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Ultra-high pressure homogenisation of milk: effects on cheese-making

Tesis doctoral

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Publications

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Rennet coagulation properties of whole bovine milk treated by ultra-high pressure homogenisation. AFE-Tech Workshop. Barcelona, Spain. 2006.

Effects of ultra-high pressure homogenization on cheesemaking properties of milk. European COST D30 workshop - Trends in High Pressure Protein Sciences. Montpellier, France. 2005.

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Section I, Chapter 1

Interest of the study

1. Interest of the study

Food technology research is very much focused on fulfilling social demands. The main social concern has been and will always be food security. In that sense, research aims at guaranteeing food safety by the improvement of both food processing and monitoring. However, questions of food quality and healthy nutrition are becoming increasingly important and are socially reflected as an increase in the demand of "fresh like" and natural products.

Preservation of foodstuff has been traditionally performed through thermal processing. However, high temperatures required for the inactivation of foodborne pathogens may cause undesirable effects, e.g., changes in flavour and colour via Maillard reactions or destruction of essential compounds such as vitamins. Hence, during the last decades, food research has focused in the development of non-thermal technologies.

Among the emerging technologies, ultra-high pressure homogenisation is a promising technology for liquid foods such as milk owing to the fact that it is a continuous process (vs. high hydrostatic pressure); it performs two separated processes at once, i.e., pasteurisation and homogenisation; it can deal with volumes usual at pilot-scale with a view to go for larger volumes such as those at production-scale; it can provoke high temperatures but at such a short time that heat effect can be minimised; and finally, it requires the same equipments as a common homogeniser (vs. pulsed electric fields or ionising radiation).

Ultra-high pressure homogenisation, as a food technology alternative to conventional heat-treatments, is currently investigated in a wide range of liquid foods. Research not only aims at evaluating the effects on the liquid food itself, e.g., milk for human consumption, but also the effects on the capacity in obtaining manufactured products and the quality of the final products, e.g., yogurt or cheese.

Section I, Chapter 2

Background

2. Background

2.1. Starter-free fresh cheese

2.1.1. General information and technological aspects of cheese-making

In Latin American, Middle Eastern and some European countries, fresh rennet cheeses, such as Queso Blanco, Queso Fresco, Italian fresh cheese and Halloumi, are produced with little or no culture. Queso fresco de Burgos, which is one of the main fresh cheeses in Spain, is characterised by an intense whiteness, softness and high moisture content. Due to being slightly salted and with mild taste and fresh-milk flavour, this variety of cheese is commonly used as ingredient for salads and sauces. But it can also be consumed as a dessert, especially with sweet accompaniments such as honey.

In literature, few studies have dealt with Burgos cheese, and were focused in either characterising the chemical composition (Marcos et al., 1983, 1985; García et al., 1987; Millán et al., 1990; Medina et al., 1992) or the microbiology (Chavarri et al., 1985; García et al., 1987). As a consequence, reviews are rather scarce (Compairé, 1975; Yubero, 1988; Altamirano, 1994; Prieto et al., 1998). However, as a marketing tool, some studies conducted by distribution companies on commercial cheeses have been published online (Consumer, 2002, 2007).

Originally elaborated with ewe's raw milk, pasteurised cow's milk, or a mixture of both, is nowadays exclusively used due to Spanish regulations and economic reasons. Milk for cheese manufacture is generally pasteurised at 70-80 °C for 15-40 s. Higher temperatures have adverse effects on curd formation, namely longer coagulation times and weaker gels (Guinee et al., 1997; Singh & Waungana, 2001), and on curd syneresis; that is, higher moisture content (Walstra et al., 1985; Pearse & Mackinlay, 1989; Rynne et al., 2004). Nevertheless, the effect of high heat treatment has received considerable attention owing to its potential for improving cheese yield through incorporation of whey proteins into cheese curd (Lucey, 1995; Singh & Waungana, 2001). In addition, pasteurisation reverses the undesirable effects of

cold storage on the cheese-making properties of milk (Fox & McSweeney, 2004). Before cheese production, milk cooled to 4 ºC may be held at about this temperature for several days on the farm and at the factory. Cold storage not only affects the native microbiota of raw milk by selecting the development of undesirable psychrotrophic microbiota, but also causes physico-chemical changes, such as shifts in calcium phosphate equilibrium and some dissociation of casein micelles, which have undesirable effects on the cheese-making properties of milk. However, these changes are reversed on heating and are of no practical significance (Fox & McSweeney, 2004).

Cheese manufacture usually involves an initial standardisation of milk fat and protein contents. Moreover, the addition of caseinate and/or skim milk powder into milk before pasteurisation is commonly used in order to increase yield and improve textural characteristics of cheeses. Besides, milk can present seasonal differences on native calcium; owing to the importance of Ca^{2+} in various aspects of cheese manufacture and quality, which will be later discussed, deficiencies are overcome by supplementing milk after pasteurisation with calcium chloride (Banks & Horne, 2002). Salting of the cheeses was traditionally performed through brining for only 10-30 min or 1-2 h depending on the temperature, size of the cheese and salt concentration of the brine; but nowadays adding sodium chloride directly to milk before coagulation is widely used since it results in a better homogeneity of salt content within the final product and lower microbial contamination by eliminating one of the critical control points of the HACPP. Moreover, due to the recent social concerns on health and the role of salt in some diseases, “low salt” cheeses are being produced without addition of sodium chloride.

Coagulation of milk was traditionally performed with calf rennet, but the increasing demand has provoked a shift to the use of microbial enzymes, e.g. from *Rhizomucor miehei* (Fox & McSweeney, 2004). Coagulation temperature and time range from 28 to 32 ºC and 15 to 45 min depending on the characteristics of the rennet used. Since lactic starters are generally not used, the coagulation is entirely by rennet at the natural pH of milk. However, in some cases, in order to simulate the effect of native microorganisms present in

raw milk, little amounts of culture is added to the pasteurised milk. The mechanism of enzymatic coagulation can be divided into two main phases: a primary enzymatic phase and a secondary aggregation stage of gel formation (Banks & Horne, 2002). Briefly, the enzyme of the coagulant preparation cleaves κ-casein at the surface of casein micelles, which reduces the net negative charge and steric repulsion between micelles, destabilising them sufficiently to allow coagulation. Rennet-hydrolysed micelles thus become susceptible to aggregation and a three-dimensional gel network is formed (McMahon & Brown, 1984). The slight decrease in pH, provoked by the addition of calcium chloride, promotes the primary enzymatic phase of renneting, while the secondary stage of aggregation is enhanced by calcium salts (Banks & Horne, 2002). Fat globules participate to a relatively limited extent in formation of the protein network, being largely physically entrapped therein, but the fat globule membrane may play a structural role in gels (Michalski et al., 2002).

Post-coagulation manufacturing procedures aim at the dehydration of curd and the formation of grains. Under quiescent conditions, a rennet-coagulated milk gel show syneresis, i.e., expel whey, because the gel (curd) contracts (Dejmek & Walstra, 2004). However, syneresis may be enhanced if the curd is cut and stirred. The rate and extent of syneresis is influenced by intrinsic factors, i.e., Ca^{2+} and casein concentrations, and pH of the curd, as well as by extrinsic factors, i.e., size of the cut pieces, rate of stirring, temperature of the process, and of course time (Dejmek & Walstra, 2004). The role of added calcium chloride has not been so far elucidated; the balance between calcium ion activity, which enhances syneresis, and colloidal calcium phosphate, which diminishes syneresis, must be considered. However, adding calcium chloride provokes a slight decrease of the pH, which results in a higher syneresis rate. Cutting the rennet-coagulated milk gel into pieces creates a free surface through which syneresis can occur. Syneresis is not only enhanced by increasing the amount of free surface through cutting small pieces, but also by the fact that small pieces of curd shrink more than large ones. Since fresh cheeses are characterised by high moisture content, grains are cut rather large. Stirring enhances syneresis by preventing sedimentation of the curd particles,

and by provoking external pressure and intermittent deformation of the curd grains. Increasing the rate of stirring provokes greater syneresis but grains, especially large ones, tend to break resulting in fine losses. Temperature greatly affects syneresis by enhancing it as temperature is increased. During the manufacture of low-moisture cheeses, e.g., Parmigiano-Reggiano, cooking of the curd is performed by increasing the temperature up to ~55 ºC (Fox & McSweeney, 2004). In contrast, for fresh cheeses, the temperature is usually kept at the same value at which coagulation is performed.

Fresh cheese shaping is carried out by pouring the obtained grains directly into moulds of different sizes depending on the format. Whey drainage is achieved without pressing through gravitation at 7-10 ºC. In the case of large cheeses (500 g), drained curds are turned upside down to facilitate the drainage of the upper part of the cheese. Unmoulding and compensated vacuum-packing are performed after cheese cooling in a cold chamber at 2 ºC. During storage at 4 ºC before commercialisation, syneresis carries on resulting in a block of cheese embedded within expelled whey. Although some syneresis is desired in order to maintain the wet aspect to which consumers are used, when excessive, it can worsen the quality of the product. Hernando et al. (2000) observed that the addition of alginate, locust beam gum, pectin, microcrystalline cellulose or guar gum decreased syneresis in Burgos cheeses. Indeed, stabilisers or thickeners, i.e., gelatine, starches, citrates, phosphates, alginates, agar and gums, are allowed in the production of fresh cheeses under the Codex Standard 221-2001 (FAO/WHO, 2001). Although a derogated regulation allowed almost all the stabilisers or thickeners previously mentioned (BOE, 1985), the current Spanish regulations, i.e., Real Decreto 142/2002 (BOE, 2002) and modifications (BOE, 2004a,b, 2007), are much stricter; only phosphates are allowed to be used but gelatine is not considered an additive but a facultative ingredient of fresh cheeses, such as calcium chloride and skim milk powder (BOE, 2006).

The composition of fresh cheeses strongly depends on the technological conditions applied by the manufacturer. In general, Burgos cheeses are characterised by high moisture content (60-75%), slightly higher amounts of fat than protein on a dry basis (45-55% vs. 35-45%), rather low salt content on

a dry basis (1-1.5%), and high pH (6.5-6.8) (Millán et al., 1990; Medina et al., 1992; Hernando, 1998; Consumer, 2002, 2007). Under European Directive 2000/13/EC (European Commission, 2000) and Spanish Real Decreto 1334/1999 (BOE, 1999) regulations, the content of a specific ingredient must be labelled only if it is essential in the definition of the product and to distinguish it from other products. However, the provisions of Codex Standard 221-2001 for unripened cheese including fresh cheese (FAO/WHO, 2001) recommend the labelling of fat content (**FDM**). Burgos-type cheeses fall between full- (45% ≤ FDM < 60%) and medium-fat (25% ≤ FDM < 40%) categories of Codex Standard 221-2001 (FAO/WHO, 2001) and Real Decreto 1113/2006 (BOE, 2006). A recent study on Burgos cheeses commercialised in Spain has shown that many producers use erroneously the category labels (Consumer, 2007). Since Burgos cheese is a fresh type, proteolysis is rather low (García et al., 1987; Marcos et al., 1983, 1985). As already mentioned, coagulation is usually achieved with microbial rennet, although traditionally calf rennet was used. Recombinant chymosin is commonly used at the laboratory level and has been proven not to change yield, texture, ripening or flavour development of cheeses, since recombinant chymosins are biochemically and genetically identical to calf rennet (Teuber, 1990). Medina et al. (1992) studied the compositional differences upon using calf rennet or recombinant chymosin in Burgos-type cheeses. No differences were observed in yield, moisture content, pH, soluble nitrogen or flavour, but residual levels of α_{s1}- and β-caseins were higher when using recombinant chymosin.

Concerning general microbiological criteria of cheeses, the Spanish regulation limited populations of *Enterobacteriaceae* to 4 log cfu g^{-1}, *Escherichia coli* and enterotoxigenic *Staphylococcus aureus* to 3 log cfu g^{-1}, and *Salmonella* spp. or *Shigella* spp. absent in 25 g (BOE, 1985). However, since this normative was derogated by the current national regulation Real Decreto 1113/2006 (BOE, 2006), in which no microbiological criteria are taken into account, the European regulations on the topic must be complied. Directives 92/46/EEC and 94/71/EC (European Commission, 1992, 1994), on the health rules for the production and placing on the market of raw milk, heat-treated milk and milk-based products, were taken into account when stipulating the regulations.

General and specific rules on the hygiene of foodstuffs are dictated in Regulations 852/2004 and 853/2004, and the amending Regulations 1662/2006 and 1020/2008 (European Commission, 2004a,b, 2006, 2008): '*Food business operators manufacturing dairy products must initiate procedures to ensure that, immediately before being heat treated and if its period of acceptance specified in the HACCP-based procedures is exceeded: (a) raw cows' milk used to prepare dairy products has a plate count at 30 °C of less than 300 000 per ml; and (b) heat treated cows' milk used to prepare dairy products has a plate count at 30 °C of less than 100 000 per ml*'. '*When raw milk, colostrum, dairy or colostrum-based products undergo heat treatment, food business operators must ensure that this satisfies the requirements laid down in Chapter XI of Annex II to Regulation (EC) No 852/2004. In particular, they shall ensure, when using the following processes, that they comply with the specifications mentioned: (a) Pasteurisation is achieved by a treatment involving: (i) a high temperature for a short time (at least 72 ºC for 15 seconds); (ii) a low temperature for a long time (at least 63 ºC for 30 minutes); or (iii) any other combination of time-temperature conditions to obtain an equivalent effect, such that the products show, where applicable, a negative reaction to an alkaline phosphatase test immediately after such treatment*'. Concerning the microbiological criteria for foodstuffs, Regulation 2073/2005 and the amending Regulation 1441/2007 (European Commission, 2005, 2007), which modifies the standard method for the identification of coagulase positive staphylococci, stipulate that for "*Ready-to-eat foods able to support the growth of L. monocytogenes, other than those intended for infants and for special medical purposes*" the limit of *Listeria monocytogenes* is 100 cfu g^{-1} (n = 5, c = 0), for '*cheeses made from milk or whey that has undergone heat treatment*' those of *E. coli*, used as an indicator for the level of hygiene, are m = 100 cfu g^{-1} and M = 1,000 cfu g^{-1} (n = 5, c = 2), and for '*unripened soft cheeses (fresh cheeses) made from milk or whey that has undergone pasteurisation or a stronger heat treatment*', those of coagulase-positive staphylococci, m = 10 cfu g^{-1} and M = 100 cfu g^{-1} (n = 5, c = 2), where n = number of sample units comprising the sample; m = threshold value for the number of bacteria considered satisfactory if the number of bacteria in all sample units does not exceed "m"; M = maximum value for the number of bacteria considered unsatisfactory if the number of bacteria in one or more

sample units is "M" or more; c = number of sample units where the bacteria count may be between m and M, the sample being considered acceptable if the bacteria count of the other sample unit is m or less.

Fresh cheeses such as Burgos cheese are particularly susceptible to contamination due to their relatively high pH and moisture content, as well as the absence of competing starter populations. Pasteurisation of the milk used in the manufacture of such cheeses generally kills pathogens that are present in the starting milk substrate. However, this does not alleviate concerns regarding post-processing contamination, such as improper handling or storage by the manufacturer or the consumer. Among human pathogens that may be found in dairy products, *Staphylococcus aureus*, in particular strains producing enterotoxins, is of paramount importance because heat-treated milk is more suitable for its growth and enterotoxin production than raw milk; improper heating regimen or post-pasteurisation contamination are the main causes for its occurrence (Özer, 1999). *Salmonella* spp. is widespread in the environment and appears in a broad range of dairy products, thus posing a great problem for the industry; almost any milk or milk product may be contaminated with salmonellae as a consequence of mishandling or improper hygiene (Özer, 1999). *Listeria monocytogenes* is the most notorious pathogen associated with cheese-related outbreaks of disease, because its optimum pH range for growth is pH 6-9, and it can grow in the presence of 10% NaCl solution and at 1-5 ºC; although it may be able to survive pasteurisation and grow during refrigerated storage, post-pasteurisation contamination is its main source (Özer, 1999). Burgos cheese is usually held at the dairy premises for 24 h under refrigeration, marketed the day after manufacture and consumed within the next 3-7 days, although the labelled expiring date is usually of 14 days. During early 1980s, a survey on 94 market samples of Burgos cheese showed that most of the samples did not meet the obsolete Spanish microbiological standards (Chavarri et al., 1985); mean counts (log cfu g^{-l}) of 5.83 for staphylococci (25.1% of coagulase-positive strains) and 5.01 for coliforms (16.3% of faecal coliforms) were reported. The authors noted an inadequate storage at retail with an average temperature of 11.4 ºC. Although great advances have been made in food production, packaging, and storage that have enhanced food safety and

reduced or eliminated a number of contamination concerns, two recent surveys conducted by a distribution company (Consumer, 2002, 2007) showed that 1 brand out of 7-8, of traditionally produced Burgos cheese, had coliform counts above 3 log cfu g^{-1}; although these microorganisms are not of particular concern from a safety point of view, they indicate either an insufficient pasteurisation treatment of milk or a broken cold chain during storage.

In conclusion, a part from the sensory characteristics, two main aspects are of major importance for the quality of fresh cheeses, such as Burgos cheese: syneresis or amount of whey expelled during storage and microbiological characteristics. Since the general trend in the commercialisation of such products involves longer shelf-life, both handicaps have to be tackled.

2.1.2. Overcoming microbiological and textural handicaps of starter-free fresh cheeses

- The use of preservatives in commercial cheeses

Owing to the fact that fresh cheeses without additional preservatives can spoil in a matter of days, preservatives are commonly used and regulated. Under the Codex Standard 221-2001 (FAO/WHO, 2001), sorbic acid (E-200) and its potassium (E-202) and calcium (E-203) salts, nisin (E-234), propionic acid (E-280) and its sodium (E-281), calcium (E-282) and potassium (E-283) salts are allowed to be used in the production of fresh cheese. However, the Spanish regulations, i.e., Real Decreto 142/2002 (BOE, 2002) and modifications (BOE, 2004a,b, 2007), are much restrictive and only sorbic acid (E-200) and its salts (E-202 and E-203) are allowed in a maximum dose of 1,000 mg kg^{-1}, individually or together, expressed as sorbic acid. Although, labelling of additives is compulsory under European Directive 2000/13/EC (European Commission, 2000) and Spanish Real Decreto 1334/1999 (BOE, 1999), a recent study on Burgos cheeses commercialised in Spain has shown that many producers infringe the rule by omitting them in the labelling (Consumer, 2007).

Sorbic acid and its potassium, calcium, or sodium salts are collectively known as sorbates. The effectiveness of sorbic acid is greatest in the undissociated

state and as the pH decreases below 6.5 (Davidson et al., 2002). Sorbates inhibit some species of food-related yeasts, e.g., *Brettanomyces* and *Candida*, and moulds, e.g., *Mucor* and *Penicillium*; but, certain species of yeast are more resistant, and some acquire a resistance to sorbates, e.g., *Saccharomyces* (Davidson et al., 2002). The inhibitory effect of sorbates on yeasts and moulds may be lethal as well as static. Moreover, sorbates have been found to inhibit the growth of *Escherichia coli* O157:H7 and *Salmonella* in fresh cheese (Kasrazadeh & Genigeorgis, 1994, 1995). The mechanism by which sorbic acid inhibits microbial growth may partially be due to its effect on enzymes, e.g., inactivation of microbial dehydrogenases involved in fatty acid oxidation (Davidson et al., 2002).

- Ultrafiltration as an alternative cheese-making process

Ultrafiltration (UF) of milk is a concentration process based on membrane technology which removes water, lactose and most water-soluble minerals and vitamins. It is generally conducted at ~50 ºC by running the feed under pressure (less than 1,000 kPa) tangentially across an UF membrane with a molecular weight cut-off of 1-200 kDa. Low molecular weight materials, i.e. water, lactose, soluble minerals and vitamins, pass through the membrane and form the permeate (or ultrafiltrate) stream. The membrane retains the remaining components, i.e., proteins, fat and colloidal salts, and this mass, called retentate (or concentrate), is used for cheese-making. The concentration of the retentate is varied by continually recycling the feed across the membrane until the desired concentration of milk proteins is achieved or by using a very large surface area of membrane, as in large commercial operations.

As a technology for cheese manufacture, UF has been extensively investigated and reviewed (Ernstrom & Anis, 1985; Zall, 1985; Lelievre & Lawrence, 1988; Lawrence, 1989). However, to date, many aspects and drawbacks of the technology have not been yet elucidated (Pouliot, 2008). Since the early studies of 1970s, UF attracted the attention of cheese and equipment manufacturers, not only because of its potential to increase yield through the recovery of whey proteins in the cheese curd, but also due to its potential to reduce production costs and to produce new varieties with different textural and functional

characteristics. Nowadays, UF is been applied in cheese-making technology at three different levels: (a) protein standardisation of milk prior to cheese manufacture, applicable to most cheese varieties, (b) concentration of gelled milk as an alternative to centrifugation in commercial production of fresh acid cheeses, e.g., Quarg, and (c) production of liquid *pre-cheeses* for the manufacture of high-moisture, unripened cheeses, e.g., Feta and Burgos-type cheeses.

Liquid *pre-cheese* refers to the concentrate obtained by UF with a composition close to that of the finished cheese. The pre-cheese concept is based on the process commonly known as the MMV, patented by Maubois, Mocquot and Vassal (1969), which was originally developed for Camembert cheese. This process is continuous and fully mechanised. Milk is usually pasteurised prior to UF mainly for microbial destruction, since bacteria cells remain within the retentate, but also to provoke some protein denaturation. After UF, the obtained retentate is cooled to a temperature not higher than 40 ºC, and dosed with calcium chloride, rennet and salt with a depositor under UV light. Filled containers are sealed and coagulation takes place at ~30 ºC for 45 min.

The retention in cheese of the whole whey protein in its native form produces a theoretical increase of yield in the conversion of milk into cheese for 12-15 % (Mistry, 2002). However, this theoretical increase is less than the one actually found; the suppression of drainage not only obviates the loss of fine particles of casein, but also causes the retention of glycomacropeptide (5 % by weight of milk caseins) in cheese made from ultrafiltered milk. Besides increasing, the yield of cheese by as much as 32 %, the use of the MMV process leads to a radical simplification of the technology (with a corresponding reduction in investment costs) since pre-cheese coagulation takes place directly in the container in which the product is sold. Moreover, since the amount of rennet needed to obtain a given coagulation time is independent of the concentration of proteins in the pre-cheese; a saving of up to 80 % of this enzyme may be achieved (Kosikowski, 1974). However, when UF takes place, colloidal mineral salts are concentrated in the same proportion as proteins, as shown by Brulé et al. (1974). Pre-cheese mineralisation must therefore be adjusted to the

desired value, which is characteristic for each type of cheese, to avoid manufacturing defects due to an increase in buffering power of the cheese substance, such as highly acid and metallic taste frequently associated with bitterness.

Uniformity of quality, composition and, especially of weight makes it possible to considerably reduce safety margins for cheeses sold by the unit. Moreover, UF of cheese milk and renneting the retentate allows the manufacture of curd in such a way that almost no syneresis occurs (Dejmek & Walstra, 2004). However, the chief difficulty is that MMV process provokes radical changes at the cheese texture; UF results in a close and homogeneous texture which has nothing to do with the structured or "curdy textured" cheeses conventionally manufactured.

To conclude, although both the use of preservatives and UF cheeses are generally accepted by consumers, there is an increasing social concern about, on one hand, the possible effects of additives, especially those labelled as E-, and on the other, on the loss of regionality through traditional foodstuff. Hence, the research applied to cheese manufacturing focuses on the development of new technologies or processes which would ensure longer shelf-life without changing the main specific characteristics of the cheese.

2.2. Conventional homogenisation and cheese-making

Conventional homogenisation, developed by Gaulin in 1899, has been widely adopted by the dairy industry. Homogenisation is usually performed at 60 °C with pressures of up to 20 MPa (Wilbey, 2002), and the milk is processed to break milk fat globules into fine lipid droplets, preventing cream separation, thereby increasing stability and shelf life of milk emulsion. Two-stage homogenisation is commonly used, in which the primary stage reduces the size of fat globules and the secondary stage disrupts clusters that may be formed.

Although homogenisation of whole milk has detrimental effects on curd forming properties (Emmons et al., 1980) and curd syneresis (Humbert et al., 1980; Green et al., 1983), it improves rennet action (Humbert et al., 1980;

Robson & Dalgleish, 1984) and increases cheese yield due to better fat recovery (Peters, 1956; Peters & Moore, 1958; Jana & Upadhyay, 1992). These effects will be meticulously described and discussed throughout the thesis since conventional homogenisation will be considered as the main reference treatment. Briefly, homogenisation affects protein structure and causes casein micelles and whey proteins to become associated with the fat globule membrane (Michalski et al., 2002). These interactions between fat and proteins lead to lower curd firmness during rennet coagulation, curd shattering during cutting, and improper curd matting (Peters, 1956; Peters & Moore, 1958; Humbert et al., 1980; Green et al., 1983; Jana & Upadhyay, 1992; Tunick et al., 1993).

2.3. Ultra-high pressure homogenisation

2.3.1. General considerations

In recent years, homogenisation equipment design has been modified to achieve far greater pressures. Although the principle of ultra-high pressure homogenisation (UHPH) is similar to that of conventional ball-and-seat homogenisers, current developments in the design (e.g., the Stansted valve) allow homogenisation at pressures of up to 350 MPa (see 2.3.2. Ultra-high pressure homogenisation equipment).

Applications of high-pressure homogenisation are mainly found in the pharmaceutical and biotechnology sectors where the technique is used to emulsify, disperse, and mix (Floury et al., 2000). However, there has been increasing interest in its application in food technology. Studies of UHPH processing for food applications, namely for producing milk, dairy products and vegetable milks have been recently supported by national or European research projects. As a novel technology, UHPH is actually studied in the food with a view to assess inactivation of microorganisms, enzymes or viruses (Moroni et al. 2002; Vachon, et al., 2002; Diels et al., 2005a,b; Hayes et al., 2005; Picart et al., 2006; Briñez et al., 2006a,b,c), as well as to fragment

particles in dispersions and emulsions (Floury et al., 2000; Keck & Muller, 2006) and has recently been reviewed by Donsì et al. (2009).

Forces encountered during UHPH include cavitation, friction, turbulence, high velocity, and shear (Floury et al., 2004a,b), and result in the heating of the homogenised liquid (Floury et al., 2000; Hayes & Kelly, 2003a; Thiebaud et al., 2003). This technology effectively allows a more efficient particle reduction (than the classical homogenisation) with a concomitant reduction of microbial load (Hayes & Kelly, 2003a; Thiebaud et al., 2003). When UHPH is applied to a fluid containing microorganisms, some of them are inactivated during the compression step, but mostly they are disrupted by the process. When the fluid passes through the narrow gap of the high pressure valve the sudden pressure drop, torsion and shear stresses, turbulence, impingement, cavitation phenomena, shock waves and temperature increase act against bacteria (Middelberg, 1995; Thiebaud et al., 2003; Diels et al., 2005a; Donsì et al., 2009b).

Inactivation is affected by: composition of bacterial cell walls (Middelberg, 1995; Wuytack et al., 2002); shape of the microorganisms (Moroni et al., 2002; Saboya et al., 2003); inlet temperature (Vachon et al., 2002; Diels et al., 2004; Briñez et al., 2006a,b; Donsì et al., 2009b); pressure level and number of passes (Moroni et al., 2002; Vachon et al., 2002; Wuytack et al., 2002, Thiebaud et al., 2003; Diels et al., 2005a; Picart et al., 2006; Donsì et al., 2009b); vicosity of the fluid (Diels et al., 2004; Floury et al., 2004b); initial load (Moroni et al., 2002; Vachon et al., 2002; Diels et al., 2005b); substrate and water activity (Vachon et al., 2002; Diels et al., 2005a; Briñez et al. 2006a,c); presence of inhibitors (Vanini et al., 2004; Diels et al., 2005b; Iucci et al., 2007). Typical inactivation values of resistant pathogenic cells are approximately 3-4 log cfu mL^{-1} for 300 MPa treatments and less resistant cells reach up to 9-log cfu mL^{-1} (Wuytack et al., 2002; Diels et al., 2003, 2004; Briñez et al., 2006a,c). Finally, scarce information is available regarding the presence of sublethally injured cells caused by UHPH treatments or inactivation of spores, being this a subject which deserves to be explored in detail.

Moreover, as will be later discussed, studies with whole and skimmed milk have shown that UHPH produces fine emulsion particles (Hayes & Kelly, 2003a; Thiebaud et al., 2003; Hayes et al., 2005), modifies protein structure and characteristics (Hayes & Kelly, 2003a; Hayes et al., 2005; Sandra & Dalgleish, 2005), and inactivates enzymes (Hayes & Kelly, 2003b; Datta et al., 2005; Pereda et al, 2007), all of which could have indirect effects on the coagulation properties of milk and the microstructural properties of cheese.

2.3.2. Ultra-high pressure homogenisation equipment

Basically, a high-pressure homogeniser consists of a high-pressure generator and a homogenising valve assembly designed for this specific high-pressure application. The processed liquid in any type of homogeniser valve passes under high pressure through a convergent section called the "homogenising gap". Middelberg (1995), Pandolfe (1999) and Miller (2002), reviewed the physical processes responsible for the disruption of fat globules and microorganisms in classical high-pressure homogenisers, e.g., APV-Gaulin and Rannie. In the classical valve design, the fluid is fed axially into the valve seat and then accelerates radially into the small region between the valve and the valve seat (Fig. I.2.1.a). Once the fluid leaves the gap (10-30 μm), it becomes a radial jet that stagnates on an impact ring before leaving the homogeniser at atmospheric pressure (Kleinig & Middelberg, 1997).

Stansted high-pressure homogenisers (Stansted Fluid Power Ltd., Harlow, UK) have modified this configuration, allowing steady working pressures of 400 MPa, from year 2006. Stansted homogenising valves consists of ceramic material, which is known to withstand to ultra-high pressure levels. Moreover, geometry of the valve has been modified compared with a classical one from APVGaulin (Fig. I.2.1.b). The fluid is first fed axially along the mobile part of the valve and then accelerates radially through the narrow gap between the valve and valve seat. The size of the slit (~2.0-2.5 μm) and the resulting stream velocity and the pressure of the liquid ahead of the valve depend on the force acting on the valve piston, which can be adjusted to regulate the homogenising intensity. The pressure drop of the liquid in the valve is called the homogenising pressure (Floury et al., 2004a,b).

Figure I.2.1. Homogenising valves from (a) Manton-Gaulin APV, and (b) Stansted Fluid Power (reproduced with permission from Donsì et al., 2009a).

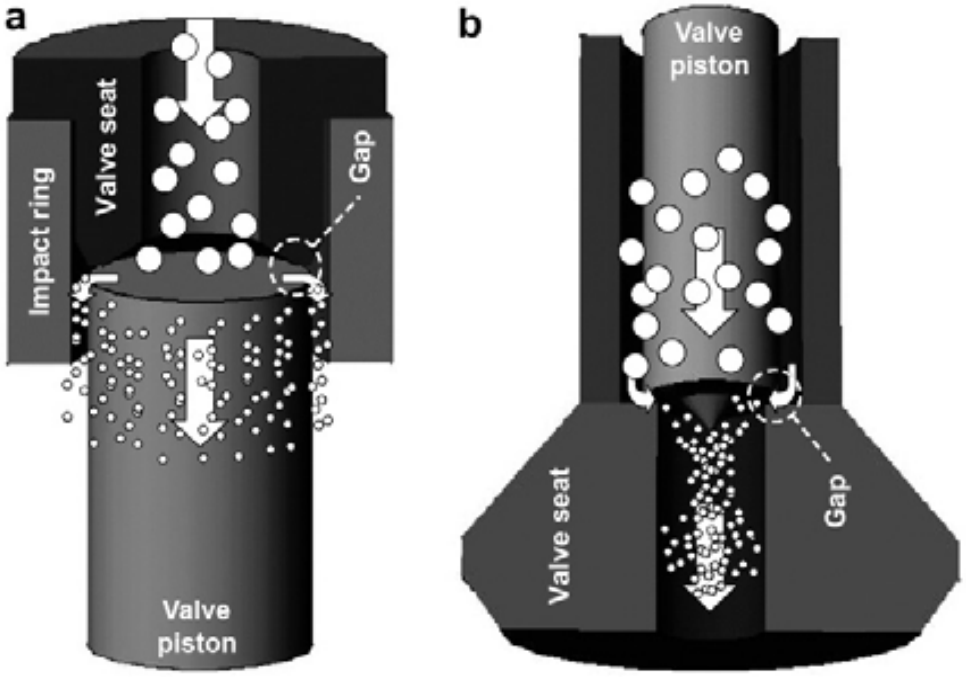
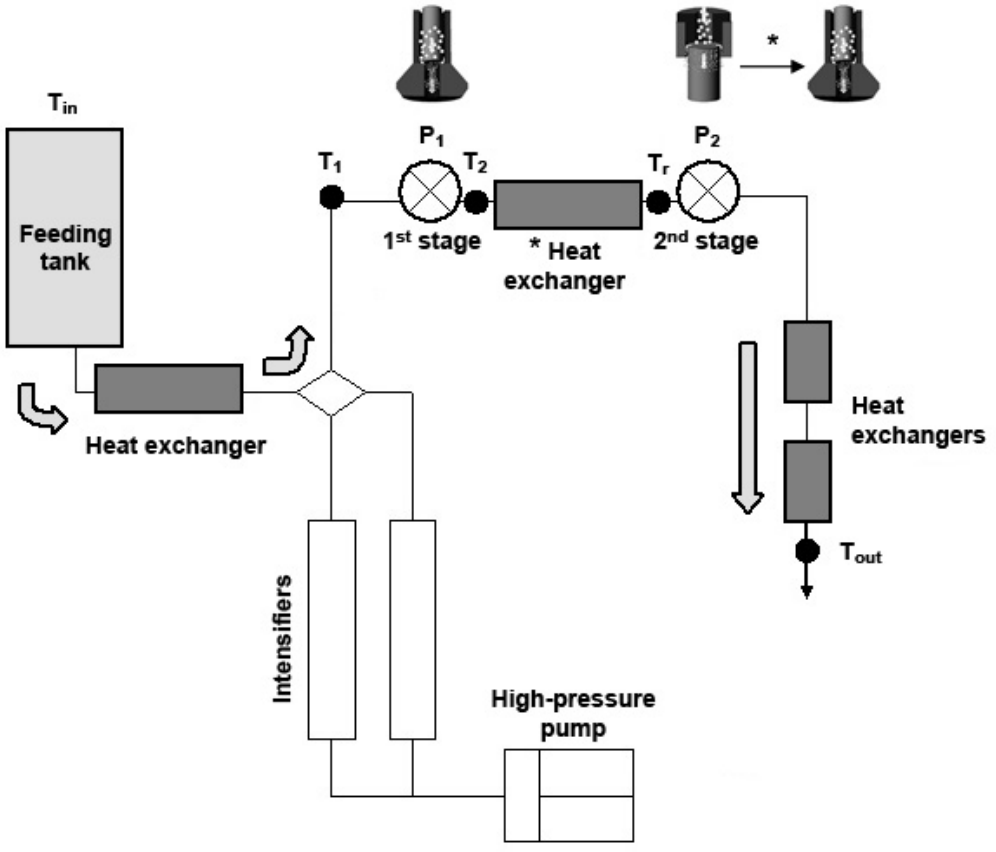


Figure I.2.2. Scheme of the ultra-high pressure homogenisation equipment model FPG11300 (* indicates a modification).



Throughout the present thesis, three different Stansted high-pressure homogenisers were used. The study on the coagulation properties of milk (Section II Chapter 1) was carried out with a first version of the model FPG11300 owned by CERPTA. This homogeniser comprised a high pressure valve made of ceramics able to support 350 MPa and a second pneumatic valve able to support up to 50 MPa located behind the first one (Fig. I.2.2). The high pressure system consisted of two intensifiers driven by a hydraulic pump. The flow rate of milk in the homogeniser was 120 L h^{-1}. Inlet temperature ($\mathbf{T_{in}}$) of milk was kept at the desired temperature by a heat exchanger located behind the feeding tank (Garvía S.A., Barcelona, Spain). Temperature thermocouples and pressure gauges placed at the two valves measured temperature ($\mathbf{T_1}$ and $\mathbf{T_2}$) and pressure changes during processing. In order to minimise temperature retention after treatment, two spiral type heat-exchangers (Garvía S.A.) located behind the second valve were used.

Some upgrades at the homogeniser led to a modified version of the model FPG11300 (Fig. I.2.2). The main difference between the two versions was a replacement of the conventional second-stage valve by a Stansted valve. In addition, a refrigeration system between the two valves was incorporated in order to minimise the heat effect of the treatment, which allowed an instant cooling and a concomitant decrease of the outlet temperature. Finally, although not used in any of the present studies, a tubular system, which could be incorporated to the device when necessary, provided the possibility of increasing temperature retention time of the treatment.

For the study on protein interactions within the curd carried out at the University College of Cork (Section II Chapter 2), the UHPH homogeniser (nm-Gen 7400 H model) consisted of a much smaller device with a flow rate of 300 mL min^{-1}. The primary homogenising valve (Stansted valve) was surrounded by a constant flow of water at ambient temperature to minimise rapid expansions or contractions of this valve. Since this device did not have a cooling system, treated milk was cooled with ice water when collected in receiving containers.

In order to avoid contamination, both cleaning and sanitisation protocols were stipulated. The cleaning protocol, which was fully carried out after and partially before UHPH treatment, consisted in (a) washing out the remaining product with hot water with some pressure (~50 MPa) on the equipment in order to prevent the contraction of metallic parts, which could entrap residual product, (b) cleaning with a hot solution of universal neutral detergent, and (c) a cold solution of enzymatic detergent, both cycled for ~1 h. Disinfectants such as peracetic acid are commonly used on food processing equipments, especially in dairy plants. However, since they are corrosive, sanitisation of the UHPH equipment was mainly performed by running water-vapour into the pipes at downstream of the valve. In addition, in order to avoid cross-contamination, UHPH was always carried out from the highest conditions (pressure and temperature) to the lowest.

2.3.3. Ultra-high pressure homogenisation and milk

Microbial inactivation of milk has been assessed by different authors. Thiebaud et al. (2003) homogenised raw whole bovine milk purchased at a local farm at T_i of 4, 14 and 24 ºC (model FPG7400 H, Stansted Fluid Power Ltd). UHPH treatments at pressures of 200 MPa and inlet temperatures of 24 ºC caused total bacterial count reductions similar to those of pasteurisation processes (30 min at 63 ºC) and almost 3 log cycles after 300 MPa. However, it was observed a shift in the distribution of the population with respect to raw milk. 200 MPa treated samples presented an increased proportion of halotolerant bacteria and decreased psychrotrophic cells. 300 MPa treated samples consisted of heat resistant and halotolerant bacteria. Picart et al. (2006) obtained the same levels of inactivation for total microbial counts. Pereda et al. (2007) also obtained approximately 3 and 4 reduction cycles of total bacterial counts for Ti of 30 and 40 ºC, respectively psychrotrophic and lactococci count presented similar reductions. Coliforms, lactobacilli and enterococci were completely destroyed by UHPH treatment. UHPH milk was granted a refrigerating shelf life of 18 days.

The potential of UHPH as alternative to heat pasteurisation to inactivate foodborne pathogens has been also demonstrated in milk (Diels et al., 2005a;

Vachon et al., 2002; Briñez et al., 2006a,c). About 5 log reductions of *Escherichia coli* were obtained in skim milk at 300 MPa with Ti of 25 ºC; whereas with whole milk under the same conditions, higher inactivation were observed (~8 log cycles) and was speculated to be due to an increase of piezosensitivity of microorganisms induced by fat (Briñez et al., 2006c). *Listeria innocua* was reduced by ~4 log cycles at 300 MPa with Ti of 20 ºC (Briñez et al., 2006a), but *L. innocua* counts increased during storage at 4 ºC (Briñez et al., 2006a) and 37 ºC (Vachon et al., 2002). Complete inactivation of *Salmonella enterica* serotype Enteritidis was reported by Vachon et al. (2002) after 5 homogenisation cycles at 200 MPa.

According to Picart et al. (2006) for T_i of 24 ºC, size distribution of fat globules changed from dimodal (3.31 μm main peak and small shoulder around 1 μm for raw milk) to multimodal (0.63 and 0.16 μm main peaks) after 100 MPa treatment. UHPH treatment induced a progressive decrease at 200 and 250 MPa. Increasing the pressure up to 300 MPa, caused the 0.16 μm peak to increase at the expense of the 0.63 μm one. Fat globules with a diameter <0.36 μm represented 78% and 93% of the total volume at 200 and 300 MPa, respectively. It was also confirmed the presence of very small fat globules of 40-60 nm. No fat aggregates were observed in these experiments. Recycling at 200 MPa decreased the mean diameters and also narrowed the size distribution. Similar findings were reported by Hayes and Kelly (2003a), and Hayes et al. (2005). The use of a second stage (10 % pressure balance of the first valve) could stop or decrease the recoalescence of broken uncoated fat globules (Hayes & Kelly, 2003a). Milk inlet temperatures have a significant effect on the reduction of fat globule size. This was informed by Thiebaud et al. (2003) and Hayes and Kelly (2003a) and confirmed by Datta et al. (2005) who stated that milk inlet temperature is determinant in the resulting globule size.

Skim milk subjected to two-stage UHPH at pressures ~150 MPa (T_i of 5-7 ºC; model 'nm-GEN' 7400H, Stansted Fluid Power Ltd.) showed no change in casein micelle size, and a 5 % decrease at higher pressures, i.e., from 180.75 to 170.65 nm (Hayes & Kelly, 2003a). Sandra and Dalgleish (2005) found no significant change in the average micelle casein size as a result of single-stage

41 MPa treatment pressures (T_i of 25 ºC; Emulsiflex C-5, Avestin, Otawa, Canada), but increasing the pressure to 186 MPa diminished casein micelle size significantly. The number of passes at the same pressure level also caused its size to diminish. According to the authors, UHPH up to 200 MPa seems to partially remove the surfaces of κ- and α_{s1}-caseins, but do not disrupt completely the micelles.

The effect of UHPH treatment on whey milk proteins has been subject of some controversy. Hayes and Kelly (2003a), found no significant denaturation of whey proteins in samples subjected to single or two stage UHPH treatments (T_i of 5-7 ºC, up to 225 MPa). When T_i is raised up to 45 ºC whey protein denaturation was observed, being more extensive the denaturation of β-lactoglobulin (**β-LG**) than that of α-lactalbumin (**α-LA**). Compared to literature values, UHPH denaturation appeared to be higher than thermal alone (Hayes et al., 2005). Datta et al. (2005) confirmed these results and proved that α-LA was not denatured, and for outlet temperatures ≤ 65 ºC, β-LG denaturation was not observed. Pereda et al. (2009) obtained similar levels of α-LA denaturation in UHPH-treated milk at 200 and 300 MPa with T_i of 30 and 40 ºC than in HTST-pasteurised milk. However, UHPH treatments provoked lower levels of β-LG denaturation than HTST treatment (32-37% vs. 47 %).

Dispersions of whey protein isolate containing 6% or 10% (w/w) protein at pH 6.5 were processed using a 15 L h^{-1} UHPH homogeniser with a Stansted valve immediately followed by cooling heat exchangers (Gràcia-Juliá et al., 2008). UHPH did not induce protein aggregation below 225 MPa. Photon correlation spectroscopy (PCS) revealed protein aggregation at 250-300 MPa for both dispersions. PCS (in particle number frequency) indicated a main population of aggregates at 7, 26 or 50 nm, at, respectively, 250, 275 or 300 MPa and 6% protein, while a monomodal distribution at 26 nm was observed at the 3 pressure levels and 10% protein, resulting in controlled protein aggregation without gelation. The effects of mechanical forces predominated over those of short-time heating, since more insoluble protein was found after UHPH of the 6% protein dispersion at 275-300 MPa (maximum temperature 71-74.6 ºC) than after control assays of continuous heating (4 s) at 71-74.6 ºC and atmospheric

pressure. Atomic force microscopy and electrophoresis confirmed protein aggregation at 250 MPa which occurred mainly through hydrophobic interactions.

Concerning indigenous enzymatic activities in milk and UHPH, lactoperoxidase activity was lowered by UHPH treatments (135, 180 and 225 MPa) to 91, 34 or 0% of the activity shown by raw milk samples (Hayes et al., 2005). Datta et al. (2005) found that UHPH causes stronger inactivation than thermal treatments alone (even observed at outlet UHPH temperatures of 60 ºC). Plasmin activity was decreased in samples treated at ≥ 135 MPa. Nevertheless, it seems UHPH-treated milk would always retain a proportion of its original activity for T_i of 5-7 ºC (Hayes & Kelly, 2003b). Picart et al. (2006) observed an increase of alkaline phosphatase activity for T_i of 4 ºC and 200-250 MPa. This corresponded to a maximum temperature of 58 ºC after the high pressure valve, which might help to release enzyme bound to the fat globule membrane or unfold the enzyme and making active sites more accessible to the substrate. Above 200 MPa, inactivation to 6% is mostly due to mechanical forces rather than to thermal effects. Higher lipase activity was observed in UHPH-treated milk samples (240 % of raw milk lipase activity) for outlet temperatures of approximately 57 ºC. UHPH treatment caused complete inactivation of lipase when the outlet temperature was higher than 71 ºC (Datta et al., 2005).

It is important to bear in mind that discrepancies in the obtained results might be due to the fact that Stansted homogenisers are prototypes and can be rather different from one to the other. Hence, the PhD research on milk carried out by Pereda (2008c) with the same UHPH-equipment used in the present thesis (model FPG11300, Stansted Fluid Power Ltd.) will be the main reference work. Briefly, UHPH-treatments at 200-300 MPa were found to be as effective as pasteurisation in reducing the microbial population (Pereda et al., 2006, 2007), but unlike the heat treatment this technology avoided the overheating of the product as shown by thermal indicators (Pereda et al., 2009). In relation to enzymes, the alkaline phosphatase was completely inactivated by UHPH (Pereda et al, 2007). However, lactoperoxidase and plasmin were not completely inactivated under certain UHPH conditions, e.g., 200 MPa with T_i of 30 ºC

(Pereda et al., 2007, 2008b). The residual activity of plasmin together with the reduction of particle size in UHPH-treated milk led to great hydrolysis of casein. Concerning lipolysis and oxidation, milk behaviour depended on the applied pressure. At 200 MPa, lipolysis was enhanced resulting in an increase of free fatty acids during storage and was detected by sensory analysis (Pereda et al., 2008a; Pereda et al., 2008c). In contrast, at 300 MPa no lipolysis phenomena was detected but UHPH resulted in the oxidation of milk (Pereda et al., 2008a). However, the majority of panellists did not perceive oxidation and described UHPH-treated milk at 300 MPa as milk without defects (Pereda et al., 2008c).

2.3.4. Ultra-high pressure homogenisation and cheese

Previous studies on the cheese-making characteristics of UHP-treated milk have focused on the rennet coagulation properties. To date, the effects of UHPH on the manufacture of cheese, in order to evaluate UHPH as an alternative to pasteurisation for cheese production, have not yet been studied.

In the study conducted by Hayes and Kelly (2003a) raw whole milk was subjected to single or two-stage (90% of pressure on the primary homogenising valve and the balance on the secondary valve) UHPH at 50, 100, 150 or 200 MPa (model 'nm-GEN' 7400H, Stansted Fluid Power Ltd.) at Ti of ~5-7 ºC. Gel time, maximum curd firming rate and gel firmness after 2400 s renneting generally decreased, increased or increased, respectively, as homogenisation pressure was increased. The use of the second-stage provoked little differences, with the exception of samples treated at 200 MPa. Samples treated by two-stage UHPH at this pressure had a higher maximum curd firming rate, greater curd firmness at 2400 s and shorter gel time than samples treated by single-stage UHPH at the same pressure.

Sandra and Dalgleish (2007) studied the coagulation properties of heated and non-heated skim milk treated with single-stage UHPH at 179 MPa (±7 MPa), for 6 passes, with Ti of 25 ºC (EmulsiFlex C-5, Avestin). The effects of UHPH on the rennet-induced coagulation of skim milk were small but consistent:

UHPH-treatment provoked a decrease in the gelation time, presumably due to removal of some κ-casein from the surface of the casein micelles.

More recently, Lodaite et al. (2009) focused on the rheological properties of rennet gels from skim and standardised milk (3.6% fat w/w) treated by UHPH at 100, 200 and 300 MPa with a secondary stage pressure of 5 MPa at Ti of 55 ºC (model 'nm-GEN' 7400H, Stansted Fluid Power Ltd.). For both skim and standardised milk samples, UHPH treatment with pressure ≥ 200 MPa, the gel formation time was reduced with increasing homogenising pressure. Independently of fat content, the storage modulus of rennet-induced milk gels increased with increasing pressure; for skim milk, the highest values were observed for samples containing smaller casein particles. UHPH had little effect on the fracture properties of rennet-induced skim milk gels; in contrast, for gels from standardised milk, UHPH provoked higher apparent fracture stress and lower apparent strain at fracture than those of untreated milk samples.

In conclusion, so far, UHPH has been proven to enhance rennet coagulation properties of milk.

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Section I, Chapter 3

Objectives and working plan

3. Objectives and working plan

The present research work is enclosed within the Spanish national project AGL2004-01943 entitled "Aplicación del tratamiento de ultrahomogenización en leches de vaca y cabra para la obtención de quesos frescos y madurados", and included in the European project CRAFT-512626 entitled "Development and optimisation of a continuous ultra-high pressure homogeniser for application on milks and vegetable milks".

3.1. General objective

The general objective of the present research work was to assess the suitability of ultra-high pressure homogenisation as an alternative to conventional processes for the manufacture of starter-free fresh cheeses.

3.2. Specific objectives

In order to achieve the general objective, UHPH-treatment was compared with the conventional processes used in the industry for milk intended to manufacture fresh cheese, i.e., pasteurisation and homogenisation-pasteurisation. The work dealt with the following specific objectives:

- To determine the optimal conditions, i.e., pressure and temperature, of UHPH treatment for cheese milk, by studying the effects of UHPH on the rennet coagulation behaviour of milk.
- To identify changes at protein-protein interactions within rennet gels and the protein composition of the milk fat globules membrane due to UHPH treatment, in order to better understand the overall effect of UHPH on the coagulation properties and cheese-making properties of milk.
- To evaluate the suitability of UHPH-treated milk for the manufacture of fresh cheeses, by producing starter-free fresh cheeses at a pilot-scale under the optimal UHPH conditions previously determined.
- To determine the shelf-life of produced fresh cheeses, by following their microbiological, compositional and biochemical evolution during cold storage.
- To evaluate the texture, rheology, microstructure and colour of produced fresh cheeses and their aptness for consumption through a sensory analysis.

3.3. Working plan

The dissertation is divided into two main parts corresponding to Sections II and III of the present thesis:

- Laboratory experiences including (a) study of milk rennet coagulation properties and other related characteristics (Fig. I.3.1), (b) study on protein-protein interactions within rennet curds (Fig. I.3.2), and (c) study on the protein composition of milk fat globule membrane (Fig. I.3.3).
- Fresh cheese production at pilot-scale and subsequent analyses (Fig. I.3.4) including (a) cheese production, (b) microbiological, (c) compositional, (d) textural, (e) biochemical and (f) sensorial analyses.

Figure I.3.1. Working plan corresponding to the study on rennet coagulation.

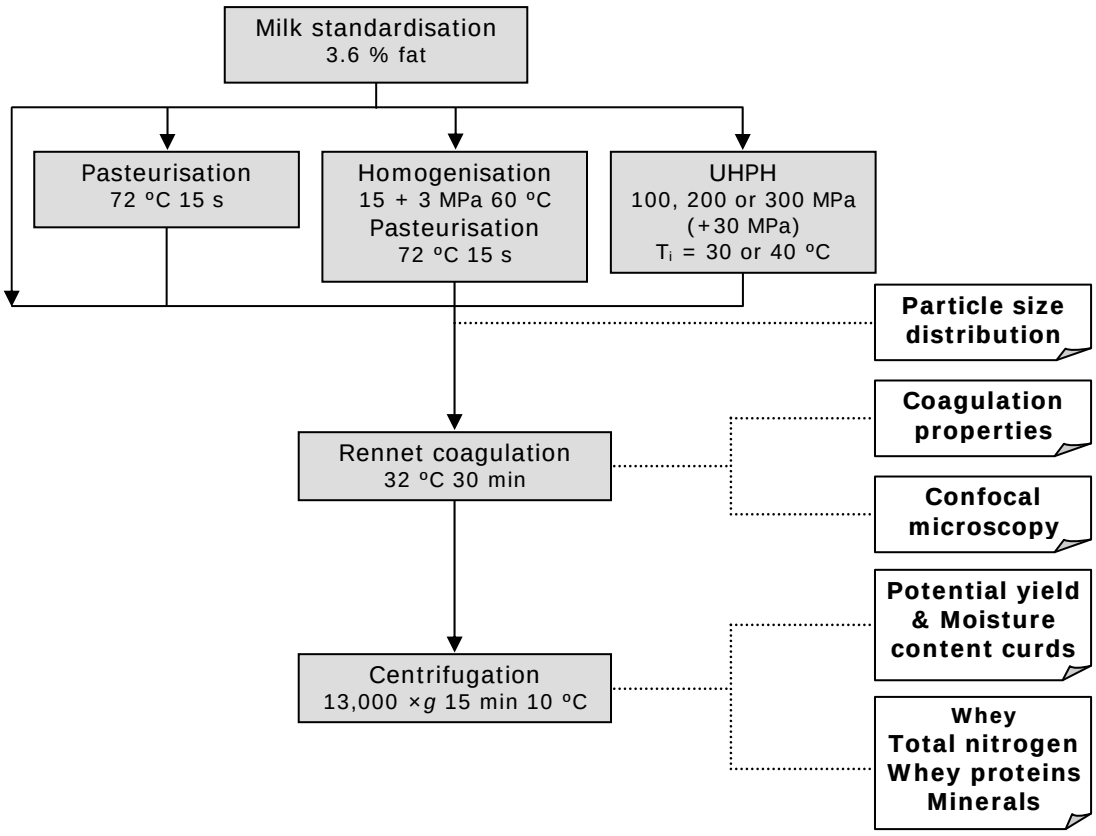


Figure I.3.2. Working plan corresponding to the study on protein interactions within the curds.

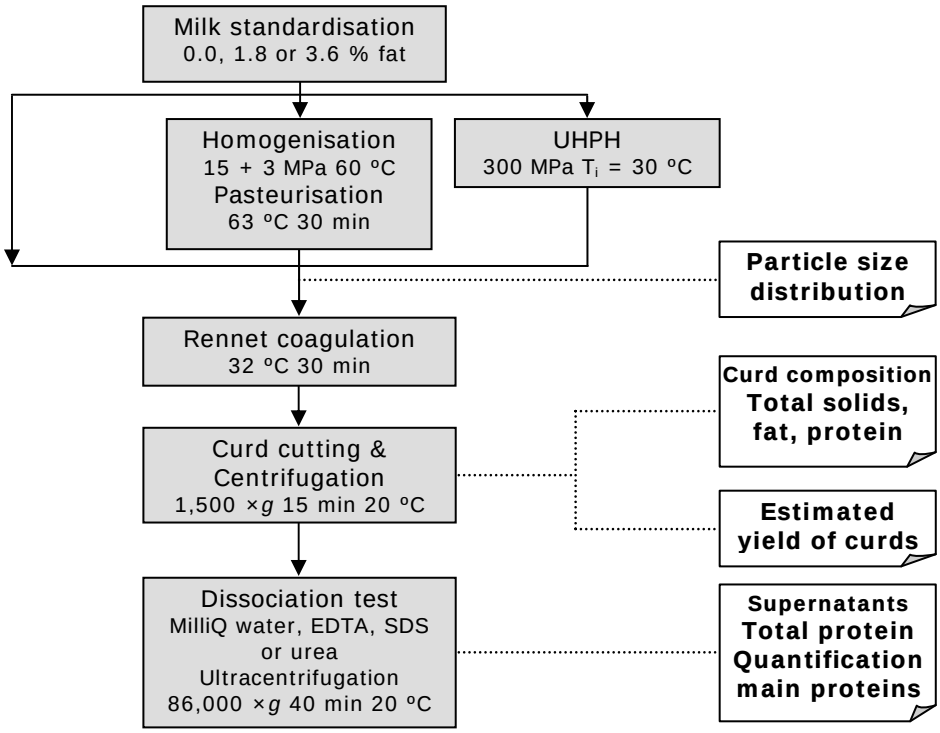


Figure I.3.3. Working plan corresponding to the study on the fat globule membrane (MFGM) composition.

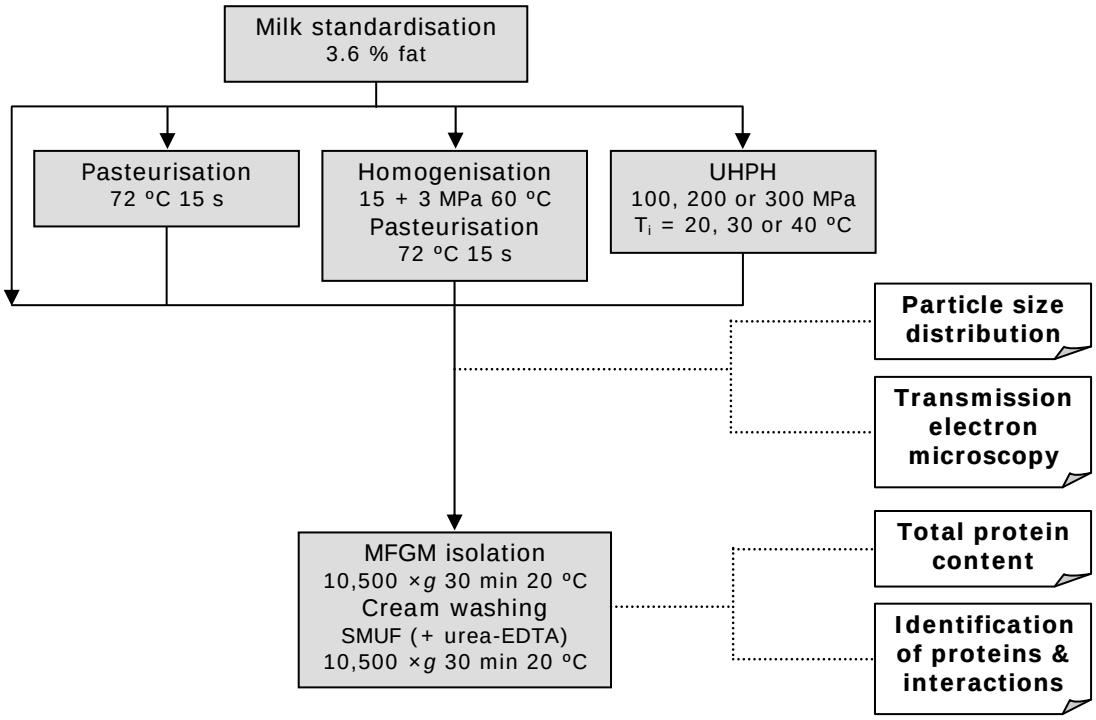
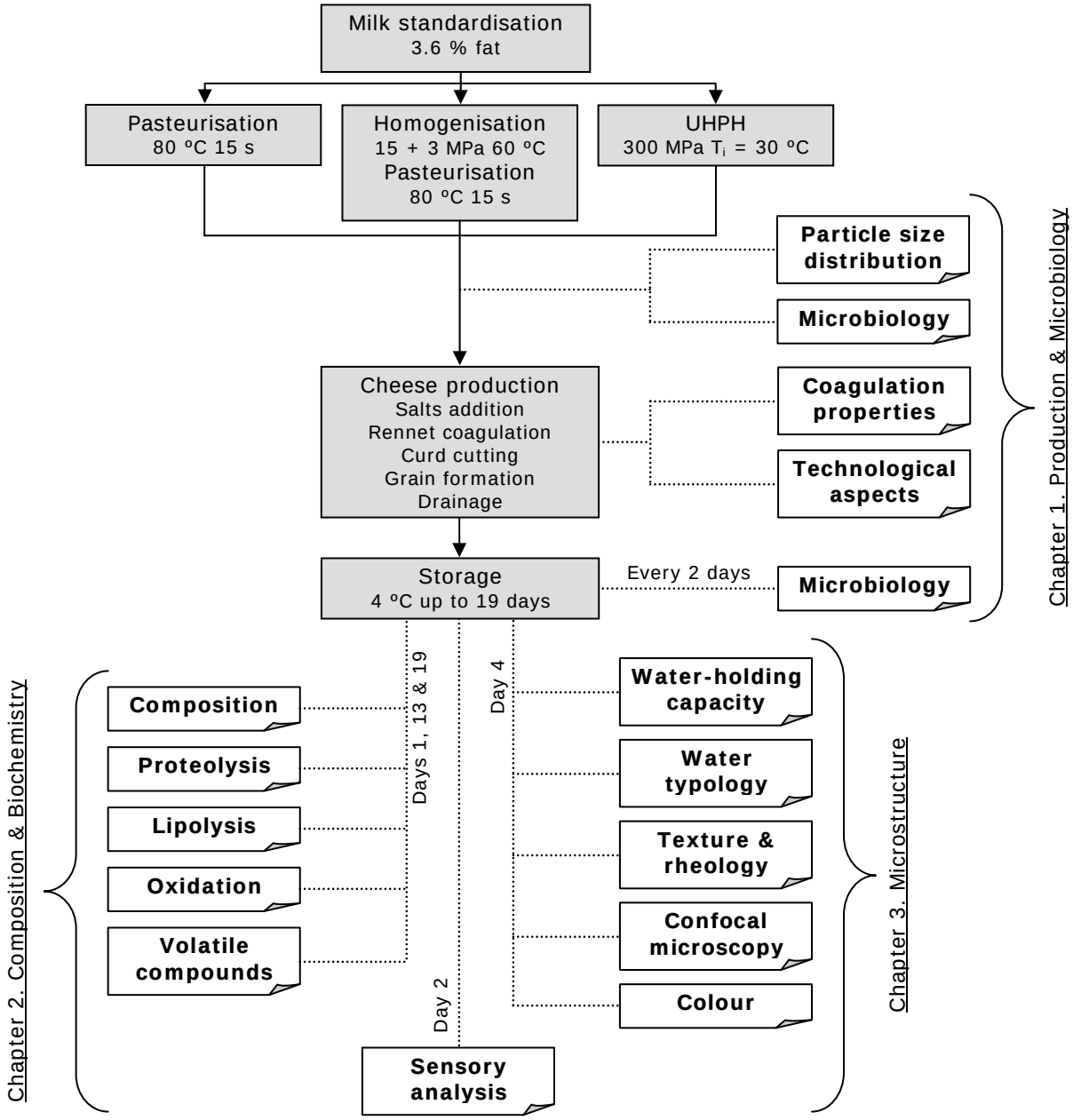


Figure I.3.4. Working plan corresponding to the studies on fresh cheese.



Section II, Chapter 1

Effects of ultra-high pressure homogenisation on the coagulation properties of milk

The effects of single- or two-stage ultra-high pressure homogenisation (100 to 300 MPa) at inlet temperatures of 30 and 40 ºC on the cheese-making properties of bovine milk were investigated. Effects were compared with those from raw, pasteurisation and conventional homogenisation-pasteurisation treatments. Rennet coagulation time, rate of curd firming, curd firmness, wet yield, and moisture content of curds were assessed. Results of particle size and distribution of milk, whey composition, and gel microstructure observed by confocal laser scanning microscopy were analysed to understand the effect of UHPH. Single-stage UHPH at 200 and 300 MPa enhanced rennet coagulation properties. However, these properties were negatively affected by the use of the UHPH secondary stage. Increasing the pressure led to higher yields and moisture content of curds. The improvement in the cheese-making properties of milk by UHPH could be explained by changes to the protein-fat structures due to the combined effect of heat and homogenisation.

1. Effects of ultra-high pressure homogenisation on the coagulation properties of milk

1.1. Introduction

At the present time, data to describe the technological aptitude of milk treated by UHPH are scarce. As previously mentioned in Section I Chapter 2, so far, UHPH has been proven to enhance the coagulation properties of milk (Hayes & Kelly, 2003a; Sandra & Dalgleish, 2007; Lodaite et al., 2009). However, discrepancies between studies have been reported on whey proteins denaturation (Hayes & Kelly, 2003a; Hayes et al., 2005; Datta et al., 2005; Pereda et al., 2009) and enzymes inactivation (Hayes & Kelly, 2003b; Datta et al., 2005; Hayes et al., 2005; Picart et al., 2006; Pereda et al, 2007). Such discrepancies could be attributed to the fact that Stansted UHPH homogenisers are prototypes and can be rather different from one to the other.

The two main goals of the present work was (a) to determine the effect of UHPH treatment on the coagulation properties of milk by comparing this new technology with conventional treatments, and (b) to establish the optimal coagulation conditions for cheese manufacture.

1.2. Material and methods

1.2.1. Supply and treatment of milk

Raw whole bovine milk was obtained from a local dairy farm (S.A.T. Can Badó, Roca del Vallès, Spain). Milk was standardized at 3.5 ± 0.2% (w/v) fat and kept overnight at 4 °C. Before all treatments, the milk was warmed to approximately 20 °C. Ultra-high pressure homogenisation was carried out by subjecting milk to single- or two-stage UHPH (100, 200, and 300 MPa on the primary valve and 30 MPa on the secondary valve) using the non-modified high-pressure homogeniser (see Section I Chapter 2) at inlet temperatures (T_i) of 30 and 40 °C. The milk temperatures measured for the conditions used in this study are shown in Table II.1.1.

Table II.1.1. Temperatures (ºC) reached during ultra-high pressure homogenisation treatments[1].

	Treatment	T_{in}	T_1	T_2	T_{out}
30 ºC	100 MPa	29.6 ± 0.5 ^{c}	33 ± 1 ^{f}	54 ± 3 ^{h}	26 ± 1 ^{d}
	100 + 30 MPa	29.6 ± 0.5 ^{c}	34 ± 1 ^{f}	58 ± 6 ^{g,h}	27 ± 1 ^{d}
	200 MPa	29.6 ± 0.4 ^{c}	37 ± 1 ^{e}	75 ± 3 ^{f}	34 ± 1 ^{a,b}
	200 + 30 MPa	29.6 ± 0.2 ^{c}	37 ± 1 ^{e}	78 ± 7 ^{e,f}	34 ± 1 ^{a,b}
	300 MPa	29.8 ± 0.3 ^{c}	41 ± 1 ^{d}	93 ± 0 ^{b,c}	36 ± 2 ^{a}
	300 + 30 MPa	29.9 ± 0.2 ^{c}	41 ± 1 ^{d}	88 ± 2 ^{c,d}	35 ± 3 ^{a}
40 ºC	100 MPa	39.7 ± 0.3 ^{b}	43 ± 1 ^{c}	62 ± 2 ^{g}	28 ± 3 ^{d}
	100 + 30 MPa	39.8 ± 0.3 ^{b}	43 ± 1 ^{c}	64 ± 7 ^{g}	28 ± 3 ^{c,d}
	200 MPa	39.9 ± 0.4 ^{b}	46 ± 1 ^{b}	84 ± 1 ^{d,e}	33 ± 3 ^{a,b}
	200 + 30 MPa	39.8 ± 0.4 ^{b}	47 ± 1 ^{b}	89 ± 8 ^{c,d}	32 ± 3 ^{a,b,c}
	300 MPa	40.6 ± 0.3 ^{a}	50 ± 1 ^{a}	101 ± 3 ^{a}	28 ± 4 ^{d}
	300 + 30 MPa	40.6 ± 0.1 ^{a}	50 ± 2 ^{a}	98 ± 7 ^{a,b}	30 ± 3 ^{b,c,d}

$^{a-l}$ Mean value ± s.d.; n = 3; values without common superscripts were significantly different ($P < 0.05$).

[1] T_{in}: real inlet temperature; T_1: temperature before the 1st valve; T_2: temperature after the 1st valve; T_{out}: outlet temperature after the cooling system.

UHPH treatments were compared with raw and heat-treated milk samples. Pasteurised milk (72 ºC for 15 s) and homogenised-pasteurised milk (15 + 3 MPa at 60 ºC, 72 ºC for 15 s) were chosen as typical treatments of cheese milk in different cheese varieties (fresh or ripened). Two-stage homogenisation and pasteurisation of raw milk were performed with a Niro Soavi homogeniser (model X68P Matr. 2123, Niro Soavi, Parma, Italy) and a Finamat heat exchanger (model 6500/010, GEA Finnah GmbH, Ahaus, Germany), respectively.

1.2.2. Particle size and distribution

The particle size distribution in milk samples was determined using a Beckman Coulter laser diffraction particle size analyser (LS 13 320 series, Beckman Coulter, Fullerton, CA, USA). Milk samples were diluted in distilled water until an appropriated obscuration was obtained in the diffractometer cell. An optical model based on the Mie theory of light scattering by spherical particles was applied by using the following conditions: real refractive index, 1.471; refractive index of fluid (water), 1.332; imaginary refractive index, 0; pump speed, 21%. The diameter below which 90% of the volume of particles are found (**D_v0.9**), the diameter below which 50% of the volume of particles are found (**D_v0.5**), the volume-weighted mean diameter [**D(4,3)**], and the surface-weighted mean diameter [**D(3,2)**] were determined.

1.2.3. Rennet coagulation properties

Milk was warmed to 32 ºC, and recombinant rennet (chymosin with a declared activity of 180 International Milk Clotting Units mL^{-1}, Maxiren 180, DSM Food Specialties, Seclin Cedex, France) was added at 0.074% (v/v). Coagulation was carried out at 32 ºC for 30 min. Rennet coagulation properties, i.e., rennet coagulation time (**RCT**), rate of curd firming (**RCF**), and curd firmness at 30 min (**CF**), were assessed in triplicate by the Optigraph system (Ysebaert Inc., Frepillon, France). This device passes an infrared beam through a sampling tube containing milk. A sensor on the other side measures the amount of light absorbed by the milk as it coagulates; the changes are analysed in real time by a computer that converts them into directly usable data.

1.2.4. Evaluation of yield and moisture content of curds

The potential yield of cheese curd was estimated in quadruplicate as described by Macheboeuf et al. (1993). Milk samples (270 mL) were warmed to 32 ºC and recombinant rennet (chymosin with a declared activity of 180 International Milk Clotting Units mL^{-1}, Maxiren 180, DSM Food Specialties) at 0.074% (v/v) was added. Portions of the renneted milk samples (30 g) were transferred into centrifuge tubes and allowed to coagulate at 32 ºC for 30 min. The coagulum was centrifuged at 13,000 $\times g$ for 15 min at 10 ºC. Wet yield of curds, expressed as grams of retained curd per one hundred grams of milk, was determined by weighing the obtained pellets.

Curds were analysed in duplicate for total solids (**TS**) content (IDF, 1987) to calculate their moisture content (100 – TS) and the yield of total curd solids (wet yield × TS/100).

1.2.5. Whey composition: total nitrogen, whey proteins, and minerals content

The total nitrogen content of whey was analysed in duplicate by the Dumas combustion method (IDF, 2002). Reversed-phase HPLC analysis of whey proteins in rennet curd whey was performed using an automated system (LCM1, Waters Corporation, Milford, MA, USA). Separations were carried out in

a 250 × 4.6 mm column packed with C8-bonded silica gel with a particle diameter of 5 μm and pore width of 3,000 nm (Tracer Excel, Teknokroma, Sant Cugat del Vallès, Spain) at a constant temperature of 40 ºC, following the method of Resmini et al. (1989). Residual levels of α-lactalbumin (**α-LA**) and β-lactoglobulin (**β-LG**) were measured as total area of the respective peaks.

Calcium, magnesium, and phosphate in rennet whey were determined in triplicate by inductively coupled plasma optical emission spectroscopy with a Perkin-Elmer inductively coupled plasma spectroscopy unit (model 4300, Perkin-Elmer, Shelton, CT) with axial plasma viewing. The spectroscopy operating conditions were as follows: power = 1.3 kW; argon plasma flow rate = 15 L min^{-1}; argon auxiliary flow rate = 0.2 L min^{-1}; argon nebuliser flow rate = 0.74 L min^{-1}; sample uptake rate = 1.5 mL min^{-1}; wavelengths (nm) for Ca, P, and Mg = 317.925, 213.611, and 285.213, respectively. Whey samples of 1 mL were transferred to a 25-mL volumetric flask and nitric acid and deionised water were added to reach a final concentration of 0.2% (v/v) nitric acid. Standard solutions from 1 mg mL^{-1} stock solution of Ca, P, and Mg were used to prepare the calibration curves.

1.2.6. Confocal laser scanning microscopy of rennet gels

Confocal laser scanning microscopy observations were performed in fluorescence mode essentially as Michalski et al. (2002) described. The protein matrix of renneted milk samples was stained by the fluorescent dye, fluorescein isothiocyanate (**FITC**; Fluka, Steinheim, Germany), and the fat globules were stained by Nile red (Sigma, Steinheim, Germany). The FITC and Nile red were dissolved in ethanol at a concentration of 2 and 1 mg mL^{-1}, respectively. Milk samples (10 mL) warmed at 32 ºC were dyed with two drops of FITC and three drops of Nile red. Recombinant rennet (Maxiren 180, DSM Food Specialties) at a concentration of 0.074% (v/v) was added to the dyed milk samples. Then, 3 to 4 drops of the labelled renneted milk samples were transferred to microscope slides with concave cavities, covered with a coverslip, sealed to prevent evaporation, and incubated in a temperature-controlled incubator at 30 ºC for 30 min. The preparations were cooled and kept at 4 ºC for a maximum of 3 h.

The confocal microscope (Leica TCS SP2 AOBS, Heidelberg, Germany) was equipped with an oil-coupled Leica objective with a 63× augmentation and a numerical aperture of 1.4. Fluorescence from the samples was excited with the 488 nm line of an argon laser. Images were acquired in 2 channels simultaneously (501 to 549 nm and 574 to 626 nm) as 1,024 × 1,024 pixel slides in the horizontal *x–y* plane along the *z* plane at constant gain and offset. Three-dimensional images were obtained by the average projection of 4 slides with Leica software.

1.2.7. Statistical analysis

The complete experiment was repeated on 3 independent occasions. Data were processed by multifactor analysis of variance (ANOVA) using the general linear models procedure of Statgraphics (Statgraphics Inc., Chicago, IL, USA), taking into account both treatment and production factors, as well as their interaction except for data on temperatures recorded during UHPH treatment. LSD test was used for comparison of sample data, and evaluations were based on a significance level of $P < 0.05$.

1.3. Results

1.3.1. Particle size and distribution

Four parameters [$D_v0.9$, $D_v0.5$, D(4,3), and D(3,2)] as well as the distribution patterns were taken into consideration to see the effects of UHPH on particle size and distribution (Table II.1.2). The size distribution of particles in raw milk was characterised by a main peak at 3.8 μm and a second lower peak around 0.2 μm, which corresponded to fat globules and casein micelle particles, respectively. Pasteurised milk showed a similar pattern. As expected, the size distribution of homogenised milk samples changed markedly; their main peaks were between 0.1 and 0.3 μm for UHPH-treated milk samples, and approximately 0.4 μm for homogenised-pasteurised milk. Milk samples treated with single-stage UHPH at 300 MPa and T_i of 40 ºC showed two other peaks, lower but much wider at 2 and 20 μm. Samples undergoing two-stage UHPH also showed such distribution pattern at pressures of 300 MPa with both T_i and 200 MPa with T_i of 40 ºC.

Significant differences ($P < 0.05$) between pasteurised and raw milk samples were found for $D_v0.5$ and D(3,2). Homogenised-pasteurised samples showed values between those of pasteurised and raw milk samples, on one side, and those of UHPH-treated milk samples, on the other. At 30 ºC, increasing the pressure of UHPH significantly decreased all 4 parameters, except for milk treated with two-stage UHPH at 300 MPa; in contrast, with T_i of 40 ºC, the lowest values were observed in milk samples treated with single-stage UHPH at 200 MPa. Indeed, samples treated at 300 MPa showed higher $D_v0.9$ values compared with raw milk. Above 100 MPa, the two-stage homogenised samples showed higher $D_v0.5$ and D(3,2) values than their counterparts treated by single-stage UHPH. In addition, two-stage UHPH affected greatly both $D_v0.9$ or D(4,3) at 300 MPa resulting in higher values than those of raw milk, especially with T_i of 40 ºC.

1.3.2. pH and rennet coagulation properties of milk

Both conventional treatments, i.e., pasteurisation and homogenisation-pasteurisation, did not affect the pH of milk. However, the influence of UHPH on the pH highly depended on T_i and the applied pressure; below 300 MPa for T_i of 30 ºC and 200 MPa for T_i of 40 ºC, the values were significantly lower (Table II.1.3).

Rennet coagulation times were very much dependent on the treatment, although generally two-stage homogenisation did not seem to affect it, except for the most extreme treatment at 300 MPa with T_i of 40 ºC (Table II.1.3). Samples treated at 100 MPa had significantly lower RCT than raw milk. In the case of milk treated at pressures ≥ 200 MPa, the effect of UHPH depended on T_i. At 200 MPa with T_i of 30 ºC, RCT were also significantly lower than that of raw milk but similar to those obtained with homogenised-pasteurised milk. Increasing T_i to 40 ºC resulted in an increase of RCT down to values similar to those of raw and pasteurised milk samples. However, temperature increase at 300 MPa provoked a decrease of RCT from values similar to those of raw and pasteurised milk samples down to those of homogenised-pasteurised milk.

Table II.1.2. Particle size (nm) of raw, pasteurised, homogenised-pasteurised, and ultra-high pressure homogenised milk samples[1].

Treatment[2]	$D_v0.5$	$D_v0.9$	D(3,2)	D(4,3)
Raw	3,110 ± 35 [b]	5,071 ± 27 [c]	626 ± 29 [a]	2,904 ± 50 [b]
PA	3,156 ± 12 [a]	5,162 ± 36 [c]	589 ± 14 [b]	2,936 ± 14 [b]
HP	389 ± 5 [c]	1,104 ± 35 [d]	315 ± 12 [c]	504 ± 11 [d]
UH T_i = 30 ºC				
100 MPa	338 ± 3 [d]	804 ± 33 [d,e]	277 ± 8 [d]	444 ± 7 [d,e]
100 + 30 MPa	310 ± 9 [e]	680 ± 10 [d,e,f]	250 ± 11 [e]	403 ± 6 [e,f]
200 MPa	214 ± 25 [h]	430 ± 15 [e,f]	187 ± 22 [i]	246 ± 19 [g,h]
200 + 30 MPa	250 ± 14 [g]	491 ± 13 [e,f]	210 ± 14 [g,h]	307 ± 27 [f,g]
300 MPa	147 ± 4 [j]	292 ± 12 [f]	169 ± 3 [k]	134 ± 5 [h,i]
300 + 30 MPa	254 ± 10 [g]	5,817 ± 354 [b]	215 ± 7 [f,g]	2,013 ± 138 [c]
UH T_i = 40 ºC				
100 MPa	255 ± 5 [f,g]	487 ± 4 [e,f]	206 ± 5 [g,h]	279 ± 4 [g]
100 + 30 MPa	247 ± 4 [g]	505 ± 10 [e,f]	200 ± 3 [h]	335 ± 31 [f,g]
200 MPa	122 ± 2 [k]	232 ± 1 [f]	112 ± 1 [l]	137 ± 1 [i]
200 + 30 MPa	177 ± 8 [i]	453 ± 40 [e,f]	157 ± 6 [j]	470 ± 88 [d,e]
300 MPa	153 ± 1 [j]	451 ± 14 [e,f]	143 ± 1 [k]	2,083 ± 120 [c]
300 + 30 MPa	266 ± 9 [f]	12,226 ± 1,731 [a]	224 ± 6 [f]	3,704 ± 354 [a]

[a-l] Mean value ± s.e.; n = 9; values without common superscripts were significantly different ($P < 0.05$).

[1] $D_v0.5$: diameter below which 50% of the volume of particles are found; $D_v0.9$: diameter below which 90% of the volume of particles are found; D(3,2): surface-weighted mean diameter; D(4,3): volume-weighted mean diameter.

[2] PA: pasteurisation; HP: homogenisation-pasteurisation; UH: ultra-high pressure homogenisation at inlet temperatures (T_i) of 30 and 40 ºC.

Table II.1.3. pH and rennet coagulation properties of raw, pasteurised, homogenised-pasteurised, and ultra-high pressure homogenised milk samples1.

Treatment[2]	pH	RCT (min)	RCF (mA/min)	CF (mA)
Raw	6.75 ± 0.02 [a,b]	7.50 ± 0.12 [a,b,c]	1.35 ± 0.04 [d]	12.74 ± 0.38 [d]
PA	6.77 ± 0.02 [a]	7.48 ± 0.19 [a,b,c]	1.51 ± 0.03 [c]	13.54 ± 0.24 [c]
HP	6.75 ± 0.03 [a,b]	7.08 ± 0.21 [d]	1.08 ± 0.06 [e]	10.25 ± 0.26 [h]
UH T_i = 30 ºC				
100 MPa	6.52 ± 0.08 [f]	5.44 ± 0.27 [g]	1.07 ± 0.10 [e]	11.51 ± 0.71 [g]
100 + 30 MPa	6.51 ± 0.09 [f]	5.18 ± 0.27 [g]	0.77 ± 0.07 [g]	10.32 ± 0.54 [h]
200 MPa	6.68 ± 0.05 [c,d]	6.91 ± 0.29 [d,e]	1.84 ± 0.07 [a]	15.00 ± 0.31 [a]
200 + 30 MPa	6.69 ± 0.03 [b,c]	7.02 ± 0.09 [d]	1.32 ± 0.05 [d]	11.99 ± 0.27 [e,f]
300 MPa	6.75 ± 0.03 [a,b]	7.69 ± 0.09 [a]	1.72 ± 0.02 [b]	14.46 ± 0.16 [b]
300 + 30 MPa	6.74 ± 0.01 [a,b,c]	7.68 ± 0.11 [a]	1.32 ± 0.05 [d]	11.75 ± 0.33 [f,g]
UH T_i = 40 ºC				
100 MPa	6.61 ± 0.04 [e]	6.40 ± 0.16 [f]	1.07 ± 0.05 [e]	10.65 ± 0.43 [h]
100 + 30 MPa	6.62 ± 0.03 [d,e]	6.53 ± 0.24 [e,f]	0.54 ± 0.07 [g]	7.64 ± 0.46 [i]
200 MPa	6.74 ± 0.01 [a,b,c]	7.48 ± 0.23 [a,b,c]	1.81 ± 0.06 [a]	15.41 ± 0.24 [a]
200 + 30 MPa	6.75 ± 0.02 [a,b]	7.22 ± 0.30 [b,c,d]	1.36 ± 0.08 [d]	12.22 ± 0.30 [e]
300 MPa	6.74 ± 0.01 [a,b,c]	7.13 ± 0.29 [c,d]	1.86 ± 0.12 [a]	14.43 ± 0.42 [b]
300 + 30 MPa	6.76 ± 0.02 [a]	7.57 ± 0.11 [a,b]	1.13 ± 0.03 [e]	10.45 ± 0.18 [h]

[a-i] Mean value ± s.e. (for pH, mean value ± s.d.); n = 9 (n = 3); values without common superscripts were significantly different ($P < 0.05$).

[1] RCT: rennet coagulation time; RCF: rate of curd firming; CF: curd firmness.

[2] PA: pasteurisation; HP: homogenisation-pasteurisation; UH: ultra-high pressure homogenisation at inlet temperatures (T_i) of 30 and 40 ºC.

Pasteurisation treatment increased RCF compared with raw milk, but no differences on CF were observed. In contrast, conventional homogenisation-pasteurisation provoked a decrease of both RCF and CF. The effect of UHPH depended mainly on the applied pressure. At 100 MPa, UHPH triggered a decrease of RCF and CF down to values similar to those of homogenised-pasteurised milk samples. However, at pressures ≥ 200 MPa, both RCF and CF were drastically increased. Two-stage homogenisation in all UHPH treatments significantly ($P < 0.05$) diminished both RCF and CF in relation to their homologues treated by single stage.

1.3.3. Curd yield and moisture content

Wet yields and moisture content of the curds obtained from the UHPH-treated milk samples were significantly greater than those of homogenised-pasteurised, pasteurised, and raw milk samples (Table II.1.4). Increasing the pressure from 100 to 300 MPa with T_i of 30 ºC increased wet curd yield by approximately 33-65%, and moisture content by approximately 11-18% compared with raw milk. Below 300 MPa, UHPH treatments with Ti of 40 ºC resulted in higher wet yields and moisture content of curds in relation to their homologue treatments at T_i of 30 ºC.

Conventional pasteurisation and homogenisation-pasteurisation increased the yield of total curd solids by ~15% compared with raw milk (data not shown). Higher increases were obtained with UHPH treatments above 200 MPa at both T_i. The use of the second stage at 200 MPa increased the yield of total curd solids; in contrast, two-stage UHPH provoked lower increases at 100 and 300 MPa than their single-stage homologue treatments.

1.3.4. Whey composition

All treatments significantly ($P < 0.05$) decreased the amount of total N in whey (Table II.1.5). The UHPH samples showed a decrease of approximately 2 to 27% correlated to the increase of pressure. Above 200 MPa, the effect of UHPH was much higher than those of conventional pasteurisation and homogenisation-pasteurisation treatments (data not shown). Increasing T_i to

Table II.1.4. Wet yield and moisture content (%) of curds from raw, pasteurised, homogenised-pasteurised, and ultra-high pressure homogenised milk samples.

Treatment[1]	Wet yield (n = 9)	Moisture content (n = 6)
Raw	20.72 ± 0.13 [o]	65.62 ± 0.66 [k]
PA	22.71 ± 0.95 [n]	63.53 ± 0.48 [l]
HP	27.34 ± 0.49 [m]	70.10 ± 0.20 [j]
UH T_i = 30 ºC		
100 MPa	28.39 ± 0.57 [l]	71.92 ± 0.22 [i]
100 + 30 MPa	29.01 ± 0.51 [k]	73.03 ± 0.15 [h]
200 MPa	31.47 ± 0.76 [h]	74.35 ± 0.24 [e]
200 + 30 MPa	32.76 ± 0.57 [g]	75.10 ± 0.16 [d]
300 MPa	35.35 ± 0.71 [a]	76.42 ± 0.09 [a]
300 + 30 MPa	34.34 ± 0.64 [b]	75.95 ± 0.09 [b]
UH T_i = 40 ºC		
100 MPa	29.94 ± 0.11 [j]	73.43 ± 0.17 [g]
100 + 30 MPa	30.81 ± 0.09 [i]	73.84 ± 0.10 [f]
200 MPa	33.75 ± 0.13 [e]	75.18 ± 0.12 [d]
200 + 30 MPa	34.14 ± 0.11 [c]	75.39 ± 0.27 [d]
300 MPa	33.98 ± 0.11 [d]	75.70 ± 0.17 [c]
300 + 30 MPa	33.40 ± 0.19 [f]	75.66 ± 0.31 [c]

[a-o] Mean value ± s.e.; values without common superscripts were significantly different ($P < 0.05$).

[1] PA: pasteurisation; HP: homogenisation-pasteurisation; UH: ultra-high pressure homogenisation at inlet temperatures (T_i) of 30 and 40 ºC.

Table II.1.5. Differences in nitrogen and mineral content (calcium, phosphate and magnesium) of whey from ultra-high pressure homogenised milk samples (Δ% of raw milk whey).

Treatment[1]	N	Ca	P	Mg
T_i = 30 ºC				
100 MPa	-2.33 ± 0.47 [a]	4.29 ± 0.69 [a,b]	6.19 ± 1.27 [a,b]	1.28 ± 0.81 [a,b]
100 + 30 MPa	-4.11 ± 1.09 [b]	7.42 ± 1.23 [a]	8.37 ± 1.65 [a]	2.13 ± 0.70 [a]
200 MPa	-12.62 ± 2.01 [d]	2.61 ± 0.64 [b]	2.83 ± 1.39 [b,c]	-1.11 ± 0.87 [c,d]
200 + 30 MPa	-18.07 ± 4.52 [e]	0.90 ± 1.48 [b]	1.70 ± 1.04 [c,d]	-0.37 ± 0.44 [b,c]
300 MPa	-21.63 ± 0.49 [g]	-5.98 ± 1.97 [c,d]	-4.69 ± 1.01 [g,h]	-5.36 ± 0.32 [f,g]
300 + 30 MPa	-21.61 ± 1.45 [g]	-4.30 ± 0.42 [c]	-3.56 ± 0.42 [f,g,h]	-5.46 ± 0.64 [g]
T_i = 40 ºC				
100 MPa	-9.21 ± 1.28 [c]	-7.04 ± 1.37 [c,d]	0.75 ± 1.78 [c,d,e]	-0.63 ± 0.79 [b,c]
100 + 30 MPa	-9.85 ± 2.67 [c]	-4.78 ± 1.54 [c]	-1.02 ± 0.67 [d,e,f]	-1.44 ± 0.35 [c,d,e]
200 MPa	-21.99 ± 1.55 [g]	-8.42 ± 0.64 [d,e]	-2.64 ± 1.39 [e,f,g]	-2.74 ± 0.92 [d,e]
200 + 30 MPa	-19.29 ± 2.44 [e,f]	-9.38 ± 1.23 [d,e,f]	-5.74 ± 0.97 [g,h]	-3.35 ± 0.61 [e,f]
300 MPa	-26.56 ± 0.74 [h]	-11.27 ± 0.64 [e,f]	-6.99 ± 0.95 [h]	-6.15 ± 0.58 [g,h]
300 + 30 MPa	-20.58 ± 0.78 [f,g]	-12.97 ± 1.64 [f]	-10.89 ± 1.72 [i]	-7.65 ± 1.34 [h]

[a-i] Mean value ± s.e.; n = 9; values without common superscripts were significantly different ($P < 0.05$).

[1] T_i: inlet temperature of milk.

40 ºC resulted in lower N recovery in relation to their homologue treatments at T_i of 30 ºC. The effect of the second-stage in UHPH depended on T_i. Either a significant decrease or no effect were observed with T_i of 30 ºC; in contrast at pressures ≥ 200 MPa with T_i of 40 ºC, higher N contents were observed for two-stage UHPH treatments compared with their single-stage homologues.

Similar results were observed for β-LG content of whey (Table II.1.6). For α-LA, although no statistical differences were found between whey from raw, pasteurised, and homogenised-pasteurised milk samples, all UHPH treatments showed a significant decrease of α-LA in whey compared with raw milk. Denaturation of β-LG was more important than that of α-LA; the highest levels were obtained at 300 MPa with approximately 35% denaturation for β-LG, and around 12% for α-LA.

Table II.1.6. Residual α-lactalbumin and β-lactoglobulin (measured as total area $\times 10^5$ of the respective peaks) in whey of raw, pasteurised, homogenised-pasteurised, and ultra-high pressure homogenised milk.

Treatment[1]	α-Lactalbumin	β-Lactoglobulin
Raw	41.09 ± 1.55 [a]	107.71 ± 5.99 [a]
PA	40.52 ± 1.52 [a]	102.76 ± 5.66 [c]
HP	39.88 ± 1.50 [a]	94.65 ± 4.82 [d]
UH T_i = 30 ºC		
100 MPa	38.41 ± 0.56 [b]	103.16 ± 3.04 [b,c]
100 + 30 MPa	38.43 ± 0.39 [b]	103.88 ± 4.06 [b]
200 MPa	37.55 ± 0.29 [b,c]	85.65 ± 1.60 [e]
200 + 30 MPa	36.75 ± 0.72 [c,d]	86.25 ± 1.30 [e]
300 MPa	36.08 ± 0.22 [c,d]	70.04 ± 2.07 [f]
300 + 30 MPa	36.86 ± 0.42 [d]	69.28 ± 1.86 [f]

[a-f] Mean value ± s.e.; values without common superscripts were significantly different ($P < 0.05$).

[1] PA: pasteurisation; HP: homogenisation-pasteurisation; UH: ultra-high pressure homogenisation at inlet temperature (T_i) of 30 ºC.

Whey from pasteurised and homogenised-pasteurised milk samples showed lower amounts of Ca and Mg and similar amounts of P compared with raw milk (data not shown). The mineral content of whey from UHPH-treated milk samples markedly depended on T_i (Table II.1.5). At 30 ºC, treatments at 100 MPa increased significantly the amount of the three mineral salts compared to raw milk; in contrast, whey from milk UHPH treated at 300 MPa had lower amounts. Increasing T_i from 30 to 40 ºC lead to lower mineral content in whey especially at pressures ≥ 200 MPa.

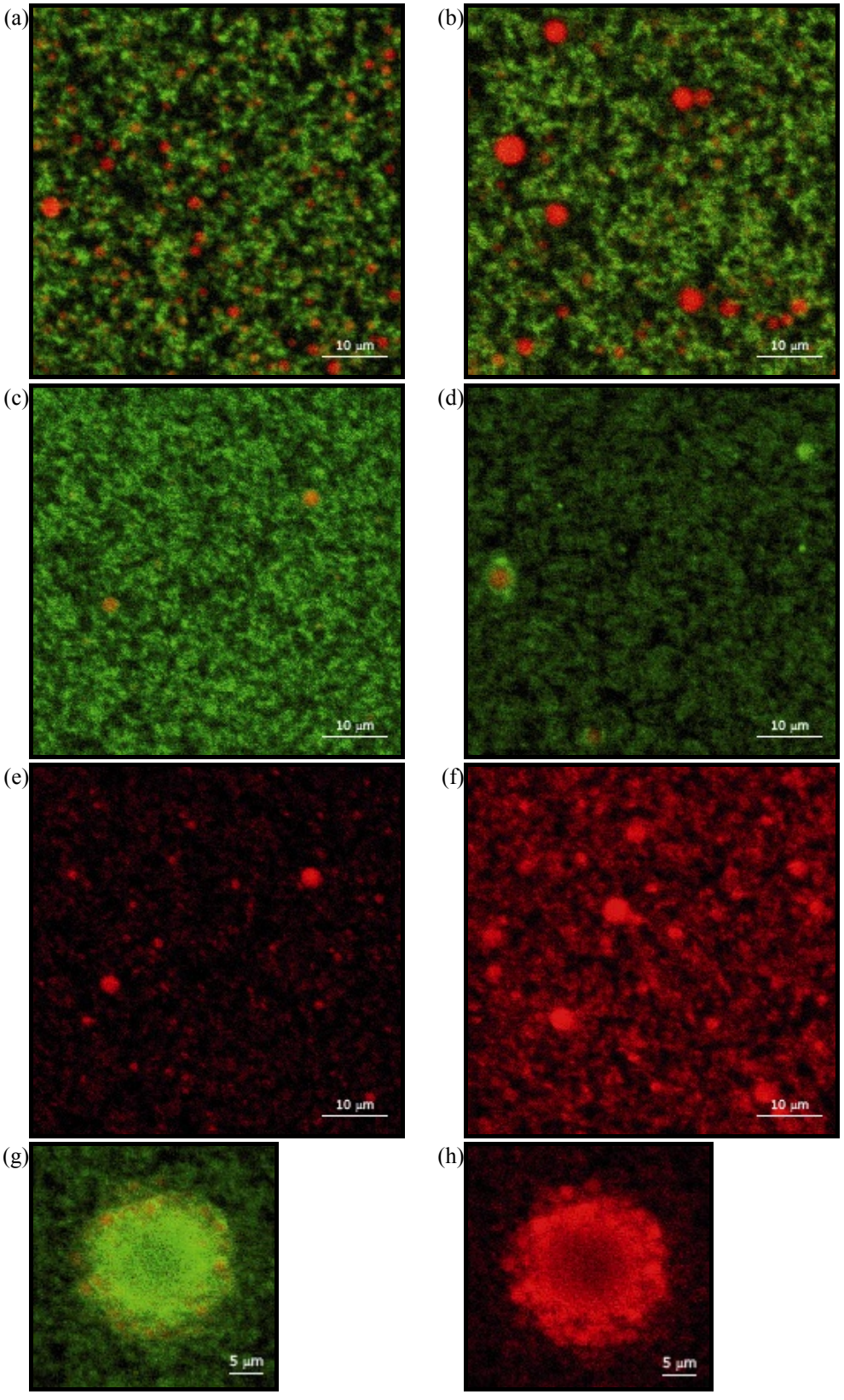
1.3.5. Confocal laser scanning microscopy of rennet gels

Confocal micrographs of rennet gels revealed the existence of visual differences between treatments in the proteinaceous matrix and fat globule size as well as at their interaction (Fig. II.1.1). Rennet gels from pasteurised milk were similar to those obtained from raw milk. The micrographs showed a porous structure of the casein network with native milk fat globules mainly located in the serum pores of the gels (results not shown).

When milk was homogenised-pasteurised, the rennet gels presented open matrices; serum pores were large, irregular, and delimited by thick and lumpy strands. Nile red fluorescence revealed that fat globules had different locations depending on their size (Fig. II.1.1.a). The smallest fat globules (< 0.5 μm) became part of the proteinaceous network, explaining the thickness of the strands. The gels presented many strands that ended with midsized fat globules (~1 μm). Larger fat globules (~1.5 to 2 μm), which accounted for a very small number, were retained in the serum pores.

Although UHPH treatments at 100 MPa with both T_i of 30 and 40 ºC showed a greater amount of smaller fat globules and few larger globules (~4 to 6 μm), the general aspect of the matrix was rather similar to that of homogenised-pasteurised milk samples (Fig. II.1.1.b). The second stage at 100 MPa reduced the size of the largest fat globules (~3 μm). Moreover, the structure of the gel was smoother with smaller pores. However, the smallest fat globules were also embedded within the proteinaceous network.

Figure II.1.1. Confocal laser scanning micrographs of rennet curds from: (a) homogenised-pasteurised milk; ultra-high pressure homogenised milk samples with an inlet temperature of 30 ºC at (b) 100 MPa, (c) 200 MPa, and (d) 300 MPa; Nile red fluorescence (fat) of curds from milk treated by ultra-high pressure homogenisation at (e) 200 MPa, and (f) 200 + 30 MPa; aggregates of fat globules died with (g) Nile red and FITC, and (h) Nile red.



Micrographs of gels from single-stage treatments above 200 MPa showed that Nile red fluorescence at the level of the proteinaceous network was markedly weaker (Figs. II.1.1.c & d). Rennet gels from milk treated at 200 MPa revealed tight matrices. It should be mentioned that micrographs from UHPH-treated milk at 300 MPa not only had lower levels of Nile red fluorescence but also lower FITC fluorescence (Fig. II.1.1.d). The second stage above 200 MPa provoked the coating of midsized fat globules; this phenomenon was more visible as the pressure at the first valve was increased. Moreover, the proteinaceous matrices, which were more lax than those of rennet gels from single-stage treated milk samples, were strongly stained by Nile red (Figs. II.1.1.e & f). Two-stage UHPH treatment at 300 MPa, especially with T_i of 40 ºC, provoked the formation of spherical protein aggregates surrounding a large number of non-coated, midsized fat globules (~2 μm; Figs. II.1.1.g & h).

1.4. Discussion

1.4.1. Effects of heat and conventional homogenisation treatments

Mild heat treatments are considered to have no or little effect on the whey proteins of milk, although there are reports that heat pasteurisation (72 °C for 20 s or 73 °C for 15 s) could cause denaturation of approximately 7% of the whey protein fraction of milk (Jelen & Rattray, 1995). Our results showed that pasteurisation heat treatment of 72 °C for 15 s was sufficient to reduce ~5% of the total N of whey, with levels of residual β-LG and α-LA being ~5 and 1.4% lower, respectively, in whey from pasteurised milk compared with untreated milk.

Furthermore, heating has a marked effect on the milk salts equilibrium and their interaction with casein. It is generally agreed that heating leads to a decrease in diffusible calcium and inorganic phosphate, due to precipitation of calcium phosphate, which may be reversed depending on the intensity of the treatment (Gaucheron, 2005). Under our experimental conditions, the decrease of Ca, P, and Mg in whey from pasteurised milk suggests a mineral transfer from soluble to colloidal phase of milk.

Milk pasteurisation has only minor effects on the formation and physical properties of rennet-induced milk gels (Lucey, 1995). In the present study, pasteurised milk had no significantly different RCT and CF in relation to untreated milk. However, more severe heating conditions impair renneting milk properties (Dalgleish & Banks, 1991; Guinee et al., 1996, 1997; Singh & Waungana, 2001). The causes have been broadly investigated even though they are not yet fully understood. Both the enzymatic and non-enzymatic phases of rennet clotting are delayed and the RCT is longer than that of unheated milk. The strength of renneted milk gels is also adversely affected in heated milk. It has been established that when heated, β-LG and κ-casein form a complex by sulfydryl-disulfide interchange at the micelle surfaces that reduces the accessibility of the rennet to the κ-casein and provides steric hindrance to close approach and fusion of paracasein micelles. Moreover, heat induces the deposition of calcium phosphate and the consequent reduction in native calcium phosphate, which is important for cross-linking paracasein micelles.

Incorporation of denatured whey protein in the curd from pasteurised milk did not increase the moisture content of the curd compared with raw milk. However, the yield of TS of the curd from pasteurised milk was ~15% higher than that from raw milk, which is probably due to the incorporation of denatured whey proteins into the curd. According to Lau et al. (1990) pasteurisation (63 °C for 30 min) has little effect on fat recovery in cheese but N recovery is improved, and approximately 5% of the whey proteins are associated with casein micelles after pasteurisation, resulting in an increased theoretical cheese yield.

During conventional homogenisation, the fat globule size is reduced, the fat surface area increases markedly, and a new adsorbed layer consisting of milk proteins (mainly casein micelles and casein subunits and whey proteins) is formed around the fat globules (Cano-Ruiz & Richter, 1997). The results of the present study showed a marked reduction of both volume- and surface-weighted mean diameters, from 2.9 to 0.5 μm and 0.6 to 0.3 μm, respectively.

It has been reported that homogenisation processes do not affect the distribution of calcium in milk (Robson & Dalgleish, 1984). In our study, the

amount of Ca, P, and Mg in whey of homogenised-pasteurised milk samples did not differ from those of pasteurised milk samples.

Homogenised-pasteurised milk samples presented lower RCT than raw and pasteurised milk samples, results that have also been observed by other authors (Robson & Dalgleish, 1984; Ghosh et al., 1994; Guinee et al., 1997). The lower RCT of homogenised milk samples could be explained by the fact that most κ-casein is located on the micelle surface. As the casein enrobes the fat globules, the κ-casein level is effectively diluted and a smaller critical level of κ-casein hydrolysis is required to start coagulation (Guinee et al., 1997). Furthermore, homogenisation increases the surface area of casein by a spreading process, κ-casein being more available for chymosin action, and thus, reducing RCT (Ghosh et al., 1994).

In the current study, the CF of homogenised milk samples was reduced by approximately 24% compared with untreated milk. The weaker gels from homogenised-pasteurised milk samples could be attributed, according to different authors (Humbert et al., 1980; Robson & Dalgleish, 1984; Ghosh et al., 1994), to a greater dispersion of fat in the curd, to a reduced number of casein particles available to form a strong network because some of the casein is tied to the surface of the new formed fat globules, or to the small fat globules that are entrapped in the gel disrupting the continuity of gel structure and acting as weak centers in the gel. Confocal laser scanning microscopy revealed fat globules embedded within the protein matrix resulting in thick and lumpy strands and a concomitant coarser texture.

Compared with single pasteurisation, the homogenisation-pasteurisation treatment produced higher ($P < 0.05$) amounts of denatured β-LG and significantly increased the moisture content of the curd. Homogenisation of milk resulting in slower whey drainage of the curd has been observed by several researchers (Humbert et al., 1980; Green et al., 1983; Ghosh et al., 1994). The effects of homogenisation on the moisture content of the curd have been attributed to the higher incorporation of denatured β-LG and the alteration in the protein-fat structure of the curd.

1.4.2. Effects of UHPH treatments

In accordance with Hayes and Kelly (2003a), smaller fat globules were obtained by two-stage UHPH at pressures below 200 MPa. Thus, the second valve would act as the secondary stage of a normal homogeniser; that is, stopping or decreasing coalescence. Many studies have shown that above a critical pressure, there is an increased susceptibility of fat globule coalescence (Floury et al., 2000, 2004; Desrumaux & Marcand, 2002). Above 200 MPa, the secondary stage not only increased the average size of particles but also widened the distribution; that is, higher heterogeneity, compared with single-stage homogenisation. This may be due to partial agglomeration of very small, insufficiently coated globules that collide within the second valve. Thiebaud et al. (2003) detected very small fat globules (40 to 60 nm droplets) in single-stage UHPH-treated milk at 200 and 300 MPa, and the impact forces that act on the droplets as the result of a collision have been determined as sufficient to cause disruption of the interfacial membranes (Floury et al., 2000). A broadening of the size distributions was observed for single-stage UHPH of warmed milk (Thiebaud et al., 2003), and model oil-in-water emulsions (Floury et al., 2000) at 300 MPa. The formation of large particles was attributed to unfolding and aggregation of whey proteins of the newly created droplets. In our case, two-stage UHPH-treated milk samples at 300 MPa showed much broader size distributions. Confocal laser scanning microscopy of rennet gels revealed that this phenomenon was due to the aggregation of well-defined small fat globules within dense proteinaceous structures (Figs. II.1.1.g & h).

Milk samples UHPH-treated below 200 MPa showed similar gel strength compared with homogenised-pasteurised milk. In fact, the protein matrices of the rennet gels observed by confocal microscopy were very similar to those of homogenised-pasteurised gels; that is, thick and lumpy strands giving a rough texture to the matrix (Figs. II.1.1.a & b). However, their coagulation times were lower than those of homogenised-pasteurised milk samples. This decrease could be attributed to the lower pH of UHPH-treated milk samples, which would enhance chymosin performance. At these pressures, the temperature

during processing never exceeded 60 °C. Thus, the decrease in pH could be attributed to the action of residual indigenous lipoprotein lipase after UHPH treatment. Treatment with UHPH increased the interfacial fat surface by decreasing the average size of particles, which would lead to a greater potential for lipolysis to occur (Hayes & Kelly, 2003a; Hayes et al., 2005).

Mineral equilibrium in milk is very dependent on physicochemical parameters; that is, pH and temperature. The distribution of ions between the different fractions of milk (diffusible and non-diffusible) is defined by the balance between these factors. The pH of milk samples treated at 100 MPa, especially with T_i of 30 ºC, could be, to some extent, responsible for the higher amounts of minerals in their whey. As pH decreases, the acido-basic groups of milk constituents become more protonated; hence, micellar calcium phosphate and the small amount of magnesium associated to casein micelles are dissolved (Gaucheron, 2005).

Single-stage UHPH above 200 MPa produced the smallest particles with the narrowest distributions, except for 300 MPa with T_i of 40 ºC. A further reduction of fat globule size and the increase in interfacial fat surface would lead to a higher adsorption of casein and whey proteins to the newly formed fat globules. Sandra and Dalgleish (2005) reported a decreased micelle average size in skimmed milk by increasing UHPH pressure. They suggested that UHPH would not cause complete disruption of the casein micelles but rather dissociate parts of their surfaces. The obtained particle distributions corroborated that casein micelle fragments, rather than intact casein micelles, would surround fat globules (Hayes et al., 2005). Thus, very small fat globules would behave as casein micelles rather than embedded fat globules observed in normal homogenisation or lower UHPH pressures. Such structures could enhance gel firmness and rate of aggregation by increasing the amount of particle associations; hence, leading to the higher RCF and CF values observed for milk UHPH-treated at 200 and 300 MPa. Confocal micrographs of rennet milk samples treated at 200 and 300 MPa showed lower levels of Nile red fluorescence at the level of the proteinaceous network (Figs. II.1.1.c & d). This

could be explained by the fact that more than 50% of their particles were beyond the resolution threshold (0.23 μm $pixel^{-1}$).

In early studies on UHPH, no denaturation of whey proteins was reported (Hayes and Kelly, 2003a; Sandra & Dalgleish, 2005). However, the temperature of the process in these studies never exceeded 55 °C. Our temperature values during UHPH treatments were much higher (from ~55 to 95 °C), presumably because of different experimental designs, i.e., a much higher flow rate and relatively larger volumes of milk being processed. If only heat effect is considered, at ~65 °C, whey proteins start to denature and interact with casein micelles (Singh, 1993). However, in UHPH, simultaneous heating and homogenisation processes exist. In fact, Hayes et al. (2005), treating warmed milk up to 250 MPa that reached 83.6 °C, suggested that the physical forces experienced by whole milk during UHPH also denatured β-LG. Our results showed that the amount of denatured β-LG was much greater (17%) for UHPH-treated at 200 MPa with T_i of 30 ºC, which reached approximately 75 °C for a very short time (~0.7 s), than for pasteurised milk at 72 °C for 15 s. Such results corroborate the idea that not only heat but also homogenisation forces induce the denaturation of whey proteins. Increasing the pressure led to higher recovery of N in curd with lower levels of residual β-LG and α-LA in the whey.

As previously mentioned, both the pH of the milk and the temperature reached during the treatment affected the mineral equilibrium. Moreover, UHPH produces partial disruption of casein micelles (Sandra & Dalgleish, 2005) that could lead to a transfer of calcium and inorganic phosphate from the micellar to the diffusible fraction. The balance between these factors could explain the differences observed between the treatments at 200 and 300 MPa. The fact that whey from milk treated at 200 MPa (T_i of 30 ºC) showed higher amounts of calcium than whey from homogenised-pasteurised milk could be explained by both the release due to disruption of casein micelles and its slightly lower pH value. In contrast, the amount of minerals in whey from milk UHPH-treated at 300 MPa, especially with T_i of 40 ºC, which was lower than those of the other

treatments, suggests a mineral transfer from soluble to colloidal phase due to heat during UHPH treatment.

As pressure was increased, the RCT of milk samples was prolonged. The differences between RCT at 200 and 300 MPa could be explained by the relative effect of the following factors: 1) the spreading of κ-casein; that is, higher availability and lower critical level for chymosin action; 2) denaturation of β-LG; that is, steric hindrance; 3) the pH of milk; and 4) changes in the concentration of calcium between soluble and colloidal phases.

Increasing UHPH pressure led to a lower recovery of N with lower levels of residual β-LG and α-LA in whey, and higher TS yield and moisture content of curds. The observed differences between treatments could be explained by variations in 1) the association of denatured whey proteins to the surface of casein micelles, 2) the reduction of fat globule size, 3) the incorporation of denatured whey proteins and casein micelle fragments at the fat globule membrane, and 4) the microstructure of the resulting gels. The association of whey proteins at the micelle surface by heat sterically impedes the fusion of rennet-altered micelles resulting in less shrinkage of the paracasein network (Singh & Waungana, 2001). Moreover, the incorporation of denatured whey proteins into the gel matrix increases the water-binding capacity of the paracasein-whey network (Singh & Waungana, 2001). The reduction of fat globule size implies a dispersion of fat into an increased number of smaller globules. The newly built surfaces are modified by the presence of adhering casein particles and become part of the paracasein network, thus hindering shrinkage of the network (Walstra et al., 1985). The water-holding capacity of curds is directly linked to the microstructure of the gels; that is, porosity or permeability (Green et al., 1983; Walstra et al., 1985; Lucey et al., 2001). Green et al. (1983) observed that curds from conventionally homogenised milk had a less coarse protein network, which retained moisture more effectively than curds from non-homogenised milk samples. Greater firmness, attributed to greater protein content and cross-linking of casein by denatured whey proteins, leads to higher volume of the network relative to that of the interstices, and

thus a reduction of the relative ease of movement of the strands in the protein network (Green et al., 1983; Lucey et al., 2001).

As already stated, two-stage UHPH above 200 MPa led to greater average particle size and higher heterogeneity than single-stage treatments. The obtained rennet gels showed similar firmness to those of homogenised-pasteurised and 100 MPa UHPH-treated milk samples. Confocal microscopy revealed a higher number of fat globules embedded within the proteinaceous matrix giving a rougher texture to the gels than in single-stage UHPH (Figs. II.1.1.e & f). These results corroborate the hypothesis that 1) embedded fat globules, which lead to thicker strands and a concomitant coarser matrix, are responsible for weaker gels, and 2) the presence of very small fat globules behaving as casein micelles results in stronger rennet gels.

1.5. Conclusions

The results of this study show that UHPH treatment of milk reduced fat globule size, increased the wet yield of curd and its moisture content, and decreased the protein content of whey. The rennet coagulation properties were enhanced by single-stage UHPH at 200 and 300 MPa. However, taking curd firmness into account, the application of a secondary stage produced weaker gels similar to those obtained by conventional homogenisation-pasteurisation. The improvement of cheese-making properties of milk by UHPH could be attributed to the combined effect of homogenisation (i.e., reduction of particle size) and heat (i.e., denaturation of whey proteins) on the protein-fat structures of the milk.

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Section II, Chapter 2

Effect of fat content and homogenisation under conventional or ultra-high-pressure conditions on interactions between proteins in rennet curds

Interactions between proteins within drained rennet curds were studied by measuring the dissociating capacity of different chemical agents as affected by two factors: fat content of milk (0.0, 1.8 or 3.6%) and technological treatment (conventional or ultra-high pressure homogenisation, UHPH). Increasing fat content of raw milk increased levels of unbound whey proteins and calcium-bonded caseins in curds; in contrast, hydrophobic interactions and hydrogen bonds were inhibited. Both homogenisation treatments enhanced the incorporation of unbound whey proteins in the curd, and of caseins through ionic bonds involving calcium salts; however, UHPH increased the amount of unbound caseins. Conventional homogenisation-pasteurisation also enhanced interactions between caseins through hydrogen bonds and hydrophobic interactions; in contrast, UHPH impaired hydrogen bonding, and led to the incorporation of both whey proteins and caseins through hydrophobic interactions. Both homogenisation treatments provoked changes in the protein interactions within rennet curds; however, the nature of the changes depended on the homogenisation conditions.

2. Effect of fat content and homogenisation under conventional or ultra-high-pressure conditions on interactions between proteins in rennet curds

2.1. Introduction

Homogenisation of milk reduces the size of fat globules and increases the moisture content of cheese and cheese yield, due to increased fat recovery (Peters, 1956; Jana & Upadhyay, 1992). However, homogenisation also affects protein structure and causes casein micelles and whey proteins to become associated with the fat globule membrane (Michalski et al., 2002). These interactions between fat and proteins lead to lower curd firmness during rennet coagulation, curd shattering during cutting, and improper curd matting (Peters, 1956; Green et al., 1983; Jana & Upadhyay, 1992). It has been reported that UHPH also affects the coagulation properties of milk; however, conflicting results have been obtained depending on both the applied pressure and the milk fat content (see Section I Chapter 2). In general single-stage UHPH enhances rennet coagulation properties by increasing the rate of curd-firming resulting in a concomitant increase of gel strength (Hayes & Kelly, 2003; Sandra & Dalgleish, 2007; Lodaite et al., 2009; see Section II Chapter 1). The improvement of cheese-making properties of milk by UHPH has been attributed to the combined effect of homogenisation (i.e., reduction of particle size) and heat (i.e., denaturation of whey proteins) on the protein-fat structures of the milk (see Section II Chapter 1).

One strategy for understanding the interactions between proteins within cheese curd involves using different chemical dissociating agents which disrupt specific types of bond or interaction (Lefebvre-Cases et al., 1998). For example, hydrophobic interactions and hydrogen bonds can be disrupted by sodium dodecyl sulfate (**SDS**) and urea, respectively, while ionic bonds involving calcium salts are broken by the chelating effect of ethylenediamine-tetraacetic acid (**EDTA**). The amount of proteins dissociated by dispersing curds in the presence of the appropriate agent, followed by ultracentrifugation, indicates the action of the agent on the network, and thus the presence and relative importance of specific interactions. Subsequent identification of

dissociated proteins by electrophoresis can identify which proteins are involved in each type of interaction.

The aim of the current study was thus to identify the differences in the protein-protein interactions in curds from conventionally homogenised-pasteurised and UHPH-treated milk, with different fat contents, compared to those produced from raw milk.

2.2. Material and methods

2.2.1. Milk supply and treatments

Raw whole milk was obtained from a local dairy farm and skimmed at pilot-scale (final fat content of 0.05% ± 0.01 w/v); fat contents were determined using a Milkoscan FT 120 (IDF, 2000; Foss Electric, Hilerod, Denmark). Skimmed and whole milk were then mixed for standardisation to fat contents of 1.8 ± 0.1% w/v and 3.6 ± 0.1% w/v. Conventional treatment consisted of two-stage homogenisation at 60 ºC (15 MPa first stage, 3 MPa second stage; Model APV 1000, APV homogenisers AS, Albertslund, Denmark) followed by batch pasteurisation (63 ºC for 30 min). UHPH treatment was done by a single-stage process at 300 MPa (nm-Gen 7400 H model, Stansted Fluid Power Ltd., Harlow, UK) at an inlet temperature of 30 ºC. Milk samples were immediately cooled to 4 ºC in iced water. Skimmed and standardised raw milk samples were also collected for further analysis.

2.2.2. Particle size distribution of milk samples

The particle size distribution in milk samples was determined by light-scattering using a Mastersizer model S (Malvern Instruments, Malvern, UK), equipped with a 3000F (reverse Fourier) lens and a He-Ne laser (λ of 633 nm). Milk samples were diluted in deionised water by stirring in a sample-dispersion unit. The polydisperse optical model was applied by using the following conditions: real refractive index of 1.46, refractive index of fluid (water) of 1.33, and imaginary refractive index of 0.00. Volume- [**D(4,3)**] and surface-[**D(3,2)**] weighted mean diameters were determined.

2.2.3. Sample preparation

Milk samples were warmed to 32 ºC and recombinant chymosin (180 International Milk Clotting Units mL^{-1}, Maxiren 180, DSM Food Specialties, Seclin Cedex, France) was added at 0.074% (v/v). After coagulation at 32 °C for 30 min, curds were cut and centrifuged at 1,500 × g for 15 min at 20 ºC (Beckman J2-21 with rotor JA-14, Beckman Instruments France S.A., Gagny, France) and pellets were recovered, after decanting whey, for yield estimation and for further analysis.

2.2.4. Composition analysis of curds

Protein and fat content of drained curds were determined in triplicate using the Kjeldahl method (IDF, 1993) and the van Gulik method (ISO, 1975), respectively. Curds were analysed in triplicate for total solids (**TS**; IDF, 2004) to calculate their moisture content (100 – TS) and express protein and fat content on a dry basis. Estimated yield of curds on wet basis, expressed as grams of curd recovered per 100 g milk, was determined by weighing the curds obtained (Daviau et al., 2000).

2.2.5. Dissociation tests

Protein-protein interactions in drained curds were studied following a modification of the method of Lefebvre-Cases et al. (1998). Samples of drained curds (10 g) were dispersed in 40 mL of aqueous dissociating solutions containing either 2 mM EDTA (pH 10.0), 1% (w/v) SDS, or 6 M urea. For a control sample, curds were mixed with MilliQ water. Mixtures of curds and dissociating agent were dispersed for 1 min at 9000 rpm (Ultra-Turrax T 25, Janke and Kunkel, IKA Labortechnik, Staufen, Germany), and the resulting mixtures were ultracentrifuged at 86,000 × g for 40 min at 20 °C (Beckman Optima LE-80K with rotor Type 50.2 Ti).

Immediately after ultracentrifugation, supernatants were analysed for total protein using a modified version of the method of Lowry et al. (1951). Supernatants were diluted in MilliQ water [25% (v/v) for dissociation in MilliQ water or EDTA or 2.5% (v/v) for dissociation in SDS or urea]. Diluted

samples (200 μL) were mixed with 2.1 mL of Lowry Reagent D (1:1:98 mixture of 1% (w/v) copper sulphate, 1% (w/v) sodium potassium tartrate, and 2% (w/v) sodium carbonate in 0.1 M NaOH). After 10 min at room temperature, Folin-Ciocalteu's phenol reagent (250 mL of a 1:1 dilution with MilliQ water) was added and colour development was allowed to proceed for 30 min at room temperature. The absorbance at 750 nm was then measured with a spectrophotometer (model Cary 300 Bio, Varian Inc., Palo Alto, CA, USA), and the protein content quantified using a standard curve of bovine serum albumin (**BSA**) standards in the range 0-1 mg protein mL^{-1}. All chemicals were purchased from Sigma-Aldrich Chemie (Steinheim, Germany).

Both qualitative and quantification analysis of the main proteins extracted were carried out by SDS-PAGE analysis under reducing conditions using separating and stacking gels containing 14% or 4% acrylamide, respectively. Supernatants mixed with double-strength reducing buffer (Laemmli, 1970) were loaded onto the gels (20 μL for MilliQ water and EDTA, or 3 μL for SDS and urea) together with BSA standard (10 μg) and a wide-range molecular weight marker (from 6.5 to 205 kDa; M4038, Sigma-Aldrich). Gels were run at 200 V, stained using 0.1% (w/v) Coomassie Brilliant Blue R250 in a 5:1:4 mixture of methanol, acetic acid and distilled water, and destained in two steps with 5:1:4 and 7:5:88 mixtures of methanol, acetic acid and distilled water, respectively. Destained gels were scanned using a calibrated BioRad GS800 densitometer (BioRad Laboratories, Hercules, CA, USA).

2.2.6. Statistical analysis

For each type of homogenisation, the complete experiment was repeated on 3 independent occasions. Data were processed by multifactor analysis of variance (ANOVA) using the general linear models procedure of Statgraphics (Statgraphics Inc., Chicago, IL, USA), taking into account both treatment and production factors as well as their interaction. LSD test was used for comparison of sample data, and evaluations were based on a significance level of $P < 0.05$.

2.3. Results and discussion

2.3.1. Particle size distribution of milk samples

Both homogenisation treatments significantly decreased D(4,3) values compared to raw milk (Table II.2.1); conventional homogenisation-pasteurisation resulted in significantly lower values than UHPH and the effect of the former was independent of the level of fat. However, in the case of UHPH, 3.6% fat milk samples had significantly higher values than UHPH-treated milk samples with 0.0% or 1.8% fat. No significant differences in D(3,2) values were found between 0.0% fat milk samples subjected to different treatments. When fat was present, both homogenisation treatments decreased D(3,2), and UHPH yielded significantly lower values than conventional homogenisation-pasteurisation. Conventionally homogenised-pasteurised milk showed a monomodal distribution of sizes, ranging from 0.05 to 3.0 μm, with a peak at ~0.2 μm. For UHPH-treated milk samples, the observed distributions were bimodal; the principal peak was also at ~0.2 μm, but the particles ranged from 0.05 to 1.0 μm. The smallest particles (<0.1 μm) were more abundant in UHPH-treated milk than conventionally homogenised-pasteurised milk. A second peak of particles, of diameters from 2.0 to 5.0 μm, was observed, but represented less than 5% of the total particles. Such results are broadly in accordance with previous studies (Hayes and Kelly, 2003; see Section II Chapter 1).

2.1.1. Estimated yield, and moisture, fat and protein contents of curds

When fat was present, both homogenisation treatments significantly increased estimated curd yield on wet basis compared to that obtained from raw milk (Table II.2.1). However, the effect of UHPH was greater than that of conventional homogenisation-pasteurisation. The increases for 3.6 and 1.8% fat milk samples were 51 and 54% for UHPH-treated milk samples and 19 and 23% for conventionally homogenised-pasteurised milk samples, respectively. Higher estimated yields could be explained by higher moisture content, higher recovery of fat and/or the incorporation of whey proteins into the curds (see Section II Chapter 1).

Table II.2.1. Volume [D(4,3)] and surface [D(3,2)] weighted mean diameters of particles in milk and wet yield, moisture content, and fat and protein contents on a dry basis of drained curds.

Treatment[1]	D(4,3) (nm)	D(3,2) (nm)	Wet yield (g curd 100 g^{-1} milk)	Moisture content (g 100 g^{-1} curd)	Fat content (g 100 g^{-1} TS)	Protein content (g 100 g^{-1} TS)
0.0-R	944 ± 136 ^{d}	211 ± 8 ^{e,f}	18.38 ± 0.16 ^{e}	79.38 ± 0.17 ^{d}	4.41 ± 0.69 ^{g}	65.47 ± 1.92 ^{a}
0.0-H	139 ± 95 ^{f}	212 ± 6 ^{e,f}	21.02 ± 1.06 ^{e}	80.73 ± 0.21 ^{c}	7.78 ± 0.79 ^{f}	59.16 ± 1.83 ^{b}
0.0-U	684 ± 58 ^{e}	185 ± 2 ^{f}	21.62 ± 1.18 ^{e}	81.15 ± 0.35 ^{c}	4.46 ± 0.05 ^{g}	60.99 ± 1.00 ^{b}
1.8-R	3,176 ± 63 ^{b}	634 ± 11 ^{b}	25.95 ± 0.37 ^{d}	77.20 ± 0.19 ^{f}	32.38 ± 0.70 ^{d}	43.76 ± 0.88 ^{c}
1.8-H	212 ± 23 ^{f}	259 ± 2 ^{d}	31.87 ± 1.52 ^{c}	79.55 ± 0.34 ^{d}	35.02 ± 1.31 ^{c}	40.29 ± 1.18 ^{d}
1.8-U	605 ± 22 ^{e}	191 ± 2 ^{e,f}	39.99 ± 1.22 ^{b}	83.12 ± 0.24 ^{a}	29.34 ± 0.74 ^{e}	42.15 ± 0.87 ^{c,d}
3.6-R	3,614 ± 75 ^{a}	913 ± 16 ^{a}	34.57 ± 1.76 ^{c}	75.83 ± 0.22	47.61 ± 1.28 ^{a}	31.83 ± 0.66 ^{e}
3.6-H	302 ± 12 ^{f}	310 ± 4 ^{c}	41.15 ± 0.62 ^{b}	78.56 ± 0.16 ^{e}	45.78 ± 1.37 ^{a}	29.59 ± 0.67 ^{e}
3.6-U	1,277 ± 152 ^{c}	220 ± 12 ^{e}	52.32 ± 2.30 ^{a}	82.26 ± 0.27 ^{b}	42.55 ± 0.63 ^{b}	32.61 ± 1.00 ^{e}

$^{a-g}$ Mean value ± standard error; n = 6 for R, n = 3 for H and U (estimated yield) or n=18 for R, n=9 for H and U (others); values without common superscripts were significantly different ($P < 0.05$).

[1] 0.0, 1.8 and 3.6 represents the milk fat content; R stands for raw, H for conventional homogenisation-pasteurisation and U for ultra-high pressure homogenisation.

Table II.2.2. Effect of dissociating agent on protein content of supernatants (g supernatant protein 100 g^{-1} curd protein).

Treatment[1]	MilliQ water	EDTA	Urea	SDS
0.0-R	8.00 ± 0.37 ^{f}	8.74 ± 0.16 ^{e}	81.52 ± 2.82 ^{b}	98.53 ± 4.25 ^{c,d}
0.0-H	9.39 ± 0.24 ^{e}	11.36 ± 0.27 ^{c}	87.70 ± 1.41 ^{a}	104.13 ± 1.22 ^{b,c,d}
0.0-U	8.22 ± 0.24 ^{f}	10.16 ± 0.27 ^{d}	83.29 ± 1.89 ^{a,b}	116.57 ± 4.18 ^{a}
1.8-R	9.70 ± 0.16 ^{e}	11.64 ± 0.16 ^{c}	69.11 ± 1.12 ^{c,d}	97.08 ± 1.40 ^{d}
1.8-H	12.54 ± 0.60 ^{c,d}	15.38 ± 0.77 ^{b}	86.55 ± 2.75 ^{a,b}	109.12 ± 5.32 ^{a,b}
1.8-U	11.71 ± 0.25 ^{d}	15.77 ± 0.34 ^{b}	68.15 ± 1.14 ^{c,d}	106.08 ± 3.17 ^{b,c}
3.6-R	12.61 ± 0.35 ^{c}	15.75 ± 0.33 ^{b}	65.96 ± 2.27 ^{d}	99.02 ± 2.33 ^{c,d}
3.6-H	16.28 ± 0.37 ^{a}	19.58 ± 0.69 ^{a}	73.41 ± 1.60 ^{c}	107.55 ± 1.96 ^{a,b}
3.6-U	14.26 ± 0.30 ^{b}	19.50 ± 0.87 ^{a}	49.25 ± 2.11 ^{e}	83.03 ± 2.18 ^{e}

$^{a-f}$ Mean value ± standard error; n = 18 for R, n = 9 for H and U; values without common superscripts were significantly different ($P < 0.05$).

[1] 0.0, 1.8 and 3.6 represent the milk fat content; R stands for raw, H for conventional homogenisation-pasteurisation and U for ultra-high pressure homogenisation.

As milk fat content increased, both homogenisation treatments increased the moisture content of curds, by 2-4% for conventionally homogenised-pasteurised milk, and by up to 9% for UHPH-treated milk (Table II.2.1). Impaired whey drainage in curds made from homogenised milk has been previously reported (Humbert et al., 1980; Green et al., 1983; Ghosh et al., 1994; see Section II Chapter 1). The water-holding capacity of curds is directly linked to the microstructure of the gels, i.e., porosity or permeability (Walstra et al., 1985). Native fat globules act as fillers in the interstices of the network (Lopez & Dufour, 2001), thus reducing the volume of water. The effects of homogenisation on the moisture content of the curd have been attributed to the incorporation of denatured whey proteins and the alteration in the protein-fat structure of the curd (Green et al., 1983; Métais et al., 2006; see Section II Chapter 1).

As expected, the recovery of fat in the curd was significantly higher with increasing fat level in milk (Table II.2.1). Conventional homogenisation-pasteurisation significantly increased the fat content of curds made from 0.0% and 1.8% fat milk. An increase in fat in dry matter content in cheese made using homogenised milk and cream has been reported (Jana & Upadhyay, 1992; Metzger & Mistry, 1994). In contrast, UHPH decreased the recovery of fat, particularly for 3.6% and 1.8% fat milk. Lanciotti et al. (2006) reported that Caciotta cheeses made from milk high-pressure-homogenised at ≤ 100 MPa had lower fat content than those made from raw milk. Losses of fat might be due to very small fat globules which are not retained within the curd.

The protein content of curds on a dry basis did not significantly differ between treatments; increasing the fat content of milk from 0.0% to 3.6% resulted in very large differences in protein content, thus hiding the small effect of the treatments (Table II.2.1). However, both homogenisation treatments decreased the protein content of curds, except in the case of UHPH-treated 3.6% fat milk. UHPH has been proven to reduce the amount of β-lactoglobulin (**β-LG**) and, to a lesser extent α-lactalbumin (**α-LA**), in whey, by incorporation of these proteins into the curd (see Section II Chapter 1).

Table II.2.3. Levels of proteins in supernatants of curds dissociated by MilliQ water (g dissociated protein 100 g^{-1} supernatant protein).

Treatment[1]	BSA	α_s-CN	β-CN	κ-CN	β-LG	*Para* κ-CN	α-LA	γ_2-/γ_3-CN
0.0-R	1.29 ± 0.09 ^{b}	0.94 ± 0.08 ^{c}	1.33 ± 0.27 ^{b,c}	1.10 ± 0.11 ^{e}	19.83 ± 2.10 ^{c}	-	7.25 ± 0.89 ^{d}	-
0.0-H	1.30 ± 0.06 ^{b}	1.13 ± 0.14 ^{b,c}	1.72 ± 0.87 ^{a,b,c}	1.34 ± 0.10 ^{b,c,d}	20.90 ± 2.16 ^{c}	-	7.45 ± 0.65 ^{d}	-
0.0-U	1.36 ± 0.06 ^{a,b}	1.14 ± 0.13 ^{b,c}	1.67 ± 0.29 ^{a,b,c}	1.23 ± 0.10 ^{d,e}	23.24 ± 4.21 ^{b}	-	8.24 ± 1.55 ^{b,c,d}	-
1.8-R	1.47 ± 0.06 ^{b}	1.01 ± 0.10 ^{c}	1.27 ± 0.23 ^{b,c}	1.29 ± 0.10 ^{c,d}	23.47 ± 1.82 ^{b}	-	8.20 ± 0.52 ^{c,d}	-
1.8-H	1.45 ± 0.07 ^{a,b}	1.18 ± 0.14 ^{b,c}	1.33 ± 1.37 ^{a}	1.44 ± 0.10 ^{a,b,c}	23.82 ± 2.69 ^{a,b}	-	9.19 ± 1.15 ^{b,c}	-
1.8-U	1.50 ± 0.11 ^{a}	1.40 ± 0.14 ^{a,b}	1.35 ± 0.37 ^{a}	1.52 ± 0.14 ^{a}	24.47 ± 4.19 ^{a,b}	-	11.36 ± 1.47 ^{a}	-
3.6-R	1.52 ± 0.07 ^{a}	1.06 ± 0.10 ^{c}	1.24 ± 0.20 ^{c}	1.40 ± 0.12 ^{a,b,c}	24.36 ± 2.24 ^{a,b}	-	8.75 ± 0.68 ^{b,c}	-
3.6-H	1.50 ± 0.12 ^{a}	1.14 ± 0.14 ^{b,c}	2.03 ± 1.10 ^{a,b}	1.36 ± 0.14 ^{a,b,c,d}	23.27 ± 2.75 ^{b}	-	9.45 ± 1.68 ^{b}	-
3.6-U	1.50 ± 0.04 ^{a}	1.52 ± 0.25 ^{a}	1.90 ± 0.28 ^{a,b,c}	1.50 ± 0.05 ^{a,b}	25.15 ± 4.29 ^{a}	-	11.56 ± 1.24 ^{a}	-

$^{a-e}$ Mean value ± standard error; n = 6 for R, n = 3 for H and U; values without common superscripts were significantly different ($P < 0.05$).

[1] 0.0, 1.8 and 3.6 represent the milk fat content; R stands for raw, H for conventional homogenisation-pasteurisation and U for ultra-high pressure homogenisation.

Table II.2.4. Levels of proteins in supernatants of curds dissociated by EDTA (g dissociated protein 100 g^{-1} supernatant protein).

Treatment[1]	BSA	α_s-CN	β-CN	κ-CN	β-LG	*Para* κ-CN	α-LA	γ_2-/γ_3-CN
0.0-R	1.16 ± 0.08 ^{b}	3.15 ± 0.73 ^{c,d}	5.24 ± 1.74 ^{b,c}	1.45 ± 0.08 ^{a}	18.01 ± 1.74 ^{b,c}	1.25 ± 0.06 ^{a}	6.80 ± 0.70 ^{a,b}	-
0.0-H	1.24 ± 0.12 ^{a,b}	4.14 ± 1.05 ^{b,c}	6.02 ± 3.66 ^{b}	1.50 ± 0.14 ^{a}	17.74 ± 1.92 ^{c,d}	1.39 ± 0.12 ^{a}	6.52 ± 0.64 ^{a,b,c}	-
0.0-U	1.26 ± 0.04 ^{a,b}	4.23 ± 0.70 ^{b,c}	6.76 ± 1.24 ^{b}	1.62 ± 0.14 ^{a}	19.77 ± 3.38 ^{a}	1.32 ± 0.14 ^{a}	7.30 ± 1.35 ^{a}	-
1.8-R	1.31 ± 0.07 ^{a}	2.53 ± 0.65 ^{d}	3.66 ± 1.37 ^{c,d}	1.50 ± 0.12 ^{a}	19.02 ± 1.65 ^{a,b}	1.05 ± 0.10 ^{b,c}	6.33 ± 0.59 ^{b,c}	-
1.8-H	1.22 ± 0.08 ^{a,b}	4.56 ± 0.75 ^{b}	6.26 ± 3.68 ^{b}	1.58 ± 0.18 ^{a}	17.41 ± 2.69 ^{c,d}	1.30 ± 0.09 ^{a}	5.90 ± 0.90 ^{b,c,d}	-
1.8-U	1.23 ± 0.03 ^{a,b}	6.43 ± 1.38 ^{a}	11.15 ± 1.97 ^{a}	1.53 ± 0.12 ^{a}	17.58 ± 3.04 ^{c,d}	1.22 ± 0.16 ^{a,b}	6.77 ± 1.32 ^{a,b}	-
3.6-R	1.22 ± 0.06 ^{a,b}	2.82 ± 0.47 ^{d}	2.85 ± 1.14 ^{d}	1.49 ± 0.11 ^{a}	16.94 ± 1.48 ^{d}	0.95 ± 0.07 ^{c}	5.07 ± 0.54 ^{d}	-
3.6-H	1.24 ± 0.11 ^{a,b}	4.37 ± 1.25 ^{b}	5.48 ± 3.24 ^{b,c}	1.53 ± 0.18 ^{a}	15.31 ± 1.57 ^{e}	1.05 ± 0.07 ^{b,c}	4.06 ± 0.50 ^{e}	-
3.6-U	1.14 ± 0.05 ^{b}	7.38 ± 1.01 ^{a}	10.31 ± 1.51 ^{a}	1.40 ± 0.13 ^{a}	14.59 ± 2.52 ^{e}	1.23 ± 0.16 ^{a}	5.44 ± 1.00 ^{c,d}	-

$^{a-e}$ Mean value ± standard error; n = 6 for R, n = 3 for H and U; values without common superscripts were significantly different ($P < 0.05$).

[1] 0.0, 1.8 and 3.6 represent the milk fat content; R stands for raw, H for conventional homogenisation-pasteurisation and U for ultra-high pressure homogenisation.

2.1.2. Dissociation of proteins from curd

The rates of dissociation of protein from curd depended markedly on the dissociating agent used (Table II.2.2). The amount of protein extracted with MilliQ water, i.e., unbound proteins, was in the range of 8-16% of the protein content of the curds. These results were similar to those reported for rennet gels (Lefebvre-Cases et al., 1998); no differences in the distribution of the stabilising bonds have been found between rennet-induced gels and cheese grains (Hinrichs & Keim, 2007). The main unbound proteins were identified as the whey proteins β-LG and α-LA (Figs. II.2.1 and 2; Table II.2.3); no *para* κ-casein (**CN**) or γ_2-/γ_3-CN were detected. These results suggest that mechanical disruption of the network on mascerating in water released the whey entrapped in the curd. Increasing the milk fat content led to significantly higher proportions of whey proteins released into the supernatant. The presence of native fat globules may increase the amount of solids susceptible to creation of weak interactions that can be mechanically disrupted. When fat was present, both homogenisation treatments increased the dissociation of curd proteins. The homogenisation of fat globules results in a higher number of small particles which would increase the degree of interactions. The proportion of unbound α-LA in curds made from UHPH-treated milk was significantly higher than those from non-treated and conventionally homogenised-pasteurised milk. As previously mentioned, UHPH increased the moisture content of curds; in other words, more whey entrapped in the curd. To a lesser extent, caseins, i.e., α_s-CN and β-CN, were also affected. This fact could be explained because homogenisation, especially UHPH, may cause partial disintegration of the casein micelles (Sandra & Dalgleish, 2005; Roach & Harte, 2008).

EDTA, as a chelating agent, disrupts ionic bonds of proteins involving calcium salts, but reports on its dissociation capacity in the literature differ significantly. Lefebvre-Cases et al. (1998) obtained 76% dissociation of protein from rennet gels with 2 mM EDTA, but did not refer to pH adjustment when preparing the chemical agent. Gagnaire et al. (2002) subsequently showed that

Table II.2.5. Levels of proteins in supernatants of curds dissociated by urea (g dissociated protein 100 g^{-1} supernatant protein).

Treatment[1]	BSA	α_s-CN	β-CN	κ-CN	β-LG	*Para* κ-CN	α-LA	γ_2-/γ_3-CN
0.0-R	0.66 ± 0.07 ^{f}	25.98 ± 1.91 ^{f}	37.63 ± 2.20 ^{e}	3.39 ± 0.26 ^{a}	1.97 ± 0.20 ^{d}	16.08 ± 1.42 ^{a}	1.24 ± 0.08 ^{e}	4.43 ± 0.67 ^{a}
0.0-H	0.79 ± 0.10 ^{e,f}	27.23 ± 2.58 ^{e,f}	36.89 ± 2.62 ^{e}	2.43 ± 0.54 ^{c}	1.83 ± 0.19 ^{d}	14.93 ± 1.13 ^{a,b}	1.29 ± 0.15 ^{d,e}	3.15 ± 0.43 ^{b,c}
0.0-U	0.74 ± 0.10 ^{f}	26.63 ± 1.86 e,f	38.90 ± 1.45 ^{e}	2.53 ± 0.18 ^{b,c}	1.97 ± 0.24 d	12.23 ± 1.81 ^{b,c}	1.16 ± 0.11 ^{e}	3.46 ± 0.12 ^{a,b}
1.8-R	0.97 ± 0.07 ^{d,e}	31.41 ± 2.04 ^{d}	47.24 ± 4.16 ^{c,d}	2.15 ± 0.12 ^{c}	2.30 ± 0.15 ^{d}	8.94 ± 1.02 ^{d}	1.67 ± 0.16 ^{c,d}	2.31 ± 0.16 ^{c}
1.8-H	1.03 ± 0.18 ^{d}	30.86 ± 3.88 ^{d,e}	44.11 ± 4.35 ^{c,d,e}	1.99 ± 0.16 ^{c}	2.38 ± 0.36 ^{c,d}	10.71 ± 1.33 ^{c,d}	1.57 ± 0.22 ^{c,d,e}	2.11 ± 0.29 ^{c}
1.8-U	1.58 ± 0.08 ^{b}	41.04 ± 1.96 ^{b}	54.47 ± 3.60 ^{a,b}	2.63 ± 0.17 ^{b,c}	4.29 ± 0.85 ^{a}	10.35 ± 0.71 ^{c,d}	2.20 ± 0.21 ^{b}	2.57 ± 0.18 ^{b,c}
3.6-R	1.33 ± 0.10 ^{c}	37.04 ± 2.13 ^{c}	49.90 ± 2.68 ^{b,c}	2.51 ± 0.14 ^{c}	3.08 ± 0.22 ^{b}	4.79 ± 0.70 ^{e}	2.00 ± 0.12 ^{b,c}	2.57 ± 0.14 ^{b,c}
3.6-H	1.35 ± 0.22 ^{b,c}	33.67 ± 4.87 ^{c,d}	42.14 ± 3.53 ^{d,e}	2.11 ± 0.31 ^{c}	2.96 ± 0.37 ^{b,c}	3.20 ± 0.41 ^{e}	2.05 ± 0.32 ^{b,c}	2.20 ± 0.39 ^{c}
3.6-U	2.49 ± 0.12 ^{a}	49.85 ± 3.42 ^{a}	57.25 ± 5.39 ^{a}	3.32 ± 0.17 ^{a,b}	4.83 ± 0.66 ^{a}	4.61 ± 0.38 ^{e}	3.35 ± 0.44 ^{a}	3.54 ± 0.17 ^{a,b}

$^{a-f}$ Mean value ± standard error; n = 6 for R, n = 3 for H and U; values without common superscripts were significantly different ($P < 0.05$).

[1] 0.0, 1.8 and 3.6 represent the milk fat content; R stands for raw, H for conventional homogenisation-pasteurisation and U for ultra-high pressure homogenisation.

Table II.2.6. Levels of proteins in supernatants of curds dissociated by SDS (g dissociated protein 100 g^{-1} supernatant protein).

Treatment[1]	BSA	α_s-CN	β-CN	κ-CN	β-LG	*Para* κ-CN	α-LA	γ_2-/γ_3-CN
0.0-R	0.54 ± 0.03 ^{f}	22.80 ± 1.61 ^{d}	27.24 ± 1.23 ^{f}	2.71 ± 0.32 ^{b}	1.51 ± 0.11 ^{e}	14.12 ± 1.01 ^{a,b}	0.91 ± 0.07 ^{e}	2.05 ± 0.25 ^{b,c,d}
0.0-H	0.66 ± 0.10 ^{e,f}	26.05 ± 2.01 ^{c}	34.29 ± 2.38 ^{d,e}	2.80 ± 0.28 ^{b}	1.76 ± 0.17 ^{d,e}	16.72 ± 0.41 ^{a}	1.06 ± 0.10 ^{d,e}	2.50 ± 0.40 ^{b}
0.0-U	0.58 ± 0.06 ^{f}	22.25 ± 0.86 ^{d}	30.56 ± 0.85 ^{e,f}	3.58 ± 0.50 ^{a}	2.14 ± 0.19 ^{c,d,e}	12.90 ± 0.29 ^{b}	0.87 ± 0.07 ^{e}	3.06 ± 0.52 ^{a}
1.8-R	0.73 ± 0.05 ^{d,e}	26.91 ± 1.25 ^{c}	36.74 ± 1.31 ^{c,d}	2.14 ± 0.36 ^{c}	1.75 ± 0.12 ^{d,e}	14.08 ± 0.92 ^{a,b}	1.11 ± 0.07 ^{d}	1.77 ± 0.27 ^{c,d}
1.8-H	0.85 ± 0.18 ^{c,d}	29.13 ± 2.08 ^{b,c}	39.73 ± 2.30 ^{b,c}	2.21 ± 0.11 ^{b,c}	2.16 ± 0.18 ^{c,d,e}	15.41 ± 1.05 ^{a,b}	1.33 ± 0.11 ^{c}	2.08 ± 0.14 ^{b,c,d}
1.8-U	1.08 ± 0.09 ^{b}	31.03 ± 1.72 ^{b}	42.99 ± 2.26 ^{b}	1.96 ± 0.23 ^{c}	4.63 ± 0.74 ^{b}	13.41 ± 0.36 ^{a,b}	1.44 ± 0.14 ^{c}	1.66 ± 0.19 ^{d}
3.6-R	0.98 ± 0.08 ^{b,c}	31.59 ± 1.61 ^{b}	42.82 ± 2.77 ^{b}	2.20 ± 0.12 ^{c}	2.29 ± 0.23 ^{c,d}	13.77 ± 1.62 ^{a,b}	1.42 ± 0.12 ^{c}	1.79 ± 0.15 ^{c,d}
3.6-H	1.10 ± 0.19 ^{b}	32.23 ± 3.11 ^{b}	41.30 ± 3.41 ^{b}	2.35 ± 0.08 ^{b,c}	2.79 ± 0.29 ^{c}	13.62 ± 1.65 ^{a,b}	1.69 ± 0.25 ^{b}	2.08 ± 0.32 ^{b,c,d}
3.6-U	1.66 ± 0.14 ^{a}	40.09 ± 1.57 ^{a}	52.69 ± 3.23 ^{a}	2.36 ± 0.22 ^{b,c}	5.96 ± 1.06 ^{a}	12.65 ± 1.86 ^{b}	2.34 ± 0.05 ^{a}	2.20 ± 0.11 ^{b,c}

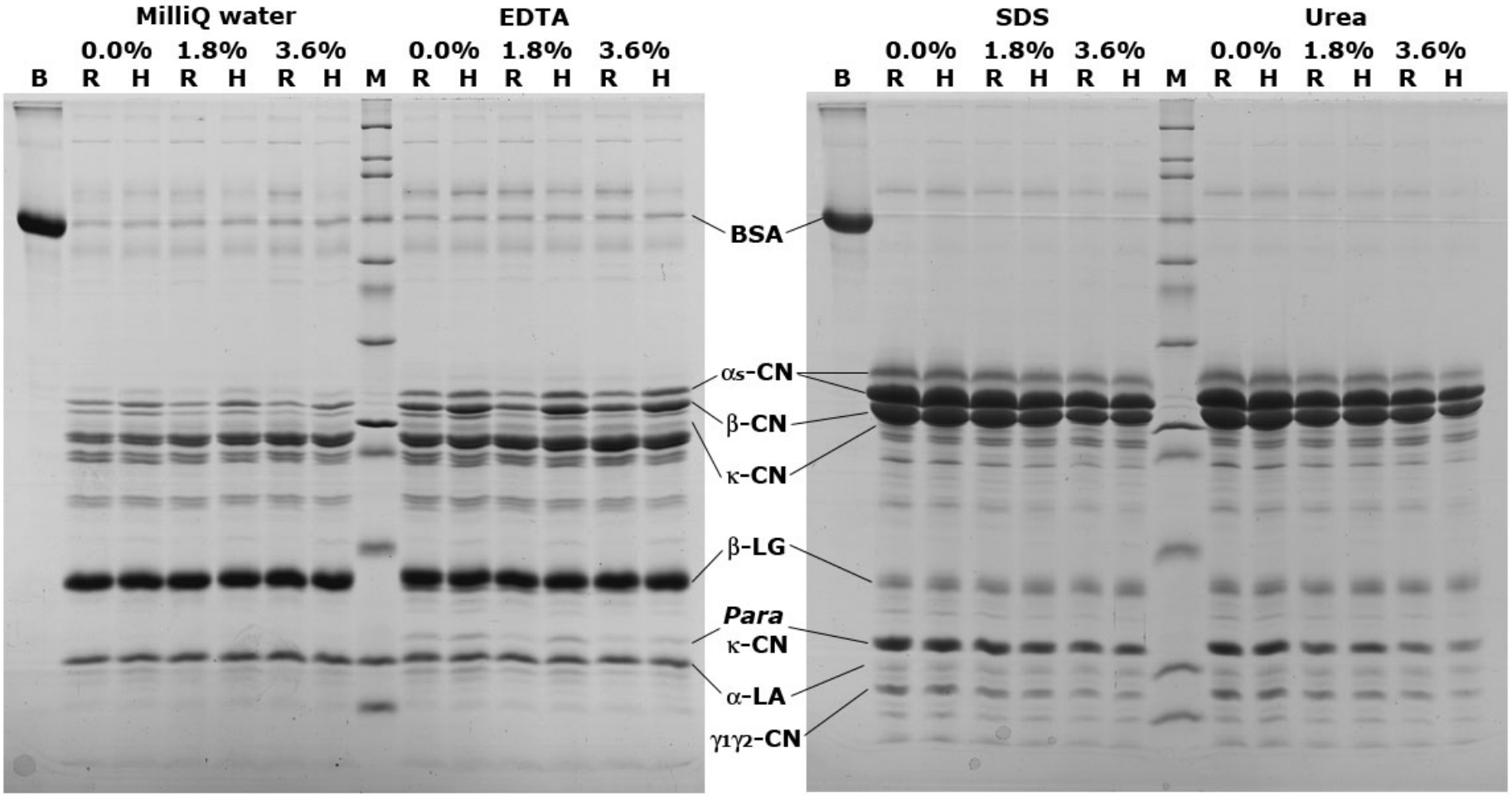
$^{a-f}$ Mean value ±standard error; n = 6 for R, n = 3 for H and U; values without common superscripts were significantly different ($P < 0.05$).

[1] 0.0, 1.8 and 3.6 represent the milk fat content; R stands for raw, H for conventional homogenisation-pasteurisation and U for ultra-high pressure homogenisation.

the rate of dissociation varied depending on EDTA concentration at pH 8.0; for 2 mM EDTA, the amount of calcium present in the supernatant was similar to that found without EDTA, and no casein was released. In a recent study, Alessi et al. (2007) evaluated the interactions in rennet gels obtained from reconstituted skim milk prepared at two different temperatures (25 or 38 ºC), and reported that the amount of protein dissociated by 2 mM EDTA varied widely; increasing temperature decreased dissociation down to the value obtained without EDTA. The results of the present study showed that the presence of EDTA slightly increased the amount of protein extracted compared to MilliQ water alone (Table II.2.2). The main proteins involved were caseins and their hydrolysis products (Figs. II.2.1 and 2; Table II.2.4). Calcium bonding is thus, to some degree, responsible for casein retention within the curd. Increasing the milk fat content significantly increased the dissociation rates obtained with EDTA. None of the identified proteins seemed to be particularly involved in the increase as, in general, their proportions in the supernatants did not vary significantly. Independent of the fat content of milk, both homogenisation treatments enhanced the dissociation of curd proteins, i.e., α_s-CN and β-CN. Calcium bonding could be enhanced by a rearrangement of the mineral balance in milk; UHPH at 300 MPa induces a transfer of minerals from the soluble to the micellar fraction (see Section II Chapter 1).

Urea disrupts hydrogen bonds by denaturing proteins through establishing strong hydrogen bonds with polypeptide groups of proteins. In agreement with previous results (Lefebvre-Cases et al., 1998; Gagnaire et al., 2002), a much higher release of protein occurred in the presence of urea (Table II.2.2) than with water or EDTA. The proteins involved were mainly caseins (Figs. II.2.1 and 2; Table II.2.5). However, the levels of dissociation varied depending on the fat content of milk. Increasing milk fat content significantly decreased the amount of protein dissociated by urea, suggesting that caseins are partly linked through hydrogen bonds within the curd, which the presence of fat could inhibit. The proportion of *para* κ-CN in the supernatants significantly decreased with increasing fat content. In model systems, native milk fat globules are considered as non-interacting particles and act as an inert filler (Métais et al., 2006). However, the inclusion of native fat globules in a drained

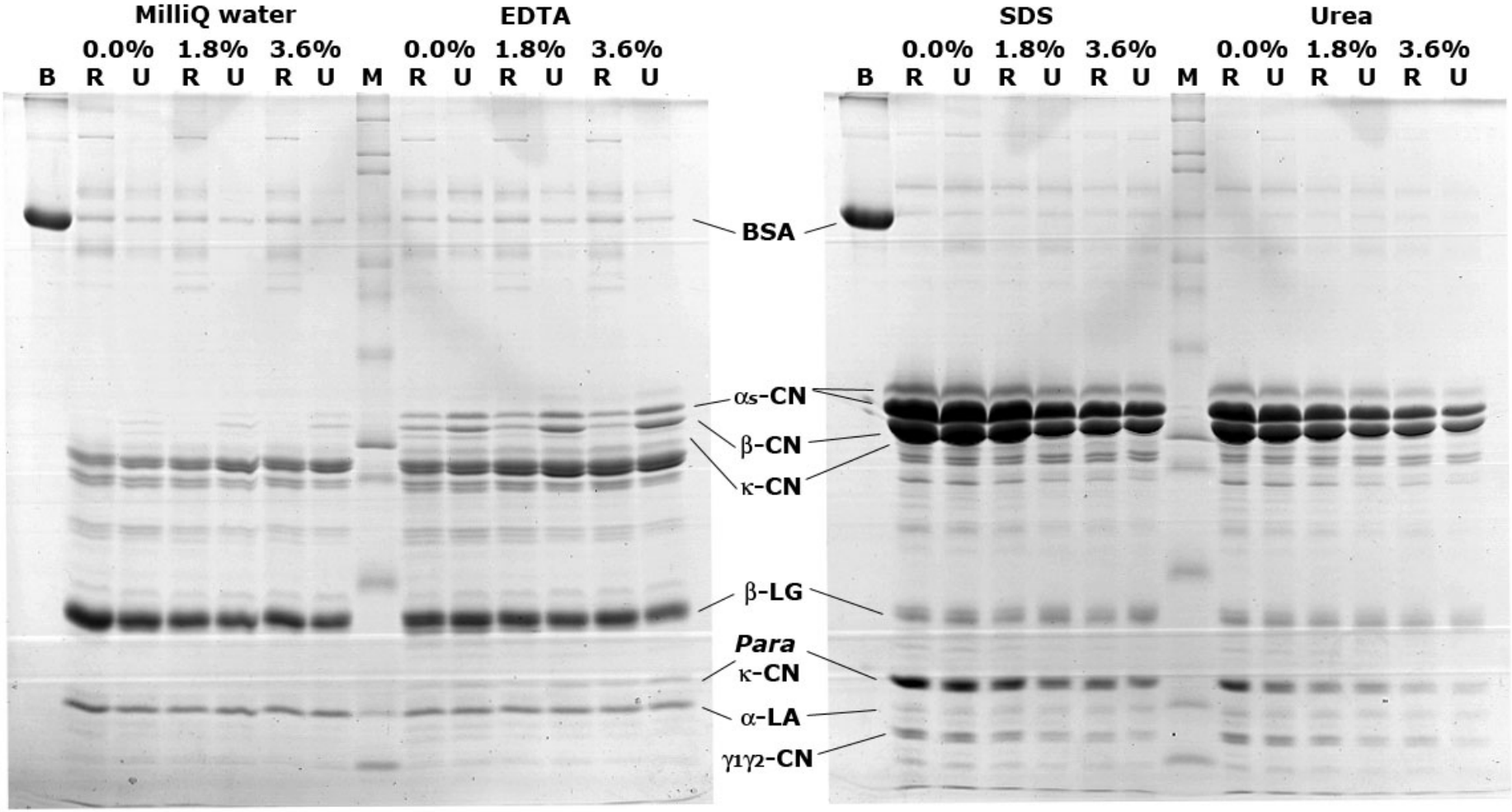
Figure II.2.1. SDS-PAGE electrophoretograms of supernatants obtained on dissociation of drained curds from conventionally homogenised milk [0.0%, 1.8% and 3.6% represent the milk fat content; R and H indicate raw and conventional homogenisation-pasteurisation; B and M are BSA standard and molecular weight markers: 205, 116, 97, 84, 66, 55, 45, 36 29, 24, 20, 14.2, and 6.5 kDa].



curd would keep casein micelles apart, thus reducing the amount of *para* κ-CN linked through hydrogen bonds. Regardless of milk fat content, conventional homogenisation-pasteurisation increased the dissociation of proteins; small fat globules may not perturb the formation of the protein network (Lopez & Dufour, 2001). However, UHPH either had no effect on the extent of dissociation for curds prepared from 1.8% fat milk, while dissociation was reduced for 3.6% fat milk. The incorporation of whey proteins, i.e., β-LG and α-LA, into the curd, by means of other bonds, e.g., hydrophobic interactions, would lead to greater difficulty in creating hydrogen bonds due to spatial competition.

SDS breaks hydrophobic interactions by inducing intramolecular electrostatic repulsions. In accordance with previous results (Lefebvre-Cases et al., 1998), the use of SDS resulted in almost complete dissociation of the curd (Table II.2.2), with no significant effect of fat level. However, both homogenisation treatments significantly affected the dissociation of protein, and the protein pattern differed depending on both the treatment and the absence or presence of fat (Figs. II.2.1 and 2; Table II.2.6). In 0.0% fat milk, conventional homogenisation-pasteurisation significantly increased the proportion of dissociated caseins, i.e., α_s-CN, β-CN, and κ-CN; for UHPH-treated skim milk, the proteins dissociated were mainly κ-CN and γ_2-/γ_3-CN. When fat was present, only the proportion of α-LA was significantly increased by conventional homogenisation-pasteurisation. In contrast, UHPH significantly increased the level of dissociation of both whey proteins (BSA, β-LG and α-LA), and caseins (α_s-CN and β-CN). Although in early studies on UHPH no denaturation of whey proteins was reported (Hayes & Kelly, 2003; Sandra & Dalgleish, 2005), these results support those obtained in the study on rennet coagulation that UHPH and, to a lesser extent, conventional homogenisation-pasteurisation caused the incorporation of denatured whey proteins into the curd (see Section II Chapter 1).

Figure II.2.2. SDS-PAGE electrophoretograms of supernatants obtained on dissociation of drained curds from UHPH-treated milk [0.0%, 1.8% and 3.6% represent the milk fat content; R and U indicate raw and ultra-high pressure homogenisation; B and M are BSA standard and molecular weight markers: 205, 116, 97, 84, 66, 55, 45, 36 29, 24, 20, 14.2, and 6.5 kDa].



2.4. Conclusions

Increasing fat content of raw milk impaired hydrogen bonding between caseins within drained curds. Both conventional homogenisation and UHPH increased the amount of unbound whey proteins and calcium-bonded caseins in curds. There were significant differences in extents of hydrogen bonding and hydrophobic interactions in curds between the two treatments. Conventional homogenisation-pasteurisation enhanced casein-casein interactions through hydrogen bonds and hydrophobic interactions, and, when fat was present, the incorporation of α-LA in the curd through hydrophobic interactions. In contrast, UHPH impaired hydrogen bonding and favoured hydrophobic interactions of whey proteins and caseins.

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Section II, Chapter 3

Changes in the surface protein of the fat globules during ultra-high pressure homogenisation of milk

Disruption of fat globules upon homogenisation provokes a reduction of their size and a concomitant increase in their specific surface area. In order to overcome this phenomenon, the milk fat globule membrane (MFGM) adsorbs non-native MFGM proteins. The aim of the present study was to examine the effects of UHPH conditions (temperature and pressure) on the milk fat globule and the surface proteins by comparison with conventional treatments applied in the dairy industry. Transmission electron microscopy and SDS-PAGE revealed major differences. In UHPH-treated milk, casein micelles were found to be adsorbed on the MFGM in a lesser extent than in conventional homogenisation-pasteurisation. However, greater adsorption of directly bonded casein molecules, released by UHPH through the partial disruption of casein micelles, was observed especially at high UHPH pressures. Adsorption of whey proteins on the MFGM of conventionally homogenised-pasteurised milk was mainly through intermolecular disulfide bonds with MFGM material, whereas in UHPH-treated milk, disulfide bonding with both indirectly and directly absorbed casein molecules was also involved.

3. Changes in the surface protein of the fat globules during ultra-high pressure homogenisation of milk

3.1. Introduction

Milk fat globules are surrounded by a membrane composed mainly of proteins, phospholipids, glycoproteins, triglycerides, cholesterol and enzymes. This membrane, which is known as the milk fat globule membrane (MFGM), consists of several distinct layers of different origins. Precursors of milk lipid globules are formed at the endoplasmic reticulum of mammary epithelial cells and are released into cytosol as lipid droplets surrounded by a monolayer coat of proteins and phospholipids. During secretion, milk fat globules gain the outer bilayer coat from the apical plasma membrane of secretory cells (Keenan & Mather, 2006).

The protein composition of the MFGM is very complex with over 40 different polypeptides, ranging in molecular weight from 15 to 240 kDa (Mather, 2000). The major MFGM proteins are xanthine oxidase (**XO**; 155 kDa), butyrophilin (**BTN**; 67 kDa), PAS 6/7 (49-50 kDa) (Mather, 2000). Since cross-links between MFGM proteins, e.g., XO-BTN, through intermolecular disulfide bonds occur naturally, the protein composition of the MFGM is usually studied with 8% polyacrylamide SDS-PAGE gels under reducing conditions. Previous studies have shown that milk processing, i.e., heating (Ye et al., 2002; Ye et al., 2004a), evaporation (Ye et al., 2004b), and homogenisation (Ye et al., 2008), does not provoke major changes on the composition of native MFGM proteins.

However, milk processing provokes interactions between MFGM components and whey protein and/or caseins (Ye et al., 2004a,b, 2008). Since whey proteins and caseins run out of 8% polyacrylamide gels due to their low molecular masses (~14-25 kDa), resolving gels of 15% polyacrylamide are usually used. In addition, interactions between MFGM and caseins might be through adsorption of casein micelles (indirect adsorption) at the fat globule surface or through direct adsorption and/or bonding of the protein molecules. In order to differentiate direct from indirect adsorption, urea-ethylenediamine-

tetraacetic acid (**EDTA**) buffer is used; washing isolated MFGM material with the buffer dissociates and washes away the casein micelles adsorbed at the fat globule surface, but protein molecules adsorbed directly at the interface of fat globules and the protein molecules bound to the interfacial protein layer via covalent bonds remain on the surface of the fat globule (Ye et al., 2004b).

The research work on rennet coagulation properties (see Section II Chapter 1) showed that ultra-high pressure homogenisation (**UHPH**) enhances the coagulation properties of milk, and such improvement was attributed to changes at the protein-fat structures. Moreover, fat content of milk was proven to greatly influence the protein-protein interactions within rennet curds from UHPH-treated milk (see Section II Chapter 2). The aim of the present study was to examine the effects of UHPH conditions (temperature and pressure) on the milk fat globule and the surface proteins. Needs to be mentioned that the testing of the methods applied in this work resulted in a published study on the MFGM composition of goat's milk (see Annex 2).

3.2. Material and methods

3.2.1. Milk supply and treatments

Raw whole bovine milk was obtained from a local dairy farm (S.A.T. Can Badó, Roca del Vallès, Spain). Milk was standardised at 3.6% fat and kept overnight at 4 ºC. Before all treatments, the milk was warmed to approximately 20 ºC. Single-stage UHPH was carried out at 100, 200 and 300 MPa with inlet temperatures ($\mathbf{T_i}$) of 20, 30 and 40 ºC using the modified model FPG11300 of Stansted Fluid Power Ltd. (see Section I Chapter 2). Milk temperature during UHPH treatment was measured with thermocouples located at different points of the equipment (see Fig. I.2.2). Real inlet temperature ($\mathbf{T_{in}}$) corresponded to milk temperature at the exit of the first heat-exchanger. Milk temperature before and immediately after the Stansted valve were referred as T_1 and T_2. The refrigeration system located between the two valves cooled down milk to T_r. Outlet temperature ($\mathbf{T_{out}}$) was measured after the cooling system which consisted of two heat-exchangers.

UHPH was compared with conventional treatments, i.e., pasteurisation (72 ºC for 15 s) and homogenisation-pasteurisation (15 + 3 MPa at 60 ºC, 72 ºC for 15 s), carried out with a Finamat heat-exchanger (model 6500/010, GEA Finnah GmbH, Ahaus, Germany), and a Niro Soavi homogeniser (model X68P Matr. 2123, Niro Soavi, Parma, Italy).

3.2.2. Mean diameter and specific surface area of fat globules

Determination of the surface-weighted mean diameter [D(3,2)] and the specific surface area (SSA) was carried out using a Beckman Coulter laser diffraction particle size analyser (LS 13 320 series, Beckman Coulter, Fullerton, CA, USA) as described in Section II Chapter 1.

3.2.3. Isolation of MFGM material

MFGM components were obtained following the method of Ye et al. (2002) with modifications. Cream was separated by centrifugation at 10,500 × *g* for 30 min at 20 ºC after addition of 28.6 g of sucrose 100 g^{-1} of milk in order to increase the difference in density between fat and serum phases (Cano-Ruiz & Richter, 1997). After cooling, the top layer (cream) was removed from the centrifuge tube using a spatula. Cream was washed twice for 1 h at room temperature in 10 volumes of simulated milk ultrafiltrate (SMUF; Jenness & Koops, 1962) or SMUF containing 6 M urea and 50 mM EDTA (Ye et al., 2002), centrifuged at 10,500 × *g* for 30 min at 20 ºC and solidified by cooling at 4 ºC in order to remove the top layer, i.e., washed cream.

3.2.4. Determination of washed cream protein content

Total protein content of the washed creams was determined in triplicates through the Dumas combustion method (IDF, 2002) by determining total nitrogen and multiplying by a factor of 6.38. Protein coverage was calculated by dividing the amount of protein per gram washed cream by the SSA of fat globules (Lee & Sherbon, 2002).

3.2.5. Analysis of MFGM protein components

Protein composition of MFGM from washed creams was determined by SDS-PAGE. Samples of washed creams (0.25 g) were dispersed in 0.5 mL Tris-HCl buffer (6% Tris 0.5 M, 10% glycerol, 2% SDS and 0.05% bromophenol blue). For non-reducing PAGE, the samples were heated at 45 ºC for 5 min. For reducing conditions, 5% β-mercaptoethanol was added to the samples before heating at 95 ºC for 5 min in a boiling water bath. In order to remove fat, the samples were centrifuged at 2.500 × *g* for 30 min. 15 µL of supernatants diluted in sample buffer (1:3 v/v) were loaded onto 15% SDS-polyacrylamide gels (37.5% Acryl-Bis at 40% in 1.5 M Tris-HCl buffer, pH 8.8, for separating gel; 10% Acryl-Bis at 40%, in 0.5 M Tris-HCl buffer, pH 6.8, for stacking gel). A molecular mass marker (wide range from 14.4 to 212 kDa, Amresco, Solon, USA) and milk protein standards, i.e., bovine serum albumin, caseins (**CN**), α-lactalbumin (**α-LA**) and β-lactoglobulin (**β-LG**) (Sigma-Aldrich Chemie, Steinheim, Germany) were applied to each gel. Gels were run at 200 V using a Pharmacia Biotech power supply unit (model EPS 3500, Pharmacia Biotech, Uppsala, Sweden). Protein bands were stained with a solution of Coomassie brilliant blue R-250. Gels were destained with a solution of methanol and glacial acetic acid at concentrations of 160 and 10 mL L^{-1}, respectively. Scanned images of the destained gels were analysed using the ImageMaster software (Amersham Pharmacia Biotech, Newcastle, UK). The apparent molecular mass (**Mr**) of the bands on the SDS-PAGE was estimated from the mobility of proteins in the gel when compared with the mobility of the molecular mass markers. Protein identification was carried out by comparison with protein standards, for caseins and whey proteins (Sigma-Aldrich Chemie), and with results from previous studies for MFGM proteins (Ye et al., 2002; Ye et al., 2004b; Singh, 2006).

3.2.6. Transmission electron microscopy

Microstructure of milk was observed by transmission electron microscopy (TEM). Milk samples were mixed with glutaraldehyde (3% final concentration) in a bijou bottle and then mixed with warm 2% low-temperature gelling agar at a 1:1 ratio. The mixture was allowed to gel and was chopped into 1 mm^3 cubes.

The cubes were then washed as follows: with 0.1 M sodium cacodylate buffer pH 7.2 for 30 min, then again twice for 1 h, with 1 mL of a solution containing 50% osmium tetroxide (2% solution) and 50% cacodylate/HCL buffer for 2 h, with 1 mL of % uranium acetate for 30 min, and finally with water. Dehydration consisted of washing with ethanol at increasing concentration and time (50%, 70%, 90%, and 100% for 5 + 30, 30, 180, and 30 + 60 min). The ethanol was poured off and the bottle was filled with incomplete resin [20 mL epoxy resin, 20 mL dodecylsuccinic anhydride (DDSA) and 1 drop of dibutyl phthalate] and placed on a rotator overnight. Incomplete resin was replaced with complete resin [incomplete formulation with addition of 0.6 mL of the plasticiser benzyldimethylamine (BDMA)] and then placed on the rotator for 4 h. Sample cubes were added to moulds containing fresh complete resin which and baked overnight at 60 ºC. After cutting (0.03-0.05 μm) with a Reichert Ultracut microtome, they were mounted on 3 mm copper grids and stained using uranyl acetate and lead citrate. Examination was performed with a Philips 201 transmission electron microscope at an accelerating voltage of 60 kV (Philips, NL-5600 MD, Eindhoven, Netherlands).

3.2.7. Statistical analysis

Data were processed by one-way ANOVA (Statgraphics Inc., Chicago, IL, USA). LSD test was used for comparison of sample data, and evaluations were based on a significance level of $P < 0.05$.

3.3. Results

3.3.1. Temperatures reached during UHPH treatments

Temperatures reached throughout the UHPH equipment are shown in Table II.3.1. Milk temperature increased by ~10 ºC between T_{in} and T_1; such increase could be attributed to adiabatic heating as result of the pressure exerted by the valve. Maximum values were observed at T_2. Indeed, high turbulence, shear and cavitation forces that are produced after the valve, through the release of pressure, increases the temperature of the fluid (Hayes & Kelly, 2003).

Table II.3.1. Temperatures (ºC) reached during ultra-high pressure homogenisation treatments[1].

Treatment		T_{in}	T_1	T_2	T_r	T_{out}
20 ºC	100 MPa	19.9	27	48	33	15.1
	200 MPa	20.2	30	65	46	17.8
	300 MPa	20.5	32	76	57	16.5
30 ºC	100 MPa	29.8	36	56	38	16.5
	200 MPa	30.2	38	72	49	18.6
	300 MPa	30.2	40	86	61	19.3
40 ºC	100 MPa	40.6	45	60	42	17.8
	200 MPa	39.0	47	81	54	20.3
	300 MPa	40.7	49	92	66	20.0

[1] T_{in}: real inlet temperature; T_1: temperature before the 1st valve; T_2: temperature after the 1st valve; T_r: temperature after the refrigeration system; T_{out}: outlet temperature after the cooling system.

Table II.3.2. Surface-weighted mean diameter [D(3,2)] and specific surface area (SSA) of fat globules[1], total protein of washed creams, and protein coverage of fat surface.

Treatment[2]		D(3,2) (nm)	SSA (m^2 mL^{-1})	Total protein (g 100 g^{-1} fat)	Protein coverage (mg m^{-2})
Raw		3,233 ± 5	1.86 ± 0.00	0.31 ± 0.05 ^{e}	1.53 ± 0.25
PA		3,214 ± 10	1.87 ± 0.01	0.43 ± 0.03 ^{e}	2.10 ± 0.14
HP		255 ± 11	23.57 ± 0.97	3.54 ± 0.07 ^{a}	1.37 ± 0.03
UH					
100 MPa	20 ºC	286 ± 10 ^{a}	20.96 ± 0.71 ^{e}	0.24 ± 0.09 ^{e}	0.10 ± 0.04 ^{d}
	30 ºC	190 ± 9 ^{b}	31.66 ± 1.52 ^{d}	1.38 ± 0.07 ^{c}	0.40 ± 0.02 ^{c}
	40 ºC	183 ± 13 ^{b}	32.87 ± 2.26 ^{d}	1.88 ± 0.14 ^{d}	0.52 ± 0.04 ^{a,b}
200 MPa	20 ºC	182 ± 9 ^{b}	32.97 ± 1.63 ^{d}	0.36 ± 0.07 ^{e}	0.10 ± 0.02 ^{d}
	30 ºC	147 ± 3 ^{d}	40.75 ± 0.85 ^{b}	1.53 ± 0.08 ^{c,d}	0.35 ± 0.02 ^{c}
	40 ºC	126 ± 5 ^{e}	47.61 ± 2.05 ^{a}	2.61 ± 0.11 ^{b}	0.50 ± 0.02 ^{b}
300 MPa	20 ºC	166 ± 7 ^{c}	36.25 ± 1.43 ^{c}	2.44 ± 0.47 ^{b}	0.62 ± 0.12 ^{a}
	30 ºC	149 ± 5 ^{d}	40.29 ± 1.16 ^{b}	2.38 ± 0.38 ^{b}	0.54 ± 0.09 ^{a,b}
	40 ºC	147 ± 4 ^{d}	40.83 ± 0.98 ^{b}	2.33 ± 0.30 ^{b}	0.52 ± 0.07 ^{a,b}

[a-e] Mean value ± s.d.; n = 3; values without common superscripts were significantly different ($P < 0.05$).

[1] Results obtained with the Beckman Coulter LS software; for raw and pasteurised milk, data correspond to the fat globule peak; for homogenised samples, the whole distribution was selected.

[2] PA: pasteurisation; HP: homogenisation-pasteurisation; UH: ultra-high pressure homogenisation.

However, T_2 values obtained in this study were ~10 ºC lower than those of previous studies (Pereda et al., 2007; see Section II Chapter 1). Moreover, plotting T_2 vs. pressure revealed linear temperature increases of 14, 15 and 16 ºC for 100 MPa at T_i of 20, 30 and 40 ºC, with r^2 of 0.985, 0.999 and 0.969, respectively (average linear temperature increase, by plotting ($T_2 - T_{in}$) vs. pressure, of 14.8 ºC per 100 MPa with $r^2 = 0.940$).

Such values were lower than those previously reported; Pereda et al. (2007) observed an increase of 19.15 ºC per 100 MPa with T_i of 40 ºC, and Thiebaud et al. (2003) obtained an increase of 18.5 ºC with T_i of 4-24 ºC. Increases of 16.6 and 17.6 ºC were observed by Hayes & Kelly (2003) working at 150-250 MPa with T_i of 45 ºC, and at 50-200 MPa with T_i of 6-10 ºC, respectively.

The improvements performed on the UHPH equipment (see Section I Chapter II) might have originated the differences observed with previous studies carried out in our laboratories; the refrigeration system inserted between the two valves contributed to lowering not only T_{out} but also T_2, probably through thermal conductivity in the metallic pipes.

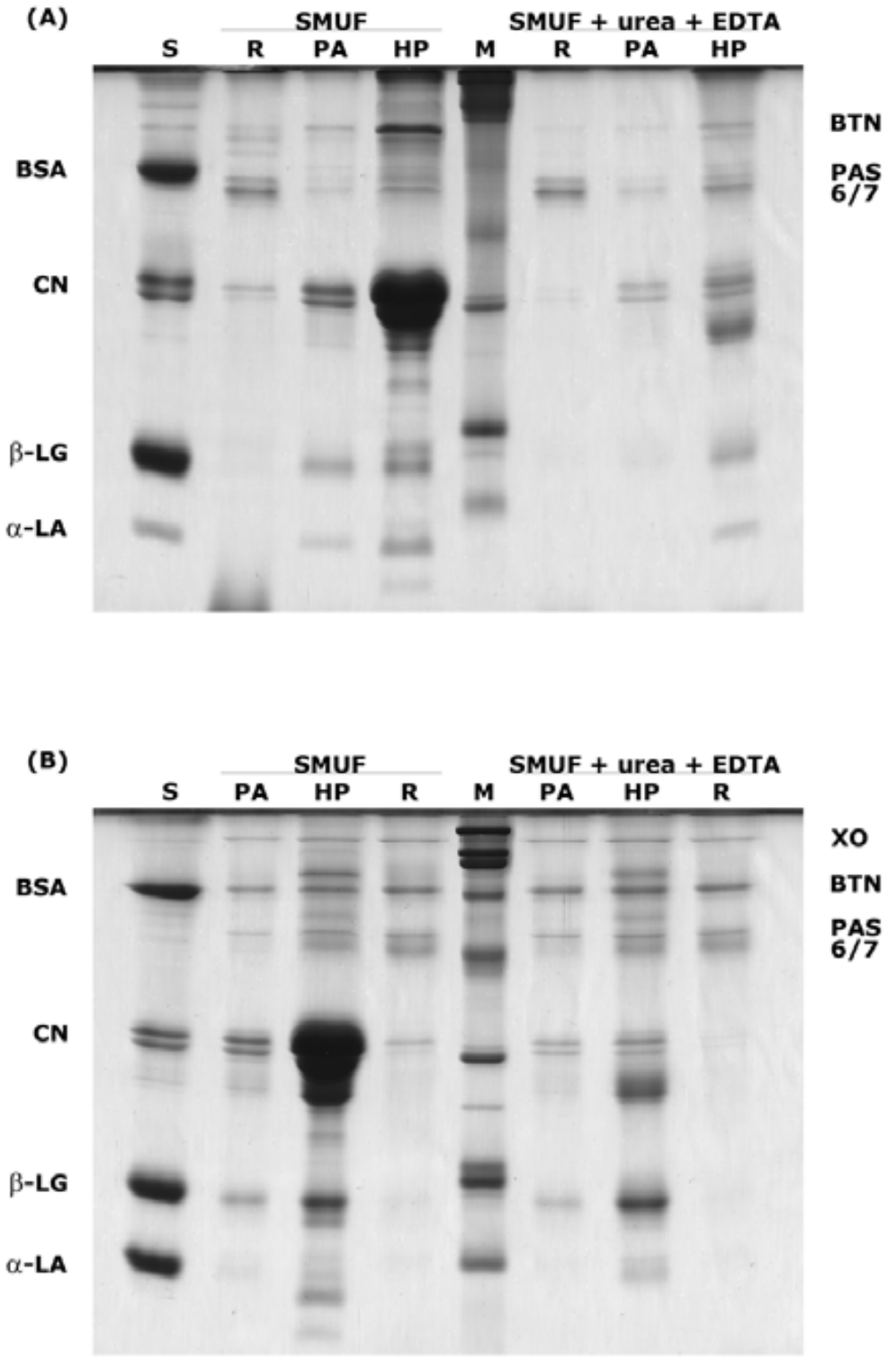
3.3.2. Mean diameter and specific surface area of fat globules

Homogenisation treatments of milk reduced the size of fat globules provoking a concomitant increase in the SSA (Table II.3.2). The effect of 100 MPa at T_i of 20 ºC on D(3,2) and SSA was similar to that of conventional homogenisation-pasteurisation. At 100 MPa, increasing T_i from 20 to 30 ºC decreased significantly the fat globule size. UHPH at 200 MPa with T_i of 20 ºC reduced D(3,2) down to values obtained at 100 MPa with T_i either of 30 or 40 ºC. At 200 MPa, further decrease was obtained as T_i was increased. The effect of 300 MPa at T_i of 20 ºC was in between those of 200 MPa at 20 and 30 ºC. Increasing the T_i from 20 to 30 ºC at 300 MPa led to a reduction of the D(3,2) comparable to that of 200 MPa at 30 ºC. However, further T_i increase did not affect the size of fat globules.

3.3.3. Protein content and fat surface coverage

Although not being statistically significant (Table II.3.2), pasteurisation treatment increased the amount of total protein in washed creams leading to higher amount of protein coverage than in raw milk. The amount of total protein in washed creams from homogenised-pasteurised milk was by far the highest. These results combined with the fact that, conventional homogenisation-pasteurisation triggered an important reduction of D(3,2) led to a great amount of protein covering the fat surface. Except for treatments at 200 and 300 MPa with a T_i of 20 ºC, UHPH triggered significant increases on the total protein

Figure II.3.1. SDS-PAGE patterns of membrane material isolated from control milk samples under (A) non-reducing and (B) reducing conditions [S: protein standards; M: molecular weight markers of 212, 116, 97, 66, 45, 31, 21, 20 and 14 kDa; R: raw milk; PA: pasteurised milk; HP: homogenised-pasteurised milk; BSA: bovine serum albumin; CN: casein; β-LG: β-lactoglobulin; α-LA: α-lactalbumin; XO: xanthine oxidase; BTN: butyrophilin].



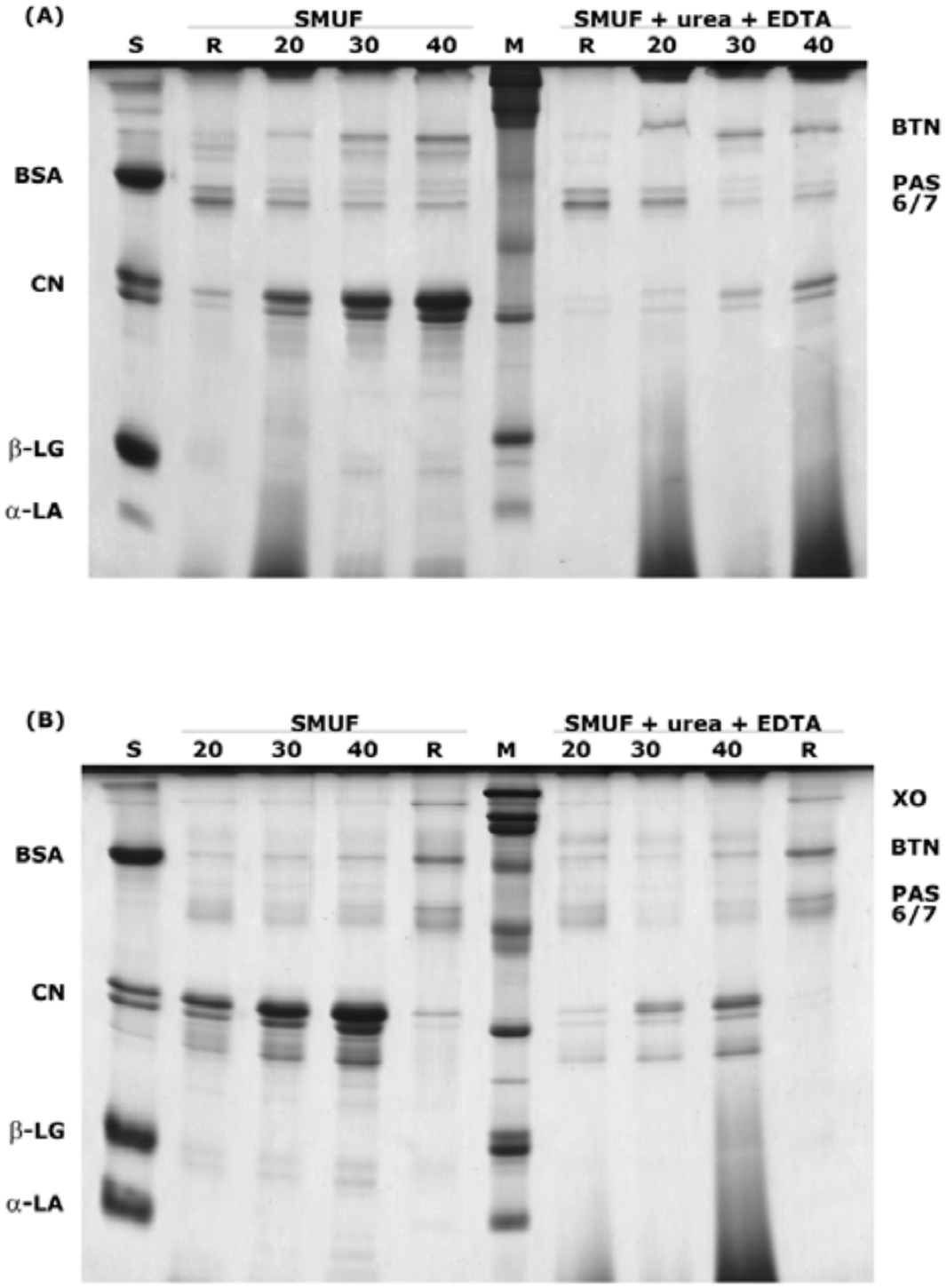
content of washed creams. Two main groups could be distinguished: 1) treatments at 100 MPa with T_i of 30 and 40 ºC and at 200 MPa with a T_i of 30 ºC, and 2) treatments at 200 MPa with T_i of 30 and 40 ºC and at 300 MPa. Increasing T_i led to a significant increase in the total protein, except for treatments at 300 MPa, which showed the same amount as that of washed creams from UHPH-treated milk at 200 MPa with T_i of 40 ºC. However, as the amount of protein covering the fat surface takes into account both the protein load as well as the SAA, UHPH treatments at 300 MPa led to the highest amounts of protein coverage, especially with T_i of 20 ºC.

3.3.4. Electrophoretic patterns

The SDS-PAGE patterns under non- and reducing conditions are shown in Figs. II.3.1-4. Only the main native MFGM proteins, i.e., XO, BTN and PAS 6/7, could be observed. Some CN, especially α_s-CN, was present in washed cream from raw milk, but were washed away with urea-EDTA buffer (Fig. II.3.1). Hence, some micelle caseins in raw milk would be adsorbed rather than covalently bound at the fat globule surface. Pasteurisation treatment of milk triggered the incorporation of CN, i.e. α_s-CN and β-CN, and whey proteins, i.e., β-LG and α-LA. After washing with urea-EDTA buffer, some CN and β-LG remained; the bands corresponding to these proteins were much obvious under reducing conditions, indicating that their incorporation involved covalent bonding to some extent. In addition, β-LG bands fainted after washing with urea-EDTA buffer; hence, some β-LG was retained in the MFGM material through interactions with casein micelles. No major differences were observed in band intensity of the main native MFGM proteins, except for a fading of PAS 6/7 bands.

Major changes were observed in the pattern of washed cream from conventionally homogenised-pasteurised milk (Fig. II.3.1). The intensity of the bands corresponding to CN and whey proteins bands was increased. Little amounts of caseins, which were greatly incorporated in the MFGM material, remained after washing with urea-EDTA buffer; thus, almost all CN was incorporated through adsorption of micelle caseins. In addition, a band with Mr of ~27 kDa, was clearly apparent. In previous studies, such band was attributed

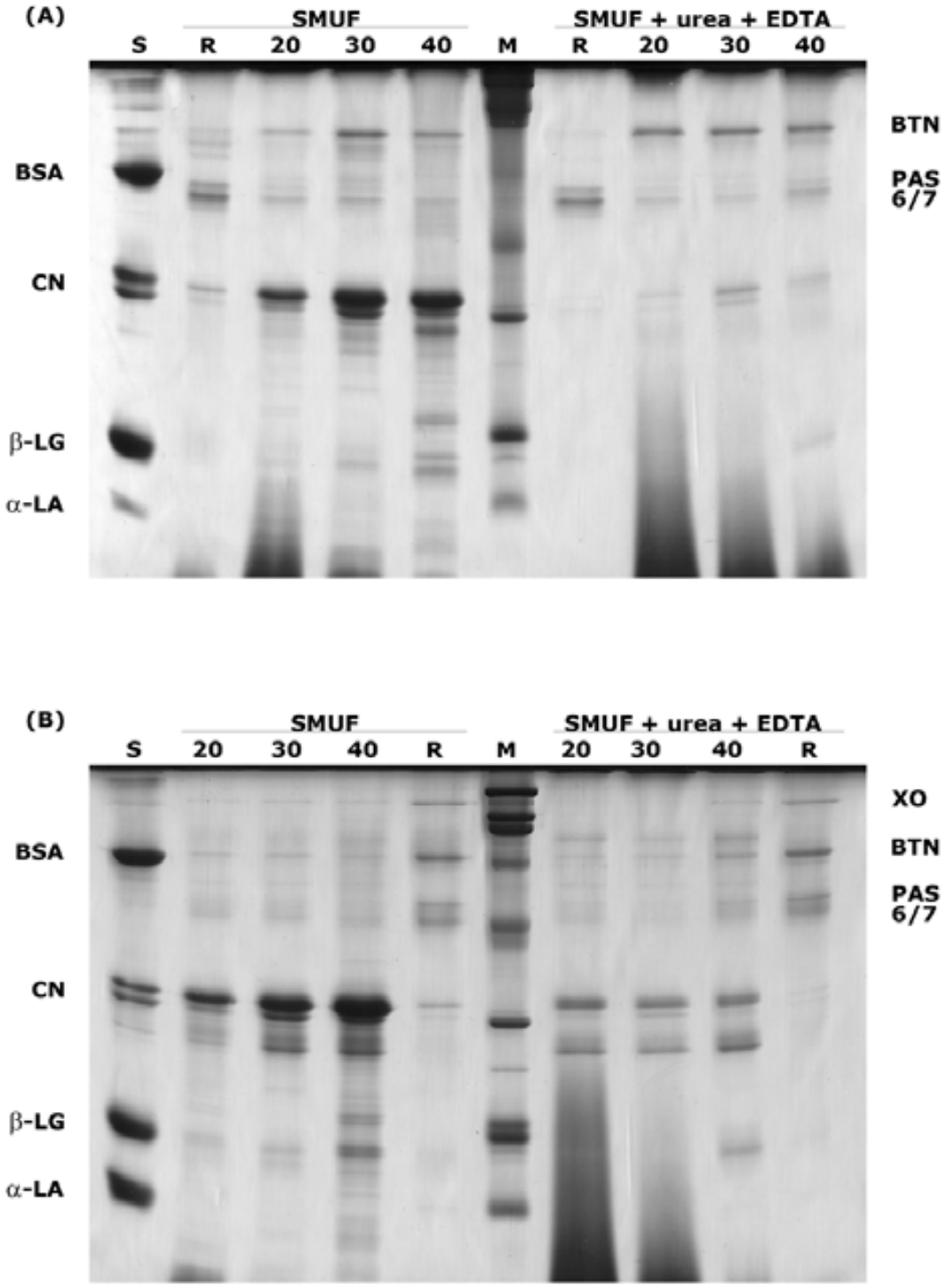
Figure II.3.2. SDS-PAGE patterns of membrane material isolated from milk treated by ultra-high pressure homogenisation at 100 MPa with different inlet temperatures (T_i) under (A) non-reducing and (B) reducing conditions [S: protein standards; M: molecular weight markers of 212, 116, 97, 66, 45, 31, 21, 20 and 14 kDa; R: raw milk; 20: T_i of 20 ºC; 30: T_i of 30 ºC; 40: T_i of 40 ºC; BSA: bovine serum albumin; CN: casein; β-LG: β-lactoglobulin; α-LA: α-lactalbumin; XO: xanthine oxidase; BTN: butyrophilin].



to κ-CN (Cano-Ruiz & Richter, 1997; Ye et al., 2004b). Moreover, no differences between non- and reducing conditions were observed in the intensity of the bands corresponding to CN, indicating that the incorporation of some CN molecules, directly adsorbed to the surface, was not through covalent bonding. Intensity of the bands corresponding to whey proteins was higher than those of washed creams from pasteurised milk. Washing with urea-EDTA buffer did not affect whey proteins bands; but under reducing conditions, the bands had higher intensity, meaning that conventional homogenisation-pasteurisation treatment triggered the incorporation of whey proteins, especially β-LG, through covalent bonding with the interfacial protein layer of the fat globules. Concerning the native MFGM proteins, no major changes were observed, except for the presence of two bands with Mr of ~75 and ~58 kDa, present only under reducing conditions. After washing with urea-EDTA buffer, no differences were observed in their intensity, suggesting that these proteins were directly adsorbed at the fat surface through covalent bonding. Bands with Mr ~75 and ~58 kDa were also observed on the fat globule surface after concentration of whole through evaporation, and were attributed to the secretory and the heavy chain immunoglobulin components, respectively (Ye et al., 2004b).

Non-reducing SDS-PAGE gels of washed creams from UHPH treated milk samples showed that the amount of dense material stuck between stacking and separation gels was greater than for the conventional homogenisation-pasteurisation treatment (Figs. II.3.2-4). The comparison of patterns between non- and reducing conditions allowed ascribing it to CN and β-LG. UHPH at 100 MPa led to the incorporation of CN, although at lower extent than conventional homogenisation-pasteurisation treatment (Fig. II.3.2). Increasing T_i led to higher incorporation of CN. After washing with urea-EDTA, the intensity of the bands faded but also darkened as T_i increased. In contrast to conventional homogenisation-pasteurisation, the main CN involved was α_s-CN. Moreover, the intensity of the bands did not vary between non- and reducing conditions, except for the band that corresponded to κ-CN; this band was almost absent under non-reducing condition, but very apparent under reducing conditions. Hence, the incorporation of CN in the MFGM material was through

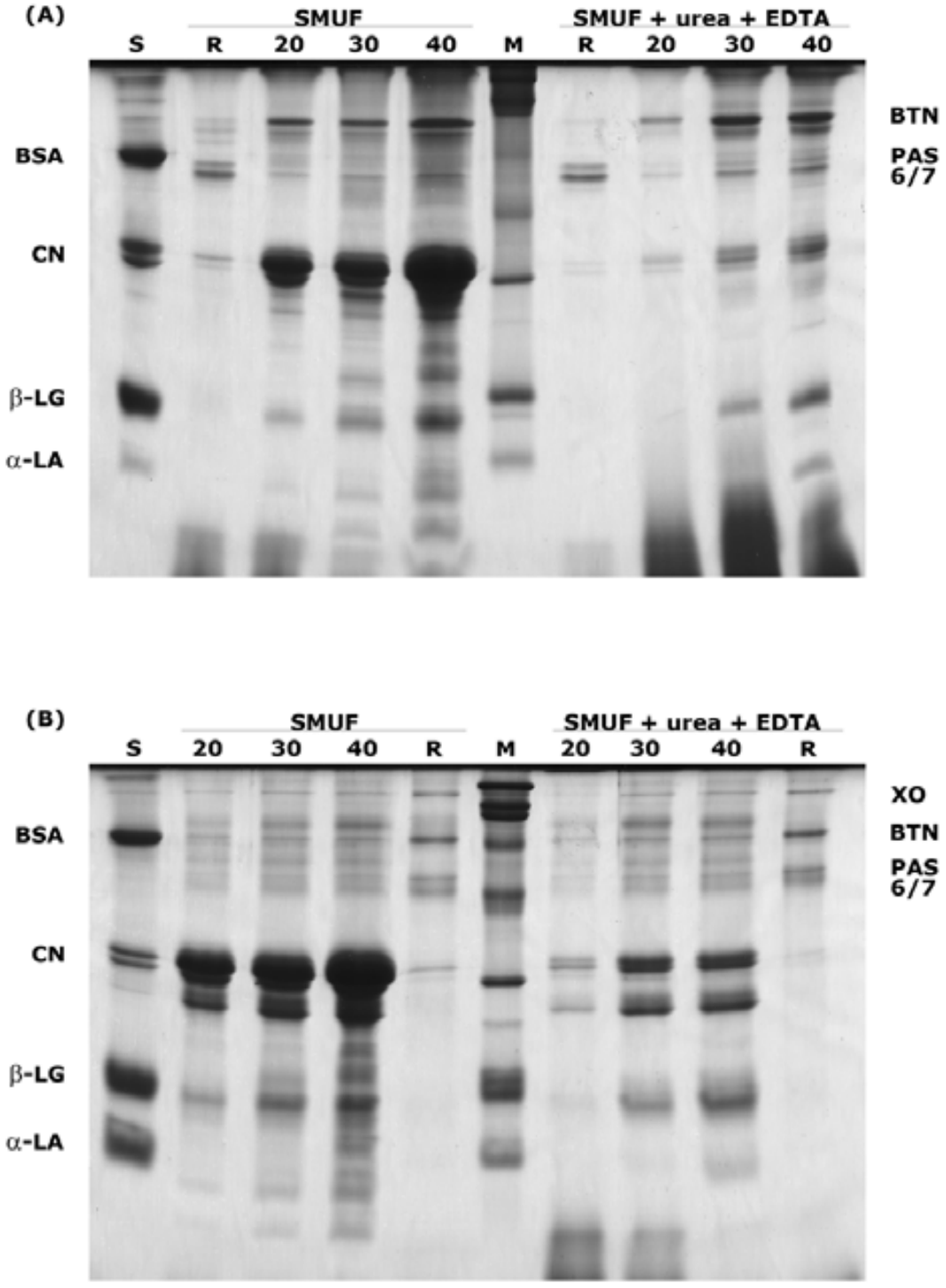
Figure II.3.3. SDS-PAGE patterns of membrane material isolated from milk treated by ultra-high pressure homogenisation at 200 MPa with different inlet temperatures (T_i) under (A) non-reducing and (B) reducing conditions [S: protein standards; M: molecular weight markers of 212, 116, 97, 66, 45, 31, 21, 20 and 14 kDa; R: raw milk; 20: T_i of 20 ºC; 30: T_i of 30 ºC; 40: T_i of 40 ºC; BSA: bovine serum albumin; CN: casein; β-LG: β-lactoglobulin; α-LA: α-lactalbumin; XO: xanthine oxidase; BTN: butyrophilin].



the adsorption of casein micelles and in a lower extent through direct adsorption of protein molecules, i.e., α_s-CN, and covalent bonding, i.e., κ-CN. A very faint band corresponding to β-LG appeared both under non- and reducing conditions. In addition, gel patterns showed some fading of the bands corresponding to the native MFGM proteins and the presence of bands with Mr of ~75 and ~58 kDa, also detected in washed creams from conventionally homogenised-pasteurised milk.

Almost no differences were observed in washed creams from UHPH-treated milk at 200 MPa compared with 100 MPa, except for an increase in β-LG at T_i of 40 ºC, which remained after washing with urea-EDTA buffer (Fig. II.3.3). In contrast, major differences in the intensity of the bands were detected in UHPH-treated milk at 300 MPa (Fig. II.3.4); much greater amounts of both CN and whey proteins were observed compared with the other UHPH treatments. The increase of T_i between 20 and 30 ºC did not affect the amount of CN in washed creams. However, after washing with urea-EDTA buffer, the amount of remaining CN was higher in MFGM material from UHPH-treated milk with a T_i of 30 ºC than that of 20 ºC. Hence, UHPH treatment at 300 MPa with a T_i of 30 ºC enhanced the direct incorporation of proteins instead of the incorporation through casein micelle adsorption. At 300 MPa with T_i of 40 ºC, UHPH treatment resulted in a further increase of CN content. After washing with urea-EDTA buffer and under reducing conditions, the intensity of CN bands did not vary between samples from T_i of 30 and 40 ºC. Thus, increasing T_i from 30 to 40 ºC did not increase the amount of directly adsorbed CN rather increased the amount of adsorbed casein micelles. As previously mentioned, UHPH treatment at 300 MPa provoked the incorporation of whey proteins, with an increase concomitant to that of Ti. Washing with urea-EDTA buffer resulted in a slight fading of whey protein bands, indicating that the incorporation of whey proteins was in a low extent through interactions with casein micelles. Considering the native MFGM proteins, some fading was also observed, but increasing T_i better revealed the bands with Mr of ~75 and ~58 kDa.

Figure II.3.4. SDS-PAGE patterns of membrane material isolated from milk treated by ultra-high pressure homogenisation at 300 MPa with different inlet temperatures (T_i) under (A) non-reducing and (B) reducing conditions [S: protein standards; M: molecular weight markers of 212, 116, 97, 66, 45, 31, 21, 20 and 14 kDa; R: raw milk; 20: T_i of 20 ºC; 30: T_i of 30 ºC; 40: T_i of 40 ºC; BSA: bovine serum albumin; CN: casein; β-LG: β-lactoglobulin; α-LA: α-lactalbumin; XO: xanthine oxidase; BTN: butyrophilin].



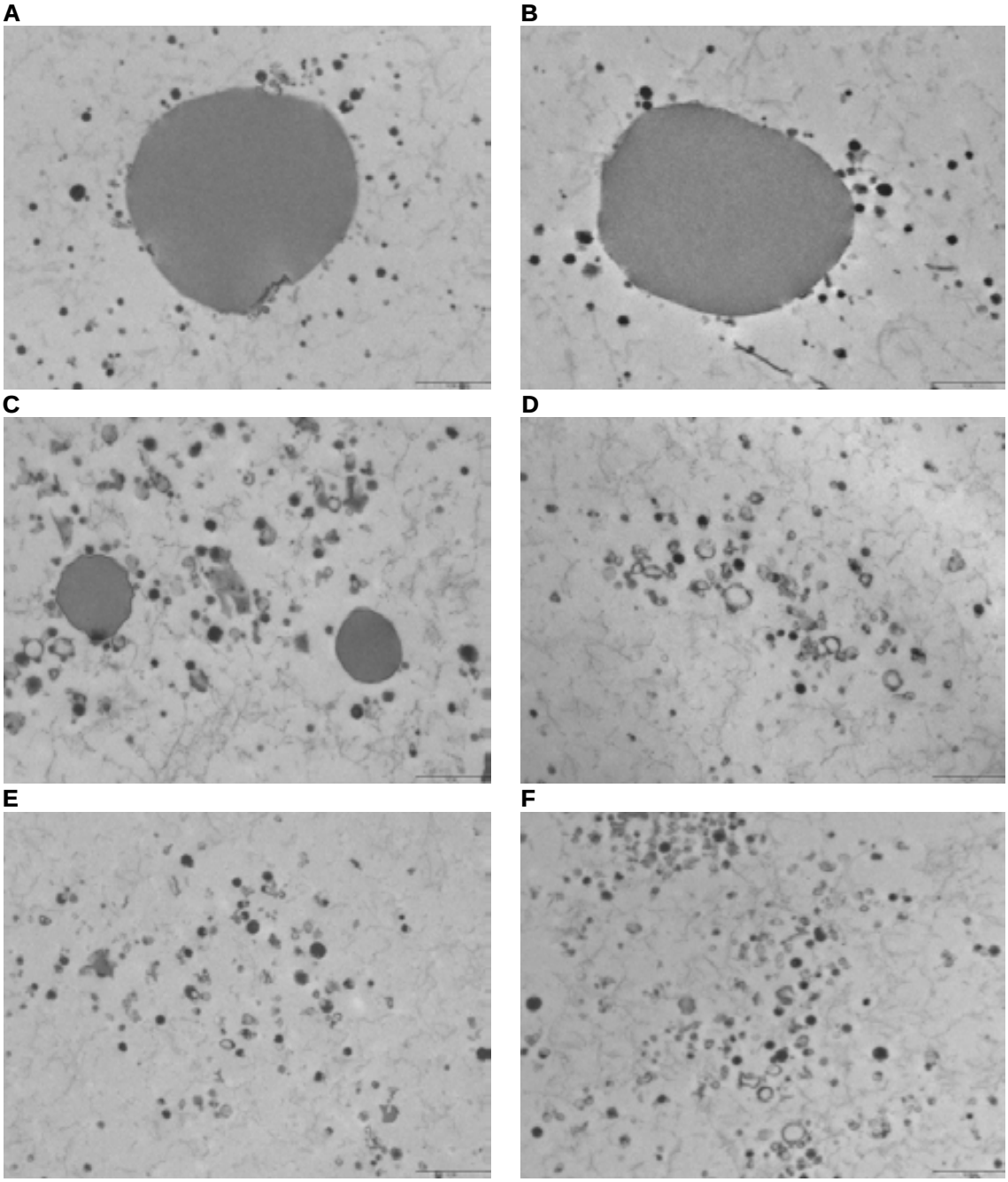
3.3.5. Electron microscopy

Electron micrographs of raw milk (Figs. II.3.5-7 A) showed casein micelles individually dispersed and large round fat globules with well-defined MFGM; both the bilayer and the monolayer could be observed at 100× augmentations (Fig. II.3.7 A). In pasteurised milk, the size of casein micelles seemed to be larger and denser compared with raw milk (Fig. II.3.5 B). Some damage at the MFGM could be observed; few globules had lost their integrity resulting in empty cores (Fig. II.3.6 B). The bilayer of the MFGM was less apparent, and fat globules were surrounded by a fuzzy and less dense material, onto which some casein micelles were adsorbed (Fig. II.3.7 B).

Conventional homogenisation-pasteurisation reduced the size of fat globules, of which two main populations could be observed (Fig. II.3.5 C): round middle size fat globules (~0.8 μm) with some casein micelles adsorbed at the MFGM (Fig. II.3.6 C), and smaller heterogeneous particles (~0.2 μm) enveloped with MFGM (Fig. II.3.7 C). The latter formed aggregates directly by MFGM interaction, which appeared more electrodense at the point of contact, or through bridges of whey protein (less electrodense material in the micrographs). Small casein micelles were adsorbed onto the MFGM of the new particles, but they did not contribute to the aggregation phenomenon.

Electron micrographs of UHPH treatments with T_i of 40 ºC (Figs. II.3.5-7 D-F) were consistent with the fat globule size distribution results; a complete reduction of fat globules, which corresponded to the obtained monomodal distributions, was observed (Fig II.3.5 D-F). Newly formed particles, which were very heterogeneous in shape and size, consisted of aggregates of very small fat globules, casein micelles and some whey proteins. Individually dispersed casein micelles were slightly smaller and less electrodense, especially at 300 MPa (Fig. II.3.6 F). Adsorption of casein micelles at the MFGM of larger fat globules was observed (Fig. II.3.7 F). However, at pressures ≥ 200 MPa an opposite phenomena occurred; middle size casein micelles were those absorbing at their surface small fat globules (Fig. II.3.7 E-F). In addition, at such pressures, some disintegrated MFGM material did not reform globular particles.

Figure II.3.5. Electron micrographs at 15× augmentations of (A) raw, (B) pasteurised, (C) homogenised-pasteurised, and ultra-high-pressure homogenised milk samples at (D) 100 MPa, (E) 200 MPa and (F) 300 MPa with an inlet temperature of 40 ºC [bar = 1 μm].



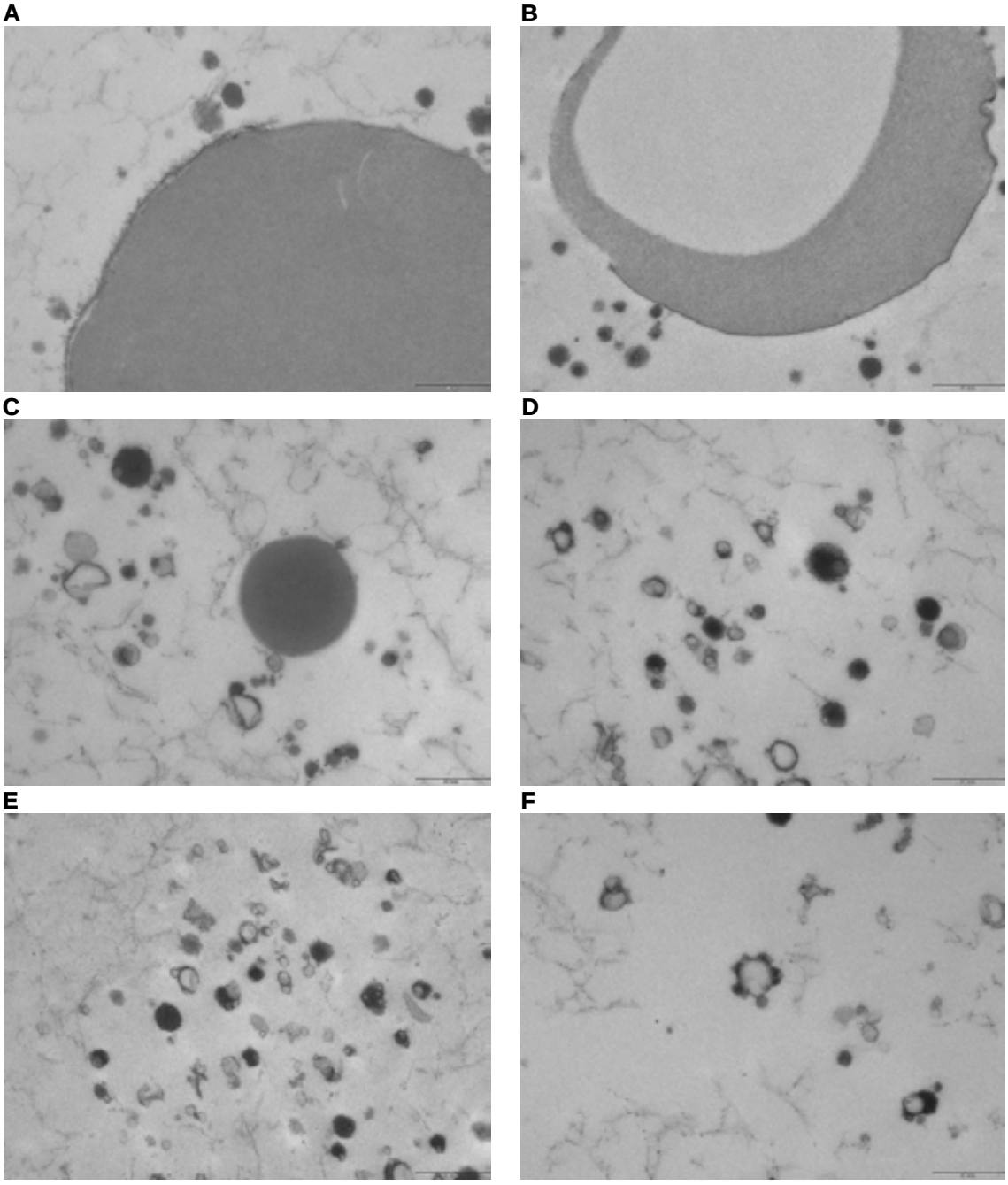
3.4. Discussion

In raw whole milk, the size of particles follows a bimodal distribution characterised by a peak corresponding to casein micelles (at 0.2 μm with 25% volume of milk particles) and a second peak to fat globules (at 3.5 μm and with 75% volume). Beckman Coulter LS software allows selecting populations of the particle distribution; the calculated SSA of the particles corresponding to the fat globules was ~1.9 m^2 mL^{-1}. But, since not all software allow the virtual segregation of casein micelles from fat globules, dissociating buffers, such as EDTA, urea and SDS, have been used prior to light scattering analysis. Upon dissociation of raw whole milk, Michalski et al. (2002a) estimated the SSA of fat globules to be 1.6 m^2 mL^{-1} on a volumic basis corresponding to 1.75 m^2 g^{-1} on a massic basis, which takes into account the density of milk fat (0.916 g ml^{-1} at 20 ºC). However, using dispersing agents, the SSA was overestimated, e.g., ~5 m^2 g^{-1} (Ye et al., 2002). In the present study, an attempt of using dissociating agents resulted in a decrease of globule diameter (from 3.5 to 2.9 μm) and casein micelles were still present (10% volume of milk particles); since the method resulted in the loss of the largest fat globules and did not ensure the complete dissociation of casein micelles, using this method was discarded.

The SSA allows calculating the surface protein load of fat globules by dividing the protein content of washed creams by the SSA on a massic basis. Hence, differences on estimated SSA results in differences in the calculated surface protein coverage. Moreover, differences on the method of MFGM isolation, which might affect the recovery of MFGM material (Mather, 2000; Danthine et al., 2000; Singh, 2006), could also contribute to differences between studies.

Upon homogenisation, globule size is reduced down to that of micelle caseins, resulting in either a bimodal distribution with a second small peak, or a monomodal distribution with a unique peak. At 100 MPa, increasing T_i from 20 to 40 ºC led to a decrease of the second peak, which corresponded to 30, 8 and 2% of particles volume in T_i of 20, 30 and 40 ºC, respectively. At 200 MPa, the peak disappeared with T_i above 20 ºC. In contrast, at 300 MPa with T_i of 40 ºC the second peak was present indicating that coalescence and aggregation

Figure II.3.6. Electron micrographs at 30× augmentations of (A) raw, (B) pasteurised, (C) homogenised-pasteurised, and ultra-high-pressure homogenised milk samples at (D) 100 MPa, (E) 200 MPa and (F) 300 MPa with an inlet temperature of 40 ºC [bar = 0.5 μm].

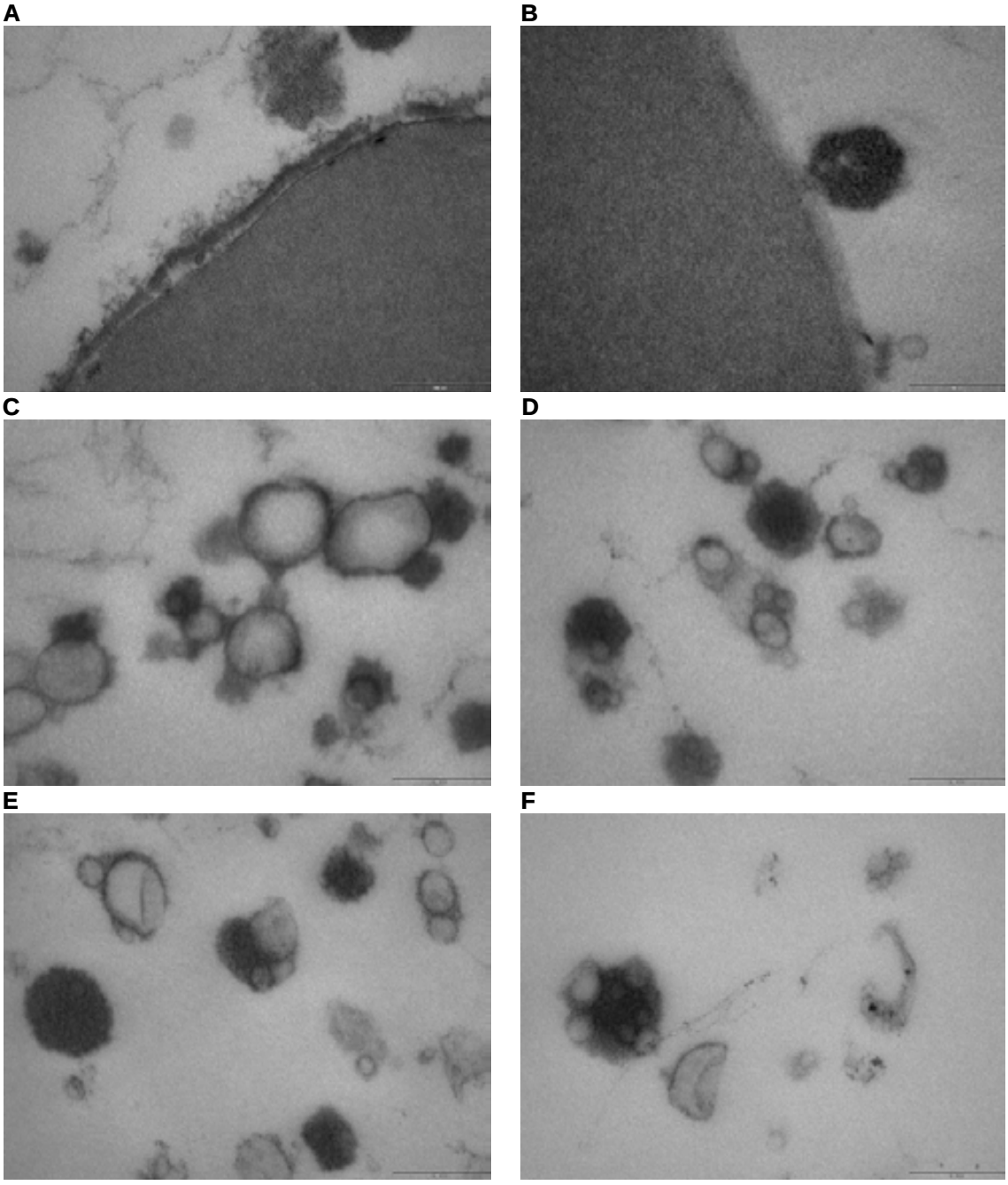


phenomena occurred. Hence, the state of the milk fat is a major factor for UHPH treatment. Transmission electron micrographs showed that conventional homogenisation-pasteurisation and UHPH treatments produced new particles which could not be accounted for either casein micelles or fat globules.

Reduction of fat globule size implied two handicaps of the method, which were unsolved neither in this study nor in previous ones. Segregation of casein micelles from fat globules with the software was not any longer feasible. In one of the first studies on MFGM, McCrae and Muir (1991) estimated the globule size in recombined milk through homogenisation as the volume per cent of fat globules above a specified threshold (0.5 μm). After the observation of transmission electron micrographs, this procedure was discarded. Corredig and Dalgleish (1996) determined the particle size distribution of fat globules after separation of milk; in the present study such determination was not possible since the amount of recovered fat after centrifugation was very little, especially for UHPH-treated samples. In a recent study, dynamic light scattering was used, together with dispersing buffers, to estimate the particle size of milk after mechanical treatments (Michalski et al., 2002a).

Since the formation of new particles during homogenisation involved the adsorption of casein micelles, the use of dissociating agents was expected to greatly influence the particle size analysis; the disappearance of casein micelles through dissociation should trigger an increase in the diameter of intact particles, i.e. fat globules. Unexpectedly, the attempt of using dissociating agents in homogenised milk samples resulted in a diminution of the particle size diameter. For homogenised-pasteurised and UHPH-treated samples showing a bimodal distribution, lower particle size was due to a reduction of the 2nd peak, which should correspond to native fat globules (particles > 1 μm), e.g. from 14 to 9% volume of milk particles in homogenised-pasteurised sample. In contrast, for UHPH-treated milk showing a monomodal distribution, the reduction in particle size was mainly due to a narrowing of the peak, i.e., 40-900 vs. 40-400 nm for non-dissociated and dissociated samples, respectively. Such results were also taken into account when discarding the dissociation method.

Figure II.3.7. Electron micrographs at 100× augmentations of (A) raw, (B) pasteurised, (C) homogenised-pasteurised, and ultra-high-pressure homogenised milk samples at (D) 100 MPa, (E) 200 MPa and (F) 300 MPa with an inlet temperature of 40 ºC [bar = 0.2 μm].



In addition, reduction of fat globule size implies a change in the density of milk fat. Although commonly found in literature (e.g. Cano-Ruiz & Richter, 1997; Lee & Sherbon, 2002; Ye et al., 2007, 2008), using the value of raw milk, for the calculation of the SSA of fat globules on a massic basis in homogenised milk samples, entails an unsolvable error. Since the fat density in homogenised milk is unknown, the SSA should be expressed as a volumic quantity; however, the protein coverage can not be calculated from the SSA on a volumic basis. In some cases, such difficulties are concealed by either not showing the results of raw milk or the SSA values of homogenised samples (e.g. Cano-Ruiz & Richter, 1997; Ye et al., 2007).

In the present study, different ways in the calculation of SSA were performed. For raw and pasteurised milk samples, fat globule SSA was estimated by selecting the peak corresponding to fat globules with the software. For homogenised milk samples, the whole distribution was taken into account; including individually dispersed casein micelles resulted in an overestimation of the fat globules SSA. Hence, the results on both SSA and protein coverage of fat globules of non-homogenised and homogenised samples can not be compared; however, the results would be comparable among homogenised samples as long as the differences in the distribution shape are taken into account.

SDS-PAGE electrophoretograms of washed creams from raw milk showed that very small amounts of CN were present at the MFGM. Dalgleish and Banks (1991) attributed it to an inadvertent partial homogenisation during the handling of the milk. It must be pointed out that the staining intensity may vary between protein species. Hence, it may not represent actual protein content, but highlights differences between samples and changes during processing.

Upon heating, the protein load of MFGM material is increased due to the incorporation, partially via covalent bonding, of whey proteins and, in a lesser extent, of CN. A number of studies have shown that β-LG associates with the MFGM (Dalgleish & Banks, 1991; Houlihan et al., 1992; Kim & Jiménez-Flores, 1995; Corredig & Dalgleish, 1996; Lee & Sherbon, 2002; Ye et al., 2002, 2004a); this association was observed in heated milk at temperatures of 60-65 ºC,

which were lower than the denaturation temperature of β-LG (78 ºC; Jelen & Rattray, 1995). Two mechanisms have been suggested for the association of β-LG with the MFGM: via disulphide bonding (Houlihan et al., 1992), or via displacement of the original MFGM material (Dalgleish & Banks, 1991). The results obtained by Corredig and Dalgleish (1996) strongly suggested that the interaction of whey proteins with intact milk fat globules was not simply displacement of the original membrane by absorbing whey protein. In the present study, the loss of β-LG by washing with dissociation buffer indicated that some β-LG was retained at the MFGM by interacting with casein micelles adsorbed onto the surface of the MFGM. Indeed, upon heating skim milk, whey proteins, especially β-LG, associate with casein micelles resulting in an increase in the casein micelle size (Anema & Li, 2003). Moreover, hair-like structures have been observed on the surface of casein micelles in heated milk samples (Davies et al., 1978; Mohammad & Fox, 1987) and have been described as κ-CN/β-LG complexes (Singh & Fox, 1987). In the present study, larger casein micelles were observed by transmission electron microscopy in pasteurised than raw milk samples.

Conflicting results have been reported with respect to the association of α-LA with the MFGM proteins. Dalgleish and Banks (1991) did not detect α-LA in the MFGM of milk that had been heated at 80 ºC. In some studies, other authors detected that the level of α-LA increased with heating time at 80 ºC (Houlihan et al., 1992; Lee & Sherbon, 2002) but, in the earliest study, α-LA was considered as an artefact (Houlihan et al., 1992). Ye et al. (2002) observed that α-LA was present in the MFGM material isolated from milk that had been heated at ≥ 65 ºC, but the band was fainter and fuzzier compared to β-LG band. In the present study, a very faint band could be discerned under reducing conditions. The association of α-LA with the MFGM probably occurs via disulphide bonding in a similar way to that of β-LG, but in a much lesser extent (Singh, 2006).

Concerning the association of individual CN proteins and/or casein micelles with the MFGM, former reports considered the presence of κ-CN as an artefact due to fat globule damage during the handling of the milk (Dalgleish & Banks,

1991; Houlihan et al., 1992). Recently, the use of both non- and dissociating buffers has allowed elucidating whether the whole casein micelle was present in the MFGM after heating (Ye et al., 2004a). In the present study, under reducing conditions, in addition to the κ-CN band, α_{s1}-, α_{s2}- and β-CN bands were also observed in pasteurised samples; under non-reducing conditions, κ-CN, β-LG and α-LA bands disappeared. These results are in accordance to those obtained by Ye et al. (2004a), and suggest that the casein micelles may be directly associated with the MFGM proteins through κ-CN, and indirectly through the interaction of κ-CN with β-LG already associated with the MFGM.

Interactions between MFGM proteins via disulphide bonds are enhanced by heat (Ye et al., 2002, 2004a). However, SDS-PAGE electrophoretograms under reducing conditions showed that pasteurisation did not affect the pattern of the main MFGM proteins, since β-mercaptoethanol breaks down disulphide bonds, except for a slight fading of the band corresponding to PAS 6/7. It has been suggested that whey protein displace the original MFGM material, either by directly competing or by filling the gaps produced by heat at the original MFGM (Dalgleish & Banks, 1991). The results of the present study, which are in accordance to those obtained by Ye et al. (2004a), do not support this contention. Kim and Jiménez-Flores (1995) interpreted the decrease of PAS 7 as a breakdown in the protein structure. Houlihan et al. (1992) observed that the loss of PAS 6/7 was related to the presence of skim milk components during heating. Ye et al. (2002) observed that in the absence of serum proteins, PAS 7 did not associate with other MFGM proteins via disulphide bonds upon heating, but this protein was found to be more heat stable than the other MFGM proteins. In a more recent study, these authors suggested two possible explanations for such behaviour: a) the alteration of the membrane structure due to the formation of β-LG/MFGM complexes or, b) the formation of direct β-LG/PAS 7 complexes could result in the removal of PAS 7 (Ye et al., 2004a).

In literature, much more interest has been focused in the effect of conventional homogenisation since this treatment provokes drastic changes on the structure of the MFGM (Keenan et al., 1983; McPherson & Kitchen, 1983; McPherson et al., 1984; Sharma & Dalgleish, 1993, 1994; Cano-Ruiz & Richter, 1997;

Michalski et al., 2002a,b; Lee & Sherbon, 2002; Ye et al, 2004b, 2008). However, it must be pointed out that the effect of homogenisation has been commonly studied in combination to that of heat. Heating has been usually performed by batch pasteurisation, and the sequence of the treatments, i.e., downstream and upstream homogenisation, has been proven to be very important (Sharma & Dalgleish, 1994; Ye et al., 2008). In the present study, two-stage homogenisation was performed before HTST pasteurisation. Two-stage homogenisation is commonly used in industry as it prevents the coalescence of homogenised fat globules. Transmission electron microscopy of homogenised-pasteurised milk showed two fat globule populations: some individually dispersed large fat globules of uncertain origin (native or coalesced fat globules), and aggregates of small fat globules.

Conventional homogenisation-pasteurisation provoked much greater MFGM protein load than the corresponding pasteurisation treatment, which is in agreement with previous studies (Sharma et al., 1996; Cano-Ruiz & Richter, 1997). During homogenisation, fat globules are reduced, and the concomitant increase in the surface area must be overcome by building a new MFGM through the adsorption of indigenous material (McPherson & Kitchen, 1983; McPherson et al., 1984; Sharma & Dalgleish, 1993, 1994; Cano-Ruiz & Richter, 1997).

The results of the present study showed that, after conventional homogenisation-pasteurisation, high amounts of CN were adsorbed onto the MFGM; indeed, several authors observed that CN were adsorbed preferentially over whey proteins in homogenised milk samples (Sharma & Dalgleish, 1993; Cano-Ruiz & Richter, 1997; Sharma et al., 1996). However, comparing the protein patterns of pasteurised and homogenised-pasteurised samples showed that the latter treatment provoked greater adsorption of whey proteins. Such differences could be indirectly due to the spreading of casein micelles during homogenisation provoking more “association sites” (Ye et al., 2008). Moreover, a recent study, on the kinetics of heat-induced association of whey proteins with MFGM, showed that their incorporation reached a plateau (Ye et al., 2004c). The damage caused at the MFGM by homogenisation, i.e., gaps which

ought to be refilled, might raise the saturating limit, resulting in greater direct association of whey proteins with the MFGM.

Before discussing the results obtained for UHPH-treated milk samples, the difficulties encountered during the isolation of MFGM material should be emphasised. The reduction of the fat globules provokes an increase of their density near to that of the serum phase; hence, the use of sucrose during the centrifugation of milk enhances fat recovery by increasing the density between the fat and serum phases (Cano-Ruiz & Richter, 1997). Indeed, after centrifugation, the top layer (cream) of conventionally homogenised-pasteurised milk was as thick as that of raw milk. However, for UHPH-treated milk samples, the layer was much thinner, especially at high pressures. In some cases, a second centrifugation step was performed, but the fat recovery was still low. Such difficulties resulted in a much laborious handling during the washing of the cream. Recently, major improvements have been focused on the washing of cream rather than on the fat recovery (Vanderghem et al., 2008; Le et al., 2009). Further research on the matter, e.g. using gradient densities, must be carried out in order to optimise the technique.

The effect of UHPH treatment on the MFGM composition depended on both the temperature of the milk and the applied pressure. Increasing T_i lead to an increase of the MFGM protein load, except for treatments at 300 MPa. Although milk UHPH-treated at 200 MPa with T_i of 30 ºC had similar particle size than those treated at 300 MPa with $T_i > 20$ ºC, differences in the MFGM protein load of washed creams were observed. In addition, UHPH-treatment at 300 MPa with T_i of 20 ºC resulted in larger particles but similar MFGM protein load. In other words, increasing T_2 from 72 to 76 ºC led to a greater incorporation of proteins at the MFGM without involving a change in the particle size. However, further increase of T_i did not affect the amount of protein associated to the MFGM.

The adsorption of whey proteins onto the MFGM do not necessary imply complete denaturation of the proteins; Ye et al. (2004a) suggested that association of β-LG occurs after the native β-LG dimer dissociates into monomers, but before the free thiol group is exposed; the thiol-disulphide

interchange reactions are initiated by free thiol groups of the MFGM proteins, which become available at lower temperatures (~60 ºC) than that of β-LG denaturation (78 ºC). Indeed, some β-LG was observed in washed creams from all UHPH-treated milk samples. However, when the temperature reached during UHPH-treatment was > 76 ºC, high levels of β-LG and some α-LA were covalently bonded to the MFGM. In fact, whey proteins have more affinity for MFGM than for the casein micellar surface (Corredig & Dlagleish, 1996). Unexpectedly, treating milk with T_i of 20 ºC at 300 MPa (T_2 = 76 ºC) resulted in association of β-LG at the MFGM through interactions with casein micelles. A possible explanation involves the effect that UHPH treatment has on casein micelles, as will be later discussed.

In contrast to conventional homogenisation-pasteurisation, UHPH treatment not only provoked direct association of κ-CN with the MFGM through covalent bonding but also that of α_s- and β-CN. The effect of UHPH on casein micelles has been recently studied (Sandra & Dalgleish, 2005; Roach & Harte, 2008). UHPH breaks up casein micelles, which results in the formation of protein complexes in the milk serum that are different from those produced by heat treatment. The effect of UHPH is to partially remove parts of the casein micellar surface, and formed protein aggregates contain mainly α_s-CN and whey proteins. Partial disruption of casein micelles could allow the binding of inner proteins through direct association with the MFGM proteins or by interacting with whey proteins which in turn associate with the MFGM.

3.5. Conclusions

UHPH provoked greater reduction of fat globule size than conventional homogenisation-pasteurisation. The concomitant SSA increase was overcome by the absorption of non-native MFGM proteins; conventionally homogenised fat globules adsorbed mainly intact casein micelles whereas in UHPH-treated milk, especially at high pressures, caseins molecules, released through partial disruption of casein micelles, were directly bonded to the MFGM material. Varying levels of whey proteins, especially β-LG, were also adsorbed onto the MFGM. In conventionally homogenised fat globules, intermolecular disulfide

bonding with MFGM material was involved. In contrast, for UHPH-treated milk, in addition to direct interaction with native MFGM proteins, whey proteins were adsorbed through disulfide bonding with both indirectly and directly absorbed casein molecules. TEM observations revealed that UHPH, especially at high pressure, provoked the formation of new chimerical particles.

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Section III, Chapter 1

Ultra-high pressure homogenisation of milk: technological aspects of cheese-making and microbial shelf life of a starter-free fresh cheese

Although the coagulation properties of milk were enhanced by ultra-high pressure homogenisation (UHPH), the cheese-making properties were somewhat altered; both conventional homogenisation and UHPH of milk provoked some difficulties at cutting the curd due to crumbling and improper curd matting due to poor cohesion of the grains. Starter-free fresh cheeses obtained from UHPH-treated milk showed less syneresis during storage and longer microbiological shelf-life than those from conventionally treated milk samples.

1. Ultra-high pressure homogenisation of milk: technological aspects of cheese-making and microbial shelf life of a starter-free fresh cheese

1.1. Introduction

Many studies have shown that homogenisation of milk affect the quality of a great variety of cheeses by altering their composition, i.e., moisture, fat and protein content, their body and texture, and their colour (see review Jana & Upadhyay, 1992). However, very few studies have taken into consideration the technological aspects of the cheese-making process. During the manufacture of Cheddar and Swiss-type cheeses, homogenising milk at increasing pressures from 3.5 to 14 MPa resulted in decreasing elasticity of curds at cutting time, slow whey expulsion and decreasing matting of curds during cooking and cheddaring (Peters, 1956; Peters & Moore, 1958; Green et al., 1983). In addition, curds prepared from homogenised milk were brittle, resulting in curd shattering during stirring (Humbert et al., 1980; Tunick et al., 1993).

Spoilage of food, which can be described as a loss of qualitative properties, is mostly caused by microorganisms such as bacteria, yeasts and moulds (Hansen & Bautista, 1999). A wide range of microorganisms are destroyed during pasteurisation of milk, thus microbiological spoilage and presence of pathogens in pasteurised-milk products are usually the result of improper heating regimen or post-pasteurisation contamination through mishandling or improper hygiene (Özer, 1999). Since starter-free fresh cheeses have high pH and moisture content, are produced without starters and stored under refrigeration, spoilage microorganisms in this cheese variety are related to those found in pasteurised milk. The principal microorganisms growing and causing spoilage in refrigerated pasteurised milk are psychrotrophs, e.g. *Pseudomonas*, *Alcaligenes* and *Flavobacterium*, which most common origin is post-pasteurisation contamination (Özer, 1999). However, some thermophilic and mesophilic bacteria, e.g. thermoduric micrococci and *Streptococcus*, or endospore-forming *Bacillus*, may cause spoilage of heat-treated milk (Özer, 1999). Concerning food-borne pathogens, European regulations specify the allowed limits of *Listeria monocytogenes* and *Staphylococcus aureus* (European Commission,

2005, 2007; see Section I Chapter 2). In addition under these regulations, *Escherichia coli* is used as an indicator for the level of hygiene in cheeses made from milk that has undergone heat treatment (European Commission, 2005, 2007).

From previous results obtained on the coagulation properties (see Section II Chapter 1), two conditions were identified as optimum for cheese production, i.e., 200 MPa at 40 ºC and 300 MPa at 30 ºC. In addition, the study on the microbiological quality of UHPH-treated milk conducted by Pereda et al. (2007) proved that both treatment conditions reduced microbiological counts down to those obtained by high-heat pasteurisation (90°C for 15 s). However, the study on lipolysis (Pereda et al., 2008) showed that milk UHPH-treated at 200 MPa with an inlet temperature of 40 ºC suffered lipolysis during storage provoking problems of rancidity. As a consequence, the UHPH conditions for the production of fresh cheeses were set at 300 MPa with an inlet temperature of 30 ºC.

The two main goals of the present study were (a) to evaluate the cheese-making properties of milk treated by ultra-high pressure homogenisation (UHPH) and (b) to determine the shelf-life of the resulting starter-free fresh cheeses.

1.2. Material and methods

1.2.1. Milk supply and treatment

Raw whole bovine milk was obtained from a local dairy farm (S.A.T. Can Badó, Roca del Vallès, Spain). Milk was standardised at 3.5 ± 0.2% (w/v) fat and kept overnight at 4 ºC. Before all treatments, the milk was warmed to approximately 20 ºC. UHPH treatment was carried out by subjecting milk to 300 MPa using the modified model FPG11300 of Stansted Fluid Power Ltd. (see Section I Chapter 2) at an inlet temperature of 30 ± 0.5 ºC. Milk temperature reached during treatment was 104 ± 1 ºC at the valve level and 47 ± 2 ºC just after the refrigeration system. The outlet temperature of milk never exceeded 20 ºC.

UHPH (**UH**) was compared with conventional treatments. Pasteurised milk (**PA**; at 80 ºC for 15 s) and homogenised-pasteurised milk (**HP**; with 15 + 3 MPa at 60 ºC, and at 80 ºC for 15 s) were chosen since these treatments are commonly used for cheese-making milk. Two-stage homogenisation and pasteurisation were performed with a Niro Soavi homogeniser (model X68P Matr. 2123, Niro Soavi, Parma, Italy) and a Finamat heat-exchanger (model 6500/010, GEA Finnah GmbH, Ahaus, Germany), respectively.

1.2.2. Particle size and distribution of milk

The particle size distribution in milk samples was determined using a Beckman Coulter laser diffraction particle size analyser (LS 13 320 series, Beckman Coulter, Fullerton, CA) as described in Section II Chapter 1. Both volume- and surface-weighted mean diameters [**D(4,3)** and **D(3,2)**] were determined.

1.2.3. Cheese production

Milk (60 L) was placed in a 150 L-vat and warmed under manual stirring until coagulation temperature was reached. The pH of the milk was recorded with a portable pH-meter (PH 25, electrode 50 54 with automatic temperature compensation, Crison, Alella, Spain) before and after adding salt (1% v/v) and 35% (w/v) calcium chloride at 0.01% (v/v) in milk. Coagulation of the milk was performed at 32 ºC for 45 min after addition of recombinant rennet chymosin (Maxiren 180, DSM Food Specialties, Seclin Cedex, France) at 0.03% (v/v). Curd was manually cut and kept undisturbed for 15 min at 37 ºC. Curd grains of about 1 cm, obtained by gently stirring for 10 min, were poured into polypropylene moulds (250 g packaging for Ricotta, ETS A. Coquard, Villefranche-sur-Saône, France). Filled moulds were allowed to drain at 7 ºC for 90 min, and collected whey was weighted. Packaged cheeses were kept at 4 ºC during the storage period. The pH of milk and whey were recorded with a portable pH-meter (PH 25, electrode 50 54 with automatic temperature compensation, Crison) in order to assess the cheese-making process.

1.2.4. Coagulation properties

Rennet coagulation properties, such as rennet coagulation time (**RCT**), rate of curd firming (**RCF**), and curd firmness at 45 min (**CF**), were assessed on-line with a Gelograph-NT (GelInstrumente, Thalwil, Sweden), which measures light transmission; photometric coagulation measurement is based on the principle of light absorption and scattering in the coagulating milk. Light in the near infra-red range is passed through the milk specimen and depending on the structure of this, it will be scattered or absorbed to a greater or lesser extent. A photo diode detects the transmitted signal and the relative transmission is evaluated electronically giving a direct measurement for the structure of the milk specimen. Simultaneously the measuring instrument calculates the first derivative of this function (change in transmission per time interval) which can be used as a measure of the rate of coagulation.

1.2.5. Cheese pH and whey expelled during storage

Both packaged cheeses and whey, which remained in the packaging after taking away the cheese for microbiological analysis, were weighted. The amount of expelled whey was calculated as percentage of the packaged cheese weight. The pH was measured with a pH meter (Micro-pH 2001, Crison) on a cheese/distilled water (1:1) slurry.

1.2.6. Microbial counts

The microbiological quality of milk samples was assessed on day 1. For cheeses, the analysis was carried out every 2 days until the total counts reached at least 6 log cfu g^{-1}. The whole cheese was transferred into a sterile plastic bag and manually crushed. Ten grams of homogenised cheese was diluted with 90 mL of sterile peptone water (Oxoid Ltd., Basingstoke, Hampshire, UK), in a stomacher bag and mixed for 1 min with a Stomacher (400 Circulator, Seward Ltd., London, UK).

Decimal dilutions of both milk and cheese homogenates were performed with peptone water, and microbiological counts of total bacteria (**TC**), psychrotrophs (**PSY**), lactococci (**LC**), lactobacilli (**LB**), coliforms (**COL**),

Escherichia coli (**EC**), yeasts & moulds (**YM**), and *Staphylococcus aureus* (**SA**) were determined. The following media and incubation conditions were used (APHA, 1992): pour-plating in Plate Count Agar (Oxoid) incubated at 30 ºC for 48 h (TC) and at 20 ºC for 72 h (PSY); pour-plating in M17 Agar and Rogosa Agar (Oxoid) incubated at 30 ºC for 48 h (LC) and 72 h (LB); pour-plating in Violet Red Bile Agar (Oxoid) and/or ColiID (bioMérieux S.A., Marcy L'Etoile, France), with a covering layer of the same medium, incubated at 37 ºC for 24 h (COL and EC); pour-plating in Rose-Bengal Chloramphenicol Agar (Oxoid) incubated at 20 ºC for 5 days (YM); pour-plating in Baird-Parker RPF Agar (Oxoid) incubated at 37 ºC for 24-48 h (SA).

1.2.7. Screening for pathogens

The design of the experiment included a sensory analysis of the cheeses, thus it was necessary to ensure that cheeses were free of pathogens. The absence of *Listeria monocytogenes* and *Salmonella* spp. in cheese on day 1 was confirmed with modified standard methods (ISO, 1996, 2002, 2004).

The method for *Listeria monocytogenes* entailed a selective pre-enrichment in half-Fraser broth followed by a selective enrichment in Fraser broth (bioMérieux) and detection with solid agar plates of Palcam (Oxoid) and ALOA (AES Chemunex, Bruz, France). Crushed cheese (25 g) and 225 mL of half-Fraser broth were transferred in a sterile plastic bag and mixed for 1 min with a Stomacher (Seward Ltd.). After 24 h incubation at 30 ºC, subsequent enrichment was done in Fraser broth and incubated at 37 ºC for 24 h. The later broth was subcultured by streaking on to the solid agar plates, which were read after incubation at 37 ºC for 24 h.

For *Salmonella* spp., the pre-enrichment was done with a non-selective broth, i.e., buffered peptone water, for 24 h at 37 ºC. Subsequent enrichment was done in two selective broths: Rappaport-Vassiliadis with soy and Muller-Kauffmann Tetrathionate-Novobiocin (bioMérieux). After 24 h incubation at 42 ºC and 37 ºC, respectively, the selective broths were subcultured by streaking on to XLD (Oxoid) and SM ID2 (bioMérieux) agar plates. Reading was carried out after incubation at 37 ºC for 24 h.

1.2.8. Statistical analysis

The complete experiment was repeated on 3 independent occasions. All analyses were carried out in duplicate. Results were processed by multifactor analysis of variance (ANOVA) using the general linear models procedure of Statgraphics (Statgraphics Inc., Chicago, IL, USA), taking into account both treatment and production factors, as well as their interaction. Either LSD or Tukey's tests were used for comparison of sample data, and evaluations were based on a significance level of $P < 0.05$.

1.3. Results and discussion

1.3.1. Milk: particle size, microbiological quality and coagulation properties

Both particle size distributions of raw and pasteurised milk samples were characterised by a main peak at ~4 μm and a lower peak at 0.2 μm, which corresponded to fat globules and casein micelle particles, respectively (data not shown). As expected, both homogenisation treatments altered markedly the size distribution of milk; in conventionally treated milk, the main peak was found at ~0.4 μm followed by a second peak at 1.5 μm. In contrast, UHPH-treated milk showed only one peak at ~0.1 μm. Consequently, both D(4,3) and D(3,2) of milk differed significantly ($P < 0.05$) depending on the treatment (Table III.1.1). UHPH resulted in significantly lower values than conventional homogenisation-pasteurisation. The obtained results were in accordance to previous studies (see Section II Chapters 1 and 3).

Table III.1.1. Particle size (nm) of raw, pasteurised, pasteurised-homogenised, and ultra-high pressure homogenised milk samples.

Treatment[1]	D(4,3)	D(3,2)
Raw	2,777 ± 17 [a]	536 ± 5 [a]
PA	2,640 ± 26 [b]	503 ± 9 [b]
HP	494 ± 1 [c]	327 ± 12 [c]
UH	155 ± 3 [d]	132 ± 3 [d]

[a-d] Mean value ± s.e.; n = 9; values without common superscripts were significantly different ($P < 0.05$) by LSD test.

[1] PA: pasteurisation; HP: homogenisation-pasteurisation; UH: ultra-high pressure homogenisation.

For total bacteria and psychrotrophs, conventional treatments provoked reductions of ~2.2 and ~2.7 log cfu mL^{-1}, respectively (Table III.1.2). Conventional homogenisation-pasteurisation of milk provoked a greater reduction of lactococci than pasteurisation (2.6 vs. 2.3 log cfu mL^{-1}). Significantly greater reductions were obtained by UHPH treatment, i.e., reductions of ~3 log cfu mL^{-1}. Lactobacilli, coliforms, *E. coli*, *S. aureus*, yeasts and moulds were not detected in either conventionally or UHPH-treated milk samples. These results are broadly in accordance with those obtained in previous studies (Pereda et al., 2007; Smiddy et al., 2007).

Table III.1.2. Milk microbiology (log cfu mL^{-1} milk).

Microbial group[1]	Treatment[2]			
	Raw	PA	HP	UH
TC	3.79 ± 0.03 [a]	1.66 ± 0.10 [b]	1.58 ± 0.09 [b]	0.79 ± 0.03 [c]
PSY	3.75 ± 0.04 [a]	0.98 ± 0.19 [b]	1.06 ± 0.05 [b]	0.71 ± 0.11 [c]
LC	3.09 ± 0.04 [a]	0.73 ± 0.12 [b]	0.46 ± 0.11 [c]	0.17 ± 0.12 [d]
LB	2.69 ± 0.10 [a]	n.d. [b]	n.d. [b]	n.d. [b]
COL	2.50 ± 0.03 [a]	0.08 ± 0.08 [b]	n.d. [b]	n.d. [b]
EC	2.13 ± 0.08 [a]	n.d. [b]	n.d. [b]	n.d. [b]
YM	2.11 ± 0.12 [a]	n.d. [b]	n.d. [b]	n.d. [b]
SA	1.75 ± 0.05 [a]	n.d. [b]	n.d. [b]	n.d. [b]

[a-d] Mean value ± s.e.; n = 6; n.d.: not detected with a detection limit of 1 cfu mL^{-1}; values in rows without common superscripts were significantly different ($P < 0.05$) by Tukey's test.

[1] TC: total bacteria; PSY: psychrotrophs; LC: lactococci; LB: lactobacilli; COL: coliforms; EC: *Escherichia coli*; YM: yeasts & moulds; SA: *Staphylococcus aureus*.

[2] PA: pasteurisation; HP: homogenisation-pasteurisation; UH: ultra-high pressure homogenisation.

Concerning the coagulation properties of milk samples, no significant differences ($P < 0.05$) were observed between conventional treatments for RCT (Table III.1.3). In contrast, UHPH decreased RCT from 17 min down to 14 min. RCT obtained in the study on rennet coagulation (see Section II Chapter 1) were much lower and were not affected by UHPH at 300 MPa with an inlet temperature of 30 ºC. Rennet concentration used in cheese production was lower than in the experimental lab studies [0.03 vs. 0.074% (v/v)], resulting in general longer coagulation time. However, the casein-spreading effect by UHPH, i.e., higher availability and lower critical level for chymosin action, resulted in lower RCT in UH-milk than conventionally treated milk samples. Moreover, conventional pasteurisation-homogenisation led to significantly

lower RCF compared with pasteurisation, which resulted in lower CF at 45 min. In contrast, UHPH increased RCF, which, combined with lower RCT, resulted in significantly higher CF compared with pasteurisation treatment. Such results are in accordance to those of the study on rennet coagulation (see Section II Chapter 1).

Table III.1.3. Coagulation properties of milk[1].

Treatment[2]	RCT (min)	RCF (% min^{-1})	CF (Δ%)
PA	17.63 ± 0.18 [a]	0.0052 ± 0.0050 [b]	4.17 ± 0.11 [b]
HP	17.18 ± 0.21 [a]	0.0014 ± 0.0035 [c]	1.19 ± 0.08 [c]
UH	13.74 ± 0.24 [b]	0.0105 ± 0.0091 [a]	8.49 ± 0.26 [a]

[a-c] Mean value ± s.e.; n = 6; values without common superscripts were significantly different ($P < 0.05$) by LSD test.

[1] RCT: rennet coagulation time; RCF: rate of curd firming; CF: curd firmness at 45 min.

[2] PA: pasteurisation; HP: homogenisation-pasteurisation; UH: ultra-high pressure homogenisation.

1.3.2. Technological aspects of cheese-making

The addition of chloride calcium and salt caused a slight decrease in pH (Table III.1.4). Rennet coagulation of milk is pH-dependent, with optimum activity for hydrolysis of the Phe_{105}-Met_{106} in κ-casein at pH 6.0 (Banks & Horne, 2002). Decreasing the natural pH of milk also encourages gel formation by solubilisation of calcium phosphate and a decrease in the charge on the casein micelles. The slight decrease in pH provoked by the addition of calcium chloride promotes the primary enzymatic phase of renneting, while the secondary stage of aggregation is enhanced by calcium addition (Banks & Horne, 2002). Since no starter was used, further acidification of the milk was not observed. Whey pH at the end of the production was similar to that of the initial milk supplemented with salt and calcium chloride. No significant differences were observed between treatments indicating that all productions were carried out under the same conditions.

The transfer of milk into the vat provoked great foaming for UH-milk, which was visually whiter and less viscous than those conventionally treated. In fact, UH-milk resembled somehow to skim milk. The analysis of

Table III.1.4. Milk and whey pH and whey drainage.

		Treatment[1]		
		PA	**HP**	**UH**
pH	Milk	6.59 ± 0.06	6.59 ± 0.02	6.66 ± 0.04
	Milk/NaCl/$CaCl_2$	6.48 ± 0.01	6.49 ± 0.05	6.53 ± 0.04
	Whey	6.50 ± 0.04	6.48 ± 0.03	6.49 ± 0.01
Drained whey (kg)[†]		3.45 ± 0.06 [a]	3.14 ± 0.28 [ab]	3.06 ± 0.29 [b]

[a-b] Mean value ± s.d.; n = 3; values without common superscripts were significantly different ($P < 0.05$) by LSD test.

[1] PA: pasteurisation; HP: homogenisation-pasteurisation; UH: ultra-high pressure homogenisation.

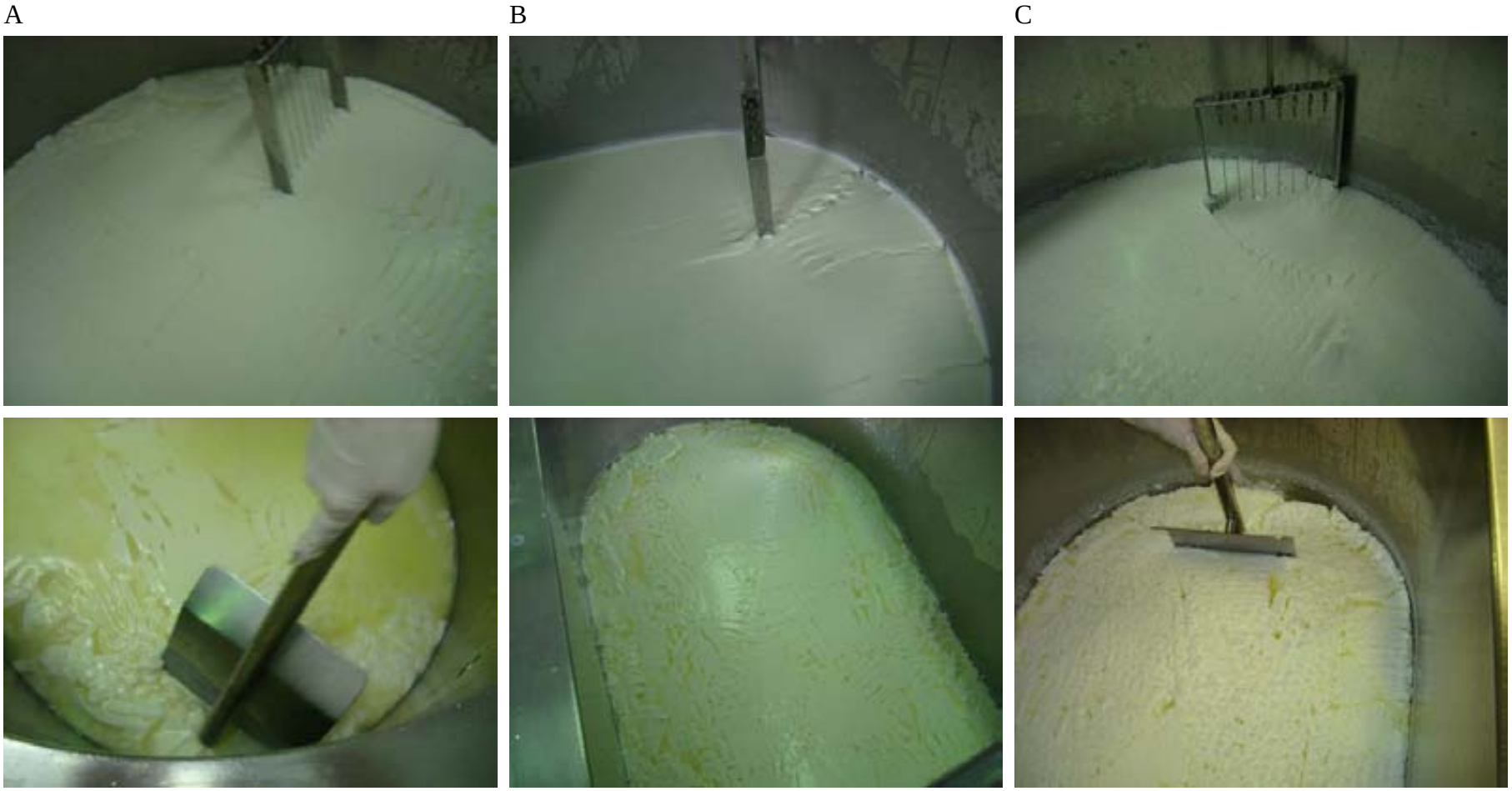
[†] Total amount of whey collected from all cheeses after 90 min of drainage.

milk colour and viscosity performed by Pereda et al. (2007) showed that UHPH at 300 MPa with an inlet temperature of 30 ºC provoked whitening of the milk by increasing the lightness and decreasing both a and b colour components, but did not affect the viscosity of milk.

After 45 min of coagulation, some spontaneous expelled whey was observed at the surface of undisturbed curds from PA-milk. Indeed, syneresis in curd occurs as coagulation takes place by a change in solubility, and rearrangement and shrinkage of the network (Walstra et al., 1985). Although gels formed from UH-milk showed analytically higher firmness, the cutting was somehow more difficult; some attempts were done in order to use both horizontal and vertical blade knives, but the former provoked great destruction of curds from both HP- and UH-samples.

The homogenisation of milk, especially UHPH, provoked lower amounts of expelled whey (Fig. III.1.1). Whey obtained from UH-milk was visually more transparent and greenish than those of conventionally treated samples. However, during stirring, the curd was somewhat brittle resulting in curd shattering and a concomitant increase of curd fines in whey. When using raw or pasteurised milk, two main reasons are responsible for curd chattering. If it occurs at an early stage, i.e., when cutting the curd, it indicates that the curd was not firm enough to be cut and a longer coagulation time would solve the problem. In contrast, if chattering occurs when stirring the curd, it indicates that the size of the blocks after cutting were too big, since big grains tend to chatter; thus reducing the size of the

Figure III.1.1. Cheese production: cutting of the curd (1st row) and end of the holding time (2nd row) from (A) pasteurised, (B) homogenised-pasteurised, and (C) ultra-high-pressure homogenised milk samples.



blade knifes would solve the problem. However, in the present study, no chattering was observed in curds from PA-milk; since it was a peculiarity of homogenised milk, no changes in the technological process itself were done.

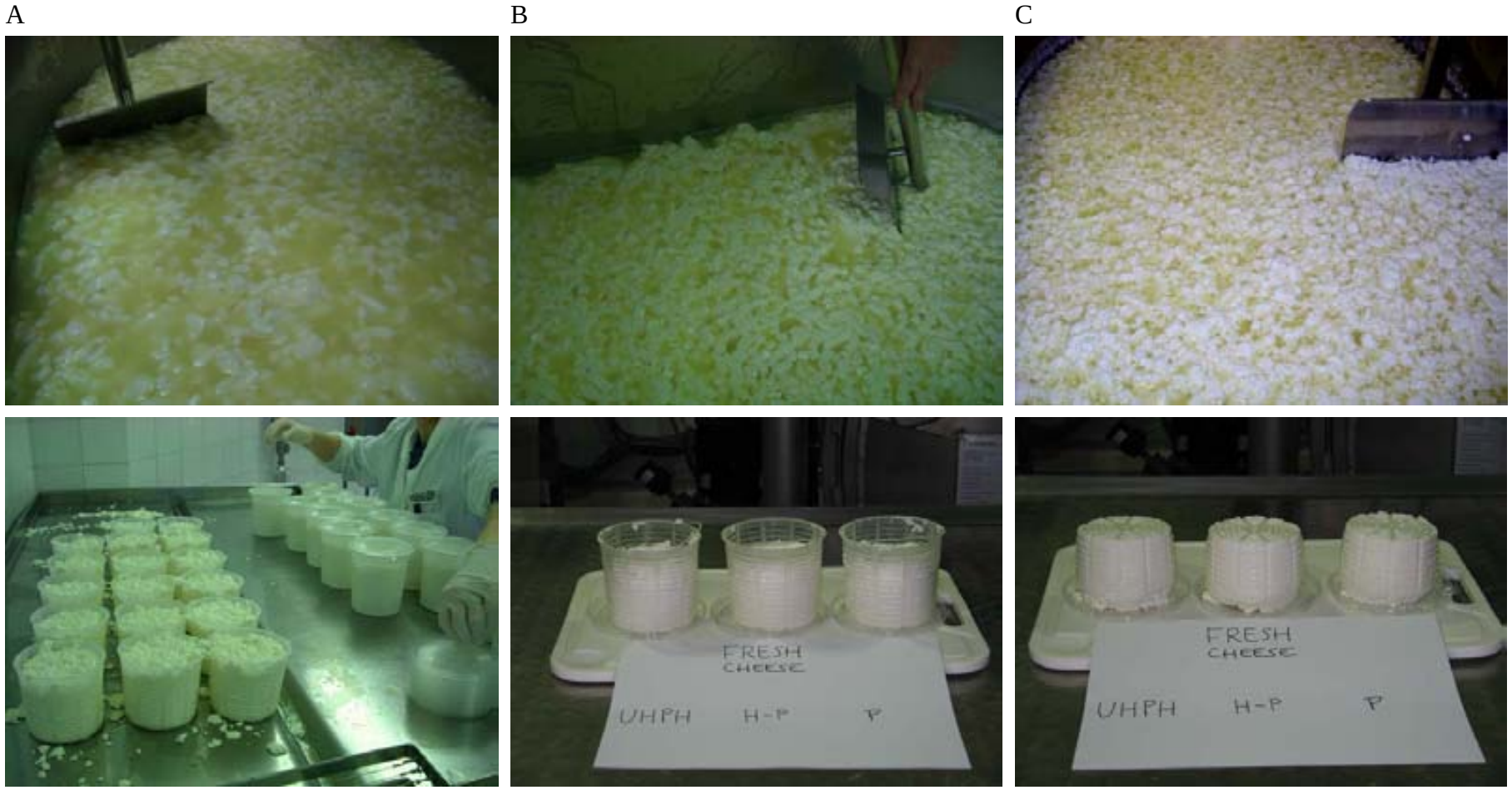
The cohesion of the grains was impaired by UHPH treatment of milk, which led to difficulties in obtaining homogeneous grains. Moreover, during moulding, grains did not get completely packed due to poor deformability. Such difficulties were also found in a lesser extent with HP- milk. Indeed, many studies have shown that the interactions between fat and casein provoked by conventional homogenisation lead to weaker rennet curd, curd shattering, and improper curd matting (Peters, 1956; Green et al., 1983; Jana & Upadhyay, 1992).

Both homogenisation treatments reduced the amount of whey expelled by gravitation before storage compared with pasteurisation treatment. However, the effect of UHPH was much important than that of conventional homogenisation-pasteurisation (11% vs. 9% reduction, respectively). Indeed, homogenisation of milk increases moisture content of grains, by decreasing the amount of whey expelled, and decreases fat losses to whey (Peters, 1956; Green et al., 1983; Jana & Upadhyay, 1992). The water-holding capacity and water typology of cheeses are studied in Section III Chapter 3.

1.3.3. Cheese pH and whey expelled during storage

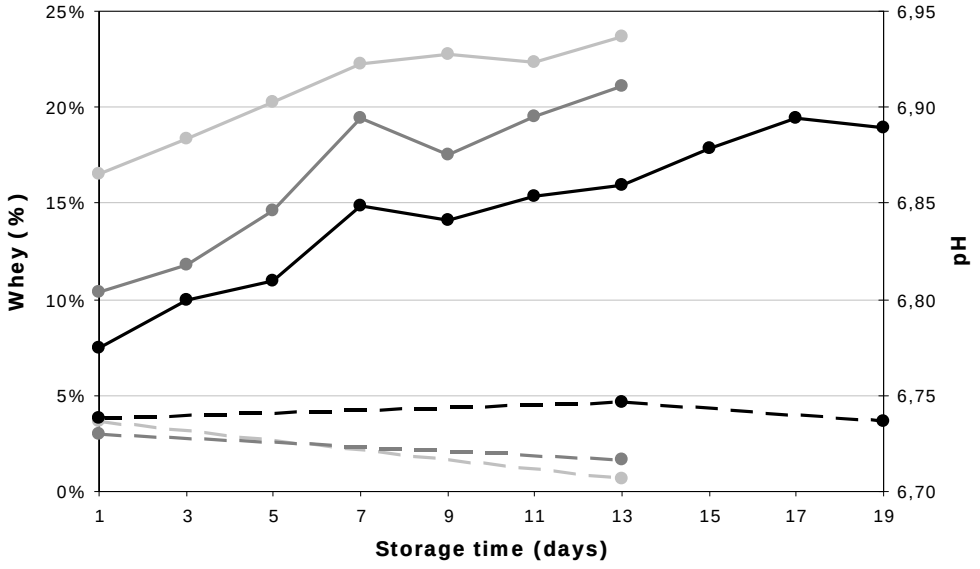
Independently of milk treatment, the initial pH of all cheeses ranged from 6.7 to 6.8. These values are in accordance to those obtained by Hernando (1998) with Burgos cheeses made from pasteurised milk. During the storage period (13 days for PA- and HP-cheeses, and 19 days for UH-cheeses), almost no changes in the pH of cheeses were observed (Fig. III.1.2). Hernando (1998) observed that the initial 6.78 pH of commercial Burgos cheeses, made from pasteurised milk, decreased after 14 days of storage at 4 ºC, which corresponded to the labelled expiring date.

Figure III.1.1. Cheese production: grain formation through stirring (1st row) from (A) pasteurised, (B) homogenised-pasteurised, and (C) ultra-high-pressure homogenised milk samples, packaging and obtained cheeses (2nd row). (continuation)



Concerning the amount of whey expelled during storage, significant differences between treatments were observed already at day 1 (Fig. III.1.2); expelled whey represented 17, 10 and 7% of the total weight in PA-, HP- and UH-cheeses, respectively. During storage, a sharp increase was observed throughout the first week; since the rate for cheeses from homogenised milk samples was slightly higher than that of PA-cheeses, smaller differences in the amount of whey expelled at day 7 were observed, especially between PA- and HP-cheeses. Moreover, a decrease in the amount of whey expelled between days 7 and 9 was observed in both HP- and UH-cheeses. Hernando (1998) estimated whey drainage by centrifugation and observed a slight decrease during the first week, followed by a great increase during the second week and no further changes beyond day 14. Although not being comparable to the results of the present study, since whey drainage was estimated by centrifugation, these results confirm that by day 7, unknown changes in the cheese, most probably related to a rearrangement of the cheese matrix, must have taken place.

Figure III.1.2. Evolution of whey drainage (solid lines; g 100 g^{-1} cheese) and pH (dotted lines) in cheeses from pasteurised (light grey), homogenised-pasteurised (dark grey), and ultra-high-pressure homogenised (black) milk samples.



1.3.4. Microbiological quality and shelf-life of cheese

Screening for pathogens confirmed that cheeses were free of *L. monocytogenes* and *Salmonella* spp. Moreover, *S. aureus* and *E. coli* were not detected throughout the storage period. UH-cheeses showed the lowest rate of growth of total bacteria and psychrotrophs (Table III.2.2 and Fig. III.1.3), with a concomitant increase in the shelf-life of cheeses, i.e., when 6 log cfu g^{-1} were reached, from 11-13 (PA- and HP-cheeses) to 17-19 days. Since cheeses were stored at ~4 ºC, psychrotrophs growth was enhanced resulting in counts close to those of total bacteria.

Comparison with reference works is difficult since studies on the microbiology of Burgos cheeses were surveys carried out during 1980s on commercial Burgos-cheeses randomly collected from retail shops (Chavarri et al., 1985; García et al., 1987). In both studies, the authors observed that microbial counts were higher than expected since the cheeses were made from pasteurised milk. Chavarri et al. (1985) detected total counts of 7.23 and 7.50 log cfu g^{-1} in April and July, respectively. Higher counts were obtained by García et al. (1987) ranging from 5.8 to 9.93 log cfu g^{-1}, with an average of 8.89 log cfu g^{-1}. Since high levels of coliforms and/or enterococci were observed in both studies, the authors concluded that cheeses were not produced under conditions assuring microbiological quality. Moreover, temperature monitoring at the retail shops showed that the temperature of stored cheeses was 11.4 ºC on average (Chavarri et al., 1985).

Concerning specific microbial populations, lactobacilli were not detected throughout the storage period regardless of the applied treatment. However, lactococci were the main population among those studied (Table III.2.2 and Fig. III.1.3). Major differences were observed between UH- and cheeses from conventionally treated milk samples; UH-cheeses showed significantly lower counts of lactococci than PA- and HP-cheeses on day 1; however, by day 5, counts did not differ. Pereda et al. (2007) also observed higher counts of lactococci in UHPH-treated milk during storage at 4 ºC from day 14. UHPH might have triggered some unknown changes in the milk which enhanced the

Table III.1.5. Cheese microbiology (log cfu g^{-1} cheese).

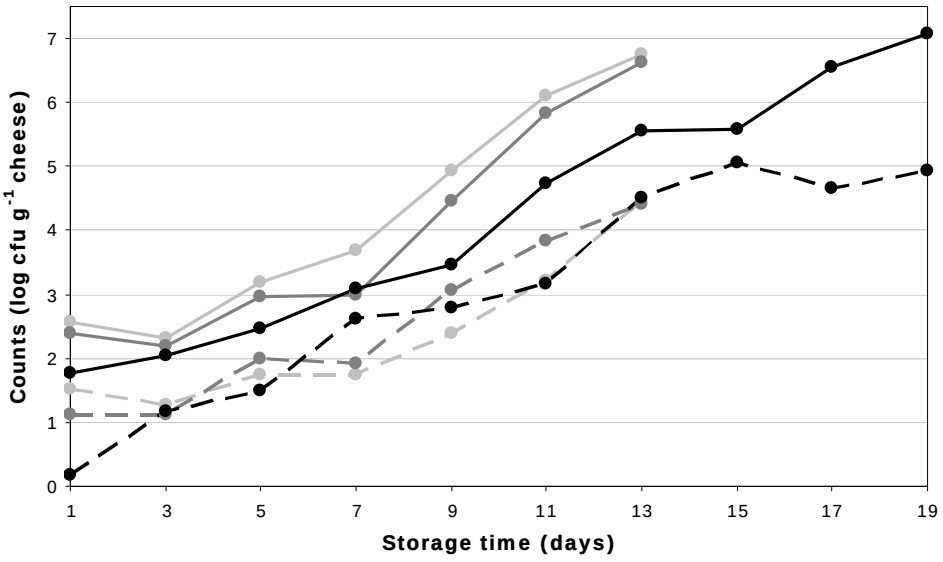
Microbial group[1]	Day	Treatment[2] PA	HP	UH
TC	1	2.56 ± 0.25 ^{h}	2.40 ± 0.14 ^{h}	1.77 ± 0.13 ^{i}
	5	3.18 ± 0.60 ^{g}	2.97 ± 0.33 ^{g}	2.48 ± 0.10 ^{h}
	9	4.93 ± 0.65 ^{d}	4.46 ± 0.54 ^{e}	3.46 ± 0.24 ^{f}
	13	6.76 ± 0.35 ^{b}	6.62 ± 0.33 ^{b}	5.55 ± 0.19 ^{c}
	19	-	-	7.07 ± 0.31 ^{a}
PSY	1	2.38 ± 0.26 ^{h,i}	2.22 ± 0.20 ^{i}	1.90 ± 0.09 ^{j}
	5	2.73 ± 0.41 ^{f,g}	2.93 ± 0.25 ^{f}	2.57 ± 0.10 ^{g,h}
	9	4.31 ± 0.88 ^{d}	4.57 ± 0.36 ^{d}	3.23 ± 0.37 ^{e}
	13	6.58 ± 0.38 ^{b}	6.39 ± 0.35 ^{b}	5.36 ± 0.17 ^{c}
	19	-	-	6.90 ± 0.28 ^{a}
LC	1	1.53 ± 0.21 ^{e,f}	1.13 ± 0.26 ^{f}	0.17 ± 0.17 ^{g}
	5	1.74 ± 0.41 ^{e,f}	2.00 ± 0.17 ^{d,e}	1.51 ± 0.33 ^{e,f}
	9	2.39 ± 0.38 ^{c,d}	3.05 ± 0.39 ^{b}	2.80 ± 0.44 ^{b,c}
	13	4.45 ± 0.42 ^{a}	4.41 ± 0.41 ^{a}	4.51 ± 0.59 ^{a}
	19	-	-	4.92 ± 0.89 ^{a}
COL	1	0.33 ± 0.21 ^{c,d}	0.17 ± 0.17 ^{c,d}	n.d. ^{d}
	5	0.82 ± 0.27 ^{b,c}	0.46 ± 0.29 ^{c,d}	0.17 ± 0.17 ^{c,d}
	9	1.21 ± 0.76 ^{a,b}	1.87 ± 0.79 ^{a}	n.d. ^{d}
	13	1.58 ± 1.00 ^{a}	1.84 ± 0.82 ^{a}	0.61 ± 0.39 ^{b,c,d}
	19	-	-	0.17 ± 0.17 ^{c,d}
YM	1	0.43 ± 0.27 ^{b,c,d,e}	0.43 ± 0.27 ^{b,c,d,e}	0.38 ± 0.25 ^{b,c,d,e}
	5	0.33 ± 0.21 ^{c,d,e}	0.50 ± 0.22 ^{b,c,d,e}	n.d. ^{e}
	9	0.90 ± 0.57 ^{b,c,d}	1.17 ± 0.60 ^{b}	0.17 ± 0.17 ^{d,e}
	13	2.22 ± 0.37 ^{a}	0.98 ± 0.46 ^{b,c}	0.50 ± 0.22 ^{b,c,d,e}
	17	-	-	0.90 ± 0.30 ^{b,c,d}

$^{a-j}$ Mean value ± s.e.; n = 6; n.d.: not detected with a detection limit of 10 cfu g^{-1}; values per microbial group without common superscripts were significantly different ($P < 0.05$) by Tukey's test.

[1] TC: total bacteria; PSY: psychrotrophs; LC: lactococci; COL: coliforms; YM: yeasts & moulds.

[2] PA: pasteurisation; HP: homogenisation-pasteurisation; UH: ultra-high pressure homogenisation.

Figure III.1.3. Microbial evolution (log cfu g^{-1} cheese) of total bacteria (solid lines) and lactococci (dotted lines) in cheeses from pasteurised (light grey), homogenised-pasteurised (dark grey), and ultra-high-pressure homogenised (black) milk samples.



growth of lactococci in cheese at an early stage of the storage period. As already mentioned, no differences in the pH of cheeses were observed, thus pH seemed not to be related to this phenomenon. Heat effect of UHPH has been proven to be lesser than that of conventional pasteurisation through the determination of thermal indicators, e.g., hydroxymethylfurfural and total sulphydryl content of milk (Pereda et al., 2009), thus UHPH could be less aggressive towards thermolabile compounds, which could act as growth factors.

Yeast and moulds presented higher variability. However, their general trends were to increase over time (Table III.2.2). Lower levels were observed in UH-cheeses especially at the end of the shelf-life. The composition analysis of the cheeses (see Section III Chapter 2) showed that UH-cheeses had higher amounts of hexanal and *t*-2-hexenal than PA- or HP-cheeses; these compounds have been proven to have an antimicrobial activity against the fungi *Saccharomyces cerevisiae* in soft drinks (Belletti et al., 2007) and *Aspergillus flavus* in model systems (Gardini et al, 2001), and the yeast *Pichia subpelliculosa* in sliced apples. In addition, although typification of the microorganisms present in cheeses was not performed, it is important to bear in mind that some bacterial volatiles, e.g., from pseudomonads, prevent fungal growth by either inhibiting spore germination or mycelium growth (Kai et al., 2009).

1.4. Conclusions

In contrast to conventional homogenisation, UHPH enhanced the coagulation properties of milk. However, both homogenisation treatments impaired to some extent the cheese-making, i.e., difficulty at cutting the curd due to crumbling and improper curd matting due to poor cohesion of the grains. During storage, cheeses from UHPH-treated milk expelled less whey than those from conventionally treated milk samples. In addition, UHPH of milk resulted in fresh cheeses with longer microbiological shelf life.

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Section III, Chapter 2

Evolution in cold storage of a starter-free fresh cheese made from milk treated by ultra-high pressure homogenisation

The aims of the present study were to evaluate the effects of using ultra-high pressure homogenisation (UHPH), as an alternative to conventional treatments applied in the production of fresh cheese, on the composition and biochemistry of starter-free fresh cheeses and to monitor their evolution during cold storage. Although both homogenisation treatments increased cheese moisture content, cheeses from UHPH-treated milk showed lower whey drainage during storage than those from conventionally homogenised-pasteurised milk. Lipolysis and proteolysis levels in cheeses from UHPH-treated milk were lower than those from conventionally treated milk samples. However, in cheeses from UHPH-treated milk, oxidation was found to be the origin of many volatile compounds which explained the metallic and cooked milk flavours detected by some panellists.

2. Evolution in cold storage of a starter-free fresh cheese made from milk treated by ultra-high pressure homogenisation

2.1. Introduction

Compositional and biochemical changes in ripened cheese are essential in order to convert during ripening fresh curds into one of many cheeses differing characteristically in appearance, taste, aroma, texture and functionality. Ripening involves three primary events, i.e., glycolysis, lipolysis and proteolysis, the products of which are modified via various biochemical reactions. Glycolisis and related events are caused by living microorganisms, while lipolysis and proteolysis are catalysed mainly by enzymes from rennet, milk or bacteria (Fox & McSweeney, 2004).

However, starter-free fresh cheeses are ready for consumption immediately after processing of the curds. In other words, possible biochemical changes provoked in the milk through processing could drastically alter the characteristics of the final product. Moreover, compositional and biochemical changes during storage lead to a decrease in the quality of fresh cheeses since many of them, e.g. lipolysis, are undesired.

The aims of the present study were to evaluate the effects of using ultra-high pressure homogenisation (UHPH), as an alternative to conventional treatments applied in the production of fresh cheese, on the initial composition and biochemistry of starter-free fresh cheeses and to monitor their evolution during cold storage.

2.2. Material and methods

Starter-free fresh cheeses were analysed on the day after production (day 1) and the expiring day (day 13 for PA- and HP-cheeses, day 19 for UH-cheeses). UH-cheeses were also analysed on day 13 in order to be compared with cheeses from conventional treatments.

2.2.1. Cheese composition

Cheeses were analysed in duplicate for fat (ISO, 1975) and in triplicate for total solids (**TS**; IDF, 2004) and total nitrogen (**TN**; IDF, 2002). Moisture content (100 – TS) and total protein (TN × 6.38) were calculated. Salt in cheese was determined in triplicate by chloride analysis (Corning 926 Chloride Analyzer, Sherwood Scientific Ltd., Cambridge, UK) following the procedure in the manual of the chloride titrator, and expressed on a dry basis.

2.2.2. Proteolysis

Water-soluble extracts were prepared according to the method of Kuchroo and Fox (1982). Water-soluble nitrogen at pH 4.6 (**WSN**), expressed as percentage of TN, was determined in duplicate by the Dumas combustion method (IDF, 2002). Total free amino acids (**FAA**) were determined in triplicate on the water soluble extracts by the cadmium-ninhydrin method described by Folkertsma and Fox (1992).

pH 4.6-insoluble fractions recovered during the WSN extraction were washed three times with 1 M sodium acetate buffer (pH 4.6), and the remaining fat was eliminated by washing with dichloromethane-sodium acetate buffer (1:1 v/v). The final protein precipitate was then lyophilised. Analyses of total and individual protein were performed by the Dumas combustion method (IDF, 2002) and capillary electrophoresis, respectively.

Capillary electrophoresis was performed in triplicate following the method of Recio and Olieman (1996) with an Agilent CE instrument (Agilent Technologies, Waldbronn, Germany) controlled by Chemstation software (Agilent). Protein separation was carried out with a fused-silica capillary column (BGB Analytik, Essen, Germany) of 0.6 m × 50 μm interior diameter with an effective length of 50 cm, by applying a linear voltage gradient from 0 to 20 kV in 3 min at 45 ºC, followed by a constant voltage of 20 kV. Electrophoregrams were obtained at 214 nm and designation of capillary electrophoresis peaks was carried out by comparing the electrophoregrams with those of pure standards (Sigma Aldrich, St Louis, MO, USA) and those of

Recio et al. (1997). Although an intensive cleaning of the pH 4.6-insoluble fraction was made, total protein content of lyophilised pellets varied significantly, thus quantification of individual protein was corrected by dividing the obtained peak areas by total protein.

2.2.3. Lipolysis

Assessment of lipolysis was carried out by qualitative and quantification analysis of free fatty acids (**FFA)** according to De Jong & Badings (1990). Briefly, 1 g of cheese was mixed in a screw-capped plastic tube with 3 g of anhydrous Na_2SO_4, 0.3 mL of H_2SO_4 (2.5 M), and 20 μL of internal standard solutions (heptanoic acid 9.18 mg mL^{-1}, and decaheptanoic acid 8.00 mg mL^{-1}; Sigma Aldrich). Then 3 mL of dry diethyl ether/heptane (1:1 v/v) were added, and the mixture was shaken for 20 s with a vortex mixer. After centrifugation at 230 × *g* for 2 min at 20 ºC, the supernatant was transferred to a screw-capped glass tube containing 1 g of anhydrous Na_2SO_4. This operation was repeated twice, and the obtained supernatants were homogenised for 10 s using a vortex mixer.

Isolation of FFA from the ether/heptane extract was done using a solid phase extraction technique with aminopropyl column Spe-ed NH2 500 mg 3 mL^{-1} (Applied Separations, Allentown, PA, USA). The aminopropyl column was conditioned with 10 mL of heptane before the lipid extract was applied to the column. Then hexane/2-propanol (20 mL; 3:2 v/v) was used to eliminate glycerides, and finally, FFA were eluted with 5 mL of dry diethyl ether containing 2% formic acid. A direct injection of this solution (1 μL) was used for gas chromatographic analysis, as described below. Two independent extractions were carried out for each sample. All organic solvents were purchased from Panreac (Barcelona, Spain), while formic and sulphuric acids were acquired from Sigma Aldrich.

Analysis of FFA for each extraction was carried out by duplicate with an HP 6890 Series II gas chromatograph (Hewlett-Packard Inc., Wilmington, DL, USA) using a fused silica capillary column (30 m × 0.32 mm, 0.25 μm thickness, DB-FFAP-coated; J&W Scientific, Folsom, California, USA), and a flame-

ionization detector. Helium, hydrogen and synthetic air were the carrier gases. The initial column temperature of 75 °C was maintained for 1 min, then raised to 240 °C at a rate of 5 °C min^{-1}, and then held at 240 °C for 21 min. Injection of 1 µl of the extracted fraction was done in splitless mode and an injector temperature of 250 °C. The detector temperature was 300 °C. Each fatty acid was identified with reference to the retention time (**RT**) of standards (Sigma Aldrich) as follows: butyric acid (C4:0; 10.624 min RT), caproic acid (C6:0; 15.459 min RT), caprylic acid (C8:0; 19.929 min RT), capric acid (C10:0; 24.014 min RT), lauric acid (C12:0; 27.795 min RT), myristic acid (C14:0; 31.313 min RT), palmitic acid (C16:0; 34.575 min RT), stearic acid (C18:0; 38.667 min RT), oleic acid (C18:1; 39.313 min RT).

Quantification was done with respect to the internal standards; heptanoic acid (C7:0; 17.674 min RT) was used for short-chain FFA (**SCFA**; C4:0-C8:0) and medium-chain FFA (**MCFA**; C10:0-C14:0), and decaheptanoic acid (C17:0; 36.289 min RT) for long-chain FFA (**LCFA**; C16:0-C18:1). The relative areas were expressed on a dry basis (µg 100 g^{-1} TS).

2.2.4. Lipid oxidation

Hexanal content of cheese determined by GC-MSD (see 3.2.5. Volatile compounds) was used as an indicator of lipid oxidation. Quantification was achieved using a standard curve ($R^2 = 0.974$) of hexanal (Sigma Aldrich) in the range 0-24 ppb with PA- and UH-cheeses, which showed the lowest and highest areas, respectively.

2.2.5. Volatile compounds

Volatile compounds in cheese were extracted in duplicate by solid phase micro-extraction with an 85 µm CAR/PDMS fibre (Supelco, Bellefonte, PA, USA). Prior to use, the fibre was preconditioned at 280 ºC for 1 h. A CombiPAL autosampler (CTC Analytics AG, Zwingen, Switzerland) and a HP 6890 Series II gas chromatograph (**GC**) equipped with a HP 5973 Mass Selective Detector (**MSD**; Hewlett-Packard Inc.) were used.

Samples with 1.5 g of cheese in sealed vials were pre-equilibrated at 80 ºC for 5 min, and the volatile compounds were extracted at the same temperature for 60 min. Thermal desorption in the GC injector in splitless mode was carried out at 280 ºC for 1 min. Between runs the fibre was cleaned for 15 min at 280 ºC. Separation of compounds was performed using a Supelcowax 10 capillary column (60 m × 0.25 mm, 0.25 μm film thickness, Supelco) with a constant helium flow of 1 mL min^{-1}. The oven temperature was programmed as follows: 40 ºC held for 10 min, heated to 110 ºC at a rate of 5 ºC min^{-1}, then to 240 ºC at 15 ºC min^{-1} with a final hold time of 15 min. Initially, the MSD was operated in the full scan mode. Electron impact ionization was used at a voltage of 70 eV, ion source temperature of 230 ºC, quadrupole temperature of 150 ºC and the transfer line was kept at 280 ºC. Mass spectra of different treated samples were obtained with a scan range m/z 33–220.

Peak identification of resulting chromatograms was performed comparing retention times and mass spectra of each compound with those of NIST 98 and Wiley 275 libraries. The retention time and a characteristic target ion were selected for each of the compounds identified.

2.2.6. Sensory analysis

Sensory evaluation of cheeses was performed after 2 days of storage, in order to ensure microbiological safety. A panel of 12-14 university faculty and staff members who were familiar with fresh-type cheese were asked to identify and quantify differences. HP- and UH-cheeses, randomly coded with three-digit numbers, were compared with PA-cheeses.

Differences in sensory attributes of appearance, texture and flavour were scored in a 9-point negative to positive scale (0 = no differences with control; 1 = minimal differences; 2 = noticeable differences; 3 = considerable differences; 4 = very considerable differences; algebraic sign, i.e., negative or positive, indicates lower or greater perception). They were also asked to list qualities and defects in a free-word table (see Annex 1).

2.2.7. Statistical analysis

The complete experiment was repeated on 3 independent occasions. Data were processed by multifactor analysis of variance (ANOVA) using the general linear models procedure of Statgraphics (Statgraphics Inc., Chicago, IL, USA), taking into account both treatment and production factors, as well as their interaction. LSD test was used for comparison of sample data, and evaluations were based on a significance level of $P < 0.05$.

For sensory data, descriptive statistics, i.e., mean, median, frequency of the median category, and cumulative frequency of either negative or positive values depending on the algebraic sign of the median, were calculated. Means were analysed for significance along with the other measurements as previously described.

2.3. Results and discussion

2.3.1. Cheese composition

The basic composition of PA-cheeses (Table III.2.1) was broadly in accordance to the composition of Burgos-type cheese (Hernando, 1998; see Section I Chapter 2). PA-cheeses were characterised by 68% moisture content, 45% fat, 40% protein and 1.5% salt contents on dry basis. During storage, their moisture content decreased due to whey drainage (see Section III Chapter 2), leading to a decrease of proteins and salt, since the latter are found as soluble forms in whey, and a concomitant increase of fat on dry basis.

Both homogenisation treatments affected in different ways the whole composition of cheeses. On one hand, conventional homogenisation triggered a drastic increase in moisture content just after production due to changes at the protein-fat structures and at the water typology (see Section III Chapter 4). The highest values in protein and salt contents of HP-cheeses at day 1 may be due to the high moisture content. Indeed, during storage, a drastic decrease of moisture with a concomitant decrease of both protein and salt content was observed. During homogenisation, globule fat size is reduced leading to lower

fat losses during cheese-making (Jana & Upadhyay, 1992). In fact, higher fat content in HP-cheeses than PA-cheeses was observed. However, during storage the fat content of the former diminished probably due to changes at the fat microstructure leading to leakage through drainage or fat degradation.

On the other hand, UHPH treatment also provoked an increase of moisture content but its level was maintained through storage at the levels of PA-cheeses at day 1. As will be extensively discussed in the chapter on texture and water typology (see Section III Chapter 4), UHPH triggered drastic changes in both the microstructure and the water typology leading to higher water-holding capacity in UH-cheeses. A part from a relatively important decrease in salt content, almost no changes were observed during storage in UH-cheeses.

Table III.2.1. Cheese composition.

Treatment[1] & day	Moisture (g 100 g^{-1} cheese)	Fat (g 100 g^{-1} TS)	Protein (g 100 g^{-1} TS)	Salt (g 100 g^{-1} TS)
PA				
1	67.39 ± 0.35 ^{c,d}	45.27 ± 1.35 ^{d}	39.47 ± 0.42 ^{b}	1.50 ± 0.02 ^{c}
13	65.19 ± 0.72 ^{e}	49.16 ± 0.96 ^{b}	37.36 ± 0.29 ^{c,d}	1.26 ± 0.04 ^{f}
19	-	-	-	-
HP				
1	69.94 ± 0.32 ^{a}	51.57 ± 0.89 ^{a}	41.33 ± 1.18 ^{a}	1.56 ± 0.04 ^{b}
13	64.21 ± 0.72 ^{f}	49.37 ± 0.41 ^{b}	36.72 ± 0.38 ^{d}	1.28 ± 0.04 ^{e}
19	-	-	-	-
UH				
1	68.61 ± 0.38 ^{b}	44.44 ± 1.77 ^{d}	38.07 ± 1.07 ^{c}	1.59 ± 0.07 ^{a}
13	67.46 ± 0.51 ^{c}	47.14 ± 0.43 ^{c}	37.76 ± 0.44 ^{c,d}	1.36 ± 0.02 ^{d}
19	66.82 ± 0.81 ^{d}	45.51 ± 2.56 ^{d}	39.29 ± 0.86 ^{b}	1.36 ± 0.03 ^{d}

$^{a-f}$ Mean value ± s.e.; n = 9 (except for fat where n = 6); values in columns without common superscripts were significantly different ($P < 0.05$).

[1] PA: pasteurisation; HP: homogenisation-pasteurisation; UH: ultra-high pressure homogenisation.

2.3.2. Proteolysis

Proteolysis can be considered as the main biochemical event in most cheese varieties. Primary proteolysis refers to the extent of native cheese proteins breakdown, while secondary proteolysis represents the further degradation leading to the formation of peptides and FAA (Beuvier & Buchin, 2004). Primary proteolysis is mainly due to the enzyme action of residual coagulant, i.e., chymosin, and the indigenous enzyme plasmin; most of the water-

insoluble peptides in cheese are produced from α_{s1}-CN by chymosin or from β-CN by plasmin (Sousa et al., 2001).

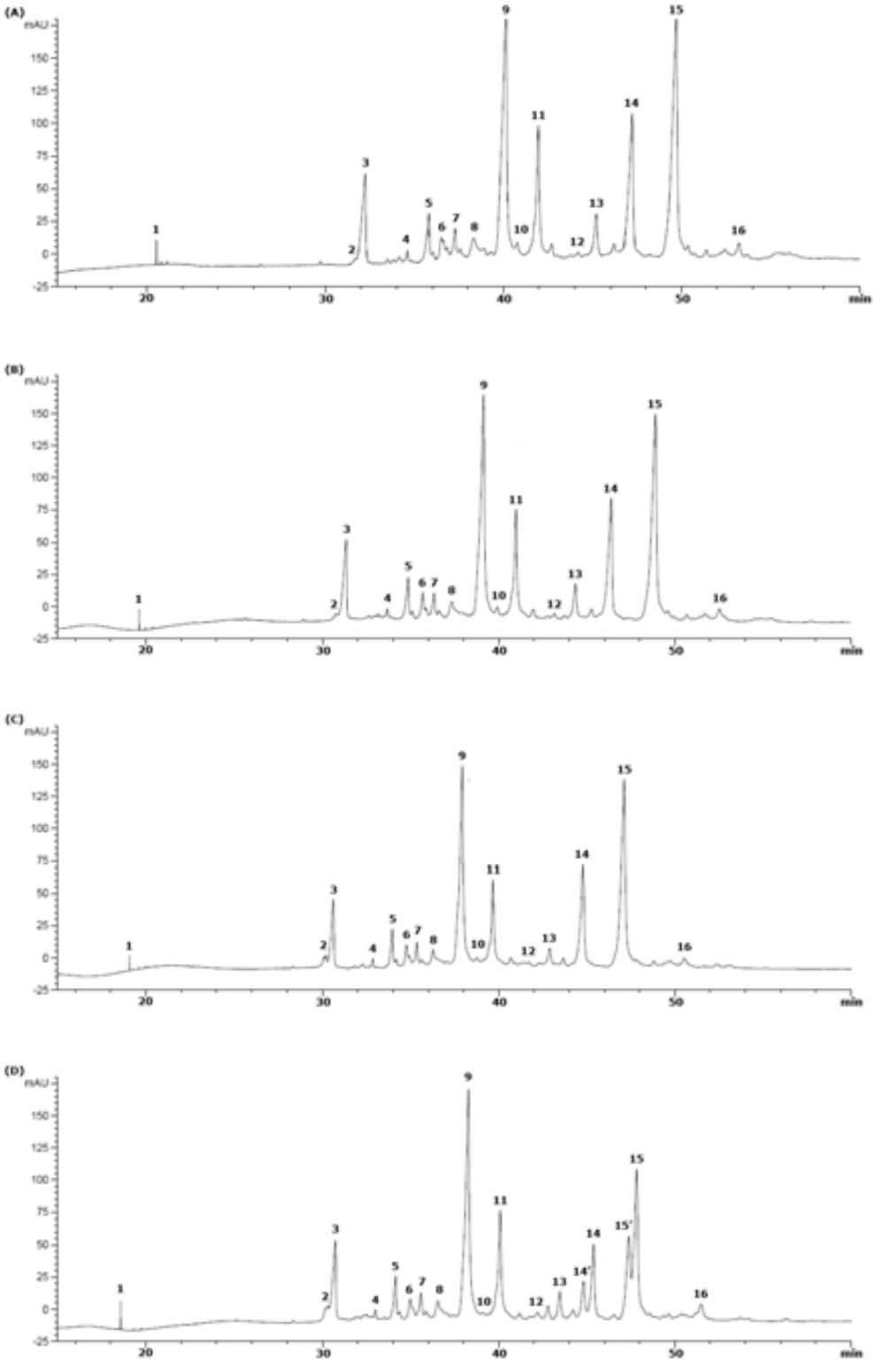
Electropherograms of the different cheeses are shown in Fig. III.2.1. During enzymatic coagulation, κ-CN (peak 12) is rapidly and specifically cleaved by chymosin to give *para*-κ-CN (peak 3), and caseinomacropeptide (Lucey, 2002). No statistical differences ($P > 0.05$) in the amount of *para*-κ-CN were observed between treatments (Table III.2.2), and very small amounts of residual κ-CN were observed in all samples.

The principal cleavage site in bovine α_{s1}-CN (peak 9) is Phe_{23}-Phe_{24}, and is readily hydrolysed by chymosin giving rise to α_{s1}-I-CN (α_{s1}-CN f(24-199); peak 16) and α_{s1}-CN f(1-23) (peak 1) (Sousa et al., 2001). The presence of both degradation products in all cheeses from day 1 showed that some proteolysis occurred at a very early stage. However, the amount of α_{s1}-CN did not vary in any of the cheeses during storage. Furthermore, no statistical differences ($P > 0.05$) on the degree of α_{s1}-CN proteolysis were observed between treatments.

Plasmin is responsible for the hydrolysis of β-CN yielding γ-CN and some proteose-peptones (Sousa et al., 2001). Peptides from β-CN, i.e., γ_1-CN (β-CN f29-209) and γ_2-CN (β-CN f106-209), were observed in all cheeses already at day 1 (peaks 7, 8, 10 and 4, 6, respectively), and no statistical differences ($P > 0.05$) in γ-CN content were observed during storage and between treatments. Around 70% of plasmin activity in milk is inactivated by UHPH treatment at 300 MPa and homogenisation-pasteurisation treatment at 90ºC for 15 s (Pereda et al., 2008a). Hence, proteolysis of β-CN by plasmin might have occurred in milk before treatment.

However, a significant decrease in β-CN between day 1 and 13 took place in all cheeses, with reductions of 35, 23 and 14% for UH, PA and HP-cheeses, respectively; two unidentified peaks were observed just before those of β-CN A1 (peak 14) and β-CN A2 (peak 15). This phenomenon was more important in UH-cheeses and the decreases of β-CN A1 and β-CN A2 were negatively correlated ($R^2 = 0.52$ and 0.74, respectively) with increases of the

Figure III.2.1. Electropherograms corresponding to cheeses at day 1 from (A) pasteurised milk, (B) homogenised-pasteurised milk, and (C) ultra-high pressure homogenised milk (UH), and to (D) UH-cheese at day 19 [1: α_{s1}-casein (CN) f(1-23); 2: β-lactoglobulin; 3: *para*-κ-CN; 4: γ_2-CN C; 5: α_{s2}-CN; 6: γ_2-CN A; 7: γ_1-CN B; 8: γ_1-CN A1; 9: α_{s1}-CN; 10: γ_1-CN A2; 11: α_{s0}-CN; 12: κ-CN; 13: β-CN B; 14: β-CN A1; 15: β-CN A2; 16: α_{s1}-I-CN; 14' and 15': β-CN A1 and β-CN A2 degradation products, respectively].



two unidentified peaks (14' and 15'). The hydrolysis of β-CN by chymosin occurs very slowly in cheese and results in a large peptide, β-CN f(1-192) and a small very hydrophobic and bitter peptide, β-CN f(193-209) (Upadhyay et al., 2004). Moreover, proteinases from lactic bacteria, such as lactocepins from a number of *Lactococcus* strains, can rapidly degrade β-CN at specific cleavage sites close to that of chymosin, i.e., Leu_{192}-Tyr_{193} (Upadhyay et al., 2004). However, almost no differences on lactococci counts were observed between treatments, although strain characterisation was not performed. The observed differences at the rate of β-CN degradation could be due to a conformational change of the protein making it more available for the enzyme.

Table III.2.2. Primary proteolysis - Caseins and other proteins or peptides (arbitrary units).

		Treatment[1]		
	Day	**PA**	**HP**	**UH**
***para*-κ-CN**	**1**	**13.38 ± 0.66** ^{a}	**12.97 ± 0.60** ^{a,b}	**12.00 ± 0.33** ^{a,b,c}
	13	**11.35 ± 1.27** ^{b,c}	**11.80 ± 0.36** ^{a,b,c}	**11.52 ± 0.45** ^{b,c}
	19	**-**	**-**	**10.51 ± 0.39** ^{c}
α_{s0}-CN	1	18.08 ± 0.59 ^{b}	18.14 ± 0.71 ^{b}	17.06 ± 0.65 ^{b,c}
	13	17.27 ± 0.85 ^{b,c}	17.45 ± 0.74 ^{b,c}	19.94 ± 0.95 ^{a}
	19	-	-	15.71 ± 0.82 ^{c}
α_{s1}-CN	1	42.94 ± 1.33 ^{a,b}	44.70 ± 1.52 ^{a}	43.50 ± 1.17 ^{a,b}
	13	39.72 ± 1.96 ^{b}	40.67 ± 2.07 ^{a,b}	39.54 ± 2.95 ^{b}
	19	-	-	41.91 ± 1.86 ^{a,b}
α_{s2}-CN	1	5.19 ± 0.44 ^{a}	5.15 ± 0.30 ^{a}	4.91 ± 0.12 ^{a,b}
	13	4.41 ± 0.28 ^{b}	4.59 ± 0.21 ^{a,b}	4.34 ± 0.30 ^{b}
	19	-	-	4.50 ± 0.15 ^{a,b}
α_s-CN	**1**	**66.22 ± 1.97** ^{a,b}	**67.99 ± 2.27** ^{a}	**65.48 ± 1.22** ^{a,b}
	13	**61.39 ± 2.52** ^{b}	**62.70 ± 2.63** ^{a,b}	**63.82 ± 2.70** ^{a,b}
	19	**-**	**-**	**62.11 ± 2.61** ^{a,b}
β-CN A1	1	22.33 ± 0.86 ^{a}	20.37 ± 1.51 ^{b}	20.85 ± 0.61 ^{a,b}
	13	17.43 ± 0.96 ^{c}	16.81 ± 0.75 ^{c}	13.15 ± 1.08 ^{d}
	19	-	-	11.20 ± 0.61 ^{e}
β -CN A2	1	50.89 ± 2.44 ^{a}	45.28 ± 2.62 ^{b}	48.59 ± 1.17 ^{a,b}
	13	38.95 ± 2.51 ^{c}	39.40 ± 2.13 ^{c}	32.27 ± 1.99 ^{d}
	19	-	-	25.71 ± 1.31 ^{e}
β -CN B	1	4.60 ± 0.36 ^{a}	4.25 ± 0.45 ^{a,b}	4.49 ± 0.50 ^{a}
	13	3.78 ± 0.54 ^{b}	3.77 ± 0.50 ^{b}	2.57 ± 0.13 ^{c}
	19	-	-	2.77 ± 0.48 ^{c}
β -CN	**1**	**77.81 ± 2.53** ^{a}	**69.89 ± 4.17** ^{b}	**73.93 ± 1.55** ^{a,b}
	13	**60.16 ± 3.89** ^{c}	**59.98 ± 2.95** ^{c}	**48.00 ± 2.99** ^{d}
	19	**-**	**-**	**39.68 ± 1.89** ^{e}

$^{a-e}$ Mean value ± s.e.; n = 9; values without common superscripts were significantly different ($P < 0.05$).

[1] PA: pasteurisation; HP: homogenisation-pasteurisation; UH: ultra-high pressure homogenisation.

Table III.2.2. Primary proteolysis - Caseins and other proteins or peptides (arbitrary units). (continuation)

		Treatment[1]		
	Day	**PA**	**HP**	**UH**
γ_1-CN A1	1	3.39 ± 0.18 [a]	3.34 ± 0.27 [a]	3.55 ± 0.32 [a]
	13	3.41 ± 0.28 [a]	3.46 ± 0.27 [a]	3.77 ± 0.14 [a]
	19	-	-	2.56 ± 0.22 [b]
γ_1-CN A2	1	1.28 ± 0.13 [a]	1.06 ± 0.11 [a,b]	0.97 ± 0.11 [a,b]
	13	1.30 ± 0.16 [a]	1.23 ± 0.17 [a]	0.75 ± 0.11 [b,c]
	19	-	-	0.54 ± 0.06 [c]
γ_1-CN B	1	2.52 ± 0.19 [a]	2.70 ± 0.19 [a]	2.60 ± 0.17 [a]
	13	2.39 ± 0.28 [a]	2.23 ± 0.20 [a]	2.65 ± 0.22 [a]
	19	-	-	2.71 ± 0.21 [a]
γ_1-CN	1	*7.19 ± 0.31* [a]	*7.10 ± 0.49* [a]	*7.12 ± 0.35* [a]
	13	*7.10 ± 0.61* [a]	*6.93 ± 0.45* [a,b]	*7.16 ± 0.33* [a]
	19	-	-	*5.82 ± 0.42* [b]
γ_2-CN A	1	3.44 ± 0.37 [a]	3.37 ± 0.16 [a,b]	2.59 ± 0.20 [b,c]
	13	3.41 ± 0.42 [a]	2.83 ± 0.26 [a,b,c]	2.21 ± 0.28 [c]
	19	-	-	2.44 ± 0.24 [c]
γ_2-CN C	1	0.93 ± 0.08 [a]	0.80 ± 0.04 [a,b,c]	0.81 ± 0.03 [a,b]
	13	0.78 ± 0.09 [b,c,d]	0.72 ± 0.07 [b,c,d]	0.67 ± 0.05 [c,d]
	19	-	-	0.66 ± 0.03 [d]
γ_2-CN	1	*4.37 ± 0.39* [a]	*4.17 ± 0.19* [a,b]	*3.40 ± 0.20* [b,c]
	13	*4.19 ± 0.48* [a,b]	*3.54 ± 0.27* [a,b,c]	*2.88 ± 0.22* [c]
	19	-	-	*3.10 ± 0.23* [c]
γ -CN	**1**	**11.56 ± 0.59** [a]	**11.27 ± 0.53** [a]	**10.53 ± 0.48** [a,b]
	13	**11.29 ± 1.00** [a]	**10.47 ± 0.62** [a,b]	**10.05 ± 0.64** [a,b]
	19	**-**	**-**	**8.92 ± 0.62** [b]
Deg. β -CN A1	1	1.34 ± 0.18 [d]	2.36 ± 0.45 [c]	1.06 ± 0.24 [d]
	13	2.07 ± 0.32 [c]	2.41 ± 0.52 [c]	3.57 ± 0.33 [b]
	19	-	-	4.28 ± 0.47 [a]
Deg. β -CN A2	1	n.d. [d]	3.85 ± 1.94 [c]	n.d. [d]
	13	4.14 ± 1.08 [c]	4.28 ± 1.16 [c]	9.30 ± 0.44 [b]
	19	-	-	11.88 ± 1.20 [a]
Deg. β -CN[†]	**1**	**1.34 ± 0.18** [d]	**6.21 ± 2.36** [c]	**1.06 ± 0.24** [d]
	13	**6.21 ± 1.37** [c]	**6.68 ± 1.63** [c]	**12.88 ± 0.74** [b]
	19	**-**	**-**	**16.17 ± 1.62** [a]
β -LG	**1**	**0.54 ± 0.05** [c]	**0.73 ± 0.05** [c]	**1.91 ± 0.25** [a,b]
	13	**0.43 ± 0.05** [c]	**0.64 ± 0.08** [c]	**1.90 ± 0.30** [b]
	19	**-**	**-**	**2.28 ± 0.10** [a]

[a-e] Mean value ± s.e.; n = 9; n.d.: not detected; values without common superscripts were significantly different ($P < 0.05$).

[1] PA: pasteurisation; HP: homogenisation-pasteurisation; UH: ultra-high pressure homogenisation.

[†] Deg.: degradation product.

Plasmin also hydrolyses α_{s2}-CN (Sousa et al., 2001) producing a peptide which peak appears just before that of γ_2-CN C (peak 4) (Recio et al., 1997). Several small peaks were observed in that position, proving that some α_{s2}-CN proteolysis already occurred in milk or at a very early stage of cheese

production. However, no differences in the content of α_{s2}-CN were observed between treatments during storage.

Denatured whey proteins can be observed in cheese eletropherograms, with the two genetic variants of β-lactoglobulin (**β-LG**) appearing as a double shoulder (peak 2) on the *para*-κ-CN peak (3) (Recio et al., 1997). The content of this protein was much higher in UH-cheeses than those of cheeses from conventionally treated milk samples. Previous studies have shown that UHPH triggers the denaturation of whey proteins and that β-LG is more affected than α-lactalbumin (**α-LA**), with 35% and 12% denaturation, respectively (see Section II Chapter 1). The amount of β-LG did not vary during storage, except for an increase in UH-cheeses between day 13 and 19, which might be due to other proteins that co-migrate with β-LG, such as forms of *para*-κ-CN (García-Risco et al., 1999).

Secondary proteolysis is the degradation of large-medium casein peptides to low molecular weight peptides and free amino acids due to proteinases and peptidases of microorganisms (Rank et al., 1985). Levels of WSN are mainly influenced by the amount of whey proteins, soluble peptides and FAA. In cheeses from day 1, no statistical differences ($P > 0.05$) were observed between treatments (Table III.2.3). However, WSN increased in PA- and UH-cheeses from days 1 to 13. In addition, UH-cheeses showed that an important decrease in WSN had occurred at some point from day 13 to day 19. The balance between various factors might have influenced WSN content in cheeses. Factors directly affecting whey drainage (see Section III Chapter 1), such as microstructure and water typology (see Section III Chapter 3), may have played an important role. Moreover, microorganisms (see Section III Chapter 1) by changing whey composition could also have contributed to the differences observed between treatments.

Considering FAA content (Table III.2.3), UH-cheeses showed lower levels compared to cheeses from conventionally treated milk samples. During storage, the later showed a trend to increase FAA content. However, in UH-cheeses, FAA content decreased from day 1 to day 13 and stayed unchanged up to day 19. The increase of FAA in PA- and HP-cheeses may reflect the

microbial peptidase activity. However, in UH-cheeses the FAA remained unchanged when the highest microbial activity was expected (from day 13 to 19; see Section III Chapter 1). FAA content results from the balance between production and degradation, which in turn depend upon microorganisms in cheese; amino acid catabolism remains to be fully characterised but recent studies on lactic bacteria have shown that two major pathways, initiated either by transamination or amino acid cleavage, seem to be involved (Yvon & Rijnen, 2001). Both series of reactions are catalysed by microbial enzymes, and the specificity of the enzymes depends upon microbial species and strain (Yvon & Rijnen, 2001). Differences in microbial populations might be at the origin of the differences observed between UH-cheeses and those from conventionally treated milk samples (see Section III Chapter 1).

Table III.2.3. Secondary proteolysis – Soluble nitrogen and free amino acids[1].

Treatment[2] & day		SN (g 100 g^{-1} TN)	FAA (mg Leu g^{-1} cheese)
PA	1	13.78 ± 0.38 ^{b}	0.123 ± 0.006 ^{d}
	13	14.98 ± 0.96 ^{a}	0.154 ± 0.007 ^{b}
	19	-	-
HP	1	13.48 ± 0.57 ^{b,c}	0.146 ± 0.004 ^{c}
	13	12.96 ± 0.48 ^{c,d}	0.191 ± 0.022 ^{a}
	19	-	-
UH	1	14.01 ± 0.64 ^{b}	0.080 ± 0.002 ^{e}
	13	14.85 ± 0.49 ^{a}	0.065 ± 0.004 ^{f}
	19	12.40 ± 0.27 ^{d}	0.061 ± 0.007 ^{f}

$^{a-f}$ Mean value ± s.e.; n = 6 for SN and n = 9 for FAA; values without common superscripts were significantly different ($P < 0.05$).

[1] SN: soluble nitrogen; FAA: free amino acids.

[2] PA: pasteurisation; HP: homogenisation-pasteurisation; UH: ultra-high pressure homogenisation.

2.3.3. Lipolysis

Lipolysis, or hydrolysis of lipids, is caused by the enzyme lipase which hydrolises the triacylglycerols. Two main types of lipases may cause problems in milk and dairy products, i.e., indigenous naturally occurring in raw milk and bacterial produced by contaminating bacteria (Deeth, 2002). Most, if not all, of the lipolytic activity in bovine milk, comes from only one lipolytic enzyme,

lipoprotein lipase (LPL). This enzyme is a glycoprotein with two *N*-linked oligosaccharides, which seem to be necessary for its activity. In milk, LPL is normally associated with the casein micelle through electrostatic and hydrophobic interactions between LPL and caseins (Deeth, 2006). Milk fat is enveloped in a biological membrane, the milk fat globule membrane (MFGM), which facilitates dispersion of the fat in the aqueous phase of milk and also segregates the fat from milk lipase. The MFGM is an effective barrier to access by LPL to the fat. A necessary condition for lipolysis is the transfer of LPL from the skim phase to the water-fat interface via attachment to the MFGM. Technological operations such as cooling, freezing or agitation of milk enhance the association of LPL to the MFGM, and together with a concomitant damage to the MFGM may result in extensive lipolysis (Deeth, 2002).

Homogenisation of milk reduces fat globule size damaging the MFGM and provoking changes on its composition (see Section II Chapter 3). Both the incorporation of proteins such as caseins, which brings LPL into intimate contact with fat, and the increase in fat surface area render fat globules more susceptible to lipolysis (Deeth, 2006). Moreover, high-pressure homogenisation appears to activate the enzyme in bovine milk (Datta et al., 2005), but the mechanism of this activation has not been elucidated. In fact, Pereda et al. (2008b) observed that an extensive lipolysis occurred in milk UHPH-treated at 200 MPa with an inlet temperature of 30 ºC. Probably, the temperature reached during treatment together with the short time at which milk was subjected to this temperature was not high enough to inactivate completely the LPL. However, higher temperatures (from 85 ºC to 100 ºC), reached by increasing either the inlet temperature of milk to 40 ºC or the pressure of the treatment to 300 MPa, resulted in a drastic decrease of lipolysis down to the values obtained for raw and homogenised-pasteurised (90 ºC for 15 s) milk samples (Pereda et al., 2008b). LPL is relatively unstable to heat. The inactivation varies according to the severity of the heat treatment; pasteurisation at 70ºC for 15 s results in a residual activity of 2% but complete inactivation is reached at 75 ºC for 15 s (Beuvier, 2004).

In the present study, statistical differences ($P < 0.05$) between treatments were observed for total FFA and for almost all individual FFA (Table III.2.4). Unexpectedly, a reduction of total FFA in both HP- and UH-cheeses (25 and 43%, respectively) was observed in comparison to PA-cheeses. This reduction could be related to the homogenisation intensity and to the changes produced by these technologies on milk fat globules. Possibly, the rearrangement of the globule surface by adsorption of casein micelles (or fragments) and whey proteins (see Section II Chapter 3) resulted in the loss of FFA by binding with milk proteins. Whey proteins β-LG and serum albumin, and some milk fat globule membrane proteins are able to bind fatty acids. In addition, LPL is inactivated by oxidising agents (Shakeel-ur-Rehman & Farkye, 2002). Indeed, greater oxidation was observed in HP- and UH-cheeses than PA-cheeses (see 2.3.4. Oxidation), thus the presence of oxidising agents in homogenised milk samples could have partly inactivated LPL resulting in lower FFA content than in PA-cheeses.

During cold storage (up to day 13) a reduction of total FFA in PA- and HP-cheeses was observed possibly due to the catabolism of FFA in other compounds such as methyl-ketones, secondary alcohols and lactones (see 2.3.5. Volatile compounds). In contrast, for UH-cheeses, the evolution of FFA concentration during storage was steady, indicating a higher storage capacity in comparison with PA- and HP-cheeses on respect to lipolysis. Similar results have been obtained with UHPH-treated milk by Pereda et al. (2008b). The incorporation of caseins and whey proteins would render fat globules less susceptible to microbial lipases, thus reducing the overall lipolysis during cheese storage. This fact could explain the lower lipolysis observed in HP-cheese related to PA-cheese, and the constant levels of FFA in UHPH-cheese.

In UHPH-treated milk (Pereda et al., 2008b), butyric (C4:0), caproic (C6:0), caprylic (C8:0), capric (C10:0), lauric (C12:0), myristic (C14:0), palmitic (C16:0), stearic (C18:0), oleic (C18:1) and linoleic (18:2) acids were identified. In the present study, SCFA and some MCFA (C4-C10) were not observed by GC analysis in cheese, but were detected at the analysis of volatile compounds by GC-MSD. Recent studies on Caciotta and Crescenza cheeses have also shown

such differences between the two analytical methods (Lanciotti et al., 2004; Lanciotti et al., 2006; Burns et al., 2008). SCFA, which are present at very low concentrations in milk (Pereda et al., 2008b), are partly soluble in water and might be lost to some extent during whey drainage. The extraction for the analysis of volatile compounds was performed at a very much higher temperature than that of FFA, 80 ºC vs. room temperature, and might have enhanced the extraction of remaining SCFA.

Table III.2.4. Free fatty acids (μg FFA 100 g^{-1} TS).

		Treatment[1]		
	Day	**PA**	**HP**	**UH**
C12:0	1	11.87 ± 3.30 [a]	6.48 ± 3.28 [b]	n.d. [c]
	13	n.d. [c]	n.d. [c]	n.d. [c]
	19	-	-	n.d. [c]
C14:0	1	31.63 ± 2.10 [a]	22.63 ± 4.77 [b]	13.32 ± 4.05 [c]
	13	33.48 ± 2.73 [a]	19.83 ± 0.68 [b]	11.23 ± 3.87 [c]
	19	-	-	3.06 ± 1.53 [d]
C16:0	1	101.03 ± 5.35 [a]	70.23 ± 3.07 [b]	57.36 ± 1.61 [c]
	13	101.64 ± 5.23 [a]	72.74 ± 3.08 [b]	53.80 ± 4.32 [c,d]
	19	-	-	50.60 ± 0.60 [d]
C18:0	1	104.48 ± 2.69 [a]	96.69 ± 3.43 [b]	99.97 ± 2.54 [a,b]
	13	83.22 ± 4.22 [c,d]	79.55 ± 6.62 [d]	87.88 ± 9.44 [c]
	19	-	-	88.51 ± 1.62 [c]
C18:1	1	52.61 ± 3.49 [b]	31.07 ± 3.91 [c]	n.d. [e]
	13	60.21 ± 4.55 [a]	30.37 ± 1.40 [c]	16.26 ± 4.32 [d]
	19	-	-	5.91 ± 3.65 [e]
Total FFA	**1**	**301.62 ± 13.93 [a]**	**227.09 ± 9.29 [c]**	**170.64 ± 7.11 [e]**
Σ (C12- C18:1)	**13**	**278.56 ± 10.41 [b]**	**202.49 ± 9.89 [d]**	**169.16 ± 19.34 [e]**
	19	**-**	**-**	**148.08 ± 4.45 [f]**

[a-f] Mean value ± s.e.; n = 9; n.d.: not detected; values per compound without common superscripts were significantly different ($P < 0.05$).

[1] PA: pasteurisation; HP: homogenisation-pasteurisation; UH: ultra-high pressure homogenisation.

Concentrations on a wet basis of identified FFA in HP-cheeses were generally in accordance with those obtained in milk (Pereda et al., 2008b), with the exception of stearic acid (C18:0); much greater values were observed in cheese than in milk, resulting in higher amounts of stearic acid (C18:0) than oleic acid (C18:1) in cheese. Although not being common, higher amounts of stearic than oleic acid were also observed in Crescenza cheeses from pasteurised milk (Burns et al., 2008). Owing to the fact that cheeses presented great variation on their moisture content (see 2.3.1. Cheese composition) and that MCFA and

LCFA are rather insoluble in water (Deeth, 2002), FFA content was expressed on a dry basis (Table III.2.4). Stearic acid (C18:0) followed by palmitic acid (C16:0) were the most abundant FFA in all cheeses. The amount of saturated FFA tended to decrease or to not change during storage. In contrast, oleic acid (18:1), a mono-unsaturated fatty acid, tended to increase in PA- and UH-cheeses. As will be afterwards discussed, the amount of FFA in cheese depends upon a balance between formation and degradation.

2.3.4. Oxidation

Lipid oxidation is a chain reaction involving initiation, propagation and termination stages. The initiation reaction involves the generation of fatty acid radicals (alkyl radicals) due to the removal of a hydrogen atom in an unsaturated fat molecule. Although the detailed mechanism of formation is not fully understood, factors such as light exposure, metal catalyst, heat, and active oxygen species influence the formation of alkyl radicals. These radicals are extremely reactive and can combine with molecular oxygen and another unsaturated fatty acid molecule to generate the corresponding hydroperoxide and a new alkyl radical. Hydroperoxides, which are the primary oxidation products, degrade to produce volatile secondary oxidation products, many of which result in off-flavours (O'Brien & O'Connor, 2002).

Methods for quantifying lipid oxidation are usually based in the measurement of hydroperoxides (peroxide value) or substances that react with thiobarbituric acid (TBARS). However, hydroperoxides may not correlate well with the level of off-flavour since during the course of lipid oxidation, peroxide values reach a peak and then decline (O'Brien & O'Connor, 2002). Many substances, such as ketones, ketosteroids, acids, esters, sugars, imides and amides, amino acids, oxidized proteins, and pyridines and pyrimidines, are TBARS (Fenaille et al., 2001). Since aldehydes, such as hexanal, are common secondary oxidation products with thresholds generally lower than ketones, their concentration might be used as an indicator of lipid oxidation and oxidised off-flavour. From day 1, UH-cheeses showed higher amounts of hexanal than cheeses from conventionally treated milk samples (Table III.2.5). A previous study on lipid oxidation in UHPH-treated milk (Pereda et al., 1008b) showed that at 300 MPa,

the amount of secondary oxidation products, such as TBARS and hexanal, was statistically higher than that of milk homogenised-pasteurised at 90 ºC for 15 s. Higher TBARS and hexanal values, together with lower hydroperoxides values, indicated in the mentioned study the progression of oxidation from a primary to a secondary state in UHPH-treated milk at 300 MPa.

Table III.2.5. Hexanal content of cheeses (ppb).

Treatment[1]	Day		
	1	13	19
PA	0.68 ± 0.26 [d]	2.45 ± 0.72 [c]	-
HP	1.37 ± 0.39 [c,d]	1.23 ± 0.24 [c,d]	-
UH	10.85 ± 1.99 [a]	8.75 ± 1.29 [b]	8.01 ± 1.07 [b]

[a-d] Mean value ± s.e.; n = 6; values without common superscripts were significantly different ($P < 0.05$).

[1] PA: pasteurisation; HP: homogenisation-pasteurisation; UH: ultra-high pressure homogenisation.

Milk fat globule membrane (MFGM) is a focal point for oxidation since phospholipids of the membrane are in close proximity to pro-oxidants. Oxidation, initiates in the MFGM, propagates into the fat globule core resulting in generalised oxidation of milk fat triglycerides. Xanthine oxidase (XO) has been proposed as partially responsible for the susceptibility of the membrane to lipid oxidation (O'Brien & O'Connor, 2002). However, the study on the MFGM composition (see Section II Chapter 3) revealed that some native MFGM proteins, such as XO, were partly removed during homogenisation through the incorporation of caseins and whey proteins. Nevertheless, both resurfacing of the fat globules, especially with caseins, and the partial transfer of unsaturated phospholipids from the MFGM to the aqueous phase inhibit lipid oxidation (Dunkley et al., 1962; Huppertz & Kelly, 2006). Indeed, the levels of hexanal increased in PA-cheeses, but did not vary in HP-cheeses during storage.

Although UHPH also triggers changes at the MFGM (see Section II Chapter 3), lipid oxidation occurs. Thus, an opposite mechanism must be at its origin. As previously mentioned, metals catalyse the formation of alkyl radicals. Small amounts of metal ions can generate large number of reaction chains by cycling

between the oxidised and reduced forms. The juxtaposition of a copper-protein complex with the phospholipids of the MFGM appears to be an important factor (O'Brien & O'Connor, 2002). Contamination with metals may be due to a transfer from the equipment pieces, e.g., non-return valves and seals, to the milk. Moreover, added metals interact less with milk proteins, which modulate their pro-oxidant effects, than indigenous metals. Since the decomposition of hydroperoxides is catalysed by pro-oxidant metals (O'Brien & O'Connor, 2002), high level of hexanal is an excellent indicator of lipid oxidation due to metal contamination (Marsili, 2002). In addition, oxygen is a requirement for oxidative deterioration of lipids. Both a modification of the equipment pieces and a removal of oxygen from milk or its replacement by an inert gas just before treatment may reduce lipid oxidation during UHPH.

The role of oxidation in both proteolysis and lipolysis has been already described (see 2.3.2. Proteolysis and 2.3.3. Lipolysis) and will be further commented when describing the volatile profiles of cheeses in the next section.

2.3.5. Volatile compounds

In UH-cheeses, forty six compounds were identified by their RT and characteristic ion, which included 5 fatty acids, 8 ketones, 9 aldehydes, 8 alcohols and 16 miscellaneous compounds. However, only 37 compounds were detected in cheeses from conventionally treated milk. In Tables III.2.6-10, the identified compounds are listed by chemical group.

Since SCFA are much volatile than MCFA and LCFA, only SCFA were identified in the headspace of cheese samples by GC-MS (Table III.2.6). All of them have been previously reported in different cheese varieties (Le Quéré & Molimard, 2002; Van Leuven et al., 2008). In general, HP-cheeses showed higher amounts than PA- and UH-cheeses. In cheeses from conventionally treated milk samples, the general trend was to increase during storage. Cold storage of milk provokes a shift in the main microbial population from lactic acid bacteria to psychrothrophic bacteria, such as pseudomonads; many of these produce extracellular lipases which can cause lipolysis (Deeth, 2002). Indeed, Pereda et al. (2008b) attributed to *Pseudomonas* spp. the increase of lipolysis in UHPH-

treated milk. Although pseudomonads were not studied in cheese as specific microbial group, at day 13, psychrotrophs were 2 log higher than lactococci in cheeses from conventionally treated milk samples, and 1 log higher in UH-cheeses (see Section III Chapter 1). Hence, microbial lipases might be the cause of the SCFA increase in PA- and HP-cheeses during storage. Moreover, the greater values obtained in HP-cheeses could be explained by a greater susceptibility of fat globules to lipolysis due to damage during homogenisation. Lanciotti et al. (2006), working with high-pressure homogenisation at 100 MPa, reported an increase in butanoic, hexanoic, octanoic and decanoic acids between day 1 and day 13 in Caciotta cheese. In contrast, in this study and for UH-cheeses, although no statistical differences were found, fatty acids tended to decrease during the first 13 days. Differences in both the temperature-pressure conditions and the type of homogeniser could explain these contradictory results. Butanoic acid, which has a rancid taste, was the volatile FFA detected with the highest concentration in all samples. Volatile acids play a role in cheese flavour by themselves (see 2.3.6. Sensory analysis) and serve as precursors of methyl-ketones, alcohols, lactones and esters (Deeth & Fitz-Gerald, 1994).

Table III.2.6. Volatile compounds – Fatty acids (arbitrary units).

		Treatment[1]		
Fatty acids	**Day**	**PA**	**HP**	**UH**
Butanoic acid	1	427.0 ± 55.3 [c]	788.0 ± 96.9 [b]	655.8 ± 155.3 [b,c]
	13	452.7 ± 59.5 [c]	1,649.5 ± 265.8 [a]	449.8 ± 64.1 [c]
	19	-	-	477.0 ± 51.8 [c]
Hexanoic acid	1	354.8 ± 27.6 [e]	750.2 ± 64.1 [b]	593.1 ± 56.6 [c]
	13	368.2 ± 114.7 [d,e]	1,530.6 ± 135.0 [a]	489.2 ± 40.3 [c,d]
	19	-	-	549.8 ± 51.4 [c]
Octanoic acid	1	155.1 ± 20.6 [c]	270.1 ± 65.6 [b]	151.4 ± 51.5 [c]
	13	188.3 ± 67.1 [b,c]	467.9 ± 47.6 [a]	96.4 ± 13.7 [c]
	19	-	-	109.7 ± 57.5 [c]
Nonanoic acid	1	101.7 ± 44.1 [a]	118.9 ± 53.3 [a]	139.5 ± 80.7 [a]
	13	229.1 ± 111.7 [a]	168.2 ± 57.7 [a]	129.5 ± 50.2 [a]
	19	-	-	104.2 ± 87.5 [a]
Decanoic acid	1	39.2 ± 4.6 [b]	74.3 ± 31.2 [a,b]	27.9 ± 19.0 [b]
	13	66.8 ± 31.3 [a,b]	112.9 ± 25.6 [a]	17.6 ± 9.0 [b]
	19	-	-	26.3 ± 18.8 [b]

[a-e] Mean value ± s.e.; n = 6; values per compound without common superscripts were significantly different ($P < 0.05$).

[1] PA: pasteurisation; HP: homogenisation-pasteurisation; UH: ultra-high pressure homogenisation.

Ketones were by far the most abundant group of volatile compounds in fresh cheese (Table III.2.7). All identified ketones have been widely reported in cheese (Le Quéré & Molimard, 2002; Hayaloglu & Brechany, 2007; Van Leuven et al., 2008; Hayaloglu, 2009). Acetone was the most abundant ketone in all cheese samples. This compound is thought to be derived from cows' feed (Gordon & Morgan, 1972). Pereda et al. (2008c) working on milk from the same farm as in this study, also reported acetone as the most abundant compound in milk. Similar to fatty acids, as the methyl-ketone chain length increased, the concentration decreased due to a decrease in the volatile character of the molecule. UH-cheeses showed higher amounts of 2-butanone, 2-pentanone and 2-heptanone than cheeses from conventionally treated milk.

Table III.2.7. Volatile compounds – Ketones (arbitrary units).

		Treatment[1]		
Ketones	**Day**	**PA**	**HP**	**UH**
Acetone	1	13,360.6 ± 2,125.2 [a]	9,958.9 ± 1,680.3 [a]	13,604.3 ± 2,734.5 [a]
	13	13,642.8 ± 2,215.6 [a]	9,062.8 ± 1,262.9 [a]	11,263.8 ± 1,989.1 [a]
	19	-	-	11,051.4 ± 2,204.4 [a]
2-Butanone	1	5,352.6 ± 1,552.6 [c]	5,263.9 ± 1,493.8 [c]	11,450.0 ± 2,185.2 [a]
	13	3,848.5 ± 283.5 [c,d]	3,428.8 ± 399.0 [d]	8,460.9 ± 1,339.7 [b]
	19	-	-	7,375.2 ± 855.3 [b]
2-Pentanone	1	5,071.1 ± 277.3 [d]	5,600.7 ± 578.2 [c,d]	7,516.5 ± 410.2 [a]
	13	7,086.8 ± 208.7 [a,b]	6,390.3 ± 380.7 [b,c]	7,415.9 ± 618.8 [a]
	19	-	-	7,851.5 ± 696.2 [a]
2-Heptanone	1	694.3 ± 50.2 [c]	1,680.2 ± 390.5 [b,c]	2,504.8 ± 523.7 [b]
	13	2,368.3 ± 621.2 [b]	2,376.4 ± 823.2 [b]	2,572.5 ± 581.5 [b]
	19	-	-	4,271.1 ± 765.6 [a]
2-Octanone	1	38.1 ± 5.3 [b]	41.1 ± 10.8 [b]	78.6 ± 16.4 [b]
	13	52.9 ± 6.3 [b]	41.1 ± 8.6 [b]	51.5 ± 8.9 [b]
	19	-	-	238.5 ± 94.8 [a]
2-Nonanone	1	176.8 ± 14.2 [b]	141.1 ± 13.9 [b]	157.6 ± 24.5 [b]
	13	427.3 ± 95.9 [b]	345.7 ± 116.8 [b]	213.5 ± 49.0 [b]
	19	-	-	1,784.3 ± 983.2 [a]
2-Undecanone	1	27.8 ± 9.9 [b,c,d]	34.0 ± 2.3 [b,c]	26.1 ± 3.2 [b,c,d]
	13	58.4 ± 9.1 [a]	13.3 ± 8.4 [c,d]	6.6 ± 4.2 [d]
	19	-	-.	45.9 ± 18.5 [a,b]
Acetophenone	1	n.d. [c]	0.5 ± 0.3 [b,c]	3.3 ± 1.6 [a]
	13	0.7 ± 0.4 [b,c]	1.9 ± 0.9 [a,b]	3.3 ± 2.1 [a]
	19	-	-	0.7 ± 0.7 [b,c]

[a-d] Mean value ± s.e.; n = 6; n.d.: not detected; values per compound without common superscripts were significantly different ($P < 0.05$).

[1] PA: pasteurisation; HP: homogenisation-pasteurisation; UH: ultra-high pressure homogenisation.

Both autoxidation and thermal oxidation reactions can generate these compounds through oxidation of free fatty acids to β-ketoacids and their subsequent decarboxylation to the corresponding methyl-ketones with one carbon atom less (Adda et al., 1982; McSweeney & Sousa, 2000). Ketones are intermediate compounds, which may be reduced to 2-alkanols, thus their levels depend on the balance between production and degradation (Beuvier & Buchin, 2004). Due to their typical odours and their low perception threshold, ketones, and especially methyl-ketones, are primarily know for their contribution to the aroma of cheese; they have been correlated with fruity, floral, musty and blue cheese notes (Curioni & Bosset, 2002).

Aldehydes were characteristic of UH-cheese profiles (Table III.2.8.). Both straight- and branched-chain aldehydes were identified. However, in cheeses from conventional treatments only straight-chain aldehydes, i.e., hexanal, octanal and nonanal, were observed, and their amounts were statistically lower than in UH-cheeses. UHPH triggers the formation of aldehydes in milk (Pereda et al., 2008c). Aldehydes, together with ketones, are the major secondary products of autoxidation of unsaturated fatty acids; straight-chain aldehydes originate from the β-oxidation of unsatured fatty acids (Beuvier & Buchin, 2004). Other aldehydes, mainly branched, originate from amino acids either by transamination, leading to an imide that can be decarboxylated, or by a non-enzymatic browning via Strecker's degradation (Le Quéré & Molimard, 2002). Aldehydes are transitory compounds in cheese; due to enzymatic activities of microorganisms, they are oxidised to acids or reduced to alcohols (Beuvier & Buchin, 2004). Due to their low perception threshold, aldehydes could play a very important role in the flavour of UH-cheeses (see 2.3.6. Sensory analysis). They are characterised by green-grass-like and herbaceous aromas (Moio et al., 1993), but become very unpleasant when their concentrations exceed certain thresholds (Curioni & Bosset, 2002). Hexanal and t-2-hexenal give the green note of immature fruit, while octanal and nonanal are described as having an aromatic note resembling orange (Le Quéré & Molimard, 2002). Branched-chain aldehydes 3-methylbutanal and 2-methylbutanal, which originate from leucine and isoleucine, respectively, are responsible for unclean and harsh flavours in Hispánico cheese (Garde et al., 2002).

Table III.2.8. Volatile compounds – Aldehydes (arbitrary units).

		Treatment[1]		
Aldehydes	**Day**	**PA**	**HP**	**UH**
Butanal	1	n.d. ^{d}	n.d. ^{d}	102.7 ± 14.9 ^{a}
	13	n.d. ^{d}	n.d. ^{d}	65.8 ± 4.0 ^{b}
	19	-	-	49.7 ± 8.5 ^{c}
2-Butenal	1	n.d. ^{c}	n.d. ^{c}	109.3 ± 41.0 ^{a}
	13	n.d. ^{c}	n.d. ^{c}	110.4 ± 20.7 ^{a}
	19	-	-	65.3 ± 13.5 ^{b}
2-Methylbutanal	1	n.d. ^{c}	n.d. ^{c}	265.5 ± 32.3 ^{a}
	13	n.d. ^{c}	n.d. ^{c}	211.3 ± 40.8 ^{b}
	19	-	-	177.7 ± 21.2 ^{b}
3-Methylbutanal	1	n.d. ^{d}	n.d. ^{d}	552.4 ± 90.7 ^{a}
	13	n.d. ^{d}	n.d. ^{d}	498.4 ± 96.9 ^{b}
	19	-	-	353.6 ± 45.5 ^{c}
Hexanal	1	134.2 ± 35.4 ^{d}	227.2 ± 52.4 ^{c,d}	1,496.6 ± 266.3 ^{a}
	13	371.9 ± 96.0 ^{c,d}	208.2 ± 32.5 ^{c}	1,216.4 ± 173.0 ^{b}
	19	-	-	1,116.4 ± 143.0 ^{b}
t-2-Hexenal	1	n.d. ^{b}	n.d. ^{b}	39.5 ± 8.6 ^{a}
	13	n.d. ^{b}	n.d. ^{b}	44.1 ± 5.0 ^{a}
	19	-	-	33.5 ± 5.2 ^{a}
2-Heptenal	1	n.d. ^{b}	n.d. ^{b}	27.3 ± 4.4 ^{a}
	13	n.d. ^{b}	n.d. ^{b}	24.7 ± 3.4 ^{a}
	19	-	-	26.9 ± 3.5 ^{a}
Octanal	1	1.6 ± 1.1 ^{c}	8.0 ± 3.3 ^{b,c}	41.6 ± 12.4 ^{a}
	13	8.9 ± 4.1 ^{b,c}	1.8 ± 0.9 ^{c}	15.6 ± 5.5 ^{b}
	19	-	-	6.5 ± 6.1 ^{b,c}
Nonanal	1	11.3 ± 2.4 ^{b}	11.6 ± 2.0 ^{b}	38.3 ± 13.0 ^{a}
	13	21.6 ± 4.3 ^{a,b}	11.3 ± 2.3 ^{b}	22.8 ± 4.5 ^{a,b}
	19	-	-	19.2 ± 2.6 ^{b}

$^{a-d}$ Mean value ± s.e.; n = 6; n.d.: not detected; values per compound without common superscripts were significantly different ($P < 0.05$).

[1] PA: pasteurisation; HP: homogenisation-pasteurisation; UH: ultra-high pressure homogenisation.

All eight identified alcohols (Table III.2.9) were also detected in two recent studies with ripened cheeses (Hayaloglu & Brechany, 2007; Hayaloglu, 2009). Primary alcohols such as 1-butanol, 1-pentanol, 1-hexanol, 1-heptanol and 1-octanol are produced mainly by the reduction of aldehydes (Arora et al., 1995; Moio & Addeo, 1998) and they impart alcoholic, sweet, and fruity notes in cheese (Barron et al., 2005). Cheeses from UHPH-treated and conventionally homogenised milk samples had higher concentration of butanol and pentanol compared with PA-cheeses. However, for the rest of alcohols, UH-cheeses presented higher amounts than cheeses from conventionally treated milk samples. The presence of precursors and a decrease of the redox potential in UH-cheeses may be the reason of such differences. Some alcohols arise either from the Erhlich's amino acid metabolism pathway or from aldehyde

degradation, which are derived from the Strecker's amino acid degradation pathway (Le Quéré & Molimard, 2002). Peptide degradation to amino acids can be achieved by aminopeptidases and carboxypeptidases (see 2.3.2. Proteolysis); oxidative deamination of amino acids is ensured by oxydoreductase, which can either be dehydrogenases or oxidases. Branched primary alcohols, are formed by the catabolism of branched-chain amino acids initiated by an aminotransferase (Atiles et al., 2000; Marilley & Casey, 2004). Morales et al. (2003) reported that the production of large amounts of branched-chain aldehydes and alcohols during manufacture and storage of fresh cheese is common when wild strains of *Lactoccocus lactis* are present. Specifically, 3-methylbutanol is formed from the conversion of the aldehydes produced from catabolism of leucine (Engels et al., 1997). This compound gives an alcoholic and fruity note and confers a pleasant aroma of fresh cheese (Moio et al., 1993). No secondary alcohols, which are formed by the enzymatic reduction of methyl-ketones (Le Quéré & Molimard, 2002), were detected.

Table III.2.9. Volatile compounds – Alcohols (arbitrary units).

		Treatment[1]		
Alcohols	**Day**	**PA**	**HP**	**UH**
1-Butanol	1	n.d. ^{b}	51.7 ± 21.3 ^{a}	55.1 ± 19.3 ^{a}
	13	n.d. ^{b}	47.6 ± 21.6 ^{a}	60.7 ± 19.2 ^{a}
	19	-	-	45.9 ± 19.2 ^{a}
1-Pentanol	1	21.7 ± 3.9 ^{b}	54.9 ± 15.2 ^{a}	71.1 ± 13.9 ^{a}
	13	57.2 ± 1.0 ^{a}	53.1 ± 11.9 ^{a}	65.4 ± 12.8 ^{a}
	19	-	-	69.2 ± 8.8 ^{a}
1-Hexanol	1	24.4 ± 2.7 ^{d}	39.5 ± 6.1 ^{d}	212.7 ± 39.8 ^{b}
	13	100.6 ± 27.4 ^{c}	43.2 ± 6.1 ^{d}	228.4 ± 51.5 ^{b}
	19	-	-	323.0 ± 53.2 ^{a}
1-Heptanol	1	7.7 ± 1.0 ^{d}	9.1 ± 1.2 ^{d}	24.7 ± 3.6 ^{a}
	13	15.6 ± 2.7 ^{c}	8.4 ± 1.6 ^{d}	20.9 ± 4.0 ^{b}
	19	-	-	24.6 ± 3.5 ^{a}
1-Octanol	1	10.7 ± 5.2 ^{b}	10.7 ± 7.0 ^{b}	81.7 ± 39.1 ^{a}
	13	14.3 ± 10.5 ^{b}	2.1 ± 2.1 ^{b}	21.5 ± 15.6 ^{b}
	19	-	-	n.d. ^{b}
2-Ethyl 1-hexanol	1	3.3 ± 2.1 ^{c}	3.9 ± 2.6 ^{c}	37.3 ± 9.0 ^{a}
	13	24.2 ± 8.8 ^{b}	13.4 ± 8.1 ^{b,c}	25.3 ± 11.1 ^{a,b}
	19	-	-	20.0 ± 12.0 ^{b}
3-Methyl 1-butanol	1	19.9 ± 3.1 ^{b}	24.7 ± 6.0 ^{b}	48.3 ± 13.4 ^{a}
	13	34.3 ± 3.1 ^{a,b}	25.2 ± 5.2 ^{b}	46.5 ± 9.2 ^{a}
	19	-	-	44.1 ± 10.4 ^{a}
2-Butoxyethanol	1	40.2 ± 16.1 ^{d}	233.1 ± 147.8 ^{b}	77.6 ± 34.3 ^{c,d}
	13	40.1 ± 22.3 ^{d}	293.5 ± 164.2 ^{a}	85.9 ± 38.8 ^{c}
	19	-	-	85.7 ± 31.7 ^{c}

$^{a-d}$ Mean value ± s.e.; n = 6; n.d.: not detected; values per compound without common superscripts were significantly different ($P < 0.05$).

[1] PA: pasteurisation; HP: homogenisation-pasteurisation; UH: ultra-high pressure homogenisation.

The synthesis of esters is accomplished by two enzymatic mechanisms, i.e., esterification and alcoholysis. The former is a reaction in which esters are formed from alcohols and carboxylic acids, while the latter, a transferase reaction in which fatty acyl groups from acylglycerols and acyl-CoA derivates are directly transferred to alcohols (Liu et al., 2004). These reactions are well known as a system for media detoxification against toxic alcohols and fatty acids. Esters appear during the early stage of ripening mainly due to yeasts, and contribute to cheese flavour by minimising sharpness of fatty acids and bitterness of amines (Le Quéré & Molimard, 2002). The only identified ester was ethyl acetate (Table III.2.10). At day 13, PA- and HP-cheeses presented higher concentrations than UH-cheeses. The microbiological analysis of cheeses (see Section III Chapter 1) showed that cheeses from conventionally treated milk samples had higher counts of yeasts than UH-cheeses; hence the differences in yeasts counts could explain the differences in ethyl acetate content.

A number of miscellaneous compounds were identified in fresh cheese (Table III.2.10), some of which have been previously reported both in milk and in cheese (Calvo & de la Hoz, 1992; Le Quéré & Molimard, 2002). Hydrocarbons are secondary products of lipid autoxidation and are precursors for the formation of other aromatic compounds (Arora et al., 1995). Benzaldehyde was the most abundant benzene compound in UH-cheeses. In contrast, in cheeses from conventionally treated milk samples the most abundant benzene compound identified was toluene. This compound originates from the degradation of β-carotene in milk (Contarini et al., 1997). Alkanes have been found in the volatile fraction of cheeses (Thierry et al., 1999), usually at low concentrations. Undecane was the only alkane identified in all samples, but it showed higher concentrations in UH-cheeses than those from conventionally treated milk. The heterocyclic aromatic compound pyrrol has not been previously reported either in milk or in cheese; in this study, the peak areas of this compound in cheeses from conventionally treated milks were close to the detection limit, but were quantified due to the fact that UH-cheeses presented large areas. As previously mentioned (see 2.3.4. Oxidation), UHPH increased lipid oxidation resulting in formation of hydrocarbons.

Table III.2.10. Volatile compounds – Other (arbitrary units).

		Treatment[1]		
	Day	**PA**	**HP**	**UH**
Esters				
Ethyl acetate	1	149.0 ± 89.5 [a]	721.3 ± 510.3 [a]	153.3 ± 138.3 [a]
	13	1,763.4 ± 708.4 [a]	1,801.7 ± 706.9 [a]	969.3 ± 619.6 [a]
	19	-	-	3,406.5 ± 3,103.8 [a]
Sulphur compounds				
Carbon disulphide	1	304.1 ± 55.9 [a]	184.5 ± 13.9 [b]	90.9 ± 16.3 [c,d]
	13	188.9 ± 56.0 [b]	111.6 ± 18.9 [c]	52.6 ± 4.1 [d]
	19	-	-	50.0 ± 12.1 [d]
Methanethiol	1	n.d. [c]	n.d. [c]	100.3 ± 26.3 [a]
	13	n.d. [c]	n.d. [c]	61.2 ± 13.5 [b]
	19	-	-	66.7 ± 13.9 [b]
Dimethylsulphide	1	225.9 ± 61.6 [a]	98.1 ± 22.7 [b]	148.7 ± 65.3 [a,b]
	13	62.6 ± 9.1 [b]	114.3 ± 43.1 [a,b]	62.0 ± 14.0 [b]
	19	-	-	72.4 ± 44.0 [b]
Dimethylsulphone	1	519.9 ± 85.4 [b,c]	656.0 ± 157.1 [a]	644.1 ± 113.3 [a]
	13	571.2 ± 68.5 [a,b]	575.2 ± 85.7 [a,b]	522.5 ± 77.5 [b,c]
	19	-	-	456.3 ± 56.0 [c]
Dimethyldisulphide	1	n.d. [d]	n.d. [d]	405.0 ± 122.7 [a]
	13	n.d. [d]	n.d. [d]	275.6 ± 81.5 [b]
	19	-	-	190.1 ± 52.1 [c]
Alkanes				
Undecane	1	12.7 ± 4.8 [b,c]	32.4 ± 16.0 [b,c]	97.8 ± 60.0 [a]
	13	7.5 ± 4.8 [c]	17.2 ± 6.9 [b,c]	47.3 ± 26.8 [b]
	19	-	-	n.d. [c]
Benzene compounds				
Toluene	1	431.2 ± 220.8 [b]	115.0 ± 37.5 [c]	250.0 ± 25.4 [b,c]
	13	702.8 ± 207.2 [a]	209.5 ± 18.4 [b,c]	309.2 ± 48.1 [b,c]
	19	-	-	371.9 ± 97.8 [b]
Xylene	1	16.1 ± 9.7 [c]	12.3 ± 6.2 [c,d]	0.5 ± 0.5 [d]
	13	60.8 ± 17.7 [a]	42.6 ± 16.1 [b]	15.4 ± 6.2 [c]
	19	-	-	20.7 ± 8.2 [c]
Styrene	1	26.9 ± 10.2 [c]	16.6 ± 5.7 [c]	20.5 ± 5.2 [c]
	13	54.3 ± 12.6 [a]	50.7 ± 23.7 [a,b]	26.6 ± 7.6 [c]
	19	-	-	32.8 ± 18.1 [b,c]
Ethylbenzene	1	18.9 ± 7.7 [d,e]	12.0 ± 5.5 [e]	27.2 ± 3.6 [c,d]
	13	55.3 ± 8.1 [a]	42.8 ± 14.8 [b]	31.2 ± 8.3 [c]
	19	-	-	21.5 ± 7.0 [c,d,e]
Benzaldehyde	1	18.9 ± 3.8 [c]	29.1 ± 5.2 [c]	841.8 ± 190.9 [a]
	13	25.9 ± 3.6 [c]	24.6 ± 4.5 [c]	680.3 ± 148.4 [b]
	19	-	-	549.9 ± 150.4 [b]
Heterocyclic aromatic compounds				
Pyrrole	1	5.8 ± 0.9 [c]	10.0 ± 1.6 [c]	83.0 ± 13.1 [a]
	13	9.2 ± 0.7 [c]	9.8 ± 1.1 [c]	57.3 ± 8.3 [b]
	19	-	-	45.8 ± 4.8 [b]

[a-e] Mean value ± s.e.; n = 6; n.d.: not detected; values per compound without common superscripts were significantly different ($P < 0.05$).

[1] PA: pasteurisation; HP: homogenisation-pasteurisation; UH: ultra-high pressure homogenisation.

Table III.2.10. Volatile compounds – Other (arbitrary units). (continuation)

		Treatment[1]		
	Day	**PA**	**HP**	**UH**
Lactones				
δ-Valerolactone	1	n.d. ^{c}	n.d. ^{c}	33.2 ± 6.3 ^{a}
	13	n.d. ^{c}	n.d. ^{c}	28.9 ± 4.8 ^{a,b}
	19	-	-	25.5 ± 2.9 ^{b}
δ-Hexalactone	1	13.5 ± 4.5 ^{d}	43.9 ± 3.7 ^{c}	46.2 ± 2.8 ^{c}
	13	49.9 ± 5.2 ^{c}	81.5 ± 4.4 ^{a}	68.0 ± 5.0 ^{b}
	19	-	-	81.2 ± 5.0 ^{a}
δ-Nonalactone	1	22.8 ± 3.0 ^{c}	47.0 ± 9.0 ^{b,c}	29.7 ± 7.4 ^{c}
	13	71.5 ± 11.7 ^{a,b}	81.7 ± 12.2 ^{a}	45.1 ± 10.5 ^{b,c}
	19	-	-	68.0 ± 18.7 ^{a,b}

$^{a-e}$ Mean value ± s.e.; n = 6; n.d.: not detected; values per compound without common superscripts were significantly different ($P < 0.05$).

[1] PA: pasteurisation; HP: homogenisation-pasteurisation; UH: ultra-high pressure homogenisation.

Volatile sulphur compounds can be generated from the thermal decomposition of sulphur-containing amino acids; denaturation of cysteine-containing proteins exposes the –SH, which is oxidised by Strecker's reaction and converted to sulphur compounds such as hydrogen sulphide and methanethiol (Al-Attabi et al., 2009). These compounds are responsible for the cooked, cabbage and sulphur flavour of heated milk. An early study by Josephson and Doan (1939) showed the existence of a link between the formation of cooked flavour and α-lactalbumin decomposition. However, subsequent studies have shown that β-lactoglobulin and proteins of the milk fat globule membrane are also involved (Thomas et al., 1976; Vazquez-Landaverde et al., 2006). Further degradation of methanethiol to dimethylsulphide and, subsequent dimethyl-disulphide, is due to chemical rather than biological reactions (Beuvier & Buchin, 2004). Both compounds methanethiol and dimethyldisulphide were only present in UH-cheeses. Indeed, previous studies have shown that UHPH provokes protein denaturation (Hayes et al., 2005; Pereda et al., 2008a; see 2.3.2. Proteolysis and Section II Chapter 1).

Lactones are cyclic compounds characterised by very pronounced fruity notes, and may be present in cheese as γ- and δ-lactones (Le Quéré & Molimard, 2002). However, in this study, only δ-lactones were identified (Table III.2.10). Delta-lactones have a generally higher detection threshold than those of γ-lactones. Hydroxylated fatty acids, which are lactone precursors, can be

present as triacylglycerols in milk; but they are relatively unstable and are readily hydrolysed from the triacylglycerol by heat or lipases. Once free, they are spontaneously cyclised through loss of water to form lactones (Marsili, 2002). The fact that lipolysis was not triggered by UHPH (see 2.3.3. Lipolysis) and that the heat effect of UHPH has been proven to be lower than that of conventional pasteurisation (Pereda et al., 2009) may indicate that another mechanism, rather than lipolysis or heat, would be at the origin of lactones.

2.3.6. Sensory analysis

The quantitative descriptive analysis showed that no statistical differences ($P > 0.05$) were found on the mean scores of aroma and flavour intensities in cheeses (Table III.2.11). However, medians of flavour intensity reflected that HP-cheeses, compared with PA-cheeses, were given more often higher scores than UH-cheeses (53% vs. 43%). At first sight, such results would be in disagreement with the volatile profiles, i.e., larger amount of all sorts of volatile compounds in UH-cheeses than HP-cheeses (see 2.3.5. Volatile compounds). However, as will be discussed later, the volatiles composition of HP-cheeses, which varied considerably compared with that of PA-cheeses, might be the main cause for the increase in flavour intensity. In addition, other factors, such as moisture, TS, and salt content, or textural characteristics, strongly influence both the release and the perception of flavour (Guinee & Fox, 2004). Indeed, UH-cheeses and HP-cheeses had higher salt content on dry basis compared with PA-cheeses (see 2.3.1. Cheese composition). However salt in HP-cheeses was statistically lower ($P < 0.05$) than in UH-cheeses.

Off-flavours were detected in UH-cheese (53% of the panellists), and to a lesser extent, in HP cheeses (30%). Statistical differences ($P < 0.05$) were observed in the off-flavour intensity, with UH-cheeses having the highest scores. The qualitative descriptive analysis showed that off-flavour in HP-cheeses was indefinable, i.e., strange. In contrast, off-flavour detected in UH-cheeses was generally described as unpleasant, or somehow bitter, and occasionally identified as cooked milk or with a metallic note.

Table III.2.11. Sensorial parameters[1].

	Treatment					
	HP			UH		
	Mean	Median (frequency)	Cumulative frequency[2]	Mean	Median (frequency)	Cumulative frequency[2]
Aroma	0.18 ± 0.12 [a]	0 (0.58)	0.30	0.20 ± 0.17 [a]	0 (0.50)	0.28
Flavour	0.48 ± 0.15 [a]	1 (0.40)	0.53	0.25 ± 0.25 [a]	0 (0.23)	0.43
Off-flavour	0.35 ± 0.09 [b]	0 (0.70)	0.30	0.93 ± 0.17 [a]	1 (0.28)	0.53

[a-b] Mean value ± s.e.; n = 40; values without common superscripts were significantly different ($P < 0.05$).

[1] Cheeses from milk treated by conventional homogenisation-pasteurisation (HP) or by ultra-high pressure homogenisation (UH) were compared with pasteurised cheeses; negative and positive values denote lower or greater perception, respectively.

[2] Cumulative frequencies of either negative or positive values depending on the algebraic sign of the mean.

Table III.2.12 summarises the main volatile compounds of HP- and UH-cheeses, which contents showed statistical differences ($P < 0.05$) compared with those of PA-cheeses. Volatile fatty acids contribute to the aroma of the cheese or to a rancid defect depending on their concentration and perception threshold (Le Quéré & Molimard, 2002). As they were present at high concentrations in HP-cheeses, their strong cheesy flavour may have contributed to the greater flavour intensity perceived by the panellists in these cheeses. In addition, 2-butoxyethanol and ethyl acetate, giving a solvent or pineapple note (Le Quéré & Molimard, 2002), may play a role in the indefinable off-flavour detected in HP-cheeses.

Volatile profiles of UH-cheeses showed much complex patterns; one of the main characteristics was the almost exclusive presence of aldehydes, which give fruity or greeney notes, in UH-cheeses. Their perception thresholds in water are rather low (9.18, 1.41, and 2.53 μg kg^{-1} for hexanal, octanal and nonanal, respectively; Le Quéré & Molimard, 2002). Hence, these compounds might have contributed to the off-flavour detected by the panellists in UH-cheeses. However, in the case of benzaldehyde, which has an aromatic note of bitter almond, the threshold is higher (350 μg kg^{-1}). Ketones were also very important in UH-cheeses; methyl-ketones have been identified as key odorants in the aroma of surface-ripened cheeses and blue-veined cheeses (Barron et al., 2005). In UH-cheeses, their presence might have contributed to the overall flavour. Most straight and branched chain alcohols have high

Table III.2.12. Distinguishing volatile compounds[1] in cheeses and characteristic notes.

	Treatment[2]		Characteristic notes[3]
	HP	UH	
Fatty acids	..... Butanoic		Rancid, cheesy, putrid, sharp, sour
	..... Hexanoic		Blue cheese, sweat, sour, pungent, goat
	..... Octanoic		Cheese, sweat, goat, soapy, waxy, musty
Ketones		..... 2-Butanone	Acetone, sweet, ethereal, slightly nauseating
		..... 2-Pentanone	Fruit, acetone, sweet, ethereal
		..... 2-Heptanone	Musty, blue cheese, pungent
		.... Acetophenone	Orange blossom
Aldehydes		..... Hexanal	Immature fruit, green apple
		....Benzaldehyde	Bitter almond
		.. 3-Methylbutanal ...	Malt, chocolate, toffee, green
		..2-Methylbutanal	Roasted coffee or cocoa, malt
		..... *others*	*Flower, green, orange, grapefruit, citrus fruit*
Alcohols	.. 2-Butoxyethanol ..		Alcohol, sweet, fruity, sharp
		..... 1-Hexanol	Winey, oily, fruity
		.....1-Octanol	-
	..... 1-Pentanol		Alcohol, sharp, harsh
	..... 1-Butanol		Winey, sweet, fruity, fusel oil
		3-Methyl 1-butanol ..	Fruit, green, alcohol
		..2-Ehtyl 1-hexanol....	-
Esters	..... Ethyl acetate		Pineapple
Sulphur compounds	..... Dimethylsulphone.....		-
		Dimethyldisulphide ..	Cauliflower, garlic, very ripe cheese
		..... Methanethiol	Cooked cabbage
Alkanes		..... Undecane	-
HAC†		..... Pyrrole	-
Lactones	.....δ-Hexalactone		Fruit
		... δ-Valerolactone	Fruit

[1] Volatile compounds showing statistical differences ($P < 0.05$) compared with cheeses from pasteurised milk, listed by categories from highest to lowest concentrations.

[2] HP: homogenisation-pasteurisation; UH: ultra-high pressure homogenisation.

[3] Flavour notes described by Barron et al. (2005), Le Quéré & Molimard (2002) and Qian & Reineccius (2002).

† HAC: Heterocyclic aromatic compounds.

perception thresholds; hence they do not contribute significantly to the flavour of many cheese varieties (Barron et al., 2005). Lactones, which were most present in UH-cheeses, are considered important for the flavour of cheese (Adda et al., 1982). However, literature provides little information on the role of individual lactones.

Bitter flavour in cheese is related to proteolysis (Sousa et al., 2001). However, it is important to bear in mind that other components such as indole, amino acids, amines, amides, long-chain ketones or monoglycerides could contribute to the bitter taste of cheese (Beuvier & Buchin, 2004). As already mentioned (see 2.3.5. Volatile compounds), cooked flavour is due to sulphur compounds generated from the thermal decomposition of sulphur-containing amino acids (Al-Attabi et al., 2009). Indeed, UH-cheeses presented higher content of sulphur compounds, specially methanethiol and dimethyldisulphide, than those from conventionally treated milk samples explaining the cooked flavour detected by the panellists in these cheeses. Finally, lipid oxidation, which was very important in UH-cheeses (see 2.3.4. Oxidation), is often characterised as metallic (Marsili, 2002).

2.4. Conclusions

Both homogenisation treatments increased the moisture content of cheeses, but during storage, whey drainage in UH-cheeses was lower than in cheeses from homogenised-pasteurised milk. Lipolysis and proteolysis levels in cheeses from UHPH-treated milk were lower than those from conventionally treated milk samples; throughout the storage period, UH-cheeses showed lower amounts of free amino acids and fatty acids. However, some degradation of β-CN was observed at the end of the storage period in UH-cheeses. Oxidation was found to be the major drawback of UHPH treatment; it resulted in a great number of volatile compounds, e.g., aldehydes, ketones and sulphur compounds, in UH-cheeses, which presence explained the metallic and cooked milk flavours detected by some panellists. Hence, by solving the oxidation problem, fresh cheeses with similar or even higher quality are expected to be obtained with UHPH-treated milk than with conventionally treated milk.

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Section III, Chapter 3

Effects of ultra-high pressure homogenisation of milk on the texture and water-typology of a starter-free fresh cheese

The aim of the study was to evaluate the effect of ultra-high pressure homogenisation (UHPH) on the texture, rheology, colour, microstructure and water typology of a starter-free fresh cheese. UHPH at 300 MPa with an inlet temperature of 30ºC was compared with conventional treatments. Major differences among treatments were revealed by both instrumental and sensorial methods. Homogenised cheeses were firmer, less elastic, grainier, pastier, whiter, and had higher water-holding capacity but lower water-mouth feeling than pasteurised cheeses. The effect of UHPH was greater than that of conventional homogenisation. Differences on the composition, i.e. water typology and protein content, and the microstructure of the cheeses explain the obtained results.

3. Effects of ultra-high pressure homogenisation of milk on the texture and water-typology of a starter-free fresh cheese

3.1. Introduction

The physical properties of fresh cheeses, i.e., texture, expressible moisture and colour, are dictated by the cheese structure and determine the consumer acceptance. As previously mentioned (see Section I Chapter 2), fresh cheese is commonly used as ingredient for salads, therefore upon cutting the integrity of the cheese pieces must be ensured. In addition, whey drainage during storage is one of the major handicaps to be tackled; excessive syneresis results in a small piece of cheese buried in whey, which makes cheese much less attractive to consumers.

Cheese structure is intrinsically related to the arrangement of cheese components within distinct micro- and macrostructure levels, e.g., proteic network or fat fraction. Ultra-high pressure homogenisation (UHPH) was found to greatly affect the protein and fat components (see Section II Chapter 2 and Chapter 3), and some technological aspects of the cheese production (see Section III Chapter 1). Hence, the use of UHPH could provoke changes on the textural characteristics of fresh cheeses.

The aim of the present study was to evaluate the textural characteristics of starter-free fresh cheeses produced with ultra-high-pressure homogenised milk by comparing them with cheeses from conventionally treated milk samples.

3.2. Material and methods

The microstructure of fresh cheeses was analysed on the 4th day after production. As for the previous chapters, cheeses from pasteurised, homogenised-pasteurised and UHPH-treated milk samples will be referred as PA-, HP- and UH-cheeses, respectively.

3.2.1. Water-holding capacity and water typology

Theoretical maximum syneresis was estimated by centrifugation following the method of Guo and Kindstedt (1995). Whole cheeses were transferred into plastic bags, allowed to reach room temperature and manually crushed. Fifty grams of the homogenised cheese were transferred into centrifuge tubs and centrifuged at 12,500 × *g* for 75 min at 25 ºC. Expelled whey was weighted and expressed as gram per 100 g of cheese.

Evaluation of the water contained in the matrix of cheeses was performed in triplicate by thermogravimetry on a TGA/SDTA815e thermobalance (Mettler-Toledo GmdH Analytical, Switzerland). Approximately 15 mg of cheese was placed in the thermobalance alumina sample pan and heated from 25 to 250 ºC at a scanning rate of 5 ºC min^{-1} in a flow of nitrogen of 60-80 mL min^{-1}. Three different water types were detected using the Mettler-Toledo STARe software, and their contents were expressed as grams per 100 g of cheese and on a proportion basis as grams per 100 g of water.

3.2.2. Confocal laser scanning microscopy of cheeses

Cheese slices ~1 mm thick were stained with a solution of Nile Blue A (Sigma, Steinheim, Germany) at 0.02% (w/v) by submerging them for 15 min. After washing them twice, the cheese portions were placed on microscope slides and covered with non-fluorescent observation medium and a coverslip.

The confocal microscope (Leica TCS SP2 AOBS, Heidelberg, Germany) was equipped with an oil-coupled Leica objective with a 63× augmentation and a numerical aperture of 1.4. Fluorescence from the samples was excited with the 488 nm line of an argon laser. Images were acquired in 2 channels simultaneously (501 to 549 nm and 574 to 626 nm) as 1,024 × 1,024 pixel slides in the horizontal *x*–*y* plane along the *z* plane at constant gain and offset. Three-dimensional images were obtained by the maximum projection of 15-16 slides with Leica software.

3.2.3. Texture and rheology analyses

Uniaxial compression test was carried out six times for each cheese on cylindrical samples. Cylinders were obtained with a punch of 25 mm inner diameter and sliced into 20 mm high discs. Cheese samples were kept at 4 ºC until compressed to 80% of their original height using a TA-TX2 Texture Analyzer (State Microsystem, Surrey, UK) with a 50 mm probe and a crosshead speed of 0.8 mm s^{-1}. Fracture stress (σ_f) and fracture strain (ε_f) parameters were calculated from the true stress-true strain curves (Calzada & Peleg, 1978).

Dynamic oscillatory test was performed with a ThermoHaake RS1 rheometer (ThermoHaake GmbH, Karlshrue, Germany). Cylindrical samples of cheese were obtained with a punch of 35 mm inner diameter. Cylinders were sliced into discs of 5.5 mm thickness using a device with parallel stainless-steel wires. The sliced specimens were kept at 4 ºC. Oscillatory testing was performed at 4 ºC in fivefold using 35 mm diameter parallel serrated plates, to avoid sample slippage, and a gap setting of 5.3 mm. Cheese samples were allowed to relax for 10 min after the measuring system reached the testing position.

A material, such as cheese, is considered viscoelastic if during (and after) deformation part of the mechanical energy supplied to it is stored in the material (elastic part) and part is dissipated (viscous part). The ratio of dissipated to stored energy depends on the time scale of the deformation and the consequence is that the material response is time-dependent (Lucey et al., 2003). In order to ensure that measurements were made in the linear viscoelastic region, the conditions of the test were determined by stress amplitude sweeps at a frequency of 1 Hz. Frequency sweeps were performed over the range 1-10 Hz at a maximum strain of 0.02%. Elastic (*G'*) and viscous (*G"*) components were collected, and shear modulus (G^*) was calculated with Rheowin software (ThermoHaake GmbH).

3.2.4. Colour analysis

Colour of cheeses was measured with a portable HunterLab spectrocolorimeter (MiniScan XETM, Hunter Associates Laboratory Inc., Reston, Virginia, USA) under Illuminant Fcw (cool white fluorescent) with a 10º observer. CIE L^*-, a^*- and b^*-values were read at six different points of the inner surface of cheeses cut in halves. The L^*-value, that ranges between 0 and 100, is a measure of lightness. Negative to positive values of a^* and b^* indicate the green–red and blue–yellow components, respectively. Chromaticity (C), expressed as $\sqrt{a^{*2}+b^{*2}}$, Hue angle, as $\tan^{-1}(b^*/a^*)$, and total colour differences (ΔE), as $\sqrt{\Delta L^{*2}+\Delta a^{*2}+\Delta b^{*2}}$, were calculated to assess the type of colour perceived.

3.2.5. Sensory analysis

Sensory evaluation of cheeses was performed as previously described (see Section III Chapter 2).

3.2.6. Statistical analysis

The complete experiment was repeated on 3 independent occasions. Data were processed by multifactor analysis of variance (ANOVA) using the general linear models procedure of Statgraphics (Statgraphics Inc., Chicago, IL, USA), taking into account both treatment and production factors, as well as their interaction. LSD test was used for comparison of sample data, and evaluations were based on a significance level of $P < 0.05$.

For sensory data, descriptive statistics, i.e., mean, median, frequency of the median category, and cumulative frequency of either negative or positive values depending on the algebraic sign of the median, were calculated. Means were analysed for significance along with the other measurements as previously described.

3.3. Results

3.3.1. Water-holding capacity and water typology

The estimation by centrifugation of the theoretical maximum syneresis showed that homogenisation of milk, both conventionally and by UHPH, reduced significantly ($P < 0.05$) the amount of expelled whey (Table III.3.1). In cheese, water is present in three main phases: free water in the serum channels, entrapped water in close proximity to the casein matrix, and bound water tightly associated to the caseins (Everett & Auty, 2008). In the thermo-gravimetrical curves from PA-cheeses, 3 temperatures of phase change could be identified, i.e., 115 ºC, 120 ºC and 130 ºC. However, for HP- and UH-cheeses, the change of phase at 115ºC was not observed. Thus, the amount of free (W_1), entrapped (W_2), and bound water (W_3) was determined by the losses of weight between 25-120 ºC, 120-130 ºC, and 130-250 ºC, respectively. Water content of each type of water was expressed as g per 100 g of cheese and as percentage of the total water content of cheeses.

Table III.3.1. Whey expelled by centrifugation and water typology characterised by TGA[1].

Treatment[2]	Whey (g 100 g^{-1} cheese)	W_1 (g 100 g^{-1} water)	W_2 (g 100 g^{-1} water)	W_3 (g 100 g^{-1} water)
PA	44.12 ± 0.40 [a]	89.71 ± 0.53 [a]	5.97 ± 0.46 [b]	4.32 ± 0.16 [c]
HP	33.29 ± 0.10 [b]	84.66 ± 0.58 [b]	8.15 ± 0.33 [a]	7.18 ± 0.42 [b]
UH	33.44 ± 0.12 [b]	82.52 ± 1.06 [c]	6.74 ± 0.48 [b]	10.74 ± 0.72 [a]

[a-c] Mean value ± s.e.; n = 9; values without common superscripts were significantly different ($P < 0.05$).

[1] W_1: free water; W_2: entrapped water; W_3: bound water.

[2] PA: pasteurisation; HP: homogenisation-pasteurisation; UH: ultra-high pressure homogenisation.

No significant differences were observed in the amount of free water, with an average of 56 g free water 100 g^{-1} of cheese (data not shown). However, when expressed on a proportion basis, both homogenisation treatments provoked a significant decrease of free water. Such decrease was due to an increase of entrapped and bound water on a concentration basis. However, in UH-cheeses, the amount of entrapped water on a proportion basis did not differ from that of PA-cheeses possibly due to a substantial increase in the amount of bound

water. Although in HP-cheeses the amount of bound water also increased compared with PA-cheeses, the extent was not as important as for UH-cheeses.

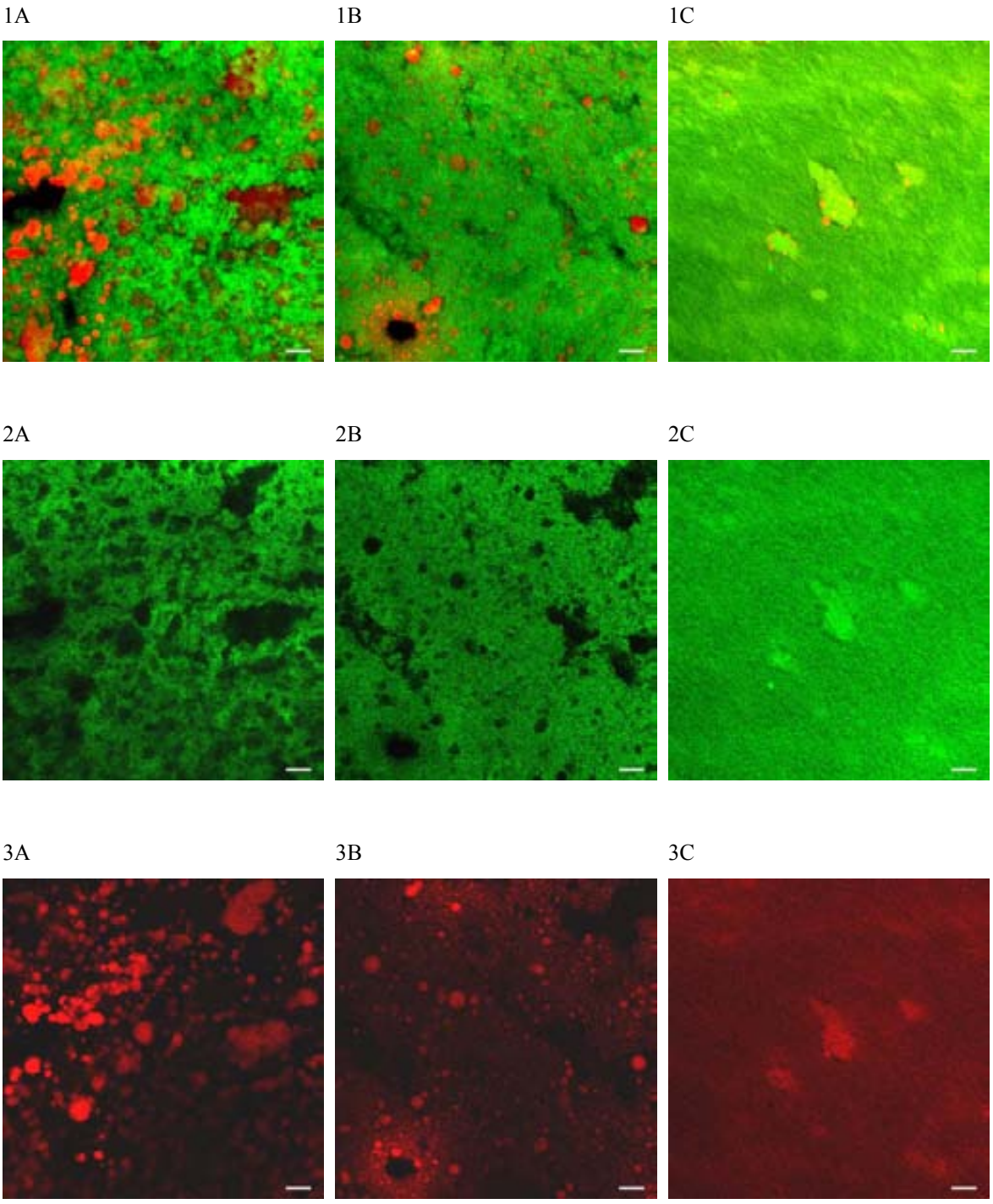
3.3.2. Microstructure

Confocal micrographs of cheeses revealed visual differences on the protein matrix stained in green, fat in red and pockets of serum phase as black areas (Fig. III.3.1). PA-cheeses were made of a highly porous matrix containing discrete serum pockets. Fat appeared to exist in three distinct states: non-globular inclusions of fat (up to ~20 μm) corresponding to free pools of fat filling the voids of the matrix, coalesced large globules (~10 μm) and native fat globules (< 5 μm) with a tendency to aggregate. In HP-cheeses, the matrix closed up but discrete serum pockets, which, in this case, were not filled with fat, were observed. Homogenised fat globules were embedded within the protein matrix. A small number of unevenly dispersed fat globules had undergone coalescence (~5 μm) and remained out of the protein matrix. For UH-cheeses, micrographs showed a continuous and dense protein matrix with no serum pockets. Fat labelling was distributed throughout the whole matrix and larger fat globules were found to associate to highly-stained proteinaceous structures, showing a high association between fat globules and proteins.

3.3.3. Texture and rheology

Fracture properties were determined by applying the uniaxial compression test at large deformations. Fracture stress (σ_f) is the force required to fracture the cheese matrix and can be used as a fracturability index; a low numerical value indicates greater fracturability. The fracture strain (ε_f) describes the deformability of cheese; higher numerical value indicates greater deformability. Low σ_f and high ε_f values of PA-cheeses showed that they were significantly ($P < 0.05$) more fracturable but more deformable than cheeses from homogenised milk (Table III.3.2). Both homogenisation treatments provoked a significant increase of σ_f and a significant decrease of ε_f. However UH-cheeses showed significantly lower fracturability and deformability compared with HP-cheeses.

Figure III.3.1. Confocal laser scanning micrographs of cheeses from (A) pasteurised, (B) homogenised, and (C) ultra-high pressure-treated milk; three-dimensional images with (1) protein in green and fat in red, (2) protein matrix, and (3) fat of a single microscopy slide [bar = 10 μm].



Viscoelastic characteristics of cheeses, i.e., storage (*G'*), loss (*G"*), and shear (G^*) moduli, were determined with frequency sweep tests. *G'*, which is related to the molecular events of elastic nature, was always higher than *G"*, related to the viscous character, showing the predominant solid character of cheeses (data not shown). Both homogenisation treatments increased G^*, which is related to the overall viscoelastic rigidity (Table III.3.2). However, UH-cheeses showed significantly higher G^* values than HP-cheeses.

3.3.4. Colour analysis

Considering L^*-value, no significant differences ($P < 0.05$) were found between PA- and UH-cheeses (Table III.3.3). However, conventional homogenisation of milk resulted in significantly lighter cheeses. Homogenisation treatments triggered a significant decrease of both red and yellow components; for UH-cheeses the reduction of the redness was such that values moved to greenness values. Differences in chromaticity, which take into account a^*- and b^*-values, were mainly due to the blue-yellow component. In contrast, differences at the Hue angle were due to the green-red component. Total colour differences, which include L^*-values, showed that UH-cheeses were much different than HP-cheeses compared with PA-cheeses.

3.3.5. Sensory analysis

The taste panel detected differences in all the sensory attributes for both HP- and UH-cheeses when compared with PA-cheeses (Table III.3.4); the medians were different from 0 in all cases. The medians of HP- and UH-cheeses had the same algebraic sign for each parameter, which means that both homogenisation treatments triggered similar effects. 75-90% of the tasters classified HP- and UH-cheeses as firmer, grainier, pastier, less deformable, less watery but whiter than PA-cheeses. However, only for elasticity and grainy character, the extent of the differences depended on the treatment; UHPH treatment resulted in less deformable and grainier cheeses than conventional homogenisation.

Table III.3.2. Textural and rheological parameters[1].

Treatment[2]	σ_f (kPa)	ε_f (–)	G^* (kPa)
PA	8.46 ± 0.17 [c]	0.572 ± 0.008 [a]	10.21 ± 1.04 [c]
HP	10.07 ± 0.56 [b]	0.383 ± 0.004 [c]	17.97 ± 1.26 [b]
UH	14.91 ± 0.54 [a]	0.402 ± 0.004 [b]	21.65 ± 1.04 [a]

[a-c] Mean value ± s.e.; n = 18 for textural parameters and n = 15 for rheological; values without common superscripts were significantly different ($P < 0.05$).

[1] σ_f: fracture stress; ε_f: fracture strain; G^*: shear modulus.

[2] PA: pasteurisation; HP: homogenisation-pasteurisation; UH: ultra-high pressure homogenisation.

Table III.3.3. Colour parameters[1].

Treat.[2]	L^*	a^*	b^*	C	Hue	ΔE
PA	95.69 ± 0.07 [b]	0.16 ± 0.03 [a]	12.12 ± 0.20 [a]	12.12 ± 0.20 [a]	89.28 ± 0.15 [c]	-
HP	96.11 ± 0.09 [a]	0.05 ± 0.05 [b]	9.75 ± 0.12 [b]	9.75 ± 0.12 [b]	89.75 ± 0.27 [b]	2.44
UH	95.68 ± 0.11 [b]	-0.46 ± 0.05 [c]	8.59 ± 0.17 [c]	8.61 ± 0.17 [c]	93.17 ± 0.35 [a]	3.61

[a-c] Mean value ± s.e.; n = 18; values without common superscripts were significantly different ($P < 0.05$).

[1] L^*: ligthness; a^*: green–red component; b^*: blue–yellow component; C: chromaticity; Hue: Hue angle in degrees; ΔE: total colour differences.

[2] Treat.: treatments; PA: pasteurisation; HP: homogenisation-pasteurisation; UH: ultra-high pressure homogenisation.

Table III.3.4. Sensorial parameters[1].

	HP			UH		
	Mean	Median (frequency)	Cumulative frequency[2]	Mean	Median (frequency)	Cumulative frequency[2]
Firmness	1.58 ± 0.21 [a]	2 (0.40)	0.85	1.13 ± 0.27 [a]	2 (0.35)	0.75
Deformability	-1.08 ± 0.19 [a]	-1 (0.35)	0.75	-1.50 ± 0.24 [a]	-2 (0.45)	0.80
Grainy	0.88 ± 0.18 [b]	1 (0.50)	0.75	1.90 ± 0.22 [a]	2 (0.35)	0.90
Pasty	1.03 ± 0.21 [a]	1 (0.43)	0.78	0.48 ± 0.25 [a]	1 (0.28)	0.58
Watery	-0.93 ± 0.19 [a]	-1 (0.48)	0.75	-1.18 ± 0.22 [a]	-1 (0.30)	0.78
Colour	-1.25 ± 0.14 [a]	-1 (0.50)	0.85	-1.38 ± 0.15 [a]	-1 (0.48)	0.90

[a-b] Mean value ± s.e.; n = 40; values without common superscripts were significantly different ($P < 0.05$).

[1] Cheeses from milk treated by conventional homogenisation-pasteurisation (HP) or by ultra-high pressure homogenisation (UH) were compared with pasteurised cheeses; negative and positive values denote lower or greater perception, respectively.

[2] Cumulative frequencies of either negative or positive values depending on the algebraic sign of the median.

2.1. Discussion

The extent of syneresis in PA- and HP-cheeses, which had the same moisture content and amount of free water on a concentration basis, but lower on a proportion basis, differed significantly, implying that differences in the cheese matrices rather than the type of water were responsible for enabling or inhibiting the release of whey. McMahon et al. (1999) found that the whey that can be expressed by centrifugation was somehow associated with the fat globules. As observed by confocal microscopy, homogenisation causes the fat globules to become part of the *para*-casein network through the incorporation of micellar casein in the surface coat of fat globules. After centrifugation, a layer of fat could be observed at the top of the supernatant from PA-cheeses but was not present in that of HP-cheeses. The release of fat in PA-cheeses, which was filling voids of the protein matrix, would allow a higher packing of the pellet, resulting in higher water release compared with HP-cheeses.

The increase of entrapped water on a concentration basis for both HP- and UH-cheeses could be explained by the fact that homogenisation enhances casein-casein interactions (see Section II Chapter 2) resulting in a tight protein matrix, as observed by confocal microscopy. For the same moisture content, a higher protein in dry matter results in cheeses that appear drier (Lemay et al., 1994). As the mastication of the cheese implies a disintegration of the cheese structure, the moisture mouth-feeling would be linked to the amount of both free and entrapped water. However, the consumer taste panel detected a lower moisture mouth-feeling for HP- and, especially, UH-cheeses compared with PA-cheeses. Thus, as in the case of expressible whey, the release of water inside the mouth during mastication would be linked to the structure of the cheese rather than the type of water.

Bound water is constituted by primary bound water molecules which are likely to be tightly bound to proteins by multiple hydrogen bonds; consequently, its amount is related to the protein content (Everett & Auty, 2008). Although the protein content on a dry basis of HP- and PA-cheeses did not differ statistically, HP-cheeses had higher content than PA-cheeses. The differences

on the amount of bound water could be explained by the fact that the type of proteins absorbed onto the membrane of fat globules influences the water holding capacity of the cheese matrix; the absorption of native phosphocaseinate and sodium caseinate reduces the micro-syneresis (Métais et al., 2006). Indeed conventional homogenisation of milk enhances interactions within the rennet curd of mainly α_s-casein (**CN**) through calcium bonds, and β-CN through calcium and hydrogen bonds (see Section II Chapter 2). UH-cheeses had higher moisture content than PA- and HP-cheeses due to a significant increase of bound water. In fact, UHPH causes the incorporation into the curd not only of caseins but also of denatured whey proteins, i.e., α-lactalbumin (**α-LA**) and β-lactoglobulin (**β-LG**), (see Section II Chapter 1) through hydrogen bonding and hydrophobic interactions (see Section II Chapter 2), resulting in significantly higher protein content. Globular proteins such as β-LG display varying degrees of hydration, depending on denaturation, aggregation, and interaction with other proteins (Kneifel & Seiler, 1993). On one hand, denaturation, which implies unfolding of the globular protein, increases its water holding capacity. On the other, dissociation of the quartenary structure of multimeric globular proteins, such as β-LG, enhances binding of water molecules by increasing the exposure to solvent of protein surfaces, which formerly interacted with each other.

Textural characteristics are related to the composition, structure and the interaction degree between the structural elements of the cheese. Some of the factors that impact upon texture include water content, state of fat, and casein matrix density or protein interactions (Everett & Auty, 2008).

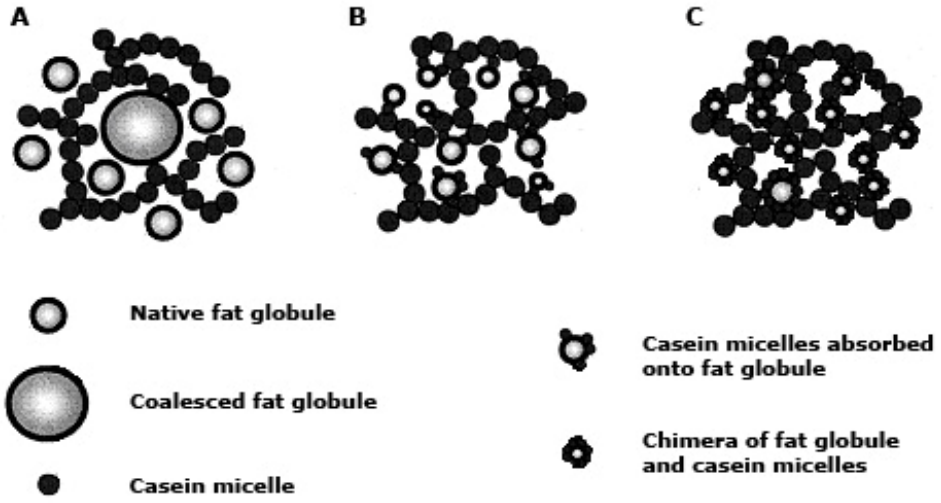
It has been well established that increasing moisture content in cheese results in a softer texture (Lucey et al., 2003), by either decreasing the volume fraction of protein or lubricating the casein matrix. However, UH-cheeses, which had significantly higher moisture content, were characterised as firmer than PA-cheeses by the consumer taste panel and the instrumental texture parameters. Since the increase of moisture in UH-cheeses was especially due to a higher retention of bound water, the type of water rather than the total moisture content determined the textural characteristics of UH-cheeses.

The role of fat on the textural properties of cheese depends on its state within the protein matrix. Native fat globules or free fat pools are easily deformed (Everett & Olson, 2003). Such phenomenon would explain the deformable character of PA-cheeses described by the consumer taste panel and the instrumental texture parameters. Moreover, coalesced fat globules and free fat pools interfere with the integrity of the gel structure (Michalski et al., 2002), leading to softer and more fracturable cheeses. When fat is homogenised two main phenomena contributing to a firmer cheese occur (Fig. III.3.2). On one hand, the size of fat globules is drastically reduced, which results in a concomitant decrease of their deformability (Everett & Olson, 2003) and their structure-breaking character (Michalski et al., 2002). On the other, the fat globule membrane suffers modifications in its protein composition (McPherson et al., 1984; Cano-Ruiz & Richter, 1997; Lee & Sherbon, 2002; see Section II Chapter 3).

Cheese firmness depends on the extent of the interconnectivity between casein strands, in other words, on the number and strength of bonds between casein particles (Lucey et al., 2003). The caseins adsorbed onto the fat globule surface after homogenisation may be able to form protein-protein bonds with the casein matrix (Fig. III.3.2; Cano-Ruiz & Richter, 1997), thus increasing the rigidity of the cheese protein matrix. As already mentioned, conventional homogenisation and, especially, UHPH enhance casein interactions within the rennet curd (see Section II Chapter 2). Moreover, UHPH triggers the denaturation and the subsequent adsorption of whey protein onto the fat globule (see Section II Chapter 3). Fat globules coated with caseins, i.e., α_{s1}-CN or β-CN, or whey proteins, i.e., α-LA or β-LG, result in a stronger cheese structure (Everett & Olson, 2003).

Colour depends on how a material reflects, absorbs or transmits light. In cheese, light penetrates the superficial layers and is scattered by milk fat globules and whey pockets (Lemay et al., 1994; Paulson et al., 1998). Homogenisation increases the number of fat globules, thus increases scattering centres resulting in higher lightness (Everett & Auty, 2008). In UH-cheeses, the loss of the red component triggering a greenish tint, which is characteristic

Figure III.3.2. Structure of cheese matrices from (A) pasteurised, (B) homogenised-pasteurised, and (C) ultra-high-pressure homogenised milk samples [reproduced from Michalski et al (2002) with modifications].



of low-fat cheeses (Fife et al., 1996), could be explained by a loss of light scattering power of fat globules, due to the drastic reduction of their size by UHPH, allowing whey colour to be revealed.

2.2. Conclusions

The results of the present study show that both conventional homogenisation and UHPH provoked changes on the textural characteristics of cheese, i.e., firmer, less deformable, with lower water-mouth feeling, and whiter. However, the effect of UHPH was greater than that of conventional homogenisation. The highest firmness and lowest deformability in cheeses from UHPH-treated milk could be explained by the reduction of fat globule size, and the incorporation of caseins and whey proteins at the milk fat globule membrane, which increased the interconnectivity within the cheese matrix. In addition, the incorporation of whey proteins into the curd led to an increase in bound water. Finally, results from the sensory analysis were greatly in accordance with those obtained with the instrumental analyses. However,

except for the dry mouth-feeling, the sensory panel described the structural attributes of UH-cheeses as their best characteristics.

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Section IV

Final conclusions

The conclusions obtained in this thesis are the following:

1. Rennet coagulation properties of milk were enhanced by single-stage UHPH at 200 and 300 MPa. UHPH treatment increased the wet yield of curd and its moisture content, and decreased the protein content of whey. The improvement of cheese-making properties of milk by UHPH could be attributed to the combined effect of homogenisation and heat on the protein-fat structures of the milk.

2. UHPH treatment of milk affected protein interactions within drained curds by increasing the amount of unbound whey proteins and calcium-bonded caseins in curds, impairing hydrogen bonding and favouring hydrophobic interactions of whey proteins and caseins.

3. UHPH greatly reduced milk fat globule size with a concomitant increase of their specific surface area overcome by the adsorption of milk proteins. On one hand, caseins molecules, released through partial disruption of casein micelles, were directly bonded to the milk fat globule membrane. On the other, whey proteins were adsorbed through direct interaction with native proteins of the milk fat globule membrane, and through disulfide bonding with both indirectly and directly absorbed casein molecules. Consequently, UHPH provoked the formation of new chimerical particles.

4. Although UHPH enhanced the coagulation properties of milk, it impaired to some extent some cheese-making processes, i.e., difficulty at cutting the curd due to crumbling and improper curd matting due to poor cohesion of the grains. In addition, UHPH reduced the syneresis of curds.

5. UHPH of milk resulted in fresh cheeses with longer microbiological shelf life by extending it from ~13 to ~19 days. Lactococci growth was enhanced at an early stage of the storage period and lactobacilli were not detected throughout the cold storage. The growth of yeasts and moulds was inhibited to some extent resulting in lower counts at the end of the storage period. Finally, pathogen microorganisms were not detected.

6. During storage, cheeses from UHPH-treated milk expelled less whey than those from conventionally treated milk samples. Syneresis is one of the major handicaps of fresh cheeses because it results in a small piece of cheese buried in whey making it much less attractive to consumers. The use of UHPH treatment was proven to be a way to tackle this problem.

7. UHPH treatment of milk resulted in cheeses with higher moisture content, and concomitantly higher salt content. However, fat and protein content on a dry basis were not affected. Proteolysis levels in cheeses from UHPH-treated milk were lower than those from conventionally treated milk. However, some degradation of β-casein was observed at the end of the storage period. In addition, UHPH treatment of milk resulted in lower lipolysis levels in cheeses during the whole storage period. In addition, free fatty acid profiles differed from those of cheeses obtained with conventionally treated milk samples.

8. Oxidation was found to be the major drawback of UHPH treatment; it resulted in a great number of volatile compounds, e.g., aldehydes, ketones and sulphur compounds, in UH-cheeses, which presence explained the metallic and cooked milk flavours detected by some panellists. The source of oxidation is presumed to be contamination with metals due to a transfer from the equipment pieces, e.g., non-return valves and seals, to the milk.

9. UHPH treatment of milk provoked changes on the textural characteristics of fresh cheese, i.e., firmer, less deformable, with lower water-mouth feeling, and whiter. Such changes could be explained by the reduction of fat globule size, and the incorporation of caseins and whey proteins at the milk fat globule membrane, which increased the interconnectivity within the cheese matrix and the amount of bound water. Except for the dry mouth-feeling, the sensory panel described the structural attributes of cheeses from UHPH-treated milk as their best characteristics.

10. **The results obtained in this study show that UHPH is a promising technology as treatment of milk for the manufacture of fresh cheese with better microbiological quality during storage in relation to conventional treatments applied in industry, obtaining longer shelf-life and the improvement of cheese characteristics such as texture and microstructure. By solving the oxidation problems produced by this treatment, fresh cheeses with similar or even higher flavour quality are expected to be obtained with UHPH-treated milk than with conventionally treated milk.**

Annex 1

Sensory analysis form

FECHA: NOMBRE DEL CATADOR:

Cátese atentamente las muestras de queso y señálese la valoración que se considere para cada carácter (comparando con la muestra control).
Para la notación de cada atributo, la siguiente escala da la magnitud de la desviación (nota hacia la parte positiva o negativa de la escala según es mayor o menor la desviación apreciada respecto a la muestra C):

Puntos

C desviación no apreciable respecto al Control

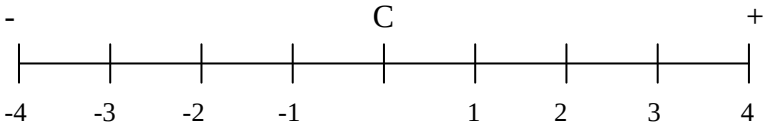
-1 y 1 desviación mínima respecto al Control

-2 y 2 desviación notable respecto al Control

-3 y 3 desviación considerable respecto al Control

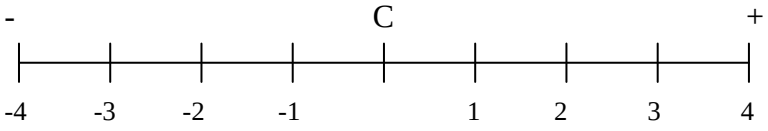
-4 y 4 desviación muy considerable respecto al Control

COLOR

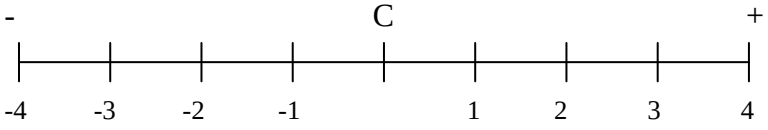


TEXTURA

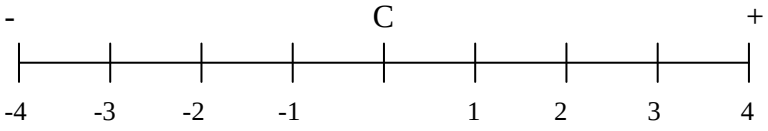
Firmeza



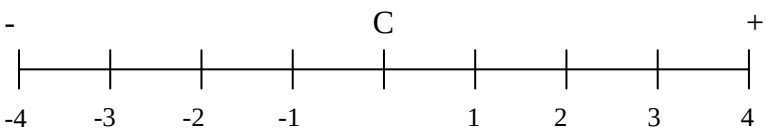
Elasticidad



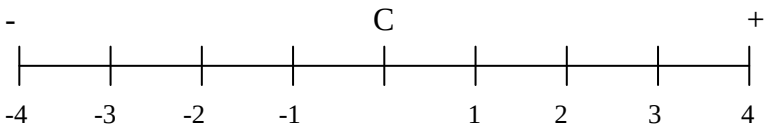
Granulosidad



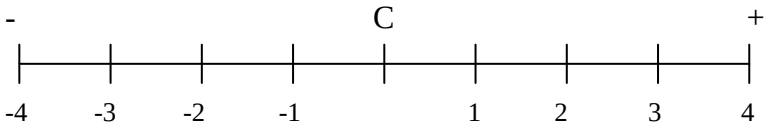
Sensación pastosa



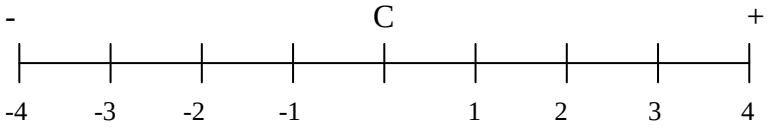
Sensación acuosa



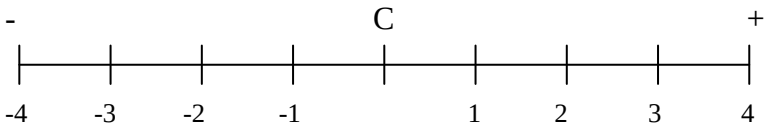
AROMA (intensidad)



FLAVOR (intensidad)



SABORES EXTRAÑOS



Observaciones (describir todo aquello que creáis conveniente de los parámetros o atributos analizados):

Describir cuáles son los aspectos (atributos o parámetros) más y menos valorados de cada tipo de queso.

Código queso	Aspectos mejores	Aspectos peores

Annex 2

Scientific outputs

J. Dairy Sci. 90:13–23

Effects of Ultra-High Pressure Homogenization on the Cheese-Making Properties of Milk

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ABSTRACT

The effects of single- or 2-stage ultra-high pressure homogenization (UHPH; 100 to 330 MPa) at an inlet temperature of 30°C on the cheese-making properties of bovine milk were investigated. Effects were compared with those from raw, heat-pasteurized (72°C for 15 s), and conventional homogenized–pasteurized (15 + 3 MPa, 72°C for 15 s) treatments. Rennet coagulation time, rate of curd firming, curd firmness, wet yield, and moisture content of curds were assessed. Results of particle size and distribution of milk, whey composition, and gel microstructure observed by confocal laser scanning microscopy were analyzed to understand the effect of UHPH. Single-stage UHPH at 200 and 300 MPa enhanced rennet coagulation properties. However, these properties were negatively affected by the use of the UHPH secondary stage. Increasing the pressure led to higher yields and moisture content of curds. The improvement in the cheese-making properties of milk by UHPH could be explained by changes to the protein–fat structures due to the combined effect of heat and homogenization.

Key words: ultra-high pressure homogenization, milk, rennet coagulation, cheese-making properties

INTRODUCTION

Research on technological processes on food is focused on 2 main goals: improving safety and quality of final products, and changing the characteristics of raw materials to obtain value-added products. However, process-induced modifications can have both beneficial and detrimental effects on technological aspects.

In dairy processes, thermal treatment of milk aims at increasing shelf life and improving food safety of the final product. Milk for cheese manufacture is generally pasteurized at 72°C typically for 15 to 35 s. Higher temperatures have adverse effects on curd formation, namely longer coagulation times and weaker gels (Guinee et al., 1997; Singh and Waungana, 2001), and on curd syneresis; that is, higher moisture content (Walstra et al., 1985; Pearse and Mackinlay, 1989; Rynne et al., 2004). Nevertheless, the effect of high heat treatment has received considerable attention owing to its potential for improving cheese yield through incorporation of whey proteins into cheese curd (Lucey, 1995; Singh and Waungana, 2001).

Conventional homogenization, developed by Gaulin in 1899, has been widely adopted by the dairy industry. Homogenization is usually performed at 60°C, and the milk is processed to break milk fat globules into fine lipid droplets, preventing cream separation, thereby increasing stability and shelf life of milk emulsion. Two-stage homogenization is commonly used, in which the primary stage reduces the size of fat globules and the secondary stage disrupts clusters that may be formed. Although homogenization of whole milk has detrimental effects on curd forming properties (Emmons et al., 1980) and curd syneresis (Humbert et al., 1980; Green et al., 1983), it improves rennet action (Humbert et al., 1980; Robson and Dalgleish, 1984) and increases cheese yield due to better fat recovery (Jana and Upadhyay, 1992).

In recent years, homogenization equipment design has been modified to achieve far greater pressures. Although the principle of ultra-high pressure homogenization (**UHPH**) is similar to that of conventional ball-and-seat homogenizers, current developments in the design (e.g., the Stansted valve) allow homogenization at pressures of up to 350 MPa. Forces encountered during UHPH include cavitation, friction, turbulence, high velocity, and shear (Floury et al., 2004a,b), and result in the heating of the homogenized liquid (Floury et al., 2000; Hayes and Kelly, 2003a; Thiebaud et al., 2003). Applications of high-pressure homogenization are mainly found in the pharmaceutical and biotechnology sectors where the technique is used to emulsify, disperse, and mix (Floury et al., 2000). However, there has been increasing interest in its application in food technology. Reports on the effect of UHPH on some pathogenic and spoilage microorganisms in model and real food systems have proved its efficiency in reducing

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microbial counts (Hayes and Kelly, 2003a; Thiebaud et al., 2003; Hayes et al., 2005; Brinez et al., 2006). Moreover, studies with whole and skimmed milk have shown that UHPH produces fine emulsion particles (Hayes and Kelly, 2003a; Thiebaud et al., 2003; Hayes et al., 2005), modifies protein structure and characteristics (Hayes and Kelly, 2003a; Hayes et al., 2005; Sandra and Dalgleish, 2005), and inactivates enzymes (Hayes and Kelly, 2003b; Datta et al., 2005), all of which could have indirect effects on the coagulation properties of milk and the microstructural properties of cheese.

At the present time, data to describe the technological aptitude of milk treated by UHPH are scarce. The aim of this work was to determine the effect of UHPH treatment on the cheese-making properties of milk by comparing this new technology with pasteurization and conventional homogenization-pasteurization treatments.

MATERIALS AND METHODS

Supply and Treatment of Milk

Raw whole bovine milk was obtained from a local dairy farm (S.A.T. Can Badó, Roca del Vallès, Spain). Milk was standardized at 3.5 ± 0.2% fat and kept overnight at 4°C. Before all treatments, the milk was warmed to approximately 20°C.

Ultra-high pressure homogenization was carried out by subjecting milk to single- or 2-stage UHPH (100, 200, and 300 MPa on the primary valve and 30 MPa on the secondary valve) using a Stansted high-pressure homogenizer (model FPG11300, Stansted Fluid Power Ltd., Essex, UK) at an inlet temperature of 30 ± 1°C. This homogenizer comprises a high pressure valve made of ceramics able to support 350 MPa and a second pneumatic valve able to support up to 50 MPa located behind the first one. The high-pressure system consisted of 2 intensifiers driven by a hydraulic pump. The flow rate of milk in the homogenizer was 120 L/h. The inlet temperature of milk was kept at 30°C by a heat exchanger located behind the feeding tank. Temperature thermocouples and pressure gauges placed at the 2 valves measured temperature and pressure changes during processing. Throughout the experiment, the range of milk temperature was 33 to 41°C at the primary valve and 54 to 94°C at the secondary valve. To minimize temperature retention after treatment, 2 spiral-type heat exchangers (Garvía S.A., Barcelona, Spain) located behind the second valve were used. The outlet temperature of milk never exceeded 40°C.

Milks from UHPH treatments were compared with raw and heat-treated milks. Pasteurized milk (72°C for 15 s) and homogenized–pasteurized milk (15 MPa + 3 MPa at 57 to 60°C, 72°C for 15 s) were chosen as typical treatments of cheese milk in different cheese varieties (fresh or ripened). Two-stage homogenization and pasteurization of raw milk were performed with a Niro Soavi homogenizer (model X68P Matr. 2123, Niro Soavi, Parma, Italy) and a Finamat heat exchanger (model 6500/010, GEA Finnah GmbH, Ahaus, Germany), respectively.

The complete experiment was repeated on 3 independent occasions.

Particle Size and Distribution

The particle size distribution in milk samples was determined using a Beckman Coulter laser diffraction particle size analyzer (LS 13 320 series, Beckman Coulter, Fullerton, CA). Milk samples were diluted in distilled water until an appropriated obscuration was obtained in the diffractometer cell. An optical model based on the Mie theory of light scattering by spherical particles was applied by using the following conditions: real refractive index, 1.471; refractive index of fluid (water), 1.332; imaginary refractive index, 0; pump speed, 21%. The diameter below which 90% of the volume of particles are found ($\mathbf{D_v0.9}$), the diameter below which 50% of the volume of particles are found ($\mathbf{D_v0.5}$), the volume-weighted mean diameter [$\mathbf{D(4,3)}$], and the surface-weighted mean diameter [$\mathbf{D(3,2)}$] were determined.

Rennet Coagulation Properties

Milk was warmed to 32°C, and recombinant rennet (chymosin with a declared activity of 180 International Milk Clotting Units/mL, Maxiren 180, DSM Food Specialties, Seclin Cedex, France) was added at 0.074% (vol/vol). Coagulation was carried out at 32°C for 30 min. Rennet coagulation properties [rennet coagulation time (**RCT**), rate of curd firming (**RCF**), and curd firmness at 30 min (**CF**)] were assessed in triplicate by the Optigraph system (Ysebaert Inc., Frepillon, France). This device passes an infrared beam through a sampling tube containing milk. A sensor on the other side measures the amount of light absorbed by the milk as it coagulates; the changes are analyzed in real time by a computer that converts them into directly usable data.

Evaluation of Yield and Moisture Content of Curds

The potential yield of cheese curd was estimated in quadruplicate as described by Mache boeuf et al. (1993). Milk samples (270 mL) were warmed to 32°C and recombinant rennet (chymosin with a declared activity of 180 International Milk Clotting Units/mL, Maxiren 180, DSM Food Specialties) at 0.074% (vol/vol) was

added. Portions of the renneted milks (30 g) were transferred into centrifuge tubes and allowed to coagulate at 32°C for 30 min. The coagulum was centrifuged at 13,000 × *g* for 15 min at 10°C. Wet yield of curds, expressed as grams of retained curd per one hundred grams of milk, was determined by weighing the obtained pellets.

Curds were analyzed in duplicate for TS content (IDF, 1987) to calculate their moisture content (100 − TS) and the yield of total curd solids (wet yield × TS/100).

Whey Composition: Total N, Whey Proteins, and Minerals Content

The total N content of whey was analyzed in duplicate by the Dumas combustion method (IDF, 2002).

Reversed-phase HPLC analysis of rennet whey was performed using an automated system (LCM1, Waters Corporation, Milford, MA). Separations were carried out in a 250- × 4.6-mm column packed with C8-bonded silica gel with a particle diameter of 5 μm and pore width of 3,000 nm (Tracer Excel, Teknokroma, Sant Cugat del Vallès, Spain) at a constant temperature of 40°C, following the method of Resmini et al. (1989). Residual levels of α-LA and β-LG were measured as total area of the respective peaks.

Calcium, Mg, and P in whey were determined in triplicate by inductively coupled plasma optical emission spectroscopy with a Perkin-Elmer inductively coupled plasma spectroscopy unit (model 4300, Perkin-Elmer, Shelton, CT) with axial plasma viewing. The spectroscopy operating conditions were as follows: power = 1.3 kW; argon plasma flow rate = 15 L/min; argon auxiliary flow rate = 0.2 L/min; argon nebulizer flow rate = 0.74 L/min; sample uptake rate = 1.5 mL/min; wavelengths (nm) for Ca, P, and Mg = 317.925, 213.611, and 285.213, respectively. Whey samples of 1 mL were transferred to a 25-mL volumetric flask and nitric acid and deionized water were added to reach a final concentration of 0.2% (vol/vol) nitric acid. Standard solutions from 1 mg/mL stock solution of Ca, P, and Mg were used to prepare the calibration curves.

Confocal Laser Scanning Microscopy of Rennet Gels

Confocal laser scanning microscopy observations were performed in fluorescence mode essentially as Michalski et al. (2002) described. The protein matrix of renneted milks was stained by the fluorescent dye, fluorescein isothiocyanate (**FITC**; Fluka, Steinheim, Germany), and the fat globules were stained by Nile red (Sigma, Steinheim, Germany). The FITC and Nile red were dissolved in ethanol at a concentration of 2 and 1 mg/mL, respectively. Milks (10 mL) warmed at 32°C were dyed with 2 drops of FITC and 3 drops of Nile red. Recombinant rennet (Maxiren 180, DSM Food Specialties) at a concentration of 0.074% (vol/vol) was added to the dyed milks. Then, 3 to 4 drops of the labeled renneted milks were transferred to microscope slides with concave cavities, covered with a coverslip, sealed to prevent evaporation, and incubated in a temperature-controlled incubator at 30°C for 30 min. The preparations were cooled and kept at 4°C for a maximum of 3 h.

The confocal microscope (Leica TCS SP2 AOBS, Heidelberg, Germany) was equipped with an oil-coupled Leica objective with a 63× augmentation and a numerical aperture of 1.4. Fluorescence from the samples was excited with the 488 nm line of an argon laser. Images were acquired in 2 channels simultaneously (501 to 549 nm and 574 to 626 nm) as 1,024 × 1,024 pixel slices in the horizontal *x*–*y* plane along the *z* plane at constant gain and offset. Three-dimensional images were obtained by the average projection of 4 slices with Leica software.

Statistical Analysis

Data were processed by ANOVA using the GLM procedure of Statgraphics (Statgraphics, Inc., Chicago, IL). Tukey's test was used for comparison of sample data. Evaluations were based on a significance level of $P < 0.05$.

RESULTS

Particle Size and Distribution

Four parameters [$D_v0.9$, $D_v0.5$, D(4,3), and D(3,2)] as well as the distribution patterns were taken into consideration to see the effects of UHPH on particle size and distribution (Table 1).

The size distribution of particles in raw milk was characterized by a main peak at 3.8 μm and a second lower peak around 0.2 μm, which corresponded to fat globules and casein micelle particles, respectively. Pasteurized milk showed a similar pattern. As expected, the size distribution of homogenized milks changed markedly; their main peaks were between 0.1 and 0.3 μm for UHPH-treated milks, and approximately 0.4 μm for homogenized–pasteurized milk. Samples undergoing UHPH treatment at 330 MPa showed a second peak, lower but much wider at 4.6 μm with a shoulder at approximately 11.9 μm.

Significant differences ($P < 0.05$) between pasteurized and raw milks were found for $D_v0.5$ and D(3,2). Homogenized–pasteurized samples showed values between those of pasteurized and raw milks, on one side, and

Table 1. Particle size (μm) of raw, pasteurized, homogenized-pasteurized, and ultra-high pressure homogenized (UHPH) milks[1]

Treatment[2]	$D_v0.9$	$D_v0.5$	D(4,3)	D(3,2)
Raw	5.07 ± 0.03[b]	3.11 ± 0.04[b]	2.90 ± 0.05[a]	0.63 ± 0.03[a]
Pasteurized	5.16 ± 0.04[b]	3.16 ± 0.01[a]	2.94 ± 0.01[a]	0.59 ± 0.01[b]
Homogenized–pasteurized	1.10 ± 0.03[e]	0.39 ± 0.00[e]	0.50 ± 0.01[e]	0.32 ± 0.01[c]
UHPH (MPa)				
100	0.60 ± 0.03[d]	0.34 ± 0.00[d]	0.44 ± 0.01[e,d]	0.26 ± 0.01[d]
130	0.68 ± 0.01[de]	0.31 ± 0.01[e]	0.40 ± 0.01[d]	0.25 ± 0.01[e]
200	0.43 ± 0.01[f]	0.21 ± 0.02[g]	0.25 ± 0.02[e]	0.19 ± 0.02[g]
230	0.49 ± 0.01[e,f]	0.25 ± 0.01[f]	0.31 ± 0.03[e]	0.21 ± 0.01[f]
300	0.29 ± 0.01[f]	0.15 ± 0.00[h]	0.17 ± 0.01[f]	0.13 ± 0.00[h]
330	5.82 ± 0.35[a]	0.25 ± 0.01[f]	2.01 ± 0.14[b]	0.22 ± 0.01[f]

[a–h]Values without common superscripts were significantly different ($P < 0.05$).

[1]Mean value ± standard error. Particle size parameters: $D_v0.9$ and $D_v0.5$ = the diameters below which 90% and 50% of the volume of particles are found, respectively; D(4,3) = the volume-weighted mean diameter; and D(3,2) = the surface-weighted mean diameter.

[2]Pasteurized (72°C for 15 s); homogenized–pasteurized (15 + 3 MPa at 57 to 60°C, 72°C for 15 s); UHPH treatments at 130, 230, and 330 MPa made using 2-stage homogenization: 100, 200, and 300 MPa in first valve and 30 MPa in second valve.

those of UHPH-treated milks, on the other. Increasing the pressure of UHPH significantly decreased all 4 parameters, except for UHPH treatment at 330 MPa. Two-stage homogenization did not affect either $D_v0.9$ or D(4,3) at pressures below 300 MPa. However, above 100 MPa, the 2-stage homogenized samples showed higher $D_v0.5$ and D(3,2) values than their counterparts treated by single-stage homogenization. Milk samples UHPH-treated at 330 MPa showed a $D_v0.9$ value significantly higher than that of raw milk, and a D(4,3) value closer to that of raw milk than to UHPH-treated milks.

Rennet Coagulation Properties

Pasteurization did not affect pH, but homogenized–pasteurized milk showed significantly lower pH than that of raw milk. The influence of UHPH on the pH highly depended on the applied pressure; below 300 MPa, the values were significantly lower (Table 2).

Rennet coagulation times were very much dependent on the treatment, although 2-stage homogenization did not seem to affect it. Samples treated at 100 to 130 MPa had significantly lower RCT than raw milk. In the case of milk treated at 200 to 230 MPa, RCT were also significantly lower than that of raw milk but similar to that obtained with homogenized–pasteurized milk. On the other hand, the RCT of the samples treated at 300 to 330 MPa were similar to those obtained with pasteurized and raw milks.

Two-stage homogenization in all UHPH treatments significantly ($P < 0.05$) diminished both RCF and CF in relation to their homologues treated by single stage. The values of RCF were either significantly lower or similar to those of raw milk. The UHPH treatments at 200 and 300 MPa and heat pasteurization significantly increased RCF compared with raw milk. However, only the UHPH treatments at 200 and 300 MPa resulted in significantly higher CF than raw milk.

Curd Yield and Moisture Content

Wet yields and moisture content of the curds obtained from the UHPH-treated milks were significantly greater than those of homogenized–pasteurized, pasteurized, and raw milks (Table 3). Increasing the pressure from 100 to 300 MPa increased wet curd yield by approximately 33 to 65%, and moisture content by approximately 11 to 18% compared with raw milk. Samples treated at 330 MPa showed significantly lower values than samples treated at 300 MPa.

Conventional pasteurization and homogenization–pasteurization increased the yield of total curd solids by approximately 7% compared with raw milk. For UHPH-treated milks, the increases were approximately 4, 6, 7, 8, 10, and 11% at 130, 100, 200, 230, 330, and 300 MPa, respectively.

Whey Composition

All treatments significantly ($P < 0.05$) decreased the amount of total N in whey (Table 4). The UHPH samples showed a decrease of approximately 2 to 22% correlated to the increase of pressure. Above 200 MPa, the effect of UHPH was much higher than those of the pasteurization and homogenization–pasteurization treatments, although the 2-stage homogenized samples did not differ from their single-stage homologues. The same results were observed for β-LG content of whey (Table 4). For α-LA, although no statistical differences were found between whey from raw, pasteurized, and homoge-

Table 2. pH and rennet coagulation properties [rennet coagulation time (RCT), rate of curd firming (RCF), and curd firmness (CF)] of raw, pasteurized, homogenized–pasteurized, and ultra-high pressure homogenized (UHPH) milks[1]

Treatment[2]	pH	RCT (min)	RCF (mA/min)	CF (mA)
Raw	6.75 ± 0.01[a]	7.43 ± 0.13[a]	1.37 ± 0.04[d]	13.33 ± 0.27[c]
Pasteurized	6.75 ± 0.01[a]	7.66 ± 0.15[a]	1.47 ± 0.03[c]	13.80 ± 0.24[bc]
Homogenized–pasteurized	6.72 ± 0.01[b]	6.91 ± 0.15[b]	0.99 ± 0.04[f]	10.21 ± 0.16[e]
UHPH (MPa)				
100	6.52 ± 0.03[e]	5.44 ± 0.27[c]	1.07 ± 0.10[e]	11.51 ± 0.71[d]
130	6.51 ± 0.03[f]	5.18 ± 0.27[c]	0.77 ± 0.07[g]	10.32 ± 0.54[e]
200	6.68 ± 0.02[d]	6.91 ± 0.29[b]	1.84 ± 0.07[a]	15.00 ± 0.31[a]
230	6.69 ± 0.01[c]	7.02 ± 0.09[b]	1.32 ± 0.05[d]	11.99 ± 0.27[d]
300	6.75 ± 0.01[a]	7.69 ± 0.09[a]	1.72 ± 0.02[b]	14.46 ± 0.16[ab]
330	6.74 ± 0.00[a]	7.68 ± 0.11[a]	1.32 ± 0.05[d]	11.75 ± 0.33[d]

[a–g]Values without common superscripts were significantly different ($P < 0.05$).

[1]Mean value ± standard error.

[2]Pasteurized (72°C for 15 s); homogenized–pasteurized (15 + 3 MPa at 57 to 60°C, 72°C for 15 s); UHPH treatments at 130, 230, and 330 MPa made using 2-stage homogenization: 100, 200, and 300 MPa in first valve and 30 MPa in second valve.

nized–pasteurized milks, all UHPH treatments showed a significant decrease of α-LA in whey compared with raw milk. Denaturation of β-LG was more important than that of α-LA; levels were obtained of up to approximately 35% for β-LG at 300 and 330 MPa, and around 12% for α-LA at 300 MPa.

Only the whey obtained from UHPH treatments performed at 100 to 130 MPa and 300 to 330 MPa had significantly higher or lower amounts, respectively, of all 3 mineral salts (Ca, P, and Mg) compared with raw milk (Table 5). Whey from milk UHPH-treated at 200 MPa had higher amounts of Ca and P and a lower amount of Mg than that of raw milk. Whey from treated milk at 230 MPa presented similar amounts of Ca and Mg and higher amounts of P than raw milk. Pasteurized and homogenized–pasteurized samples showed lower amounts of Ca and Mg and similar amounts of P compared with raw milk.

Table 3. Wet yield and moisture content of curds from raw, pasteurized, homogenized–pasteurized, and ultra-high pressure homogenized (UHPH) milks[1]

Treatment[2]	Wet yield (%)	Moisture content (%)
Raw	21.38 ± 0.38[b]	64.76 ± 0.22[b]
Pasteurized	22.00 ± 0.58[c]	63.45 ± 0.39[e]
Homogenized–pasteurized	26.68 ± 0.37[a]	69.86 ± 0.19[a]
UHPH (MPa)		
100	28.39 ± 0.57[f]	71.92 ± 0.22[f]
130	29.01 ± 0.51[e]	73.03 ± 0.15[g]
200	31.47 ± 0.76[d]	74.35 ± 0.24[d]
230	32.76 ± 0.57[c]	75.10 ± 0.16[c]
300	35.35 ± 0.71[a]	76.42 ± 0.09[a]
330	34.33 ± 0.64[b]	75.95 ± 0.09[b]

[a–g]Values without common superscripts were significantly different ($P < 0.05$).

[1]Mean value ± standard error.

[2]Pasteurized (72°C for 15 s); homogenized–pasteurized (15 + 3 MPa at 57 to 60°C, 72°C for 15 s); UHPH treatments at 130, 230, and 330 MPa made using 2-stage homogenization: 100, 200, and 300 MPa in first valve and 30 MPa in second valve.

Confocal Laser Scanning Microscopy of Rennet Gels

Confocal micrographs of rennet gels revealed the existence of visual differences between treatments in the proteinaceous matrix and fat globule size as well as at their interaction (Figure 1).

Rennet gels from pasteurized milk were similar to those obtained from raw milk. The micrographs showed a porous structure of the casein network with native milk fat globules mainly located in the serum pores of the gels (results not shown).

When milk was homogenized–pasteurized, the rennet gels presented open matrices; serum pores were large, irregular, and delimited by thick and lumpy strands. Nile red fluorescence revealed that fat globules had different locations depending on their size (Figure 1a). The smallest fat globules (<0.5 μm) became part of the proteinaceous network, explaining the thickness of the strands. The gels presented many strands that ended with midsized fat globules (~1 μm). Larger fat globules (~1.5 to 2 μm), which accounted for a very small number, were retained in the serum pores.

Although UHPH treatments at 100 MPa showed a greater amount of smaller fat globules and few larger globules (~4 to 6 μm), the general aspect of the matrix was rather similar to that of homogenized–pasteurized milks (Figure 1b). The second stage at 100 MPa reduced the size of the largest fat globules (~3 μm). Moreover, the structure of the gel was smoother with smaller pores. However, the smallest fat globules were also embedded within the proteinaceous network.

Table 4. Total N content and residual α-LA and β-LG (measured as total area ×10⁵ of the respective peaks) in whey of raw, pasteurized, homogenized–pasteurized, and ultra-high pressure homogenized (UHPH) milks[1]

Treatment[2]	Total N (%)	α-LA	β-LG
Raw	0.143 ± 0.004[a]	41.09 ± 1.55[a]	107.71 ± 5.99[a]
Pasteurized	0.135 ± 0.005[c]	40.52 ± 1.52[ab]	102.76 ± 5.66[b]
Homogenized–pasteurized	0.133 ± 0.004[d]	39.88 ± 1.50[abc]	94.65 ± 4.82[c]
UHPH (MPa)			
100	0.140 ± 0.004[b]	38.41 ± 0.56[cd]	103.16 ± 3.04[b]
130	0.137 ± 0.003[c]	38.43 ± 0.39[bcd]	103.88 ± 4.06[b]
200	0.125 ± 0.001[e]	37.55 ± 0.29[de]	85.65 ± 1.60[d]
230	0.126 ± 0.004[e]	36.75 ± 0.72[de]	86.25 ± 1.39[d]
300	0.112 ± 0.003[f]	36.08 ± 0.22[e]	70.04 ± 2.07[e]
330	0.112 ± 0.002[f]	36.86 ± 0.42[de]	69.28 ± 1.86[e]

[a–f]Values without common superscripts were significantly different ($P < 0.05$).

[1]Mean value ± standard error.

[2]Pasteurized (72°C for 15 s); homogenized–pasteurized (15 + 3 MPa at 57 to 60°C, 72°C for 15 s); UHPH treatments at 130, 230, and 330 MPa made using 2-stage homogenization: 100, 200, and 300 MPa in first valve and 30 MPa in second valve.

Micrographs of gels from single-stage treatments above 200 MPa showed that Nile red fluorescence at the level of the proteinaceous network was markedly weaker (Figures 1c and d). Rennet gels from milk treated at 200 MPa revealed tight matrices. It should be mentioned that micrographs from UHPH-treated milk at 300 MPa not only had lower levels of Nile red fluorescence but also lower FITC fluorescence (Figure 1d).

The second stage above 200 MPa provoked the coating of midsized fat globules; this phenomenon was more visible as the pressure at the first valve was increased. Moreover, the proteinaceous matrices, which were more lax than those of rennet gels from single-stage treated milks, were strongly stained by Nile red (Figures 1e and f). Two-stage UHPH treatment at 300 MPa provoked the formation of spherical protein aggregates surrounding a large number of noncoated, midsized fat globules (~2 μm; Figures 1g and h).

DISCUSSION

Effects of Heat and Conventional Homogenization Treatments

Mild heat treatments are considered to have no or little effect on the whey proteins of milk, although there are reports that heat pasteurization (72°C for 20 s or 73°C for 15 s) could cause denaturation of approximately 7% of the whey protein fraction of milk (Jelen and Rattray, 1995). Our results showed that pasteurization heat treatment of 72°C for 15 s was sufficient to reduce ~5% of the total N of whey, with levels of residual β-LG and α-LA being ~5 and 1.4% lower, re-

Table 5. Whey composition in minerals of raw, pasteurized, homogenized–pasteurized, and ultra-high pressure homogenized (UHPH) milks[1]

	Minerals (mg/L)		
Treatment[2]	Ca	P	Mg
Raw	377.33 ± 9.17[d]	435.07 ± 3.12[e]	88.24 ± 1.66[b]
Pasteurized	371.64 ± 8.98[e]	433.75 ± 1.63[e]	87.23 ± 1.62[cd]
Homogenized–pasteurized	372.23 ± 9.16[e]	436.17 ± 2.65[de]	86.91 ± 1.65[d]
UHPH (MPa)			
100	393.12 ± 7.61[b]	461.73 ± 3.10[b]	89.29 ± 1.21[a]
130	404.56 ± 6.33[a]	471.16 ± 4.79[a]	90.04 ± 1.17[a]
200	386.20 ± 5.38[c]	447.15 ± 4.36[c]	87.19 ± 1.28[cd]
230	381.21 ± 12.47[d]	442.48 ± 5.68[cd]	87.91 ± 1.72[bc]
300	356.10 ± 15.68[g]	414.91 ± 7.09[f]	83.52 ± 1.64[e]
330	361.33 ± 9.95[f]	419.60 ± 3.73[f]	83.36 ± 1.21[e]

[a–g]Values without common superscripts were significantly different ($P < 0.05$).

[1]Mean value ± standard error.

[2]Pasteurized (72°C for 15 s); homogenized–pasteurized (15 + 3 MPa at 57 to 60°C, 72°C for 15 s); UHPH treatments at 130, 230, and 330 MPa made using 2-stage homogenization: 100, 200, and 300 MPa in first valve and 30 MPa in second valve.

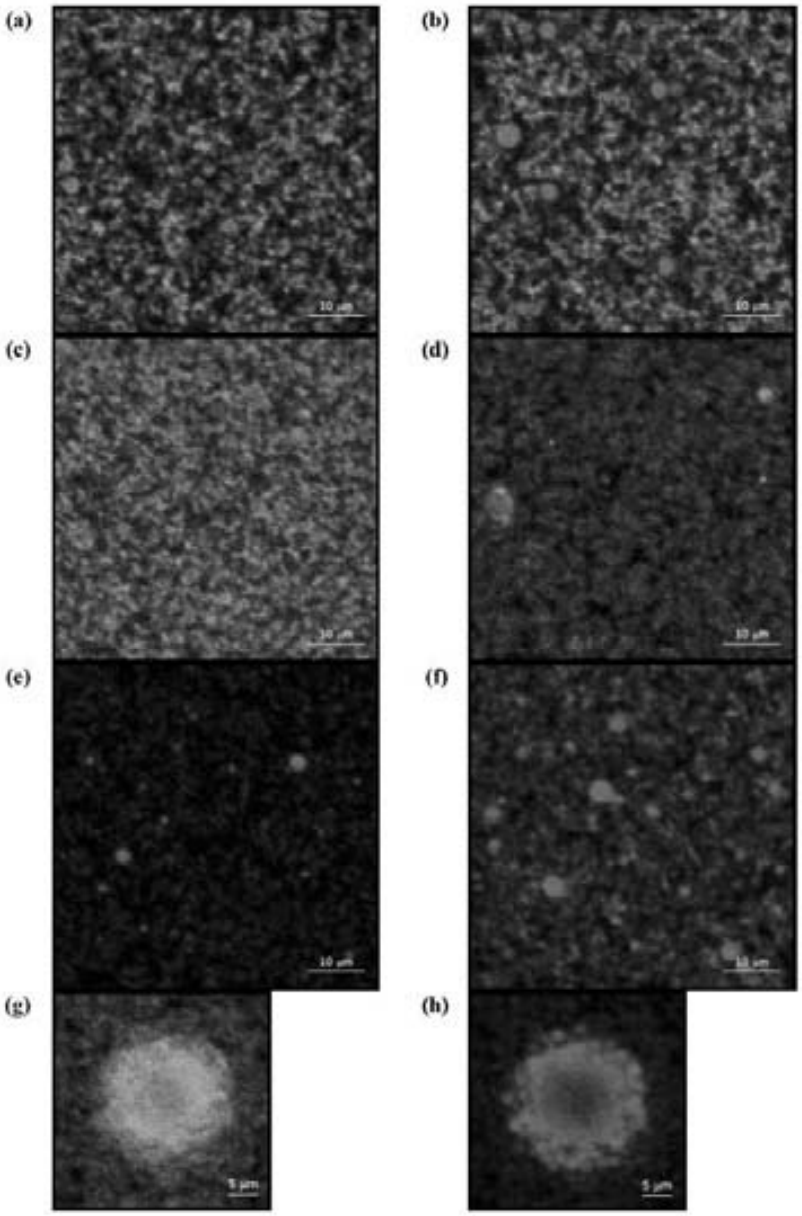

Figure 1. Confocal laser scanning micrographs of rennet curds from a) homogenized–pasteurized milk; ultra-high pressure homogenized (UHPH) milk at b) 100 MPa; c) 200 MPa; and d) 300 MPa; Nile red fluorescence (fat) from milk UHPH-treated at e) 200 MPa, and f) 230 MPa; and aggregates of fat globules dyed with g) Nile red and fluorescein isothiocyanate, and h) Nile red. Color images are available online at http://jds.fass.org/.

spectively, in whey from pasteurized milk compared with untreated milk.

Furthermore, heating has a marked effect on the milk salts equilibrium and their interaction with casein. It is generally agreed that heating leads to a decrease in diffusible calcium and inorganic phosphate, due to precipitation of calcium phosphate, which may be reversed depending on the intensity of the treatment (Gaucheron, 2005). Under our experimental conditions, the decrease of Ca, P, and Mg in whey from pasteurized milk suggests a mineral transfer from soluble to colloidal phase of milk.

Milk pasteurization has only minor effects on the formation and physical properties of rennet-induced milk gels (Lucey, 1995). In our study, pasteurized milk had no significantly different RCT and CF in relation to untreated milk. However, more severe heating conditions impair renneting milk properties (Dalgleish and Banks, 1991; Guinee et al., 1996, 1997; Singh and Waungana, 2001). The causes have been broadly investigated even though they are not yet fully understood. Both the enzymatic and nonenzymatic phases of rennet clotting are delayed and the RCT is longer than that of unheated milk. The strength of renneted milk gels is also adversely affected in heated milk. It has been established that when heated, β-LG and κ-casein form a complex by sulfydryl–disulfide interchange at the micelle surfaces that reduces the accessibility of the rennet to the κ-casein and provides steric hindrance to close approach and fusion of paracasein micelles. Moreover, heat induces the deposition of calcium phosphate and the consequent reduction in native calcium phosphate, which is important for cross-linking paracasein micelles.

Incorporation of denatured whey protein in the curd from pasteurized milk did not increase the moisture content of the curd compared with raw milk. However, the yield of TS of the curd from pasteurized milk was ~7% higher than that from raw milk, which is probably due to the incorporation of denatured whey proteins into the curd. According to Lau et al. (1990) pasteurization (63°C for 30 min) has little effect on fat recovery in cheese but N recovery is improved, and approximately 5% of the whey proteins are associated with casein micelles after pasteurization, resulting in an increased theoretical cheese yield.

During conventional homogenization, the fat globule size is reduced, the fat surface area increases markedly, and a new adsorbed layer consisting of milk proteins (mainly casein micelles and casein subunits and whey proteins) is formed around the fat globules (Cano-Ruiz and Richter, 1997). Our results showed a marked reduction of both volume- and surface-weighted mean diameters, from 2.9 to 0.5 μm and 0.6 to 0.3 μm, respectively.

It has been reported that homogenization processes do not affect the distribution of calcium in milk (Robson and Dalgleish, 1984). In our study, the amount of Ca, P, and Mg in whey of homogenized-pasteurized milks did not differ from those of pasteurized milks.

Homogenized-pasteurized milks presented lower RCT than raw and pasteurized milks, results that have also been observed by other authors (Robson and Dalgleish, 1984; Ghosh et al., 1994; Guinee et al., 1997). The lower RCT of homogenized milks could be explained by the fact that most κ-casein is located on the micelle surface. As the casein enrobes the fat globules, the κ-casein level is effectively diluted and a smaller critical level of κ-casein hydrolysis is required to start coagulation (Guinee et al., 1997). Furthermore, homogenization increases the surface area of casein by a spreading process, κ-casein being more available for chymosin action, and thus, reducing RCT (Ghosh et al., 1994).

In the current study, the CF of homogenized milks was reduced by approximately 23% compared with untreated milk. The weaker gels from homogenized–pasteurized milks could be attributed, according to different authors (Humbert et al., 1980; Robson and Dalgleish, 1984; Ghosh et al., 1994), to a greater dispersion of fat in the curd, to a reduced number of casein particles available to form a strong network because some of the casein is tied to the surface of the new formed fat globules, or to the small fat globules that are entrapped in the gel disrupting the continuity of gel structure and acting as weak centers in the gel. Confocal laser scanning microscopy revealed fat globules embedded within the protein matrix resulting in thick and lumpy strands and a concomitant coarser texture.

Compared with single pasteurization, the homogenization–pasteurization treatment produced higher ($P < 0.05$) amounts of denatured β-LG and significantly increased the moisture content of the curd. Homogenization of milk resulting in slower whey drainage of the curd has been observed by several researchers (Humbert et al., 1980; Green et al., 1983; Ghosh et al., 1994). The effects of homogenization on the moisture content of the curd have been attributed to the higher incorporation of denatured β-LG and the alteration in the protein–fat structure of the curd.

Effects of UHPH Treatments

In accordance with Hayes and Kelly (2003a), smaller fat globules were obtained by applying pressures below 200 MPa at the second valve. Thus, the second valve would act as the secondary stage of a normal homogenizer; that is, stopping or decreasing coalescence. Many studies have shown that above a critical pressure, there is an increased susceptibility of fat globule coalescence

(Floury et al., 2000, 2004b; Desrumaux and Marcand, 2002). Above 200 MPa, the secondary stage not only increased the average size of particles but also widened the distribution; that is, higher heterogeneity, compared with single-stage homogenization. This may be due to partial agglomeration of very small, insufficiently coated globules that collide within the second valve. Thiebaud et al. (2003) detected very small fat globules (40- to 60-nm droplets) in single-stage UHPH-treated milk at 200 and 300 MPa, and the impact forces that act on the droplets as the result of a collision have been determined as sufficient to cause disruption of the interfacial membranes (Floury et al., 2000). A broadening of the size distributions was observed for single-stage UHPH of warmed milk (Thiebaud et al., 2003), and model oil-in-water emulsions (Floury et al., 2000) at 300 MPa. The formation of large particles was attributed to unfolding and aggregation of whey proteins of the newly created droplets. In our case, 2-stage UHPH-treated milks at 330 MPa showed much broader size distributions. Confocal laser scanning microscopy of rennet gels revealed that this phenomenon was due to the aggregation of well-defined small fat globules within dense proteinaceous structures (Figures 1g and h).

Milks UHPH-treated below 200 MPa showed similar gel strength compared with homogenized–pasteurized milk. In fact, the protein matrices of the rennet gels observed by confocal microscopy were very similar to those of homogenized–pasteurized gels; that is, thick and lumpy strands giving a rough texture to the matrix (Figures 1a and b). However, their coagulation times were lower than those of homogenized–pasteurized milks. This decrease could be attributed to the lower pH of UHPH-treated milks, which would enhance chymosin performance. At these pressures, the temperature during processing never exceeded 60°C. Thus, the decrease in pH could be attributed to the action of residual indigenous lipoprotein lipase after UHPH treatment. Treatment with UHPH increased the interfacial fat surface by decreasing the average size of particles, which would lead to a greater potential for lipolysis to occur (Hayes and Kelly, 2003a; Hayes et al., 2005).

Mineral equilibrium in milk is very dependent on physicochemical parameters; that is, pH and temperature. The distribution of ions between the different fractions of milk (diffusible and nondiffusible) is defined by the balance between these factors. The pH of milks treated at 100 to 130 MPa could be, to some extent, responsible for the higher amounts of minerals in their whey. As pH decreases, the acido-basic groups of milk constituents become more protonated; hence, micellar calcium phosphate and the small amount of magnesium associated to casein micelles are dissolved (Gaucheron, 2005).

Single-stage UHPH above 200 MPa produced the smallest particles with the narrowest distributions. A further reduction of fat globule size and the increase in interfacial fat surface would lead to a higher adsorption of casein and whey proteins to the newly formed fat globules. Sandra and Dalgleish (2005) reported a decreased micelle average size in skimmed milk by increasing UHPH pressure. They suggested that UHPH would not cause complete disruption of the casein micelles but rather dissociate parts of their surfaces. The obtained particle distributions corroborated that casein micelle fragments, rather than intact casein micelles, would surround fat globules (Hayes et al., 2005). Thus, very small fat globules would behave as casein micelles rather than embedded fat globules observed in normal homogenization or lower UHPH pressures. Such structures could enhance gel firmness and rate of aggregation by increasing the amount of particle associations; hence, leading to the higher RCF and CF values observed for milk UHPH-treated at 200 and 300 MPa. Confocal micrographs of rennet milks treated at 200 and 300 MPa showed lower levels of Nile red fluorescence at the level of the proteinaceous network (Figures 1c and d). This could be explained by the fact that more than 50% of their particles were beyond the resolution threshold (0.23 μm/pixel).

In early studies on UHPH, no denaturation of whey proteins was reported (Hayes and Kelly, 2003a; Sandra and Dalgleish, 2005). However, the temperature of the process in these studies never exceeded 55°C. Our temperature values during UHPH treatments were much higher (from ~55 to 95°C), presumably because of different experimental designs (i.e., a much higher flow rate and relatively larger volumes of milk being processed). If only heat effect is considered, at ~65°C, whey proteins start to denature and interact with casein micelles (Singh, 1993). However, in UHPH, simultaneous heating and homogenization processes exist. In fact, Hayes et al. (2005), treating warmed milk up to 250 MPa that reached 83.6°C, suggested that the physical forces experienced by whole milk during UHPH also denatured β-LG. Our results showed that the amount of denatured β-LG was much greater (17%) for UHPH-treated at 200 MPa, which reached approximately 75°C for a very short time (~0.7 s), than for pasteurized milk at 72°C for 15 s. Such results corroborate the idea that not only heat but also homogenization forces induce the denaturation of whey proteins. Increasing the pressure led to higher recovery of N in curd with lower levels of residual β-LG and α-LA in the whey.

As previously mentioned, both the pH of the milk and the temperature reached during the treatment affected

the mineral equilibrium. Moreover, UHPH produces partial disruption of casein micelles (Sandra and Dalgleish, 2005) that could lead to a transfer of calcium and inorganic phosphate from the micellar to the diffusible fraction. The balance between these factors could explain the differences observed between the treatments at 200 and 300 MPa. The fact that whey from milk treated at 200 MPa showed higher amounts of calcium than whey from homogenized–pasteurized milk could be explained by both the release due to disruption of casein micelles and its slightly lower pH value. In contrast, the amount of minerals in whey from milk UHPH-treated at 300 MPa, which was lower than those of the other treatments, suggests a mineral transfer from soluble to colloidal phase due to heat during UHPH treatment.

As pressure was increased, the RCT of milks was prolonged. The differences between RCT at 200 and 300 MPa could be explained by the relative effect of the following factors: 1) the spreading of κ-casein; that is, higher availability and lower critical level for chymosin action; 2) denaturation of β-LG; that is, steric hindrance; 3) the pH of milk; and 4) changes in the concentration of calcium between soluble and colloidal phases.

Increasing UHPH pressure led to a higher recovery of N with lower levels of residual β-LG and α-LA in whey, and higher TS yield and moisture content of curds. The observed differences between treatments could be explained by variations in 1) the association of denatured whey proteins to the surface of casein micelles, 2) the reduction of fat globule size, 3) the incorporation of denatured whey proteins and casein micelle fragments at the fat globule membrane, and 4) the microstructure of the resulting gels. The association of whey proteins at the micelle surface by heat sterically impedes the fusion of rennet-altered micelles resulting in less shrinkage of the paracasein network (Singh and Waungana, 2001). Moreover, the incorporation of denatured whey proteins into the gel matrix increases the water-binding capacity of the paracasein-whey network (Singh and Waungana, 2001). The reduction of fat globule size implies a dispersion of fat into an increased number of smaller globules. The newly built surfaces are modified by the presence of adhering casein particles and become part of the paracasein network, thus hindering shrinkage of the network (Walstra et al., 1985). The water-holding capacity of curds is directly linked to the microstructure of the gels; that is, porosity or permeability (Green et al., 1983; Walstra et al., 1985; Lucey et al., 2001). Green et al. (1983) observed that curds from conventionally homogenized milk had a less coarse protein network, which retained moisture more effectively than curds from nonhomogenized milks. Greater firmness, attributed to greater protein content and cross-linking of casein by denatured whey proteins, leads to higher volume of the network relative to that of the interstices, and thus a reduction of the relative ease of movement of the strands in the protein network (Green et al., 1983; Lucey et al., 2001).

As already stated, 2-stage UHPH above 200 MPa led to greater average particle size and higher heterogeneity than single-stage treatments. The obtained rennet gels showed similar firmness to those of homogenized–pasteurized and 100 to 130 MPa UHPH-treated milks. Confocal microscopy revealed a higher number of fat globules embedded within the proteinaceous matrix giving a rougher texture to the gels than in single-stage UHPH (Figures 1e and f). These results corroborate the hypothesis that 1) embedded fat globules, which lead to thicker strands and a concomitant coarser matrix, are responsible for weaker gels, and 2) the presence of very small fat globules behaving as casein micelles results in stronger rennet gels.

CONCLUSIONS

The results of this study show that UHPH treatment of milk reduced fat globule size, increased the wet yield of curd and its moisture content, and decreased the protein content of whey. The rennet coagulation properties were enhanced by single-stage UHPH at 200 and 300 MPa. However, taking curd firmness into account, the application of a secondary stage produced weaker gels similar to those obtained by conventional homogenization–pasteurization. The improvement of cheesemaking properties of milk by UHPH could be attributed to the combined effect of homogenization (i.e., reduction of particle size) and heat (i.e., denaturation of whey proteins) on the protein–fat structures of the milk.

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Protein composition of caprine milk fat globule membrane

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ABSTRACT

Milk fat globule membrane proteins of individual goats were characterised using one-dimensional SDS-PAGE analysis. Many differences were observed between bulked caprine and bovine milk samples. Goat sample showed much higher content of xanthine oxidase and of minor proteins compared with cow sample. Among individuals, a high heterogeneity could be observed; 32 bands were identified of which 19 were present in all caprine samples. Several previously non-described bands were detected. No correlations between fat content of milk, average size of fat globules and total protein of washed creams were observed. However, both the amount of protein and the correlations among them could be linked to their physiological role, i.e., general or specific transport, cell signalling, metabolism and immune functions.

1. Introduction

Milk fat globules are surrounded by a membrane composed mainly of proteins, phospholipids, glycoproteins, triglycerides, cholesterol and enzymes. This membrane, which is known as the milk fat globule membrane (MFGM), consists of several distinct layers of different origins. Precursors of milk lipid globules are formed at the endoplasmic reticulum of mammary epithelial cells and are released into cytosol as lipid droplets surrounded by a monolayer coat of proteins and phospholipids. During secretion, milk fat globules gain the outer bilayer coat from the apical plasma membrane of secretory cells (Keenan and Mather, 2006).

Bovine MFGM was initially characterised during the 1970s and 1980s by isoelectric focusing with SDS-PAGE gels. The studies published during the following decade were mainly focussed on the purification of major MFGM proteins and their sequence identification through immunological methods and N-terminal sequencing. The proteins of the bovine MFGM were reviewed by Mather (2000). Technological advances in proteomics, e.g., mass spectrometry, and genetic techniques, i.e., molecular cloning and computer-assisted sequence analysis, have recently led to a better knowledge on human (Cavaletto et al., 2004) and bovine (Reinhardt and Lippolis, 2006; Fong et al., 2007) MFGM proteins.

The physiological role of MFGM proteins is not completely understood despite an extensive research has been conducted. Some of them are evidently involved in milk fat globule formation (Heid and Keenan, 2005). In recent years, several physiological benefits of these proteins have been detected (Spitsberg, 2005; Dewettinck et al., 2008). However, some of these proteins have been linked to human pathologies (Riccio, 2004). Moreover, MFGM acts as a natural emulsifying agent, preventing flocculation and coalescence of fat globules in milk and protecting the fat against enzyme action. In recent years, there has been increasing interest in investigating the potential applications of MFGM in food systems, as reviewed by Singh (2006) and Dewettinck et al. (2008).

Goat is an economically important species for which milk samples from individuals are readily available. In contrast to the extensive work on human and bovine milk,

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very few studies on MFGM have included goat's milk since the review written by Jenness in 1980. Up to date, two caprine MFGM proteins have been characterised, i.e., MUC1 (Campana et al., 1992; Sacchi et al., 2004) and xanthine oxidase (XO) (Atmani et al., 2004). The aim of the study was to characterise the protein composition of goat's MFGM.

2. Material and methods

2.1. Milk samples

Milk was collected individually from 19 Murciano-Granadina goats of the Universitat Autònoma de Barcelona farm, Bellaterra, Spain, on week 38th after parturition. Bulked milk sample of the whole herd and a bulked bovine milk sample were collected as internal indicators. Milks were kept at 4 °C for further isolation of the MFGM material.

2.2. Determination of milk fat content and globule average size

Fat content of the individual milks was determined by the routine method (ISO, 1976). Determination of the average fat globule size D(3,2) (volume-surface average mean diameter) was carried out using a Beckman Coulter laser diffraction particle size analyzer (LS 13 320 series, Beckman Coulter, Fullerton CA, USA). Milk samples were diluted in distilled water until an appropriated obscuration in the diffractometer cell was obtained. An optical model based on Mie theory of light scattering by spherical particles was applied by using the following conditions: real refractive index, 1.471; refractive index of fluid (water), 1.332; imaginary refractive index, 0; pump speed, 20%.

2.3. Isolation of MFGM material

Separation of the fat phase from the serum was done by centrifuging milks at 10,500 × g for 30 min at 20 °C after addition of 28.6 g of sucrose per 100 g of milk. After cooling, the top layer (cream) was removed from the centrifuge tube using a spatula. The isolation of MFGM material was performed according to a modified method of Ye et al. (2002). The cream was washed twice with simulated milk ultrafiltrate (SMUF; Jenness and Koops, 1962) during 1 h at room temperature and centrifuged at 10,500 × g for 30 min at 20 °C. After cooling, the top layer was collected.

2.4. Determination of washed cream protein content

Total protein content of the washed creams was determined in duplicates through the Dumas combustion method (IDF, 2002) by determining total nitrogen and multiplying by a factor of 6.38.

2.5. Electrophoresis

Protein composition of MFGM from washed creams was determined by SDS-PAGE. Samples of washed creams (0.25 g) were suspended in 0.5 mL reducing buffer (6% Tris–0.5 M, 10% glycerol, 5% β-mercaptoethanol, 2% SDS and 0.05% bromophenol blue). Samples were heated at 95 °C for 5 min and then centrifuged at 2500 × g for 30 min in order to remove the fat from the sample. Supernatants (20 or 15 μL) were loaded onto 8% and 15% SDS-polyacrylamide gels (20% and 37.5% Acryl-Bis at 40%, respectively in 1.5 M Tris–HCl buffer, pH 8.8, for separating gel; 10% Acryl-Bis at 40%, in 0.5 M-Tris–HCl buffer, pH 6.8, for stacking gel). A molecular mass marker (high range from 40 to 212 kDa or wide range from 14.4 to 212 kDa, Amresco, Solon, USA) and milk protein standards, albumin, caseins, α-lactalbumin and β-lactoglobulin (Sigma–Aldrich Chemie, Steinheim, Germany) were applied to each gel. Gels were run at 200 V using a Pharmacia Biotech power supply unit (model EPS 3500, Pharmacia Biotech, Uppsala, Sweden). Protein bands were stained with a solution of Coomassie brilliant blue R-250. Gels were destained with a solution of methanol and glacial acetic acid at concentrations of 160 and 10 mL L^{-1}, respectively. Scanned images of the destained gels were analysed using the ImageMaster software (Amersham Pharmacia Biotech, Newcastle, UK). The apparent molecular mass (Mr) of the bands on the SDS-PAGE was estimated from the mobility of proteins in the gel when compared with the mobility of the molecular mass markers. Quantification of individual proteins was done in relation to the Albumin internal standard, and expressed as percentage of the total MFGM protein.

2.6. Statistical analysis

Statistical analysis of experimental data was performed by correlation analysis using Statgraphics (Statgraphics, Inc., Chicago, IL). Significance levels used were $p < 0.01$ and 0.05.

3. Results and discussion

3.1. Fat content of milk, average size of fat globules and total protein of washed creams

Milk fat contents ranged from 3.35% to 6.00% with an average of 4.77% (Table 1). Such results are consistent with the range 4.5–6.6% that has been reported for milk of the Murciana-Granadina breed (Ramos and Juarez, 1993). The average size of globules was 3.07 μm, ranging from 2.66 to 3.70 μm, which is consistent with the results of other authors (Attaie and Richter, 2000). The average of total MFGM proteins accounted for 7.83 mg/g fat, with a range of 5.35–9.75 mg/g fat.

3.2. MFGM proteins

In general, caprine and bovine MFGM resemble each other closely (Jenness, 1980). Thus, the protein composition of caprine milks will be compared to that of bovine milk. Many differences were observed between bulked caprine and bovine samples (lane 3 and 2, Figs. 1 and 2). Goat sample showed a much higher content of a protein with Mr 150 kDa and of minor proteins compared with the bovine sample. Taking into account the protein pattern of individual samples, a high heterogeneity could be observed (lanes 4–10, Figs. 1 and 2); 32 bands were identified, of which 19 were present in all samples. However, none of the goats presented all 32 bands. Six caprine samples had 28 bands, while 1, 3, 3, 4 and 2 were the number of samples that had 23, 24, 25, 27 and 28 bands, respectively.

3.2.1. MUC 1

MUC 1 is the largest protein of the MFGM (Mather, 2000). Many studies with cow milk have shown that the apparent Mr of this protein ranges from 120 to 220 kDa depending on the breed (Mather, 2000). In sheep and cow

Table 1
Statistical summary of total MFGM protein content, fat globule size and fat content of milk.

Factor	Average (n = 19)	Minimum	Maximum	Coeff. of variation
Total protein (mg/g fat)	7.83	5.35	9.75	14.8%
D(3,2) (μm)	3.07	2.66	3.70	9.5%
Fat content (%)	4.77	3.35	6.00	15.1%

D(3,2): volume-surface average mean diameter.

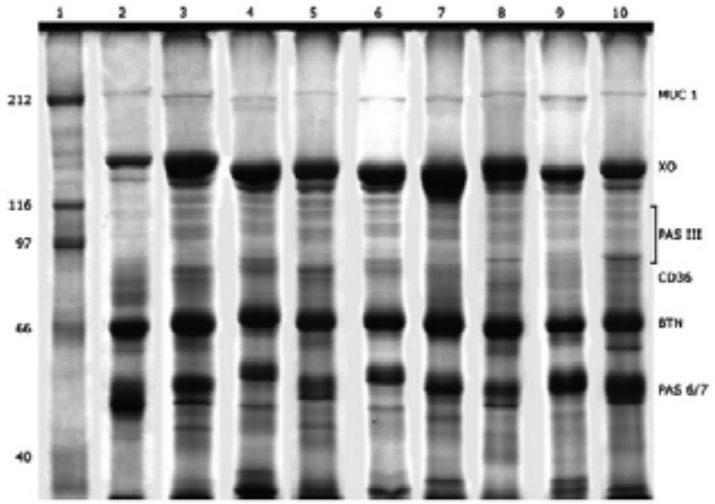

Fig. 1. 8% polyacrylamide gel of washed MFGM (lane 1: Mr standard; lane 2: bulked bovine milk; lane 3: bulked caprine milk; lanes 4–10: individual caprine milks).

this protein moves further into the running gel than in goat (Welsch et al., 1990): the goat analogue may be a considerably larger protein (Campana et al., 1992). All the caprine samples showed a band with Mr ~225 kDa both in the 8% and 15% acrylamide gels (Table 2). However, in the 8% gels, 47% of the goats showed a second band with Mr ~215 kDa. These results are in accordance with the fact that this protein is polymorphic in many species (Patton and Patton, 1990; Huott et al., 1995) and, specifically, in goats (Campana et al., 1992; Sacchi et al., 2004). Both bands accounted for 0.7% of the total MFGM protein on average. MUC 1 corresponds to 1.21% of the weight of bovine MFGM (Kvistgaard et al., 2004). However, as MUC 1 is a glycoprotein that poorly stains with Coomassie blue and is readily dissolved in the aqueous serum when cooling or stirring milk (Mather, 2000), these results should be taken as a reference for individual comparison and correlations with factors and between proteins.

3.2.2. *Xanthine oxidase*

Atmani et al. (2004) purified XO from goat's milk to study its enzymatic activity and observed that caprine XO corresponded to a single band with Mr ~150 kDa on SDS-PAGE. The gels showed that XO was the most abundant protein in washed creams, accounting for, on average almost 10% of the total protein (Table 2). In bovine milk, this protein constituted some 20% of the MFGM proteins (Mather, 2000). It must be pointed out that in caprine

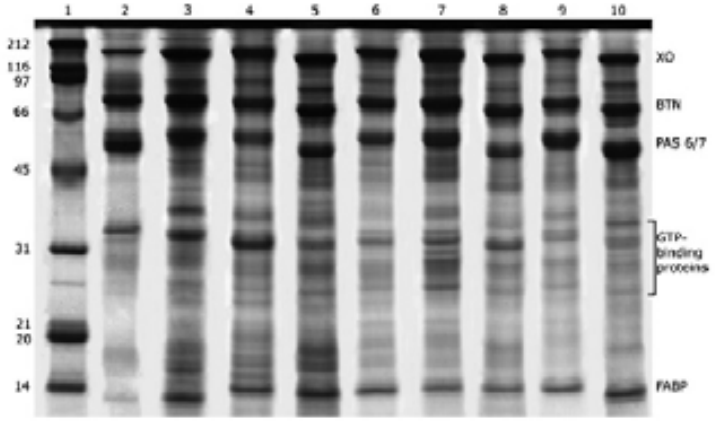

Fig. 2. 15% polyacrylamide gel of washed MFGM (lane 1: Mr standard; lane 2: bulked bovine milk; lane 3: bulked caprine milk; lanes 4–10: individual caprine milks).

Table 2
Statistical summary of bands detected in all goats.

	Molecular weight	Percentage of total protein			
		Average (n = 10)	Minimum	Maximum	Coeff. of variation
8% Acrylamide					
MUC 1	225 kDa*	0.69	0.19	1.81	69.0
XO	150 kDa	9.68	6.83	13.88	20.6
Fr135	135 kDa	2.45	1.33	4.88	35.9
Fr125	125 kDa	1.46	0.53	4.18	68.9
Fr115	115 kDa	1.07	0.64	2.07	40.5
Fr105	105 kDa	1.60	0.79	3.32	42.4
CD36	83 kDa	2.58	1.24	4.59	33.6
BTN	68 kDa	9.09	5.72	12.97	20.4
15% Acrylamide					
PAS 6/7	53 kDa	7.90	4.79	12.31	26.0
Fr44	44 kDa	1.71	0.61	4.20	58.2
Fr40	40 kDa	2.76	1.87	4.21	25.1
Fr32	32 kDa	3.42	0.97	7.92	55.1
Fr30	30 kDa	3.18	1.08	5.62	36.2
Fr26	26 kDa	1.61	0.81	2.79	31.7
Fr24	24 kDa	1.22	0.21	2.52	43.6
Fr21	21 kDa	0.72	0.26	1.51	44.0
Fr19	19 kDa	2.72	1.20	5.97	45.2
Fr18	18 kDa	2.42	0.22	7.29	92.6
FABP	14 kDa	3.71	0.19	1.81	37.0

Fr: fraction; MUC 1: mucin 1; XO: xanthine oxidase; CD36: cluster of differentiation 36; BTN: butyrophilin; PAS 6/7: periodic acud Schiff 6/7; FABP: fatty-acid binding protein.
* Bands at 215 kDa included.

MFGM this protein showed one of the lowest coefficients of variation (21%) together with that of butyrophilin (BTN). Both their high concentration and low variability between individuals could be a hint of the important biological role of these proteins. Recent studies using knock-out mice have revealed that these two proteins are indispensable for milk fat secretion (Vorbach et al., 2002; Ogg et al., 2004).

3.2.3. *Proteins of Mr 135 and 125 kDa*

All washed creams presented two bands with Mr ~135 and ~125 kDa (Table 2). The former protein showed a rather low coefficient of variation (36%) and accounted for 2.5% of the total protein. This unknown protein was also observed in cow's milk by Ye et al. (2002) and represented 3–4% of the total protein in the MFGM. Although present in all caprine MFGM samples, the band with Mr ~125 kDa showed a much higher coefficient of variation (69%), with an average ranging from 0.5% to 4.2%; this band has not been reported previously.

3.2.4. *PAS III and CD36*

In the region corresponding to an apparent Mr range of 70–120 kDa, up to 9 bands could be observed. All caprine washed creams presented 3 bands with Mr ~115, ~105 and ~83 kDa (Table 2). The latter was the protein that showed the highest content and lowest coefficient of variation among them (with an average of 2.6). In bovine milk, CD36 (Mr 75–88 kDa) constitutes ≤5% of the MFGM proteins (Mather, 2000). Hence, the band with Mr ~83 kDa could correspond to CD36 in goat's MFGM. The other proteins accounted for 1.1% and 1.6% of the MFGM proteins, respectively. PAS III, also called MUC 15, is a poorly characterised glycoprotein, which is resolved in SDS-PAGE as a diffuse band of material with an apparent Mr of 95 to over 100 kDa (Mather, 2000). This polydisperse character is thought to be due to variable amounts of carbohydrate (Pallesen et al., 2002). Antibodies specifically binding to the PAS III band of bovine MFGM cross-react with a similarly sized component in caprine MFGM (Kaetzel et al., 1987). Among the bands that were not present in all washed creams, two facts should be noticed. On one hand, proteins with Mr ~87 and ~75 kDa, which were almost present in all goats, showed rather high amounts (1.4 and 1.3%, respectively). On the other hand, a protein with Mr ~72 presented much higher amounts (3.7%), even though it was found only in 21% of the caprine milk samples (Table 3).

3.2.5. *Butyrophilin and 60 kDa protein*

BTN is the most abundant MFGM protein in cow's milk, representing from 20% to 40% on average, depending on the breed (Mather, 2000). Some studies using SDS-PAGE have shown the presence of two bands with Mr of 67 and 64 kDa, both considered as BTN and its degradation product by proteolysis, respectively (Freudenstein et al., 1979; Heid et al., 1983). Isolation of the gene and purification of its product has proved that the later was not an artefact produced during sample preparation (Cavaletto et al., 1999). Hence, this band with Mr ~60 kDa should be taken into account as a true protein and quantified separately. Our results showed that, in caprine MFGM, BTN (Mr 68 kDa) was the second most abundant protein, accounting for 9.1% (Table 2). Ye et al. (2002) showed that this protein constituted from 10% to 16% of the bovine MFGM proteins, depending on the lactation season. They also observed a faint band with Mr ~60 kDa, which was prominent in the early and late season samples, and accounted for 2–4% of the bovine MFGM proteins. In our gels, 58% of goat samples showed the Mr ~60 kDa band; when present, this protein constituted from

Table 3
Statistical summary of bands not detected in all goats.

	Number of goats (percentage)	Molecular weight	Percentage of total protein when present			
			Average	Minimum	Maximum	Coeff. of variation
8% Acrylamide						
Fr95	5 (26%)	95 kDa	0.34	0.21	0.60	48.6
Fr91	14 (74%)	91 kDa	0.77	0.23	1.65	55.0
Fr87	18 (95%)	87 kDa	1.36	0.33	3.11	50.5
Fr79	4 (21%)	79 kDa	0.76	0.44	1.06	35.5
Fr75	16 (84%)	75 kDa	1.26	0.45	2.22	39.6
Fr72	4 (21%)	72 kDa	3.71	2.94	4.36	15.8
Fr60	11 (58%)	60 kDa	2.50	0.80	5.74	55.9
15% Acrylamide						
Fr47	3 (16%)	47 kDa	0.87	0.64	1.14	29.3
Fr38	11 (58%)	38 kDa	0.76	0.31	1.09	37.5
Fr37	13 (68%)	37 kDa	0.87	0.48	1.72	45.6
Fr35	15 (79%)	35 kDa	1.79	0.44	3.59	57.8
Fr34	8 (42%)	34 kDa	2.70	1.31	5.08	46.8
Fr30	7 (37%)	30 kDa	2.07	0.50	9.12	105.6

Fr: fraction.

0.8% to 5.7% of the total protein, with an average of 2.5% (Table 3).

3.2.6. *PAS 6/7*

Separate purification of bovine glycoproteins PAS 6 and PAS 7 demonstrated that their polypeptide cores were identical, and that the difference in their Mr was due to different levels of glycosylation (Hvarregaard et al., 1996). Hence, they have often been referred as PAS 6/7. These proteins, which are the most abundant MFGM glycoproteins after BTN, have values for Mr ranging from 43 to 59 kDa (Mather, 2000). In 15% polyacrylamide gels, two bands with Mr from 50 to 55 kDa, observed in all washed creams, constituted 7.9% of the MFGM proteins and showed a rather low coefficient of variation (26%; Table 2). These results together with the above information allowed bands to be assigned as PAS 6/7.

3.2.7. *GTP-binding proteins and proteins of Mr 15–50 kDa*

Up to 15 bands were detected between 47 and 18 kDa (Tables 2 and 3). The majority of them were present in all washed creams but at different ratios. These results are in accordance with those obtained from bovine MFGM by Ye et al. (2002), who found at least 10–12 bands between 44 and 20 kDa and a number of diffuse bands with Mr 17–18 kDa. Even if some of the bands could be referred as GTP-binding proteins (Ghosal et al., 1993), the others had not been identified until recently. Fong et al. (2007) identified 3 Ig heavy chain proteins with Mr 52, 40 and 36 kDa in bovine MFGM. They also detected α_{s1}-casein, α_{s2}-casein and β-casein with Mr ~25 kDa and suggested that they were the result of some degree of contamination, possibly as a result of MFGM damage during the cream washing steps. The results showed that a special attention should be given, on one hand, to bands with Mr ~40, ~32 and ~29 kDa and, on the other, to bands with Mr ~19 and ~18 kDa, given the fact that they were abundantly present in all caprine MFGM (Table 2). Ye et al. (2002) reported that the unknown proteins with Mr 17–18 kDa constituted 2–5% of the total protein in bovine MFGM. Our results showed that, in caprine MFGM, these proteins accounted separately for 2.7 and 2.4% of the total proteins. Fong et al. (2007) detected the presence of κ-casein (19 kDa) and β-lactoglobulin (18 kDa), and identified a protein with Mr 17 kDa as proteose peptone 3.

3.2.8. *Fatty-acid-binding protein*

Fatty-acid-binding protein (FABP) is a polypeptide with Mr ~14 kDa (Mather, 2000). Ye et al. (2002) reported that this protein constituted 2–3% of bovine MFGM proteins. In caprine MFGM, it accounted for 3.7% of the total protein (Table 2), with a mid-range coefficient of variation (37%).

3.3. *Correlations*

No statistical correlations between fat content of milk, average size of fat globules and total protein of washed creams were determined. Wiking et al. (2004) found that the average diameter of cow globule milk correlated with fat production; when cows produced a high level of fat, the synthesis of membrane material was limited. However, in goat milks, no correlation was found between fat content and globule size, as reported for bovine milk by Walstra (1969).

Only XO and 47 kDa showed a positive correlation with the amount of fat, with correlation coefficients of 0.461 and 0.522, respectively ($p < 0.05$; Table 4). Mondy and Keenan (1993) found that there was no correlation between fat content of bovine milk and the percentage of XO. Studies with mice in which the XO gene was knocked out suggested that this protein has a crucial role in the secretion of lipid droplets (Vorbach et al., 2002). The expression of XO gene is tightly linked with the secretory activity of the mammary epithelial cells (McManaman et al., 2002). Although human BTN strongly correlates with total level of milk fat (Hamosh et al., 1997), bovine (Mondy and Keenan, 1993) and caprine BTN did not show such correlation.

The size of fat globules was positively correlated with the amount of XO and BTN, with correlation coefficients of 0.520 and 0.482, respectively ($p < 0.05$). Mondy and Keenan

Table 4
Main correlations between protein content (% of MFGM proteins) fat content and average fat globule diameter.

	XO	135	125	115	105	CD36	BTN	PAS6/7	47	38	35	34	21	19	FABP
Fat	0.461*								0.522*						
D(3,2)	0.530						0.482*								
XO		0.574*		0.492*			0.687**		0.564*					0.496*	
135	0.574*		0.868**	0.960**	0.643**		0.755**							0.556*	
125		0.868**		0.540*	0.578**		0.737**			0.486*				0.521*	
115	0.492*	0.660**	0.540*		0.616**		0.521*								
105		0.643**	0.578**	0.616**		0.527*	0.618**								
CD36					0.527*							−0.503*			−0.495*
BTN	0.687**	0.755**	0.737**	0.521*	0.618**					0.560*	0.515*	−0.488*	−0.475*	0.459*	
PAS6/7											−0.467*	0.768**			0.615**
47	0.564*														
38			0.486*				0.560*				0.518*	−0.583**			
35							0.515*	−0.467*		0.518*		−0.722**			0.597**
34						−0.503*	−0.488*	0.768**		−0.583**	−0.722**		0.582**		
21							−0.475*					0.582**			
19	0.496*	0.556*	0.521*				0.459*								0.667**
FABP						−0.496*		0.615**				0.597**		0.667**	

D(3,2): volume-surface average mean diameter; XO: xanthine oxidase; CD36: cluster of differentiation 36; BTN: butyrophilin; PAS 6/7: periodic acid Schiff 6/7; FABP: fatty-acid binding protein.
* $p \leq 0.05$.
** $p \leq 0.01$.

(1993) suggested that the differences in the amount of these proteins observed between two bovine breeds was due to differences in the diameter of fat globules, in other words, their surface area to volume ratio; the breed with smaller globules showed higher amounts of these proteins. If that was the case, the observed correlation in caprine MFGM should have been negative. However, our results are in accordance with those of Ye et al. (2002), who observed that fat globule diameter was largest in early season, when the levels of XO, BTN and total MFGM protein were highest.

The amount of XO and BTN were positively correlated, with a correlation coefficient of 0.687 ($p < 0.01$). Many studies have shown that, although the concentration of these proteins varies within the lactation period, they are present in constant molar proportions of about 1:4 in cow milk (Mondy and Keenan, 1993; Ye et al., 2002). However, the relative proportion of these proteins shows interspecies differences; in the case of human milk, the levels of XO are much higher than those of BTN (Freudenstein et al., 1979). Our results showed a relation of about 1:1 between these two proteins. Although Franke et al. (1981) and Keenan and Heid (1982) did not measure the protein contents, their published SDS-PAGE images of bovine and caprine MFGM confirm that XO:BTN ratio is not 1:4 for goat MFGM. Biochemical studies have proved the existence of a thiol-dependent complex between these two proteins; XO, which is a globular cytoplasmic protein, binds to the cytoplasmic C-terminal of the integral transmembrane protein BTN (Ishii et al., 1995).

Other proteins that may also interact with BTN include PAS III and, in a lesser extent, PAS 6 (Ye et al., 2002). Almost all the bands defined as PAS III correlated with BTN and fewer with XO, with correlation coefficients ranging from 0.492 to 0.755 ($p < 0.05$). However, no correlations with BTN level were found for PAS6/7. There is some evidence that fat globule secretion may be partially regulated by GTP-binding proteins through the interaction with BTN (Keenan, 2002). Some of the bands characterised as GTP-binding proteins correlated with BTN; the observed correlation coefficients between BTN and bands with Mr 38, 35, 34, 21 and 19 were 0.560, 0.515, −0.488, −0.475 and 0.459 ($p < 0.05$). The fact that XO was not found to correlate, does not support the idea that the complex XO-BTN is needed to bind other proteins (Keenan, 2002). BTN is thought to have a major role in the budding of cytoplasmic fat droplets, by acting as an integral receptor (Jack and Mather, 1990).

Spitsberg et al. (1995) demonstrated that, not only there was interaction between CD36 and FABP, but also that these two proteins were coexpressed in the bovine mammary gland epithelial cells during lactation. In a subsequent study, they demonstrated that MFGM-associated FABP was identical to the cytosolic form of the bovine mammary gland tissue, and that its association with the MFGM was through the cytoplasmic domain of CD36 (Spitsberg and Gorewit, 2002) which was in accordance with the results obtained by Rasmussen et al. (1998). However, our results showed the existence of a negative correlation with a coefficient of −0.495 ($p < 0.05$). Both proteins are supposed to be involved in fat transport (Reinhardt and Lippolis, 2006).

As already mentioned, bovine PAS III is resolved in SDS-PAGE as a diffuse band of Mr 95 to over 100 kDa.

Pallesen et al. (2002) isolated and characterised bovine PAS III as a glycoprotein with a calculated molecular mass of 33,317 Da, and an approximately 130 kDa extrapolated from the electrophoretic mobility (Pallesen et al., 2007). In caprine MFGM, the four bands ranging from 105 to 135 kDa, present in all washed creams, were found to be correlated, with coefficients ranging from 0.540 to 0.868 ($p < 0.05$). Altogether, these bands could be attributed to PAS III, although further studies are needed in order to confirm it.

If the band at 60 kDa were an artefact of BTN degradation, they would have been correlated. Proteolysis of BTN would lead to lower amounts of BTN but higher levels of 60 kDa protein, and thus to a negative correlation between these. However, the statistical analysis showed that there was no correlation between them, which supports the fact that the band with Mr 60 kDa should rather be considered as a protein on its own right.

In human milk, both mucin and lactadherin (MUC 1 and PAS 6/7 in bovine milk) correlate very significantly with the levels of phospholipids in milk (Hamosh et al., 1997). Unexpectedly, our results do not show any correlation between the levels of these two proteins in caprine milk. However, it is important to bear in mind that human mucins are larger and more abundant than bovine or caprine MUC 1 (Patton et al., 1995). Working with knock-out mice, no direct role of MUC 1 in milk-fat globule secretion was found (Spicer et al., 1995); in fact, this protein did not correlate with none of the others. However, it should be recalled that this protein stains poorly with Coomassie blue and that it is readily released in the milk serum by cooling (freezing), agitation, and age of the samples (Mather, 2000).

Several positive and negative correlations were found among the proteins with Mr ranging from 50 to 15 kDa. Those with the highest Mr (i.e., 34–44 kDa), identified as GTP-binding proteins, made up a clear correlated pool, with correlation coefficients ranging from −0.722 to 0.830 ($p < 0.01$). As already mentioned, Fong et al. (2007) have recently identified, in bovine MFGM, Ig heavy chain proteins with Mr similar to those we have observed. The fact that some of them show negative correlations suggests that the absence or low content of one of them would be supplemented by others, in order to carry on their biological role, i.e., cell signalling or immune function (Reinhardt and Lippolis, 2006). In addition, Fong et al. (2007) detected contaminating forms of casein with Mr ~25 kDa. In goat's MFGM, a band of 24 kDa was also observed in all samples. Although this protein did not show any correlation with any other protein, it showed a mid-range coefficient of variation. Such results could refute the idea that this band came from some contamination during sample preparation due to fat globule damage.

Fong et al. (2007) also detected contaminating forms of casein with Mr ~25 kDa. A band of 24 kDa, which did not show any correlation with any MFGM protein, was observed in all goats. Such results could suggest that some contamination had happened during sample preparation due to fat globule damage. However, here again, the fact that this protein was present in all goats with a mid-range coefficient of variation supported the idea that this protein came from the MFGM and not from some contamination.

4. Conclusions

Major components of bovine MFGM were also observed in caprine MFGM. However, they showed many differences, i.e., content of XO and minor proteins. Several previously non-described bands were detected. The protein composition of caprine MFGM showed high heterogeneity among individuals. Both the amount and the correlations between proteins could be linked to their physiological role.

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Title: Effect of fat content and homogenisation under conventional or ultra-high-pressure conditions on interactions between proteins in rennet curds

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Abstract: Interactions between proteins within drained rennet curds were studied by measuring the dissociating capacity of different chemical agents as affected by two factors: fat content of milk (0.0, 1.8 or 3.6%) and technological treatment (conventional or ultra-high pressure homogenisation, UHPH). Increasing fat content of raw milk increased levels of unbound whey proteins and calcium-bonded caseins in curds; in contrast, hydrophobic interactions and hydrogen bonds were inhibited. Both homogenisation treatments enhanced the incorporation of unbound whey proteins in the curd, and of caseins through ionic bonds involving calcium salts; however, UHPH increased the amount of unbound caseins. Conventional homogenisation also enhanced interactions between caseins through hydrogen bonds and hydrophobic interactions; in contrast, UHPH impaired hydrogen bonding, and led to the incorporation of both whey proteins and caseins through hydrophobic interactions. Both homogenisation treatments provoked changes in the protein interactions within rennet curds; however, the nature of the changes depended on the homogenisation conditions.

Effect of fat content and homogenisation under conventional or ultra-high-pressure conditions on interactions between proteins in rennet curds

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ABSTRACT

Interactions between proteins within drained rennet curds were studied by measuring the dissociating capacity of different chemical agents as affected by two factors: fat content of milk (0.0, 1.8 or 3.6%) and technological treatment (conventional or ultra-high pressure homogenisation, UHPH). Increasing fat content of raw milk increased levels of unbound whey proteins and calcium-bonded caseins in curds; in contrast, hydrophobic interactions and hydrogen bonds were inhibited. Both homogenisation treatments enhanced the incorporation of unbound whey proteins in the curd, and of caseins through ionic bonds involving calcium salts; however, UHPH increased the amount of unbound caseins. Conventional homogenisation also enhanced interactions between caseins through hydrogen bonds and hydrophobic interactions; in contrast, UHPH impaired hydrogen bonding, and led to the incorporation of both whey proteins and caseins through hydrophobic interactions. Both homogenisation treatments provoked changes in the protein interactions within rennet curds; however, the nature of the changes depended on the homogenisation conditions.

1. Introduction

The enzyme chymosin (the principal active agent in rennet) cleaves κ-casein at the surface of casein micelles, which reduces the net negative charge and steric repulsion between micelles, destabilising them sufficiently to allow coagulation. Rennet-hydrolysed micelles thus become susceptible to aggregation and a three-dimensional gel network is formed (McMahon and Brown, 1984). Fat globules participate to a relatively limited extent in formation of the protein network, being largely physically entrapped therein, but the fat globule membrane may play a structural role in gels (Michalski et al., 2002).

Homogenisation of milk reduces the size of fat globules and increases the moisture content of cheese and cheese yield, due to increased fat recovery (Jana and Upadhyay, 1992; Peters, 1956). However, homogenisation also affects protein structure and causes casein micelles and whey proteins to become associated with the fat globule membrane (Michalski et al., 2002). These interactions between fat and proteins lead to lower curd firmness during rennet coagulation, curd shattering during cutting, and improper curd matting (Green et al., 1983; Jana and Upadhyay, 1992; Peters, 1956).

Ultra-high pressure homogenisation (UHPH) is based on the same operating principle as conventional homogenisation, but current developments in the design of UHPH systems allow pressures of up to 350 MPa to be reached. It has been reported that UHPH also affects the coagulation properties of milk; however, conflicting results have been obtained depending on both the applied pressure and the milk fat content. The coagulation time is reduced for skimmed milk (Lodaite et al., 2009; Sandra and Dalgleish, 2007) and full-fat milk homogenised at ≤ 200 MPa (Zamora et al., 2007).

The rate of curd-firming for full-fat milk UHPH-treated at ≥ 200 MPa is increased, with a concomitant increase of gel strength (Hayes and Kelly, 2003; Zamora et al., 2007). However, rennet-induced gels from UHPH-treated skim milk show a brittle texture (Lodaite et al., 2009).

One strategy for understanding the interactions between proteins within cheese curd involves using different chemical dissociating agents which disrupt specific types of bond or interaction (Lefebvre-Cases et al., 1998). For example, hydrophobic interactions and hydrogen bonds can be disrupted by SDS and urea, respectively, while ionic bonds involving calcium salts are broken by the chelating effect of EDTA. The amount of proteins dissociated by dispersing curds in the presence of the appropriate agent, followed by ultracentrifugation, indicates the action of the agent on the network, and thus the presence and relative importance of specific interactions. Subsequent identification of dissociated proteins by electrophoresis can identify which proteins are involved in each type of interaction.

The aim of the current study was thus to identify the differences in the protein-protein interactions in curds from conventionally homogenised and UHPH-treated milk, with different fat contents, compared to those produced from raw milk.

2. Material and methods

2.1. Milk supply and treatments

Raw whole milk was obtained from a local dairy farm and skimmed at pilot-scale (final fat content of 0.05% ± 0.1 w/v); fat contents were determined using a Milkoscan FT 120 (Foss Electric, Hilerod, Denmark). Skimmed and whole milk were then mixed for standardisation to fat contents of 1.8 ± 0.1% w/v and 3.6 ± 0.1% w/v. Conventional

treatment consisted of two-stage homogenisation at 60°C (15 MPa first stage, 3 MPa second stage; Model APV 1000, APV homogenisers AS, Albertslund, Denmark) followed by batch pasteurisation (63°C for 30 min). UHPH treatment was done by a single-stage process at 300 MPa (nm-Gen 7400 H model, Stansted Fluid Power Ltd., Harlow, UK) at an inlet temperature of 30°C. Milk samples were immediately cooled to 4°C in iced water. Standardised raw milk samples were also collected for further analysis.

2.2. Particle size distribution of milk samples

The particle size distribution in milk samples was determined by light-scattering using a Mastersizer model S (Malvern Instruments, Malvern, UK), equipped with a 3000F (reverse Fourier) as described by Lodaite et al. (2009). The D(4,3) (volume-weighted mean diameter) and D(3,2) (surface-weighted mean diameter) were determined.

2.3. Sample preparation

Milk samples were warmed to 32°C and recombinant chymosin (180 International Milk Clotting Units mL^{-1}, Maxiren 180, DSM Food Specialties, Seclin Cedex, France) was added at 0.074% (v/v). After coagulation at 32°C for 30 min, curds were cut and centrifuged at 1,500 × *g* for 15 min at 20°C (Beckman J2-21 with rotor JA-14, Beckman Instruments France S.A., Gagny, France) and pellets were recovered, after decanting whey, for further analysis.

2.4. Composition analysis of curds

Protein and fat content of drained curds were determined in triplicate using the Kjeldahl method (IDF, 1993) and the van Gulik method (ISO, 1975), respectively. Curds were

analysed in triplicate for total solids (TS; IDF, 2004) to calculate their moisture content (100 − TS) and express protein and fat content on a dry basis. Wet yield of curds, expressed as g curd recovered per 100 g milk, was determined by weighing the curds obtained.

2.5. Dissociation tests

Protein-protein interactions in drained curds were studied following a modification of the method of Lefebvre-Cases et al. (1998). Samples of drained curds (10 g) were dispersed in 40 mL of aqueous dissociating solutions containing either 2 mM EDTA (pH 10.0), 1% (w/v) SDS, or 6 M urea. For a control sample, curds were mixed with MilliQ water. Mixtures of curds and dissociating agent were dispersed for 1 min at 9000 rpm (Ultra-Turrax T 25, Janke and Kunkel, IKA Labortechnik, Staufen, Germany), and the resulting mixtures were ultracentrifuged at 86,000 × *g* for 40 min at 20°C (Beckman Optima LE-80K with rotor Type 50.2 Ti).

Immediately after ultracentrifugation, supernatants were analysed for total protein using a modified version of the method of Lowry et al. (1951). Supernatants were diluted in MilliQ water (25% (v/v) for dissociation in MilliQ water or EDTA or 2.5% (v/v) for dissociation in SDS or urea). Diluted samples (200 µL) were mixed with 2.1 mL of Lowry Reagent D (1:1:98 mixture of 1% (w/v) copper sulphate, 1% (w/v) sodium potassium tartrate, and 2% (w/v) sodium carbonate in 0.1 M NaOH). After 10 min at room temperature, Folin-Ciocalteu's phenol reagent (250 mL of a 1:1 dilution with MilliQ water) was added and colour development was allowed to proceed for 30 min at room temperature. The absorbance at 750 nm was then measured with a spectrophotometer (model Cary 300 Bio, Varian Inc., Palo Alto, CA, USA), and the

protein content quantified using a standard curve of bovine serum albumin standards in the range 0-1 mg protein mL^{-1}. All chemicals were purchased from Sigma-Aldrich Chemie (Steinheim, Germany).

Both qualitative and quantification analysis of the main proteins extracted were carried out by SDS-PAGE analysis under reducing conditions using separating and stacking gels containing 14% or 4% acrylamide, respectively. Supernatants mixed with double-strength reducing buffer (Laemmli, 1970) were loaded onto the gels (20 µL for MilliQ water and EDTA, or 3 µL for SDS and urea) together with bovine serum albumin standard (10 µg) and a wide-range molecular weight marker (M4038, Sigma-Aldrich). Gels were run at 200 V, stained using 0.1% (w/v) Coomassie Brilliant Blue R250 in a 5:1:4 mixture of methanol, acetic acid and distilled water, and destained in two steps with 5:1:4 and 7.5:88 mixtures of methanol, acetic acid and distilled water, respectively. Destained gels were scanned using a calibrated BioRad GS800 densitometer (BioRad Laboratories, Hercules, CA, USA).

2.6. Statistical Analysis

The whole experiment was repeated on three independent occasions. Data were analysed by ANOVA using the GLM procedure of Statgraphics (Statgraphics, Inc., Chicago, IL, USA). LSD test was used for comparison of sample data, and evaluations were based on a significance level of $P < 0.05$.

3. Results and discussion

3.1. Particle size distribution of milk samples

Both homogenisation treatments significantly decreased D(4,3) values compared to raw milk (Table 1); conventional homogenisation resulted in significantly lower values than UHPH and the effect of the former was independent of the level of fat. However, in the case of UHPH, 3.6% fat milk samples had significantly higher values than UHPH-treated milk samples with 0.0% or 1.8% fat.

No significant differences in D(3,2) values were found between 0.0% fat milk samples subjected to different treatments. When fat was present, both homogenisation treatments decreased D(3,2), and UHPH yielded significantly lower values than conventional homogenisation. Conventionally homogenised milk showed a normal distribution of sizes, ranging from 0.05 to 3.0 µm, with a peak at ~0.2 µm. For UHPH-treated milk samples, the observed distributions were bimodal; the principal peak was also at ~0.2 µm, but the particles ranged from 0.05 to 1.0 µm. The smallest particles (<0.1 µm) were more abundant in UHPH-treated milk than conventionally homogenised milk. A second peak of particles, of diameters from 2.0 to 5.0 µm, was observed, but represented less than 5% of the total particles. Such results are broadly in accordance with previous studies (Hayes and Kelly, 2003; Zamora et al., 2007).

3.2. Wet yield, and moisture, fat and protein contents of curds

When fat was present, both homogenisation treatments significantly increased wet curd yield compared to that obtained from raw milk (Table 1). However, the effect of UHPH was greater than that of conventional homogenisation. The increases for 3.6 and 1.8% fat milk samples were 51 and 54% for UHPH-treated milk samples and 19 and 23% for

conventionally homogenised milk samples, respectively. Higher wet yields could be explained by higher moisture content, higher recovery of fat and/or the incorporation of whey proteins into the curds (Zamora et al., 2007).

As milk fat content increased, both homogenisation treatments increased the moisture content of curds, by 2 - 4% for conventionally homogenised milk, and by up to 9% for UHPH-treated milk (Table 1). Impaired whey drainage in curds made from homogenised milk has been previously reported (Ghosh et al., 1994; Green et al., 1983; Humbert et al., 1980; Zamora et al., 2007). The water-holding capacity of curds is directly linked to the microstructure of the gels, i.e., porosity or permeability (Walstra et al., 1985). Native fat globules act as fillers in the interstices of the network (Lopez and Dufour, 2001), thus reducing the volume of water. The effects of homogenisation on the moisture content of the curd have been attributed to the incorporation of denatured whey proteins and the alteration in the protein-fat structure of the curd (Green et al., 1983; Métais et al., 2006; Zamora et al., 2007).

As expected, the recovery of fat in the curd was significantly higher with increasing fat level in milk (Table 1). Conventional homogenisation significantly increased the fat content of curds made from 0.0% and 1.8% fat milk. An increase in fat in dry matter content in cheese made using homogenized milk and cream has been reported (Jana and Upadhyay, 1992; Metzger and Mistry, 1994). In contrast, UHPH decreased the recovery of fat, particularly for 3.6% and 1.8% fat milk. Lanciotti et al. (2006) reported that Caciotta cheeses made from milk high-pressure-homogenised at ≤100 MPa had lower fat content than those made from raw milk.

The protein content of curds on a dry basis did not significantly differ between treatments; increasing the fat content of milk from 0.0% to 3.6% resulted in very large differences in protein content, thus hiding the small effect of the treatments (Table 1). However, both homogenisation treatments decreased the protein content of curds, except in the case of UHPH-treated 3.6% fat milk. UHPH has been proven to reduce the amount of β-LG and, to a lesser extent α-LA, in whey, by incorporation of these proteins into the curd (Zamora et al., 2007).

3.3. Dissociation of proteins from curd

The rates of dissociation of protein from curd depended markedly on the dissociating agent used (Table 2). The amount of protein extracted with MilliQ water, i.e., unbound proteins, was in the range of 8-16% of the protein content of the curds. These results were similar to those reported for rennet gels (Lefebvre-Cases et al., 1998); no differences in the distribution of the stabilising bonds have been found between rennet-induced gels and cheese grains (Hinrichs and Keim, 2007). The main unbound proteins were identified as the whey proteins β-LG and α-LA (Figs. 1 and 2; Table 3); no *para* κ-CN or γ2-/γ3-CN were detected. These results suggest that mechanical disruption of the network on macerating in water released the whey entrapped in the curd. Increasing the milk fat content led to significantly higher proportions of whey proteins released into the supernatant. The presence of native fat globules may increase the amount of solids susceptible to creation of weak interactions that can be mechanically disrupted. When fat was present, both homogenisation treatments increased the dissociation of curd proteins. The homogenisation of fat globules results in a higher number of small particles which would increase the degree of interactions. The proportion of unbound α-LA in curds made from UHPH-treated milk was significantly higher than those from

non-treated and conventionally homogenised milk. As previously mentioned, UHPH increased the moisture content of curds; in other words, more whey entrapped in the curd. To a lesser extent, caseins, i.e., α_s-CN and β-CN, were also affected. This fact could be explained because homogenisation, especially UHPH, may cause partial disintegration of the casein micelles (Roach and Harte, 2008; Sandra and Dalgleish, 2005).

EDTA, as a chelating agent, disrupts ionic bonds of proteins involving calcium salts, but reports on its dissociation capacity in the literature differ significantly. Lefebvre-Cases et al. (1998) obtained 76% dissociation of protein from rennet gels with 2 mM EDTA, but did not refer to pH adjustment when preparing the chemical agent. Gagnaire et al. (2002) subsequently showed that the rate of dissociation varied depending on EDTA concentration at pH 8.0; for 2 mM EDTA, the amount of calcium present in the supernatant was similar to that found without EDTA, and no casein was released. In a recent study, Alexsi et al. (2007) evaluated the interactions in rennet gels obtained from reconstituted skim milk prepared at two different temperatures (25 or 38°C), and reported that the amount of protein dissociated by 2 mM EDTA varied widely; increasing temperature decreased dissociation down to the value obtained without EDTA. The results of the present study showed that the presence of EDTA slightly increased the amount of protein extracted compared to MilliQ water alone (Table 2). The main proteins involved were caseins and their hydrolysis products (Figs. 1 and 2; Table 4). Calcium bonding is thus, to some degree, responsible for casein retention within the curd. Increasing the milk fat content significantly increased the dissociation rates obtained with EDTA. None of the identified proteins seemed to be particularly involved in the increase as, in general, their proportions in the supernatants did not vary

significantly. Independent of the fat content of milk, both homogenisation treatments enhanced the dissociation of curd proteins, i.e., α_s-CN and β-CN. Calcium bonding could be enhanced by a rearrangement of the mineral balance in milk; UHPH at 300 MPa induces a transfer of minerals from the soluble to the micellar fraction (Zamora et al., 2007).

Urea disrupts hydrogen bonds by denaturing proteins through establishing strong hydrogen bonds with polypeptide groups of proteins. In agreement with previous results (Gagnaire et al., 2002; Lefebvre-Cases et al., 1998), a much higher release of protein occurred in the presence of urea (Table 2) than with water or EDTA. The proteins involved were mainly caseins (Figs. 1 and 2; Table 5). However, the levels of dissociation varied depending on the fat content of milk. Increasing milk fat content significantly decreased the amount of protein dissociated by urea, suggesting that caseins are partly linked through hydrogen bonds within the curd, which the presence of fat could inhibit. The proportion of *para* κ-CN in the supernatants significantly decreased with increasing fat content. In model systems, native milk fat globules are considered as non-interacting particles and act as an inert filler (Métais et al., 2006). However, the inclusion of native fat globules in a drained curd would keep casein micelles apart, thus reducing the amount of *para* κ-CN linked through hydrogen bonds. Regardless of milk fat content, conventional homogenisation increased the dissociation of proteins; small fat globules may not perturb the formation of the protein network (Lopez and Dufour, 2001). However, UHPH either had no effect on the extent of dissociation for curds prepared from 1.8% fat milk, while dissociation was reduced for 3.6% fat milk. The incorporation of whey proteins, i.e., β-LG and α-LA, into the curd,

by means of other bonds, e.g., hydrophobic interactions, would lead to greater difficulty in creating hydrogen bonds due to spatial competition.

SDS breaks hydrophobic interactions by inducing intramolecular electrostatic repulsions. In accordance with previous results (Lefebvre-Cases et al., 1998), the use of SDS resulted in almost complete dissociation of the curd (Table 2), with no significant effect of fat level. However, both homogenisation treatments significantly affected the dissociation of protein, and the protein pattern differed depending on both the treatment and the absence or presence of fat (Fig 1; Table 6). In 0.0% fat milk, conventional homogenisation significantly increased the proportion of dissociated caseins, i.e., α_s-CN, β-CN, and κ-CN; for UHPH-treated skim milk, the proteins dissociated were mainly κ-CN and γ_2-/ γ_3-CN. When fat was present, only the proportion of α-LA was significantly increased by conventional homogenisation. In contrast, UHPH significantly increased the level of dissociation of both whey proteins (BSA, β-LG and α-LA), and caseins (α_s-CN and β-CN). Although in early studies on UHPH no denaturation of whey proteins was reported (Hayes and Kelly, 2003; Sandra and Dalgleish, 2005), these results support those obtained in a more recent study (Zamora et al., 2007) that UHPH and, to a lesser extent, conventional homogenisation caused the incorporation of denatured whey proteins into the curd.

4. Conclusions

Increasing fat content of raw milk impaired hydrogen bonding between caseins within drained curds. Both conventional and ultra-high-pressure homogenisation increased the amount of unbound whey proteins and calcium-bonded caseins in curds. There were significant differences in extents of hydrogen bonding and hydrophobic interactions in

curds between the two treatments. Conventional homogenisation enhanced casein-casein interactions through hydrogen bonds and hydrophobic interactions, and, when fat was present, the incorporation of α-LA in the curd through hydrophobic interactions. In contrast, UHPH impaired hydrogen bonding and favoured hydrophobic interactions of whey proteins and caseins.

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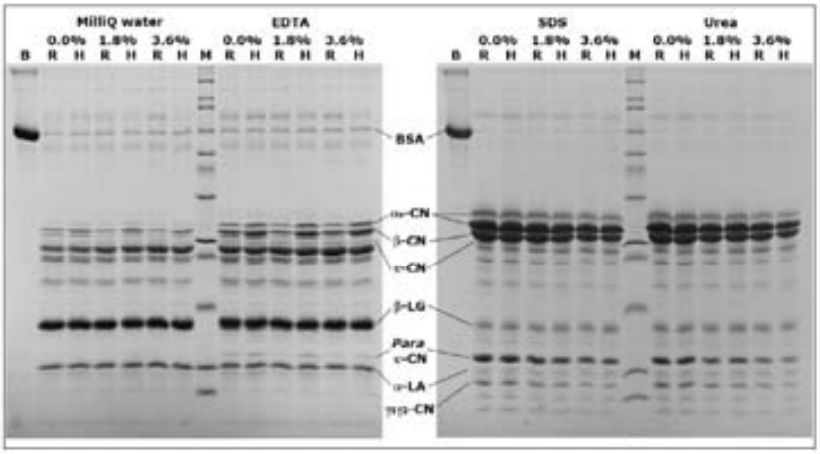
Figure legends

Fig. 1 SDS-PAGE electrophoretograms of supernatants obtained on dissociation of drained curds from conventionally homogenised milk (0.0%, 1.8% and 3.6% represent the milk fat content; R and H indicate raw and conventional homogenisation; B and M are BSA standard and molecular weight markers).

Fig. 2 SDS-PAGE electrophoretograms of supernatants obtained on dissociation of drained curds from UHPH-treated milk (0.0%, 1.8% and 3.6% represent the milk fat content; R and U indicate raw and ultra-high pressure homogenisation; B and M are BSA standard and molecular weight markers).

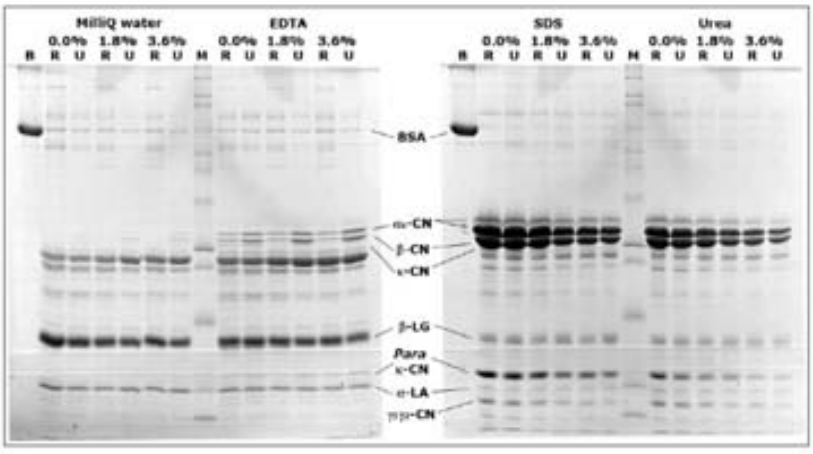
Figure

Figure 1



1

Figure 2



2

Table

Table 1 Volume [D(4,3)] and surface [D(3,2)] weighted mean diameters of particles in milk and wet yield, moisture content, and fat and protein contents on a dry basis of drained curds[a]

Treatment[b]	D(4,3) (μm)	D(3,2) (μm)	Wet yield (g curd 100 g^{-1} milk)	Moisture content (g 100 g^{-1} curd)	Fat content (g 100 g^{-1} TS)	Protein content (g 100 g^{-1} TS)
0.0-R	0.944 ± 0.136 [a]	0.211 ± 0.008 [a,b]	18.38 ± 0.16 [a]	79.38 ± 0.17 [a]	4.41 ± 0.69 [a]	65.47 ± 1.92 [a]
0.0-H	0.139 ± 0.095 [f]	0.212 ± 0.006 [a,b]	21.02 ± 1.06 [a]	80.73 ± 0.21 [c]	7.78 ± 0.79 [f]	59.16 ± 1.83 [b]
0.0-U	0.684 ± 0.058 [a]	0.185 ± 0.002 [f]	21.62 ± 1.18 [a]	81.15 ± 0.35 [c]	4.46 ± 0.05 [a]	60.99 ± 1.00 [b]
1.8-R	3.176 ± 0.063 [b]	0.634 ± 0.011 [b]	25.95 ± 0.37 [d]	77.20 ± 0.19 [f]	32.38 ± 0.70 [d]	43.76 ± 0.88 [c]
1.8-H	0.212 ± 0.023 [f]	0.259 ± 0.002 [e]	31.87 ± 1.52 [c]	79.55 ± 0.34 [d]	35.02 ± 1.31 [e]	40.29 ± 1.18 [d]
1.8-U	0.605 ± 0.022 [a]	0.191 ± 0.002 [a,f]	39.99 ± 1.22 [b]	83.12 ± 0.24 [e]	29.34 ± 0.74 [g]	42.15 ± 0.87 [c,d]
3.6-R	3.614 ± 0.075 [b]	0.913 ± 0.016 [d]	34.57 ± 1.76 [c]	75.83 ± 0.22 [g]	47.61 ± 1.28 [h]	31.83 ± 0.66 [e]
3.6-H	0.302 ± 0.012 [f]	0.310 ± 0.004 [c]	41.15 ± 0.62 [b]	78.56 ± 0.16 [f]	45.78 ± 1.37 [h]	29.59 ± 0.67 [e]
3.6-U	1.277 ± 0.152 [c]	0.220 ± 0.012 [a]	52.32 ± 2.30 [e]	82.26 ± 0.27 [h]	42.55 ± 0.63 [h]	32.01 ± 1.00 [e]

[a-f] Values without common superscripts were significantly different ($P < 0.05$)

[a] Mean value ± standard error; n=6 for R, n=3 for H and U (wet yield) or n=18 for R, n=9 for H and U (others).

[b] 0.0, 1.8 and 3.6 represents the milk fat content; R stands for raw, H for conventional homogenization and U for ultra-high pressure homogenization.

1

Table 2 Effect of dissociating agent on protein content of supernatants (g supernatant protein 100 g^{-1} curd protein)[a]

Treatment[b]	MilliQ water	EDTA	Urea	SDS
0.0-R	8.00 ± 0.37 [f]	8.74 ± 0.16 [e]	81.52 ± 2.82 [b]	98.53 ± 4.25 [c,d]
0.0-H	9.39 ± 0.24 [e]	11.36 ± 0.27 [c]	87.70 ± 1.41 [a]	104.13 ± 1.22 [b,c,d]
0.0-U	8.22 ± 0.24 [f]	10.16 ± 0.27 [d]	83.29 ± 1.89 [a,b]	116.57 ± 4.18 [a]
1.8-R	9.70 ± 0.16 [e]	11.64 ± 0.18 [c]	69.11 ± 1.12 [c,d]	97.08 ± 1.40 [d]
1.8-H	12.54 ± 0.60 [c,d]	15.38 ± 0.77 [b]	86.55 ± 2.75 [a,b]	109.12 ± 5.32 [a,b]
1.8-U	11.71 ± 0.25 [d]	15.77 ± 0.34 [b]	68.15 ± 1.14 [c,d]	106.08 ± 3.17 [b,c]
3.6-R	12.61 ± 0.35 [c]	15.75 ± 0.33 [b]	65.96 ± 2.27 [d]	99.02 ± 2.33 [c,d]
3.6-H	16.28 ± 0.37 [a]	19.58 ± 0.69 [a]	73.41 ± 1.60 [c]	107.55 ± 1.96 [a,b]
3.6-U	14.26 ± 0.30 [b]	19.50 ± 0.87 [a]	49.25 ± 2.11 [e]	83.03 ± 2.18 [e]

[a-f] Values without common superscripts were significantly different ($P < 0.05$)

[a] Mean value ± standard error; n=18 for R, n=9 for H and U.

[b] 0.0, 1.8 and 3.6 represent the milk fat content; R stands for raw, H for conventional homogenization and U for ultra-high pressure homogenization.

2

Table 3 Levels of proteins in supernatants of curds dissociated by MilliQ water (g dissociated protein 100 g^{-1} supernatant protein)[a]

Treatment[b]	BSA	α_s-CN	β-CN	κ-CN	β-LG	*Para* κ-CN	α-LA	γ_2- and γ_3-CN
0.0-R	1.29 ± 0.09 ^{b}	0.94 ± 0.08 ^{c}	1.33 ± 0.27 ^{b,c}	1.10 ± 0.11 ^{a}	19.83 ± 2.10 ^{c}	-	7.25 ± 0.89 ^{g}	-
0.0-H	1.30 ± 0.06 ^{b}	1.13 ± 0.14 ^{b,c}	1.72 ± 0.87 ^{a,b,c}	1.34 ± 0.10 ^{b,c,d}	20.90 ± 2.16 ^{c}	-	7.45 ± 0.65 ^{g}	-
0.0-U	1.36 ± 0.06 ^{a,b}	1.14 ± 0.13 ^{b,c}	1.67 ± 0.29 ^{a,b,c}	1.23 ± 0.10 ^{a,b,c}	23.24 ± 4.21 ^{b}	-	8.24 ± 1.55 ^{b,c,d}	-
1.8-R	1.47 ± 0.06 ^{b}	1.01 ± 0.10 ^{c}	1.27 ± 0.23 ^{b,c}	1.29 ± 0.10 ^{c,d}	23.47 ± 1.82 ^{b}	-	8.20 ± 0.52 ^{c,d}	-
1.8-H	1.45 ± 0.07 ^{a,b}	1.18 ± 0.14 ^{b,c}	1.33 ± 1.37 ^{a}	1.44 ± 0.10 ^{a,b,c}	23.82 ± 2.69 ^{a,b}	-	9.39 ± 1.15 ^{b,c}	-
1.8-U	1.50 ± 0.11 ^{a}	1.40 ± 0.14 ^{a,b}	1.35 ± 0.37 ^{a}	1.52 ± 0.14 ^{a}	24.47 ± 4.19 ^{a,b}	-	11.36 ± 1.47 ^{a}	-
3.6-R	1.52 ± 0.07 ^{a}	1.06 ± 0.10 ^{c}	1.24 ± 0.20 ^{c}	1.40 ± 0.12 ^{a,b,c}	24.36 ± 2.24 ^{a,b}	-	8.75 ± 0.68 ^{b,c}	-
3.6-H	1.50 ± 0.12 ^{a}	1.14 ± 0.14 ^{b,c}	2.03 ± 1.10 ^{a,b}	1.36 ± 0.14 ^{a,b,c,d}	23.27 ± 2.75 ^{b}	-	9.45 ± 1.68 ^{b}	-
3.6-U	1.50 ± 0.04 ^{a}	1.52 ± 0.25 ^{a}	1.90 ± 0.28 ^{a,b,c}	1.50 ± 0.05 ^{a,b}	25.15 ± 4.29 ^{a}	-	11.36 ± 1.24 ^{a}	-

[**] Values without common superscripts were significantly different ($P < 0.05$).

[a] Mean value ± standard error; n=6 for R, n=3 for H and U.

[b] 0.0, 1.8 and 3.6 represent the milk fat content; R stands for raw, H for conventional homogenization and U for ultra-high pressure homogenization.

3

Table 4 Levels of proteins in supernatants of curds dissociated by EDTA (g dissociated protein 100 g^{-1} supernatant protein)[a]

Treatment[b]	BSA	α_s-CN	β-CN	κ-CN	β-LG	*Para* κ-CN	α-LA	γ_2- and γ_3-CN
0.0-R	1.16 ± 0.08 ^{b}	3.15 ± 0.73 ^{c,d}	5.24 ± 1.74 ^{b,c}	1.45 ± 0.08 ^{a}	18.01 ± 1.74 ^{b,c}	1.25 ± 0.06 ^{a}	6.80 ± 0.70 ^{a,b}	-
0.0-H	1.24 ± 0.12 ^{a,b}	4.14 ± 1.05 ^{b,c}	6.02 ± 3.66 ^{b}	1.50 ± 0.14 ^{a}	17.74 ± 1.92 ^{c,d}	1.39 ± 0.12 ^{a}	6.52 ± 0.64 ^{a,b,c}	-
0.0-U	1.26 ± 0.04 ^{a,b}	4.23 ± 0.70 ^{b,c}	6.76 ± 1.24 ^{b}	1.62 ± 0.14 ^{a}	19.77 ± 3.38 ^{a}	1.32 ± 0.14 ^{a}	7.30 ± 1.35 ^{a}	-
1.8-R	1.31 ± 0.07 ^{a}	2.53 ± 0.65 ^{d}	3.66 ± 1.37 ^{c,d}	1.50 ± 0.12 ^{a}	19.02 ± 1.65 ^{a,b}	1.05 ± 0.10 ^{b,c}	6.33 ± 0.59 ^{b,c}	-
1.8-H	1.22 ± 0.08 ^{a,b}	4.56 ± 0.71 ^{b}	6.26 ± 3.68 ^{b}	1.58 ± 0.18 ^{a}	17.41 ± 2.69 ^{c,d}	1.30 ± 0.09 ^{a}	5.90 ± 0.90 ^{b,c,d}	-
1.8-U	1.23 ± 0.03 ^{a,b}	8.43 ± 1.38 ^{a}	11.15 ± 1.97 ^{a}	1.53 ± 0.12 ^{a}	17.58 ± 3.04 ^{c,d}	1.22 ± 0.16 ^{a,b}	6.77 ± 1.32 ^{a,b}	-
3.6-R	1.22 ± 0.06 ^{a,b}	2.82 ± 0.47 ^{d}	2.85 ± 1.14 ^{d}	1.49 ± 0.11 ^{a}	16.94 ± 1.48 ^{d}	0.95 ± 0.07 ^{c}	5.07 ± 0.54 ^{d}	-
3.6-H	1.24 ± 0.11 ^{a,b}	4.37 ± 1.25 ^{b}	5.48 ± 3.24 ^{b,c}	1.53 ± 0.18 ^{a}	15.31 ± 1.57 ^{a}	1.05 ± 0.07 ^{b,c}	4.06 ± 0.50 ^{a}	-
3.6-U	1.14 ± 0.05 ^{b}	7.38 ± 1.01 ^{a}	10.31 ± 1.31 ^{a}	1.40 ± 0.13 ^{a}	14.59 ± 2.52 ^{a}	1.23 ± 0.16 ^{a}	5.44 ± 1.00 ^{c,d}	-

[**] Values without common superscripts were significantly different ($P < 0.05$).

[a] Mean value ± standard error; n=6 for R, n=3 for H and U.

[b] 0.0, 1.8 and 3.6 represent the milk fat content; R stands for raw, H for conventional homogenization and U for ultra-high pressure homogenization.

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Table 5 Levels of proteins in supernatants of curds dissociated by urea (g dissociated protein 100 g^{-1} supernatant protein)[a]

Treatment[b]	BSA	α_s-CN	β-CN	κ-CN	β-LG	*Para* κ-CN	α-LA	γ_2- and γ_3-CN
0.0-R	0.66 ± 0.07 ^{f}	25.98 ± 1.91 ^{g}	37.63 ± 2.20 ^{a}	3.39 ± 0.26 ^{a}	1.97 ± 0.20 ^{g}	16.08 ± 1.42 ^{a}	1.24 ± 0.08 ^{a}	4.43 ± 0.67 ^{a}
0.0-H	0.79 ± 0.10 ^{e,f}	27.23 ± 2.58 ^{e,f}	36.89 ± 2.62 ^{a}	2.43 ± 0.54 ^{c}	1.83 ± 0.19 ^{g}	14.93 ± 1.13 ^{a,b}	1.29 ± 0.15 ^{a,b}	3.15 ± 0.43 ^{b,c}
0.0-U	0.74 ± 0.10 ^{f}	26.63 ± 1.86 ^{e,f}	38.90 ± 1.45 ^{a}	2.53 ± 0.18 ^{b,c}	1.97 ± 0.24 ^{g}	12.23 ± 1.81 ^{b,c}	1.16 ± 0.11 ^{a}	3.46 ± 0.12 ^{a,b}
1.8-R	0.97 ± 0.07 ^{d,e}	31.41 ± 2.04 ^{d}	47.24 ± 4.16 ^{c,d}	2.15 ± 0.12 ^{c}	2.30 ± 0.15 ^{d}	8.94 ± 1.02 ^{d}	1.67 ± 0.16 ^{c,d}	2.31 ± 0.16 ^{c}
1.8-H	1.03 ± 0.18 ^{d}	30.86 ± 3.88 ^{d,e}	44.11 ± 4.35 ^{c,d,e}	1.99 ± 0.16 ^{c}	2.38 ± 0.36 ^{c,d}	10.71 ± 1.33 ^{c,d}	1.57 ± 0.22 ^{c,d,e}	2.11 ± 0.29 ^{c}
1.8-U	1.58 ± 0.08 ^{b}	41.04 ± 1.96 ^{b}	54.47 ± 3.60 ^{a,b}	2.63 ± 0.17 ^{b,c}	4.29 ± 0.85 ^{b}	10.35 ± 0.71 ^{c,d}	2.20 ± 0.21 ^{b}	2.57 ± 0.18 ^{b,c}
3.6-R	1.33 ± 0.10 ^{c}	37.04 ± 2.13 ^{c}	49.90 ± 2.68 ^{b,c}	2.51 ± 0.14 ^{c}	3.08 ± 0.22 ^{b}	4.79 ± 0.70 ^{e}	2.00 ± 0.12 ^{b,c}	2.57 ± 0.14 ^{b,c}
3.6-H	1.35 ± 0.22 ^{b,c}	33.67 ± 4.87 ^{c,d}	42.14 ± 3.53 ^{d,e}	2.11 ± 0.31 ^{c}	2.96 ± 0.37 ^{b,c}	3.20 ± 0.41 ^{e}	2.05 ± 0.32 ^{b,c}	2.20 ± 0.39 ^{c}
3.6-U	2.49 ± 0.12 ^{a}	49.85 ± 3.42 ^{a}	57.25 ± 5.39 ^{a}	3.32 ± 0.17 ^{a,b}	4.83 ± 0.66 ^{a}	4.01 ± 0.38 ^{e}	3.35 ± 0.44 ^{a}	3.54 ± 0.17 ^{a,b}

$^{a-g}$ Values without common superscripts were significantly different ($P < 0.05$).

[a] Mean value ± standard error; n=6 for R, n=3 for H and U.

[b] 0.0, 1.8 and 3.6 represent the milk fat content; R stands for raw, H for conventional homogenization and U for ultra-high pressure homogenization.

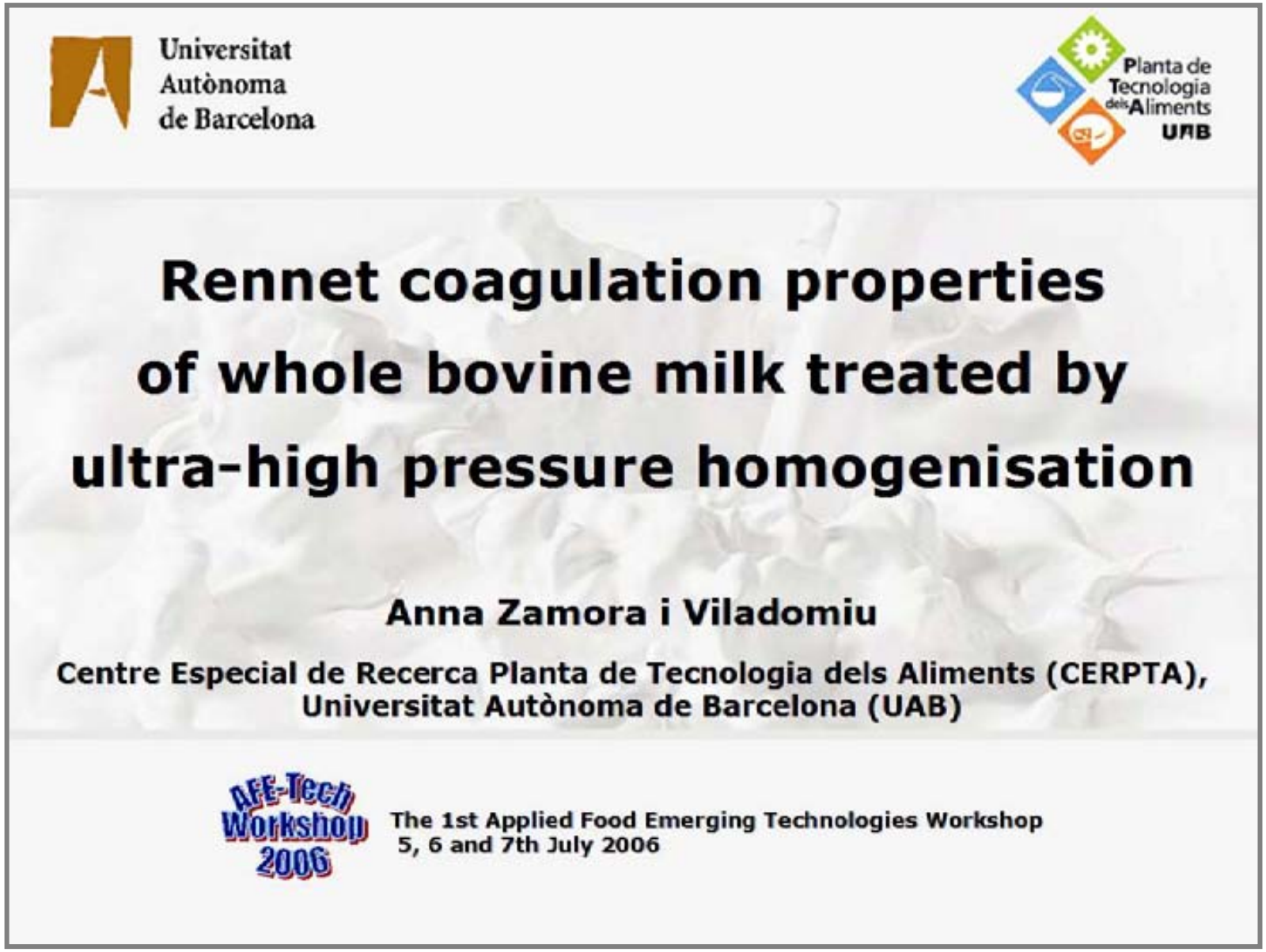
Table 6 Levels of proteins in supernatants of curds dissociated by SDS (g dissociated protein 100 g^{-1} supernatant protein)[a]

Treatment[b]	BSA	α_s-CN	β-CN	κ-CN	β-LG	*Para* κ-CN	α-LA	γ_2- and γ_3-CN
0.0-R	0.54 ± 0.03 ^{f}	22.80 ± 1.61 ^{g}	27.24 ± 1.23 ^{g}	2.71 ± 0.32 ^{b}	1.51 ± 0.11 ^{e}	14.12 ± 1.01 ^{a,b}	0.91 ± 0.07 ^{e}	2.05 ± 0.25 ^{b,c,d}
0.0-H	0.66 ± 0.10 ^{e,f}	26.05 ± 2.01 ^{e}	34.29 ± 2.38 ^{d,e}	2.80 ± 0.28 ^{b}	1.76 ± 0.17 ^{d,e}	16.72 ± 0.41 ^{a}	1.06 ± 0.10 ^{d,e}	2.50 ± 0.40 ^{b}
0.0-U	0.58 ± 0.06 ^{f}	22.25 ± 0.86 ^{g}	30.56 ± 0.85 ^{e,f}	3.58 ± 0.50 ^{a}	2.14 ± 0.19 ^{c,d,e}	12.90 ± 0.29 ^{b}	0.87 ± 0.07 ^{e}	3.08 ± 0.52 ^{a}
1.8-R	0.73 ± 0.05 ^{d,e}	26.91 ± 1.25 ^{e}	36.74 ± 1.31 ^{c,d}	2.14 ± 0.36 ^{c}	1.75 ± 0.12 ^{d,e}	14.08 ± 0.92 ^{a,b}	1.11 ± 0.07 ^{d}	1.77 ± 0.27 ^{c,d}
1.8-H	0.83 ± 0.18 ^{c,d}	29.13 ± 2.08 ^{b,c}	39.73 ± 2.30 ^{b,c}	2.21 ± 0.11 ^{b,c}	2.16 ± 0.18 ^{c,d,e}	15.41 ± 1.05 ^{a,b}	1.33 ± 0.11 ^{c}	2.08 ± 0.14 ^{b,c,d}
1.8-U	1.08 ± 0.09 ^{b}	31.03 ± 1.72 ^{b}	42.99 ± 2.26 ^{b}	1.96 ± 0.23 ^{c}	4.63 ± 0.74 ^{b}	13.41 ± 0.36 ^{a,b}	1.44 ± 0.14 ^{c}	1.66 ± 0.19 ^{d}
3.6-R	0.98 ± 0.08 ^{b,c}	31.39 ± 1.61 ^{b}	42.82 ± 2.77 ^{b}	2.20 ± 0.12 ^{c}	2.29 ± 0.23 ^{c,d}	13.77 ± 1.62 ^{a,b}	1.42 ± 0.12 ^{c}	1.79 ± 0.15 ^{c,d}
3.6-H	1.10 ± 0.19 ^{b}	32.23 ± 3.11 ^{b}	41.30 ± 3.41 ^{b}	2.35 ± 0.08 ^{b,c}	2.79 ± 0.29 ^{c}	13.62 ± 1.65 ^{a,b}	1.69 ± 0.25 ^{b}	2.08 ± 0.32 ^{b,c,d}
3.6-U	1.66 ± 0.14 ^{a}	40.09 ± 1.57 ^{a}	52.69 ± 3.23 ^{a}	2.36 ± 0.22 ^{b,c}	5.96 ± 1.06 ^{a}	12.65 ± 1.86 ^{b}	2.34 ± 0.05 ^{a}	2.20 ± 0.11 ^{b,c}

$^{a-g}$ Values without common superscripts were significantly different ($P < 0.05$).

[a] Mean value ± standard error; n=6 for R, n=3 for H and U.

[b] 0.0, 1.8 and 3.6 represent the milk fat content; R stands for raw, H for conventional homogenization and U for ultra-high pressure homogenization.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Rennet coagulation properties
of whole bovine milk treated by
ultra-high pressure homogenisation
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RENNET COAGULATION PROPERTIES OF WHOLE BOVINE MILK TREATED BY ULTRA-HIGH PRESSURE HOMOGENISATION

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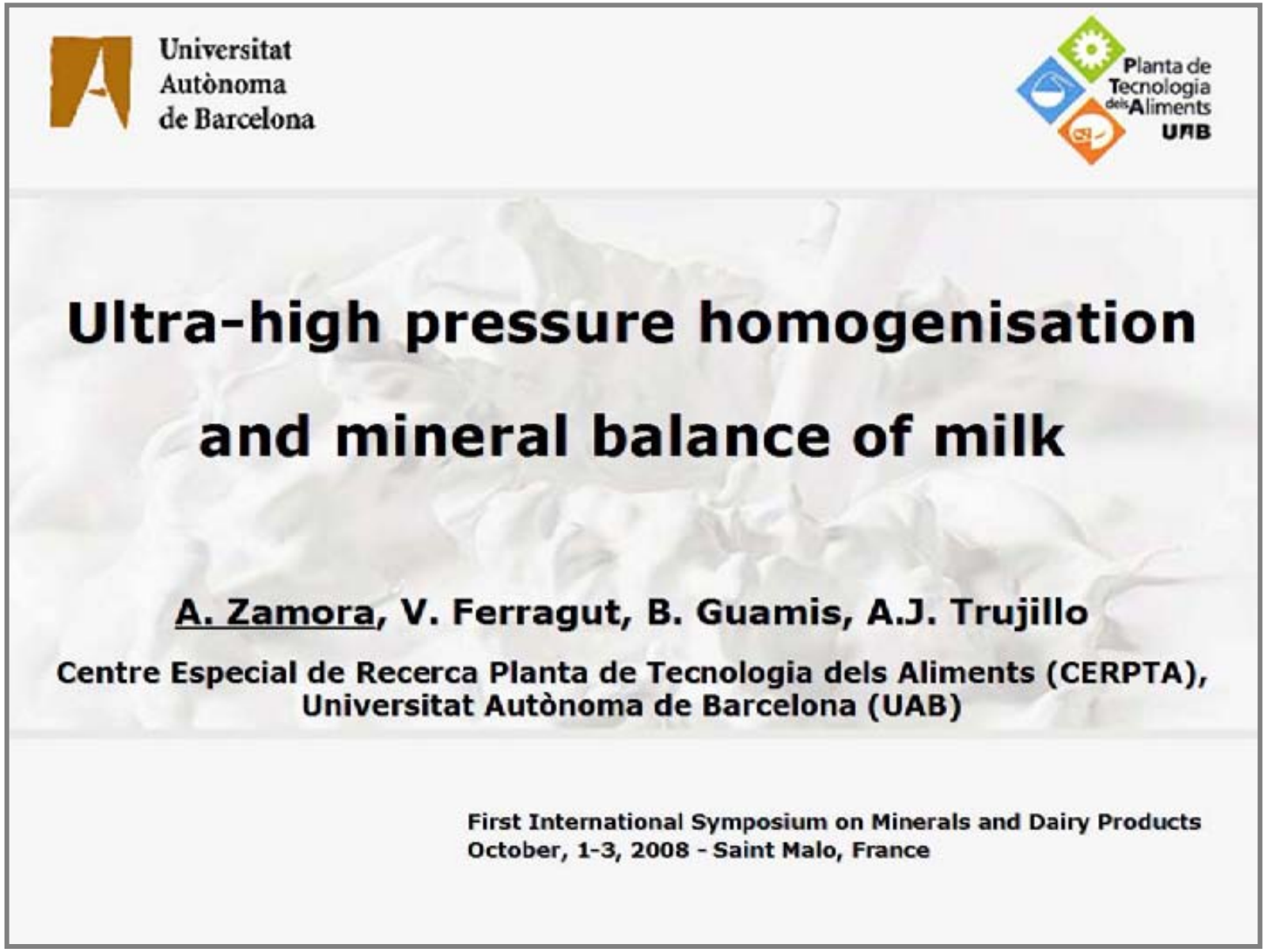
The aim of the present work was to determine the effect of Ultra-High Pressure Homogenisation (UHPH) on the rennet coagulation properties of whole bovine milk by subjecting it to single or two-stage UHPH (100, 200 and 300 MPa on the primary valve and 30 MPa on the secondary valve) using a Stansted high pressure homogeniser (model FPG11300, Stansted Fluid Power Ltd., Essex, UK) at an inlet temperature of 30ºC. Studied coagulation properties were rennet clotting time (RCT), rate of curd firming (RCF) and curd firmness (CF). Rennet gels were observed by confocal laser scanning microscopy. The results obtained from UHPH treatments were compared to those of raw, conventionally pasteurised (72ºC, 15 s) and homogenised-pasteurised (18 MPa, 60ºC; 72ºC, 15 s) milks. The range of milk temperature during UHPH treatments was 54-94ºC, with an outlet temperature below 35ºC. RCT strongly depended on the treatment, although the secondary stage of UHPH did not seem to affect it. Coagulation occurred earlier in UHPH-treated milks at 100-130 MPa. Highest RCTs were observed for raw, pasteurised and UHPH-treated milk at 300-330 MPa. The results of RCF showed a similar pattern to those of CF. Strongest gels were obtained with UHPH at 200 and 300 MPa treated milks. However, two-stage UHPH significantly diminished both RCF and CF. Micrographs revealed that rennet gels from UHPH-treated milk at 100 MPa were similar to those from homogenised-pasteurised milk. They showed open matrices with large and irregular serum pores delimited by thick and lumpy strands. Small fat globules were embedded within the proteinaceous network. Rennet gels from single-stage UHPH-treated milk at 200 and 300 MPa were much tighter. Very small fat globules were strongly associated to the protein network, which was formed by much thinner strands. In conclusion, UHPH at 200 MPa enhanced rennet coagulation properties of whole milk suggesting possible applications of UHPH technology on milk for cheese manufacture.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Ultra-high pressure homogenisation
and mineral balance of milk
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Oral presentation – Session 3 - Minerals as a function of physico-chemical conditions and technological treatments

Ultra-high pressure homogenisation and mineral balance of milk

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The aim of the present work was to determine the effect of Ultra-High Pressure Homogenisation (UHPH) on the mineral balance of milk by subjecting it to single or two-stage UHPH (100, 200 and 300 MPa on the primary valve and 30 MPa on the secondary, expressed as 130, 230 and 330 MPa) using a Stansted high pressure homogeniser (model FPG11300, Stansted Fluid Power Ltd., Essex, UK) at inlet temperatures (T_i) of 30 and 40°C. The amount of minerals (Ca, P and Mg) in whey obtained by centrifugation of rennet coagulated gels was determined in triplicate by inductively coupled plasma optical emission spectroscopy with a Perkin-Elmer unit (model 4300, Perkin-Elmer, Shelton, CT). The particle size distribution in milk samples was determined using a Beckman Coulter laser diffraction particle size analyser. Microstructure of milks was observed by transmission electron microscopy (TEM). Mineral equilibrium in milk is very dependent on physicochemical parameters such as pH and temperature. As expected, the increase of T_i and pressure led to significantly higher temperatures during UHPH treatments ($p<0.05$). Both factors also affected the pH of milks. UHPH treatments at 100-130 MPa (30 and 40°C T_i) and 200-230 MPa (30°C T_i) decreased the pH of milks. Although milk temperature during UHPH treatment at 300 MPa (40°C T_i) reached 100°C, no decrease of the pH was observed. Considering the amount of minerals, whey from milks UHPH-treated at 100-130 MPa (30°C T_i) showed significantly higher amount of Ca and P than whey from raw milk. The decrease in pH, which could be attributed to the action of residual indigenous lipoprotein lipase, leads to the transfer of calcium phosphate from the micellar to the diffusible fraction. However, for Mg no differences where observed. Such results are in accordance with the idea that this ion is associated with the surface rather than the bulk of the calcium phosphate microgranules. On the contrary, whey from milk UHPH-treated at 300-330 MPa showed significantly lower amounts of minerals than whey from raw milk. At first, this could be attributed to the increase of temperature during UHPH treatment. However, the use of the secondary valve (330 MPa, 40°C T_i) leaded to a higher transfer of minerals from the diffusible to the micellar fraction; but lower temperatures were reached during treatment compared with single stage at 300 MPa. UHPH partly dissociates casein micelles and disrupts fat globules. As observed by TEM, the increase in interfacial fat surface causes the adsorption of casein micelle fragments to the newly formed fat globules. Above 200 MPa, the collision of particles leads to coalescence and aggregation observed as a broadening of the particle size distributions. These phenomena were more evident at 330 MPa. The disruption of casein micelles followed by the adsorption of fragments onto the membrane of fat globules could result in a decrease of mineral solubility by increasing the number and/or the size of microgranules. Treatments at 200-230 MPa, as intermediate conditions, corroborated the above mentioned.

In conclusion, the effect of UHPH on the mineral balance of milk results to be more linked to the homogenisation effect than to the heat effect.

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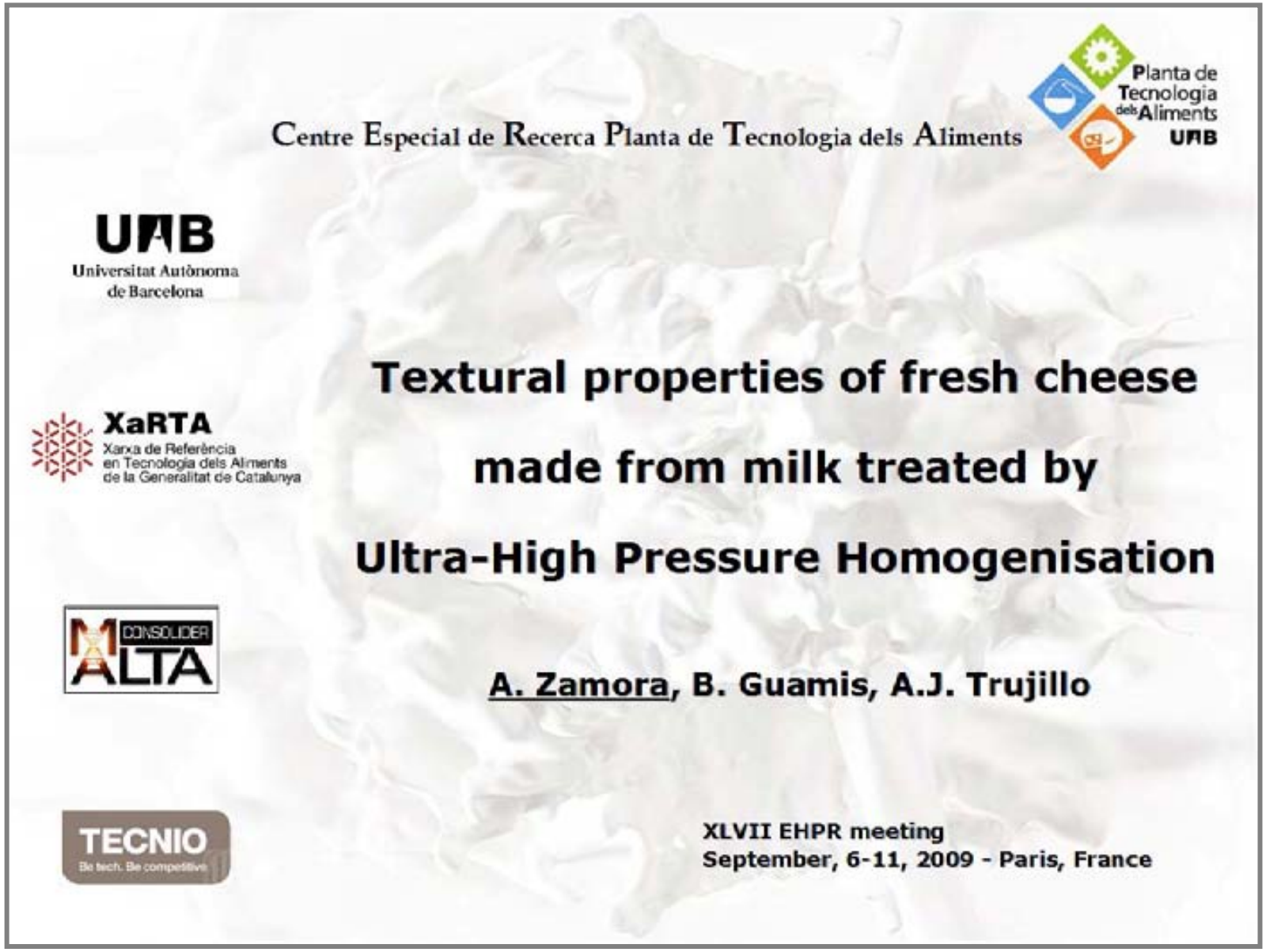
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Textural properties of fresh cheese made from milk treated by Ultra-High Pressure Homogenisation
A. Zamora, B. Guamis, A.J. Trujillo
XLVII EHPR meeting
September, 6-11, 2009 - Paris, France

Textural properties of fresh cheese made from milk treated by Ultra-High Pressure Homogenisation

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The aim of the study was to evaluate the effect of homogenising milk with Ultra-High Pressure Homogenisation (UHPH) on the textural properties of a starter-free fresh cheese. UHPH (300 MPa at 30ºC) significantly reduced the size of milk particles and improved the coagulation properties by reducing rennet clotting time and increasing optical density of curds compared to pasteurisation (PA; 80ºC for 15 s) and homogenisation-pasteurisation (HP; 18 MPa at 60ºC, 80ºC for 15 s). A sensory panel described UHPH-cheeses as crumbly, grainy and hard. Textural analysis showed that both the Young's modulus and the stress at fracture of UHPH-cheeses were much higher. However, the strain at fracture of UHPH-cheeses was similar to those of HP-cheeses. In addition, G* of the rheological analysis significantly differed; UHPH-cheeses presented the highest G* followed by HP- and PA-cheeses. Concerning the water content of cheeses, the sensory panel found UHPH-cheeses much drier than HP-cheeses. However, UHPH-cheeses showed slightly higher moisture content compared to HP-cheeses. Both homogenisations, especially UHPH, triggered a change in the typology of water retained by decreasing the amount of water type II. The temperature needed to change from one type to the other was significantly higher in UHPH-cheeses. Forced whey drainage through centrifugation showed that the water-holding capacity of UHPH-cheeses was significantly higher to that of HP- and PA-cheeses. Confocal images revealed that matrices of UHPH-cheeses were tighter with smaller interfacial spaces, thus water would be tightly entrapped in the protein network. In conclusion, UHPH triggered textural changes which were detected by sensory evaluation. Instrumental analysis helped to understand that two phenomena were at the base of the changes: the drastic decrease in size of milk particles would lead to harder and less elastic cheeses, and the change in the water typology and water-binding capacity of the cheese matrix would lead to a drier mouth-feeling.

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Keywords: Ultra-high pressure homogenisation; cheese; microstructure

Effects of ultra-high pressure homogenisation on the rennet coagulation properties of milk

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Introduction

Ultra-high pressure homogenisation is based on the same principle that of conventional ball-and-seat homogenisers, but current developments in the design allow to reach pressures of 350 MPa. There has been an increasing interest in the application of UHPH in food technology.

Objective

To determine the effect of UHPH treatment on the rennet coagulation properties of whole bovine milk by comparing this new technology with conventional pasteurisation and homogenisation-pasteurisation treatments.

Material and Methods

Raw whole milk standardised at 3.5±0.2% fat was subjected to:

- **Single or two-stage UHPH:** 100, 200, 300 MPa primary valve and 30 MPa secondary valve; Stansted high pressure homogeniser (model FPG11300, Stansted Fluid Power Ltd., Essex, UK); inlet temperature of 30±1ºC.
- **Conventional pasteurisation** (72ºC, 15 s) and **homogenisation-pasteurisation** (15+3 MPa, 60ºC; 72ºC, 15 s).

Rennet coagulation properties: rennet coagulation time (RCT), rate of curd firming (RCF) and curd firmness at 30 min (CF)) assessed by the Optigraph® system (Ysebaert Inc, Frepillon, France).

Confocal laser scanning microscopy of rennet gels: in fluorescence mode as described by Michalski et al. (2002).

Results

- UHPH-treatments at 100-130 MPa: lowest RCT, RCF and CF; gels similar to homogenised-pasteurised milk, opened protein matrices, thick and lumpy strands with embedded small fat globules.
- Single-stage UHPH above 200 MPa: highest RCFs and CFs; RCTs similar to those of control milks; tightest protein matrices with thin strands and associated very small fat globules.
- Two-stage UHPH above 200 MPa compared to single-stage: similar RCTs; significantly lower RCFs and CFs; laxer protein matrices with bigger fat globules (coalescence).

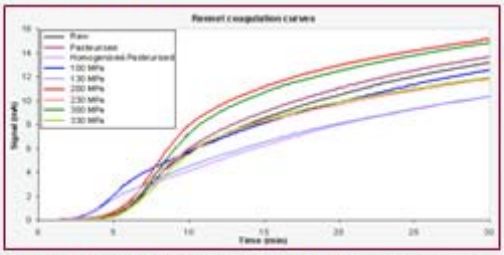

Figure 1. Coagulation curves of raw, pasteurised, homogenised-pasteurised and UHPH-treated milks (two-stage homogenisation: 130, 230 and 330 MPa).

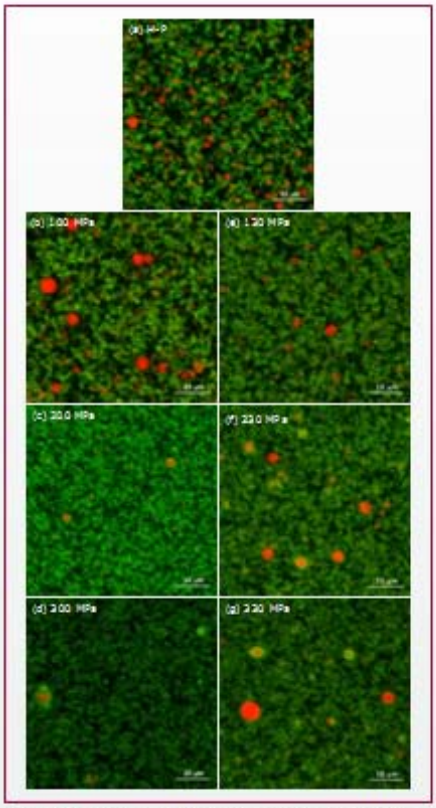

Figure 2. Confocal laser scanning micrographs (fat-Nile Red in red; proteins-FITC in green) of rennet curds from: (a) homogenised-pasteurised milk; (b, c, d) single-stage and (e, f, g) two-stage UHPH-treated milks.

Conclusions

Whole milk with an inlet temperature of 30ºC treated by single-stage UHPH at 200 MPa showed superior rennet coagulation properties, which could be attributed to the microstructure of the curd. The results suggest that UHPH could enhance the cheese-making properties of milk.

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RENNET COAGULATION PROPERTIES OF WHOLE BOVINE MILK TREATED BY ULTRA-HIGH PRESSURE HOMOGENISATION

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The aim of the present work was to determine the effect of Ultra-High Pressure Homogenisation (UHPH) on the rennet coagulation properties of whole bovine milk by subjecting it to single or two-stage UHPH (100, 200 and 300 MPa on the primary valve and 30 MPa on the secondary valve) using a Stansted high pressure homogeniser (model FPG11300, Stansted Fluid Power Ltd., Essex, UK) at an inlet temperature of 30ºC. Studied coagulation properties were rennet clotting time (RCT), rate of curd firming (RCF) and curd firmness (CF). Rennet gels were observed by confocal laser scanning microscopy. The results obtained from UHPH treatments were compared to those of raw, conventionally pasteurised (72ºC, 15 s) and homogenised-pasteurised (18 MPa, 60ºC; 72ºC, 15 s) milks. The range of milk temperature during UHPH treatments was 54-94ºC, with an outlet temperature below 35ºC. RCT strongly depended on the treatment, although the secondary stage of UHPH did not seem to affect it. Coagulation occurred earlier in UHPH-treated milks at 100-130 MPa. Highest RCTs were observed for raw, pasteurised and UHPH-treated milk at 300-330 MPa. The results of RCF showed a similar pattern to those of CF. Strongest gels were obtained with UHPH at 200 and 300 MPa treated milks. However, two-stage UHPH significantly diminished both RCF and CF. Micrographs revealed that rennet gels from UHPH-treated milk at 100 MPa were similar to those from homogenised-pasteurised milk. They showed open matrices with large and irregular serum pores delimited by thick and lumpy strands. Small fat globules were embedded within the proteinaceous network. Rennet gels from single-stage UHPH-treated milk at 200 and 300 MPa were much tighter. Very small fat globules were strongly associated to the protein network, which was formed by much thinner strands. In conclusion, UHPH at 200 MPa enhanced rennet coagulation properties of whole milk suggesting possible applications of UHPH technology on milk for cheese manufacture.

Effects of Ultra-High Pressure Homogenisation on the Rennet Coagulation Properties of Milk

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INTRODUCTION

Ultra-high pressure homogenisation (UHPH) is based on the same principle that of conventional ball-and-seat homogenisers, but current developments in the design allow to reach pressures of 350 MPa. There has been an increasing interest in the application of UHPH in food technology.

AIM: *To determine the effect of UHPH treatment on the rennet coagulation properties of whole bovine milk by comparing this new technology with conventional pasteurisation and homogenisation-pasteurisation treatments.*

MATERIALS & METHODS

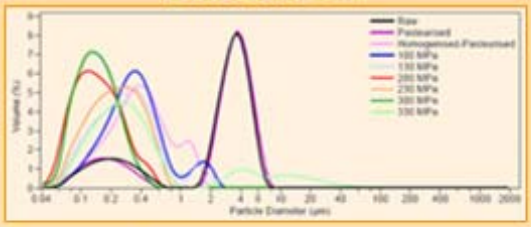
Raw whole milk standardised at 3.5±0.2% fat was subjected to:

- Single or two-stage UHPH: 100, 200, 300 MPa primary valve and 30 MPa secondary valve; Stansted high pressure homogeniser (model FPG11300, Stansted Fluid Power Ltd., Essex, UK); inlet temperature of 30±1°C.
- Conventional pasteurisation (72°C, 15 s) and homogenisation-pasteurisation (15+3 MPa, 60°C; 72°C, 15 s).

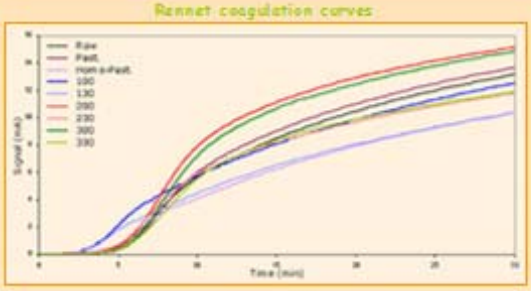
Particle size distribution: Beckman Coulter laser diffraction particle size analyser (LS 13 320 series, Beckman Coulter, Fullerton CA, USA).

Rennet coagulation properties: rennet coagulation time (RCT), rate of curd firming (RCF) and curd firmness at 30 min (CF)) assessed by the Optigraph® system (Ysebaert Inc, Frepillon, France).

Particle size distribution



Rennet coagulation curves



RESULTS

Temperature of treatment and pH of milk

	T1 (°C)	T2 (°C)	pH
Raw	-	-	6.75 (±0.03)
Pasteurised	-	-	6.75 (±0.02)
Homogenised-Pasteurised	-	-	6.72 (±0.02)
100 MPa	33.2 (±0.4)	53.8 (±1.9)	6.52 (±0.07)
130 MPa	33.6 (±0.5)	57.5 (±4.7)	6.51 (±0.08)
200 MPa	36.8 (±0.4)	75.2 (±1.9)	6.68 (±0.04)
230 MPa	37.0 (±0.7)	77.4 (±4.8)	6.69 (±0.02)
300 MPa	41.0 (±0.7)	84.2 (±1.6)	6.75 (±0.03)
330 MPa	40.4 (±0.5)	88.8 (±1.3)	6.74 (±0.01)

T1 and T2: temperatures reached before and after the primary valve, respectively.

DISCUSSION

UHPH-treatments at 100-130 MPa: particle size distributions rather similar to homogenised-pasteurised milk; lowest RCT, RCF and CF.

Single-stage UHPH above 200 MPa: smallest particles; highest RCFs and CFs; RCTs similar to those of control milks.

Two-stage UHPH above 200 MPa vs. single-stage: coalescence and aggregation of small fat globules; similar RCTs; significantly lower RCFs and CFs.

CONCLUSION

Whole milk with an inlet temperature of 30°C treated by single-stage UHPH at 200 MPa showed superior rennet coagulation properties, which could be attributed to smaller fat globules. The results suggest that UHPH could enhance the cheese-making properties of milk.

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Effects of ultra-high pressure homogenisation on the rennet coagulation properties of milk

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Ultra high-pressure homogenization (UHPH) has been proposed as an effective alternative to pasteurisation in milk sanitization [1]. In addition, UHPH creates smaller fat particles and modifications in the functional properties of proteins [2,3], and hence it can modify the rheological and/or coagulation properties of milk or dairy emulsions. However, renneting properties of UHPH-treated milk have received little attention. Our objective was to determine the effect of UHPH treatment (100-330 MPa) on the rennet coagulation properties of bovine milk. UHPH was carried out by subjecting milk to single or two-stage UHPH (100, 200 and 300 MPa on the primary valve and 30 MPa on the secondary valve) using a Stansted high pressure homogeniser (model FPG11300, Stansted Fluid Power Ltd., Essex, UK) at an inlet temperature of 30°C. Rennet coagulation properties such as rennet coagulation time (RCT), rate of curd firming (RCF) and curd firmness (CF) of milk were assessed by the Optigraph (Ysebaert Inc, Frepillon, France) in relation to controls: conventionally pasteurised milk (72°C for 15s), conventionally pasteurised-homogenised milk (72°C for 15s, 18 MPa at 57-60°C), and raw milk. The range of milk temperature during UHPH treatments was 33-41°C at the primary valve and 54-94°C at the secondary valve. However, milk outlet temperature was kept under 35°C. Rennet clotting time was very much dependant on the treatment although two-stage homogenisation did not seem to affect it. It was observed that coagulation occurred early in homogenized milks up to 230 MPa than in control milks. On the other hand, two-stage homogenisation significantly diminished both RCF and CF. The strongest gels were obtained with 200 and 300 MPa treated milks. Increasing pressure at the primary valve decreased particle size of milk. However, milk treated with two-stage homogenisation at pressures higher than 200 MPa showed larger particles than the controls. This phenomenon was observed by microscopic images as aggregations of small fat globules. These differences could explain the rennet coagulation properties of UHPH-treated milks. In conclusion, milk with an inlet temperature of 30°C treated at 200 MPa (single stage) showed superior rennet coagulation properties suggesting possible applications of UHPH treatment of milk for cheese manufacture.

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Effects of ultra-high pressure homogenisation on the rennet coagulation properties of milk

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Abstract
The aim of the study was to determine the effect of ultra-high pressure homogenisation (UHPH) on the rennet coagulation properties of bovine milk. Milk was subjected to single or two-stage UHPH (100, 200 and 300 MPa on the primary valve and 30 MPa on the secondary valve) using a Stansted high pressure homogeniser at an inlet temperature of 30ºC. Rennet coagulation properties were assessed by the Optigraph device and compared to conventionally pasteurised milk (72ºC for 15 s), pasteurised-homogenised milk (72ºC for 15 s, 18 MPa at ~60ºC), and raw milk. Temperature during UHPH treatments ranged between 54-94ºC. RCT was dependent on the treatment although two-stage UHPH did not seem to affect it. It was observed that coagulation occurred earlier in milks treated up to 200 MPa than in control milks. Two-stage UHPH significantly diminished both RCF and CF. The strongest gels were obtained with 200 and 300 MPa treated milks. Increasing pressure at the primary valve decreased particle size of milk. However, milk treated with two-stage UHPH above 200 MPa showed large particles, observed by confocal microscopy as aggregations of small fat globules. These differences could explain the rennet coagulation properties of UHPH-treated milks. In conclusion, milk with an inlet temperature of 30ºC treated at 200 MPa showed superior rennet coagulation properties suggesting the application of UHPH on milk for cheese manufacture.
Key words: Ultra-high pressure homogenisation, rennet coagulation of milk.

Introduction
Ultra-high pressure homogenisation is based on the same principle that of conventional ball-and-seat homogenisers, but current developments in the design allow to reach pressures of 350 MPa. There has been an increasing interest in the application of UHPH in food technology. Reports on the effect of UHPH on some pathogenic and spoilage microorganisms in model and real food systems have proved its efficiency in reducing microbial counts (Hayes and Kelly, 2003; Briñez et al., 2006). The aim of this work was to determine the effect of UHPH treatment on the rennet coagulation properties of whole bovine milk by comparing this new technology with conventional pasteurisation and homogenisation-pasteurisation treatments.

Material and Methods
Raw whole milk standardised at 3.5±0.2% fat was subjected to single or two-stage UHPH (100, 200 and 300 MPa on the primary valve and 30 MPa on the secondary valve) using a Stansted high pressure homogeniser (model FPG11300, Stansted Fluid Power Ltd., Essex, UK) at an inlet temperature of 30±1ºC. UHPH treatments were compared to raw, pasteurised (72ºC, 15 s) and homogenised-pasteurised (15+3 MPa, 60ºC; 72ºC, 15 s) milks. The particle size distribution in milk samples was determined using a Beckman Coulter laser diffraction particle size analyser (LS 13 320 series, Beckman Coulter, Fullerton CA, USA). The coagulation of warmed milks by recombinant rennet was carried out at 32ºC for 30 min.

Rennet coagulation properties (rennet coagulation time (RCT), rate of curd firming (RCF) and curd firmness at 30 min (CF)) were assessed in triplicate by the Optigraph® system (Ysebaert Inc, Frepillon, France). Data were processed by analysis of variance (ANOVA) using the general linear models procedure of Statgraphics®. Tukey's test was used for comparison of sample data. Evaluations were based on a significance level of $P < 0.05$. Confocal laser scanning microscopy (CLSM) observations of rennet gels were performed in fluorescence mode essentially as Michalski et al. (2002) described.

Results and Discussion

Rennet clotting time (RCT) was dependent on the treatment, although the secondary stage of UHPH did not seem to affect it. However, two-stage UHPH significantly diminished both the rate of curd firmness (RCF) and curd firmness (CF) in relation to their homologues treated by single stage.

Samples treated at 100-130 MPa had significantly lower RCT than the rest of milks. This could be attributed to lower pH-values, which would enhance chymosin performance, due to the action of residual indigenous lipoprotein lipase after UHPH treatment (Hayes and Kelly, 2003). Milks UHPH-treated at 100-130 MPa and homogenised-pasteurised showed the lowest RCF and CF. The particle size distributions showed that milks treated at 100-130 MPa were rather similar to homogenised-pasteurised milk. Moreover, confocal micrographs showed that the gels obtained from these milks were also rather similar. They presented open protein matrices, with large and irregular pores delimited by thick and lumpy strands, within middle-sized fat globules were embedded. Fat globules entrapped into the gel would disrupt the continuity of gel structure acting as weak centres (Gosh et al., 1994).

Milks UHPH-treated at 200 and 300 MPa (single-stage) showed RCT similar to those of the controls. However, their RCF and CF were much higher. Single-stage UHPH above 200 MPa provoked the smallest particles. The micrographs showed that gels presented tight protein matrices with thin strands and associated very small fat globules.

UHPH treatments at 230 and 330 MPa (two-stage) resulted in significantly lower RCF and CF. The particle size distributions showed that above 200 MPa, the secondary stage provoked the coalescence of the smallest fat globules resulting in distributions much closer to those of milks UHPH-treated at 100-130 MPa and homogenised-pasteurised. The micrographs showed that their gels presented much laxer protein matrices than those from single-stage treated milks. Moreover, the secondary stage above 200 MPa provoked a broadening of the distributions, with particles larger than 40 μm that corresponded to aggregates of small fat globules.

Conclusions

Whole milk with an inlet temperature of 30ºC treated by single-stage UHPH at 200 MPa showed superior rennet coagulation properties, which could be attributed to smaller fat globules. The results suggest that UHPH could enhance the cheese-making properties of milk.

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Effect of ultra-high pressure homogenisation on microbial and rennet coagulation properties of goats milk

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Introduction

Ultra-high pressure homogenisation is based on the same principle that of conventional ball-and-seat homogenisers, but current developments in the design allow to reach pressures of 350 MPa. There has been an increasing interest in the application of UHPH in food technology as a minimal process for the production of a wide variety of safe and nutritious foods. Possible uses of this technology for the dairy industry include reduction of fat globule size, inactivation of enzymes, and destruction of bacteria (Pereda et al., 2007; Zamora et al., 2007).

Objective

To determine the effect of UHPH on the microbiology and rennet coagulation properties of goat's milk.

Material and Methods

Raw goat milk was subjected to:

- Single or two-stage UHPH: 100, 200, 300 MPa primary valve and 30 MPa secondary valve; Stansted high pressure homogeniser (model FPG11300, Stansted Fluid Power Ltd., Essex, UK); inlet temperatures of 30 and 40±1ºC.
- Conventional pasteurisation (72ºC, 15 s)

Microbiological analysis: total bacteria (CT), psychrotrophic bacteria (PSI), coliform (VRBL), lactobacilli (LB), lactococci (LC) and enterococci (EC) (Pereda et al., 2007).

Particle size distributions and rennet coagulation properties: particle size distributions were determined using a Beckman Coulter laser diffraction particle size analyser (LS 13 320 series, Beckman Coulter, Fullerton CA, USA) and rennet coagulation time (RCT), rate of curd firming (RCF) and curd firmness at 30 min (CF)) assessed by the Optigraph® system (Ysebaert Inc, Frepillon, France).

Results

- **At 100 MPa:**
  - Microbial counts were higher than the acceptable limit.
  - Inlet temperature strongly affected particle size.
  - Similar RCF, CF and RCT to those of raw milk.
- **Above 200 MPa:**
  - From important reductions up to complete destruction depending on the microorganism.
  - Secondary stage enhanced coalescence.
  - At 200 MPa: Smallest particles; highest RCF and CF.
  - At 300 MPa: Lowest RCF and CF; longest RCT.

Table 1. Microbial populations (log cfu/ml) of raw and UHPH milks.

Microbial group	Raw	100 MPa 30ºC	200 MPa 30ºC	300 MPa 30ºC	100 MPa 40ºC	200 MPa 40ºC	300 MPa 40ºC
CT	6,41 ± 1,36	5,54 ± 0,57	1,80 ± 0,52	1,39 ± 0,15	5,22 ± 0,18	1,48 ± 0,52	1,59 ± 0,58
PSI	6,49 ± 1,36	5,62 ± 0,67	1,34 ± 0,21	1,21 ± 0,36	5,23 ± 0,17	1,59 ± 0,30	1,64 ± 0,58
LC	6,35 ± 1,46	5,41 ± 0,54	1,62 ± 0,56	0,95 ± 0,15	5,22 ± 0,24	1,30 ± 0,56	1,30 ± 0,53
LB	3,64 ± 0,36	3,12 ± 0,22	Nd	Nd	2,72 ± 0,32	Nd	Nd
VRBL	5,01 ± 1,08	2,79 ± 1,21	Nd	Nd	1,53 ± 0,37	Nd	Nd
EC	4,81 ± 0,03	4,53 ± 0,08	Nd	Nd	4,08 ± 0,47	0,09 ± 0,12	0,20 ± 0,28

Figure 1. Particle size distributions (differential volume).

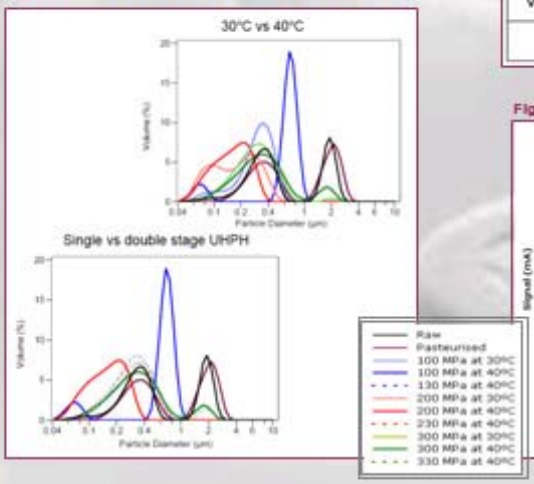
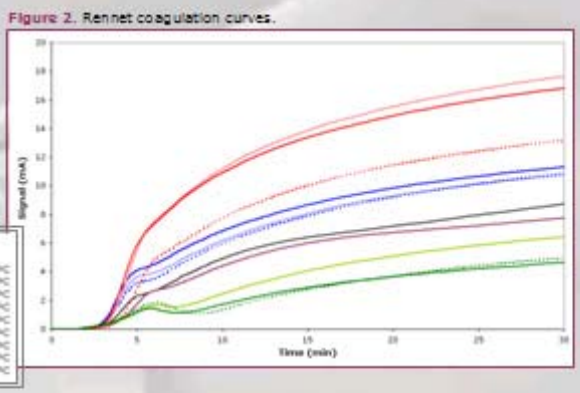


Figure 2. Rennet coagulation curves.



Conclusions

Single-stage UHPH at 200 MPa was efficient in reducing the studied bacterial populations and enhanced the rennet coagulation properties suggesting possible applications of UHPH technology for cheesemaking.

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II-P121: Effect of Ultra-high Pressure Homogenisation on Microbial and Rennet Coagulation Properties of Goats Milk

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Summary

The effect of ultra-high pressure homogenisation (UHPH) on microbial and rennet coagulation properties of goat milk was studied.

Milk was subjected to single or two-stage UHPH (100/200/300 MPa and 30 MPa on the primary and secondary valves, respectively) using a Stansted High Pressure homogeniser with inlet temperatures of 30ºC and 40ºC. The microbiological quality of raw and UHPH-treated milks was studied by enumerating total bacteria, psychrotrophic bacteria, coliforms, lactococci, lactobacilli and enterococci. Particle size distributions and pH of UHPH-treated milks were compared to raw and conventionally treated milks. Studied coagulation properties were rennet clotting time (RCT), rate of curd firming (RCF) and curd firmness (CF).

From the analyses performed, it can be deduced that single-stage UHPH at 200 MPa was the best treatment to enhance the microbial and technological properties of goat milk.

1. Introduction

Ultra-high pressure homogenisation is based on the same principle that of conventional ball-and-seat homogenisers, but current developments in the valve design and materials allow to reach pressures of 350 MPa. There has been an increasing interest in the application of UHPH in food technology as a minimal process for the production of a wide variety of safe and nutritious foods. Possible uses of this technology for the dairy industry include reduction of fat globule size, inactivation of enzymes, and destruction of bacteria (Briñez et al., 2006; Pereda et al., 2007; Zamora et al., 2007).

The aim of this work was to determine the effect of UHPH on the microbiology and rennet coagulation properties of goat's milk.

2. Material and methods

Ultra-high pressure homogenisation of raw goat milk was performed with a Stansted high-pressure homogeniser (model FPG11300, Stansted Fluid Power Ltd., Essex, UK). Milk was UHPH-treated under the following conditions: single or two-stage UHPH (100/200/300 MPa and 30 MPa on the primary and secondary valves, respectively) with inlet temperatures of 30ºC and 40ºC. Microbial and rennet coagulation properties of treated samples were compared to those of raw and conventionally treated milks (pasteurisation at 72ºC for 15 s and homogenisation-pasteurisation at 18 MPa and ~60ºC, and 15 s at 72ºC).

Microbiological analysis

The microbiological quality of treated and untreated milk was assessed by enumerating total bacteria, psychrotrophic bacteria, coliform, lactobacilli, lactococci and enterococci as Pereda described elsewhere (2007).

Particle size distributions and rennet coagulation properties

The particle size distribution in milk samples was determined using a Beckman Coulter laser diffraction particle size analyser (LS 13 320 series, Beckman Coulter, Fullerton CA, USA). The coagulation of warmed milks by recombinant rennet was carried out at 32ºC for 30 min. Rennet coagulation properties (rennet coagulation time (RCT), rate of curd firming (RCF) and curd firmness at 30 min (CF)) were assessed in triplicate by the Optigraph® system (Ysebaert Inc, Frepillon, France).

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3. Results and discussion

UHPH treatments at 100 MPa were able to obtain a significant reduction in the counts of all microorganisms studied compared to raw milk, which initial counts were 6,5 log cfu/ml for total bacteria, psychrotrophic and lactococci, 3,6 for lactobacilli and 5 for coliforms and enterococci. However, the reductions were small (~3 log cfu/ml concerning coliforms and <1 log cfu/ml for the other microorganisms). Above 200 MPa, important reductions were achieved (4.6 – 5.2 log cfu/ml) in relation to psychrotrophic, lactococci and total bacteria. In addition, coliforms, lactobacilli and enterococci were completed destroyed.

Although pasteurised milk showed similar RCF and CF to that of raw milk, its RCT was higher than that of raw milk. Both milks showed similar pH and particle distributions. Conventionally homogenised-pasteurised milks showed the lowest CF. Concerning UHPH, the difference in inlet temperature affected only the distribution of milks treated at 100 MPa. The results of RCF showed a similar pattern to those of CF. Even if milks treated at 100 MPa showed lower pH, their rennet coagulation properties were similar to those of raw milk. Strongest gels were obtained by single-stage UHPH-treated milks at 200 MPa. However, two-stage UHPH diminished both RCF and CF. Particle distributions showed that the secondary stage at 200 MPa enhanced coalescence. Although UHPH-treated milks at 300 MPa showed similar RCT to that of pasteurised milk, their CF were much lower.

These results are similar to those obtained in previous studies with UHPH-treated cow milk (Pereda et al., 2007; Zamora et al., 2007).

4. Conclusion

Single-stage UHPH at 200 MPa was efficient in reducing the studied bacterial populations and enhanced the rennet coagulation properties suggesting possible application of UHPH technology for cheesemaking from goat milk.

References

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Identification of interactions within rennet curds prepared from raw and UHPH-treated milks using dissociating chemical agents

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INTRODUCTION

Ultra-high pressure homogenisation (UHPH) is based on the same operating principle as conventional homogenisation, but current developments in the design of UHPH systems allow pressures of up to 350 MPa. It has been observed that UHPH improves rennet coagulation of milk by reducing clotting time and increasing gel strength in full-fat milks (Zamora et al., 2007). However, rennet-induced gels from UHPH-treated skim milk showed a more brittle texture (Lodaite et al., in press). One strategy for understanding the interactions between proteins within the curd underpinning these changes involves using different chemical dissociating agents to explain the effect of UHPH on the cheese-making capacity of UHPH-treated milk.

AIM: *To determine the effect of UHPH on protein–protein interactions within rennet curds with different fat content.*

MATERIALS & METHODS

From raw milk to drained curds: Raw milk was standardised at different fat contents (0.0%, 1.8% or 3.6%) and single-stage UHPH-treated at 300 MPa (nm-Gen 7400 H model, Stansted Fluid Power Ltd., Essex, UK) at inlet temperature of 30ºC; milk was rennet coagulated for 30 min at 32ºC, followed by cutting and centrifugation at 1,500 x *g* for 15 min at 20ºC to recover curds .

Quantification of total protein in curds: was by the Kjeldahl method.

Dissociation through chemical agents: The approach of Lefebvre-Cases et al. (1998) was used; drained curds were dispersed separately in MilliQ water, EDTA (to disrupt ionic bonds with Ca salts), SDS (to disrupt hydrophobic interactions) or urea (to disrupt hydrogen bonds); mixtures of curds and dissociating agent were homogenised with an Ultra-Turrax and soluble and insoluble proteins were separated by ultracentrifugation (86,000 x *g* for 40 min at 20ºC) .

Quantification and identification of proteins in extracts: Soluble proteins were quantified with modified Lowry method and identified by SDS-PAGE gels under reducing conditions.

RESULTS & DISCUSSION

Increasing fat content resulted in:

- Increased level of proteins dissociated in MilliQ water and EDTA.
- Decreased dissociation of proteins in urea and SDS.

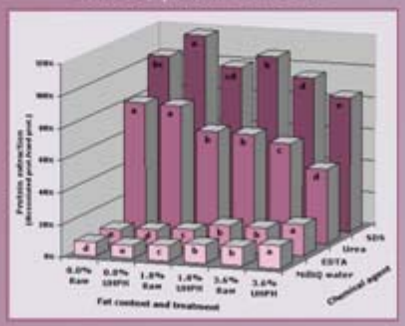
➢ Therefore, the presence of fat enhanced formation of ionic bonds involving calcium salts, but impaired hydrophobic interactions and hydrogen bonds.

UHPH treatment resulted in:

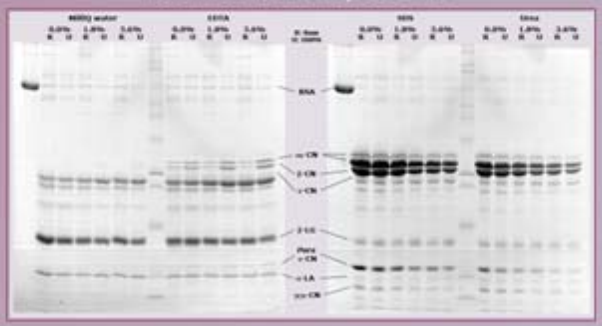
- Increased dissociation of proteins in MilliQ water and EDTA (particularly α_{s}-CN and β-CN).
- For milk with 0.0% and 1.8% fat, higher dissociation in SDS, but no differences with urea.
- For 3.6% fat milk, decreased dissociation in urea and SDS.

➢ Therefore, UHPH increased free and calcium-bonded α_{s}-CN and β-CN and enhanced hydrophobic interactions in curds from low-fat milks, but impaired both hydrogen bonding and hydrophobic interactions of caseins with full-fat milks.

Levels of proteins extracted



Protein identification by SDS-PAGE



CONCLUSIONS: *UHPH significantly changes the nature of protein interactions within rennet curds in a manner depending on milk fat content. Such changes could help in understanding of the improvement or worsening of rennet coagulation properties following UHPH of milk.*

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Lodaite et al. in press. J. Dairy Res.
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Effect of high pressure homogenization on the microbial safety, shelf-life and sensorial properties of fluid foods and ingredients

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Identification of interactions within rennet curds prepared from raw and UHPH-treated milks using dissociating chemical agents

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Single-stage ultra-high pressure homogenisation (UHPH) of standardised milks (0.0, 1.8 or 3.6% fat) was performed at 300 MPa (nm-Gen 7400 H model, Stansted Fluid Power Ltd., Essex, UK) at an inlet temperature of 30°C. Raw and UHPH-treated milks were coagulated by rennet addition, and curds were cut and centrifuged. Total protein content of drained curds was determined using the Kjeldahl method. The interactions between proteins were studied by measuring the extent of extraction possible using MilliQ water, EDTA, SDS or urea, following the method of Lefebvre-Cases et al. (1998). Soluble proteins were quantified using a modified Lowry method and identified by SDS-PAGE gels. For both raw and UHPH-treated milks, increasing the amount of fat led to an increase of proteins dissociated in water and EDTA, but decreased dissociation in urea and SDS. The main proteins dissociated by water and EDTA in both raw and UHPH curds were the whey proteins β-LG and α-LA, and breakdown products from αs-CN and β-CN. UHPH significantly increased the amount of proteins dissociated in water and EDTA; the proteins involved were mainly αs-CN, β-CN and their degradation products and, in the case of EDTA, some k-CN and *para*-k-CN were dissociated. The main proteins dissociated by urea and SDS were αs-CN, β-CN, para-k-CN, some β-LG and the degradation products of αs-CN and β-CN. UHPH significantly decreased the amount of protein dissociable by urea, but increased that dissociable by SDS. In conclusion, fat enhanced the formation of ionic bonds with calcium salts, but impaired hydrophobic interactions and hydrogen bonds. However, UHPH increased free and calcium-bonded αs-CN and β-CN, enhanced hydrophobic interactions, but impaired hydrogen bonding of caseins.

Effect of ultra-high pressure homogenisation on shelf-life of a starter-free fresh cheese

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Introduction

Ultra-high pressure homogenisation (UHPH) is based on the same principle that of conventional ball-and-seat homogenisers, but current developments in the design allow to reach pressures of 350 MPa. There has been an increasing interest in the application of UHPH in food technology as a minimal process for the production of a wide variety of safe and nutritious foods.

Material and Methods

Material: standardised bovine milk (3.6% fat).

Treatments: UHPH at 300 MPa (UH; FPG11300, Stansted Fluid Power Ltd.), conventional pasteurisation (PA; 80ºC 15 *s*) or homogenisation-pasteurisation (HP; 18 MPa 60ºC, 80ºC 15 *s*).

Cheese-making: 45 min rennet coagulation, 15 min cutting, 10 min stirring, moulding, 90 min drainage, packaging.

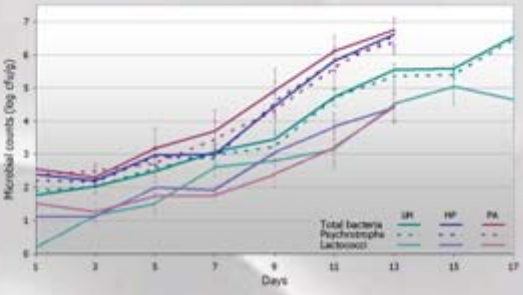
Cheese composition: total solids & moisture (IDF Standard 004:2004), fat (ISO Standard 3433:1975), protein (IDF Standard 185:2002).

Microbiology: total bacteria (TC) & psychrotrophs (PSY): Plate Count Agar (30ºC 48 h & 20ºC 72 h); lactococci (LC): M17 (30ºC 48 h); lactobacilli (LB): Rogosa (30ºC 72 h); coliforms (COL): Violet Red Bile Agar (37ºC 24 h); *E. coli* (EC): ColiID (37ºC 24h); yeasts & fungi (YF): Rose-Bengal Chloramphenicol Agar (20ºC 5 d); *S. aureus* (SA): Baird-Parker RPF Agar (37ºC 48 h); on day 1: *L. monocytogenes* (UNE-EN ISO 11290-1) & *Salmonella* spp. (UNE-EN ISO 6579).

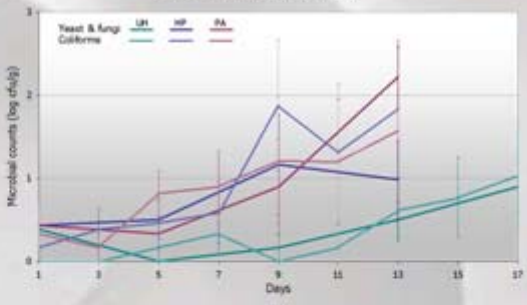
Cheese composition

(g/100 g cheese)	Total solids	Fat	Protein
UH	30.04 ± 0.32 ^{c}	13.9 ± 0.4 ^{c}	11.92 ± 0.28 ^{c}
HP	32.61 ± 0.35 ^{b}	15.5 ± 0.3 ^{b}	12.42 ± 0.35 ^{b}
PA	31.39 ± 0.38 ^{a}	14.8 ± 0.4 ^{b}	12.87 ± 0.20 ^{a}

Total bacteria, psychrotrophs & lactococci



Yeast or fungi & coliforms



Results & Discussion

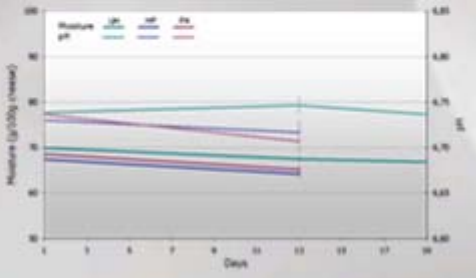
- Lactobacilli, coliforms, *E. coli*, and yeast & moulds were not detected in either conventionally or UHPH-treated milks. However, the effect of UHPH on total mesophiles, psychrotrophs and lactococci was significantly greater than that of conventional treatments.
- In cheeses, none of the pathogens were detected at day 1, nor were lactobacilli and *E. coli* throughout the storage period.
- UHPH-cheeses showed the lowest rate of growth of total mesophiles and psychrotrophs, with a concomitant increase in the shelf-life of cheeses (6 log cfu/g reached at day 13 *vs.* 17).
- UHPH triggered some unknown changes in the milk which enhanced the growth of lactococci at an early stage of the storage period. No differences in the pH of cheeses were observed, thus pH seemed not to be related to this phenomenon. UHPH has been proven to be less aggressive towards thermolabile compounds, which could act as growth factors.
- Yeast and moulds were mainly detected in PA-cheeses; UHPH-cheeses had higher moisture content, which could enhance bacterial growth thus inhibiting the growth of yeast and moulds.

Milk microbiology

	Raw	PA	HP	UH
TC	3.79 ± 0.03 ^{a}	1.66 ± 0.10 ^{b}	1.58 ± 0.09 ^{b}	0.79 ± 0.03 ^{c}
PSY	3.75 ± 0.04 ^{a}	0.98 ± 0.19 ^{b}	1.06 ± 0.05 ^{b}	0.71 ± 0.11 ^{c}
LC	3.09 ± 0.04 ^{a}	0.73 ± 0.12 ^{b}	0.46 ± 0.11 ^{c}	0.17 ± 0.12 ^{d}
LB	2.69 ± 0.10 ^{a}	ND ^{b}	ND ^{b}	ND ^{b}
COL	2.50 ± 0.03 ^{a}	0.08 ± 0.08 ^{b}	ND ^{b}	ND ^{b}
EC	2.13 ± 0.08 ^{a}	ND ^{b}	ND ^{b}	ND ^{b}
YF	2.11 ± 0.12 ^{a}	ND ^{b}	ND ^{b}	ND ^{b}
SA	1.75 ± 0.05 ^{a}	ND ^{b}	ND ^{b}	ND ^{b}

ND: not detected

Moisture content & pH



Conclusions

Fresh cheeses from UHPH-treated milks showed longer shelf-life than those from conventionally treated milks due to both a greater reduction of microorganisms and a lower rate of growth.

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Poster 40

EFFECT OF ULTRA-HIGH PRESSURE HOMOGENISATION ON SHELF-LIFE OF A STARTER-FREE FRESH CHEESE

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The aim of the study was to evaluate the effect of homogenising milk with ultra-high pressure homogenisation (UHPH) on the microbiological shelf-life of a starter-free fresh cheese. UHPH (300 MPa at 30ºC; model FPG11300, Stansted Fluid Power Ltd) was compared with conventional pasteurisation (PA; 80ºC for 15 s) and homogenisation-pasteurisation (HP; 18 MPa at 60ºC, 80ºC for 15 s). Microbiological quality of cheeses

stored at 4ºC was studied by enumerating total aerobic mesophiles, psychrotrophs, lactococci, lactobacilli, coliforms, *E. coli*, and yeast and moulds. The analysis was carried out every 2 days until the total counts reached 6 log cfu/g. Treated milks and pathogens in cheeses (*L. monocytogenes*, *Salmonella* spp. and *S. aureus*) were analysed on day 1.
Coliforms, *E. coli*, lactobacilli, and yeast and moulds were not detected in either conventionally or UHPH-treated milks. However, the effect of UHPH on total mesophiles, psychrotrophs and lactococci was significantly greater than that of conventional treatments. In cheeses, none of the pathogens were detected at day 1, nor were lactobacilli and *E. coli* throughout the storage period. UHPH-cheeses showed the lowest rate of growth of total mesophiles and psychrotrophs, with a concomitant increase in the shelf-life of cheeses (6 log cfu/g reached at day 13 *vs.* day 17). Moreover, UHPH triggered some unknown changes in the milk which enhanced the growth of lactococci at an early stage of the storage period. No differences in the pH of cheeses were observed, thus pH seemed not to be related to these phenomena. UHPH has been proven to be less aggressive towards thermolabile compounds, i.e., vitamins, which could act as growth factors. Yeast and moulds were mainly detected in PA-cheeses; both UHPH- and HP-cheeses had higher moisture content, which could enhance bacterial growth thus inhibiting the growth of yeast and moulds.