The Effect of Salt on the Structure of Individual Fat Globules and the Microstructure of Dry Salted Cheddar Cheese

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Abstract

Salting is an essential step in the production of Cheddar and other cheese varieties and is a well-studied process but the effect of salt addition on the microstructure of the milk ingredients and resulting cheese is not well known. This study provides insights into how the primary components in milk and the cheese matrix respond to salting. High concentrations of salt (15-25% (w/w) NaCl) disrupted fat globules due to the increased osmotic pressure. This led to fat coalescence, resulting in large fat globules $>10\text{ }\mu\text{m}$ in diameter, together with submicron sized fat globules $\sim 120\text{-}500\text{ nm}$ in diameter. Salt addition also prevented the visualization of the milk fat globule membrane when added at high concentrations (25% (w/w) NaCl) and induced asymmetry in liquid ordered domains at lower concentrations (10% (w/w) NaCl). The microstructure of the surface of the milled curd was compacted by salt, appearing coarse with 5% (w/w) NaCl or more hydrated with a denser protein structure with 2.5% (w/w) NaCl. After pressing, the curd junctions were fine and thin within the unsalted sample but coarse and thick where 5% (w/w) NaCl was added. Such coarse junctions appear to reduce binding between curd particles leading to a less cohesive cheese. Our results show that NaCl can significantly impact on the structure of fat and protein matrix of the curd surface if salt is not evenly distributed during dry salting. High concentrations of salt can also change the microstructure and texture of the cheese, resulting in a more heterogeneous product.

Keywords: Cheese; Fat globule; Microstructure; Salt

Introduction

Sodium chloride (NaCl) is used in cheese making to control moisture and to moderate microbial and enzymatic activity [1]. NaCl also contributes a salty taste, enhances flavour and affects the texture and the functionality of the final cheese [2, 3]. NaCl content can vary between the cheeses produced from different vats or between the different micro-environments within a cheese [4], leading to varied cheese texture and quality. Best practice is achieved when the salt uptake by the curd is optimised, leading to a uniform distribution within each cheese and the production of a minimal volume of salty waste product.

NaCl can be added to cheese in dry form or as an aqueous solution of NaCl, known as brine. The final concentration of NaCl in different cheese varieties is quite broad and ranges from 0.7% (w/w) in Swiss cheese to 6% (w/w) in Domiati cheese [5]. Previous studies have shown that the moisture, curd pH and salt concentration, defined here as the mass of salt per unit mass of curd, can be used to control the efficiency of NaCl uptake [1, 6]. In general, an increase in the moisture content of Cheddar curd, a lower pH or a very high concentration of NaCl decreases the efficiency of NaCl uptake. Consequently, the addition of limited salt to a drier curd at high pH can be used to optimise NaCl uptake. Multiple additions of NaCl at milling can also offer further improvements in salt uptake compared to a single addition [1].

Whilst there have been many studies examining the effect of salt in Cheddar production that have focused on cheese texture and proteolysis [7-9], to date these have not focused on the fundamental changes induced by salt addition, leaving large gaps in our understanding of how salt impacts on the primary components in milk and how salt changes the resulting product microstructure. Nor have prior studies systematically examined the potential effect of different concentrations of NaCl on the microstructure of the fat globule and its surrounding milk fat globule membrane (MFGM) and the microstructure of the milled curd and the final cheese product, despite the importance of the salting procedure.

Salting may impact on the integrity of the fat globule, potentially affecting coalescence, which can increase fat loss during production [10] or induce the rupture into smaller globules with a higher surface area potentially leading to excessive lipolysis [11]. Both cases can also lead to an undesirable flavor and texture within the cheese.

The interfacial MFGM layer is central to the integrity of the fat globule. This 10-20 nm thick layer [12], consists of three sub-layers containing phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (SM), cholesterol and a minor fraction of proteins [13, 14]. The MFGM layer is of important nutritional value, helps stabilize fat within emulsions and helps protect the fat globule from enzymatic attack [15]. Removal of the MFGM may lead to rancidity in a matured Cheddar cheese [16], whilst the addition of buttermilk powder/concentrate containing MFGM results in reduced free oil formation [17]. The addition of further MFGM components can also lead to an improved cheese body and texture [17] and improved microstructure in low fat Cheddar cheese [18]. Consequently changes to the MFGM may affect the properties of the final Cheddar cheese.

NaCl can induce several changes to lipid bilayers including a reduced mobility of the phospholipid molecules [19], an increased ordering of the lipid acyl chains and the formation of a more compressed membrane [20, 21]. Diffusion through the membrane is also reduced [19]. Studies have examined the effect of NaCl on phosphatidylethanolamines and phosphatidylcholine using synthetic lipid bilayers [19, 21, 22] but the effect of

NaCl on the trilayered MFGM, which differs in structure, composition and properties, has not yet been reported. NaCl addition to cheese may also directly or indirectly affect the properties of the internal triacylglyceride fat core of both intact and disrupted fat globules.

Salting is also known to impact on the proteins present in milk, with different protein behavior observed as a function of salt concentration [23]. Low concentrations of 75-850 mM (or 0.4-4.8% w/w) do not alter casein micelle size but do reduce the net charge due to charge shielding [24], altering protein-protein interactions. Higher salt concentrations have also long been used for the precipitation and separation of proteins in other fields [23, 25] but to date the effect of the high concentrations of salt involved in salting cheese proteins has not been well examined. Specifically the effect of NaCl on coagulated proteins and the surface microstructure of the milled curd and its potential impact on the final cheese microstructure and textural properties have not been examined.

This study sought to investigate how NaCl affects the primary components in cheese-milk, with a focus on the fat globule and the milk fat globule membrane (MFGM). It also aimed to examine the effect of NaCl addition on the microstructure of curd and cheese and to explore the possible link between the surface microstructure of the curd and cheese texture. This approach studying ingredients, intermediates and products provides insight into how the individual components and the cheese matrix respond to salting. It is expected that such insights will help manufacturers to better understand salting and to apply control strategies to improve the consistency of the final cheese product.

Materials and Methods

Experimental Design

The effect of different concentrations of NaCl on the size and microstructure of fat globules and the MFGM of these fat globules in milk was investigated. NaCl was also added to the milled curd during Cheddar cheese making at a concentration of 2.5% and 5% weight of curd. The moisture content of the curd before NaCl addition was ~40% (w/w). The concentration of salt in moisture (S/M) on the surface of the milled curd if all of the NaCl added was dissolved was estimated to be ~16% (w/w) or ~33% (w/w), as shown in Online Resource 1. In practice, however, the dry NaCl usually only partially dissolves in the available moisture on the surface of the curd chips. The brine formed on the surface then sets up a concentration gradient with the moisture at the center of the curd, inducing NaCl diffusion. Osmotic pressure also causes the moisture from the center of the curd to migrate out towards the surface of the curd chips, dissolving more NaCl on the surface. This briny solution can affect nearby curd chips. This makes the estimation of the concentration of NaCl at the surface of a curd chip in a dry salting system difficult. The brine concentration used for salting cheese such as Edam or Gouda typically ranges from 18-25% (w/w) NaCl in water [5] and the concentration of NaCl around the cheese is more constant compared to a dry salting system, especially if the brine solution is circulated and the strength is adjusted frequently.

A series of NaCl concentrations between 0-25% (w/w) in milk were selected in this study, to be applicable to a wide range of salting systems. Only one salting temperature was chosen (38 °C), as this temperature is typical for hard cheese production, including Cheddar production. The NaCl (Science Supply, Mitcham, Australia) was added to a pasteurized unhomogenized milk with a composition of 4.1% (w/w) fat,

3.6% (w/w) protein and 4.7% (w/w) lactose (Parmalat, South Brisbane, Australia) to obtain a NaCl concentration of 6%, 10%, 15%, 20% or 25% (w/w) equivalent to 1.1M or 1.8M or 2.9M or 3.9M or 5.1 M respectively. Prior to NaCl addition, the milk was tempered to 38 °C for 5 min in a water bath.

In the second part of the study, the effect of NaCl addition (0%, 2.5%, 5% (w/w) of curd) on the microstructure of the milk fat and protein within a curd or cheese matrix was analyzed in pilot scale Cheddar cheese making experiments. These concentrations represent the range of salt for the manufacture of Cheddar and other dry salted cheese [26]. The equivalent concentration of salt in moisture (S/M) on the surface of the milled curd under these conditions if all of the NaCl added had dissolved was estimated to be ~16% (w/w) for 2.5% (w/w) NaCl addition and ~33% (w/w) for 5% (w/w) NaCl addition.

Light Scattering Analysis of Milk Fat Globule

The effect of different NaCl concentrations on the size distribution of fat globules within milk was measured by light scattering (LS) using a Mastersizer (Malvern Instrument, Malvern, UK), as described by Ong et al. [27], where the refractive index was set at 1.46 and 1.33 for milk fat and water respectively. The volume weighted mean (D_{43}) was calculated using the Mastersizer 2000 software (Malvern Instrument). Milk samples with different concentrations of NaCl were mixed in a thermo-mixer (Thermoline, Wetherill Park, Australia) maintained at 38 °C for 20 minutes at 1000 revolutions per minute (rpm) in order to dissolve the dry NaCl. An equal volume of 50 mM ethylene di-amine tetra acetic acid (EDTA) was then added to the samples to dissociate the casein micelles prior to LS measurements.

CLSM of Fat Globule and MFGM

The milk samples with different concentrations of NaCl were prepared for CLSM by staining with either Nile Red (Sigma-Aldrich, St Louis, USA) to observe the triacylglycerol core of fat globules [28], N-(Lissamine rhodamine B sulfonyl) dioleoylphosphatidyl ethanolamine (Rd-DOPE, Avanti polar lipids, Birmingham, England) to observe the phospholipids of the MFGM of fat globules [29] or fast green FCF (Sigma-Aldrich) to observe the protein.

The staining was performed as follows: stock solutions of Nile red (1 mg mL^{-1}) and Rh-DOPE (1 mg mL^{-1}) were prepared in dimethyl sulfoxide (DMSO) and chloroform respectively (both solvents from Chem Supply, Gillman, Australia). The stock solution of Fast green (1 mg mL^{-1}) was prepared in purified water (Millipore MilliQ). The milk samples (1 mL) were then mixed with 10 μL of Nile red stock solution and 10 μL of Fast green stock solution and incubated for 15 minutes at room temperature prior to CLSM observation. The Rh-DOPE stock solution (40 μL) was evaporated under nitrogen, just prior to mixing with 1 mL of milk sample and incubated for at least 1 hour at room temperature, for CLSM observation.

The microstructure of the fat and MFGM was analyzed using an inverted confocal laser scanning microscopy from Leica Microsystem (Heidelberg, Germany) [27]. Both Nile red and Rh-DOPE were excited at a wavelength of 488 nm using an argon laser and the emission filter was set at 520-590 nm. Fast green was excited at a wavelength of 633 nm and the emission filter was set at 660-790 nm.

Cheese Making

The milk for cheese making was provided by an Australian commercial cheese plant. The milk was standardized to a protein to fat ratio of 0.83 and pasteurized at 72 °C for 15 s in the factory. The standardized and pasteurized cheesemilk had a composition of 4.8% (w/w) fat, 4.0% (w/w) protein and 4.7% (w/w) lactose. The milk was used within 3 days of receipt. The cheese making procedure has been detailed in a previous study [30]. In brief, freeze dried direct vat set mesophilic starter culture (Chr. Hansen, Bayswater, Victoria) was added to the milk at 32 °C and allowed to ripen until the pH of the milk reached pH 6.5. Rennet (690 IMCU mL⁻¹, Chr. Hansen) was added to the milk to allow for milk coagulation to take place for 45 minutes before cutting of the coagulated gel with a wire knife to produce sections approximately 1 cm³ in size. The gel was cooked from 32 °C to 38 °C in 60 minutes with stirring at 40 revolutions per minute and maintained at 38 °C until the pH reached 6.2. The whey was drained and the curd was subjected to a cheddaring process until the curd reached pH 5.4. The curd was then milled into ~ 6 x 1 x 1 cm³ chips. Three vats of curds were prepared; the first vat proceeded without NaCl addition, where the curd chips were stirred by hand for 20 minutes at 38 °C before hooping in a cheese mold for overnight pressing at 689 kPa. The second and third vats were made with 2.5% and 5% (w/w) NaCl added respectively. The NaCl was divided into two portions of equal mass and added in two salting applications, with the second addition of NaCl occurring 5 minutes after the first NaCl addition. All curds were continuously stirred by hand for a total of 20 minutes after the first NaCl addition before hooping into cheese molds for pressing. All cheeses, with weight ~2 kg, were vacuum-packed and stored at 4 °C.

Milled curd samples were collected before and after salting and analyzed immediately. The cheese samples were analyzed 1 week after storage at 4 °C.

Composition and Texture of Cheese

The cheese was analyzed for fat, protein, moisture and NaCl content using standard methods, as described previously [30]. The fat in dry matter (FDM) was calculated as the concentration of fat in the dry matter of the cheese. The salt in moisture (S/M) was the concentration of salt in the moisture phase of cheese. The NaCl retention in the cheese or NaCl loss in the whey was calculated as a percentage of the amount of NaCl added to curd. The NaCl recovery is the sum of the NaCl loss and NaCl retention. Two samples were analysed for each treatment in each trial. The results presented are the mean of 4 data points from two independent trials (i.e. n = 4).

The texture analysis of the cheese was carried out using a texture analyser from Stable Micro Systems (Goldaming, UK) following a method described in a previous study. Cylindrical samples (2 cm in height x 2 cm in diameter) were compressed by 50% in a two-bite test using a crosshead speed of 2 mm s⁻¹. Six cheeses were analyzed for each treatment in each trial and averaged to give one data point. The hardness and cohesiveness of the cheese were reported as the average of two means from two independent trials (n = 2).

Preparation of Curd and Cheese for Cryo SEM and CLSM

Milled curd

The milled curd was prepared for cryo SEM analysis following the illustration in Fig. 1. This method is a modification of a previously optimized technique (Ong et al., 2011) that allows both the surface structure and the internal structure to be observed. The curd sample before or after salting was cut with a surgical blade to about 1 mm thick including the curd surface, as shown in Fig. 1. The thin piece of curd was then placed in a cryo sample holder with the position of the curd surface perpendicular to the well on the cryo sample holder. A thin piece of curd was also obtained from the middle of the curd chip (~30 mm from the long axes and ~4 mm from the short axes of the curd). The samples were then subjected to rapid freezing, freeze-fracturing, sublimation and metal coating, as described previously [31], followed by observation using a Quanta field emission scanning electron microscope from FEI (FE-SEM; Hillsboro, USA) operated in cryo mode. Correct positioning of the curd surface on the cryo sample holder before fracturing allows the direct examination of the microstructure of the curd surface. Fig. 1a and 1b shows an example of the curd surface (imaged from the surface to a depth of ~ 50 μm) and the microstructure within the center of the curd. At least two images were taken of the curd surface and center of the curd for each treatment in each trial and the images presented are representative of these replicates.

Milled curd samples were also collected for CLSM observation. The curd was cut into thin pieces ~2 mm x 2 mm x 1 mm in size, then stained and observed using CLSM following a method reported previously [31]. The curd samples were analysed using cryo SEM and CLSM at least 24 hours after sample collection and completed within 48 hours.

Cheese

Cheeses samples for both CLSM and cryo SEM were cut from the center of the cheese 1 week after pressing. The length of time after pressing prior to analysis allows for a more uniform salt distribution within the cheese samples. The method of preparation of cheese samples for CLSM and cryo SEM has previously been described by Ong et al. [31].

Image Analysis

Three dimensional (3D) CLSM images were reconstructed using Imaris image analysis software (Bitplane, South Windsor, CT, USA). The 3D image consists of 40 layers of 512 x 512 pixels images that are stacked together with separation between layers set at 0.25 μm . The sphericity of the fat globules and porosity of the curd sample examined was calculated using ImageJ software (Research Services Branch, National Institute of Health and Medicine, USA). The image analysis procedure follows a previously published protocol [30].

Statistical Analysis

Minitab statistical package (Minitab Inc., State College, USA) was used to analyze data from compositional and textural analysis. One way analysis of variance and Tukey paired comparison were used to compare differences between means with a significance level of $\alpha = 0.05$.

Results and Discussion

A series of experiments were performed to assess the effect of salt addition on the properties of the milk fat globule and to provide insight into the effect of salt on these globules within a curd or cheese matrix. NaCl was initially added to milk (0-25% (w/w)) or to cheese curd (0-5% (w/w)) at 38 $^{\circ}\text{C}$; these conditions were

selected to represent the wide range of salt concentrations that occur during different salting conditions, the latter being typical for the production of dry salted Cheddar and similar cheeses.

Light Scattering and CLSM Analysis of Milk Fat Globule

Low concentrations of salt (6% (w/w) or 10% (w/w)) did not significantly alter the size of fat globules, which had an average D_{43} of $3.22 \pm 0.02 \mu\text{m}$, (Fig. 2a) consistent with previous reports for raw milk [32-34]. In contrast, high concentrations of 15-25% (w/w) NaCl significantly altered the size distribution of the fat globules present in milk (Fig. 2a) resulting in fat globule rupture and coalescence. Fewer fat globules with a diameter of $\sim 3.2 \mu\text{m}$ were observed in the presence of high concentrations of salt (15% (w/w), 20% (w/w) or 25% (w/w) NaCl). Instead, a shoulder was observed corresponding to globules $\sim 10\text{-}20 \mu\text{m}$ in diameter and a peak corresponding to droplets $\sim 100 \mu\text{m}$ in diameter or larger was observed representing 10%, 12% and 47% of fat volume for samples with 15% (w/w), 20% (w/w) and 25% (w/w) NaCl respectively. A new peak corresponding to smaller fat globules, $\sim 0.2 \mu\text{m}$ in diameter was also observed.

CLSM analysis confirmed the presence of different sized fat globules in the presence of high concentrations of 15-25% (w/w) NaCl (Fig. 2c, d, e), whereas fat globules exposed to lower concentrations of 6% or 10% (w/w) NaCl appeared similar in shape and size to fat globules in the control that were not exposed to salt (Fig. 2b). Under conditions of high salt large fat globules $\sim 10 \mu\text{m}$ in diameter (Fig. 2c) and $\sim 100 \mu\text{m}$ in diameter (Fig. 2d) were observed, consistent with light scattering data. Small droplets $\sim 0.2 \mu\text{m}$ in size could also be observed under these conditions (Fig. 2e), which appear to arise from the salt induced breakup of larger fat globules. Such sub-micron sized fat globules have previously been reported in homogenized or ultrasonicated milk samples [35]. However, intermediate sized fat globules ($\sim 1 \mu\text{m}$) were not observed here, unlike these past studies that used methods other than salt addition to cause disruption.

The addition of 25% (w/w) NaCl in milk causes a significant increase in the osmotic pressure, which reaches $\sim 207 \text{ atm}$ at 20°C and up to $\sim 220 \text{ atm}$ at 38°C . This is significantly higher than the total osmotic pressure generated by the normal solutes in milk, which is $\sim 7 \text{ atm}$, close to the osmotic pressure of cow blood [36]. A higher osmotic pressure generates a pressure gradient between the aqueous and fat phases of the milk. This can then lead to the dehydration of the lipid membrane, which has previously been reported to cause membrane rupture [37]. Such changes to the structural integrity of the milk fat globule membrane would reduce the stability and emulsification of the lipid core, potentially leading to droplet breakup.

Similar pressure gradients are generated in the homogenizing valve of a homogenizer and are known to lead to intermediate sized fat globules, while the local anisotropic turbulence produces smaller sub-micron fat droplets [30, 35, 38]. Our observations here suggest that the high osmotic pressure induced by high concentrations of salt is equally disruptive to the fat globule. Spontaneous emulsification has also been observed to occur due to interfacial turbulence resulting from the Marangoni effect [39]. The addition of salt to the milk sample may cause rapid changes in local salt concentration and in the surface tension at the oil/water interface [40]. These changes create local interfacial tension that could lead to interfacial turbulence and potentially the breakup of fat droplets.

The temperature of 38°C selected to represent the temperature used in hard cheese production appears to contribute to salt induced disruption of fat globules. At a lower temperature of 25°C and in the presence of the highest concentration of 25% (w/w) NaCl, the fat droplets appeared misshapen (Fig. 3b) but the change in shape

and size was less than at 38 °C (Fig. 3c). Mixing at 25°C in the absence of NaCl did not change the microstructure of the fat globule (Fig. 3a). This difference is consistent with the calculated differences in osmotic pressure for these two conditions (~207 vs ~220 atm) for mixing at 25°C and 38°C respectively in the presence of 25% (w/w) NaCl. The fat droplet being predominantly liquid at 38°C may also be more susceptible to disruption.

The presence of NaCl also led to observable changes in the MFGM of fat globules. Circular non-fluorescent regions have previously been observed in the MFGM of fat globules in bovine milk [29, 41]. These regions are thought to correspond to the highly packed liquid ordered (Lo) domains, where Rh-DOPE cannot insert its hydrophobic tail [29, 42] resulting in a non-fluorescent region. Under the control conditions examined here, where no NaCl is added, such ‘dark’ domains are indeed observed (Fig. 4a, b). They are circular in shape, which minimizes the energy associated with the domain boundary. In the presence of a low concentration of NaCl (10% (w/w)), however, these non-fluorescent domains appear asymmetric (Fig. 4c, d). They are difficult to detect at all in the presence of high concentration of NaCl (25% (w/w)) (Fig. 4e, f), where fat droplets are disrupted.

The occurrence of the irregular shaped Lo domains in the MFGM of fat globules in the presence of NaCl is possibly due to the changes in the properties of the MFGM induced by the addition of NaCl, as has been reported for synthetic lipid bilayer membranes [19-21]. According to Böckmann et al. [19], sodium ions can bind to the carbonyl oxygens of the phospholipid molecules in the lipid membrane [19], increasing their size, leading to a lower mobility and reduced diffusion through the lipid bilayer [19]. This interaction also leads to the reorientation of the phospholipid head groups and an increase in the ordering of the lipid acyl chains, resulting in a more tightly packed membrane [20, 21].

The absence of detectable Lo domains in the MFGM in the presence of high NaCl is consistent with the disruption of fat globules and reduced stability of the milk emulsion under these conditions. However, an alternative explanation could be that changes in the MFGM packing and fluidity prevent insertion of the Rh-DOPE probe or the fluorescence of the probe may be quenched.

High concentrations of NaCl also led to the formation of a free fat layer that was visible at the surface of the milk sample (Fig. 4g). It is known that the charge potential of an oil droplet decreases with increasing NaCl concentration [43]. The reduced charge potential at the interface promotes the adsorption of colloidal particles in the case of pickering emulsions [43]. These particles can include silica, clay minerals or calcium carbonate, which contributes to emulsion stability. Here in a milk system, the reduction in the charge potential of the fat droplets leads to fat globule coalescence and the formation of a free fat layer. The addition of a large amount of NaCl to milk (i.e. 25% (w/w)) also increases the density of the sample from 1.029 g/mL to 1.170 g/mL, inducing faster separation of fat. The observation of a free fat layer is consistent with the large fat globules observed by LS and CLSM. Fig. 4f also indicates that the free fat structure is made up of completely or partially fused fat globules.

The response of milk fat globules to high concentrations of salt observed here illustrates another potential avenue for the manipulation of milk fat properties. Such lysed fat globules would behave similarly to those generated by other mechanical treatments. The use of salt for fat disruption would also involve a food safe ingredient that is relatively cheap. In Cheddar cheese production, however, fat disruption is undesirable and is typically avoided. Relatively high concentrations of salt were required to induce the observed changes,

suggesting that a second unit operation would also be required to remove the fat from the salt if this process was used; the lysed milk product was also less stable as an emulsion. Further studies would also be required to assess the potential of milk-fat pre-treated with salt, as the salt is expected to inhibit enzymatic and microbial activity associated with the formation of many dairy products.

The NaCl added to the milk is also expected to impact on the proteins present within the milk. While some insight can be obtained by studying the effect on individual proteins, the subsequent changes to these proteins as a result of coagulation and aggregation during cheese making makes their study less relevant than the study of individual fat globules that can survive intact during cheese making, we therefore chose to study the effect of NaCl on proteins that had already been coagulated by examining the structure of curd and cheese.

CLSM and Cryo SEM of Milled Curd and Cheese

The effect of different concentrations of NaCl on the microstructure of milled curd and cheese was examined by CLSM using a modification of a previous technique, allowing both the surface and internal structure of the milled curd particle to be observed. The surface microstructure of the milled curd (Fig. 5) was significantly affected by the addition of NaCl. Specifically the addition of 2.5% (w/w) NaCl (equivalent to a local concentration of ~16% (w/w) of salt in moisture within 1 mm of the curd surface) led to a denser area of protein at the surface of the curd compared to the unsalted control (Fig. 5a, b, as indicated by the arrows), when the surface was imaged to a depth of ~ 50 μ m.

The addition of 5% (w/w) NaCl (equivalent to a local concentration of ~33% (w/w) of salt in moisture within 1 mm of the curd surface) did not increase the protein density at the surface but the surface structure was coarser or rougher than the control (Fig. 5c, d). It is not clear whether these changes arise from i) the behavior of fat at these interfaces, ii) the depletion of fat or whether iii) these changes are dominated by protein hydration. The microstructure of the center of the curd particles was not perturbed by the addition of salt at either of these concentrations and the structure appeared unaltered within the center of the curd chip (Online Resource 2, Fig. 1). Salt is expected to have diffused to the center of the chip (~5 mm) by the time of the analysis (~24-48 hours) [44] but the concentration is expected to be relatively homogenous at this centre and much less than the initial local NaCl concentration experienced at the surface of the curd chip, potentially explaining the lack of differences in the microstructure of the curd center. The dynamic observation of dry salting or the future study of a model aggregated protein system may be able to indicate the extent of protein hydration and the effect of salt on water activity in order to decouple such effects.

A consequence of the salt induced changes in curd surface microstructure was that the protein within the samples treated with 5% NaCl did not fuse well at the surface of the milled curd particles prior to pressing, resulting in a highly porous surface structure for salted samples. The porosity, as measured by image analysis, significantly increased ($P < 0.05$) from 0.04 ± 0.04 (no NaCl) or 0.09 ± 0.01 , (2.5% (w/w) NaCl) to 0.21 ± 0.06 in curd with 5% (w/w) added NaCl.

No previous studies could be found on the effect of dry NaCl addition on the microstructure of milled curd. Several studies, however, have examined at the microstructure within cheeses exposed to different concentrations of NaCl in brine [6, 45, 46]. A barrier layer is known to form at the surface of a cheese block immersed in saturated NaCl or 18% (w/w) NaCl. During brining, water diffuses out from the cheese, as the salt diffuses into the cheese, resulting in a net loss of cheese weight, especially at the surface of the cheese block.

The area of the microstructure that was previously occupied by water is replaced by protein until water no longer leaves the cavity in the microstructure, creating a protein dense area described as a surface barrier layer [45]. The poorly fusing structure observed here appears similar to these surface barrier layers.

The structure of the fat was irregular where 5% (w/w) NaCl was added to the surface of the curd (Fig. 5c, d) compared to the round globules observed within the curd without NaCl or where 2.5% (w/w) NaCl was added. The sphericity of the fat globules, as assessed by image analysis (where sphericity of a sphere is 1 and non-spherical particle has a sphericity < 1), reduced slightly from 0.74 ± 0.01 or 0.75 ± 0.01 (without NaCl and with 2.5% (w/w) NaCl added respectively) to 0.71 ± 0.02 ($P < 0.05$) in curd with 5% (w/w) NaCl added. The changes in the sphericity of the fat globules with high salt could be due to the reduction in the volume of the curd as it expels more moisture with NaCl addition, which may squeeze and alter the shape of the fat globules. Alternatively it may be due to the increase in osmotic pressure near the fat globules. Previously, we have shown that fat globules in milk with a high concentration of NaCl (15-25% (w/w)) were more deformed than fat not exposed to NaCl (Figure 2 and 3). The 5% (w/w) NaCl (equivalent to ~33% (w/w) of salt in moisture concentration within 1 mm of the curd surface) investigated here is beyond the normal concentration of NaCl added during Cheddar cheese making but do provide insights into conditions when the dry salt is not well distributed, which can occur during production.

Following pressing, curd junctions were coarse and thick in the sample with 5% (w/w) NaCl added compared to the thinner junctions in the absence of salt (Fig. 6, indicated by the arrows). These junctions occupy only a small volume fraction of each particle; corresponding to ~0.2% or 0.1% of the total volume of each merged curd particle respectively resulting from a thickness of 20 μm or 10 μm in the presence or absence of salt respectively. Nevertheless, they are a significant structural feature that covers the surface of the curd particle prior to pressing and that facilitates the binding between these particles, potentially affecting the properties of the finished cheese. The microstructure of the cheese sections between the curd junctions was not significantly altered by salt treatments (Online Resource 2, Fig. 2), illustrating the importance of the salted surface as a source of structural heterogeneity within the cheese. It is important to point out, however, that the curd junctions were strongly merged in all the samples examined here.

A few studies have reported on the structure of curd junctions in cheese [28, 47-49] but the structure of fat close to the curd junction has rarely been examined due to the defatting procedure required in some conventional scanning electron microscopy techniques. Visually, the structure of the fat globules appeared qualitatively more irregular in the images for the cheese treated with high concentrations of salt. The sphericity of the fat globules around the curd junction in the cheese, however, was not found to be significantly different by image analysis ($P > 0.05$, Fig. 6); measuring 0.61 ± 0.02 , 0.65 ± 0.03 or 0.64 ± 0.02 in cheeses without NaCl added, with 2.5% (w/w) and 5% (w/w) NaCl added respectively. There were 30% fewer fat globules, however, around the curd junctions in the cheese with 5% (w/w) NaCl added (quantified from a sample area of ~400 μm x 400 μm) compared to the control sample (Fig. 6), suggesting rearrangement of the fat near the milled surface prior to pressing or the loss of fat from these surfaces prior to pressing, consistent with the protein rich surface on the outside of salted curd particles.

The difference in the microstructure of the curd junctions could possibly create different micro-environments for the cheese micro-flora that may affect the cheese sensory properties during ripening. The different structures are also expected to impact on the textural properties of the cheese.

Composition of Cheese

As expected, the moisture content of the cheese without added NaCl was significantly higher than in the salted cheeses ($P < 0.05$, Table 1), as NaCl is added during cheese production to assist moisture expulsion, allowing the target moisture content of the cheese to be reached. The higher moisture content of the unsalted cheese led to a lower percentage of fat and protein but the fat in dry matter of the cheeses was similar ($P > 0.05$), as would be expected.

The typical salt concentration of 2.5% (w/w) gave better salt retention (i.e. 66%) (Table 2) than the higher concentration of 5% (w/w) (i.e. 39%) and the addition of more salt may not provide any further advantage, as the majority of the salt was drained with the salty whey. Both treatments also gave a S/M greater than 4.5% (w/w), required for Cheddar cheese to prevent any detrimental effect to cheese quality and higher than the 5% (w/w) NaCl required to reduce any bitter flavors [50].

Texture of Cheese

The hardness of the cheeses increased significantly following NaCl addition ($P < 0.05$, Fig. 7), likely due to the reduction in the cheese moisture content (Table 1). The cheese made with 5% (w/w) NaCl was less cohesive than the cheese made without NaCl or with 2.5% (w/w) NaCl (Fig. 7), indicating that the microstructural features observed in Fig. 4 and 5, including the coarser surface microstructure and thicker curd junctions observed with 5% (w/w) NaCl, impact on the textural properties of the cheese. While the volume occupied by the curd junction is small, the heterogeneity introduced by changes to this structure appears to impact on the textural properties, potentially due to poorer bonding of the curd particles during pressing. These data therefore illustrate the important role salt plays in altering the microstructure of the curd and how these structural changes together with the associated changes in moisture content, impact on cheese properties.

Conclusion

The addition of 15-25% (w/w) NaCl to milk under conditions relevant to cheese-making (38°C) caused fat globule disruption and coalescence, as indicated by an increased number of large fat globules and the appearance of submicron sized fat globules ~120 nm to 500 nm in diameter. At high NaCl concentrations (25% (w/w) NaCl), Rh-DOPE did not appear to stain the surface of the fat globules, indicating the possible absence of the MFGM or changes induced by the high osmotic pressure. Low concentrations (10% (w/w) NaCl) led to asymmetric non-circular regions in the MFGM, indicating a change within the membrane layer. Salting also influenced the surface of the milled curd with 2.5% (w/w) NaCl addition resulting in a protein rich layer. In contrast, the addition of 5% (w/w) NaCl, resulted in a coarser surface structure with significantly increased porosity. These insights could be obtained using a cryo-SEM technique that allows both the surface and the internal structure of the curd particle to be visualized. After pressing, the curd junctions were coarse and thick within the sample salted with 5% (w/w) NaCl or fine and thin within the unsalted or 2.5% (w/w) NaCl samples. Salt retention was optimal at 2.5% (w/w) compared to 5% (w/w), as the majority of the salt was drained with the salty whey with higher salt concentrations. The changes in the surface microstructure of the curd also affected the fusing of curd particles during pressing, leading to poor bonding of salted curd particles and a lower measure of cohesiveness for these cheese samples.

While NaCl addition is important in cheese making, high concentrations can occur locally if salt is not evenly distributed. These data show that such high concentrations (e.g. 5% w/w of curd) not only lead to poorer salt retention but also lead to changes in the microstructure and texture of the cheese due to the effect on individual fat globules and larger surface features, such as the curd junction. Manufacturers therefore need to ensure that NaCl is distributed evenly during dry salt addition to improve the quality and consistency of the final cheese product.

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Fig. 1 Sample preparation technique for Cryo SEM observation of a milled and salted curd chip. Cryo SEM of the salted curd surface (a) and the center of the curd chip (b). FG = fat globule. The scale bars are 20 μm and 50 μm in length for images (a) and (b) respectively.

Fig. 2 The effect of NaCl addition to fat globules in milk (a) Light scattering analysis of milk with different concentrations of added NaCl. The data obtained for the control milk without NaCl(—), the milk with 6% (w/w) NaCl(---) and with 10% (w/w) NaCl(—) are similar and appear superimposed. Milk with higher concentrations of NaCl are 15% (---), 20% (—) or 25% (w/w) (· —). Representative CLSM images are shown for milk without added NaCl (b), with 20% (w/w) NaCl added (c, e) and with 25% (w/w) NaCl added (d). The Nile red stained triacylglyceride core appears red and the Fast green stained protein appears green in these images, the scale bars are 10 μm in length for images (b), (c) and (e) and 100 μm in length for image (d).

Fig. 3 CLSM images of the effect of temperature and salt on milk fat globule shape. Milk samples were mixed at 1000 rpm for 20 minutes at 25°C with no NaCl (a), at 25°C with 25% (w/w) NaCl (b) or at 38°C with 25% (w/w) NaCl (c). The arrows in Figure (b) indicate misshapen fat globules that are a possible effect of the high osmotic pressure caused by the addition of salt. The fat core labelled with Nile red appears red and the Fast green stained protein appears green in these images. The scale bar is 20 μm in length in (a) and (b) and 40 μm in length in (c).

Fig. 4 CLSM images of the microstructure of the milk fat globule membrane (MFGM) in milk without NaCl (a-b), in the presence of 10% (w/w) NaCl (c-d) and in the presence of 25% (w/w) NaCl (e, f) after mixing at 38°C, 1000 rpm for 20 minutes. Image of duplicate samples taken with a digital camera (g) showing the separation of fat in the sample when 25% (w/w) NaCl is added. White arrows indicate non-circular non-fluorescent regions of the MFGM in (c) and (d). The MFGM labelled with Rh-DOPE appears red in images (a-f). The scale bar is 3 μm (b, d), 5 μm (a, c) or 10 μm (e, f) in length. The microstructure observed was representative of two separate trials.

Fig. 5 The microstructure near the surface of the milled curd without the addition of NaCl (a) and after the addition of 2.5% (w/w) NaCl (b) or 5% (w/w) NaCl (c, d) observed using cryo SEM and 2D- or 3D-CLSM. The scale bars are 20 μm in length for the cryo SEM images and 3D-CLSM images and 10 μm in length for the 2D-CLSM images. FG indicates a fat globule and the arrows indicate the dense protein structure present at the surface of the milled curd particle. The fat appears red and the protein appears green in these CLSM images. The microstructure observed was representative of two separate trials.

Fig. 6 Curd junction within the microstructure of cheese without added NaCl (a), after the addition of 2.5% (w/w) NaCl (b) and after the addition of 5% (w/w) NaCl (c) observed using CLSM (left and middle images) or cryo SEM (right images). The scale bar is 50 μm , 25 μm or 20 μm in length for the left, middle and right images respectively. The white arrows indicate curd junctions that contain a dense protein structure. The fat appears red and the protein appears green in these CLSM images. The microstructure observed was representative of two separate trials.

560 **Fig. 7** The hardness (—) and cohesiveness (---) of cheese without NaCl or following the addition of 2.5% (w/w)
561 or 5% (w/w) NaCl. The data are the mean $\pm$ the standard deviation of the mean (n = 2 data points).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Table 1. Composition of the cheese made with different concentrations of NaCl added[#]

NaCl added (% w/w curd)	Fat in cheese (% w/w)	Protein in cheese (% w/w)	Moisture in cheese (% w/w)	FDM (% w/w)
0	33.5 ± 0.0 ^a	22.7 ± 0.0 ^a	38.6 ± 0.3 ^a	54.5 ± 0.2 ^a
2.5	34.7 ± 1.1 ^b	25.5 ± 0.4 ^b	35.6 ± 0.6 ^b	53.8 ± 1.4 ^a
5.0	37.2 ± 0.2 ^c	24.9 ± 0.6 ^b	32.4 ± 0.3 ^c	54.9 ± 0.2 ^a

[#] Results are presented as the mean ± the standard deviation of the mean (n = 4 from 2 independent trials). ^{abc}Data in the sample column with different superscripts are significantly different ($P < 0.05$). FDM = fat in dry matter.

Table 2. The percentage of salt lost to the salty whey or retained in the cheese[#]

NaCl added					
(% w/w curd)	NaCl in cheese (% w/w)	NaCl in whey (% w/w)	S/M (% w/w)	NaCl retention (% w/w)	NaCl lost (% w/w)
2.5	1.8 ± 0.1 ^a	7.7 ± 0.8 ^a	5.1 ± 0.3 ^a	66.1 ± 3.8 ^a	30.8 ± 1.7 ^a
5.0	2.3 ± 0.1 ^b	14.5 ± 0.2 ^b	7.1 ± 0.3 ^b	38.6 ± 1.5 ^b	47.7 ± 1.7 ^b

[#] Results are presented as the mean ± the standard deviation of the mean (n = 4 from two independent trials). S/M = salt (NaCl) in moisture.

Salting Affects the Structure of Individual Fat Globules and the Microstructure of Dry Salted Cheddar Cheese

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Online Resource 1

The cheese-making process described in the Material and Methods resulted in milled curds with an average moisture content of 40% w/w. The average dimension of each curd chip after milling was ~6 cm x 1 cm x 1 cm. The average weight of each curd chip was ~10 g and the average density was 1.67 g cm⁻³.

Two concentrations of NaCl were examined, 2.5% w/w and 5% w/w. The estimated concentrations of NaCl per unit weight of curd penetrated by NaCl and the concentration of NaCl per weight of moisture within the curd assumed to be penetrated by NaCl were calculated as follows:

Weight of curd penetrated by NaCl (1):

The total volume of each curd chip was 6 cm³. The volume of curd penetrated by the salt was 2.3 cm³, assuming the salt penetrated to a depth of 0.1 cm uniformly across the curd surface. The weight of curd penetrated by NaCl is 3.8 g, obtained by multiplying the penetrated curd volume of 2.3 cm³ by the density of 1.67 g cm⁻³.

Weight of NaCl added to each curd chip (2):

When 2.5% w/w NaCl or 5% w/w NaCl was added to the curd mass, each 10 g curd chip was exposed to ~0.25 g or ~0.5 g of NaCl respectively.

The estimated concentration (%w/w) of NaCl per unit weight of curd penetrated by NaCl (3):

$$\frac{\text{Weight (2)}}{\text{Weight (1)}} \times 100$$

For 2.5% w/w NaCl addition this gives ~6.6 % and for 5% w/w NaCl addition this gives ~13.1%.

The estimate for the percentage of salt in moisture (S/M) or concentration of NaCl per weight of moisture within the curd penetrated by NaCl, where the moisture is 40% (4):

$$= \frac{\text{Weight (2)}}{\text{Weight (1)} \times 40 \% } \times 100$$

For 2.5% w/w NaCl addition this gives ~16.4 % S/M and for 5% w/w NaCl addition this gives ~32.8% S/M.

These calculations provide an estimate of the concentration of NaCl in the initial 0.1 cm surface layer based on the assumption that all salt dissolves evenly on the surface of the curd chip. In practice, not all curd chips are exactly identical in shape and size, not all the salt dissolves, some dry salt also sticks to the surface of the cheese vat. These make the accurate estimation of the concentration of NaCl at the surface of a curd chip in a dry salting system difficult and have been excluded from these calculations.

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Online Resource 2 Fig. 1 The microstructure of the center of milled curd particles without NaCl added (a), with 2.5% (w/w) added NaCl (b) or with 5% (w/w) added NaCl (c) as visualized by CLSM. The scale bar is 10 μm in length. The fat appears red and the protein appears green in these images.

Online Resource 2 Fig. 2 The microstructure of cheese in an area without curd junctions (i.e. in the centre of the fused milled particles), as determined by CLSM. The cheese was made without added NaCl (a), with 2.5% (w/w) added NaCl (b) or with 5% (w/w) added NaCl (c). The scale bars are 10 μm in length. The fat appears red and the protein appears green in these samples.