtered through a 0.45-μm filter (no. 28145–503, VWR, Radnor, PA). Analysis of samples was performed on an Agilent capillary electrophoresis system (model G1600AX) controlled by an Agilent 3D-CE ChemStation software (Agilent Technologies Deutschland GmbH & Co. KG, Waldbronn, Germany). Separations were performed using a fused-silica capillary with an internal diameter of 50 μm and a length of 56 cm (no. G1600–61211) and a 50-μm alignment interface (no. G1600–60210). Separations were carried out at 45°C with a linear gradient voltage from 0 to 25 kV for 3 min, followed by a constant voltage of 25 kV for 40 min. Before each run, the capillary was flushed with 0.1 *M* NaOH for 5 min, and subsequently flushed and conditioned with run buffer for 20 min. Each sequence comprised 12 injections interrupted by a flush with run buffer before every injection. Skim milk and retentate samples were injected at a pressure of 3.45 kPa for 20 s. Due to lower concentrations of proteins in permeates, the injection duration of permeates was increased to 60 s to intensify the absorbance. Proteins were detected by a UV detector at 214 nm. Each sample was prepared twice and each sample preparation was distributed on 3 vials to obtain a total of 6 measurements of each sample. Electropherograms were integrated with a valley-to-valley approach (Miralles et al., 2003). Peaks were identified

according to Heck et al. (2008); however, the identification of α_{S1}-CN was based on the fact that this protein only has 2 phosphorylation states (Farrell et al., 2004). Figure 2 shows the identification of peaks in electropherograms of skim milk, retentate from MF with the 0.05-μm membrane, and permeates from MF with the 0.05- and 0.20-μm membranes. Electropherograms of retentates from MF with different membrane pore sizes were similar, and electropherograms of permeates from MF with 0.05- and 0.10-μm membranes were similar. The peak identification was verified by comparing the electropherograms of the samples with electropherograms of a milk sample or a permeate sample added standards of caseins and whey proteins (Sigma-Aldrich, St. Louis, MO). The relative concentration (%) of proteins in a sample was calculated by dividing the peak area by total peak area and adjusting for migration time (Heck et al., 2008).

The quantification of proteins based on capillary electrophoresis results could be performed with standard curves of each protein. However, the impurity of protein standards makes this difficult and could potentially result in over- or underestimation of the protein concentration. A new calculation procedure that eliminates the need for standard curves is therefore presented. The protein composition (%) of a sample was calculated by adjusting the relative concentration of a protein (1) with the TP content (Kjeldahl method) of that particular sample, and (2) with the molar extinction coefficient of that particular protein. Molar extinction coefficients ($1/M$ cm) of proteins were calculated according to Kuipers and Gruppen (2007): α-LA = 300,395; β-LG = 293,362; α_{S2}-CN = 401,482; α_{S1}-CN = 421,781; κ-CN = 332,759; and β-CN = 423,992. The new calculation procedure overestimates the content of α-LA and β-LG as it does not include other serum proteins (e.g., lactoferrin, immunoglobulins, BSA). However, in further use of this procedure, the full spectrum of serum proteins can be included.

Physical Analyses

Viscosity was measured on fresh samples using a rheometer type MCR 301 with a bob (CC27/Ti) and cup (CC27/T200/Ti) geometry (Anton Paar GmbH, Graz, Austria). The cup was tempered to the filtration temperature (50 or 60°C) used during production of the sample. Sample (4°C) was added to the cup and the measurement started when the temperature had been sufficiently stabilized. The spindle rotated at a constant shear rate of 100 1/s for 500 s with 100 measurement points. During the first period of the measurement, the viscosity of the samples decreased as a function of time. Further investigations revealed that the steady-state

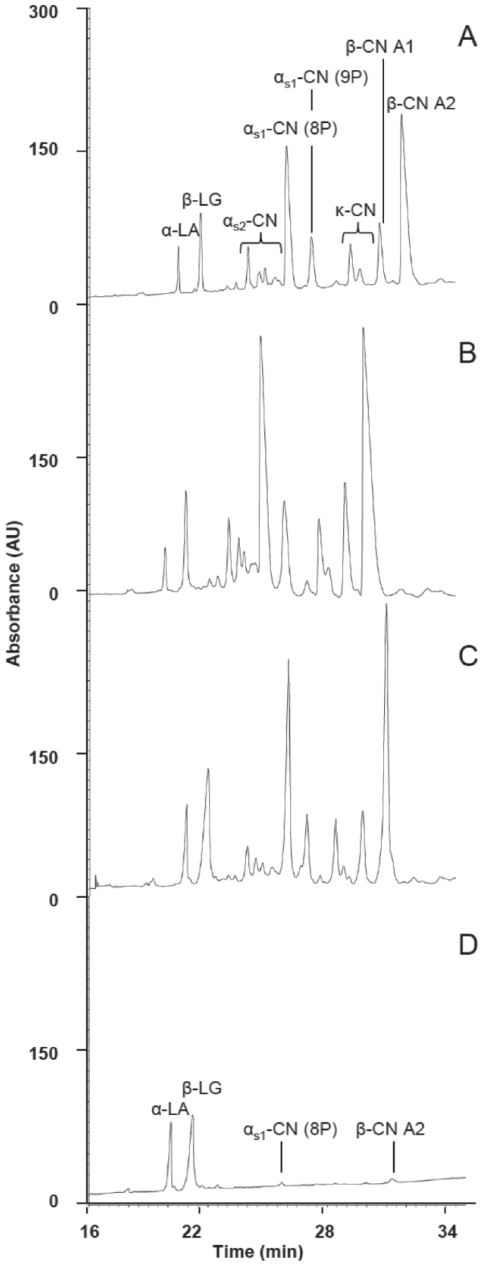

Figure 2. Electropherograms of (A) pasteurized skim milk, (B) retentate from microfiltration with a 0.05-μm membrane, (C) permeate from microfiltration with a 0.20-μm membrane, and (D) permeate from microfiltration with a 0.05-μm membrane.

temperature of the system was reached after about 300 to 500 s of measurement (results not shown). With the use of Matlab (The MathWorks Inc., Natick, MA) the asymptotic viscosity value, which means the viscosity at infinite time, was predicted according to the least square method (Schüller et al., 2010). The asymptotic value represents the viscosity of the sample at the given final steady-state filtration temperature.

Particle size distribution of fresh samples from MF at 0.20 μm was determined by dynamic light scattering. Measurements were performed using a Zetasizer 3000HS particle size analyzer equipped with a 633 nm laser (Malvern Instruments Ltd., Malvern, UK). Milk samples were mixed with filtered (0.22 μm) simulated milk ultrafiltrate (Jenness and Koops, 1962) in the ratio 1:240. The mixture with simulated milk ultrafiltrate and sample was filtered (0.8 μm) into a plastic cuvette and measured at a scattering angle of 90° at 25°C. The particle size distribution represented the casein micelle size distribution because the sample was mainly composed of caseins.

Statistical Treatment

For statistical treatment of data the R version 3.0.1 (The R Project for Statistical Computing; https://www.r-project.org/) was applied. First, it was investigated whether the random factors, replicate block and production day, significantly influenced any selected responses (CP, TP, CN, and NWP). Replicate block corresponds to the day of milk delivery (7 milk deliveries), and production day corresponds to the day of filtration (MF the day of milk delivery or the following day). It was only possible to check the potential significance of replicate block on responses from experiments with 0.05- and 0.20-μm membranes, because experiments with these pore sizes both were run on the same milk deliveries (same replicate blocks). Data from these experiments were fitted to a linear ANOVA model with pore size as a fixed factor and replicate block and production day as random factors. Replicate block had no significant effect on the selected responses. Further statistical analysis was therefore done by fitting data to an ANOVA model with pore size or filtration temperature as a fixed factor and production day as a random factor. Tukey's studentized range test was applied to confirm significant differences ($P < 0.05$) between sample means. Paired *t*-test (Minitab 17, Minitab Ltd., Coventry, UK) was applied to determine whether a statistically significant difference was obtained between the protein adjusted protein concentration and the protein and absorbance adjusted protein concentration based on capillary electrophoresis results.

RESULTS

Effect of Pore Size on Composition of Retentates and Permeates

The chemical composition and physical characteristics of retentates and permeates from MF of skim milk with different pore sizes at filtration temperature 50°C are presented in Table 3. The significantly lower concentration of TS in the retentate from MF with the 0.20-μm membrane than in the retentates from MF with the 0.05- and 0.10-μm membranes revealed that additional components permeated the 0.20-μm membrane. The significantly lower TS content in the retentate was due to the significantly lower concentration of protein (CP and TP), as no significant differences were found in fat and lactose content between the retentates from MF with different pore sizes. The permeate from MF with the 0.20-μm membrane had a significantly higher concentration of proteins (CP, TP, CN, and NWP) and a higher pH compared with the permeates from MF with smaller pore sizes. Kjeldahl analysis showed that caseins permeated through the 0.20-μm membrane, causing an opaque whitish appearance. The casein micelle size distribution in the permeate was significantly smaller than in the retentate. The absence of casein micelles in permeates from MF with 0.05- and 0.10-μm membranes gave these permeates translucent yellowish appearances.

The content of TP in retentates from MF with the 0.05- and 0.10-μm membranes did not differ; however, the membrane with 0.05-μm pore size retained significantly more native whey proteins than the 0.10-μm membrane. The significantly lower content of native whey proteins in the permeate from the 0.05-μm membrane supported this observation. As expected, the levels of calcium and phosphorus in retentates and permeates tended to follow the casein content, due to the presence of these minerals in the casein micelles. Significantly higher concentrations of calcium and phosphorus were detected in the permeate when using the 0.20-μm membrane. Also significantly more magnesium and copper passed through the 0.20-μm membrane compared with the membranes with the smaller pores. The retentate from MF with the 0.05-μm membrane contained significantly more copper than the retentate from MF with the 0.10-μm membrane.

Capillary electrophoresis was used to separate and quantify proteins. Table 4 gives an overview of the different calculations used to calculate the protein compositions based on the results from capillary electrophoresis of skim milk, retentate, and permeate from MF with the 0.10-μm membrane at 50°C. The protein composition as found with capillary electrophoresis

Table 3. Characterization (mean ± SD, n = 3 or 4) of pasteurized skim milk, retentates, and permeates from microfiltration (MF) of pasteurized skim milk at 50°C with ceramic membranes with pore sizes of 0.05, 0.10, and 0.20 μm

		MF retentates 50°C			MF permeates 50°C		
Item	Pasteurized skim milk	0.05 μm	0.10 μm	0.20 μm	0.05 μm	0.10 μm	0.20 μm
TS (%)	9.14 ± 0.12	13.31 ± 0.09^{a}	13.25 ± 0.11^{a}	11.40 ± 0.12^{b}	5.75 ± 0.44^{a}	6.16 ± 0.14^{a}	7.39 ± 0.45^{b}
CP (%)	3.64 ± 0.01	7.89 ± 0.08^{a}	7.96 ± 0.06^{a}	5.93 ± 0.05^{b}	0.44 ± 0.04^{a}	0.57 ± 0.04^{b}	2.07 ± 0.05^{c}
True protein (%)	3.46 ± 0.01	7.72 ± 0.09^{a}	7.78 ± 0.06^{a}	5.75 ± 0.05^{b}	0.27 ± 0.03^{a}	0.39 ± 0.02^{b}	1.88 ± 0.04^{c}
Casein (%)	2.92 ± 0.00	6.69 ± 0.08^{a}	6.92 ± 0.06^{b}	5.13 ± 0.07^{c}	0.03 ± 0.01^{a}	0.01 ± 0.00^{a}	1.38 ± 0.03^{b}
Native whey protein (%)	0.54 ± 0.01	1.03 ± 0.01^{a}	0.86 ± 0.01^{b}	0.62 ± 0.01^{c}	0.24 ± 0.03^{a}	0.39 ± 0.01^{b}	0.50 ± 0.01^{c}
Fat (%)	0.06 ± 0.01	0.12 ± 0.02^{a}	0.12 ± 0.02^{a}	0.10 ± 0.01^{a}	0.03 ± 0.01^{a}	0.04 ± 0.00^{a}	0.05 ± 0.02^{a}
Lactose (mmol/kg)	147.6 ± 1.0	134.2 ± 1.1^{a}	132.1 ± 4.8^{a}	139.1 ± 2.0^{a}	143.5 ± 7.3^{a}	147.7 ± 5.7^{a}	143.4 ± 7.4^{a}
Calcium (g/kg)	1.16 ± 0.03	2.25 ± 0.06^{a}	2.33 ± 0.06^{a}	1.80 ± 0.00^{b}	0.30 ± 0.03^{a}	0.32 ± 0.01^{a}	0.67 ± 0.01^{b}
Phosphorus (g/kg)	1.04 ± 0.01	1.83 ± 0.02^{a}	1.89 ± 0.01^{b}	1.52 ± 0.01^{c}	0.42 ± 0.03^{a}	0.44 ± 0.00^{a}	0.71 ± 0.01^{b}
Potassium (g/kg)	1.67 ± 0.00	1.70 ± 0.00^{a}	1.73 ± 0.06^{a}	1.67 ± 0.06^{a}	1.53 ± 0.10^{a}	1.63 ± 0.06^{a}	1.60 ± 0.00^{a}
Sodium (g/kg)	0.39 ± 0.01	0.40 ± 0.02^{a}	0.40 ± 0.02^{a}	0.40 ± 0.01^{a}	0.37 ± 0.03^{a}	0.38 ± 0.00^{a}	0.39 ± 0.01^{a}
Magnesium (g/kg)	0.13 ± 0.00	0.18 ± 0.01^{a}	0.18 ± 0.01^{a}	0.16 ± 0.01^{b}	0.09 ± 0.01^{a}	0.09 ± 0.00^{a}	0.11 ± 0.00^{b}
Copper (mg/kg)	0.09 ± 0.03	0.18 ± 0.02^{a}	0.13 ± 0.02^{b}	0.12 ± 0.01^{b}	0.02 ± 0.01^{a}	0.01 ± 0.00^{a}	0.05 ± 0.01^{b}
pH	6.77 ± 0.01	6.77 ± 0.03^{a}	6.76 ± 0.06^{a}	6.75 ± 0.01^{a}	6.64 ± 0.03^{a}	6.65 ± 0.04^{a}	6.73 ± 0.04^{b}
Viscosity (mPa·s)	1.12 ± 0.05	1.42 ± 0.11^{a}	1.59 ± 0.09^{a}	1.40 ± 0.13^{a}	0.96 ± 0.00^{a}	0.98 ± 0.04^{a}	1.00 ± 0.14^{a}
Casein micelle size (nm)	172 ± 8	NA[1]	NA	186 ± 7	NA	NA	130 ± 8
Appearance	Opaque white	Opaque white	Opaque white	Opaque white	Translucent yellowish	Translucent yellowish	Opaque whitish

[a–c]Samples with different superscript letters within same sample type differ according to Tukey's pairwise comparison ($P < 0.05$).
[1]Not analyzed.

was reported as a relative concentration by dividing peak area by total peak area, as shown in the first subcolumn of each sample type. However, if the TP content of the sample is known, the relative concentration can be protein adjusted to find the real protein composition and concentration of the sample, as shown in the second subcolumn of each sample type. Based on the fact that proteins in milk absorb UV light at 214 nm to a varying extent, the protein content can be adjusted with the molar extinction coefficient of the respective protein, as shown in the third subcolumn of each sample type. By adjusting the protein content for both TP content and molar extinction coefficient, the protein concentration in pasteurized skim milk and retentate differed significantly compared with the values obtained by only adjusting for TP content.

Figure 3 shows the protein composition of pasteurized skim milk and retentates and permeates from MF of pasteurized skim milk using membranes with different pore sizes. The protein composition is adjusted for both TP content in the respective sample and molar extinction coefficient of the specific protein. A significantly higher amount of β-LG, α_{S2}-CN, α_{S1}-CN, κ-CN, and β-CN were retained with the 0.05 and 0.10 μm membranes than with the 0.20-μm membrane, supporting the findings based on the Kjeldahl analysis. The retentate from MF of skim milk with the 0.10-μm membrane contained significantly less α-LA and β-LG and significantly more α_{S1}-CN than the retentate from MF of skim milk with pore size of 0.05 μm. The lower retention of α-LA and β-LG with the 0.10-μm membrane resulted in a permeate with a significantly higher concentration of these whey proteins. The casein distribution (α_{S2}-CN: α_{S1}-CN: κ-CN: β-CN) of the permeate from MF with membrane pore size of 0.20 μm (10: 38: 10: 42) was almost similar to that of pasteurized skim milk (13: 37: 8: 42), whereas negligible amounts of caseins were found in permeates from MF with 0.05- and 0.10-μm membranes.

Effect of Filtration Temperature on Composition of Retentates and Permeates

The chemical composition and physical characterization of retentates and permeates from MF of skim milk at filtration temperatures of 50 and 60°C and pore size 0.10 μm is presented in Table 5. The MF of skim milk at 60°C gave significantly less CP and casein in the retentate compared with filtration at 50°C. Significantly lower concentrations of native whey proteins and calcium permeated the membrane at 60°C than at 50°C. The viscosity of retentates and permeates were lower at 60°C than at 50°C, but the difference in viscosity was only significant for the permeates. Figure 4 shows

the protein composition of pasteurized skim milk and retentates and permeates from MF at 50 and 60°C. The protein composition is adjusted for both TP content in the respective sample and molar extinction coefficient of the specific protein. The capillary electrophoresis analysis did not show any significant differences in the content of α-LA, β-LG, α_{S2}-CN, α_{S1}-CN, κ-CN, and β-CN between the retentates from MF at the different temperatures. However, the permeate from MF at 50°C had significantly higher concentrations of α-LA and β-LG than the permeate from MF at 60°C. Minor levels of α_{S1}-CN and β-CN were detected in the permeates from MF with 0.10 μm at both temperatures.

TMP and Protein Transmission During MF

Figure 5 presents the TMP during MF of pasteurized skim milk both at 50°C with different membrane pore sizes (0.05, 0.10, and 0.20 μm) and at 60°C with membrane pore size of 0.10 μm. The TMP at time 0 increased with decreasing membrane pore size. When comparing the effect of filtration temperature on TMP development, the initial TMP for MF at 60°C was lower compared with MF at 50°C with the 0.10-μm membrane, although with increasing filtration time, the TMP of MF at 60°C approached the TMP level of MF at 50°C. The slopes of the linear curves revealed that MF at 60°C with membrane pore size 0.10 μm resulted in the steepest curve, whereas filtration with membrane pore size 0.20 μm at 50°C only gave a slight increase in TMP with filtration time.

The transmission of proteins during MF of pasteurized skim milk decreased with decreasing pore size (Figure 6). The transmission of proteins through the 0.20 μm membrane was quite constant, whereas the transmission of proteins through 0.05- and 0.10-μm membranes decreased with filtration time. The MF of skim milk with the 0.10-μm membrane pore size at 60°C gave a larger decline in protein transmission with filtration time compared with MF at 50°C.

DISCUSSION

Choosing an appropriate ceramic membrane pore size is important in an industrial MF process. For instance, for some industrial MF applications it will be of great importance to obtain a permeate purely containing whey proteins with the absence of caseins. This study showed that permeates from MF with 0.05- and 0.10-μm membranes contained whey proteins with negligible amounts of caseins, whereas the permeate from MF with the 0.20-μm membrane contained both whey proteins and caseins. These results are in accordance with results presented by Punidadas and Rizvi (1999) who reported that caseins permeated through a membrane with pore size 0.20 μm, whereas a membrane with pore size 0.05 μm was able to retain almost all caseins. However, they used gradient mem-

Table 4. Calculations of protein compositions in skim milk, microfiltration (MF)-retentate and -permeate based on capillary electrophoresis results: relative concentration (%), protein concentration (%) as adjusted for true protein content in the sample, and protein concentration (%) as adjusted for true protein content in the sample and molar extinction coefficient (absorbance) of the protein[1]

	Pasteurized skim milk			Retentate			Permeate		
Item	Relative conc.[2] (%)	Protein adj. protein conc.[3] (%)	Protein and absorbance adj. protein conc.[4] (%)	Relative conc.[2] (%)	Protein adj. protein conc.[3] (%)	Protein and absorbance adj. protein conc.[4] (%)	Relative conc.[2] (%)	Protein adj. protein conc.[3] (%)	Protein and absorbance adj. protein conc.[4] (%)
α-LA	3.19	0.11[a]	0.14[b]	1.76	0.14[a]	0.18[b]	27.24	0.11[a]	0.11[a]
β-LG	9.17	0.32[a]	0.42[b]	5.71	0.44[a]	0.60[b]	70.11	0.27[a]	0.28[a]
α_{S2}-CN	11.57	0.40[a]	0.39[b]	12.06	0.94[a]	0.93[a]	ND[5]	ND	ND
α_{S1}-CN	32.32	1.12[a]	1.04[b]	34.35	2.67[a]	2.53[b]	1.07	0.004[a]	0.003[a]
κ-CN	6.94	0.24[a]	0.28[b]	7.87	0.61[a]	0.73[b]	ND	ND	ND
β-CN	36.80	1.28[a]	1.18[b]	38.26	2.98[a]	2.80[b]	1.58	0.006[a]	0.004[b]
Sum	100.00	3.47	3.47	100.00	7.78	7.78	100.00	0.39	0.39

[a,b]Means of protein concentrations within the same row and same sample type with different superscript letters differ according to paired *t*-test ($P < 0.05$).

[1]Calculations are presented for samples (n = 3) of pasteurized skim milk, retentate, and permeate from MF of pasteurized skim milk at 50°C with a 0.10-μm membrane.

[2]Relative concentration = peak area divided by total peak area.

[3]Protein adjusted protein concentration = adjustment of relative concentration with true protein content of sample.

[4]Protein and absorbance adjusted protein concentration = adjustment of relative concentration with true protein content of sample and molar extinction coefficient of protein.

[5]Not detected.

branes and no permeate pump (no UTP). According to Zulewska et al. (2009), a higher casein proportion is expected to permeate gradient membranes compared with ceramic membranes in a UTP system. In the present study, the effect of ceramic membrane pore sizes in a UTP MF system was investigated. The permeation of caseins through the 0.20-μm membrane resulted in an opaque whitish permeate with casein distribution (α_{S2}-CN: α_{S1}-CN: κ-CN: β-CN) similar to that of pasteurized skim milk. Due to the permeation of caseins, the protein concentration in the retentate from MF with the 0.20-μm membrane did not reach the protein

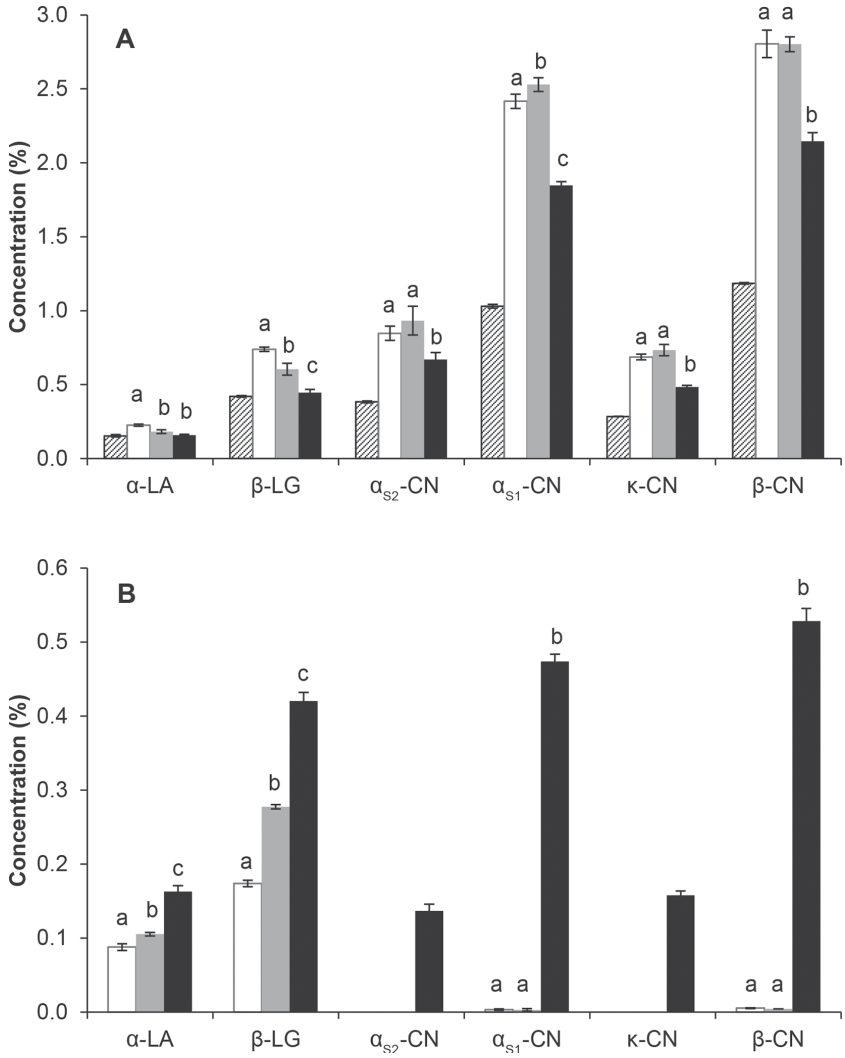

Figure 3. Protein composition (mean ± SD, n = 3 or 4) of (A) pasteurized skim milk (diagonal striped) and retentates, and (B) permeates from microfiltration of pasteurized skim milk at filtration temperature 50°C with different pore size membranes: (white) 0.05 μm, (gray) 0.10 μm, and (black) 0.20 μm. Means within the same protein type with different letters (a–c) differ according to Tukey's pairwise comparison ($P < 0.05$). Note the different scale values of the y-axes.

Table 5. Characterization (mean ± SD, n = 3 or 4) of pasteurized skim milk, retentates, and permeates from microfiltration (MF) of pasteurized skim milk with 0.10 μm ceramic membrane at 50 and 60°C

Item	Pasteurized skim milk	MF retentates 0.10 μm		MF permeates 0.10 μm	
		50°C	60°C	50°C	60°C
TS (%)	9.25 ± 0.02	13.25 ± 0.11[a]	13.38 ± 0.06[a]	6.16 ± 0.14[a]	5.82 ± 0.64[a]
CP (%)	3.65 ± 0.01	7.96 ± 0.06[a]	7.89 ± 0.08[b]	0.57 ± 0.04[a]	0.50 ± 0.01[a]
True protein (%)	3.47 ± 0.01	7.78 ± 0.06[a]	7.71 ± 0.09[a]	0.39 ± 0.02[a]	0.33 ± 0.02[a]
Casein (%)	2.91 ± 0.01	6.92 ± 0.06[a]	6.84 ± 0.08[b]	0.01 ± 0.00[a]	0.01 ± 0.01[a]
Native whey protein (%)	0.54 ± 0.00	0.86 ± 0.01[a]	0.87 ± 0.03[a]	0.39 ± 0.01[a]	0.32 ± 0.01[b]
Fat (%)	0.06 ± 0.00	0.12 ± 0.02[a]	0.06 ± 0.07[a]	0.04 ± 0.00[a]	0.03 ± 0.01[a]
Lactose (mmol/kg)	147.3 ± 1.2	132.1 ± 4.8[a]	135.2 ± 1.3[a]	147.7 ± 5.7[a]	151.9 ± 5.0[a]
Calcium (g/kg)	1.20 ± 0.00	2.33 ± 0.06[a]	2.37 ± 0.06[a]	0.32 ± 0.01[a]	0.29 ± 0.01[b]
Phosphorus (g/kg)	1.05 ± 0.00	1.89 ± 0.01[a]	1.90 ± 0.02[a]	0.44 ± 0.00[a]	0.43 ± 0.01[a]
Potassium (g/kg)	1.68 ± 0.02	1.73 ± 0.06[a]	1.67 ± 0.06[a]	1.63 ± 0.06[a]	1.60 ± 0.00[a]
Sodium (g/kg)	0.38 ± 0.01	0.40 ± 0.02[a]	0.40 ± 0.01[a]	0.38 ± 0.00[a]	0.39 ± 0.01[a]
Magnesium (g/kg)	0.13 ± 0.00	0.18 ± 0.01[a]	0.17 ± 0.01[a]	0.09 ± 0.00[a]	0.09 ± 0.00[a]
Copper (mg/kg)	0.07 ± 0.01	0.13 ± 0.02[a]	0.15 ± 0.04[a]	0.01 ± 0.00[a]	0.01 ± 0.00[a]
pH	6.78 ± 0.00	6.76 ± 0.06[a]	6.77 ± 0.02[a]	6.65 ± 0.04[a]	6.60 ± 0.04[a]
Viscosity (mPa·s)	1.08 ± 0.06	1.59 ± 0.09[a]	1.31 ± 0.26[a]	0.98 ± 0.04[a]	0.85 ± 0.02[b]
Appearance	Opaque white	Opaque white	Opaque white	Translucent yellowish	Translucent yellowish

[a,b]Samples with different superscript letters within same sample type differ according to Tukey's pairwise comparison ($P < 0.05$).

concentration of the retentates from MF with 0.05 or 0.10-μm membranes at the given volume concentration factor (2.5). On the other hand, Vadi and Rizvi (2001) reported that 0.20-μm membranes applied in a UTP MF system retained all caseins. The conflicting results with the present study could be due to differences in mean casein micelle size of the milk microfiltered. Casein micelles are polydisperse, and the diameter of the casein micelles as measured with electron microscopy varies from 50 to 500 nm (Fox and Kelly, 2004). The mean micelle diameter as measured with dynamic light scattering at 20°C has previously been reported to be in the range 149 to 222 nm for Norwegian Red Cattle (Devold et al., 2000). In the present study of milk from the same breed, the mean casein micelle diameter of the skim milk was ~172 nm. The MF with the 0.20-μm membrane divided the milk into a retentate with increased casein micelle size (~186 nm) and a permeate with smaller casein micelles (~130 nm). Permeation of caseins through a 0.20-μm membrane (ceramic gradient) was also reported by Punidadas and Rizvi (1999). Theoretically, components smaller than 200 nm are able to permeate a 0.20-μm membrane. However, the given pore size of a membrane should be considered to be more an indication than a precise definition of the separating ability of the membrane. Because the casein micelle diameter of the skim milk was 172 nm, casein micelles on the smaller tail of the distribution were expected to permeate the 0.20-μm membrane.

The MF of pasteurized skim milk with the 0.05-μm membrane gave a significantly higher retention of native whey proteins compared with the 0.10-μm membrane. The β-LG has a molecular weight of approximately 18 kDa in its monomeric form (Léonil et al., 1995). At the pH of milk (pH 6.7) and at a temperature of 50°C, around 50% of the β-LG exist in the dimeric form and has a hydrodynamic radius close to 2.5 nm (Aymard et al., 1996). Due to the size of β-LG at the given filtration temperature (50°C), a similar transmission of β-LG through the 0.05- and 0.10-μm pore size membranes was expected. Further investigations are necessary to explain the lower transmission of whey proteins at MF with the 0.05-μm membrane. It can be questioned whether the lower constant flux at MF with membrane pore size of 0.05 μm (44 L/m^2·h) compared with the flux at MF with membrane pore size 0.10 μm (59 L/m^2·h) influenced the protein transmission. However, the approximately same slow and constant increase in TMP with filtration time for both pore sizes indicated that both membranes were run above the critical flux (Howell, 1995; Brans et al., 2004). The increase in TMP during MF with 0.05- and 0.10-μm membranes was accompanied with a slow and constant decrease in protein transmission. The reduced transmission of proteins as a consequence of increased membrane resistance due to fouling has previously been reported (Le Berre and Daufin, 1996; Gésan-Guiziou et al., 1999; Gésan-Guiziou et al., 2000; Jimenez-Lopez et al., 2008; Lawrence et al., 2008; Adams et al., 2015b). The TMP and protein transmission measured during MF of skim milk with the 0.20-μm membrane were approximately constant, suggesting that the MF operation was run below the critical flux. The flux used during MF with the 0.20-μm membrane could have been

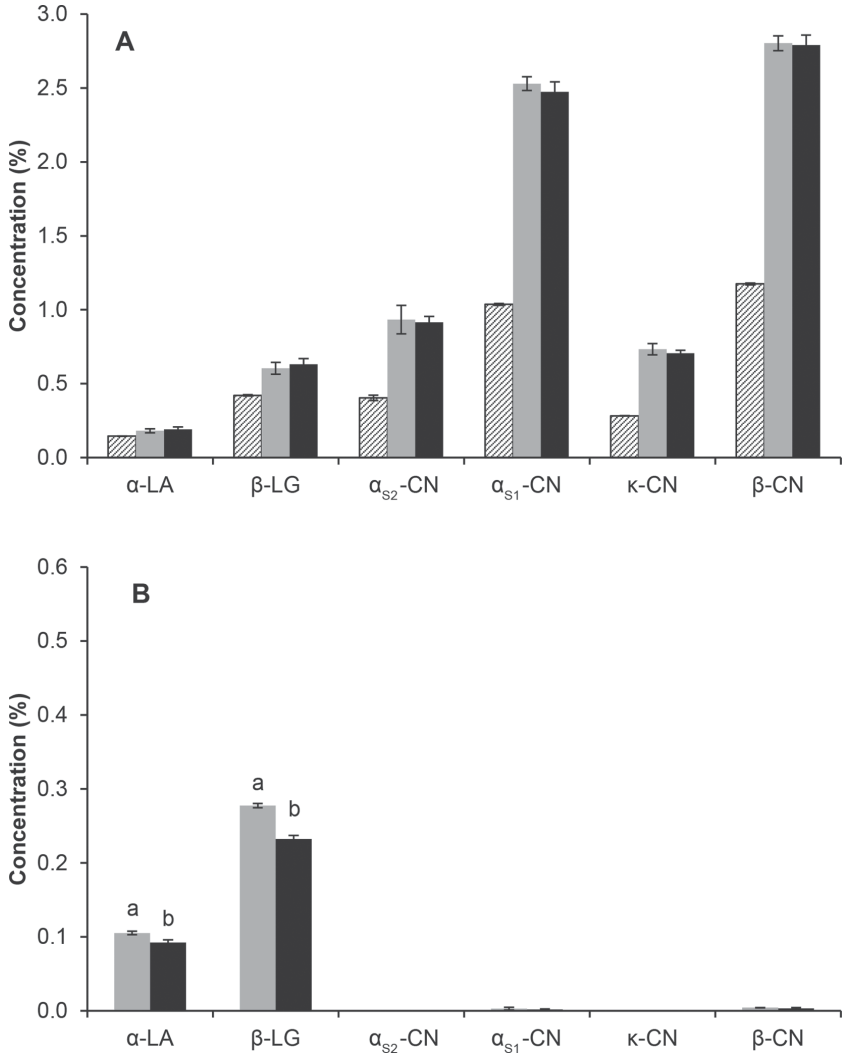

Figure 4. Protein composition (mean ± SD, n = 3) of (A) pasteurized skim milk (diagonal striped) and retentates, and (B) permeates from microfiltration of pasteurized skim milk with a 0.10-μm membrane at different filtration temperatures: (gray) 50°C, and (black) 60°C. Means within the same protein type with different letters (a,b) differ according to Tukey's pairwise comparison ($P < 0.05$). Note the different scale values of the y-axes.

increased to ensure operation of MF above the critical flux as for MF with the 0.05- and 0.10-μm membranes. From an industrial point of view, the flux should be as high as possible to maximize the utilization of the membrane area. However, fouling is a limiting factor in MF of skim milk, and an MF process with a steeper increase in TMP with filtration time requires more frequent cleaning cycles. Lower constant flux values at MF with the 0.05- and 0.10-μm membranes could give longer processing times. Further work is needed to

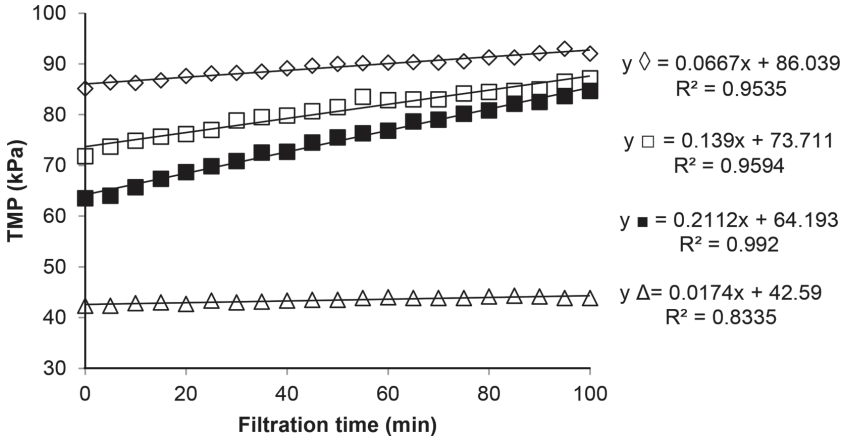

Figure 5. Transmembrane pressure (TMP; mean, n = 3 or 4) during microfiltration of pasteurized skim milk at 50°C with ceramic membranes with pore sizes: (◊) 0.05 μm, (□) 0.10 μm; and (Δ) 0.20 μm, and at 60°C with a ceramic membrane with pore size (■) 0.10 μm. Trends are indicated by linear regression lines. R^2-values report how closely the estimated values for the linear regression line correspond to the actual data.

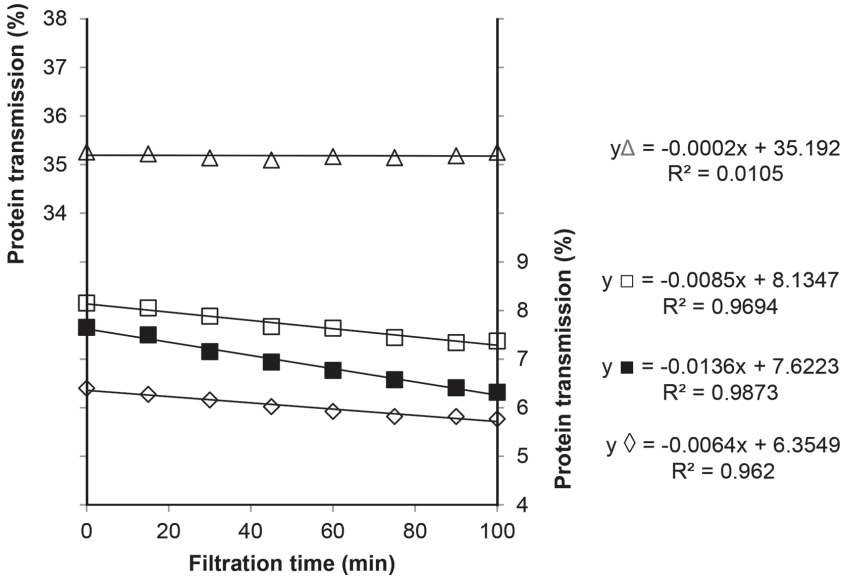

Figure 6. Transmission of proteins (mean, n = 3 or 4) as measured with MilkoScan (Foss, Hillerød, Denmark) during microfiltration of pasteurized skim milk at 50°C with ceramic membranes with pore sizes: (◊) 0.05 μm, (□) 0.10 μm, and (Δ) 0.20 μm, and at 60°C with a ceramic membrane with pore size (■) 0.10 μm. Note the different scale values of the y-axes. Trends are indicated by linear regression lines. R^2-values report how closely the estimated values for the linear regression line correspond to the actual data.

find the optimal stable MF operating conditions with these membranes to achieve stable TMP and protein transmission, and consequently longer operating times.

The retentate from MF of skim milk with the 0.05-μm membrane contained significantly higher trace amounts of copper than the retentate from MF with the 0.10-μm membrane. This observation could be explained by the increased concentration of α-LA and β-LG and their ability to chelate copper (Baumy and Brule, 1988). A correlation plot revealed a linear correlation between content of native whey protein and copper in retentates and permeates (correlation plot not shown). As expected, a linear correlation was observed between the content of calcium and phosphorus and the content of casein in retentates and permeates (correlation plot not shown). Concentration of caseins results in a simultaneous concentration of calcium and phosphorus because two-thirds of the calcium and one-half of the phosphate in milk are present in the casein micelles (de la Fuente, 1998; Gaucheron, 2005). The MF of skim milk with the 0.10-μm membrane at 60°C resulted in a permeate with significantly less native whey proteins and calcium compared with the permeate from MF at 50°C. At the same time, a higher content of calcium in the retentate from MF at 60°C than at 50°C was observed (not significant), indicating a higher retention of calcium at 60°C. The increased retention of calcium at higher temperatures could most probably be explained by the reduced solubility of calcium as reported by Pouliot et al. (1988). They observed a reduction in soluble calcium and phosphate of roughly 30 and 25%, respectively, when heating skim milk from 4 to 60°C for 60 min.

The significantly lower transmission of native whey proteins (both α-LA and β-LG) with elevated filtration temperature could be due to a certain degree of denaturation. Although the denaturation of α-LA and β-LG is reported to take place at temperatures above 70°C (Anema, 2009), conformational changes have been reported to begin at lower temperatures (Qi et al., 1995, 1997). However, the content of native whey protein was the same in retentates from MF at both filtration temperatures. If a filtration temperature of 60°C was causing denaturation, a difference in native whey protein content between retentates from MF at 50 and 60°C would have been expected. Additionally, denatured whey proteins would aggregate and precipitate with casein during Kjeldahl sample preparation, resulting in an increased amount of casein. Thus, if denaturation took place, a lower content of native whey protein and a higher content of casein in the retentate from MF at 60°C than at 50°C would have been expected. The results indicate that the lower transmission of native whey proteins with elevated filtration temperature was not solely caused by denaturation. A more likely explanation for the reduced amount of native whey proteins in the permeate from MF at 60°C is the possible interaction of whey proteins with deposited casein micelles on the membrane surface, as proposed by Jimenez-Lopez et al. (2008). The significantly lower concentration of casein observed in the retentate from MF at 60°C than at 50°C could be due to fouling. This assumption was supported by the more rapid increase in TMP with filtration time at 60°C than at 50°C. According to Jimenez-Lopez et al. (2008), casein micelles are the most important contributor to the initial deposit build-up during MF of skim milk at 48°C. They suggested that a high concentration of casein micelles in the deposit could increase the electrostatic interactions between the casein micelles and the soluble protein, thereby causing a decreased soluble protein transmission. Thus, in the present study, the potential higher concentration of caseins deposited on the membrane at 60°C than at 50°C could be the reason for the higher retention of native whey proteins through increased electrostatic interactions.

Hurt et al. (2015) also reported a decrease in native whey protein transmission when increasing filtration temperature from 50 to 65°C, but they did not observe a change in the ratio of β-LG and α-LA when SDS-PAGE was used for analysis. However, potential differences in β-LG and α-LA concentrations may have been present, but may be difficult to unveil if the result is given as relative concentrations or ratios. The new calculation procedure presented in the present study could be used to quantify proteins analyzed with capillary electrophoresis or reversed-phase HPLC. Real values give the possible advantage to observe significant differences that are difficult to detect with relative concentrations or ratios. For instance, the relative concentration of β-LG in permeates from MF at 50 and 60°C was 70.1 and 69.5%, respectively (results not shown). By conversion of the relative concentrations to real concentration values, a significant difference in the β-LG content of the permeates was revealed (at 50°C, 0.28%; and at 60°C, 0.23%). The protein composition of skim milk after protein and absorbance adjustment was close to the composition of milk as reported by Farrell et al. (2004).

CONCLUSIONS

Ceramic membrane pore size and filtration temperature significantly influenced the protein composition of retentates and permeates when skim milk was microfiltered in a UTP system to a volume concentration factor of 2.5. The 0.10-μm membrane was the most suitable membrane for protein fractionation of skim milk into a casein-rich retentate and a permeate with native whey proteins. A higher amount of native whey proteins

permeated the 0.20-μm membrane than the 0.05- and 0.10-μm membranes (0.50, 0.24, and 0.39%, respectively), but also significant amounts of smaller casein micelles permeated this membrane (1.4%). A permeate free from casein can be beneficial in the production of native whey protein concentrates and in applications where transparency is an important functional characteristic. Increasing temperature of MF from 50 to 60°C when using the 0.10-μm ceramic membrane caused a reduction in native whey protein permeation and a steeper TMP increase during filtration, probably caused by interaction of whey proteins with deposited casein micelles on the membrane surface. Further work is needed to find the optimal flux for longer MF processing times with 0.10-μm membrane at 50°C.

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Improving the structure and rheology of high protein, low fat yoghurt with undenatured whey proteins

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ABSTRACT

The objective of this study was to investigate the effects of whey protein denaturation and whey protein:casein-ratio on the structural, rheological and sensory properties of high protein (8% true protein), low fat (<0.5% fat) yoghurt. Yoghurt milk bases were made by adding undenatured whey proteins from native whey protein concentrate (NWPC) to casein concentrate in different whey protein:casein-ratios. The degree of whey protein denaturation was then controlled by the temperature treatment of the yoghurt milk bases. Addition of NWPC in low (whey protein:casein-ratio 25:75) or medium levels (whey protein:casein-ratio 35:65) in combination with heat treatment at 75 °C for 5 min gave yoghurts with significantly lower firmness, lower storage modulus (G′), and better sensory properties (less coarse and granular and more smooth), compared with corresponding yoghurts produced from yoghurt milk bases heat-treated at 95 °C for 5 min or with control yoghurts (no addition of NWPC).

1. Introduction

Yoghurt products are of high economic importance to the dairy industry worldwide, and constantly, new yoghurt varieties and concepts are launched. In particular, high protein yoghurts have gained increased popularity over the latest years. Increased consciousness about health benefits of dairy proteins, and an increasing amount of scientific documentation claiming health promotional effects of protein intake (Mellentin, 2013), bring along expanded market opportunities.

In yoghurt technology, denaturation of whey proteins, and their consequently covalent association to casein micelles, is regarded as one of the premises to obtain a good yoghurt structure (Lucey & Singh, 1998; Robinson, Lucey, & Tamime, 2006). Conventional heat treatment, i.e., 95 °C for 5 min or 80 °C for 30 min, gives almost 100% denaturation of β-lactoglobulin (Dannenberg & Kessler, 1987) and approximately 75% denaturation of α-lactalbumin (Anema, 2001). Another important step in yoghurt manufacture is the increase of total solids content to avoid too weak a yoghurt gel. This may be obtained by adding milk powder or by concentrating the milk by evaporation or by membrane filtration (Robinson et al., 2006). The yoghurt can also be concentrated after fermentation with the use of a cloth bag, mechanical separation, or membrane filtration (Özer, 2006).

From a technological point of view, skim milk powder can be replaced by whey protein concentrate (WPC) powder, and an increased yoghurt gel strength is reported by the addition of whey proteins (Krzeminski, Grosshable, & Hinrichs, 2011; Kücükcetin, 2008; Lucey, Munro, & Singh, 1999; Puvanenthiran, Williams, & Augustin, 2002). In contrast, Guzmán-González, Morais, Ramos, and Amigo (1999) reported less firm yoghurt with the addition of WPC. Other reported effects of the addition of whey proteins to yoghurt are increased fermentation time, reduced whey drainage, less viscous yoghurt gels, increased visual roughness and increased yoghurt particle size (Krzeminski et al., 2011; Kücükcetin, 2008; Lucey et al., 1999; Puvanenthiran et al., 2002). Guggisberg, Eberhard, and Albrecht (2007) and Patocka, Cervenkova, Narine, and Jelen (2006) studied the effect of adding whey proteins to yoghurt milk after heat treatment to retain the whey proteins in their undenatured state. When whey proteins were added to heat-treated yoghurt milk before fermentation, a reduction in the storage modulus of the fermented yoghurt was observed (Guggisberg et al., 2007; Patocka et al., 2006). When the whey proteins were added after fermentation, a rapid breakdown of the yoghurt gel was

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observed, resulting in two separate phases comprising fluid whey and a coagulated protein mass (Patocka et al., 2006).

In most studies where the effects of whey proteins addition on yoghurt properties have been examined, the whey protein source has been a powder or a concentrate from cheese whey. However, microfiltration (MF) of milk using pore size 0.05–0.2 μm (Brans, Schroën, van der Sman, & Boom, 2004) may give a permeate with undenatured whey proteins, also referred to as native whey (Heino, Uusi-Rauva, Rantamäki, & Tossavainen, 2007), virgin whey (Marcelo & Rizvi, 2008), or ideal whey (Maubois, 2002). Native whey is free from somatic cells, lactic acid bacteria, bacteriophages, remnants of rennet (Maubois, 2002), cheese fines and the glycomacropeptide from κ-casein, and has a neutral pH and taste. The native whey fraction can be further concentrated by ultrafiltration (UF) (Kumar et al., 2013) to a native whey protein concentrate. Heino et al. (2007) reported significantly higher gel strength of dispersions of native whey protein powders made from MF of milk compared with dispersions of whey protein powders from cheese whey, explained by the lack of glycomacropeptide and the high amount of native whey proteins.

Whey proteins have been reported to have beneficial nutritional effects. For instance the ability of whey proteins to increase the plasma amino acids (Boirie et al., 1997; Hall, Millward, Long, & Morgan, 2003) and trigger muscle protein synthesis (Garlick, 2005; Tipton et al., 2007), makes whey proteins an interesting ingredient in tailor-made products addressed to infant-, elderly- or sports nutrition.

The objective of this study was to investigate the effects of whey protein denaturation and whey protein:casein-ratio on the structural, rheological and sensory properties of high protein, low fat yoghurt. Yoghurt milk bases were obtained by adding native whey protein concentrate to casein concentrate from MF of skim milk. Different temperature/time at heat treatment of yoghurt milk bases made it possible to study the effect of whey protein denaturation on the yoghurt quality.

2. Materials and methods

2.1. Production of ingredients and yoghurts

2.1.1. Experimental design

Stirred and set yoghurts were produced using four whey protein:casein-ratios (10:90, 25:75, 35:65 or 45:55) and two heat treatment temperatures (75 or 95 °C for 5 min) leading to a total of eight factor combinations. Two replicate blocks gave a total of 16 yoghurt batches.

2.1.2. Production of ingredients

Milk was obtained from the university farm. The milk was separated (Westfalia separator AG, SA 1-01-175, Oelde, Germany) at 63 °C. Unpasteurized skim milk was temporarily collected in a double-O vat (Landteknikk A/L, Trondheim, Norway) and kept at 50 ± 2 °C until MF in an MF pilot plant (APV Anhydro AS, Silkeborg, Denmark). MF was performed at a uniform transmembrane pressure with a module containing seven ceramic membranes, each with pore size 0.14 μm and 0.303 m^2 filter area (INSIDE CéRAM™, TAMI Industries, Nyons, France). Filtration temperature varied between 55 and 58 °C with an average of 56.5 °C. The permeate was cooled to an average temperature of 51.7 °C using a separate cooling system according to the International patent WO 2011/115498 A1 (Hoffmann, 2011). MF retentates, hereafter named casein concentrates (CC), with average volume concentration factors of 1.6 and 2.9 were produced. Minimal variations in the skim milk composition (0.14 ± 0.01% fat, 3.69 ± 0.01% protein, 4.88 ± 0.02% lactose) made it possible to use the same volume concentration factors in the two replicate blocks. The CCs were pasteurized (type A3-HRB, Alfa Laval, Nakskov, Denmark) at 75 °C for 15 s. The MF permeate was concentrated on an UF pilot plant Alfa Laval UFS-4 (Alfa Laval) containing a single spiral wound membrane (GR60PP-6338/48, Alfa Laval) with 25 kDa cut-off. The filtration temperature during UF was kept between 45 and 50 °C with an average of 47.8 °C. The UF retentate, hereby named native whey protein concentrate (NWPC), had an average volume concentration factor of approximately 18.8. Fig. 1 gives a flow chart of the production of the concentrates and yoghurts. The chemical composition of the pasteurized CCs and NWPC is shown in Table 1.

2.1.3. Production of yoghurts

NWPC and CC 1.6 and 2.9 were blended in different quantities in order to achieve yoghurt milk bases with true protein contents of 8% and whey protein:casein-ratios of: 10:90 (C: control, no addition of NWPC), 25:75 (L: low addition of NWPC), 35:65 (M: medium addition of NWPC) and 45:55 (H: high addition of NWPC). The yoghurt milk base with whey protein:casein-ratio 10:90 was chosen as a control to show the effect of no addition of NWPC. The chemical composition of the yoghurt milk bases is presented in Table 2. In a randomized order, the yoghurt milk bases were homogenized (Rannie Machine Works Ltd., Albertslund, Denmark) at 180 bar at 55 °C and subsequently heat-treated at 75 or 95 °C for 5 min (Δ55 °C-75 °C = 30 s; Δ55 °C-95 °C = 140 s) in a double-jacketed 5 L heating tank connected to steam and cold water (Norwegian University of Life Sciences, Ås, Norway). The homogenized and heat-treated yoghurt milk bases were precooled and filled at 40–45 °C on sterilized 5 L stainless steel containers with lid and stirrer and tempered to 43 °C in a water bath equipped with a thermostat control. The yoghurt milk bases were inoculated with 0.02% (w/w) yoghurt culture (F-DVS YC-183, Chr. Hansen, Hørsholm, Denmark) consisting of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. A quantity of the inoculated yoghurt milk base was transferred to four 150 mL sterilized glass jars with lids. Set yoghurts for texture analyses were produced in three of the jars and pH was monitored (Radiometer Copenhagen, Nerliens Kemisk Tekniske AS, Oslo, Norway) in the fourth jar. At pH 4.60 ± 0.01, yoghurts in the 5 L containers were subjected to standardized stirring for 1 min and subsequently cooled in an ice water bath. Every tenth min, yoghurts placed in ice water bath were stirred in a standardized way for 15 s until a yoghurt temperature of 19 ± 2 °C was reached after approximately 40 min. Yoghurts were then filled in suitable smaller containers (70 mL or 300 mL plastic cups with lids) and placed in a cold room (4 °C) until analysis. The 150 mL glass jars with the set yoghurts were transferred directly to the cold room at pH 4.60 ± 0.01.

The eight different yoghurts are hereby named in the form X–Y, where X refers to the added level of NWPC and Y refers to the heat treatment temperature of the yoghurt milk base, i.e., L-75; low addition of NWPC and heat treatment at 75 °C for 5 min.

2.2. Chemical analyses

Analyses were performed on fresh samples. The samples for determination of undenatured whey proteins were however freshly frozen and thawed prior preparation. The prepared samples were frozen and thawed prior injection into the high performance liquid chromatography (HPLC) instrument. The content of total solids was determined according to oven drying at 102 ± 2 °C for 48 h (IDF, 2010a). The ash content was determined by ignition of the dried sample in a muffle furnace at 650 ± 25 °C for 4 h (NMKL, 2005). Fat content was determined according to the Röse Gottlieb principle (IDF, 2010b). Minerals (Ca, P, Na, Mg, K, Fe, Cu, Zn) were quantified by adding sub-boiled, concentrated HNO_3 to 2.5–3.5 g

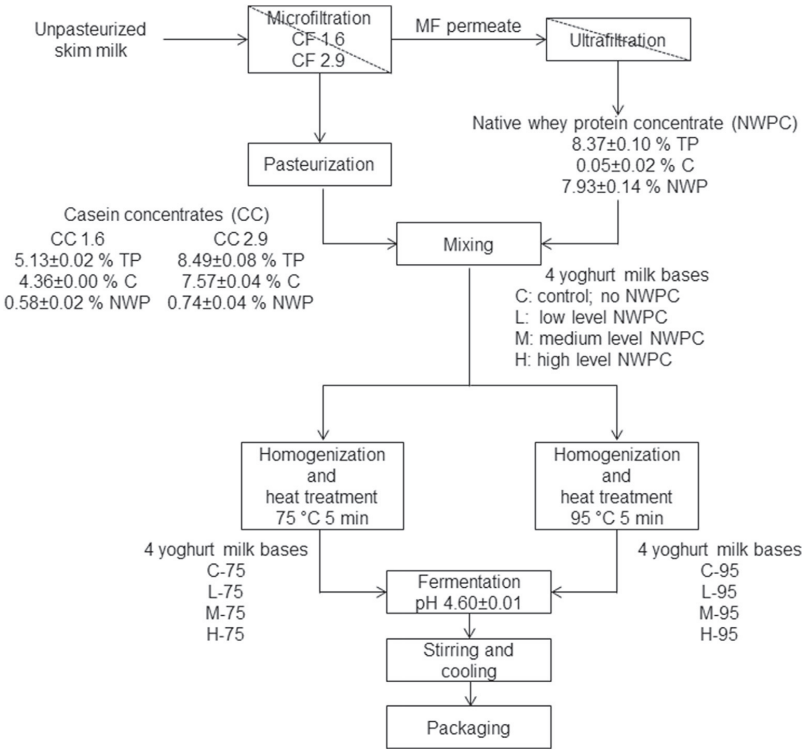

Fig. 1. Flow chart illustrating the production of stirred yoghurt based on microfiltration and ultrafiltration fractions of unpasteurized skim milk. For details of chemical composition see Tables 1 and 2. Abbreviations; CF, concentration factor; CC, casein concentrate; NWPC, native whey protein concentrate; TP, true protein; C, casein; NWP, native whey protein.

liquid sample. Samples were decomposed at 260 °C in an UltraClave (Milestone S.r.l., Sorisole, Italy) and diluted to 50 mL with deionized water. Minerals were detected by 8800 Triple Quadrupole ICP-MS (Agilent Technologies, Tokyo, Japan). ERM®-BD150 and CRM 063R (Institute for Reference Materials and Measurements, Geel, Belgium) were used as reference materials for quantification of minerals. Total nitrogen (TN) and crude protein (CP) were determined by Kjeldahl principle (IDF, 2014). Non-protein nitrogen (NPN) and non-casein nitrogen (NCN) were determined according to IDF (2001) and IDF (2004), respectively. True protein (TP) was calculated by subtracting NPN from TN. Casein (C) was calculated by subtracting NPN and NCN from TN. Native whey protein (NWP) was calculated by subtracting NPN from NCN. A multiplying factor of 6.38 was used to calculate the content of the protein components. Quantitative determination of undenatured α-lactalbumin and β-lactoglobulin A and B was performed according to a modification of the method described by Beyer (1990) (Svanborg, Johansen, Abrahamsen, & Skeie, 2014). The method was further modified by using a 0.45 μm syringe filter (25 mm) (Sarstedt, Hatfield, PA, USA).

Lactic acid and lactose in yoghurt milk bases and yoghurt samples (6 or 7 d of age) were analyzed by the method described by Moe, Porcellato, and Skeie (2013). pH in yoghurt samples were measured at 20 °C after 7 ± 1 d of storage (4 °C) (Radiometer Copenhagen).

Table 1
Chemical composition of pasteurized (75 °C 15 s) casein concentrates; CC 1.6 and CC 2.9, and native whey protein concentrate; NWPC.[a]

Components	CC 1.6	CC 2.9	NWPC
Total solids (%)	10.52 ± 0.13	13.90 ± 0.09	13.28 ± 0.08
Crude protein (%)	5.32 ± 0.02	8.66 ± 0.09	8.77 ± 0.09
True protein (%)	5.13 ± 0.02	8.49 ± 0.08	8.37 ± 0.10
Casein (%)	4.36 ± 0.00	7.57 ± 0.04	0.05 ± 0.02
Native whey protein (NWP) (%)	0.58 ± 0.02	0.74 ± 0.04	7.93 ± 0.14
NWP:casein (%/%)	11.7:88.3	8.9:91.1	99.4:0.6
Fat (%)	0.22 ± 0.01	0.47 ± 0.18	0.11 ± 0.04
Lactose (mmol kg^{-1})	130.6 ± 5.9	122.2 ± 7.7	121.1 ± 2.5
Ash (%)	0.90 ± 0.01	1.14 ± 0.07	0.58 ± 0.11
Calcium (g kg^{-1})	1.7 ± 0.1	2.7 ± 0.1	0.5 ± 0.0
Phosphorus (g kg^{-1})	1.3 ± 0.0	2.0 ± 0.1	0.5 ± 0.0

[a] Values are means ± SD from the mean (n = 2); 1.6 and 2.9 are average volume concentration factors.

2.3. Physical analyses

2.3.1. Firmness and thickness

The texture of the set and stirred yoghurts (7 or 8 d old) were analyzed with a Texture analyzer model TA-XT plus (Stable Micro Systems Ltd., Godalming, UK) with a 5 kg load cell. Set yoghurts in 150 mL glass jars or stirred yoghurts in 300 mL plastic cups were taken directly from the fridge (4 °C) and placed under a 25 mm cylindrical perspex probe with 490 mm^2 contact area. The probe was moved with a test speed at 1 mm s^{-1} to the surface of the yoghurt. At a trigger force of 0.05 N the probe continued to

Table 2
Chemical composition of yoghurt milk bases.[a]

Components	NWPC[b]	T[b]	C-75	C-95	L-75	L-95	M-75	M-95	H-75	H-95
Total solids[c] (%)	ns	ns	13.49 ± 0.04	13.40 ± 0.12	13.50 ± 0.23	13.56 ± 0.32	13.30 ± 0.16	13.60 ± 0.39	13.50 ± 0.29	13.35 ± 0.17
Crude protein[d] (%)	ns	na	8.18 ± 0.01	8.18 ± 0.01	8.33 ± 0.05	8.33 ± 0.05	8.32 ± 0.01	8.32 ± 0.01	8.36 ± 0.04	8.36 ± 0.04
True protein[d] (%)	ns	na	8.02 ± 0.04	8.02 ± 0.04	8.09 ± 0.07	8.09 ± 0.07	8.02 ± 0.02	8.02 ± 0.02	8.09 ± 0.07	8.09 ± 0.07
Casein[d] (%)	*	na	7.10 ± 0.00^{A}	7.10 ± 0.00^{A}	6.00 ± 0.09^{B}	6.00 ± 0.09^{B}	5.08 ± 0.01^{C}	5.08 ± 0.01^{C}	4.35 ± 0.07^{D}	4.35 ± 0.07^{D}
Native whey protein (NWP)[d] (%)	*	na	0.75 ± 0.06^{A}	0.75 ± 0.06^{A}	1.86 ± 0.01^{B}	1.86 ± 0.01^{B}	2.65 ± 0.02^{C}	2.65 ± 0.02^{C}	3.48 ± 0.02^{D}	3.48 ± 0.02^{D}
NWP:casein[d] (%/%)	*	na	9.6:90.4^{A}	9.6:90.4^{A}	23.7:76.3^{B}	23.7:76.3^{B}	34.3:65.7^{C}	34.3:65.7^{C}	44.5:55.5^{D}	44.5:55.5^{D}
UD α-LA[c] (mg mL^{-1})	*	*	0.77 ± 0.03^{B}	0.28 ± 0.06^{A}	2.13 ± 0.03^{C}	0.21 ± 0.00^{A}	2.61 ± 0.09^{D}	0.16 ± 0.02^{A}	2.97 ± 0.08^{E}	0.11 ± 0.00^{A}
UD α-LA[c] (%)	*	*	75.0 ± 3.4^{C}	27.6 ± 6.44^{D}	53.6 ± 1.1^{B}	5.4 ± 0.1^{A}	45.2 ± 2.4^{B}	2.7 ± 0.2^{A}	44.2 ± 1.3^{B}	1.6 ± 0.2^{A}
UD β-LG B[c] (mg mL^{-1})	*	*	1.51 ± 0.15^{D}	0.01 ± 0.01^{A}	5.48 ± 0.61^{C}	0.05 ± 0.01^{A}	6.39 ± 0.16^{BC}	0.06 ± 0.01^{A}	7.02 ± 0.12^{B}	0.05 ± 0.00^{A}
UD β-LG B[c] (%)	ns	*	48.4 ± 6.4^{B}	0.5 ± 0.34^{A}	42.3 ± 5.76^{B}	0.4 ± 0.1^{A}	34.4 ± 1.4^{B}	0.3 ± 0.0^{A}	33.1 ± 1.6^{B}	0.2 ± 0.0^{A}
UD β-LG A[c] (mg mL^{-1})	*	*	1.42 ± 0.01^{A}	0.01 ± 0.01^{A}	5.40 ± 1.04^{B}	0.04 ± 0.01^{A}	6.09 ± 0.52^{B}	0.05 ± 0.01^{A}	6.68 ± 0.60^{B}	0.04 ± 0.00^{A}
UD β-LG A[c] (%)	ns	*	55.1 ± 4.9^{B}	0.5 ± 0.3^{A}	50.5 ± 7.2^{B}	0.4 ± 0.1^{A}	39.8 ± 0.2^{B}	0.3 ± 0.0^{A}	38.0 ± 3.4^{B}	0.2 ± 0.0^{A}
Fat[c] (%)	*	ns	0.43 ± 0.17	0.43 ± 0.17	0.37 ± 0.14	0.39 ± 0.16	0.32 ± 0.13	0.32 ± 0.13	0.28 ± 0.10	0.29 ± 0.11
Lactose[c] (mmol kg^{-1})	ns	ns	118.0 ± 3.3	118.7 ± 1.9	119.5 ± 3.6	124.7 ± 5.1	122.8 ± 5.1	118.9 ± 4.9	124.4 ± 8.7	112.7 ± 8.0
Ash[c] (%)	*	ns	1.07 ± 0.04^{A}	1.04 ± 0.02^{AB}	1.04 ± 0.01^{AB}	1.05 ± 0.01^{AB}	0.94 ± 0.04^{AB}	0.99 ± 0.02^{AB}	0.89 ± 0.03^{B}	0.96 ± 0.04^{AB}
Calcium[c] (g kg^{-1})	*	ns	2.6 ± 0.1^{A}	2.6 ± 0.1^{A}	2.2 ± 0.0^{B}	2.3 ± 0.0^{B}	2.0 ± 0.0^{C}	2.0 ± 0.0^{C}	1.8 ± 0.1^{D}	1.8 ± 0.1^{D}
Phosphorus[c] (g kg^{-1})	*	ns	1.9 ± 0.0^{A}	1.9 ± 0.0^{A}	1.7 ± 0.0^{B}	1.7 ± 0.0^{B}	1.5 ± 0.0^{C}	1.5 ± 0.0^{C}	1.4 ± 0.0^{D}	1.4 ± 0.0^{D}

[a] Denotations C, L, M and H refer to the added level of native whey protein concentrate in terms of whey protein:casein-ratios in the yoghurt milk bases; control (no addition, 10:90); low (25:75); medium (35:65); and high (45:55), respectively. Denotations 75 and 95 refer to heat treatment of yoghurt milk bases; 75 °C 5 min; and 95 °C 5 min, respectively. Values are means ± SD from the mean (n = 2). Milk bases with no common letters within same component differ according to Tukey's pairwise comparison ($P < 0.05$).

[b] Effect of experimental factor identified by analysis of variance; NWPC = native whey protein concentrate addition; T, heat treatment temperature; * indicates significant effect ($P < 0.05$); ns, no significant effect; na, not analyzed (measured in milk bases before heat treatment, therefore not included in the analysis of variance).

[c] Measured in milk bases after heat treatment. UD, undenatured; LA, lactalbumin; LG, lactoglobulin. UD (%) is the percentage share of undenatured whey protein left in milk base after heat treatment compared to the amount of undenatured whey protein in milk base before heat treatment.

[d] Measured in milk bases before heat treatment.

penetrate to a depth of 20 mm at 1 mm s^{-1}. Then the probe returned to the original position with a post speed of 2 mm s^{-1}. The measurements were performed in triplicate in new glass jars or plastic cups each time. The maximum compression force was found using Exponent software (Stable Micro Systems Ltd.) and this value represented the firmness of the set gel as described by Amatayakul, Halmos, Sherkat, and Shah (2006) and the thickness of the stirred gel.

2.3.2. Storage modulus

The storage modulus (G′) of stirred yoghurts was measured using a rheometer type MCR 301 with smooth parallel plate–plate geometry (PP50) (Anton Paar GmbH, Graz, Austria). Yoghurt samples (5 d old) were taken directly from the fridge and a teaspoon of yoghurt was gently placed on the tempered plate (11 °C). The upper plate was slowly lowered to a 2 mm gap. The measurement involved four consecutive intervals; 1) amplitude sweep with controlled strain 0.05–100% and angular frequency 10 rad s^{-1} with 14 measurement points; 2) rotation at a constant shear rate (50 s^{-1}) for 30 s with 3 measurement points; 3) rest under non-destructive oscillating conditions, constant strain 0.05% for 5 min with 60 measurement points; and 4) a second amplitude sweep with equivalent settings as interval 1. Rheoplus/32 V3.40 software (Anton Paar GmbH) was used to detect the upper boundary of the linear viscoelastic range (LVER) in both interval 1 and 4. The storage modulus (G′) at the point where the LVER ended, that means when the G′ value was less than 5% of the plateau value of G′, was detected. In the first interval the storage modulus (G′) value represented the solid-like properties of yoghurt under rest conditions, hereby named non-destructed yoghurt. In interval 4, the G′ value reflected the solid-like properties of the yoghurt after a destructive rotation (interval 2) followed by a period of rest (interval 3), allowing the yoghurt structure to rebuild, hereby named de- and reconstructed yoghurt.

2.3.3. Coagulum particle size

Coagulum particle size distribution in stirred yoghurt samples (5 d old) were measured by laser light diffraction with a Mastersizer 3000 (Malvern Instruments Limited, Malvern, UK). Yoghurt samples (4 °C) were gently added to a 600 mL Hydro LV chamber containing distilled water to an obscuration of 3–10%. Yoghurt and water were blended by stirring at 3500 rpm for 10 s prior measurement. Immediately after stirring the diluted sample was pumped through the wet cell and 10 consecutive measurements (each run 20 s) were done while stirring at 1500 rpm. Dispersant refractive index was set to 1.33 (water) and particle refractive index was set to 1.51. Mie theory of light scattering was used to calculate the size of particles based on the scattering intensity data. Duplicate runs were performed resulting in a total of 20 software-approved measurements of each sample. The mass median diameter, D_{50}, was chosen to represent the coagulum particle size distribution. D_{50} reports the coagulum particle size at which 50% of the coagulum particles in the sample are smaller and 50% are larger.

2.4. Confocal laser scanning microscopy

Microstructure of stirred yoghurt samples was examined in the fluorescent mode as described by Blonk and van Aalst (1993). Dual labelling of the sample was performed by staining protein and fat with fluorescein 5-isothiocyanate (FITC; Sigma–Aldrich, St Louis, MO, USA) and Nile Red (Nile Blue A Oxazone, Sigma–Aldrich) respectively. The yoghurt sample was carefully placed on an object glass, stained and stored for 24 h at 4 °C. Confocal laser scanning microscopy (CLSM) was performed with an inverted microscope Leica TCS SP5 (Leica Microsystems CMS GmbH, Mannheim, Germany) equipped with Ar/DPPS laser with excitation wavelengths of 488 nm (protein) and 561 nm (fat), and emission wavelengths of 498–532 nm (protein) and 592–611 nm (fat). An oil immersion objective (HCX PL APO lambda blue, Leica Microsystems CMS GmbH) with 63 × magnification (numerical aperture 1.4) was used. Five to seven areas of each sample were examined. A representative image (resolution 1024 × 1024) of each sample was acquired (Leica Confocal Software version 2.00, Leica Microsystems CMS GmbH).

2.5. Sensory profiling

Sensory profiling was performed with six or seven experienced assessors from the R&D department of the dairy company TINE SA (Oslo, Norway). Prior sensory profiling the panel was trained to obtain agreement on how to use the continuous scale from 1 to 9. In the training session the assessors individually evaluated four yoghurt products; two extremity points from the yoghurt manufacture and two commercial products. In lack of available commercial, filtrated, high protein yoghurt, plain quark (1.0% fat, 4.3% carbohydrate, 12% protein) was used as one of the training products. The other commercial product was plain yoghurt (3.4% fat, 5.6% carbohydrate, 4.1% protein). Attributes evaluated are presented and defined in Table 3. After the training session the sensory profiling was performed using EyeQuestion Software (version 3.9.6., Logic8 BV, Elst, The Netherlands). Stirred yoghurt samples (7 ± 1 d, stored at 4 °C) were stirred 15 times with a tablespoon, transferred to serving cups and stored for 5 to 10 min at 4 °C in the dark until serving. Each assessor analyzed all eight yoghurt samples in unique randomized orders in two subsequent sessions. The temperature of the yoghurts ranged from 6 to 12 °C during sensory profiling.

2.6. Statistical treatment

R version 3.0.1 (The R foundation for statistical computing) was used for statistical treatment of data. Data were fitted to a linear analysis of variance (ANOVA) model; response = amount of NWPC + temperature + amount of NWPC*temperature + r (replicate block), with amount of NWPC and temperature as fixed factors and replicate block as a random factor. Tukey's studentized range test was used to confirm significant differences ($P < 0.05$) between sample means. Correlation plots were made in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) to investigate the relation between two variables.

3. Results

3.1. Chemical composition of ingredients and yoghurt milk bases and pH development in yoghurts

Kjeldahl analysis of yoghurt milk bases before heat treatment revealed NWP:casein-ratios ranging from 9.6:90.4 (control, no addition of NWPC) to 44.5:55.5 (high addition of NWPC) (Table 2). Denatured whey proteins potentially present in yoghurt milk bases before heat treatment would aggregate and precipitate with casein during sample preparation, and therefore be included in the calculated amount of casein. The concentration (mg mL^{-1}) of undenatured α-lactalbumin and undenatured β-lactoglobulin A and B found in the yoghurt milk bases after heat treatment were significantly higher in milk bases heat-treated at 75 °C for 5 min than 95 °C for 5 min. The amount of undenatured β-lactoglobulin left in heat-treated yoghurt milk bases (calculated as a percentage share of the amount in unheated yoghurt milk bases) showed that 95 °C for 5 min caused an almost complete denaturation of β-lactoglobulin A and B. However, some undenatured α-lactalbumin was still present in yoghurt milk bases heated at 95 °C for 5 min. The percentage share of undenatured α-lactalbumin left in yoghurt milk base C-75 (control) was significantly higher compared with the other yoghurt milk bases heat-treated at 75 °C for 5 min. The content of calcium and phosphorus significantly decreased with increasing NWP:C-ratio (Table 2). The content of fat and lactose in the yoghurt milk bases varied between 0.28 and 0.43 % and 112.7–124.7 mmol kg^{-1} respectively, however no significant differences between yoghurt milk bases with different NWP:C-ratios were observed.

The time needed for the yoghurt to reach pH 4.6 decreased with increasing NWP:C-ratio (Table 4). The acidification was significantly faster in H-75 than in the other yoghurts. The final pH in yoghurts measured after 7 ± 1 d of storage varied between 4.45 and 4.56, indicating a slight post-acidification.

3.2. Firmness and thickness

The effects of temperature and addition of NWPC on the firmness and thickness of set and stirred yoghurts respectively, are shown in Fig. 2. In yoghurts added NWPC (L, M, H), heat treatment at 95 °C for 5 min gave significantly firmer set yoghurts and thicker stirred yoghurts compared with heat treatment at 75 °C for 5 min. Addition of NWPC in set and stirred yoghurts produced from milk bases heat-treated at 75 °C for 5 min caused a significant lower firmness in set yoghurts and lower thickness of stirred yoghurts compared with controls (no addition of NWPC). On the contrary, addition of NWPC to set and stirred yoghurts where the milk bases had been heat-treated at 95 °C for 5 min resulted in increased firmness and thickness compared with the control. The firmness of set yoghurts and the thickness of stirred yoghurts added NWPC seemed to increase with increased NWP:C-ratio. However,

Table 3
Sensory attributes and definitions.

Property	Attribute	Definition
Appearance	Coarse	Visible amount of large protein aggregates in size range 1–5 mm, from no visible to a great number (very irregular surface)
	Granular	Visible amount of smaller protein aggregates below 1 mm seen on the back of the spoon, from no visible to a great number
	Shiny	Degree of glossy surface showing bright reflection, from not shiny (dull) to very shiny
	Ropy/stringy	Degree in which the yoghurt sticks together when falling from a spoon, from non-ropy to very ropy
	Thickness	Visible flow resistance, from thin to thick
Consistency	Viscosity	Flow resistance in the mouth, from watery to creamy
	Mealy	Sensation of flour-like particles in the mouth, from non-mealy to very mealy (example: certain potatoes)
	Gritty	Sensation of grains of sand, from non-gritty to very gritty
	Smooth	Absence of particles, from non-smooth to very smooth
flavor	Yoghurt	Intensity of yoghurt flavor, from no aromatic yoghurt flavor to intense aromatic yoghurt flavor
	Acid	Intensity of basic taste acid, from no acid flavor to intense acid flavor
	Bitter	Intensity of basic taste bitter, from no bitter flavor to intense bitter flavor
	Whey	Intensity of whey flavor, from no whey flavor to intense whey flavor
	Oxidized	Intensity of oxidized flavor initiated by light induction, mostly associated with protein oxidation, from no oxidized flavor to intense oxidized flavor
	Off-flavor	Intensity of atypical flavor, often associated with deterioration or transformation of the product, from no off-flavor to intense off-flavor

Table 4
Characterization of stirred yoghurts; time for yoghurts to reach pH 4.60 ± 0.01, final pH and lactic acid content in yoghurts measured after 7 ± 1 d storage (4 °C).[a]

Parameter	C-75	C-95	L-75	L-95	M-75	M-95	H-75	H-95
Time to reach pH 4.60 ± 0.01 (min)	440 ± 5^{A}	445 ± 5^{A}	400 ± 0^{B}	410 ± 0^{B}	365 ± 5^{C}	385 ± 10^{D}	340 ± 5^{E}	355 ± 0^{C}
Final pH	4.56 ± 0.00^{A}	4.54 ± 0.01^{AC}	4.54 ± 0.01^{AC}	4.48 ± 0.01^{BC}	4.51 ± 0.02^{ACD}	4.48 ± 0.02^{ACD}	4.51 ± 0.02^{ACD}	4.45 ± 0.02^{BD}
Lactic acid (mmol kg^{-1})	138.0 ± 8.3	140.4 ± 6.9	130.8 ± 0.7	130.3 ± 1.1	126.4 ± 1.8	123.8 ± 1.1	121.5 ± 2.5	119.4 ± 3.8

[a] Denotations C, L, M and H refer to the added level of native whey protein concentrate in terms of whey protein:casein-ratios in the yoghurt milk bases; control (no addition, 10:90); low (25:75); medium (35:65); and high (45:55), respectively. Denotations 75 and 95 refer to heat treatment of yoghurt milk bases; 75 °C 5 min; and 95 °C 5 min, respectively. Values are means ± SD from the mean (n = 2); time values are rounded to the nearest 5 min. Yoghurts with no common letters within same characteristic differ according to Tukey's pairwise comparison ($P < 0.05$).

significant differences were only observed at heat treatment 95 °C for 5 min between set yoghurt L-95 and H-95, and at heat treatment 75 °C for 5 min between stirred yoghurt L-75 and H-75. The firmness of control set yoghurts was significantly higher when the yoghurt milk bases were heat-treated at 75 °C for 5 min compared with 95 °C for 5 min.

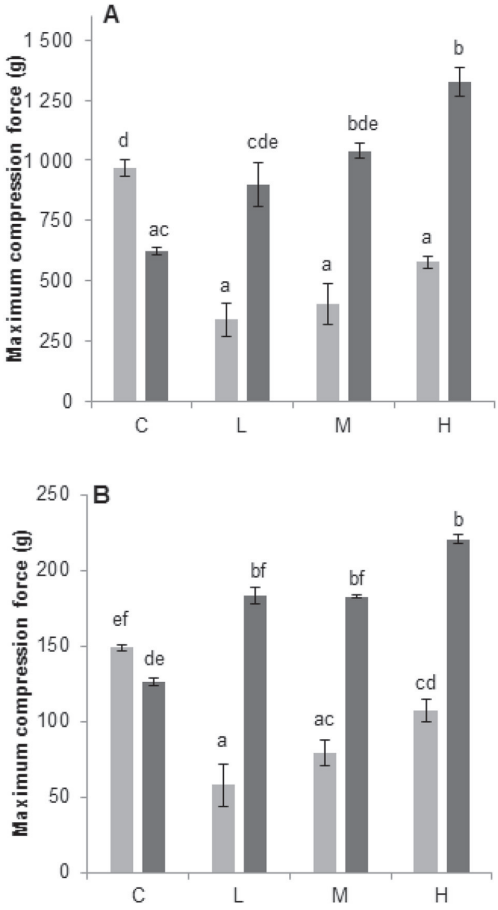

Fig. 2. Firmness (mean ± SD from the mean, n = 2) of set yoghurts (A) and thickness of stirred yoghurts (B) measured with texture analyzer. Yoghurt milk bases were heated at (■) 75 °C for 5 min or (■) 95 °C for 5 min. Denotations C, L, M and H refer to the added level of native whey protein concentrate in terms of whey protein:casein-ratios in the yoghurt milk bases; control (no addition, 10:90); low (25:75); medium (35:65); and high (45:55), respectively. Yoghurts with no common letters differ according to Tukey's pairwise comparison ($P < 0.05$). Note the different scale values of the y-axes.

3.3. Storage modulus

The storage modulus (G′) of stirred yoghurts was measured in non-destructed yoghurt and de- and reconstructed yoghurt (Fig. 3).

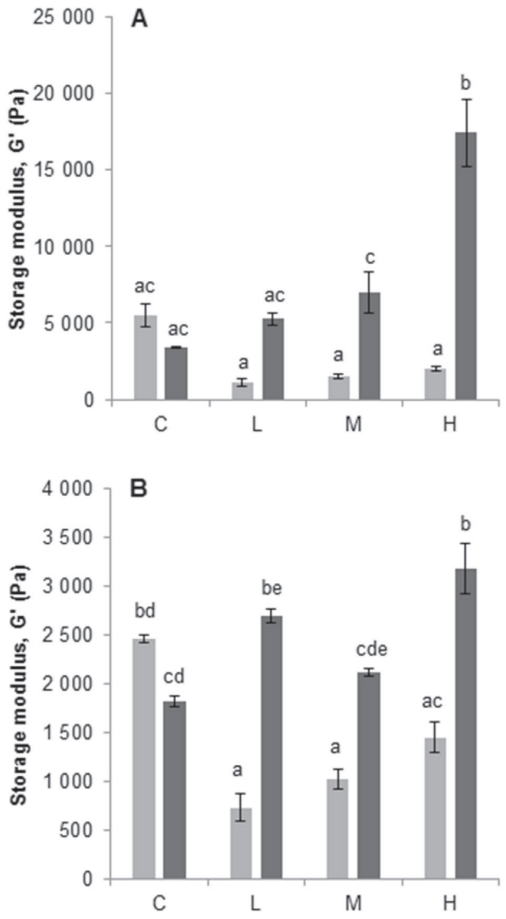

Fig. 3. Storage modulus (G′) (mean ± SD from the mean, n = 2) in stirred yoghurts measured where linear viscoelastic range ends (when the G′ value is less than 5% of the plateau value of G′) in (A) interval 1 (non-destructed yoghurt) and (B) interval 4 (de- and reconstructed). Yoghurt milk bases were heated at (■) 75 °C for 5 min or (■) 95 °C for 5 min. Denotations C, L, M and H refer to the added level of native whey protein concentrate in terms of whey protein:casein-ratios in the yoghurt milk bases; control (no addition, 10:90); low (25:75); medium (35:65); and high (45:55), respectively. Yoghurts with no common letters differ according to Tukey's pairwise comparison ($P < 0.05$). Note the different scale values of the y-axes.

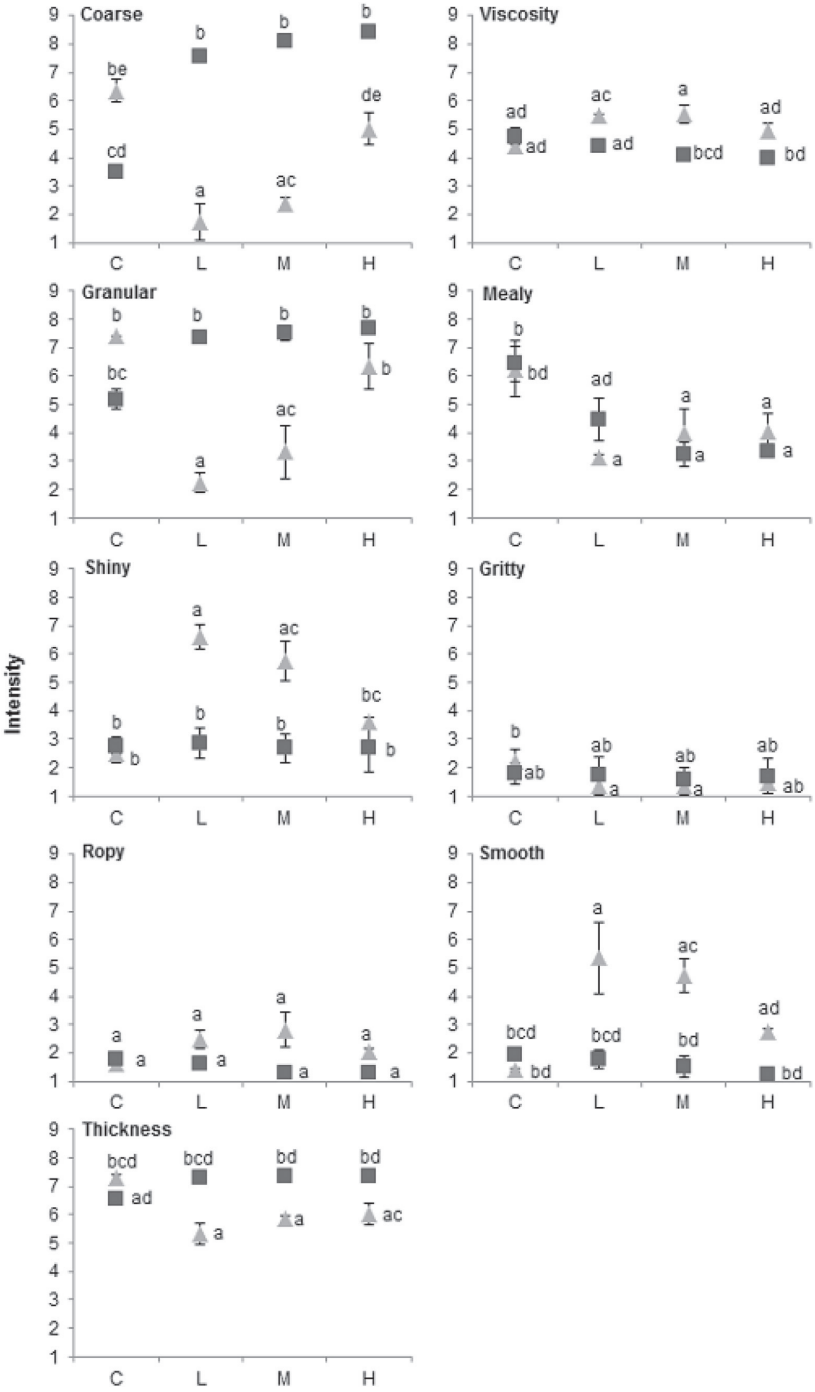

Fig. 4. Sensory profiling of stirred yoghurts produced from yoghurt milk bases heated at (▲) 75 °C for 5 min and (■) 95 °C for 5 min; intensity (mean ± SD from the mean, n = 2) on a continuous scale from 1 to 9 of appearance attributes (coarse; granular; shiny; ropy; thickness) and consistency attributes (viscosity; mealy; gritty; smooth). Denotations C, L, M and H refer to the added level of native whey protein concentrate in terms of whey protein:casein-ratios in the yoghurt milk bases; control (no addition, 10:90); low (25:75); medium (35:65); and high (45:55), respectively. Yoghurts with no common letters differ according to Tukey's pairwise comparison ($P < 0.05$).

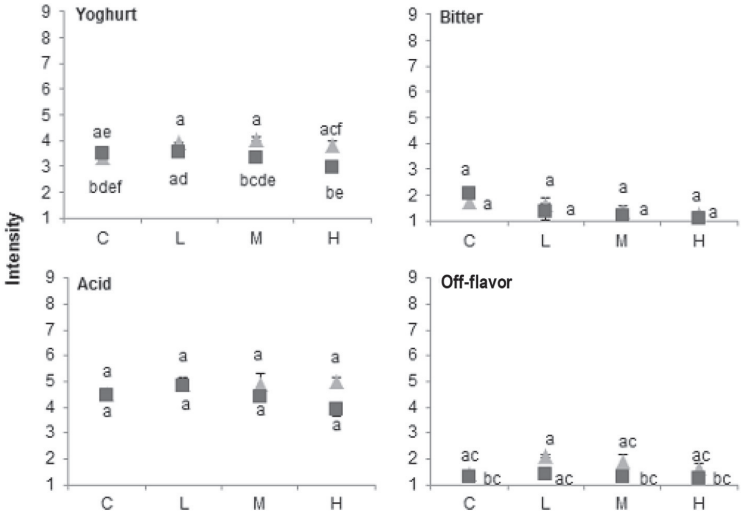

Fig. 5. Sensory profiling of stirred yoghurts produced from yoghurt milk bases heated at (▲) 75 °C for 5 min and (■) 95 °C for 5 min; intensity (mean ± SD from the mean, n = 2) on a continuous scale from 1 to 9 of flavor attributes (yoghurt; acid; bitter; off-flavor). Denotations C, L, M and H refer to the added level of native whey protein concentrate in terms of whey protein:casein-ratios in the yoghurt milk bases; control (no addition, 10:90); low (25:75); medium (35:65); and high (45:55), respectively. Yoghurts with no common letters differ according to Tukey's pairwise comparison ($P < 0.05$).

The results showed that non-destructed yoghurts had higher G′ than de- and reconstructed yoghurts, revealing that the original solid-like properties of non-destructed yoghurts could not be restored under the given measurement conditions. The yoghurt milk base with high addition of NWPC (H) and heat-treated at 95 °C for 5 min gave a yoghurt with significantly higher G′ than the other yoghurts in their non-destructed state. De- and reconstructed yoghurts produced from yoghurt milk bases heat-treated at 75 °C and added NWPC (L, M or H) had significantly lower G′ than the yoghurts produced from yoghurt milk bases heat-treated at 95 °C and with the same amounts of NWPC added. The storage modulus of de- and reconstructed yoghurts seemed to be influenced by the heat treatment temperature and NWPC addition similarly as the firmness and thickness as measured with texture analyzer (Fig. 2). An increased heat treatment temperature of yoghurt milk bases caused significantly higher G′ of yoghurts where NWPC were present. The opposite was observed for control yoghurts, where the lowest temperature treatment of yoghurt milk bases gave higher G′, however the differences were not significant.

3.4. Coagulum particle size

D_{50} was chosen to represent the coagulum particle size distribution in yoghurts (Fig. 6). The smallest coagulum particle size distribution appeared in yoghurts produced from yoghurt milk bases with added NWPC (L, M or H) and heat-treated at 75 °C for 5 min. The coagulum particle size of yoghurts produced from yoghurt milk bases heat-treated at 95 °C for 5 min significantly increased with increasing addition of NWPC. For the control yoghurt heat treatment at 95 °C for 5 min gave significantly smaller coagulum particles than 75 °C for 5 min.

Fig. 7 presents the correlation between thickness of stirred yoghurts and G′, particle size and coarseness. A strong linear relationship was found between G′ and thickness ($R^2 = 0.93$), and between coarseness and thickness ($R^2 = 0.91$). A good exponential correlation was found between coagulum particle size and thickness ($R^2 = 0.93$).

3.5. Microstructure

The effects of heat treatment and addition of NWPC on the microstructure of stirred yoghurts analyzed by CLSM are shown in

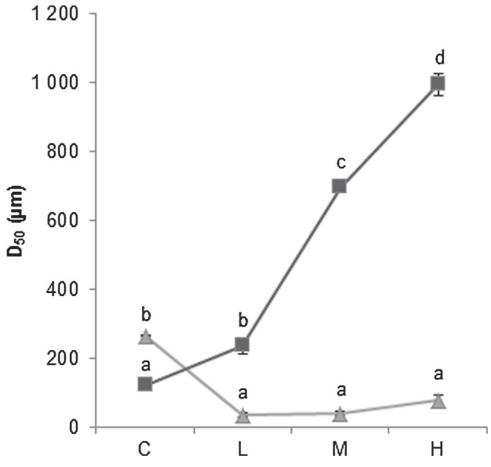

Fig. 6. Coagulum particle size distribution (mean ± SD from the mean, n = 2) of yoghurt samples produced from yoghurt milk bases heated at (▲) 75 °C for 5 min and (■) 95 °C for 5 min; D_{50} (particle size at which 50% of the particles in the sample are smaller and 50% are larger). Denotations C, L, M and H refer to the added level of native whey protein concentrate in terms of whey protein:casein-ratios in the yoghurt milk bases; control (no addition, 10:90); low (25:75); medium (35:65); and high (45:55), respectively. Yoghurts with no common letters differ according to Tukey's pairwise comparison ($P < 0.05$).

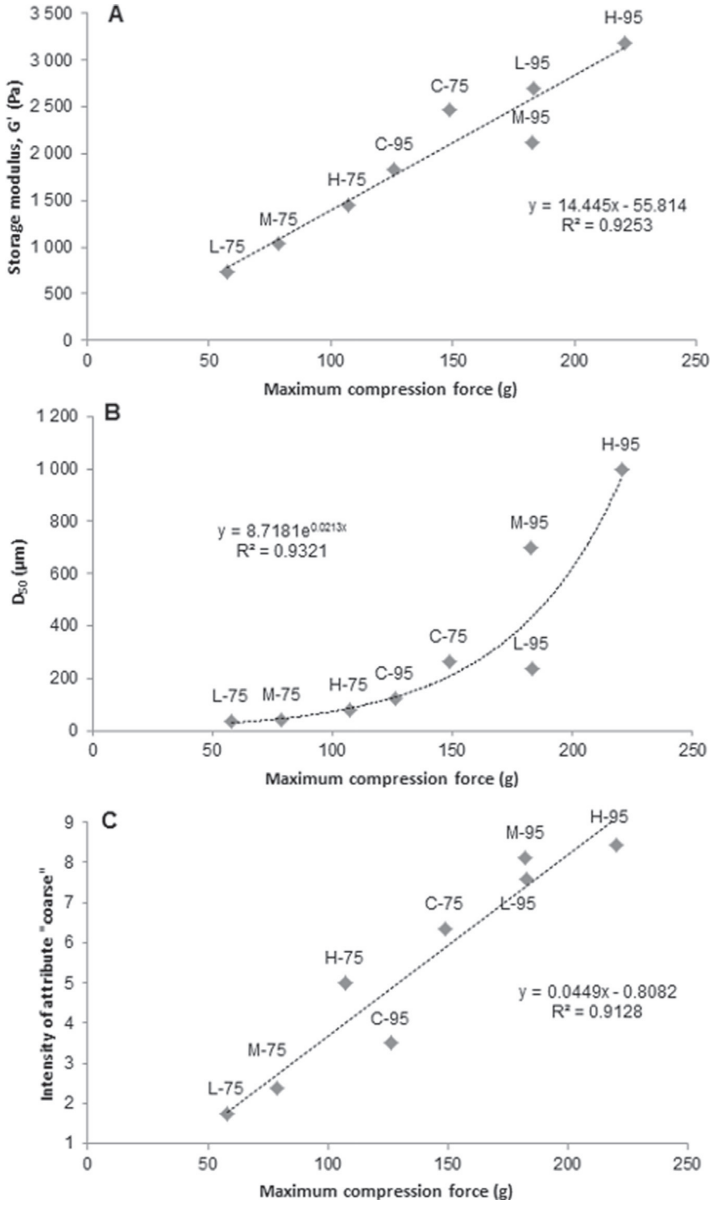

Fig. 7. Correlation plots of (A) storage modulus (G′) measured where the linear viscoelastic range ends in interval 4 (after rotation and rest), (B) coagulum particle size distribution D_{50} (particle size at which 50% of the sample is smaller and 50% is larger), and (C) intensity of consistency attribute "coarse", all as functions of maximum compression force measured in stirred yoghurts with texture analyzer. Denotations C, L, M and H refer to the added level of native whey protein concentrate in terms of whey protein:casein-ratios in the yoghurt milk bases; control (no addition, 10:90); low (25:75); medium (35:65); and high (45:55), respectively. Denotations 75 and 95 refer to heat treatment of yoghurt milk bases; 75 °C for 5 min; and 95 °C for 5 min, respectively. Trends are indicated by linear or exponential trendlines. R^2-values reveal how closely the estimated values for the trendline correspond to the actual data.

Fig. 8. Yoghurts produced from milk bases heat-treated at 95 °C for 5 min appeared to have a densely clustered and discontinuous network. Yoghurts from milk bases heat-treated at 75 °C for 5 min with low or medium levels of NWPC had a more branched and continuous network.

3.6. Sensory properties

High heat treatment temperature of yoghurt milk bases added NWPC (L, M or H) resulted in significantly more coarse, granular and thick yoghurts than low temperature (Fig. 4). Increased

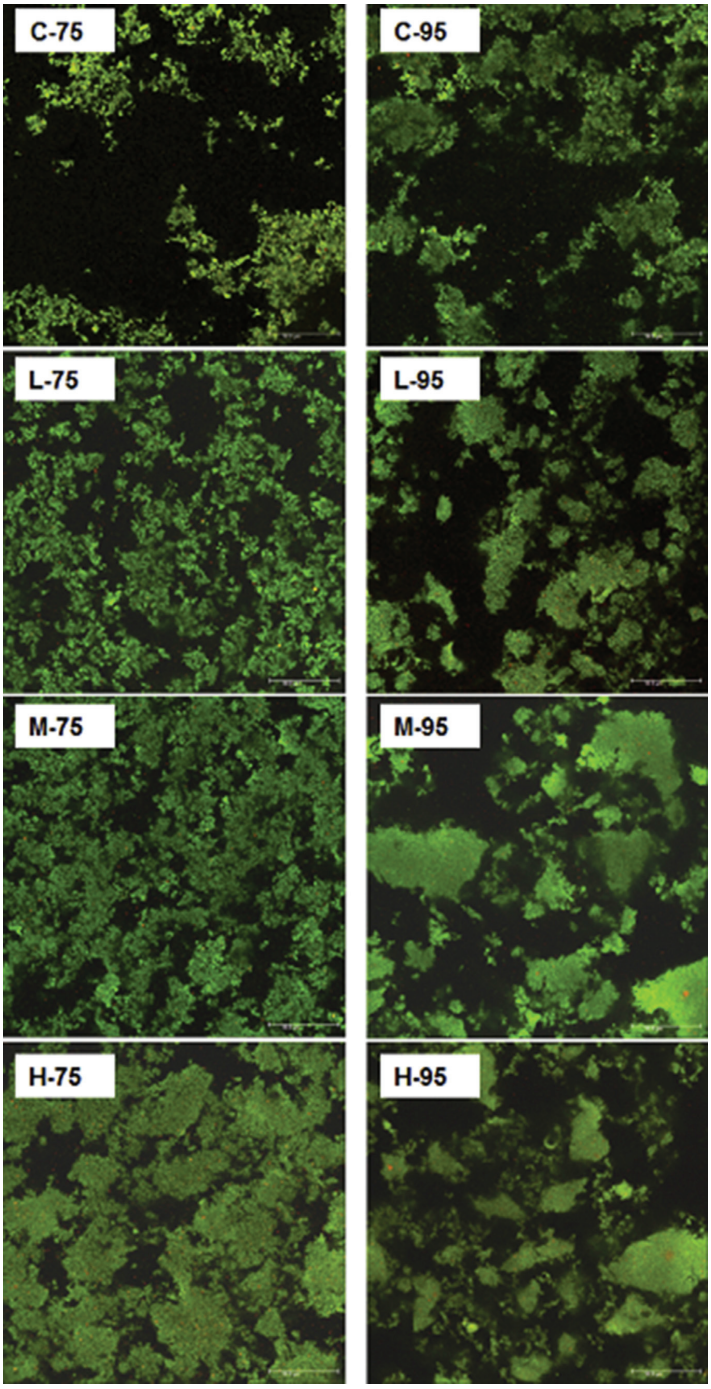

Fig. 8. Microstructure of stirred yoghurt samples; confocal laser scanning microscopy images of protein structure. Denotations C, L, M and H refer to the added level of native whey protein concentrate in terms of whey protein:casein-ratios in the yoghurt milk bases; control (no addition, 10:90); low (25:75); medium (35:65); and high (45:55), respectively. Denotations 75 and 95 refer to heat treatment of yoghurt milk bases; 75 °C for 5 min; and 95 °C for 5 min, respectively. Bar = 50 μm.

addition of NWPC only seemed to increase the intensity of these attributes when the heat treatment was set to 75 °C for 5 min. Yoghurts produced from milk bases added low (L) or medium (M) levels of NWPC and heat-treated at 75 °C for 5 min had significantly shinier appearance and significantly smoother consistency than the corresponding yoghurts from milk bases heat-treated at 95 °C for 5 min. Heat treatment and addition of NWPC did not affect the attributes ropy and gritty, while mealy consistency was reduced by the addition of NWPC compared with control yoghurt. The experimental factors did only scarcely affect the flavor attributes; yoghurt flavor, acid, bitter and off-flavor (Fig. 5). Oxidized flavor and whey flavor were absent in the yoghurts (Fig. 5).

4. Discussion

The concentration of caseins by MF with 0.14 μm pore size ceramic membranes resulted in a concurrent increase in calcium and phosphorus in the casein concentrates. Two-third of the calcium and half of the phosphate in milk are present in the casein micelles (de la Fuente, 1998; Gaucheron, 2005), thus a concentration of caseins results in a simultaneous concentration of these minerals. Consequently, yoghurt milk bases with an increased NWP:C-ratio contained lower concentrations of calcium and phosphorus.

Yoghurts with increased whey protein content reached pH 4.6 faster than yoghurts with higher casein content. Salaün, Mietton, and Gaucheron (2005) also reported increased buffering capacity of a casein micelle suspension with increased micellar casein concentration. When the pH of milk is reduced due to bacterial production of lactic acid, the buffering compounds of milk, like soluble calcium phosphate, citrate and acidic and basic amino acid side chains of caseins, become protonated and the micellar calcium phosphate dissolve into the serum phase (Gaucheron, 2005). An increased concentration of buffering compounds (i.e., amino acids of casein) will therefore increase the buffering capacity (Gaucheron, 2005), and consequently lead to a reduced rate of acidification when measured as changes in pH. The observed increased concentration of lactic acid (however not significant) in the yoghurts with the highest amount of caseins, support these observations.

An almost complete denaturation of β-lactoglobulin was observed after heat treatment at 95 °C for 5 min, which is in agreement with the results reported by Dannenberg and Kessler (1987). According to their results, heat treatment of skim milk at 75 °C for 5 min would lead to a denaturation degree of β-lactoglobulin B of about 20%. However, a higher denaturation degree was detected in the present study. Depending on the milk base, 33–48% undenatured β-lactoglobulin was present after heat treatment at 75 °C for 5 min. The observed increased percentage share of undenatured whey proteins in yoghurt milk bases with reduced NWP:C-ratio can be due to the chaperon activity of the caseins (Holt, Carver, Ecroyd, & Thorn, 2013). Spiegel (1999) studied the influence of the lactose concentration in whey protein concentrates on the denaturation degree of β-lactoglobulin. A higher lactose content resulted in a lower degree of denaturation, explained by the hydration effect of the lactose on the protein molecule. Increased level of lactose will increase the ordering of the water structure around the β-lactogloublin molecules, and favour the associated (dimeric form) and undenatured state of β-lactoglobulin (Anema, Lee, & Klostermeyer, 2006). In the present study the lactose content was kept close to or below (approximately 117 mg mL^{-1} equivalent to 4%) the regular lactose content in milk while the protein concentration was increased to 8% true protein, presumably resulting in a yoghurt milk base more prone to heat denaturation. The importance of lactose to protein ratio in high protein, low fat yoghurts manufactured by membrane filtration prior acidification was not the focus in this study, and should be further investigated.

Some of the α-lactalbumin was undenatured after heat treatment at 95 °C for 5 min, being more resistant to irreversible denaturation than β-lactoglobulin (Anema, 2009). A higher percentage share of undenatured α-lactalbumin was present after heat treatment at both 75 °C and 95 °C for 5 min of the yoghurt milk base with the highest casein content (C) compared with the milk bases added NWPC (L, M, H). This is in accordance with the results of Oldfield, Singh, and Taylor (2005), who reported that whey protein depleted milk had the slowest rate of α-lactalbumin denaturation compared with whey protein enriched milk or skim milk. As the concentration of whey proteins was increased the amount of available free thiol groups of β-lactoglobulin was concurrently increased. Calvo, Leaver, and Banks (1993) reported that the aggregation (irreversible denaturation) of α-lactalbumin is dependent upon the concentration of free thiol groups present in other whey proteins.

Plain yoghurt with high consumer acceptance should be smooth and fine-bodied with a typical flavor, and free from defects such as granular, lumpy, and whey separation (Lucey, 2004; Lucey & Singh, 1998). Yoghurts produced from yoghurt milk bases heat-treated at 95 °C for 5 min and added NWPC (L, M or H) were more coarse and granular than yoghurts made from milk bases heat-treated at 75 °C for 5 min and added NWPC. Schmidt, Sistrunk, Richter, and Cornell (1980) reported that heat treatment at 90 °C for 30 min resulted in a “grainy” body of set yoghurt, while heat treatment at 80 or 85 °C for 30 min gave a “smooth and firm” bodied yoghurt with minimal visible syneresis. The same denaturation degree is expected at heat treatment at 85 °C for 30 min as for heat treatment at 95 °C for 5 min (Dannenberg & Kessler, 1987). A smooth and firm yoghurt texture could therefore be expected in the yoghurts produced from milk bases heat-treated at 95 °C for 5 min in this study. However, the content of total solids and protein in the set yoghurts produced by Schmidt et al. (1980) were 18% and 6.4%, respectively. Higher protein content (8%) and different composition (low lactose) of yoghurts in the present study could enhance the interaction properties during heating and acidification, resulting in a more robust gel network and a more coarse and granular yoghurt appearance. Remeuf, Mohammed, Sodini, and Tissier (2003) observed an increased graininess in stirred yoghurt when the yoghurt was added WPC compared with skim milk powder, and when the heating time at 90 °C was extended from 1 to 5 min. The higher denaturation degree of whey proteins in the milk bases added NWPC and heat-treated at 95 °C for 5 min would result in increased formation of β-lactoglobulin/κ-casein, β-lactoglobulin/β-lactoglobulin and β-lactoglobulin/α-lactalbumin complexes (Gezimati, Creamer, & Singh, 1997; Oldfield, Singh, Taylor, & Pearce, 1998; Vasbinder, Alting, & de Kruif, 2003). As the pH in the yoghurt milk base is reduced and approaches the pI of the unfolded and aggregated whey proteins (pI ≈ 5.3 for β-lactoglobulin), the proteins in the yoghurt milk base start to form a network, due to reduction in electrostatic repulsions (Bryant & McClements, 1998). Increased amount of complexes and connecting points leads to increased branching, and consequently gels with higher G′ (Lucey, Teo, Munro, & Singh, 1997). Measured G′ in de- and reconstructed yoghurts were higher for yoghurts produced from milk bases heated at 95 °C for 5 min and added NWPC, compared with yoghurts with corresponding NWPC-levels and heat-treated at 75 °C for 5 min. The G′ of de- and reconstructed yoghurts correlated well with the firmness of stirred yoghurts (R^2 = 0.93). The effects of temperature treatment and NWPC addition on the firmness of set yoghurts were similar with the effects on stirred yoghurts, although set yoghurts in general

obtained higher maximum compression force values. This result is in accordance with Lee and Lucey (2006), who reported a high correlation between G′ of intact gels (set) and viscosity of stirred gels, revealing that the structure of the initial gel network greatly influenced the physical and sensory attributes of the stirred yoghurts.

An exponential correlation was found between firmness and coagulum particle size of stirred yoghurt. When shearing is applied to a yoghurt gel, the gel network is broken into smaller gel particles. If the firmness of a yoghurt gel is too high, the shearing applied could potentially be insufficient to break the gel network into smaller coagulum particles, and result in a coarse and granular yoghurt gel with low degree of smoothness. The coagulum particle size was smaller for yoghurts produced from milk bases added NWPC and heat-treated at 75 °C for 5 min compared with 95 °C for 5 min. The smallest coagulum particle size was measured in yoghurts added low or medium levels of NWPC, and these yoghurts were also smoother and shinier than the other samples. The findings were supported by the microstructures as revealed by CLSM, showing more branched and continuous yoghurt structures with smaller whey pockets in yoghurts added low or medium levels of NWPC compared with the remaining yoghurts. Cayot, Schenker, Houzé, Sulmont-Rossé, and Colas (2008) also reported a strong relationship between smoothness and particle size in stirred yoghurt. For yoghurts produced from yoghurt milk bases heat-treated at 95 °C for 5 min, addition of NWPC resulted in increased coagulum particle size, and these yoghurts were perceived as more coarse than the others. Krzeminski et al. (2011) and Kücükcetin (2008) also reported increased particle size with increased whey protein:casein-ratio in stirred yoghurt. The yoghurts produced by Krzeminski et al. (2011) were also sensory perceived as more grainy by Tomaschunas, Hinrichs, Köhn, and Busch-Stockfisch (2012) in their evaluation of the yoghurt samples. A high protein content or a low fat content are other factors previously reported to increase particle size and increase graininess in yoghurts (Brauss, Linforth, Cayeux, Harvey, & Taylor, 1999; Johansen, Laugesen, Janhøj, Ipsen, & Frost, 2008; Krzeminiski et al., 2011; Tomaschunas et al., 2012).

The firmness and thickness, measured by texture analyzer, of set and stirred yoghurts added NWPC (L, M or H) seemed to increase with increased NWP:casein-ratio, although significant differences were only observed between L-95 and H-95 for set yoghurts and L-75 and H-75 for stirred yoghurts. Increased gel strength of yoghurts by the addition of whey proteins was also reported by Krzeminski et al. (2011), Kücükcetin (2008), Lucey et al. (1999), and Puvanenthiran et al. (2002). A higher amount of denatured whey proteins could increase the number of whey proteins and casein micelle complexes formed during heat treatment. This will increase the potential for intermolecular interaction and branching during acidification, and thus increase the firmness of set yoghurt and the thickness and solid-like properties (G′) of the final stirred yoghurt. A too firm yoghurt gel due to high amount of denatured whey proteins could probably explain the higher resistance to gel degradation during stirring, causing increased coagulum particle size of these yoghurts. A similar explanation was proposed by Chever, Guyomarc'h, Beaucher, and Famelart (2014) who investigated the effect of protein composition and heat treatment on high protein, fat free acid milk gels.

According to Krzeminski et al. (2011), Kücükcetin (2008), Lucey et al. (1999), and Puvanenthiran et al. (2002), lower firmness and lower storage modulus of yoghurts were expected with decreasing whey protein:casein-ratio. Therefore, by extrapolation of these data, the firmness and storage modulus of yoghurt without addition of NWPC (C) was expected to be lower compared with the yoghurts added NWPC (L, M, H). In contrast, the firmness, thickness and storage modulus of the control yoghurt made from milk base heat-treated at 75 °C for 5 min were higher compared with the yoghurts produced from milk bases added NWPC and heat-treated at 75 °C for 5 min. The conflicting results could be due to the higher protein content in the yoghurts produced in the present study. Chever et al. (2014) also reported higher firmness of a stirred acid milk gel supplemented with calcium-caseinate compared with gels where parts of the calcium-caseinate were replaced with whey protein isolate. A possible explanation for this phenomenon could be that when the ratio of whey protein to casein is too low (i.e., 10:90), and the denaturation degree of the present whey proteins is fairly low (i.e., 50%), only a minor part of the casein micelles will be covered by denatured whey proteins. When the pH reaches the pI of the casein micelles (pI 4.6), the aggregation of the casein micelles will mainly occur as a result of the reduced net negative charge of the casein micelles (Fox & Kelly, 2004). Since the solubilization of the colloidal calcium phosphate is completed at a higher pH (i.e., 4.9) than the pI of the casein micelles, the internal weakening of the network, caused by dissolution of colloidal calcium phosphate as underlined by Lucey (2004), will not take place.

5. Conclusion

This study showed that the denaturation status of the whey proteins and the ratio of whey protein to casein influenced the yoghurt structure and thereby the rheological and sensory properties of the yoghurt without giving any flavor defects. An increased whey protein:casein-ratio (25:75-35:65) in combination with a low temperature treatment (75 °C, 5 min) of the yoghurt milk base gave a smooth and viscous high protein, low fat yoghurt where a considerable amount of the whey proteins were present in their undenatured form.

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Fractionation by microfiltration: Effect of casein micelle size on composition and rheology of high protein, low fat set yoghurt

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ABSTRACT

The effects of casein micelle size on rheological properties of high protein (5.6% crude protein), low fat (≤0.2%) set yoghurt were investigated. Microfiltration with 0.20 μm membranes was used to fractionate skim milk with an average casein micelle size of ~174 nm into a retentate and a permeate containing "large" (~183 nm) and "small" (~129 nm) casein micelles, respectively. The permeate containing the small casein micelles was further concentrated with 0.10 μm membranes. Yoghurt milk bases with large or small casein micelles were subjected to heat treatment at two different temperatures; 95 °C or 75 °C for 5 min. Yoghurt milk base with small casein micelles gave set yoghurts with higher storage modulus (G′) and higher firmness than yoghurt milk base with large casein micelles. Increased gelation capacity can be attributed to an increased amount of κ-casein in the yoghurt milk base containing small casein micelles.

1. Introduction

Thickness and viscosity are important drivers of liking of low fat yoghurt (Desai, Shepard, & Drake, 2013; Frøst & Janhøj, 2007). In low fat yoghurts the thickness is typically increased by elevating solid nonfat content of the yoghurt milk base by evaporation, membrane filtration or addition of skim milk powder, adding stabilizers, using strains of yoghurt bacteria producing extracellular polysaccharides, and/or concentrating the fermented yoghurt (Robinson, Lucey, & Tamime, 2006). With the rise in popularity of high protein dairy products with a minimum of additives ("clean label") (Mellentin, 2013), concentrated yoghurt or Greek yoghurt found its way to the consumers. An increase in the protein content of low fat yoghurt by membrane concentration of the yoghurt milk base benefits the thickness and may exclude the need of stabilizers or other additives. Protein is, however, a valuable component of milk and more economical ways of processing a thick, "clean label" yoghurt are of interest to the dairy industry.

Conventional heat treatment of a yoghurt milk base, i.e., 95 °C for 5 min, gives close to 100% denaturation of β-lactoglobulin (β-LG) (Anema, 2000; Dannenberg & Kessler, 1987) and approximately 75% denaturation of α-lactalbumin (α-LA) (Anema, 2001). During heat treatment, the reactive thiol group of β-LG is exposed and can form disulphide bonds with other β-LG molecules or other cysteine-containing proteins, or with proteins containing disulphide-bridges [α-LA, κ-casein (κ-CN) or α_{S2}-CN]. According to Vasbinder, Alting, and de Kruif (2003), 65% of the β-LG and 50% of the α-LA are associated with the casein micelle after heat treatment of skim milk at 90 °C for 10 min, whereas approximately 25% of both whey proteins exist as soluble aggregates, mainly composed of whey proteins. During acidification of a heat-treated yoghurt milk base, the surface charge of whey protein coated casein micelles is decreased. At the pI of β-LG (~5.3), the whey protein coated casein micelles start to aggregate and form a network (Lucey, 2004).

In yoghurt milk bases with a high protein content (8%) and a low fat content (<0.5%), a reduced heat treatment temperature (75 °C for 5 min) has been reported to improve the sensory properties of stirred type yoghurts (Jørgensen et al., 2015). The positive effect of a reduced heat treatment temperature was however only observed for yoghurts with an increased whey protein:casein-ratio (25:75–35:65). A conventional heat treatment of these yoghurt milk bases gave yoghurts that were too thick, granular and coarse according to the sensory assessors. In addition, these yoghurts had high storage moduli (G′) and high maximum compression forces (thickness).

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Casein micelles are polydisperse, and the diameter of the casein micelles as measured with electron microscopy varies from 50 to 500 nm (Fox & Kelly, 2004). Smaller casein micelles have a higher κ-CN content of total casein (Dalgleish, Horne, & Law, 1989; Davies & Law, 1983; Donnelly, McNeill, Buchheim, & McGann, 1984; O'Connell & Fox, 2000). In cheese production, smaller casein micelles have been reported to produce firmer rennet gels at a faster rate than larger casein micelles (Gustavsson et al., 2014; Logan et al., 2015; Walsh et al., 1998). This has been explained by the increased surface area of the smaller casein micelles, and thereby a greater availability of sites for enzymatic cleavage of the κ-CN from the casein micelle surface. The increased total surface area of paracasein micelles of milk with smaller casein micelle size distribution allows for more interaction points and a more compact arrangement of proteins.

Heat treatment of a yoghurt milk base with smaller casein micelles (high amount of κ-CN), could possibly give a higher association (>65%) of β-LG on the casein micelles and consequently more aggregation points during acid gelation. Based on previous findings on the effect of casein micelle size on rennet gels, we hypothesized that the casein micelle size could influence the rheology of yoghurt, with smaller casein micelles giving a firmer yoghurt gel. Horne (2003) observed no effect of casein micelle size on stiffness of acidified gels. These gels were however made by glucono-δ-lactone-acidification of non-heat-treated milk.

Industrial applicable technology, like microfiltration (MF), can be used to obtain casein concentrates with different casein micelle size distributions. According to Jørgensen et al. (2016), MF with 0.20 μm ceramic membranes renders it possible to divide skim milk with an average casein micelle size of ~172 nm into a retentate and a permeate containing "large" (~186 nm) and "small" (~130 nm) casein micelles, respectively.

The objective of this work was to investigate the possible effects of casein micelle size on rheological properties of high protein, low fat set yoghurt. Set yoghurt was chosen as a model system since previous research has shown a high correlation between storage modulus (G′) of the set yoghurt and viscosity of the stirred yoghurt (Lee & Lucey, 2006).

2. Materials and methods

2.1. Experimental design

Yoghurt milk bases were produced from MF retentates with large or small casein micelles (Fig. 1). The yoghurt milk bases were heat-treated at two different temperatures (75 or 95 °C for 5 min) leading to a total of 4 factor combinations. Three replicate blocks gave a total of 12 yoghurt batches.

2.2. Casein micelle size fractionation and concentration

Milk from the university herd of Norwegian Red Cattle was separated (Westfalia Separator AG, MSD50-01-076, Oelde, Germany) at 63 °C to a fat content of 0.09 ± 0.05%. The skim milk was pasteurized (Alfa-Laval, M6-MFMC, Lund, Sweden) at 73 °C for 15 s, cooled to 45 °C and temporarily collected in a double-O-vat (Landteknikk A/L, Trondheim, Norway). The skim milk was cascade microfiltered on a pilot-scale MF system (Membranteknikk AS, MTCVV 3-25, Flekkefjord, Norway) according to Fig. 1, and as described by Jørgensen et al. (2016). In short, three 0.20 μm ceramic membranes (Atech innovations GmbH, Gladbeck, Germany) were used to fractionate the skim milk into a retentate (retentate 1) containing the large casein micelles and a permeate (permeate 1) containing the small casein micelles. MF of pasteurized skim milk (3.53 ± 0.03% protein) was performed at a uniform transmembrane pressure with constant permeate flux of 58.3 L h^{-1} m^{-2} to a volume concentration factor of 2.5. The crossflow velocity during filtration was 6.9 m s^{-1} and the filtration temperature was 50.5 ± 0.3 °C. The permeate (permeate 1) was collected in a double-jacketed 300 L tank (De Danske Mejeriers Maskinfabrik, Kolding, Denmark) connected to cold water and steam and cooled to 4 °C. The following day, the permeate was gently heated under continuously stirring to 45 °C, and concentrated by MF to a volume concentration factor of 3.0 using 0.10 μm ceramic membranes (Orelis Environnement SAS, Salindres, France). Filtration was performed at a crossflow velocity of 6.9 m s^{-1} with a constant permeate flux of 58.8 L h^{-1} m^{-2} and a filtration temperature of 50.0 ± 0.1 °C. The obtained retentate (retentate 2) contained concentrated small casein micelles (SCM).

2.3. Production of set yoghurt

Set yoghurts were produced according to Jørgensen et al. (2015). The procedure was modified by excluding the homogenization step due to low fat content in the yoghurt milk bases (0.1–0.2%). The content of native whey proteins (NWP) differed in the two retentates (Table 1). To achieve yoghurt milk bases with equal NWP:casein-ratios, a recombined solution of NWP powder (Prolacta® 95, Lactalis Ingredient, Bourgbarré, France) was added to retentate 1 with the large casein micelles (LCM). The NWP powder was produced by cold MF (<10 °C) of skim milk according to information from the producer. Fig. 1 gives a flow chart of the production of yoghurt milk bases. Each of the two yoghurt milk bases (LCM and SCM) were heat-treated at two temperatures; 75 or 95 °C for 5 min and inoculated with 0.02% (w/w) concentrated and frozen yoghurt culture (F-DVS YC-183, Chr. Hansen, Hørsholm, Denmark). The inoculated yoghurt milk was filled in 150 mL sterilized glass jars with lids and fermented at 43 °C. At pH 4.60 ± 0.01 the yoghurts were transferred directly to the cold room (4 °C).

2.4. Chemical analyses

Samples for chemical analyses were immediately frozen after sampling and thawed the day of analysis. Total solids (TS) content was determined after oven drying at 102 ± 2 °C for 24 h (IDF, 2010a). Minerals (Ca, P, K, Na, Mg) were quantified by ICP-MS as described by Jørgensen et al. (2015). Fat content was determined by Fourier transform infrared (FTIR) analytical technology (MilkoScan FT1, Foss, Hillerød, Danmark). Previous measurements of milk and concentrated milk samples containing 0.02–0.2% fat showed a good correlation (R^2 = 0.83, unpublished results) between FTIR and the Röse-Gottlieb method (IDF, 2010b). Lactose was quantified by high performance liquid chromatography (HPLC) as described by Moe, Porcellato, and Skeie (2013). The Kjeldahl method was used to determine total nitrogen (TN) (IDF, 2014), crude protein (CP) (IDF, 2014), nonprotein nitrogen (NPN) (IDF, 2001), and noncasein nitrogen (NCN) (IDF, 2004). True protein (TP) was calculated by subtracting NPN from TN. Casein was calculated by subtracting NCN from TN. NWP was calculated by subtracting NPN from NCN. A multiplying factor of 6.38 was used to calculate the content of the various protein components. The pH of yoghurt milk bases and yoghurts was measured with a pH meter equipped with a temperature sensor (Radiometer Copenhagen, Nerliens Kemisk Tekniske AS, Oslo, Norway). Capillary electrophoresis was used to separate and quantify milk proteins; α-LA, β-LG, α_{S2}-CN, α_{S1}-CN, κ-CN, and β-CN, as described by Jørgensen et al. (2016). The method was modified by adding three times more dithiothreitol (DTT) to the sample buffer. The

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quantification method used, includes only the content of α-LA and β-LG, and not the other serum proteins (e.g., lactoferrin, immunoglobulins, bovine serum albumin).

2.5. Physical analyses

2.5.1. Casein micelle size distribution

Particle size distribution of unheated yoghurt milk bases was measured with dynamic light scattering as described by Devold, Brovold, Langsrud, and Vegarud (2000). The particle size distribution represented the average casein micelle size because the samples were mainly composed of caseins. Samples were stored at 4 °C until the day of analysis and room tempered for 5–6 h before measurement to partially reverse solubilization of minerals (Schmitt, Saulnier, Malhautier, & Linden, 1993) and β-CN leakage from the casein micelle (Liu, Weeks, Dunstan, & Martin, 2013; Rose, 1968) caused by cold storage. Investigations in our lab showed the same average casein micelle size in freshly drawn milk samples and in the same milk samples stored at 4 °C and subsequently room tempered for 5–6 h ($R^2 = 0.92$). Measurements were performed using a Zetasizer 3000HS particle size analyzer with a 633 nm laser (Malvern Instruments Ltd., Malvern, Worcestershire, UK). The yoghurt milk base was mixed with filtered (0.22 μm) simulated milk ultrafiltrate (SMUF) (Jenness & Koops, 1962) in the ratio 1:240. The mixture with SMUF and sample was filtered (0.8 μm) into a plastic cuvette, incubated in a heating block (25 °C) for 10 min, and measured at a scattering angle of 90° at 25 °C.

2.5.2. Firmness of yoghurt

Texture of set yoghurt was measured at 4 °C with a Texture analyzer model TA-XT plus (Stable Micro Systems Ltd., Godalming, Surrey, UK) as described by Jørgensen et al. (2015). The maximum compression force and the maximum adhesive force were found using Exponent software (Stable Micro Systems Ltd., Godalming, Surrey, UK). The maximum compression force represented the firmness of the set gel as described by Amatayakul, Halmos, Sherkat, and Shah (2006).

2.5.3. Storage modulus of yoghurt

The elastic property of set yoghurt was measured at 11 °C as described by Jørgensen et al. (2015). The oscillatory test was performed with a rheometer type MCR 301 with smooth parallel plate–plate geometry (PP50) (Anton Paar GmbH, Graz, Austria) with the following instrumental set up; amplitude sweep with controlled strain 0.05–100% and angular frequency 10 rad s^{-1} with 14 measurement points. The storage modulus (G′) at the point where the linear viscoelastic range (LVER) ended was found by using Rheoplus/32 V3.40 software (Anton Paar GmbH, Graz, Austria), and represented the solid-like properties of yoghurt under rest conditions.

2.5.4. Coagulum particle size

Coagulum particle size distribution in yoghurt samples was measured by laser light diffraction with Mastersizer 3000 (Malvern Instruments Ltd., Malvern, Worcestershire, UK) as described by Jørgensen et al. (2015). Before the yoghurt sample was added to the wet cell, the set yoghurt (4 °C) was broken and stirred with a hand mixer (Bosch 350 W, type CNHR 17) at speed 1 for 15 s. D_{50} and D_{90} report the particle size at which 50% or 90% of the coagulum particles in the sample are smaller, and 50% or 10% of the particles are larger, respectively.

2.6. Statistical treatment

Two-sample *t*-test (Minitab 17, Minitab Ltd., Coventry, West Midlands, UK) was used to identify statistically significant differences in chemical compositions between; 1) retentates, and 2) yoghurt milk bases. Two-sample *t*-test was also applied to identify statistically significant differences in physical properties of yoghurts from yoghurt milk bases heat-treated at the same temperature. In the two-sample *t*-tests, equal variances were assumed due to reasonably similar standard deviations between populations. R version 3.0.1 (The R foundation for statistical computing) was applied to investigate the overall effect of casein micelle size and heat treatment of the yoghurt milk base on physical properties of set yoghurt. Data were fitted to a linear ANOVA model with casein micelle size and heat treatment of yoghurt milk base, and interaction of these two factors, as fixed factors.

3. Results and discussion

3.1. Composition of retentates with small and large casein micelles

Two retentates with different casein micelle size distributions were obtained from the same milk by the use of cascade MF (Fig. 1). The TS content differed significantly between the two retentates (Table 1). Retentate 1 contained significantly more casein and significantly less NWP than retentate 2, because of the relatively high permeation of NWP during MF with 0.20 μm membranes (Jørgensen et al., 2016). The ratios of α_{S2}-CN, α_{S1}-CN, κ-CN, and β-CN to total casein, respectively, were all significantly different between retentate 1 and retentate 2. Retentate 2 contained higher ratios of κ-CN: casein and α_{S1}-CN: casein than retentate 1. The content of calcium was also significantly different between the two retentates; however, the ratio of casein (%) to calcium (g kg^{-1}) was similar (~2.65) ($P = 0.98$). This can be explained by the fact that two third of the calcium in milk exist as micellar casein (Gaucheron, 2005), and would be concurrently concentrated with the caseins during MF.

3.2. Composition of yoghurt milk bases with small and large casein micelles

To achieve yoghurt milk bases with equal NWP:casein-ratios, a recombined solution of NWP powder was added to retentate 1 (Fig. 1). The recombined solution of NWP powder contained 5.1% TS, 4.7% TP, a NWP:casein-ratio of 82:18, and minor levels of minerals and lactose. By the addition of NWP solution to retentate 1, yoghurt milk bases with equal TP content and NWP:casein-ratios were obtained (Table 2). Both TP content and NWP:casein-ratios influence the rheology of yoghurt (Abrahamsen & Holmen, 1980; Biliaderis, Khan, & Blank, 1992; Jørgensen et al., 2015; Krzeminski, Grosshable, & Hinrichs, 2011; Kücükcetin, 2008; Lucey, Munro, & Singh, 1999). Thus, equal TP content and NWP:casein-ratios of the yoghurt milk bases were prerequisites to investigate the effect of casein micelle size on yoghurt rheology.

The average casein micelle size of the two yoghurt milk bases differed significantly (Table 2), with the LCM having an average micelle size of ~183 nm and the SCM an average micelle size of ~129 nm. During the first MF step with 0.20 μm membranes, milk components smaller than 200 nm are able to permeate the membrane. Previous research on milk from the same breed, showed average micelle diameters of milk from individual cows ranging from 149 to 222 nm as measured with dynamic light scattering (Devold et al., 2000). In the present study, the average casein micelle size of the skim milk was 174 ± 4 nm. Thus, casein micelles on the smaller tail of the distribution could permeate the membrane. Punidadas and Rizvi (1998) also reported considerable permeation of caseins through a ceramic gradient

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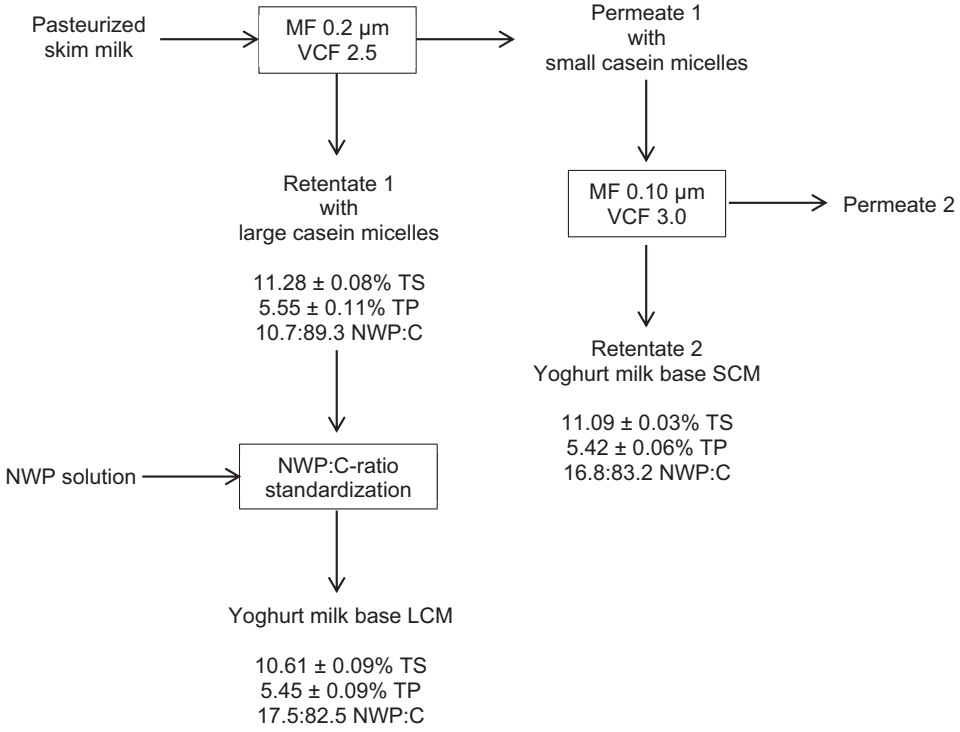

Fig. 1. Flow chart illustrating the production of yoghurt milk bases with large and small casein micelles, LCM and SCM respectively. Abbreviations are: MF, microfiltration; VCF, volume concentration factor; TS, total solids; TP, true protein; NWP, native whey protein; C, casein.

membrane with 0.20 µm pore size; however, they did not report casein micelle size distributions of the two fractions. Van Hekken and Holsinger (2000) obtained retentates and permeates with significantly different particle sizes, 159 and 132 nm respectively, with the use of lab scale MF at 4 °C with a 0.2 µm membrane. Casein micelle size fractionation has previously been done in lab scale by differential centrifugation (Anema & de Kruif, 2013; Dalgleish et al., 1989; Davies & Law, 1983; Horne, 2003; Liu et al., 2013; O'Connell & Fox, 2000) and column chromatography (Donnelly et al., 1984; Griffin & Anderson, 1983; McGann, Kearney, & Donnelly, 1979). Results from the present study show that casein concentrates with different casein micelle size distributions could be produced by the use of cascade MF, which is an industrial applicable technology.

Permeate 2 (Fig. 1), obtained from the second MF step with 0.10 µm membranes, was translucent and contained negligible amounts of caseins according to the Kjeldahl method and the analysis by capillary electrophoresis (results not shown). This was expected, because the majority of caseins in milk exist as micelles at the temperature (50 °C) the MF was performed (Liu et al., 2013).

The distributions of α-LA, β-LG, α_{S2}-CN, α_{S1}-CN, κ-CN, and β-CN in yoghurt milk bases were determined by capillary electrophoresis (Table 2) and quantified by the procedure described by Jørgensen et al. (2016). As expected, the κ-CN content in the SCM yoghurt milk base was significantly higher than in the LCM yoghurt milk base. Because the κ-CN preferentially is located on the surface of the casein micelles, a fraction of milk with smaller casein micelles will have a larger total surface area, and thus a higher proportion of κ-CN to total casein (Anema & de Kruif, 2013; Dalgleish et al., 1989; Davies & Law, 1983; Donnelly et al., 1984; O'Connell & Fox, 2000). In milk of individual cows, Bijl, de Vries, van Valenberg, Huppertz, and van Hooijdonk (2014) and De Kruif and Huppertz (2012) found no correlation between different average casein micelle sizes and the κ-CN content of the same samples. Bijl et al. (2014) did however report a strong correlation between average casein micelle size and amount of glycosylated κ-CN of total protein.

The increased κ-CN content in the SCM yoghurt milk base was almost balanced by a decreased content of α_{S2}-CN and β-CN. The decrease was only significant for the α_{S2}-CN content, however the decline in the proportions of α_{S2}-CN and β-CN to total casein were both significant. Anema and de Kruif (2013), Dalgleish et al. (1989), Davies and Law (1983), and Donnelly et al. (1984) also reported a decrease in the relative amount of β-CN in smaller casein micelles. Anema and de Kruif (2013) and Donnelly et al. (1984) reported a decrease in the relative α_S-CN (α_{S2}- and α_{S1}-CN) content with decreasing micelle size, while Davies and Law (1983) only observed a decrease in the proportion of α_{S2}-CN and not α_{S1}-CN. On the other hand, Dalgleish et al. (1989) reported that the proportions of both α_{S2}- and α_{S1}-CNs were independent of micelle size. In the present study, the reduction in α_{S2}-CN was balanced by an increase in α_{S1}-CN. Thus, the total content of α_S-CN and the proportion of α_S-CN to casein was the same in the two yoghurt milk bases, 2.25% and ~50% respectively, which is consistent with the findings of Dalgleish et al. (1989).

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Table 1
Comparison of composition of retentate 1 and 2 with large and small casein micelles, respectively.[a]

Component	Retentates		*P*-value
	1	2	
Total solids (%)	11.28 ± 0.08	11.09 ± 0.03	* (0.018)
Crude protein (%)	5.73 ± 0.10	5.64 ± 0.02	n.s. (0.203)
True protein (%)	5.55 ± 0.11	5.42 ± 0.06	n.s. (0.157)
Casein (%)	4.95 ± 0.08	4.51 ± 0.08	* (0.002)
Native whey protein (NWP, %)	0.60 ± 0.05	0.91 ± 0.05	* (0.001)
NWP:casein	10.7:89.3	16.8:83.2	* (0.001)
Fat (%)	0.20 ± 0.12	0.09 ± 0.01	n.s. (0.182)
Lactose (mmol kg^{-1})	133.3 ± 1.0	136.5 ± 1.2	* (0.023)
Calcium (g kg^{-1})	1.87 ± 0.06	1.70 ± 0.00	* (0.008)
Phosphorus (g kg^{-1})	1.52 ± 0.07	1.40 ± 0.08	n.s. (0.133)
Potassium (g kg^{-1})	1.73 ± 0.06	1.77 ± 0.06	n.s. (0.519)
Sodium (g kg^{-1})	0.38 ± 0.02	0.39 ± 0.01	n.s. (0.330)
Magnesium (g kg^{-1})	0.15 ± 0.00	0.14 ± 0.01	n.s. (0.105)
α-Lactalbumin (%)	0.16 ± 0.01	0.22 ± 0.01	* (0.003)
β-Lactoglobulin (%)	0.44 ± 0.02	0.69 ± 0.05	* (0.002)
α_{S2}-Casein (%)	0.67 ± 0.02	0.56 ± 0.01	* (0.001)
α_{S1}-Casein (%)	1.78 ± 0.03	1.69 ± 0.02	* (0.033)
κ-Casein (%)	0.52 ± 0.01	0.60 ± 0.05	n.s. (0.071)
β-Casein (%)	1.99 ± 0.07	1.66 ± 0.04	* (0.002)
α-Lactalbumin: NWP (% of NWP)	26.4 ± 2.1	24.0 ± 2.7	n.s. (0.293)
β-Lactoglobulin: NWP (% of NWP)	73.4 ± 3.1	74.9 ± 4.0	n.s. (0.646)
α_{S2}-Casein (% of casein)	13.4 ± 0.2	12.5 ± 0.1	* (0.002)
α_{S1}-Casein (% of casein)	35.9 ± 0.5	37.5 ± 0.6	* (0.030)
κ-Casein (% of casein)	10.4 ± 0.5	13.4 ± 1.1	* (0.015)
β-Casein (% of casein)	40.1 ± 0.8	36.8 ± 0.6	* (0.004)

[a] Values for retentates are means ± SD (n = 3). Significance of difference between yoghurt milk bases was identified with two-sample *t*-test:* indicates significant difference ($P < 0.05$); n.s., no significant difference; *P*-values in parentheses.

Table 2
Comparison of composition of yoghurt milk bases with large casein micelles (LCM) and small casein micelles (SCM).[a]

Component	Yoghurt milk bases		*P*-value
	LCM	SCM	
Total solids (%)	10.61 ± 0.09	11.09 ± 0.03	* (0.001)
Crude protein (%)	5.64 ± 0.07	5.64 ± 0.02	n.s. (0.919)
True protein (%)	5.45 ± 0.09	5.42 ± 0.06	n.s. (0.639)
Casein (%)	4.50 ± 0.09	4.51 ± 0.08	n.s. (0.876)
Native whey protein (%)	0.95 ± 0.01	0.91 ± 0.05	n.s. (0.183)
Native whey protein:casein	17.5:82.5	16.8:83.2	n.s. (0.275)
Fat (%)	0.20 ± 0.11	0.09 ± 0.01	n.s. (0.153)
Lactose (mmol kg^{-1})	121.0 ± 2.0	136.5 ± 1.2	* (0.000)
Calcium (g kg^{-1})	1.70 ± 0.00	1.70 ± 0.00	n.s. (1.000)
Phosphorus (g kg^{-1})	1.40 ± 0.04	1.40 ± 0.08	n.s. (0.998)
Potassium (g kg^{-1})	1.60 ± 0.00	1.77 ± 0.06	* (0.008)
Sodium (g kg^{-1})	0.35 ± 0.01	0.39 ± 0.01	* (0.004)
Magnesium (g kg^{-1})	0.14 ± 0.01	0.14 ± 0.01	n.s. (0.230)
pH	6.73 ± 0.01	6.72 ± 0.01	n.s. (0.293)
Casein micelle size (nm)	182.6 ± 5.9	129.1 ± 5.5	* (0.000)
α-Lactalbumin (%)	0.24 ± 0.02	0.22 ± 0.01	n.s. (0.203)
β-Lactoglobulin (%)	0.72 ± 0.01	0.68 ± 0.06	n.s. (0.363)
α_{S2}-Casein (%)	0.60 ± 0.01	0.56 ± 0.01	* (0.031)
α_{S1}-Casein (%)	1.65 ± 0.01	1.69 ± 0.04	n.s. (0.116)
κ-Casein (%)	0.49 ± 0.01	0.60 ± 0.06	* (0.033)
β-Casein (%)	1.76 ± 0.06	1.66 ± 0.04	n.s. (0.061)
α-Lactalbumin: NWP (% of NWP)	24.8 ± 1.8	24.0 ± 2.7	n.s. (0.708)
β-Lactoglobulin: NWP (% of NWP)	75.3 ± 0.7	74.9 ± 4.0	n.s. (0.906)
α_{S2}-Casein (% of casein)	13.2 ± 0.2	12.5 ± 0.1	* (0.003)
α_{S1}-Casein (% of casein)	36.6 ± 0.6	37.5 ± 0.6	n.s. (0.158)
κ-Casein (% of casein)	11.0 ± 0.1	13.4 ± 1.1	* (0.021)
β-Casein (% of casein)	39.1 ± 0.6	36.8 ± 0.6	* (0.010)

[a] Values are means ± SD (n = 3). Significance of difference between yoghurt milk bases was identified with two-sample *t*-test:* indicates significant difference ($P < 0.05$); n.s., no significant difference; *P*-values given in parentheses.

It could be questioned whether the decrease in β-CN:casein-ratio in SCM could be due to potential plasmin activity. Because the plasmin is concentrated with the caseins during MF (Aaltonen & Ollikainen, 2011), the two yoghurt milk bases probably contained the same amounts of plasmin (equal casein concentrations). However, the production of LCM and SCM lasted for 1 and 2 days, respectively; hence, the β-CN in the SCM was more exposed to potential plasmin proteolysis than the β-CN in the LCM. Some peaks of unidentified proteins were observed in all electropherograms of LCM and SCM. The relative amount of unidentified protein to the total peak area was significantly higher ($P = 0.02$) in SCM than in LCM as identified with two-sample *t*-test (results not shown). In addition, the relative amount of unidentified protein and β-CN to total peak area showed a lower nominal value in SCM than in LCM, however the difference was not significant ($P = 0.14$). This may indicate that, if some of the unidentified protein was γ-CN from proteolysis of β-CN, then the lower β-CN:casein-ratio in SCM (Table 2) could partly be ascribed to degradation of β-CN by plasmin. Further work is, however needed to reveal the composition of the unidentified proteins.

Secondly, it could be questioned whether the addition of NWP solution to retentate 1 influenced the ratios of proteins as found with capillary electrophoresis. The capillary electrophoresis analysis revealed that the NWP solution contained minor amounts of the various caseins (results not shown). For the identification of possible statistically significant differences in the ratios of α-LA and β-LG to NWP and α_{S2}-CN, α_{S1}-CN, κ-CN, and β-CN to casein between retentate 1 and LCM (retentate 1 with added NWP solution), a paired *t*-test was used (results not shown). Only the β-CN:casein-ratio was significantly different ($P = 0.02$) between retentate 1 and LCM. The ratio decreased from 40.1% to 39.1% by the addition of NWP solution to retentate 1 (Tables 1 and 2). The decrease in the β-CN:casein-ratio in LCM by the addition of NWP solution to retentate 1, probably influenced the relative amount of unidentified protein and β-CN to the total peak area. The data supports that the significantly lower β-CN:casein-ratio in SCM (Table 2) in fact arises from the different content of β-CN in small and large casein micelles, and not solely from a potential higher degree of proteolysis in SCM.

The contents of calcium and phosphorus were the same in the two yoghurt milk bases. Dalgleish et al. (1989) and Devold et al. (2000) also reported a constant content of calcium in milk samples with different average casein micelle sizes. Dalgleish et al. (1989) observed, however, a slight reduction in inorganic phosphate content with decreasing casein micelle size. The contents of potassium and sodium were significantly lower in LCM than in

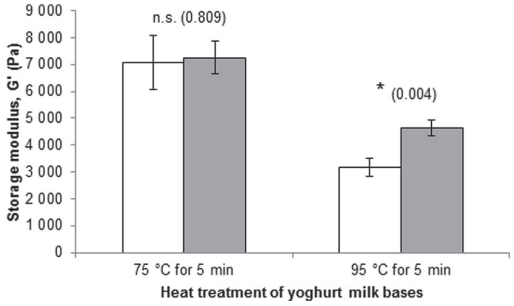

Fig. 2. Storage modulus (G′) (mean ± SD; n = 3) of yoghurts measured where the linear viscoelastic range ends (when the G′ value is less than 5% of the plateau value of G′). Yoghurt milk bases contain (□) large casein micelles or (■) small casein micelles. Significance of difference between yoghurts having undergone the same heat treatment is identified with two-sample *t*-test: *, significantly different ($P < 0.05$); n.s., not significantly different; *P*-values in parentheses.

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SCM, possibly due to the minor mineral content in the NWP solution that was added retentate 1 (LCM). The content of TS was 10.6% in LCM and 11.1% in SCM. The significant different content of TS was due to the significant difference in lactose content between the two yoghurt milk bases. The lactose content was 121 mmol kg^{-1} (~4.1%) in LCM and 137 mmol kg^{-1} (~4.7%) in SCM. McKenna and Anema (1993) reported a positive correlation between denaturation of whey proteins and firmness of yoghurt. The denaturation degree of whey proteins is, among other factors, influenced by lactose content, however the observed different content of lactose between LCM and SCM were not expected to give various denaturation degrees of whey proteins (Anema, 2000; Anema, Lee, & Klostermeyer, 2006). Thus, it was assumed that the yoghurt rheology was unaffected by the difference in lactose content between LCM and SCM.

3.3. Effect of casein micelle size and heat treatment on set yoghurt properties

Yoghurt milk base LCM and SCM were heat-treated at two different temperatures; 95 °C for 5 min or 75 °C for 5 min. The effect of casein micelle size on yoghurt undergone the conventional heat treatment temperature, 95 °C for 5 min, will be discussed first.

The storage modulus (G′) (Fig. 2) and the firmness and the adhesive force (Fig. 3) were significantly influenced by the size of the casein micelles in the yoghurt milk bases heat-treated at 95 °C for 5 min. Small casein micelles gave set yoghurts with significantly higher storage modulus (G′) and firmness than large casein micelles. In the yoghurt milk bases heat-treated at 95 °C for 5 min in the present study, a mixture of micellar bound and soluble aggregates were probably present according to the results reported by Anema (2000, 2001), Anema, Lee, Lowe, and Klostermeyer (2004), Dannenberg and Kessler (1987), and Vasbinder et al. (2003). Donato, Guyomarc'h, Amiot, and Dalgleish (2007) suggested that the heat induced reaction between whey proteins and κ-CN in milk preferentially takes place on the surfaces of the casein micelles, and that soluble aggregates of κ-CN and whey protein are formed and subsequently dissociate into the serum. Interestingly, they observed a higher content of soluble complexes of κ-CN and whey proteins in milk samples with a naturally higher content of κ-CN. Based on these findings, it was likely that a higher content of soluble κ-CN and whey protein complexes was formed in the SCM than in the LCM due to the higher κ-CN content in SCM. According to Anema et al. (2004) and Guyomarc'h, Queguiner, Law, Horne, and Dalgleish (2003), micellar aggregates have a significant effect on

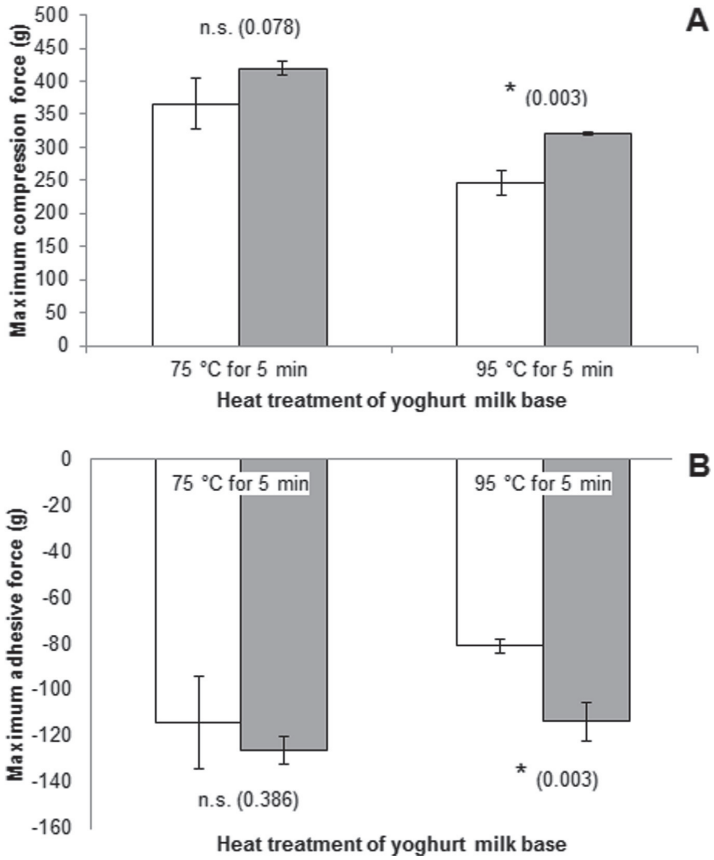

Fig. 3. Firmness (A) and adhesive force (B) (mean ± SD; n = 3) of yoghurts produced from yoghurt milk bases with (□) large casein micelles or (■) small casein micelles. Significance of difference between yoghurts having undergone the same heat treatment is identified with two-sample *t*-test: *, significantly different ($P < 0.05$); n.s., not significantly different; *P*-values in parentheses.

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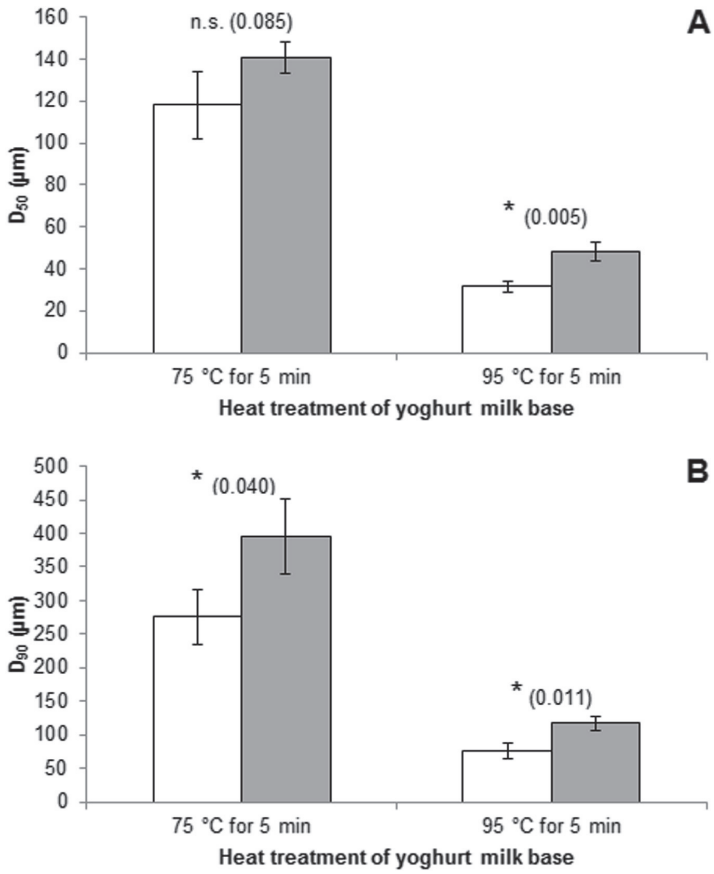

Fig. 4. Coagulum particle size distributions D_{50} (A) and D_{90} (B) (mean ± SD; n = 3) of broken set yoghurts produced from yoghurt milk bases with (□) large casein micelles or (■) small casein micelles. Significance of difference between yoghurts having undergone the same heat treatment is identified with two-sample *t*-test: *, significantly different ($P < 0.05$); n.s., not significantly different; *P*-values in parentheses.

the final storage modulus (G′) of an acid gel. However, the soluble aggregates more dominantly contribute to an increase in the storage modulus (G′) than the micellar aggregates. Based on this, it is proposed that the higher storage modulus (G′) and firmness of set yoghurt of SCM than of LCM could be ascribed the potential higher content of soluble aggregates in SCM. Both micelle bound aggregates and soluble aggregates of κ-CN and whey proteins provide points of attachment during the acid gelation (Dalgleish & Corredig, 2012). However, a potential higher amount of soluble aggregates increases the number and density of the gelling protein particles, and thereby increases the complexity and points of attachments during acidification, and finally increases the storage modulus (G′) and firmness of the set yoghurt (Anema et al., 2004; Guyomarc'h et al., 2003).

The coagulum particle size distribution (Fig. 4) was measured after breaking the set yoghurt by standardized mixing. The coagulum particle sizes, both D_{50} and D_{90}, of the broken SCM set yoghurt were significantly larger than coagulum particles of LCM when a heat treatment of 95 °C for 5 min was used. This observation reflects the increased points of attachments in the SCM set yoghurt than in the LCM set yoghurt, and is in congruence with the higher firmness and storage modulus (G′) measured for the SCM set yoghurt. The coagulum particle size measured in the present study can however not be linked to sensory perception of smoothness or graininess (Cayot, Schenker, Houzé, Sulmont-Rossé, & Colas, 2008; Jørgensen et al., 2015; Krzeminski et al., 2013), because the measurement was performed on a gently stirred set type yoghurt (stirred for 15 s) and not on a real stirred type yoghurt. Further work should reveal how small and large casein micelles affect sensory perception of stirred type yoghurts.

The firmness (Fig. 3) and the coagulum particle sizes, D_{50} and D_{90} (Fig. 4), tended to be affected by the size of the casein micelles in the yoghurt milk bases heat-treated at 75 °C for 5 min. The SCM set yoghurt had significantly higher D_{90} than the LCM set yoghurt.

ANOVA was used to investigate the overall effect of casein micelle size and heat treatment on physical properties of set yoghurt (Table 3). A reduced heat treatment temperature (75 °C for 5 min) gave set yoghurts with significantly higher storage modulus (G′), firmness, adhesive force, and coagulum particle sizes. At this heat treatment, considerable amounts of β-LG and α-LA are kept in their undenatured form (Anema, 2000, 2001; Dannenberg & Kessler, 1987). In a previous study, using the same method of

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Table 3
Effects of casein micelle size (CMS) (small or large casein micelles) and heat treatment (HT; 95 °C or 75 °C for 5 min) of yoghurt milk base, and interaction of these two factors, on physical properties of high protein, low fat set yoghurt.[a]

	Experimental factors		
	Casein micelle size	Heat treatment	Interaction CMS and HT
Storage modulus, G′ (Pa)	n.s. (0.051)	↑ (<0.001)	n.s. (0.108)
Maximum compression force (g)	↑ (0.001)	↑ (<0.001)	n.s. (0.469)
Maximum adhesive force (g)	↑ (0.010)	↑ (0.010)	n.s. (0.156)
Coagulum particle size, D_{50} (μm)	↑ (0.006)	↑ (<0.001)	n.s. (0.573)
Coagulum particle size, D_{90} (μm)	↑ (0.004)	↑ (<0.001)	n.s. (0.090)

[a] Significant effects of factors were identified by ANOVA: ↑ indicates significant effect ($P < 0.05$); n.s., no significant effect; *P*-values in parentheses. The arrow indicates that the physical property is increased (↑) by reduced casein micelle size or reduced heat treatment temperature.

heat treatment as in the present study, heat treatment at 75 °C for 5 min left approximately 50% of the β-LG and 75% of the α-LA in their undenatured form when the yoghurt milk base had a NWP:casein-ratio of 10:90 (measured prior to heat treatment) (Jørgensen et al., 2015). Less micellar and soluble aggregates of κ-CN and whey proteins are therefore formed during heat treatment at 75 °C for 5 min than at 95 °C for 5 min. Vasbinder et al. (2003) observed that a reduction in heat treatment temperature caused a reduction in aggregation of κ-CN and whey proteins, and a shift to a lower gelation pH (from pH > 5.4 at 90 °C for 10 min to pH ~5.0 at 75 °C for 10 min). Colloidal calcium phosphate (CCP) is solubilized during acidification. At pH ~5.2–5.1, the solubilization of inorganic phosphate is complete, while 10% of the calcium remains in the casein micelles and is not completely solubilized until about pH 4.8 (Heertje, Visser, & Smits, 1985; Singh, Roberts, Munro, & Teo, 1996). The solubilization of CCP from the inside of the casein particles causes a loosening of the network, which in turn impacts the texture of the yoghurt (Lucey, 2004). Set yoghurts from yoghurt milk bases heat-treated at 75 °C for 5 min in the present study probably had a lower gelation pH (Vasbinder et al., 2003). Gelation pH was, however, not measured in the present study. In gels with a lower gelation pH, a considerable amount of the CCP is already dissolved before the onset of gelation. Thus, the internal weakening, due to CCP solubilization, probably proceeded to a lesser extent in the yoghurts from yoghurt milk bases heat-treated at 75 °C for 5 min than in the yoghurts from yoghurt milk bases heat-treated at 95 °C for 5 min. Also, the reduced NWP:casein-ratio (~17:83) in the set yoghurts in this study, and the lower degree of whey protein denaturation in yoghurt milk bases heat-treated at 75 °C for 5 min, may lead to aggregation and network formation caused by a combination of attraction of casein micelles at the pI of the casein micelles (~4.6) and attraction of complexes of κ-CN and whey proteins at the pI of β-LG (~5.3). However, if the NWP:casein-ratio of the set yoghurts had been higher (e.g., 25:75), a reduced heat treatment temperature (75 °C for 5 min) of the yoghurt milk bases would probably give set yoghurts with a lower firmness than heat treatment at 95 °C for 5 min according to the observations by Jørgensen et al. (2015). In a yoghurt milk base with sufficient amounts of whey proteins, the aggregation and network formation is exclusively caused by attraction of complexes of κ-CN and whey proteins. In yoghurt milk bases with higher NWP:casein-ratios, an increased heat treatment temperature and increased denaturation of whey proteins give more complexes of κ-CN and whey proteins, which in turn provides more points of attraction during acidification and a firmer yoghurt gel is obtained (Guyomarc'h et al., 2003). The influence of casein micelle size on physical properties of set yoghurts was independent of the heat treatment temperature (no interaction effect) (Table 3). Further investigations should be performed to find the minimum protein content needed in SCM yoghurt milk base to obtain set yoghurts with the same firmness and storage modulus as that obtained by the LCM yoghurt milk base.

4. Conclusions

Cascade MF can be used to obtain casein concentrates with different casein micelle size distributions. Yoghurt milk base with small casein micelles (~129 nm) gave high protein, low fat set yoghurts with higher storage modulus (G′) and higher firmness than yoghurt milk base with large casein micelles (~183 nm). Increased gelation capacity of small casein micelles can be explained by the increased amount of κ-CN and thereby increased content of soluble κ-CN and whey protein complexes, which in turn provides more points of attraction during acidification. Casein concentrates with smaller casein micelles can be beneficial in production of "clean label" set and stirred yoghurts with increased firmness and thickness. Further work is needed to reveal how small and large casein micelles affect sensory perception of set and stirred type yoghurts.

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(54) Yoghurt with native whey proteins and processes for production thereof

(57) The present invention relates to yoghurt products of good quality, both with regard to physical and sensory properties, comprising high amounts of proteins including high amounts of native whey proteins, and methods for manufacturing such yoghurt products.

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Description

Field of the invention

[0001] The present invention relates to yoghurt products comprising high amounts of proteins including high amounts of native whey proteins, and methods for manufacturing such yoghurt products.

Background of the invention

[0002] Yoghurt products are of high economic importance to dairy industry worldwide, and constantly new yoghurt varieties and concepts are presented to consumers. High protein yoghurts have been capturing more and more consumers over the latest years. Consumers' increased consciousness about health benefits of dairy proteins, and increasing amount of scientific documentation claiming health promotional effect of protein intake, bring along expanded market opportunities.

[0003] The main proteins of dairy products are casein and whey proteins. Approximately 20 % of the proteins in cow's milk are whey proteins and 80 % are caseins.

[0004] Whey proteins have been reported to; stimulate muscle protein metabolism and accretion (Tipton et al., 2007), assist in long-term maintenance of body weight (Baer et al., 2011), reduce body weight gain (Pichon et al., 2008), lower systolic blood pressure and improve vascular function in overweight and obese individuals (Pal & Ellis, 2009), and be more satiating due to increased postprandial circulating levels of amino acids, and the hormones cholecystokinin and glucagon-like peptide 1 (Hall, Millward, Long, & Morgan, 2003; Lam, Moughan, Awati & Morton, 2009).

[0005] Furthermore, preliminary results suggest that amino acids from native whey protein concentrate fractionated directly from skim milk are better absorbed in blood than amino acids from microparticulated whey proteins, low-fat milk, hydrolyzed whey proteins or whey protein concentrate (WPC 80) (Laahne, 2013).

[0006] Almaas et al. (2006) studied in vitro digestion of bovine milk by human proteolytic enzymes. They reported a faster digestion of unheated milk than heated milk. The result was explained by the probable structural changes in proteins caused by heat denaturation and aggregation of whey proteins.

[0007] Therefore, yoghurts with a high amount of whey proteins, particularly in native form, are desirable.

[0008] However, in yoghurt technology denaturation of whey proteins, and their consequently covalent association to casein micelles, is regarded as one of the premises to obtain a good yoghurt structure (Lucey & Singh, 1998; Robinson, Lucey & Tamine, 2006). Conventional heat treatment, 95 °C for 5 minutes or 80 °C for 30 minutes, give close to 100 % denaturation of β-lactoglobulin (Dannenberg & Kessler, 1987) and 75 % denaturation of α-lactalbumin (Anema, 2001).

[0009] Another important factor in yoghurt manufacture is the increase of total solids content to avoid a too weak yoghurt gel. This can be obtained by adding milk powder or by concentrating the milk by evaporation or membrane filtration (Robinson, Lucey & Tamime, 2006). From economical, nutritional and/or technological points of view skim milk powder can be replaced by whey protein concentrate powder.

[0010] Traditionally, yoghurt is prepared as follows:

- providing raw milk;
- separating the raw milk into a cream fraction and a skim milk fraction;
- optionally, standardizing the skim milk fraction to desired level of fat content;
- mixing the skim milk fraction which may have been fat standardized, with a milk powder and thereby obtain a composition with increased dry matter;
- subjecting the composition with increased dry matter to homogenization and thereby obtain a homogenized composition with increased dry matter;
- subjecting the composition with increased dry matter to heat treatment for a period of 5 minutes at 95 °C or for a period of 30 minutes at 80 °C and thereby obtain a heat treated homogenized composition with increased dry matter; and
- adding a starter culture to the heat treated homogenized composition with increased dry matter and thereby obtaining a fermented mixture.

[0011] The yoghurt product obtained by the traditional method above typically comprises:

- content of solids-non fat from 12 to 18 g/mL;
- whey protein:casein ratio of 20:80;
- 90-100% of the β-lactoglobulins in denaturated form;
- 60-90% of the α-lactoglobulins in denaturated form.

[0012] International patent application no. WO 2012/110705 discloses a whey protein product having a ratio of whey protein to casein in the range from about 25:75 to about less than 50:50, a total protein content of at least 20% on dry matter basis, and a protein content of about 2.5 to about 8% by weight, based on the weight of the product. A method of producing the said whey protein product, using microfiltration and ultrafiltration, is described. It is suggested that the whey protein product may be used in the preparation of sour milk products like yoghurt. However, nothing is mentioned about the process conditions for producing the sour milk products. Consequently, a person skilled in the art will assume that traditional manufacturing methods and conditions should be used.

[0013] Patocka, Cervenkova, Narine & Jelen (2006) and Guggisberg, Eberhard & Albrecht (2007) studied the effect of adding whey proteins to yoghurt milk after heat treatment to retain the whey proteins in their undenaturated and native state. When whey proteins were added to heat treated yoghurt milk before fermentation a reduction in storage modulus of the yoghurts was observed (Patocka, Cervenkova, Narine & Jelen, 2006; Guggisberg, Eberhard & Albrecht, 2007). As the whey proteins were added after fermentation a rapid breakdown of yoghurt gel was observed, resulting in two separate phases comprising fluid whey and a coagulated protein mass (Patocka, Cervenkova, Narine & Jelen, 2006).

[0014] It is a challenge to find a way to produce yoghurts of good quality comprising considerable amounts of native whey proteins.

[0015] It is now surprisingly found that by modifying the temperature and optionally the time period of the heat treatment step, high quality yoghurt comprising high amounts of native whey proteins, is obtained.

Summary of the invention

[0016] It is a main object of the present invention to provide yoghurt of good quality, both with regard to physical and sensory properties, comprising high amounts of native whey proteins, i.e. from 4 to 70 mg/mL.

[0017] Another object of the present invention is to provide yoghurt of good quality wherein the whey protein:casein ratio is higher in whey protein and lower in casein compared to traditional yoghurts.

[0018] Still another object of the present invention is to provide a method for manufacturing the yoghurts mentioned above.

[0019] These and other objects are obtained by the yoghurt and method as defined in the accompanying claims.

Definitions

[0020] As used herein, the terms "yoghurt" and "yoghurt product" mean concentrated fermented milk (ref. Codex standard for fermented milks). Concentrated fermented milk is fermented milk where the protein content has been increased prior to fermentation to minimum 5.6 %. That is, products such as ymer, ylette, labneh and kefir which in addition to yoghurt are included in the definition of the above-mentioned concentrated fermented milk, are within the scope of the terms "yoghurt" and "yoghurt products" as used herein. The different concentrated fermented milks are characterized by the starter culture used for fermentation.

[0021] As used herein, the term "protein" means crude protein (CP). Crude protein is total nitrogen multiplied by 6.38, not adjusted for the non-protein nitrogen (NPN). NPN and CP are analyzed by standardized IDF methods (Kjeldahl), IDF 2001 and IDF 2014, respectively.

[0022] As used herein, true protein (TP) is calculated by subtracting non-protein nitrogen (NPN) from true nitrogen (TN), and multiplying with a factor of 6.38. NPN and TN are analyzed by standardized IDF methods (Kjeldahl), IDF 2001 and IDF 2014, respectively.

[0023] As used herein, casein (C) is calculated by subtracting NPN and non-casein nitrogen (NCN) from TN, and multiplying with a factor of 6.38. NCN is analyzed by a standardized IDF method (Kjeldahl), IDF 2004.

[0024] As used herein, the term "whey protein" includes denatured and undenatured whey proteins. Undenatured whey protein is also referred to as native whey protein.

[0025] Native whey proteins consist mainly of α-lactalbumin, β-lactoglobulin A and β-lactoglobulin B. Native whey protein is directly extracted/removed from milk (preferably unpasteurized) with the use of microfiltration or ultrafiltration. Native whey protein has not been subjected to the cheese making process, and is therefore free from remnants of rennet, lactic acid bacteria, bacteriophages, somatic cells, cheese fines, and glycomacropeptide. Native whey has a neutral pH and the whey proteins are undenatured as long as the milk has not been exposed to severe heat treatment or pressure. As used herein, native whey protein (NWP) was calculated by subtracting NPN from NCN, and multiplying with a factor of 6.38. NPN and NCN are analyzed by standardized IDF methods (Kjeldahl), IDF 2001 and IDF 2004, respectively. Native α-lactalbumin, and native β-lactoglobulin A and B were quantitatively determined according to the method described by Beyer (1990).

[0026] Denatured whey protein is whey protein where the native protein molecule conformation has been changed irreversible. The native protein molecule conformation of the main whey proteins α-lactalbumin and β-lactoglobulin is globular.

[0027] As used herein, the term "native whey protein" is equivalent to the term "undenaturated whey protein".

[0028] As defined herein milk is any type of milk produced by any milk animals. Most common milk animals are cattle or cows, buffaloes, goats, sheep and camels. Less common milk animals, but included herein, are yaks, horses, reindeers and donkeys.

[0029] The major proteins in milk are whey protein and casein. In milk from cattle the ratio of whey protein to casein is typically 20 to 80.

Detailed description of the invention

[0030] The present invention provides a yoghurt comprising a content of protein in the range from 5.6 % to 20 % (w/w), a content of native whey protein in the range from 4 to 70 mg/mL, and a weight ratio of whey protein (i.e. native and denatured whey proteins) to casein in a range from 22:78 to 50:50 (w/w).

[0031] In a preferred embodiment of the invention, the protein content is in the range from 5.6 % to 18 % (w/w). In a more preferred embodiment of the invention, the protein content is in the range from 5.6 % to 15 % (w/w). In another preferred embodiment of the invention, the protein content is in the range from 6 % to 18 % (w/w). In a more preferred embodiment of the invention, the protein content is in the range from 6.5 % to 18 % (w/w). In another preferred embodiment of the invention, the protein content is in the range from 7 % to 17 % (w/w). In still another preferred embodiment of the invention, the protein content is in the range from 7 % to 15 % (w/w). In a more preferred embodiment of the invention, the protein content is in the range from 7 % to 13 % (w/w). In another preferred embodiment of the invention, the protein content is in the range from 7 % to 12 % (w/w). In yet another preferred embodiment of the invention, the protein content is in the range from 8 % to 12 % (w/w), and in a most preferred embodiment of the invention, the protein content is in the range from 8 % to 11 % (w/w).

[0032] In a preferred embodiment of the invention, the content of native whey protein of the yoghurt is in the range from 4 mg/mL to 65 mg/mL. In another preferred embodiment of the invention, the content of native whey protein of the yoghurt is in the range from 4 mg/mL to 55 mg/mL. In a more preferred embodiment of the invention, the content of native whey protein of the yoghurt is in the range from 4.5 mg/mL to 65 mg/mL. In another preferred embodiment of the invention, the content of native whey protein of the yoghurt is in the range from 5 mg/mL to 60 mg/mL. In still another preferred embodiment of the invention, the content of native whey protein of the yoghurt is in the range from 5 mg/mL to 55 mg/mL. In a more preferred embodiment of the invention, the content of native whey protein of the yoghurt is in the range from 5 mg/mL to 45 mg/mL. In yet a preferred embodiment of the invention, the content of native whey protein of the yoghurt is in the range from 5 mg/mL to 40 mg/mL. In another preferred embodiment of the invention, the content of native whey protein of the yoghurt is in the range from 5.5 mg/mL to 40 mg/mL. In still another preferred embodiment of the invention, the content of native whey protein of the yoghurt is in the range from 6 mg/mL to 40 mg/mL. In a more preferred embodiment of the invention, the content of native whey protein of the yoghurt is in the range from 6 mg/mL to 30 mg/mL. In yet a preferred embodiment of the invention, the content of native whey protein of the yoghurt is in the range from 8 mg/mL to 40 mg/mL. In another preferred embodiment of the invention, the content of native whey protein of the yoghurt is in the range from 8 mg/mL to 30 mg/mL. In a more preferred embodiment of the invention, the content of native whey protein of the yoghurt is in the range from 10 mg/mL to 40 mg/mL, and in a most preferred embodiment of the invention, the content of native whey protein of the yoghurt is in the range from 10 mg/mL to 30 mg/mL.

[0033] In a preferred embodiment of the invention, the whey protein: casein weight ratio of the yoghurt is from 25:75 to 45:55 (w/w), more preferable from 25:75 to 40:60 (w/w), and most preferable from 25:75 to 35:65 (w/w).

[0034] In another preferred embodiment of the invention, 30 to 70 % of the total content of whey protein of the yoghurt is in native state.

[0035] Furthermore, the present invention provides a method for manufacturing a yoghurt as defined above, comprising the steps of:

a) providing raw milk;
b) separating the raw milk into a cream fraction and a skim milk fraction;
c) subjecting the skim milk fraction to microfiltration (MF) adapted to separate casein from whey protein and thereby obtaining a casein-rich fraction and a fraction of native whey protein;
d) providing a native whey protein concentrate by subjecting the fraction of native whey protein to ultrafiltration (UF) adapted to separate native whey protein from water;
e) mixing the casein-rich fraction and the native whey protein concentrate and thereby obtaining a mixture of casein and native whey protein wherein the whey protein to casein ratio is increased compared to the ratio of whey protein to casein in the raw milk;
f) subjecting the mixture of casein and native whey protein to heat treatment and thereby obtaining a heat treated mixture of casein and whey protein;
g) adding a starter culture to the heat treated mixture of casein and whey protein to provide a fermented mixture; and

h) cooling the fermented mixture;
wherein:

- the mixture of casein and native whey protein obtained in step e) has a weight ratio of whey protein to casein in a range from 22:78 to 50:50; and
- the heat treatment of step f) is carried out by heating the mixture of casein and native whey protein at a temperature and for a time period sufficient to obtain denaturation of 30 to 70 % of the native whey protein of the mixture.

[0036] The raw milk provided in step a) is optionally unpasteurized.

[0037] The casein-rich fraction obtained in step c) is the retentate resulting from microfiltration. Herein, it is also mentioned MF retentate or MFR.

[0038] The fraction of native whey protein obtained in step c) is the permeate resulting from microfiltration. This fraction is also mentioned MF permeate or MFP herein.

[0039] The native whey protein concentrate obtained in step d) is the retentate resulting from ultrafiltration of the microfiltration permeate obtained in step c). Herein, it is also mentioned UF retentate or UFR.

[0040] In a preferred embodiment of the method according to the invention, the skim milk fraction obtained in step b) is subjected to microfiltration or bactofugation adapted to separate microorganisms and spores from the skim milk fraction and thereby obtain a microorganism-rich fraction and a partly sterilized skim milk fraction before subjecting the partly sterilized skim milk fraction to the microfiltration of step c).

[0041] In another preferred embodiment of the method according to the invention, the caseinrich fraction obtained in step c) has been concentrated to a volume concentration factor (CF) in the range from 1.5 to 6, preferably 1.5 to 4.5, and more preferable 1.5 to 3.5.

[0042] The microfiltration in step c) is carried out by using membranes with pore size from 0.05 μm to 0.2 μm.

[0043] The ultrafiltration in step d) is carried out by using membranes with cut-off from 1 kDa to 35 kDa.

[0044] According to one embodiment of the method of the invention, the casein-rich fraction obtained in step c) is pasteurized before subjected to mixing with the whey protein concentrate in step e).

[0045] According to another embodiment of the method of the invention, the mixture of casein and native whey protein obtained in step e) is fat standardized with addition of cream before heat treatment in step f).

[0046] According to yet another embodiment of the method of the invention, the mixture of casein and native whey protein obtained in step e) is subjected to homogenization and thereby obtaining a homogenized casein/ native whey protein mixture to be heat treated in step f).

[0047] The starter culture added in step g) to provide fermentation, is a yoghurt culture comprising *Streptococcus* subsp. *thermophilus* and *Lactobacillars delbrueckii* subsp. *bulgaricus*. Other suitable starter cultures can be used to obtain other characteristic concentrated fermented milk products, e.g. ymer or kefir (ref. Codex standard for fermented milks).

[0048] The starter culture is added in conventional amounts.

[0049] The fermentation in step g) is carried out in a time sufficient to obtain proper acidification. That is, to obtain pH about 4.6 or lower, e.g. pH 4.5.

[0050] According to one embodiment of the method of the invention, the fermented mixture obtained in step g) is stirred before cooling in step h).

[0051] The ratio of protein to lactose of the mixture that is heat treated in step f) affects the temperature/time combination at heat treatment needed to obtain denaturation of 30 to 70 % of the native whey protein in the mixture. Lactose protects the native whey proteins from being denaturated by heat. That is, if the content of lactose increases in relation to the content of native whey proteins, a higher temperature and/or time will be needed to achieve the desired denaturation. A higher lactose content results in a lower degree of denaturation, explained by the hydration effect of the lactose to the protein molecule (Spiegel (1999)).

[0052] In a preferred embodiment of the invention, the heat treatment of step f) is carried out by heating the mixture of casein and native whey protein at a temperature of 75 °C for a time period of 5 minutes, or another combination of temperature and time period providing equivalent denaturation degree as obtained by the temperature 75°C for 5 minutes.

[0053] In another preferred embodiment of the invention, the heat treatment of step f) is carried out by heating the mixture of casein and native whey protein at a temperature of 70°C for 15 minutes. In still another preferred embodiment of the invention, the heat treatment of step f) is carried out by heating the mixture of casein and native whey protein at a temperature of 80°C for 1 minute. In a further preferred embodiment of the invention, the heat treatment of step f) is carried out by heating the mixture of casein and native whey protein at a temperature of 85°C for 30 seconds. In another preferred embodiment of the invention, the heat treatment of step f) is carried out by heating the mixture of casein and native whey protein at a temperature of 90°C for 10 seconds. In yet another preferred embodiment of the invention, the heat treatment of step f) is carried out by heating the mixture of casein and native whey protein at a temperature of 95°C for 6 seconds. In another preferred embodiment of the invention, the heat treatment of step f) is carried out by heating

the mixture of casein and native whey protein at a temperature of 100°C for 3 seconds, and in another preferred embodiment of the invention, at a temperature of 110°C for 2 seconds.

[0054] Typically, a short heating time is applicable when the temperature is high and vice versa.

[0055] Thus, a person known in the art will realize that other temperatures and time periods than those indicated in the preferred embodiment above, will be useful.

[0056] The method of present invention prepares a casein rich fraction (retentate) and a native whey protein fraction (permeate) by microfiltration of skim milk, and further concentrating the native whey protein fraction by ultrafiltration to a concentrate of native whey protein. This gives the option to mix a casein rich fraction and a concentrate of native whey protein in different ratios to obtain yoghurt with increased total protein content as well as increased whey protein content compared to the ratio of whey proteins and casein in regular raw milk. In addition, a modified temperature-time combination during heat treatment allows a considerable amount of the whey proteins to be retained in their native state.

[0057] In another aspect the present invention provides an alternative method for manufacturing a yoghurt as defined above, comprising the steps of:

a) providing raw milk;
b) separating the raw milk into a cream fraction and a skim milk fraction;
c) subjecting the skim milk fraction to ultrafiltration (UF) adapted to obtain concentrated milk rich in casein and whey protein in the UF retentate;
d) providing a powder or concentrate of native whey protein;
e) mixing the protein-rich UF retentate obtained in step c) and the native whey protein powder or concentrate from step d) and thereby obtaining a mixture of casein and native whey protein;
f) subjecting the mixture of casein and native whey protein to heat treatment and thereby obtaining a heat treated mixture of casein and whey protein;
g) adding a starter culture to the heat treated mixture of casein and whey protein to provide fermentation of the mixture; and
h) cooling the fermented mixture;
wherein:

- the mixture of casein and native whey protein obtained in step e) has a weight ratio of whey protein to casein in a range from 22:78 to 50:50; and
- the heat treatment of step f) is carried out by heating the mixture of casein and native whey protein at a temperature and for a time period sufficient to obtain denaturation of 30 to 70 % of the native whey protein of the mixture.

[0058] In still another aspect the present invention provides a further alternative method for manufacturing a yoghurt as defined above, comprising the steps of:

a) providing raw milk;
b) separating the raw milk into a cream fraction and a skim milk fraction;
c) subjecting the skim milk fraction to microfiltration (MF) adapted to separate casein from whey protein and thereby obtaining a casein-rich fraction and a fraction of native whey protein;
d) providing a powder of native whey protein;
e) mixing the casein-rich fraction and the powder of native whey protein and thereby obtaining a mixture of casein and native whey protein;
f) subjecting the mixture of casein and native whey protein to heat treatment and thereby obtaining a heat treated mixture of casein and whey protein;
g) adding a starter culture to the heat treated mixture of casein and whey protein to provide fermentation of the mixture; and
h) cooling the fermented mixture;
wherein:

- the mixture of casein and native whey protein obtained in step e) has a weight ratio of whey protein to casein in a range from 22:78 to 50:50; and
- the heat treatment of step f) is carried out by heating the mixture of casein and native whey protein at a temperature and for a time period sufficient to obtain denaturation of 30 to 70 % of the native whey protein of the mixture.

[0059] In yet another aspect the present invention provides another further alternative method for manufacturing a yoghurt as defined above, comprising the steps of:

a) providing raw milk;
b) separating the raw milk into a cream fraction and a skim milk fraction;
c) subjecting the skim milk fraction to microfiltration (MF) adapted to separate casein from whey protein and thereby obtaining a casein-rich fraction and a fraction of native whey protein;
d) providing a powder of native whey protein which is added to the fraction of native whey protein obtained in step c);
e) mixing the casein-rich fraction obtained in step c) and the native whey protein concentrate obtained in step d) and thereby obtaining a mixture of casein and native whey protein;
f) subjecting the mixture of casein and native whey protein to heat treatment and thereby obtaining a heat treated mixture of casein and whey protein;
g) adding a starter culture to the heat treated mixture of casein and whey protein to provide fermentation of the mixture; and
h) cooling the fermented mixture;
wherein:

- the mixture of casein and native whey protein obtained in step e) has a weight ratio of whey protein to casein in a range from 22:78 to 50:50; and
- the heat treatment of step f) is carried out by heating the mixture of casein and native whey protein at a temperature and for a time period sufficient to obtain denaturation of 30 to 70 % of the native whey protein of the mixture.

[0060] The raw milk provided in step a) of the alternative methods is optionally unpasteurized.

[0061] According to one embodiment of the alternative methods, the casein-rich fraction obtained in step c) is pasteurized before subjected to mixing with the native whey protein in step e).

[0062] According to another embodiment of the alternative methods of the invention, the mixture of casein and native whey protein obtained in step e) is fat standardized with addition of cream before heat treatment in step f).

[0063] According to yet another embodiment of the alternative methods of the invention, the mixture of casein and native whey protein obtained in step e) is subjected to homogenization and thereby obtaining a homogenized casein/native whey protein mixture to be heat treated in step f).

[0064] The starter culture added in step g) to provide fermentation, is a yoghurt culture comprising *Streptococcus* subsp. *thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. Other suitable starter cultures can be used to obtain other characteristic concentrated fermented milk products, e.g. ymer or kefir (ref. Codex standard for fermented milks).

[0065] The starter culture is added in conventional amounts.

[0066] The fermentation in step g) is carried out in a time sufficient to obtain proper acidification. That is, to obtain pH about 4.6 or lower, e.g. pH 4.5.

[0067] According to one embodiment of the alternative methods of the invention, the fermented mixture obtained in step g) is stirred before cooling in step h).

[0068] In a preferred embodiment of the alternative methods of the invention, the heat treatment of step f) is carried out by heating the mixture of casein and native whey protein at a temperature of 75 °C for a time period of 5 minutes, or another combination of temperature and time period providing equivalent denaturation degree as obtained by the temperature 75°C for 5 minutes.

[0069] In another preferred embodiment of the alternative methods of the invention, the heat treatment of step f) is carried out by heating the mixture of casein and native whey protein at a temperature of 70°C for 15 minutes. In still another preferred embodiment of the alternative methods of the invention, the temperature is 80°C for 1 minute. In a further preferred embodiment of the alternative methods of the invention, the temperature is 85°C for 30 seconds. In another preferred embodiment of the alternative methods of the invention, the temperature is 90°C for 10 seconds. In yet another preferred embodiment of the alternative methods of the invention, the temperature is 95°C for 6 seconds. In another preferred embodiment of the alternative methods of the invention, the temperature of 100°C for 3 seconds, and in another preferred embodiment of the alternative methods of the invention, the temperature is 110oC for 2 seconds.

[0070] Typically, a short heating time is applicable when the temperature is high and vice versa, and a person known in the art will realize that other temperatures and time periods than those indicated in the preferred embodiment above, will be useful.

[0071] All the methods provided by the present invention renders it possible to manufacture qualitatively acceptable yoghurt containing considerably amounts of native whey proteins (α-lactalbumin, β-lactoglobulin B, and β-lactoglobulin).

[0072] In the examples below it is shown that yoghurts of 8 % protein, a whey protein to casein ratio from 25:75 to 35:65 and high amounts of native whey proteins (i.e. α-lactalbumin, 2.13-2.61 mg mL^{-1}; β-lactoglobulin B, 5.48-6.39 mg mL^{-1}; β-lactoglobulin A, 5.40-6.09 mg mL^{-1}) have been prepared by mixing concentrates of casein and native whey protein and modifying the heat treatment of yoghurt milk.

[0073] It is shown in the examples below that addition of native whey protein concentrate positively influence sensory

attributes when added in low (NWP:C-ratio 23.7:76.3) or medium levels (NWP:C-ratio 34.3:65.7).

[0074] Furthermore, the examples of the present invention show that qualitatively acceptable high protein yoghurt (i.e. 8% protein) containing native whey proteins (α-lactalbumin, 2.13-2.61 mg mL^{-1}; β-lactoglobulin B, 5.48-6.39 mg mL^{-1}; β-lactoglobulin A, 5.40-6.09 mg mL^{-1}) can be produced by addition of low (whey:casein ratio 25:75) or medium (whey:casein ratio 35:65) levels of native whey protein concentrate in combination with a reduction in temperature from 95 °C for 5 minutes to 75 °C for 5 minutes.

[0075] The present invention provides yoghurt products with high amounts of native whey protein having positive nutritional and health effects.

[0076] Furthermore, the present invention provides methods of preparing nutritious and healthy yoghurt products without producing acid whey which is an unwanted by-product of low value and difficult to dispose.

Detailed description of the drawings

[0077]

Fig. 1 shows a flow chart illustrating the production of yoghurt based on microfiltration and ultrafiltration fractions from unpasteurized skim milk according to the present invention.

Fig. 2 shows pictures of stirred yoghurts with no addition of UFR and heat treated at 75 °C for 5 minutes (C-75), with no addition of UFR and heat treated at 95 °C for 5 minutes (C-95), added low (L) level of UFR and heat treated at 75 °C for 5 minutes (L-75), added low (L) level of UFR and heat treated at 95 °C for 5 minutes (L-95), added medium (M) level of UFR and heat treated at 75 °C for 5 minutes (M-75), and added medium (M) level of UFR and heat treated at 95 °C for 5 minutes (M-95).

Fig. 3 shows a flow chart illustrating the production of yoghurt based on microfiltration and ultrafiltration fractions of unpasteurized skim milk wherein UFR is added after heat treatment according to Example 6 (reference example).

Fig. 4 shows pictures of stirred yoghurts with addition of UFR before heat treatment at 75 °C for 5 minutes and fermentation (Control), addition of UFR before heat treatment at 75 °C for 5 minutes and fermentation (Extra), added low level of UFR after heat treatment at 95 °C for 5 minutes but before fermentation (BFL), added high level of UFR after heat treatment at 95 °C for 5 minutes but before fermentation (BFH), added high level of UFR after heat treatment at 95 °C for 5 minutes and fermentation (AFH).

[0078] The invention is explained in more detail in the examples below. The examples are only meant to be illustrative and shall not be considered as limiting.

Examples

Example 1

[0079] Stirred and set yoghurts were produced using three whey protein:casein ratios (10:90, 25:75 or 35:65) and two heat treatment temperatures (75 or 95 °C for 5 minutes) leading to a total of six factor combinations. Two replication blocks gave a total of 12 yoghurt batches.

[0080] Cow's milk (Norwegian red cattle) was obtained from the university farm. The milk was separated (Westfalia separator AG, SA 1-01-175, Oelde, Germany) at 63 °C. Unpasteurized skim milk was temporary collected in a double-O vat (Landteknikk A/L, Trondheim, Norway) and kept at 50 $\pm$ 2 °C until microfiltration (MF) in an MF pilot plant (APV Anhydro AS, Silkeborg, Denmark). Microfiltration was performed at a uniform transmembrane pressure with a module containing seven ceramic membranes, each with pore size 0.14 μm and 0.303 m^2 filter area (INSIDE CéRAM™, TAMI Industries, Nyons, France). Filtration temperature varied between 55 and 58 °C with an average temperature of 56.5 °C. The permeate circulation was cooled to an average temperature of 51.7 °C using a separate cooling system according to the International patent WO 2011/115498 A1 (Hoffmann, 2011). Microfiltration retentates (MFR) with average volume concentration factors of 1.6 and 2.9 were produced. The MFRs were pasteurized (type A3-HRB, Alfa Laval, Nakskov, Denmark) at 72 °C for 15 seconds. The MF permeate was concentrated on an ultrafiltration (UF) pilot plant Alfa Laval UFS-4 (Alfa Laval, Nakskov, Denmark) containing a single spiral wound membrane (GR60PP-6338/48, Alfa Laval, Nakskov, Denmark) with 25 000 Da cut-off. Filtration temperature was kept between 45 and 50 °C with an average temperature of 47.8 °C. The ultrafiltration retentate (UFR) had an average volume concentration factor of approximately 18.8. The chemical composition of the pasteurized MFRs and UFR is shown in Table 1, while Fig. 1 gives a flow chart of the production of fractions and yoghurts. It should be noted that the terms "MFR 1.6" and MRF 2.9" in Table 1 and

Fig. 1 indicate that the microfiltration retentates (MFR) has been concentrated with volume concentration factors (CF) 1.6 and 2.9, respectively.

Table 1: Chemical composition (mean±SD from the mean, n=2) of pasteurized (72 °C 15 sec) microfiltration retentates; MFR 1.6 and MFR 2.9, and ultrafiltration retentate; UFR.

Components	MFR 1.6	MFR 2.9	UFR
Total solids (%)	10.52±0.13	13.90±0.09	13.28±0.08
CP[1] (%)	5.32±0.02	8.66±0.09	8.77±0.09
TP[1] (%)	5.13±0.02	8.49±0.08	8.37±0.10
C[1] (%)	4.36±0.00	7.57±0.04	0.05±0.02
NWP[1] (%)	0.58±0.02	0.74±0.04	7.93±0.14
NWP:C[1] (%/%)	11.7:88.3	8.9:91.1	99.4:0.6
Fat (%)	0.22±0.01	0.47±0.18	0.11±0.04
Lactose (mmol kg^{-1})	130.6±5.9	122.2±7.7	121.1±2.5
Ash (%)	0.90±0.01	1.14±0.07	0.58±0.11
Calcium (g kg^{-1})	1.7±0.1	2.7±0.1	0.5±0.0
Phosphorus (g kg^{-1})	1.3±0.0	2.0±0.1	0.5±0.0

[2]CP=crude protein; TP=true protein; C=casein; NWP=native whey protein.

[0081] MFR 1.6 and 2.9 and UFR were blended in suitable quantities in order to achieve mixtures with a true protein content of 8 % and native whey:casein ratios of: 10:90 (control, no addition of UFR (C)); 25:75 (low addition of UFR (L)); 35:65 (medium addition of UFR (M)). The chemical composition of the mixtures is presented in Table 2.

Table 2: Chemical composition (mean±SD from the mean, n=2) of yoghurt mixtures. Denotations C, L and M refer to level of ultrafiltration retentate added; control (no addition); low; and medium respectively. Denotations 75 and 95 refer to heat treatment; 75 °C for 5 minutes; and 95 °C for 5 minutes respectively.

Components	C-75	C-95	L-75	L-95	M-75	M-95
Total solids[1] (%)	13.49±0.04	13.40±0.12	13.50±0.23	13.56±0.32	13.30±0.16	13.60±0.39
CP[2] (%)	8.18±0.01	8.18±0.01	8.33±0.05	8.33±0.05	8.32±0.01	8.32±0.01
TP[2] (%)	8.02±0.04	8.02±0.04	8.09±0.07	8.09±0.07	8.02±0.02	8.02±0.02
C[2] (%)	7.10±0.00	7.10±0.00	6.00±0.09	6.00±0.09	5.08±0.01	5.08±0.01
NWP[2] (%)	0.75±0.06	0.75±0.06	1.86±0.01	1.86±0.01	2.65±0.02	2.65±0.02
NWP:C[2] (%/%)	9.6:90.4	9.6:90.4	23.7:76.3	23.7:76.3	34.3:65.7	34.3:65.7
Native α-LA[1] (mg mL^{-1})	0.77±0.03	0.28±0.06	2.13±0.03	0.21±0.00	2.61±0.09	0.16±0.02
Native α-LA[1] (%)	75.0±3.4	27.6±6.44	53.6±1.1	5.4±0.1	45.2±2.4	2.7±0.2
Native P-LG B[1] (mg mL^{-1})	1.51±0.15	0.01±0.01	5.48±0.61	0.05±0.01	6.39±0.16	0.06±0.01
Native β-LG B[1] (%)	48.4±6.4	0.5±0.34	42.3±5.76	0.4±0.1	34.4±1.4	0.3±0.0
Native β-LG A[1] (mg mL^{-1})	1.42±0.01	0.01±0.01	5.40±1.04	0.04±0.01	6.09±0.52	0.05±0.01
Native β-LG A[1] (%)	55.1±4.9	0.5±0.3	50.5±7.2	0.4±0.1	39.8±0.2	0.3±0.0
Fat[1] (%)	0.43±0.17	0.43±0.17	0.37±0.14	0.39±0.16	0.32±0.13	0.32±0.13
Lactose (mmol kg^{-1})	118.0±3.3	118.7±1.9	119.5±3.6	124.7±5.1	122.8±5.1	118.9±4.9
Ash[1] (%)	1.07±0.04	1.04±0.02	1.04±0.01	1.05 ± 0.01	0.94 ± 0.04	0.99 ± 0.02
Calcium[1] (g kg^{-1})	2.6±0.1	2.6±0.1	2.2±0.0	2.3±0.0	2.0±0.0	2.0±0.0

(continued)

Components	C-75	C-95	L-75	L-95	M-75	M-95
Phosphorus[1] (g kg^{-1})	1.9±0.0	1.9±0.0	1.7±0.0	1.7±0.0	1.5±0.0	1.5±0.0

[1]Measured in mixtures after heat treatment. LA=lactalbumin, LG=lactoglobulin. Native α-LA (%) is the percentage share of native whey protein left in mixture after heat treatment compared to the amount of native whey protein in mixture before heat treatment.
[2]Measured in mixture before heat treatment. CP=crude protein; TP=true protein; C=casein; NWP=native whey protein.

[0082] In a randomized order, the mixtures were homogenized (Rannie Machine Works Ltd., Albertslund, Denmark) at 180 bar at 55 °C and subsequently heat treated at 75 or 95 °C for 5 minutes in a double-jacketed 5 L heating tank connected to steam and cold water (Norwegian University of Life Sciences, Ås, Norway). The homogenized and heat treated mixtures were precooled and filled at 40-45 °C on sterilized 5 L stainless steel cans with lid and stirrer and tempered to 43 °C in a water bath equipped with a thermostat control. The mixtures were inoculated with 0.02 % (w/w) yoghurt culture (F-DVS YC-183, Chr. Hansen, Hørsholm, Denmark) consisting of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. At pH 4.60 ± 0.01 yoghurts in the 5 L cans were subjected to stirring for 1 minute and subsequently cooled in an ice water bath. Every tenth minute, yoghurts placed in ice water bath were stirred for 15 seconds until a yoghurt temperature of 19 ± 2 °C was reached after approximately 40 minutes. Yoghurts were then filled in suitable smaller containers (70 mL or 300 mL plastic cups with lids) and placed in a cold room (4 °C) until analyzing.

Example 2

[0083] The stirred yoghurts prepared in Example 1 were sensory profiled with regard to appearance and consistency. Intensity of appearance attributes (coarse; granular; shiny; ropy; thickness) and consistency attributes (viscosity; mealy; gritty; smooth) of stirred yoghurts heat treated at 75 °C for 5 minutes and 95 °C for 5 minutes were analyzed on a continuous scale from 1 to 9, see Table 3. Denotations C, L and M refer to level of ultrafiltration retentate added; control (no addition); low; and medium respectively. Increased temperature (95 °C for 5 minutes) at heat treatment of yoghurts added UFR (L or M) resulted in higher intensity for the appearance related attributes coarse, granular and thickness. The addition of UFR only seemed to increase the intensity of these attributes when the heat treatment was set to 75 °C for 5 minutes. Yoghurts added low (L) or medium (M) levels of UFR and heat treated at 75 °C for 5 minutes were shinier than the corresponding yoghurts heat treated at 95 °C for 5 minutes. Lower heat treatment of yoghurts added low or medium levels of UFR gave smoother consistency than heat treatment at 95 °C for 5 minutes. Heat treatment and addition of UFR did not affect the attributes ropy and gritty. Mealy consistency was reduced by the addition of UFR (L or M) compared to control.

Table 3

Yoghurt	Coarse	Granular	Shiny	Ropy	Thickness	Viscosity	Mealy	Gritty	Smooth
C-75	6.4	7.4	2.5	1.6	7.3	4.4	6.2	2.2	1.4
C-95	3.5	5.2	2.8	1.8	6.5	4.8	6.4	1.8	2.0
L-75	1.7	2.2	6.6	2.5	5.3	5.5	3.1	1.4	5.4
L-95	7.6	7.3	2.9	1.6	7.3	4.4	4.5	1.7	1.8
M-75	2.4	3.3	5.7	2.8	5.8	5.5	4.0	1.4	4.7
M-95	8.1	7.5	2.7	1.3	7.3	4.1	3.2	1.6	1.5

Example 3

[0084] The yoghurts prepared in Example 1 were sensory profiled with regard to flavor. Intensity of flavor attributes (yoghurt flavor; acid; bitter; off-flavor; whey; oxidized) of stirred yoghurts heat treated at 75 °C for 5 minutes and 95 °C for 5 minutes; was evaluated on a continuous scale from 1 to 9, see Table 4. Denotations C, L, and M refer to level of ultrafiltration retentate added; control (no addition); low; and medium respectively.

[0085] The flavor attributes; yoghurt flavor, acid, bitter and off-flavor, were scarcely affected by the addition of UFR and heat treatment. Oxidized flavor or whey flavor were absent in the yoghurts.

Table 4

Yoghurt	Yoghurt	Acid	Bitter	Off-flavor	Whey	Oxidized
C-75	3.3	4.5	1.7	1.4	1.1	1.1
C-95	3.5	4.5	2.1	1.3	1.0	1.0
L-75	3.9	4.9	1.6	2.1	1.1	1.1
L-95	3.5	4.8	1.4	1.4	1.1	1.1
M-75	4.1	4.9	1.4	1.9	1.0	1.0
M-95	3.3	4.4	1.2	1.3	1.0	1.0

Example 4

[0086] The yoghurts prepared in Example 1 were studied with regard to particle size distribution.

[0087] Particle size distribution of yoghurt samples heat treated at 75 °C for 5 minutes and 95 °C for 5 minutes was measured with the use of Mastersizer 3000; D_{50} represents the particle size (μm) at which 50 % of the particles in the sample is smaller and 50 % is larger, see Table 5. Denotations C, L and M refer to level of ultrafiltration retentate added; control (no addition); low; and medium respectively.

[0088] The smallest particle size distribution appeared in yoghurts added UFR (L or M) and heat treated at 75 °C for 5 minutes. The particle size of yoghurts heat treated at 95 °C for 5 minutes increased with increasing addition of UFR, leading to largest particles in yoghurt with the highest addition of UFR. For the control yoghurt (no addition of UFR) heat treatment at 95 °C for 5 minutes gave smaller particles than 75 °C for 5 minutes.

Table 5

Yoghurt	D_{50}
C-75	265,5
C-95	121,8
L-75	35,4
L-95	237,1
M-75	39,4
M-95	696,8

Example 5

[0089] Qualitative evaluation of the yoghurts prepared in Example 1 was performed. Appearance, odor/flavor and consistency were evaluated on a scale from 1 to 5. The mean intensity of the yoghurts added low (L) and medium (M) levels of UFR and heat treated at 75 °C were above 3.6, which indicates that the quality is acceptable.

[0090] Fig. 2 shows pictures of the yoghurts indicating the superior appearance attributes of the yoghurts added low (L) and medium (M) levels of UFR and heat treated at 75 °C for 5 minutes compared to the corresponding yoghurts heat treated at 95 °C for 5 minutes and yoghurts not added UFR and treated at 75 and 95 °C.

Example 6 (reference example)

[0091] Microfiltration retentates (MFR 1.6 and MFR 2.9) were produced by microfiltration (0.14 μm) of unpasteurized skim milk with volume concentration factors (CF) 1.6 and 2.9 according to the process of Example 1. The microfiltration permeate was ultrafiltered (25 kDa) to an ultrafiltration retentate (UFR) according to the process of Example 1.
The chemical composition of the pasteurized MFRs and UFR is shown in Table 6, while Fig. 3 gives a flow chart of the production of fractions and yoghurts. The terms "MFR 1.6" and "MRF 2.9" indicate that the microfiltration retentates (MFR) has been concentrated with volume concentration factors (CF) 1.6 and 2.9, respectively.

Table 6: Chemical composition of pasteurized (72 °C 15 sec) microfiltration retentates; MFR 1.6 and MFR 2.9, and ultrafiltration retentate; UFR.

Components	MFR 1.6	MFR 2.9	UFR
Total solids (%)	10.50	14.07	12.64
CP[1] (%)	5.42	9.01	8.09

(continued)

Components	MFR 1.6	MFR 2.9	UFR
TP[1] (%)	5.23	8.83	7.77
C[1] (%)	4.39	7.81	0.04
NWP[1] (%)	0.65	0.83	7.43
NWP:C[1] (%/%)	12.9:87.1	9.6:90.4	99.4:0.6
Fat (%)	0.22	0.34	0.06
Lactose (mmol kg^{-1})	145.7	137.5	148.0
Ash (%)	0.87	1.19	0.58

[2] CP=crude protein; TP=true protein; C=casein; NWP=native whey protein.

[0092] A control mixture, representing a yoghurt sample prepared according to the present invention, was made by blending UFR with the MFRs to a protein content of about 8 % and whey protein:casein ratio 30:70. The control mixture was homogenized and heat treated at 75 °C for 5 minutes, fermented to a pH of 4.60, stirred and cooled. Mixtures named before fermentation high (BFH) and before fermentation low (BFL) were made by homogenizing and heat treating mixtures of MFRs at a heat treatment temperature of 95 °C for 5 minutes. High or low amount of UFR was added to the heat treated mixtures to a whey protein:casein ratio of 30:70 or 20:80 respectively. Subsequently the mixtures were fermented to pH 4.60, stirred and cooled. A mixture named after fermentation high (AFH) was made in the same way as BFH, but instead of adding the UFR to the mixture before fermentation, it was added after fermentation but before stirring and cooling. The whey protein:casein ratio in AFH was theoretically 30:70. The chemical composition of the mixtures is presented in Table 7.

Table 7: Chemical composition of yoghurt mixtures. Denotations 75 and 95 refer to heat treatment; 75 °C for 5 minutes; and 95 °C for 5 minutes respectively.

Components	Control-75	BFH-95	BFL-95	AFH-95
Total solids[1] (%)	13.24	13.37	13.43	13.48[3]
CP[2] (%)	8.33	8.43[3]	8.43[3]	8.43[3]
TP[2] (%)	8.07	8.23[3]	8.23[3]	8.23[3]
C[2] (%)	5.47	7.23[3]	7.23[3]	7.23[3]
NWP[2] (%)	2.35	0.81[3]	0.81[3]	0.81[3]
NWP:C[2] (%/%)	30.1:69.9	10.0:90.0[3]	10.0:90.0[3]	10.0:90.0[3]
Native α-LA[1] (mg mL^{-1})	4.06	3.85	2.23	0.37[3]
Native β-LG B[1] (mg mL^{-1})	14.17	12.71	6.61	0.05[3]
Native β-LG A[1] (mg mL^{-1})	8.93	7.60	3.86	0.02[3]
Fat[1] (%)	0.25	0.27	0.27	0.30[3]
Lactose[1] (mmol kg^{-1})	136.3	137.7	135.7	135.9[3]
Ash[1] (%)	0.97	0.92	0.95	1.04[3]

[1]Measured in mixtures after heat treatment. LA=lactalbumin, LG=lactoglobulin.
[2]Measured in mixtures before heat treatment. CP=crude protein; TP=true protein; C=casein; NWP=native whey protein.
[3]Chemical composition of mixture before addition of UFR.

Example 7

[0093] The stirred yoghurts prepared in Example 6 were sensory profiled with regard to appearance and consistency. In addition to the yoghurts described in Example 6, a yoghurt named "Extra" was made in the same way as the yoghurt named "Control", but a whey protein:casein ratio of 20:80 was obtained. Intensity of appearance attributes (coarse; granular; shiny; ropy; thickness) and consistency attributes (viscosity; mealy; gritty; smooth) of stirred yoghurts was

analyzed on a continuous scale from 1 to 9, see Table 8.

Table 8

Yoghurt	Coarse	Granular	Shiny	Ropy	Thickness	Viscosity	Mealy	Gritty	Smooth
Control	1.0	1.2	7.9	2.4	4.3	5.2	2.2	1.1	6.9
BFH	3.4	4.6	4.0	3.4	6.0	4.9	6.5	1.4	3.0
BFL	2.5	4.9	3.5	3.7	6.6	5.0	6.7	1.5	2.5
AFH	6.5	6.8	2.8	3.4	6.5	4.6	7.3	1.9	1.7
Extra	2.1	3.1	4,0	3.4	5.8	5.3	5.9	1.6	3.6

[0094] Control yoghurt obtained the lowest intensity of the attributes coarse, granular and mealy, and the highest intensity of the attributes shiny and smooth. Yoghurts BFH, BFL and AFH were perceived as granular and mealy. Also yoghurt Extra obtained relatively high intensity of the attribute mealy. Yoghurt Control and Extra were perceived as most viscous (consistency) of all the yoghurts.

Example 8

[0095] The yoghurts prepared in Example 6 were sensory profiled with regard to flavor. Intensity of flavor attributes (yoghurt; acid; bitter; off-flavor; whey; oxidized) of stirred yoghurts was evaluated on a continuous scale from 1 to 9, see Table 9.

Table 9

Yoghurt	Yoghurt	Acid	Bitter	Off-flavor	Whey	Oxidized
Control	4.2	5.1	2.1	2.4	1.7	1.2
BFH	4.0	5.4	2.5	2.7	1.8	1.1
BFL	3.8	5.1	2.0	2.3	1.4	1.1
AFH	3.9	4.8	2.3	2.4	1.3	1.1
Extra	4.1	5.4	2.0	2.2	1.2	1.1

[0096] The flavor attributes; yoghurt, acid, bitter, whey, oxidized and off-flavor, were scarcely affected by the time of UFR-addition or the heat treatment temperature. Low intensities of the flavor attributes oxidized and whey were obtained.

Example 9

[0097] The yoghurts prepared in Example 6 were studied with regard to particle size distribution measured by Mastersizer 3000, see Table 10. D_{50} reports the particle size (μm) at which 50 % of the particles in the sample are smaller and 50 % are larger.

Table 10

Yoghurt	D_{50}
Control	18.1
BFH	57.7
BFL	71.0
AFH	141.9

[0098] The smallest particle size distribution was measured in control yoghurt, and the largest particle size distribution was found in yoghurt AFH.

Example 10

[0099] Fig. 4 shows pictures of the yoghurts prepared in Example 6.

[0100] The shiny appearance of yoghurt Control can be seen in Fig. 4. Yoghurt AFH appears as coarse and granular.

BFH and BFL were perceived as mealy in the sensory analysis, however this cannot be visually judged by studying Fig. 4. BFH and BFL could visually be perceived as less shiny than yoghurt Control.

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Claims

1. A yoghurt comprising a content of protein in the range from 5.6 % to 20 % (w/w), a content of native whey proteins in the range from 4 to 70 mg/mL, and a weight ratio of whey protein to casein in a range from 22:78 to 50:50 (w/w).

2. The yoghurt of claim 1, wherein the protein content is in the range chosen from 6 % to 18 % (w/w), 5.6 % to 15 % (w/w), 6 % to 18 % (w/w), 6.5 % to 18 % (w/w), 7 % to 17 % (w/w), 7 % to 15 % (w/w), 7 % to 13 % (w/w), 7 % to 12 % (w/w), 8 % to 12 % (w/w), or 8 % to 11 % (w/w).

3. The yoghurt of claim 1, wherein the content of native whey proteins is in the range chosen from 4 to 65 mg/mL, 4 to 55 mg/mL, 4.5 to 65 mg/mL, 5 to 60 mg/mL, 5 to 55 mg/mL, 5 to 45 mg/mL, 5 to 40 mg/mL, 5.5 to 40 mg/mL, 6 to 40 mg/mL, 6 to 30 mg/mL, 8 to 40 mg/mL, 8 to 30 mg/mL, 10 to 40 mg/mL, or 10 to 30 mg/mL.

4. The yoghurt of claim 1, wherein the whey protein:casein ratio is in the range chosen from 25:75 to 45:55 (w/w), 25:75 to 40:60 (w/w), or 25:75 to 35:65 (w/w).

5. A method for manufacturing a yoghurt as defined in any one of claims 1 to 4, comprising the steps of:

   a) providing raw milk;
   b) separating the raw milk into a cream fraction and a skim milk fraction;
   c) subjecting the skim milk fraction to microfiltration (MF) adapted to separate casein from whey protein and thereby obtaining a casein-rich fraction and a fraction of native whey protein;
   d) providing a native whey protein concentrate by subjecting the fraction of native whey protein to ultrafiltration (UF) adapted to separate native whey protein from water;
   e) mixing the casein-rich fraction and the native whey protein concentrate and thereby obtaining a mixture of casein and native whey protein wherein the whey protein to casein ratio is increased compared to the ratio of whey protein to casein in the raw milk;
   f) subjecting the mixture of casein and native whey protein to heat treatment and thereby obtaining a heat treated mixture of casein and whey protein;
   g) adding a starter culture to the heat treated mixture of casein and whey protein to provide a fermented mixture; and
   h) cooling the fermented mixture;
   characterized in that

   - the mixture of casein and native whey protein obtained in step e) has a weight ratio of whey protein to casein in a range from 22:78 to 50:50; and
   - the heat treatment of step f) is carried out by heating the mixture of casein and native whey protein at a temperature and for a time period sufficient to obtain denaturation of 30 to 70 % of the native whey protein of the mixture.

6. The method of claim 5, wherein the skim milk fraction obtained in step b) is subjected to microfiltration or bactofugation

adapted to separate microorganisms from the skim milk fraction and thereby obtaining a microorganisms-rich fraction and a partly sterilized skim milk fraction before subjecting the partly sterilized skim milk fraction to the microfiltration of step c).

7. The method of claim 5, wherein the MF in step c) is carried out by using membranes with pore size from 0.05 μm to 0.2 μm.

8. The method of claim 5, wherein the UF in step d) is carried out by using membranes with cut-off from 1 kDa to 35 kDa.

9. A method for manufacturing a yoghurt as defined in any one of claims 1 to 4, comprising the steps of:

a) providing raw milk;
b) separating the raw milk into a cream fraction and a skim milk fraction;
c) subjecting the skim milk fraction to ultrafiltration (UF) adapted to obtain concentrated milk rich in casein and whey protein in the UF retentate;
d) providing a powder or concentrate of native whey protein;
e) mixing the protein-rich UF retentate obtained in step c) and the native whey protein powder or concentrate from step d) and thereby obtaining a mixture of casein and native whey protein;
f) subjecting the mixture of casein and native whey protein to heat treatment and thereby obtaining a heat treated mixture of casein and whey protein;
g) adding a starter culture to the heat treated mixture of casein and whey protein to provide fermentation of the mixture; and
h) cooling the fermented mixture;
characterized in that

- the mixture of casein and native whey protein obtained in step e) has a weight ratio of whey protein to casein in a range from 22:78 to 50:50; and
- the heat treatment of step f) is carried out by heating the mixture of casein and native whey protein at a temperature and for a time period sufficient to obtain denaturation of 30 to 70 % of the native whey protein of the mixture.

10. A method for manufacturing a yoghurt as defined in any one of claims 1 to 4, comprising the steps of:

a) providing raw milk;
b) separating the raw milk into a cream fraction and a skim milk fraction;
c) subjecting the skim milk fraction to microfiltration (MF) adapted to separate casein from whey protein and thereby obtaining a casein-rich fraction and a fraction of native whey protein;
d) providing a powder of native whey protein;
e) mixing the casein-rich fraction and the powder of native whey protein and thereby obtaining a mixture of casein and native whey protein;
f) subjecting the mixture of casein and native whey protein to heat treatment and thereby obtaining a heat treated mixture of casein and whey protein;
g) adding a starter culture to the heat treated mixture of casein and whey protein to provide fermentation of the mixture; and
h) cooling the fermented mixture;
characterized in that

- the mixture of casein and native whey protein obtained in step e) has a weight ratio of whey protein to casein in a range from 22:78 to 50:50; and
- the heat treatment of step f) is carried out by heating the mixture of casein and native whey protein at a temperature and for a time period sufficient to obtain denaturation of 30 to 70 % of the native whey protein of the mixture.

11. A method for manufacturing a yoghurt as defined in any one of claims 1 to 4, comprising the steps of:

a) providing raw milk;
b) separating the raw milk into a cream fraction and a skim milk fraction;
c) subjecting the skim milk fraction to microfiltration (MF) adapted to separate casein from whey protein and

thereby obtaining a casein-rich fraction and a fraction of native whey protein;
d) providing a powder of native whey protein which is added to the fraction of native whey protein obtained in step c);
e) mixing the casein-rich fraction obtained in step c) and the native whey protein concentrate obtained in step d) and thereby obtaining a mixture of casein and native whey protein;
f) subjecting the mixture of casein and native whey protein to heat treatment and thereby obtaining a heat treated mixture of casein and whey protein;
g) adding a starter culture to the heat treated mixture of casein and whey protein to provide fermentation of the mixture; and
h) cooling the fermented mixture;
characterized in that

- the mixture of casein and native whey protein obtained in step e) has a weight ratio of whey protein to casein in a range from 22:78 to 50:50; and
- the heat treatment of step f) is carried out by heating the mixture of casein and native whey protein at a temperature and for a time period sufficient to obtain denaturation of 30 to 70 % of the native whey protein of the mixture.

12. The method of any one of claims 5, 9, 10 or 11, wherein the casein-rich fraction obtained in step c) is pasteurized before subjected to mixing with the native whey protein concentrate in step e).

13. The method of any one of claims 5, 9, 10 or 11 wherein the mixture of casein and native whey protein obtained in step e) is subjected to homogenization and thereby obtaining a homogenized mixture of casein and native whey protein to be heat treated in step f).

14. The method of any one of claims 5, 9, 10 or 11, wherein the heat treatment of step f) is carried out by heating the mixture of casein and native whey protein at a temperature of 75 °C for a time period of 5 minutes, or another temperature and time combination providing equivalent denaturation degree as 75°C for 5 minutes.

15. The method of claim 14, wherein the heat treatment of step f) is carried out by heating the mixture of casein and native whey protein at a temperature and for a time period selected from the group consisting of: 70°C for 5 minutes, 80°C for 1 minute, 85°C for 30 seconds, 90°C for 10 seconds, 95°C for 6 seconds, 100°C for 3 seconds, and 110°C for 2 seconds.

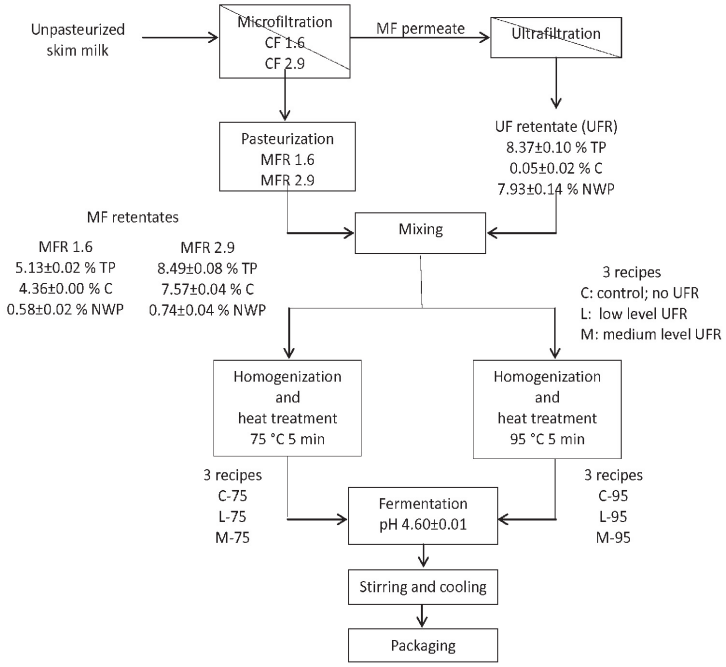
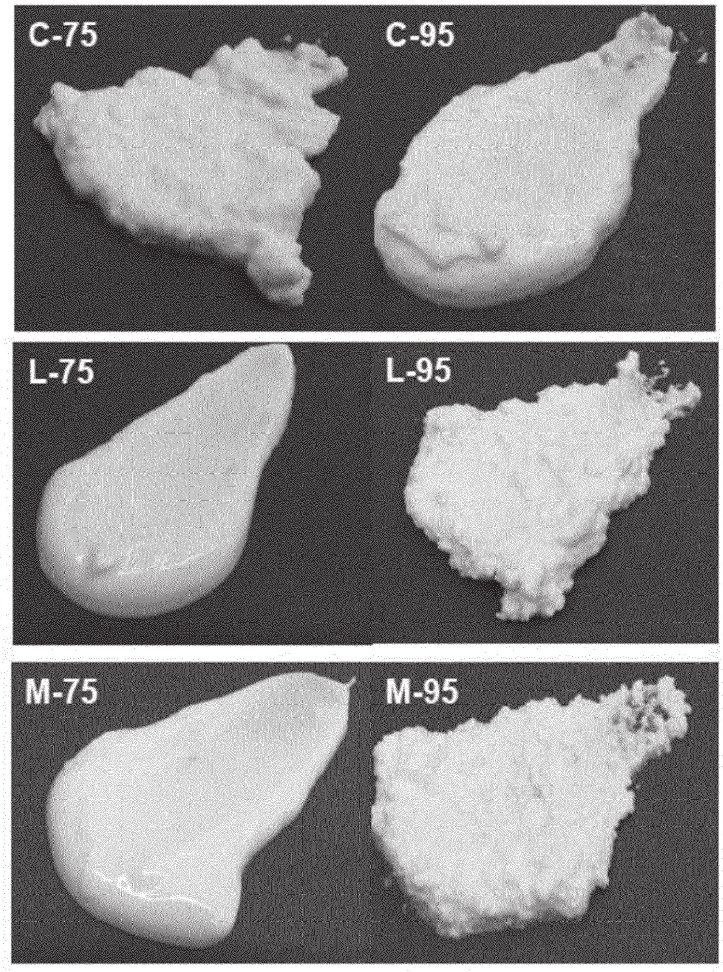

Unpasteurized skim milk
Microfiltration
CF 1.6
CF 2.9
MF permeate
Ultrafiltration
Pasteurization
MFR 1.6
MFR 2.9
UF retentate (UFR)
8.37±0.10 % TP
0.05±0.02 % C
7.93±0.14 % NWP
MF retentates
MFR 1.6
5.13±0.02 % TP
4.36±0.00 % C
0.58±0.02 % NWP
MFR 2.9
8.49±0.08 % TP
7.57±0.04 % C
0.74±0.04 % NWP
Mixing
3 recipes
C: control; no UFR
L: low level UFR
M: medium level UFR
Homogenization and heat treatment 75 °C 5 min
Homogenization and heat treatment 95 °C 5 min
3 recipes
C-75
L-75
M-75
3 recipes
C-95
L-95
M-95
Fermentation pH 4.60±0.01
Stirring and cooling
Packaging

Fig. 1

Fig. 2



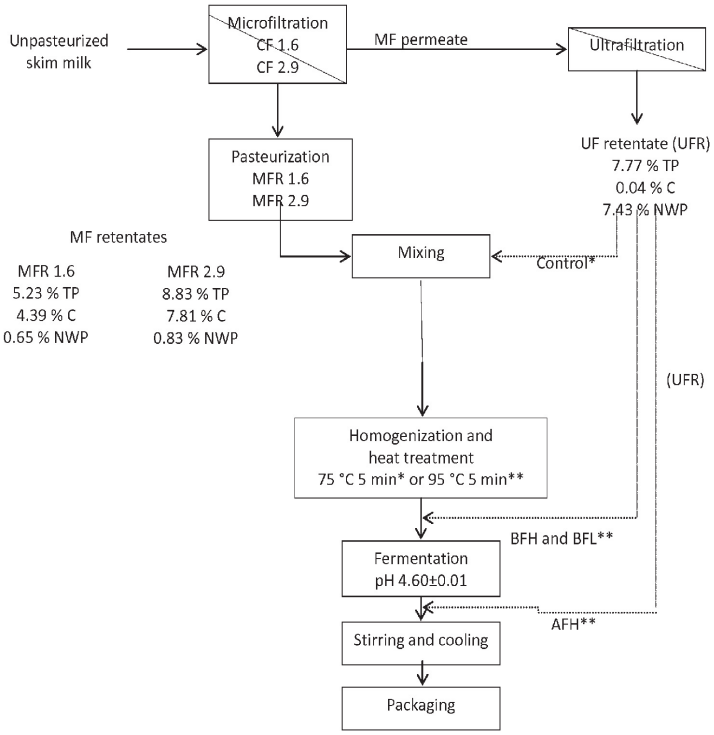
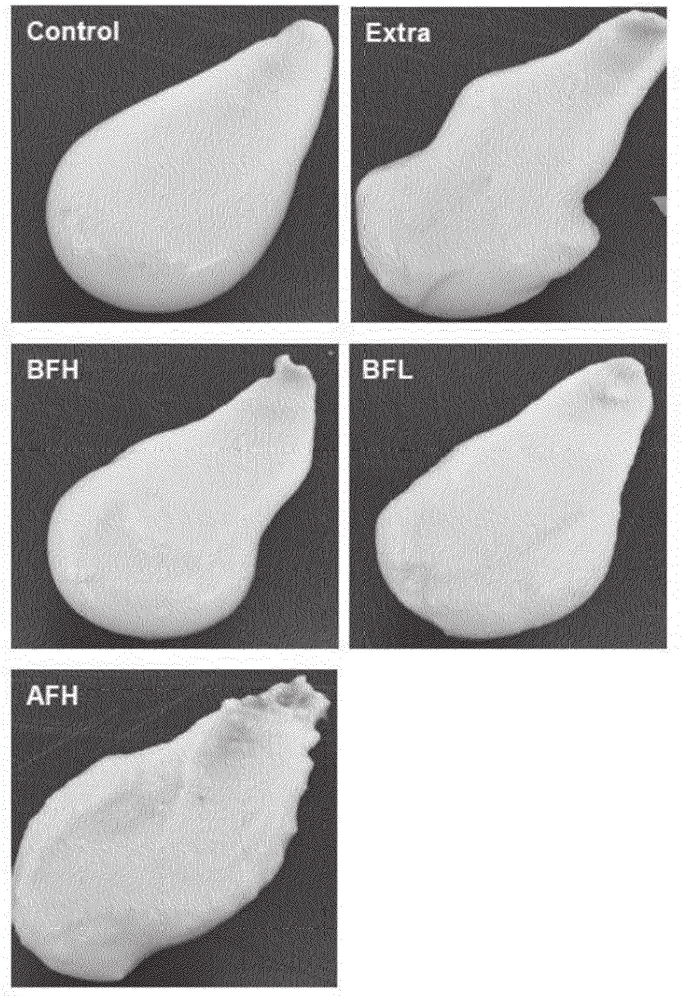

Unpasteurized skim milk
Microfiltration
CF 1.6
CF 2.9
MF permeate
Ultrafiltration
UF retentate (UFR)
7.77 % TP
0.04 % C
7.43 % NWP
Pasteurization
MFR 1.6
MFR 2.9
MF retentates
MFR 1.6
5.23 % TP
4.39 % C
0.65 % NWP
MFR 2.9
8.83 % TP
7.81 % C
0.83 % NWP
Mixing
Control*
(UFR)
Homogenization and heat treatment
75 °C 5 min* or 95 °C 5 min**
BFH and BFL**
Fermentation
pH 4.60±0.01
AFH**
Stirring and cooling
Packaging

Fig.3

Fig. 4



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EUROPEAN SEARCH REPORT

Application Number
EP 14 19 9350

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A	* tables 1,2 * * figure 1 * * paragraph [02.1] * * paragraph [0005] *	5-15	
A	EP 1 046 344 A2 (SNOW BRAND MILK PROD CO LTD [JP]) 25 October 2000 (2000-10-25) * paragraphs [0002], [0008] - [0009], [0015], [0034] - [0035], [0046] - [0047] *	1-15	
A,D	US 2014/017332 A1 (TIKANMAEKI REETTA [FI] ET AL) 16 January 2014 (2014-01-16) * paragraphs [0005], [0011] - [0012], [0015], [0019] - [0020], [0028] - [0045] * * examples 1,2 *	1-15	TECHNICAL FIELDS SEARCHED (IPC) A23L A23C

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	10 April 2015	Couzy, François

CATEGORY OF CITED DOCUMENTS

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L : document cited for other reasons

& : member of the same patent family, corresponding document

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EUROPEAN SEARCH REPORT

Application Number
EP 14 19 9350

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A	US 2014/308398 A1 (MERRILL RICHARD K [US]) 16 October 2014 (2014-10-16) * figure 3A * * paragraphs [0002] - [0003], [0010] - [0011], [0036] - [0038], [0045] - [0046] *	1-15	

The present search report has been drawn up for all claims

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