Formation of Cheddar cheese analogues using canola oil and ultrasonication – a comparison between single and double emulsion systems

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Abstract

Cheddar cheese analogues were produced from skim milk in which canola oil was emulsified using ultrasound to form either single (O/W) or double emulsions (W1/O/W2). The double emulsion cheese analogues (DECH) had a distinct microstructure and retained small skim milk droplets, dispersed in the fat phase, for more than 7 months of aging at 4 ºC. The single emulsion cheese analogues (SECH), prepared with the same fat content as control cheeses, produced comparable yields of cheese and whey, with similar composition, although the fat droplets were more spherical and showed greater coalescence. The DECH cheese with skim milk encapsulated in the oil droplets, was harder, melted less and showed more free fatty acid development over 7 months of aging than the control cheeses. The SECH cheeses were softer than the control and also melted less effectively but did not show greater free fatty acid development.

Keywords: Microstructure, free fatty acids, texture, canola oil, cheese analogues, emulsification

1. Introduction

Developing reduced-fat cheeses with comparable sensory attributes to full-fat cheese is challenging but has considerable commercial potential. One promising approach is the use of double emulsions (Garti, 1997), in which emulsion droplets themselves contain an inner emulsion of the opposite phase. For reduced fat cheeses, a water-in-oil-in-water type emulsion (W1/O/W2) can be used to displace some of the fat present in cheese with water. In such a system, water droplets can be emulsified within emulsified fat droplets that are themselves surrounded by the aqueous phase of the cheese.

Simply reducing the fat content by omission detrimentally affects texture, making the cheese firmer. By including water within the fat droplets, double emulsions can potentially reduce fat without significantly altering cheese structure. A double emulsion droplet can effectively occupy the same volume as a fat droplet in a regular emulsion, but with the calorific content reduced due to displacement of the oil with internalised water droplets. Double emulsions have been proposed to reduce the fat content of various food products including salad dressings (Gaonkar, 1994), Gauda cheese (Felfoul, Bornaz, Baccouche, Sahli, & Attia, 2015) and white fresh cheeses (Lobato-Calleros, Rodriguez, Sandoval-Castilla, Vernon-Carter, & Alvarez-Ramirez, 2006; Lobato-Calleros et al., 2007; Lobato-Calleros et al., 2008). However, achieving stability is a key challenge in the development of double emulsion products. The need for large amounts of surfactant to stabilise both the inner and outer phases (Matsumoto, Kita, & Yonezawa, 1976) has restricted the use of double emulsions in the dairy industry due to the cost and the use of non-dairy emulsifiers such as Span 80, Tween 80 (Felfoul et al., 2015), DATEM (Lobato-Calleros et al., 2008). There are ongoing efforts, however, to use natural emulsifiers like proteins and polysaccharides as replacements for synthetic surfactants (Benichou, Aserin, & Garti, 2002; Shanmugam & Ashokkumar, 2014).

Recently, an approach that reduces the amount of synthetic emulsifier needed for stable double emulsions was reported by Leong, Zhou, Kukan, Ashokkumar, and Martin (2017) where double emulsions were prepared using sunflower oil, in which the internal water droplets were stabilized by small amounts of lipophilic emulsifier and the outer oil droplets were stabilized by proteins in the skim milk. As the bulk aqueous phase in these preparations is skim milk, this ingredient has potential to be readily transformed into cheese-like products. In principle, these emulsions could be produced with droplets of similar size to fat globules in cheese milk. This modified milk stream could then be used to create a cheese microstructure similar to full-fat cheese but with the fat content reduced by the skim milk entrapped in the internal phase.

Ultrasonication is an effective method to create double emulsions with a comparable efficiency to conventional homogenization and rotor stator type devices (Walstra, 1993). An advantage of ultrasound is that the applied energy density can be readily tuned to tailor the droplet size and enable a high yield of encapsulation in the double emulsions (Tang & Sivakumar, 2012; Tang, Sivakumar, & Nashiru, 2013). The ability to control droplet size could also help improve the sensory quality of reduced fat products

(Goudédranche, Fauquant, & Maubois, 2000). The ability to produce small double emulsion droplets at a high yield with a high displaced volume of fat, would provide complementary benefits due to the increased effective fat volume and increased surface area. Ultrasonication can also provide synergistic effects, such as partial denaturation of proteins that can help improve emulsion stability (Shanmugam & Ashokkumar, 2014). The effects of ultrasonication on protein denaturation (Chandrapala & Leong, 2015; Stathopulos et al., 2004) and/or lipid oxidation (Chemat et al., 2004; Juliano et al., 2014), however, need to be characterized further in the context of finished cheese products, as these factors can potentially affect cheese functionality and flavour development.

Double emulsions can be used to displace fat in cheese and also to replace dairy fat with less expensive oils of plant and vegetable origin. For example, canola oil is less expensive than milk fat and has a higher proportion of healthy polyunsaturated and ω-3 fatty acids. The use of non-dairy liquid oils in cheese production has to date been limited. So far, olive oil (Felfoul et al., 2015) has been used for the production of Gouda cheese, while canola oil has been evaluated in the production of soft white cheese (Lobato-Calleros et al., 2007). No study has yet evaluated the use of ultrasonically prepared double emulsions for the creation of Cheddar-cheese analogues. Furthermore, there is, to our knowledge, no prior studies that have evaluated the use of ultrasonication to produce simple emulsions formulated with liquid vegetable oils that can be converted into hard cheese-like products.

This study investigates the properties of pressed Cheddar Cheese analogues created from single and double emulsions of canola oil using ultrasonication. The cheese analogues are compared to an established Cheddar cheese model using milk fat from cream. The effects of the ultrasonic treatment on the protein and fat content of the milk used to make cheese, the cheese itself and the whey was assessed. The microstructure and functional properties of the cheeses, including melting capability and texture were analysed. This study provides information that will be useful for developing new cheese products with a reduced and modified fat content.

2. Materials and methods

2.1 Materials

Canola oil (Woolworths, Bella Vista, Australia) was used in the single and double emulsions. Polyglycerol polyricinoleate (PGPR) (kindly provided by Mondelez, South Melbourne, Australia) was used at a fixed loading of 2 wt % of the oil phase to stabilise the inner W1/O emulsion of the double emulsion (Leong, Zhou, Zhou, Ashokkumar, & Martin, 2018). Pasteurised and homogenised skim milk (Pauls Dairy, Lactalis, South Brisbane, Australia) containing 4.2% total protein and <0.1% w/v fat was used as the bulk phase of the cheese milk (i.e. it was the base of the milk formulation used for the production of the model and analogue cheeses. However, it should be noted that the skim milk composition may be subjected to

seasonal variability. Thickened heavy cream (Gippsland Dairy, Dandenong South, Australia) with a fat content of ~55 %, was used as the fat source for the control cheese milk. Rennet (200 IMCU/mL Hannilase, Chr. Hansen, Bayswater, Australia) and cheese salt (Crown Pure Dried Vacuum salt, supplied by Cheetham Salt, Melbourne, Australia) were provided by an Australian dairy company.

2.2 Ultrasonic emulsification procedure

Double emulsions were prepared via two-step emulsification (Leong et al., 2017). Firstly, a 30% w/w skim milk-in-canola oil emulsion was produced using a 20 kHz Hielscher disruption horn (1 kW, Hielscher, Teltow, Germany). The concentration of PGPR used in the oil phase was 2 wt% of the oil phase unless otherwise stated. Sonication was performed at 100 W calorimetric power (using an amplitude setting of 80%) for 10 min (specific energy ~ 55 J/g), ensuring a homogenous emulsion was formed without any un-emulsified aqueous phase. The horn tip was fixed above the visible oil/water interface, approximately 40-50 mm from the bottom of the container.

The formed W1/O emulsion was then added to an external skim milk phase at an overall loading of 5.5 wt%, resulting in a final fat concentration of 3.87 wt%. The total mass of emulsion formed was 20 kg. The W1/O emulsion was first coarsely dispersed into the skim milk using an Ultra-Turrax mixer (Ika, Staufen, Germany) at 13,500 rpm for 20 minutes. The emulsion was then fed through a continuous flow-through cell attached to the Hielscher 1 kW disruption horn at a rate of 0.5 L/min. The power setting was maintained at 100 W calorimetric power input (80 % amplitude setting). The emulsion was passed through the cell, collected, pooled and subjected to a second pass at the same ultrasonic and flow settings. The calorimetric energy delivered during the emulsification of the W1/O emulsion into the milk to make the double emulsion was *ca* 24 J/g.

A single-step emulsification process was used to form the single emulsion. The single emulsion did not contain any foreign surfactants and was entirely stabilized by the proteins in the skim milk. The concentration of fat in the single emulsion was 4.76 wt%. The flow and ultrasonic settings were the same as those used to create the secondary emulsion for the double emulsion i.e. ~24 J/g calorimetric energy.

20 kg batches of formulated milk were used for cheese making. The control milk was standardised using cream and skim milk to achieve a target fat content of 4.76 wt% using the protein and fat compositions specified by the manufacturer. The single emulsion cheese milk was formulated to match the fat content of the control. The target fat content in the double emulsion cheese milk was lower (3.87 wt%) relative to the single emulsion and control preparation (4.76 wt%) and was based on the assumption that approximately 15-20 % of the fat droplet volume would be occupied by the encapsulated water phase (Leong et al., 2017; Leong et al., 2018).

2.3 Cheese making

Cheeses were prepared using a Cheddar cheese model adapted from Ong et al. (Ong, Dagastine, Kentish, & Gras, 2012, 2013) The experimental design consisted of 3 cheeses, denoted as the control Cheddar (CONCH), single emulsion Cheddar (SECH) and double emulsion Cheddar (DECH) cheeses. Batches of two different cheese types were made each day for 3 separate days, resulting in duplicate batches (denoted by the suffixes 1 and 2) of each type of cheese. The prepared emulsions and milk samples, referred to here as cheese milk, were fed into a 20 kg capacity stainless steel vat and warmed to 31 °C. A *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* (Type A Starter, Cheese Links, Lara, Australia) starter culture was added at a rate of 0.05 g/kg. Milk was ripened until the pH dropped by 0.1 units. Rennet was added at 100 µL/kg. Gelation proceeded for a minimum of 40 minutes at 33 °C. Gels were cut into ~1 cm cubes once a firm gel was formed.

Curds were cooked in the vat for at least 80 min while the stirring rate and temperature were increased gradually from 12 rpm to 30 rpm and 33 °C to 38 °C, respectively. The curds were drained at completion of cooking (when the pH decreased to ~6.1) and the composition of the sweet whey was determined. Cheddaring of the cooked curd was performed for at least 90 min until a pH of 5.5 was reached. Curds were milled into ~5 cm chips and salted at a dosage rate of 2.5% w/w. Salted chips were packed into a cheesecloth-lined mould and pressed overnight at 50 psi. Salty whey was collected for compositional analysis. Finished cheeses were vacuum packed and stored at 4 °C.

2.4 Fat analysis by Babcock method

The fat content of the cheese and whey samples was analysed using the standard AOAC Babcock method for cream/cheese (AOAC Official Method 920.111B-C) and for milk (AOAC Official Method 989.05) respectively. Samples of defined weight were measured (9 g or 18 g for cheese and whey respectively) and transferred into Babcock bottles (Thermo Fischer Scientific, Scoresby, Australia). Sulphuric acid (90%, RCI Labscan, Bangkok, Thailand) was added (19.6 mL) into the bottles to digest proteins in the samples. Samples were incubated at 60 °C and then centrifuged for 5 min at 166 x g (755 rpm) and 50 °C using a Funke Gerber SuperVario-N centrifuge (Funke Gerber, Berlin, Germany) with a Head A rotor (Thermo Fischer Scientific, Waltham, Massachusetts, USA). Warm water (60 °C) was added to the tube to reach the top of the bulb and centrifuged for a further 2 minutes. Warm water was then added to the tube to maintain the level within 12 mm of the highest reading value on the tube and centrifuged for a further 1 minute. Tubes were incubated at 60 °C with the water level near the height of the reading. The fat content was determined by reading the level of fat present in the tube.

2.5 Protein analysis by Dumas Combustion method

A LECO Trumac CNS analyser (LECO Corporation, St Joseph, Michigan, USA) was used to quantify the protein content of the samples, using an ISO 1489 standard method. Accurately weighed samples of whey

(~ 2 mL) or cheese (~ 0.1 g) were dried overnight at 104 °C in nickel-lined ceramic boats and combusted at 1100 °C within the furnace of the LECO analyser.

2.6 Solids and moisture content

The solids and moisture contents of milk and cheese samples were determined by thermogravimetric analysis according to the AOAC standard method (Horwitz, 1975). Samples were weighed on a sample holder and dried overnight in an oven at 104 °C. The dried samples were then re-weighed to determine the moisture loss.

2.7 Fatty acid profiling by gas chromatography (GC) analysis

Lipids from the finished cheeses were extracted for fatty acid profiling following a previous method (De Jong & Badings, 1990). The extracted lipid was transesterified as follows. Approximately 10 mg of the lipid was added to 4 mL of chloroform/methanol (1:2 v/v ratio) and vortexed to dissolve. Solvents were supplied by RCI Labscan, Bangkok, Thailand. The dissolved lipids were transferred to a vial to which 100 µL of 10 wt% H_2SO_4 in methanol was added. A stirring bead was added to the vial and stirred at 55 °C in a water bath on the magnetic stirrer (ISG Group, Norfolk, UK) for 3 hours. An aliquot (500 µL) of 25 wt% $KHCO_3$ (Univar, Downers Grove, Illinois, USA) in methanol was then added to the lipid solution to neutralize the acid and undergo basic transesterification. The reaction proceeded for 2 hours at 55 °C with stirring. The final solution (1 mL) was filtered through a 0.22 µm filter (to remove precipitates/salts into a GC vial. The fatty acids were analyzed using a gas chromatography unit (GC-2010 plus with AOC-20i Auto-injector, Shimadzu, Kyoto, Japan) fitted with a wax column (Econo-cap Carbowax, 30 m x 0.25 mm x 0.25 µm, Alltech) and flame ionization detector. The total gas flowrate through the injection port was 34.5 mL/min, with He as the carrier gas. The column flow was 1.50 mL/min and the purge flow (N_2) was 3.0 mL/min. Gas flow in the flame ionization detector was set at 30 mL/min (N_2), 40 mL/min (H_2) and 400 mL/min (air). The injection volume was 1 µL and the elution time was 30 min. Peaks were identified by matching against a supplied standard curve.

2.8 Assessment of free fatty acid (FFA) development

The free fatty acid content in the extracted lipids was determined by titration. A known quantity of lipid was dissolved in ethanol, to which several drops of phenolphthalein indicator (Sigma Aldrich, St. Louis, Missouri, USA) was added. The dissolved lipids were titrated with sodium hydroxide (Chem Supply, Gillman, Australia) until a pale pink colour was noted. The amount of base required was used to calculate the titratable acidity present in the dissolved lipid. The FFA content was calculated on a basis that it consisted primarily of oleic acid.

2.9 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE gels were run using pre-cast 12% Criterion TGX 18-well gels in a Bio-Rad Criterion cell (Bio-Rad Laboratories, Hercules, California, USA). The running buffer was a tris/glycine/SDS buffer (Bio-Rad Laboratories) prepared fresh on the day of running. Samples were first diluted to a standard nitrogen content

of 1 mg/mL using Milli Q water and then combined with 2 x Laemmli sample buffer (Bio-Rad Laboratories) and mercapto-ethanol (Sigma Aldrich, St. Louis, Missouri, USA) at a ratio of 1:1. Samples were then heated at 90°C for 10 min to completely denature the proteins in the sample. Samples (20 µL) were loaded into the gel, along with a broad-range protein standard (Bio-Rad Laboratories). Gels were run for ~43 minutes at 200 V. Staining was achieved by first washing the gels with water, then staining in Coomassie Biosafe (Bio-Rad Laboratories) for 60 min in a container with gentle mixing. De-staining was achieved by gentle mixing with water for 30 min. Stained gels were then loaded into a Bio-Safe Gel-Doc (Bio-Rad Laboratories) unit for imaging.

2.9 Confocal laser scanning microscopy (CLSM)

The microstructure of the cheese samples was analysed using a Leica SP8 inverted confocal microscope (Leica Microsystem, Heidelberg, Germany), following Ong, Dagastine, Auty, Kentish and Gras (2011). Nile red (1 mg/mL in dimethylsulphoxide ; Sigma Aldrich, St. Louis, Missouri, USA) was diluted 10 times with distilled water (purified to 18.2 mΩ) and added directly to the cheese samples that were cut into 2 mm cubes with a surgical blade. Excess stain was removed after 5 min with a pipette followed by the addition of Fast Green FCF (0.1 mg/mL in distilled water; Sigma Aldrich, St. Louis, Missouri, USA) for 5 min. After excess FCF stain was removed, the samples were placed on a microscope slide and secured with a 0.17 mm coverslip (Pro Sci Tech, Thuringowa, Australia). Fast green and Nile red were excited at wavelengths of 633 nm and 488 nm, respectively, with the emission filters set at 660-750 nm and 520-590 nm, respectively.

2.10 Texture analysis

A TA.HD plus Texture Analyser (Stable Microsystems, Surrey, UK), with 50 kg load cell, was used to measure the texture properties of the cheese samples. Cheese samples were cored using a 25 mm diameter corer and cut into cylinders of 25 mm height. The cylindrical samples were equilibrated to ~19 °C inside an enclosed container. Samples were measured using a 35 mm aluminium cylinder attachment (part P/35). The analysis was based on the inbuilt TPA test template, for determination of hardness. At least 4 replicate measurements were made per cheese sample.

2.11 Melting properties

A modified Schreiber melt test was used to measure the spread radius of melted cheeses. Cylindrical discs of cheese with a diameter of 36 mm and 10 mm height were prepared using a cheese corer. Samples were stored at 4 °C in a sealed container prior to testing. Cheeses placed on a glass petri dish were heated in an oven at 130 °C for 10 min. The radius of the outer rim of the melted circle of cheese was measured at 6 different points using a pre-drawn measuring grid.

The melting points of the cheeses were assessed using temperature sweep tests performed on a rheometer. Cylindrical discs of cheese with thicknesses of ~ 5 mm and diameters of 40 mm were prepared using an

egg slicer. Discs were positioned onto a parallel plate geometry with a gap of 4900 μm on an AR-G2 rheometer (TA Instruments, New Castle, Delaware, USA). The temperature of the cheese was increased from 10 °C to 90 °C with temperature steps of 5 °C with a 3 min holding time at each temperature point. Measurements were performed using a constant strain of 0.5%. The melting point was assessed as the temperature at which the storage and loss moduli (G' and G'') were of equal value.

2.12 Particle size measurements

The size of particles (fat/emulsion droplets and casein micelles) in the cheese milk samples diluted with distilled water was measured using a Malvern Mastersizer 3000 (Malvern Panalytical, Malvern, UK) fitted with a Hydro-G3000 accessory. Refractive index and absorption valves of 1.462 and 0.001, respectively were inputted into the software.

2.13 Statistical analysis

The statistical significance of the data was assessed where appropriate, using a one-way ANOVA combined with the Tukey test in Minitab 17 (Minitab Pty. Ltd., State College, Pennsylvania, USA) with a 95% confidence interval.

3. Results and discussion

3.1 Cheese appearance, composition and yield

The three cheeses (CONCH, SECH and DECH) behaved similarly during production, with no notable differences in milk ripening, renneting or cooking steps. During cheddaring, the cooked curds of the emulsion (DECH and SECH) cheeses initially appeared to undergo less complete matting, as also occurs when Cheddar cheese is made with homogenized milk, where the fat differs in structure to unhomogenised milk (Peters, 1956). After pressing, however, the curds of the emulsion cheeses adhered satisfactorily.

The cheeses differed in appearance after pressing, as shown in Fig. 1, where the CONCH cheese appeared more yellow than the paler cheeses made using canola oil (SECH and DECH) due to the presence of β-carotene in the milk fat, which is lacking in the canola oil.

The aroma and texture of the emulsion cheeses were also noticeably different to those of the control cheeses. While not unpleasant, the canola oil cheeses lacked the buttery aroma of the control cheese, presumably due to the lack of short-chain fatty acids (see section 3.5 below) in the canola oil. As canola oil is liquid at room temperature, the single and double emulsion cheeses also felt more 'greasy' on handling than the control. The texture and functionality are further assessed in section 3.3.

The cheese yield (reported as the mass of cheese produced relative to the mass of cheese milk used) of SECH cheese was similar to that of the CONCH cheese. Whilst the moisture content was higher for the DECH cheese (4% higher than CONCH and SECH), the overall cheese yield was significantly lower than

for the CONCH cheese and SECH cheese (Table 1), consistent with the higher loss of fat into the sweet whey during production (Table 1).

The cooked curds made from the DECH preparation consistently contained more moisture before pressing (data not shown). This higher moisture content could be attributed, at least in part, to the skim milk aqueous phase retained within the oil droplets of the DECH preparation (the difference in water content corresponded to approximately 15% of the oil volume). The protein content of the DECH cheese was also ~4% higher than in the CONCH and SECH cheese. In contrast, the moisture, protein and fat composition of the CONCH cheese were within the typical range expected for the Cheddar cheese model (Ong, Dagastine, Auty, Kentish, & Gras, 2011).

After pressing, the DECH cheese had a fat content that was ~¾ the level of fat in the full fat CONCH cheese (Table 1). The formulation was designed to reduce the fat content of the DECH cheese milk by ~20% compared to the full-fat CONCH and SECH milk. The fat content of the DECH cheese was slightly lower still following cheesemaking, due to the increased loss of fat in the sweet whey. As expected, the SECH cheese had a similar moisture, protein and fat content to the CONCH cheese.

The increased loss of fat in the sweet whey of the DECH does not appear to result solely from differences in fat droplet size between the milk preparations, as the size distribution of the oil droplets in the DECH emulsion appeared to contain droplets of a similar size range (~ 1-20 μm) to those in the COCH preparation with some larger particles (~20-60 μm) , as also seen in the SECH emulsion (Fig.2). The presence of PGPR in the oil phase of DECH may have resulted in altered interactions between the casein matrix and the oil phase during casein aggregation and gel network formation, compared to protein stabilized oil in SECH. The effect of PGPR containing oil in the kinetics of rennet gel formation and fat retention would need further investigation to elucidate the reason for higher fat loss in DECH cheese. Nonetheless, preliminary trials performed with increased sonication energy producing emulsions with smaller droplets appeared to reduce the amount of fat present in the sweet whey (results not shown), suggesting this could be a promising route to further optimisation.

3.2 Cheese microstructure

The microstructure of the three cheeses was examined using confocal laser scanning microscopy (Fig. 3) approximately 1 week after production and then again after 3 months and 7 months of refrigerated storage at 4 °C.

The SECH and DECH cheeses contained large pores, or areas of black coloration, present in the CLSM images collected at 1 week; these areas correspond to regions that are not stained by the protein or fat specific dyes, where the serum phase or air pockets are present (Ong et al., 2012). Similar large pores were not observed in the CONCH cheeses, which have a microstructure typical of this Cheddar cheese model (Ong et al., 2011). The pores were largest in the DECH cheeses (Fig. 3, marked by 'L') but were not

present after 3 months, although there were potentially more small pores remaining in the SECH and DECH cheeses than in the control cheeses. The disappearance of the very large pores could be due to the protein structure rearranging over time to redistribute the serum phase within the curd matrix (Hassan & Awad, 2005; Ramkumar, Creamer, Johnston, & Bennet, 1997) and fill the spaces occupied by air .

The oil droplets in the emulsion-based DECH and SECH cheeses appeared more spherical than the fat globules in the control CONCH cheeses, possibly due to canola oil being liquid at room temperature, which would allow the droplets to remain spherical during pressing, minimising surface area. In addition, there appeared to be more very small oil droplets (<2 μm) in the SECH and DECH cheeses, similar small droplets were seen in the particle size distributions for the SECH preparation but not the DECH preparation shown in Fig. 2. With aging, the larger oil droplets present in the SECH and DECH appear to have coalesced, whereas the smaller droplets remained dispersed throughout the protein matrix. Less coalescence was observed in the CONCH cheeses.

The overall microstructure of the fat in the SECH and DECH cheeses appears similar. Upon closer examination, however, it is evident that some of the oil droplets in the DECH cheese contain very small dark spots (Fig. 3), representing the encapsulated droplets of skim milk that comprises the inner phase of the double emulsion. After aging for 3 months, the smaller dark droplets remain (see inset of Fig. 3, DECH month 3) but the larger dark droplets (Fig.3, white arrow) that are observable in the fat after 1 week, are no longer present. This reflects the high stability of the smaller encapsulated skim milk droplets dispersed within the oil droplets. The larger encapsulated skim milk droplets were less stable and were eventually lost due to inter-droplet coalescence and droplet growth/shrinkage due to osmotic pressure (Florence & Whitehill, 1981).

Although there is less fat in the DECH cheese than the SECH and CONCH cheeses, the apparent volume occupied by the droplets was not lower (Fig. 3) due to the encapsulated skim milk, which increases the apparent volume of the fat droplets. Hence double emulsions provide the benefit of enhancing the effective fat volume in the microstructure. Furthermore, due to the presence of smaller oil droplets distributed in the emulsion cheeses, there may be additional sensory advantages such as creating a ‘creamier’ sensation (Goudédranche et al., 2000), although this would require confirmation by sensory analysis.

3.3 Cheese texture

The influence of the type of fat and the resulting changes in microstructure on the texture of the cheese was assessed. The SECH cheese formed with the canola oil was noticeably easier to cut than the CONCH cheese. Texture analysis confirmed that the hardness of the SECH cheese was significantly lower than the CONCH cheese (Fig.4). Given the composition in the two types of cheese were comparable (Table 1), the relative softness of the SECH (Fig. 4) can be attributed to the oil, which is liquid at room temperature, as well as differences in the microstructure (Fig. 3).

The DECH cheese was the hardest of all the cheeses tested (Fig. 4) and has the lowest fat content (Table 1). Reduced fat cheeses generally have a harder texture than their full fat counterparts, as there is proportionally more protein matrix to enhance the physical strength (Bryant, Ustunol, & Steffe, 1995). The DECH cheese was ~43% harder than the SECH cheese and had a fat content of 26%, compared with 35% fat in the SECH cheese, both of which had the same liquid oil. According Bryant et al., a reduced-fat Cheddar with a fat content of ~26% would be expected to be ~60% harder than the control full fat cheese (Bryant et al., 1995), although this relationship is also influenced by the moisture content of the cheese. The difference between the expected hardness and the hardness observed for DECH may be due to the encapsulated skim milk expanding the effective volume of the fat droplets thereby diminishing the effects of the reduction in fat. Whilst a higher water content can soften a cheeses (Bryant et al., 1995), the increased water content in the DECH (Table 1) can be accounted for by the encapsulated skim milk that would not soften the protein matrix. The combination of the liquid oil and the skim milk encapsulation meant that the DECH was also only 19% harder than the CONCH despite having much lower fat than this dairy control sample, in which the fat content was solid.

3.4 Melting properties

The meltability of the cheeses was assessed using a modified Schreiber test (Fig. 5). Relative to the control, the emulsion cheeses had lower melt radii. Interestingly, the emulsion cheeses tended to form a 'skin' during heating that appeared to limit the spreadability of the cheese upon melting. This could be due to homogenised oils in the emulsion cheeses being coated with casein, causing the oil droplets to interact more strongly with the protein network that forms during cheese making. The increased interaction can potentially restrict the melting and stretching of the cheese (Lelievre, Shaker, & Taylor, 1990). The large number of small droplets present in the emulsion cheeses (Fig. 3) would potentially have resulted in many such interactions.

A temperature sweep test was also performed using a rheometer to determine the melt temperature of the cheeses. Sample temperature sweeps for the different cheeses are presented in Fig. 6. During the temperature sweep, the CONCH cheeses had higher storage and loss moduli compared to the DECH and SECH at low temperatures. The crystallised native milk fat globules in cold CONCH cheeses could be the reason for the higher elasticity observed at low temperatures. The liquid canola oil droplets in DECH and SECH could also have reduced the elasticity of these samples. With an increase in temperature, however, the moduli of the CONCH cheeses decreased rapidly due to the conversion of milk fat from a solid to liquid state. The measured melting points were ~55 °C CONCH, ~65 °C DECH and ~80 °C SECH. A possible reason for the higher melting point of the SECH cheese is the presence of more small oil droplets relative to the DECH cheese. These droplets, as noted, may be highly bonded within the structure, potentially resisting melting. One possible way to minimise the effect of fat integration within the protein structure would be to coat the oil droplets with phospholipids (e.g. from lecithin), which will displace some of the protein at the oil-water interface (Lelievre et al., 1990). A coating of phospholipid would more closely

mimic the natural milk fat globule membrane (King, 1955), which consists of a tri-phospholipid layer (Lopez, Madec, & Jimenez-Flores, 2010), potentially increasing meltability.

3.5 Assessment of protein and lipid quality

The use of ultrasonicated milk in the production of cheese has been limited to date. The effects of ultrasonic treatment on the proteins and lipids within the cheese should therefore be assessed in more detail. In particular, the encapsulation of water within oil droplets of double emulsions may accelerate rancidity development.

One of the concerns with the use of ultrasonication in dairy processing is that the intense acoustic cavitation may modify the milk proteins (Chandrapala, Zisu, Palmer, Kentish, & Ashokkumar, 2011; Villamiel & de Jong, 2000) thereby compromising product quality. As the sonicated emulsions were readily converted into cheese using a standardised cheese making process, the native ability of the casein to undergo rennet gelation was not significantly compromised. Although the rate of rennet gelation did not appear to be significantly affected in the current study (data not shown), other studies have indicated that sonication of cheese milk at much higher intensities (286 J/g compared with 24 J/g in the current study) prior to rennet induced gelation can accelerate the rate of rennet gelation (Liu, Juliano, Williams, Niere, & Augustin, 2014).

The protein in the control and emulsion cheese milk and the sweet and salty whey were assessed using SDS PAGE to assess the potential impact of sonication and possible protein aggregation in these samples (Fig. 7). The protein profile in the ultrasonically treated emulsions was not significantly different to the control, which was not treated by ultrasonication. The protein in the sweet whey of the CONCH cheese was also similar to the protein in the whey from the DECH and SECH cheeses, indicating that sonication did not have a significant impact on the protein in these preparations.

Interestingly, the salty whey from the DECH preparation contained noticeably more casein than the CONCH and SECH preparations. As skim milk was encapsulated within the oil droplets of the DECH preparation, the presence of salt in the salty whey will act as an osmotic driving force that will draw skim milk, including casein, out of the oil droplets and into the salty whey. This observation was consistently observed across a number of trials (data not shown).

The lipids in cheese contribute significantly to the overall flavour profile. The presence of large amounts of free fatty acids can also indicate the development of rancid off-flavours (Woo, Kollodge, & Lindsay, 1984). Lipids extracted from the cheeses were analysed for fatty acid profiled using gas chromatography (GC) and free fatty acid content by titratable acidity.

The fatty acid profile of the milk fat extracted from the CONCH cheese is expectedly very different to that of the canola oil emulsions, which were identical to the native canola oil. The control cheese contains more short chain and saturated fatty acids, which are more volatile and are able to contribute a buttery aroma.

Canola oil has more long chain polyunsaturated fatty acids and ω-3 fatty acids (Fig. S1). The fatty acid profile was also assessed for lipids extracted from the cheeses after 7 months, with no change observed (data not shown).

Free fatty acid development was assessed by measuring the titratable acidity of the extracted lipids (Fig. 8). After 7 months there was considerably more FFA in the DECH cheese than in the starting native canola oil or the SECH cheese. This can be explained by the presence of water encapsulated within the fat droplets of the DECH cheese, which is expected to increase the rate of rancidity development. In general, pre-existing lipases in cheese milk and those formed by the cheese culture convert milk fat triglycerides into glycerol and fatty acids (some of which become rancid) during ripening (Fox, Guinee, Cogan, & McSweeney, 2000).The increased water activity in the cheese microenvironment, enabled by the encapsulated of water, may have improved the moisture-dependent lipase activity (Han, Walde, & Luisi, 1990; Park, 2001) and the subsequent formation of fatty acids.

After 7 months, the amount of FFA in the CONCH cheese was statistically similar to in the DECH ($p = 0.318$), the FFA in the CONCH cheese was also much higher than the SECH. The higher amount of free fatty acid in the aged CONCH cheese compared to the SECH could be due to the greater amount of free fatty acid in the butter than the canola oil (Fig. 8), together with differences in the respective fatty acid profiles.

Strategies to decrease the water activity of the encapsulated water phase could help reduce rancidity development in DECH cheeses. One possibility would be to raise the salt concentration of the encapsulated water phase, which is also compatible with increasing the encapsulation stability as described above. The benefits of this approach may therefore be two-fold in improving the quality of DECH cheese.

4. Conclusions

This study illustrates how the type, distribution of fat within the cheese microstructure and the emulsification route used to form fat droplets influence the properties of the cheese produced, both immediately after pressing and on maturation. The double emulsion (DECH) cheeses had a distinct microstructure with a skim milk phase encapsulated within the emulsified oil droplets. Small dispersed skim milk droplets remained within double emulsion droplets, even after 7 months cheese aging. Compared to control cheeses, DECH chesses with a lower fat content was harder, had lower meltability, and a similar free fatty acid development during maturation . The single emulsion cheese (SECH) analogues prepared with the same fat content as control cheeses, produced comparable yields of cheese and whey, with a similar composition to the control. Fat in both SECH and DECH cheeses appeared more spherical in shape compared to the control. The SECH cheeses were softer than the control, but had a reduced meltability similar to the DECH. Despite having a distinct aroma attributed to the canola oil, SECH cheeses did not display high FFA development during maturation. Our results suggest that using ultrasonication to produce

single and double emulsions, could be a route to design new types of emulsion cheese analogues with properties that are distinct from conventional Cheddar.

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Declaration of Interest: None.

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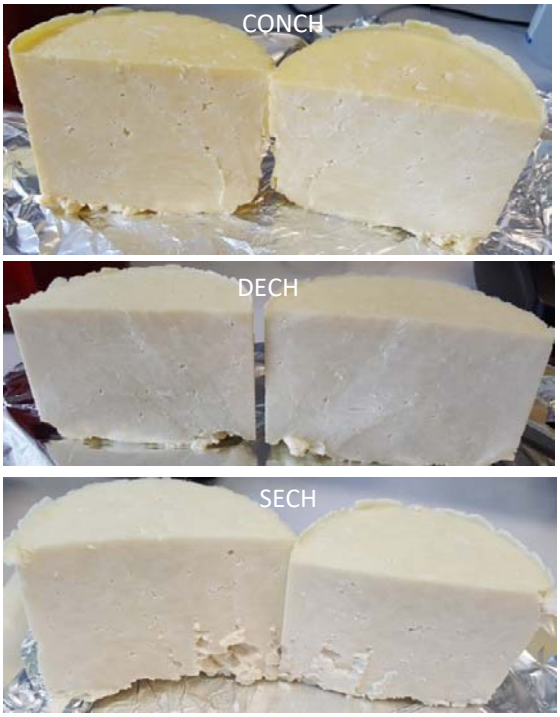
556

557

558 Table 1: Cheese composition. Standard deviations are from duplicate measurements. Different letters
559 denote a significantly different result across each column.

Sample	Moisture %	Protein %	Fat %	Fat loss in sweet whey %	Yield[1] %	FDM[2] %	PDM[3] %
CONCH-1	35.7 ± 0.1[a]	24.7 ± 0.5[a]	36.8 ± 0.4[a]	8.8 ± 0.0[a]	10.9[a]	57.1 ± 0.5[a]	38.3 ± 0.8[a]
CONCH-2	35.6 ± 0.6[a]	25.4 ± 0.3[a]	36.8 ± 0.4[a]	7.9 ± 1.2[a]	10.6[a]	57.1 ± 0.5[a]	39.5 ± 0.4[a]
DECH-1	39.0 ± 0.3[b]	28.9 ± 0.4[b]	25.8 ± 0.4[b]	13.8 ± 0.8[b]	9.2[b]	42.2 ± 0.6[b]	47.3 ± 0.7[b]
DECH-2	39.8 ± 0.5[b]	29.7 ± 0.5[b]	25.8 ± 0.4[b]	22.4 ± 0.0[c]	9.0[b]	42.8 ± 0.6[b]	49.4 ± 0.8[b]
SECH-1	34.0 ± 0.1[a]	25.6 ± 0.6[a]	35.0 ± 0.0[c]	7.1 ± 0.0[a]	10.5[a]	53.0 ± 0.0[c]	38.8 ± 0.8[a]
SECH-2	35.1 ± 1.0[a]	25.9 ± 0.2[a]	34.3 ± 0.4[c]	9.0 ± 0.0[a]	10.2[a]	52.8 ± 0.5[c]	39.9 ± 0.3[a]

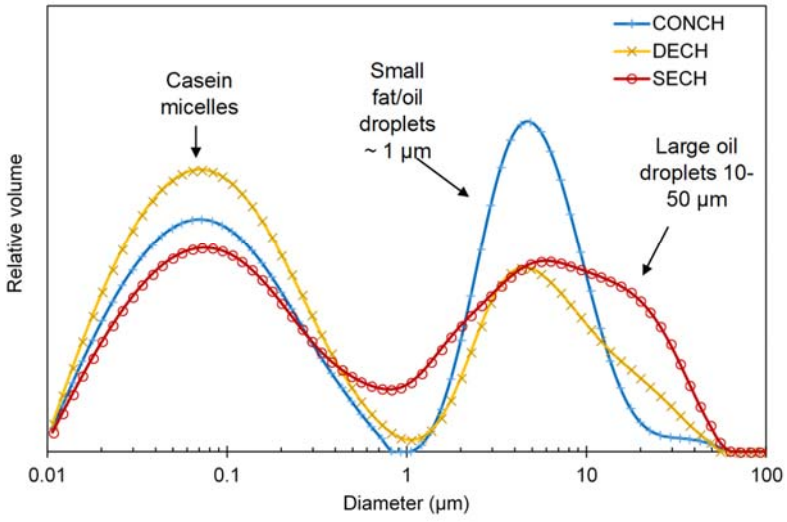
560 [1]Yield % is mass of cheese produced as a proportion of the initial mass of cheese milk
561 [2]FDM is fat % as proportion of the dry weight
562 [3]PDM is protein % as a proportion of the dry weight
563



564

565 **Fig.1** Visual appearance of Cheddar cheese made using milk fat (CONCH) and canola oil (SECH and
566 DECH).

567



568

569 **Fig. 2** Sample particle size distributions for the CONCH, DECH and SECH cheese milk preparations
570 used for cheese making.

571

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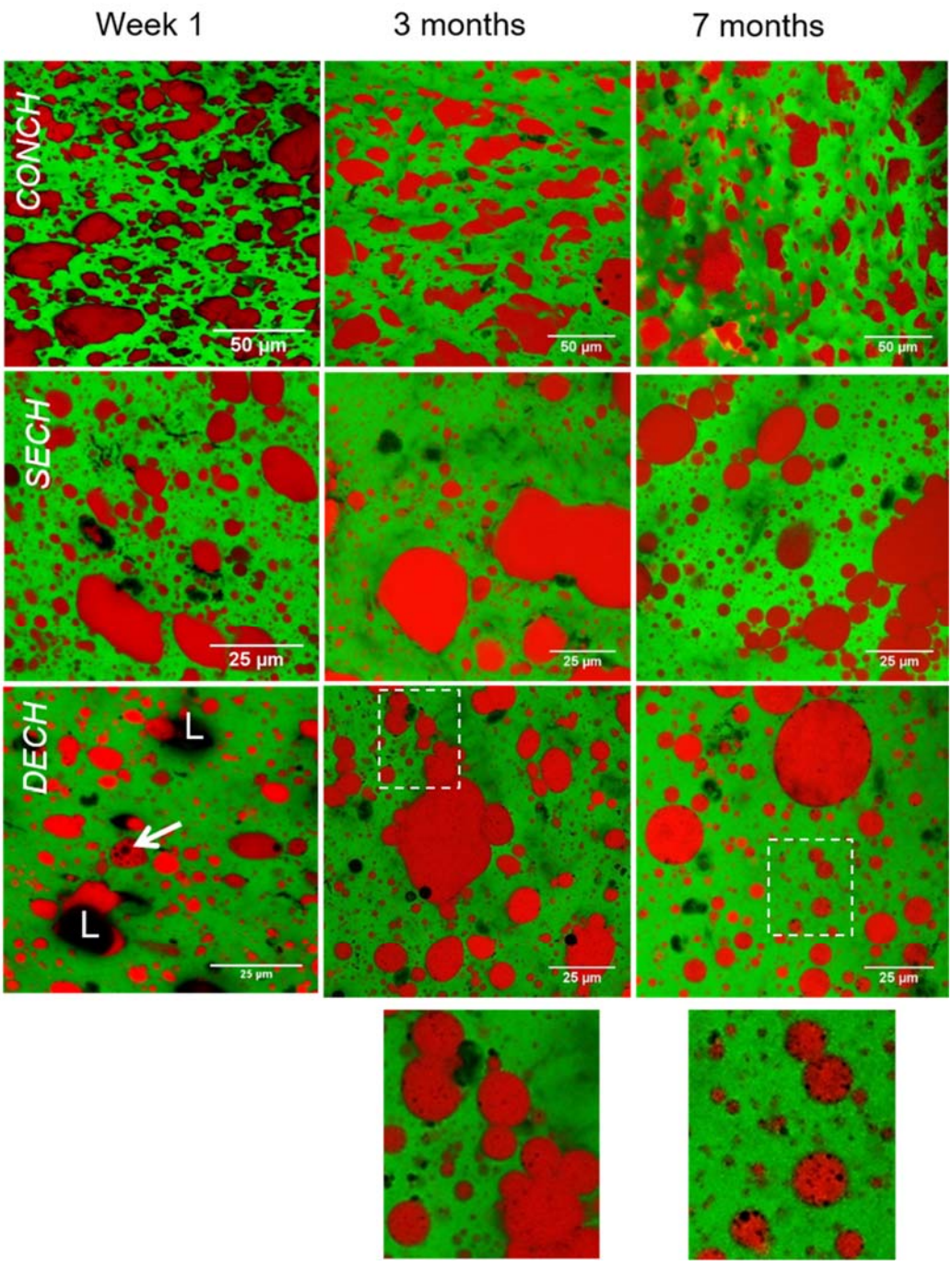


573

574 **Fig. 3** Confocal scanning micrographs for CONCH, SECH and DECH cheeses at 1 week, 3 months or 7
575 months after production. Nile red-stained fat/oil appears red and FCF Fast Green-stained protein appears
576 green. The white arrow indicates larger encapsulated skim milk droplets; 'L' indicates large pores and the
577 dotted boxes correspond to the magnified images presented on the bottom row.

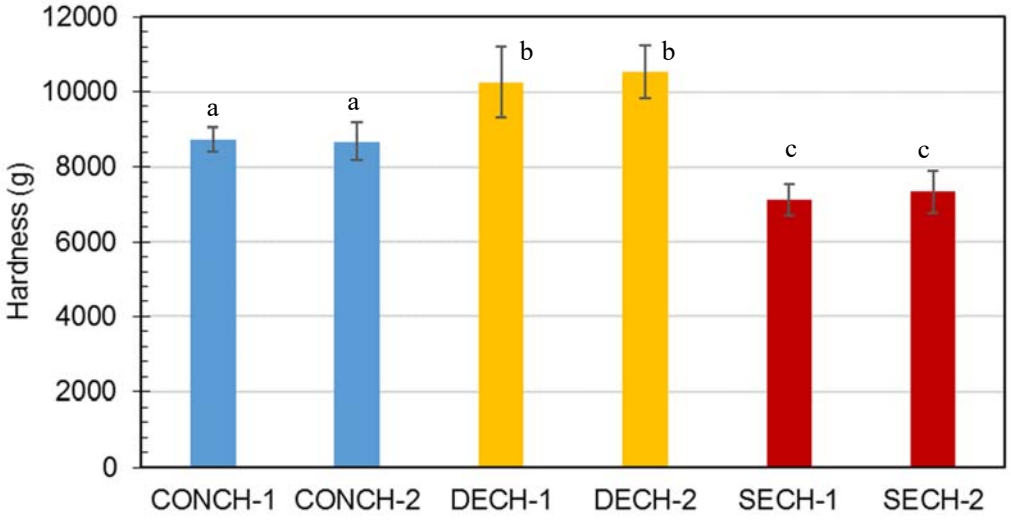


Fig. 4 Hardness as measured by TPA test using a Stable Microsystems texture analyser for cheese samples equilibrated at 19 °C. Statistically different values are depicted by letters a-c. Error bars are the standard deviation of 4 repeated measurements.

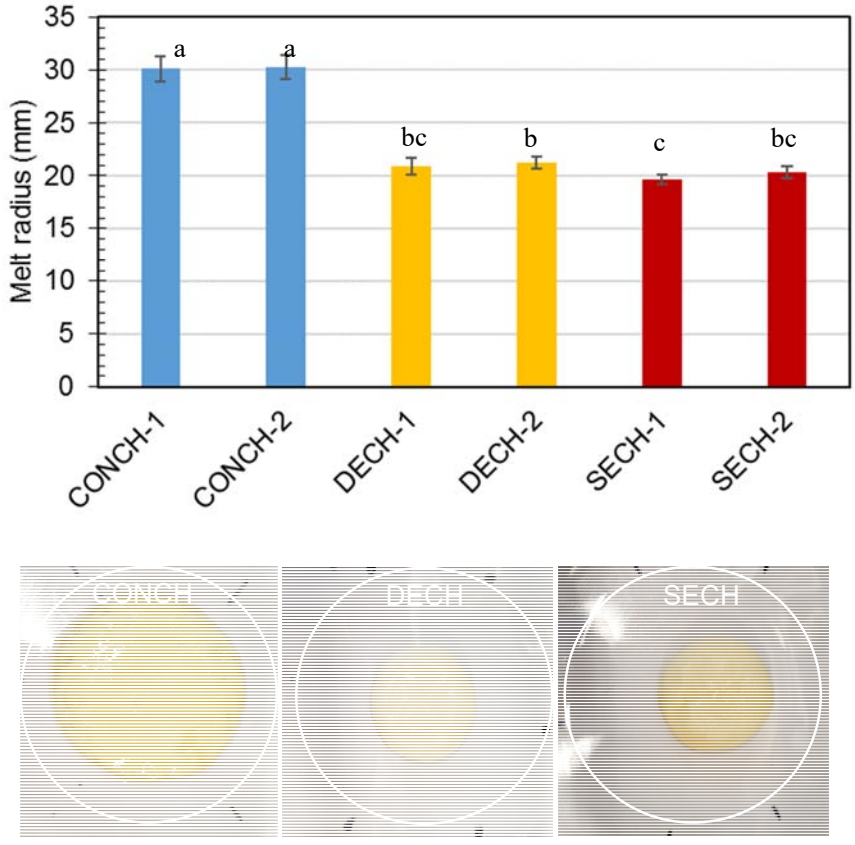


Fig. 5 Schreiber melt test performed on cheese samples. Top panel: radius of cheese melt. Bottom panel: images of melted cheese after 10 minutes in an oven at 130 °C. Different letters indicate significantly different values. Error bars are the standard deviation of 6 measurements.

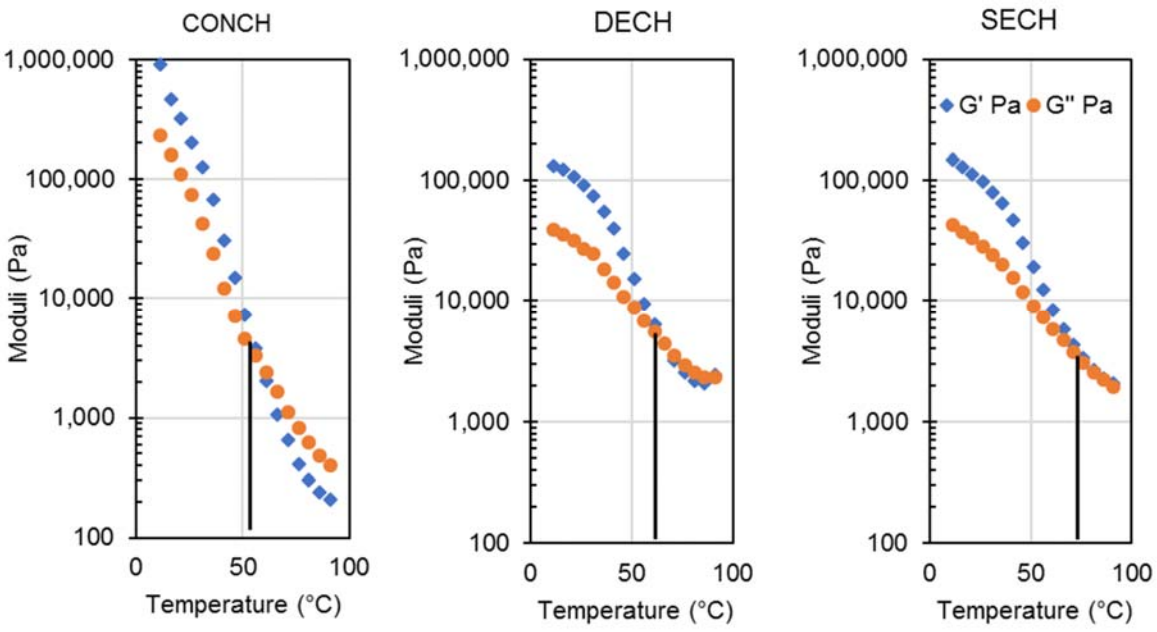


Fig. 6 Sample temperature sweep tests performed for the control and emulsion cheeses. The cross-over points indicative of melting temperatures are indicated by black vertical lines.

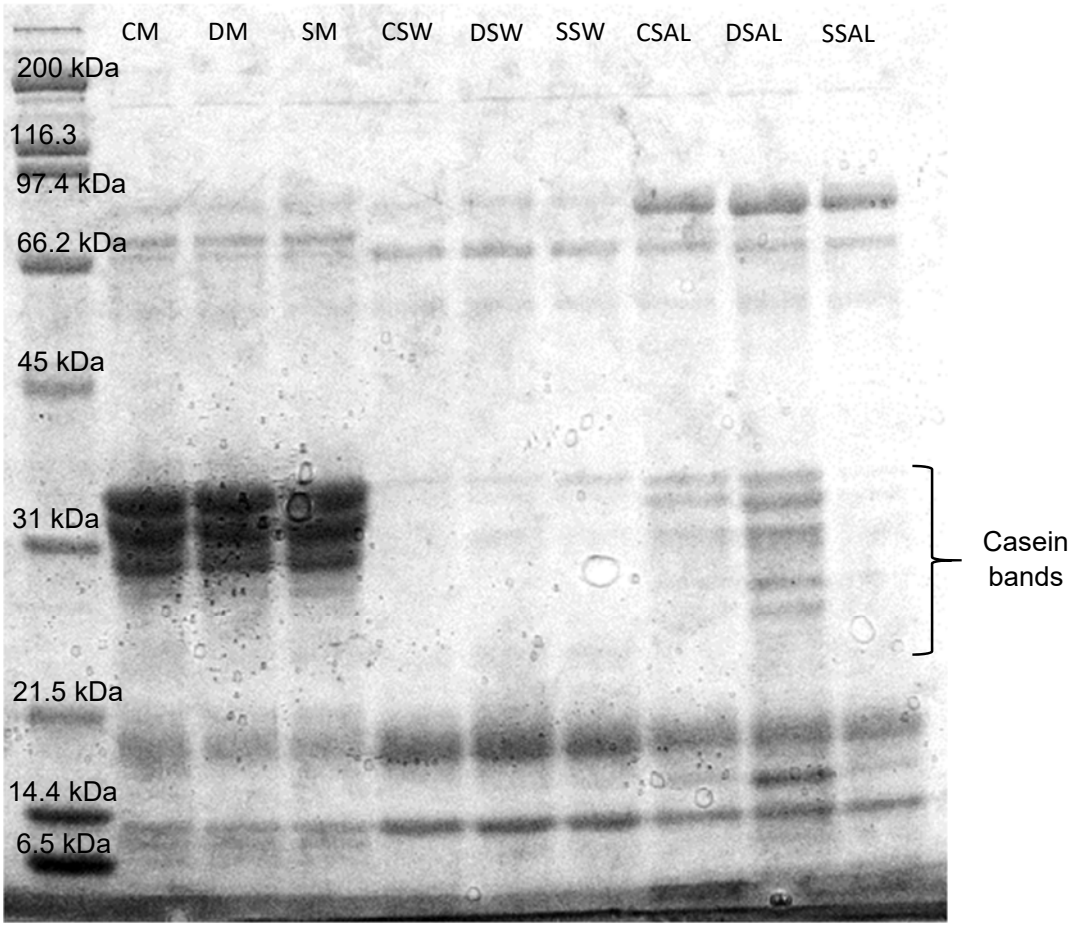
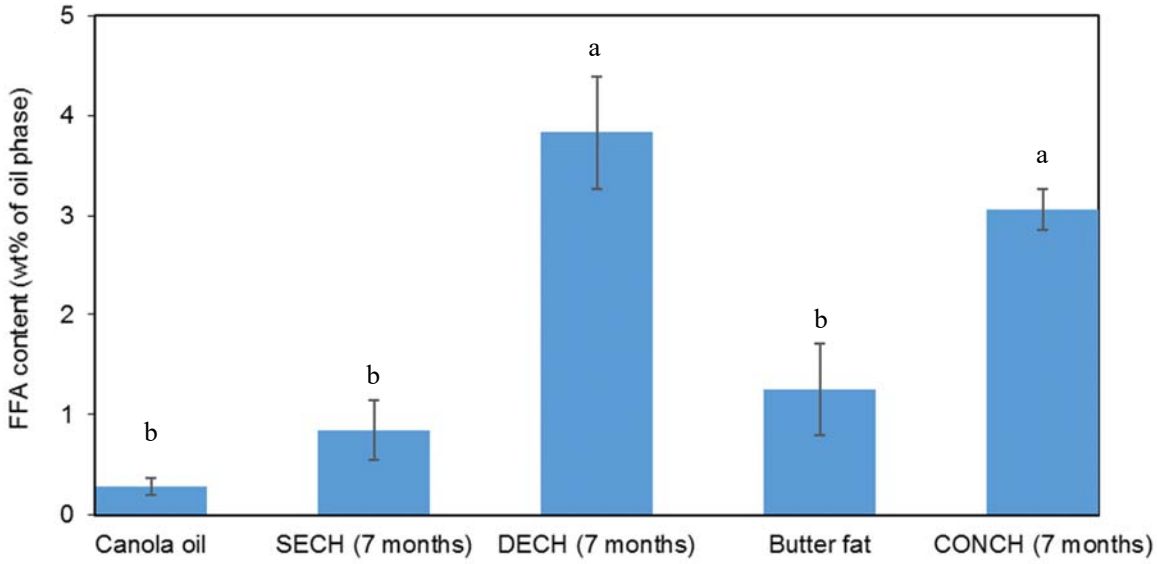


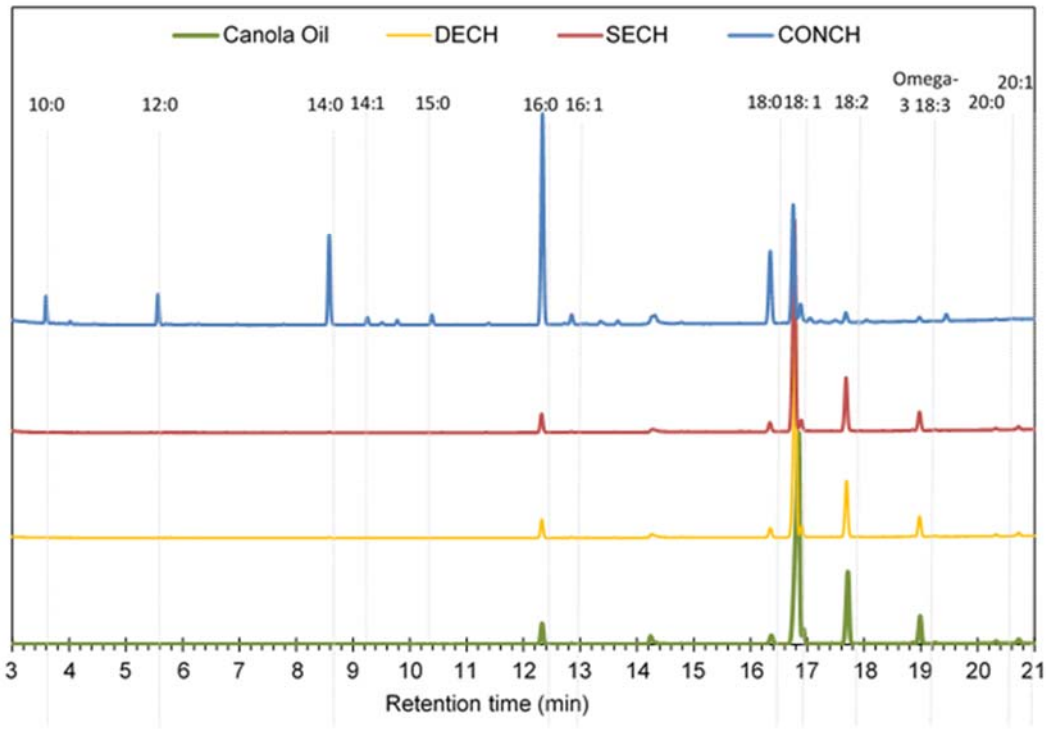
Fig. 7 SDS PAGE gel of proteins in the cheese milk, sweet whey and salty whey from the various cheeses. Each lane was normalized to ~1 mg of protein/mL. CM = control milk, DM = double emulsion milk, SM = single emulsion milk, CSW = control sweet whey, DSW = double emulsion sweet whey, SSW = single emulsion sweet whey, CSAL = control salty whey, DSAL = double emulsion salty whey, SSAL = single emulsion salty whey. Note that the analyses depicted are from a single representative replicate.



602

603 **Fig. 8** FFA content of the canola oil, butter fat, and lipids extracted from cheese after 7 months. Titratable
604 acidity reported as a wt% of the lipids. Subscript letters indicate significant difference.

605 Supplementary Data



606

607 **Fig. S1** Fatty acid profiles of lipids extracted from the CONCH, DECH and SECH cheese and canola oil.
608 Note that the intensities of the fatty acids are normalised to the maximum peak value measured for each
609 sample.

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