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The effect of milk protein concentration on the microstructure and textural properties of full fat Cheddar cheese during ripening

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Short title:

Microstructure of cheese during ripening

ABSTRACT

The effect of varying the milk protein concentration using low concentration factor ultrafiltration retentate (~3.7%, 4%, 4.8%, 5.8% w/w protein) on Cheddar cheese during ripening was investigated. Quantitative analysis of the fat microstructure observed using confocal laser microscopy showed that the effect of the milk protein or time on the microstructure of the fat was minimal. Analysis of the protein sections showed a decrease in the branching of the protein network with time, as denoted by the smaller number of intersections (vertices), which correlated significantly with the level of trichloroacetic soluble nitrogen ($r = -0.66$, $P<0.001$) as well as the cohesiveness of the cheese ($r = 0.70$, $P<0.001$). The hardness was different in cheese made with different milk protein concentration during ripening. At Week 26 of the maturation period, the cheeses made from the 4.8% w/w and 5.8% w/w milk protein were significantly harder than the cheese with no ultrafiltration retentate (UF) addition, which might appeal for particular end uses and selected consumers. This observation correlates with the thicker protein network observed for the cheeses with higher milk protein observed in the cryo-scanning electron microscopy images. Our findings indicate that milk protein concentration has little effect on the cheese microstructure and texture especially when an adequate ripening period is provided, suggesting that high cheese manufacturing throughput may be obtained via this method if maturation is adequate.

KEY WORDS: Cheese, ripening, microstructure, milk protein

1. INTRODUCTION

Bovine milk is subject to seasonal variations arising from changes in diet and the stage of lactation (Broome et al. 1998b; Halmos et al. 2003; Kefford et al. 1995; O'Keeffe 1984). This variability makes it difficult to produce cheese of consistent quality (Halmos et al. 2003) and different methods are routinely employed to standardise milk, including the addition of skim milk, cream, ultrafiltration retentate or milk powder and the removal of fat by centrifugation or natural creaming (Fox et al. 2000).

While the effect of adding ultrafiltered milk retentate to cheese-milk has been studied extensively (Green et al. 1981a; Guinee et al. 2006; Guinee et al. 1994), only one study appears to have looked at the effect of milk protein on the microstructure of the cheese during ripening (Green et al. 1981b), with most studies focusing on the yield, composition and quality of fresh cheeses made using milk with different concentrations of milk protein.

Several studies have noted lower proteolysis for cheese made from high protein milk when the ratio of rennet to milk volume was kept constant (Green 1985; Guinee et al. 1996). Green et al. (1981a) increased the concentration of milk protein 1.7 to 4 fold. The casein concentration during Cheddar maturation indicated a slightly lower proteolysis with increasing milk protein concentration but the rate of lipolysis was similar. Broome (1998a) used milk with 4-5% w/v protein using a low-concentration-ratio ultrafiltration stream. Cheddar cheeses made with high protein milk contained less trichloroacetic acid-soluble nitrogen, indicating lower proteolysis during a 12 month maturation, which was attributed to lower moisture in fat-free substance. These cheeses were also harder with increased gumminess and chewiness (Broome et al. 1998a).

Other studies examining mature Parmesan cheese have observed no significant difference in proteolysis, free fatty acids or sensory analysis between cheese made with high or low protein milk (5% c.f. 3.26%), possibly due to a constant rennet: solids ratio (Govindasamy-Lucey et al. 2004). Proteolysis however increases significantly during ripening period, as expected.

The study of cheese microstructure can provide insights into the impact of milk protein concentration during the process of ripening, including changes in proteolysis and texture development. Confocal laser scanning microscopy (CLSM), for example, can be used to produce 3D reconstructions of fluorescently labelled protein and fat within the microstructure

(Blonk and van Aalst 1993; Everett and Auty 2008) and has been successfully used to study the maturation of Emmental, Mozzarella and processed cheese from Cheddar (Auty et al. 2001; Brickley et al. 2007; Lopez et al. 2007).

In some cheeses, including goat's milk cheeses made from pasteurised, pressure-treated or raw goat's milk, changes have been observed in the microstructure of the samples during ripening. It was found that the microstructure of cheese made from raw milk resembled the microstructure of cheese made using pressure-treated milk, with smaller and more uniform fat globules, when compared to the cheese made using pasteurised milk. A more dense and compact protein network was also observed in these cheeses after 9 Weeks of maturation (Buffa et al. 2001). In another study, CLSM was used to show that only minor changes occurred in the microstructure of Cheddar cheese with a fat content between 3% w/w to 33% w/w, as the ripening time increased from 2 Weeks to 24 Weeks (Rogers et al. 2010).

There is little information on the changes of the microstructure of Cheddar cheese made using different milk protein concentrations during the process of ripening and no published information on cheese produced and ripened at a pilot manufacturing scale. The only study used traditional scanning and transmission electron microscopy to observe changes at Weeks 5 and 28 in maturing Cheddar cheese made from milk concentrated four times using ultrafiltration (Green et al. 1981b). The cheeses made from more concentrated milk appeared to be less homogeneous, with a coarser protein network. Light microscopy was also used in the study to observe the fat distribution and it was found that large areas of fat were present in the curd and cheese made from the concentrated milk. The use of traditional electron microscopy techniques is, however, limited by the need for chemical fixation and ethanol dehydration steps used in sample preparation which may create artefacts. Cryo-scanning electron microscopy (SEM) was employed instead in this study to observe the cheese samples. Cryo-SEM has been used previously to study the structure of cheeses such as Emmental (Famelart et al. 2002; Rousseau and Le Gallo 1990), Karish and Feta (Hassan et al. 2003) and Cheddar (Ong et al. 2011; Ong et al. 2012).

During ripening, the various metabolic pathways that occur will change the cheese chemistry and specifically the concentrations of organic acids and sugars. These changing concentrations will have both a direct and indirect impacts on flavour development and hence quality of the cheese (Zeppa et al. 2001). During ripening, the residual lactose present in Cheddar cheese is normally rapidly metabolised to lactate by the starter bacteria; this is predominantly L – lactate, with some D –lactate (McSweeney 2004). Starter and non starter lactic acid bacteria will also cause the conversion of lactose to sugars, such as glucose and galactose (Upreti et al. 2006).

The aims of the current research, which combine cryo-SEM and CLSM were twofold. Firstly, we aimed to determine the changes to the microstructure and biochemistry of Cheddar cheese during the process of ripening using cheese produced within a pilot scale manufacturing plant (200 kg vats). Secondly, we aimed to investigate the effects of higher milk protein concentration during ripening to establish whether a link exists between Cheddar microstructure and cheese properties. The organic acid content, sugar content and microbial populations were monitored as part of this investigation to determine whether there were significant changes to the cheese chemistry or microflora as ripening progressed.

2. MATERIALS AND METHODS

2.1 Cheese Making

Twelve batches of cheeses were made in total over the course of three trials carried out at Murray Goulburn Co-Operative Co. Limited (Cobram, Victoria, Australia) using a method for manufacture described in a previous study (Ong et al. 2013). In each trial, 4 batches of cheese were made with different milk protein concentrations. The cheese was made with 235 L, 200 L, 160 L or 135 L of pasteurized and standardized cheese-milk with 3.7%, 4%, 4.8% or 5.8% w/w milk protein respectively. The cheese was made in a randomised order. The protein to fat ratio was maintained at 0.84 for all experiments.

Raw milk was mixed with ultrafiltered milk retentate and cream to produce cheese-milk, which was then pasteurized at 72°C for 15s. The cheese-milk was warmed to 33°C before inoculation with 25 Units of freeze dried mixed strain direct vat set (DVS) mesophilic starter culture (Chr. Hansen, Vic, Australia). The ripening time was varied so as to ensure coagulation occurred at pH 6.5; once this pH was reached, rennet (Hannilase, 690 IMCU/ mL; Chr. Hansen) was added at a concentration of 0.06 mL kg^{-1} of milk. Different set times were used to ensure the cheese reached similar gel strength. The gel was then cut over a period of 15 min by blades rotating at 10 rpm for 5 minutes, generating curd particles approximately 1 cm^3 in size, followed by stirring at 12 rpm. Cooking was performed by gradually increasing the temperature at a rate of approximately 1°C every 9 minutes until a maximum temperature of 38°C was reached over 45 minutes. The curd was allowed to cook at this temperature for another half an hour or until the pH dropped to around 6.2. The whey was then drained off (a process lasting 10 minutes) followed by a cheddaring step (lasting 90 minutes). When the pH reached approximately 5.3, the curd was milled and salted with 770 g of salt before being pressed for 24 hours. The cheese was then stored at 8°C for ripening.

2.2 Microscopy techniques – CLSM

Cheese samples were analysed during ripening at Weeks 1, 4, 13, 26 and 39 by confocal laser scanning microscopy (CLSM, Leica TCS SP2; Leica Microsystems, Baden-Wurttemberg, Germany), using a method described in a previous study (Ong et al. 2011). A total of 6 images and 3 three-dimensional pictures were collected for each cheese made using each of the different protein concentrations at each time point during ripening. Analysis of the images was then carried out using Imaris image processing software (Bitplane, Connecticut, USA), as described previously for the three dimensional quantitative analysis of Cheddar cheese microstructure (Ong et al. 2012). Parameters obtained from the image analysis included the number of fat globules per unit volume, the mean volume and diameter of fat globules and their sphericity (the dimensionless ratio of the surface area of a sphere with the same volume as the globule to the surface area of the globule; $0 < \text{sphericity} < 1$, and 1 is the sphericity of a perfect sphere (Hakon 1935). Other parameters included the number of vertices in the protein network and the porosity (the ratio of the volume of the pores with respect to the volume of the sample used for image analysis). The number of vertices has been normalised to a volume fraction of 140000 μm^3.

2.3 Microscopy techniques – Cryo-SEM

Cheese samples were analysed with cryo scanning electron microscopy, cryo-SEM (Quanta; Fei Company, Oregon, United States of America (USA)) using a method described in a previous study (Ong et al. 2011). A total of 6 images were taken at magnification 2000x, 4000x or 8000x from one representative cheese sample selected randomly for each treatment at each time-point at Weeks 1, 4, 13, 26 and 52 during ripening.

2.4 Determination of pH during ripening

To determine the pH of each cheese sample, grated cheese (20 g) was homogenised with 12 mL of distilled water to create a cheese slurry (Australian Standard 1989). The pH of the slurry was then measured using an electrode pH meter (Orion 720A, Orion Pacific Pty Limited., Vic, Australia) and the results presented as the mean of the data obtained from the three batches of cheese made from the same milk protein concentration.

2.5 Proteolysis analysis

The water soluble extract (WSE) of cheese samples was obtained using a method described in a previous study (Ong et al. 2006). The level of water soluble nitrogen (WSN) and trichloroacetic acid (TCA)-soluble nitrogen were determined using the Kjeldahl method (AOAC 1990). The TCA-soluble nitrogen was determined from 9 mL of filtrate obtained when the WSE was precipitated with 12% w/v TCA (Sigma- Aldrich). The results are presented as a mean of triplicate results obtained per cheese treatment at each time point during ripening.

2.6 Texture analysis

A texture analyser TA-XT Plus (Stable Micro Systems, Surrey, England) was used to obtain the texture profile of cheese samples using a method described in a previous study (Ong et al. 2012), with the slight modification that cheese samples of 2.0 cm in diameter were used. Six samples were obtained for each individual cheese and the mean of these six pseudo replicates was calculated for each cheese treatment in each trial. The data were then presented as a mean of the three results collected per cheese treatment over 3 trials. Hardness is the peak force required to obtain the first deformation (Chevanan et al. 2006) and cohesiveness is a measure of the extent of that deformation that occurs before any rupture occurs (Chevanan et al. 2006).

2.7 Microbiological analysis

Grated cheese (5 g) was homogenised (Polytron Homogeniser, Lucerne, Switzerland) for 1 minute in sterilized tri-sodium citrate (2% w/w, Oxoid, Vic, Australia; final volume adjusted to 50 mL). Serial dilutions in peptone solution were pour-plated in M17 agar (Merck, NSW, Australia) and incubated (3 days, 30 °C) and, in parallel, Rogosa agar (Merck) incubated anaerobically for 3 days at 30°C). The anaerobic atmosphere was maintained through the use of anaerobic gas jars (Oxoid) and AnaeroGen system (Oxoid). Colony counts are presented as the mean of data obtained from the three batches of cheese made from the same milk protein concentration.

At least three colonies of bacteria were individually selected from the cultures grown on either of the two media for each cheese treatment and each trial (in total, for each cheese treatment, at least 9 colonies of bacteria were selected for each cheese of different milk protein level) and purified via the streak method three times. These colonies were subjected to bacterial identification with a Matrix-assisted laser desorption/ionisation – Time of flight (MALDI-TOF) biotyper (Bruker, Massachusetts, USA).

A solution made up of 50 v/v % Acetonitrile (Honeywell, New Jersey, USA), 2.5 v/v% Trifluoroacetic acid (Merck) and 47.5 v/v % water (purified to a resistivity of 18 Mega Ohm) was prepared. The solution (100 µL) was then mixed with 1 µg of HCCA (a-Cyano-4-hydroxy-cinnamic acid) powder (Bruker) to create the matrix used for analysis.

The bacteria were smeared on the target support using a toothpick. Formic acid (70%, 1 µL, Fluka Analytical, NSW, Australia) was added to each colony of bacteria, and dried followed by 1 µl of the matrix, which was then allowed to dry again before analysis using the MALDI-TOF biotyper.

2.8 Sugar and organic acid analyses

Sugars (lactose, galactose, glucose) were isolated from the WSE and analysis performed using a HPLC method described in a previous study (Gosling et al. 2009) on a Shimadzu Prominence system equipped with a RID-10A refractive index detector and a 300 × 7.8 mm Rezex RCM-Monosaccharide Ca^{2+} column (Phenomenex, NSW, Australia).

Organic acid analysis was carried out using high performance liquid chromatography with a Shimadzu Prominence system equipped with PDA UV detector and a Bio-Rad Aminex HPX 87H cation exchange column connected to a cation H+ guard column (Bio-Rad Laboratories

Inc, California, USA) as described by Ong et al. 2006 except that 1.55 M nitric acid (700 µL) was used. The three organic acids were detected at wavelength 2210, 220 or 285 nm.

2.9 Statistical analysis

All experimental results were analysed using a split plot design based on 2 way analysis of variance. The model was General Analysis of Variance in Genstat (VSN International Limited, Hertfordshire, UK). The treatment was protein, time and interaction between protein and time (protein*time). Protein refers to the milk protein concentration (3.7, 4, 4.8, 5.8 % w/w) and time refers to the ripening period (Weeks 1, 4, 13, 26, 39). There were a total of 12 batches of cheeses made from the 3 trials (4 protein concentrations made in triplicate). Each batch of cheese was treated independently over the ripening period. An additional parameter ID (1 to 12) was assigned to each initial batch of cheese and this allowed the software to link ripened cheese samples to the original 12 batches of fresh cheese when analysing the effects of time. If milk protein concentration or time were significant, the data were further analysed using Tukey's test, with a significance level of 0.05. The correlation between data points was also analysed using Genstat (VSN International Limited).

3. RESULTS AND DISCUSSION

The microstructure of Cheddar cheese was assessed during ripening for standard Cheddar cheese (4% w/w protein standardised with UF retentate) and a series of cheeses made from milk with no UF (3.7% w/w protein) or milk with a higher protein concentration obtained from UF addition (4.8% w/w or 5.8% w/w protein). These cheeses had similar protein, calcium and salt content but some difference in their moisture content. The calcium content of all four cheese treatments was ~ 800 mg Ca/ 100g cheese. The higher milk protein cheeses (4.8% w/w and 5.8% w/w milk protein cheeses) had lower moisture content than the 3.7% and 4% w/w milk protein cheeses. The pressed cheese made from higher milk protein concentration were also significantly harder than the cheese made from lower milk protein, due to the lower moisture content (Ong et al. 2013). The composition and yield data is provided in **Supplementary Table 1**. During ripening, the extent of proteolysis, sugar concentration, organic acid content and texture properties were also assessed.

3.1 Microstructure of Cheddar cheese throughout ripening

The pressed cheese, observed with cryo-SEM, (**Figure 1 A-D**) has a relatively close and dense structure. Small gaps, indicated by the black regions, appear at the interface between the fat and protein phases by Week 13 (**Figure 1 E-H**), which could arise from proteolysis of the protein network leading to a weaker structure that behaves differently during microscopy sample preparations. That is, the interface of the protein and fat could be considered as the weakest point within the structure. As such, gaps are more likely to appear in the aged cheese at the fat-protein interface. The appearance of these gaps in all treatments suggests proteolysis is proceeding at a similar rate. At 26 Weeks, the protein network for the cheese made from the higher milk protein preparations tended to be qualitatively thicker (**Figure 1K and Figure 1L**) than the cheeses made from 3.7% w/w and 4% w/w milk protein concentration (**Figure 1I and Figure 1J**) but only minor structural differences were observed after 52 Weeks of ripening.

The milk protein concentration does not appear to have any effect on the structure of the cheese at any period of ripening when observed by CLSM in 2D images (**Figure 2**). A number of observations can be made, however, when quantitative image analysis was applied.

Generally, as the ripening time increases, the protein network is expected to break down. The percentage volume of protein stained by FCF fast green was significantly affected by time ($P<0.05$, **Table 1**). In **Figure 3a**, a decrease in protein volume between Weeks 1 and 39 can be observed. This is consistent with ongoing proteolysis in these cheeses. However, these images cannot distinguish without further analysis between the disappearance of protein (proteolysis), potential conformational changes (syneresis) or localised moisture migration.

A change in the branching of the protein network, as indicated by the number of protein intersections (or vertices) in the network was also observed with ripening ($P<0.05$, **Table 1**), with a slight decrease with time and with the data for Weeks 26 and 39 data being significantly lower than the earlier time points ($P <0.05$, **Figure 3b**). The decrease in protein vertices also suggests the protein is solubilising, indicating an increase in proteolysis with time.

The porosity increased slightly with time, with the Week 39 data being significantly higher than the other data points ($P<0.05$, **Figure 3c**, **Table 1**). Statistical analysis also suggested that these changes in porosity were greater for the samples of lower protein concentration (M*T $P<0.05$, **Table 1**). This result is consistent with the cryo SEM images where more gaps were observed within the cheese after ripening.

The fat globules are largely coalesced as a result of cheese-making, as can be seen in the Week 1 cheese (**Figure 2 A-D**). The fat globules differed in their sphericity, which ranged from 0.72-0.84, where a sphericity of 1 is a perfect sphere, but there was no trend in this variability (**Supplementary Figure 1a**). Quantitative analysis shows that the equivalent diameter of the fat globules was significantly affected by the ripening time ($P<0.05$, **Table 1**, **Figure 3d**). No obvious trend could be observed, however and the diameter of the globules after ripening for 39 Weeks was statistically similar to the value observed in Week 1. The sphericity and number of fat globules were also found to be significantly unaffected by the milk protein concentration or the ripening time ($P>0.05$, **Table 1**, **Supplementary Figures 1a, b**). Past studies investigating the effect of fat content on the microstructure of Cheddar cheese have stated that no further clumping of the fat molecules was expected during ripening due to the solid nature of the fat at the ripening temperature used (7 °C) (Guinee et al. 2000). The quantitative analysis here provides further evidence that the fat globules only experience minor changes during the maturation period.

Another interesting observation is the mineral deposits within the cheese at Week 13 (**Supplementary Figure 2**), which were similar to those identified in previous studies as calcium phosphate via Scanning Electron Microscopy – Energy Dispersive X-ray Spectroscopy (SEM-EDX) analysis (Pommert et al. 1988). The mineral deposit was randomly spotted within the microstructure of the cheese and thus no relationship could be drawn between the number of mineral deposits and the milk protein concentration.

3.2 Assessment of proteolysis in cheese during ripening

The water soluble nitrogen (WSN) increased significantly over the ripening period ($P<0.05$, **Figure 4a,** **Supplementary Table 2**). Approximately 8% of the total nitrogen was present as WSN, a value which increased rapidly to approximately 35% after 39 Weeks of ripening for all treatments (**Figure 4a**). The milk protein concentration, however, did not affect the proteolysis rate significantly. Guinee et al. (1994) found that the WSN increased during ripening but that the rate of this increase was much less for cheeses made from a higher concentration of protein. Cheeses made using milk with 45 g / L (~ 4.5 % w/v) or 82 g / L (~8.2 % w/v) protein had 21% or 30% less WSN when compared to a control cheese made using milk with 30 g / L (~ 3.0 % w/v) protein after 270 days. The WNS/TN here was also lower than past studies, where the ratio was observed to be around 51% for Cheddar cheese ripened at 8^{o}C for 24 Weeks (Ong and Shah 2008).

The ratio of TCA soluble to total nitrogen (TCA-SN/TN) (**Figure 4b**) was found to increase significantly over the ripening period for the cheeses (P<0.05, **Supplementary Table 2**). This ratio was also significantly affected by the milk protein concentration, with the ratio for 5.8 % w/w protein increasing more slowly than at lower protein levels. A further significant negative correlation was observed between this ratio and the number of protein vertices in the protein microstructure (r =-0.66, P<0.001) reflecting increasing proteolysis with time. The values obtained for the TCA-SN to TN ratio in this study appear consistent with those found in literature where the TCA-SN to TN ratio was ~ 13% for Cheddar cheese ripened at 8^{o}C for 24 Weeks (Ong and Shah 2008) compared to 17 ± 1 % for the 4% w/w milk protein cheese (control) at Week 26 in this study.

3.3 Texture Analysis Profile of Cheese during Ripening

As the cheese ripened in this study, a significant softening of the cheese was observed (P<0.05, **Figure 5a, Supplementary Table 2**). A 50 ± 19% and 53 ± 28% reduction in hardness occurred in the control and 5.8% w/w milk protein cheese at Week 39 when compared to the cheeses at Week 1. The link between proteolysis and textural properties has been well studied (Lawrence et al. 1987) and the softening effect observed here is likely due to proteolysis. Casein is known to break down into mostly water soluble peptides, which lower the ability of the casein to contribute to the protein network (Lawrence et al. 1987). The solubilisation of colloidal calcium phosphate, which occurs during ripening, may also contribute to this effect (Chevanan and Muthukumarappan 2007; Hassan et al. 2004; O'Mahony et al. 2005; O'Mahony et al. 2006).

Our results show that the milk protein concentration significantly affects the hardness of the cheese (P<0.05, **Supplementary Table 2**). The cheeses made with a higher protein concentration of 4.8% w/w or 5.8% w/w were generally harder than the two other cheese treatments (**Figure 5a**), most likely as a result of the lower moisture content caused by the use of higher milk protein (**Supplementary Table 1**). At 26 Weeks, cheeses made with a higher concentration of milk protein (4.8% w/w or 5.8% w/w) were observed to be significantly harder ($P < 0.05$) than the cheese made with no UF. This increase in hardness was consistent with the slightly lower porosity (**Figure 3c**) and the microstructure images of the cheese where thicker protein network was observed in the higher milk protein cheese (**Figure 1**). A significant correlation also exists between the number of vertices of the protein network obtained using the 3D image analysis and the hardness measured ($r = 0.41$, $P = 0.002$). This trend is consistent with a past study by Broome et al. (1998a) who observed that cheeses made from milk with 4.5% w/v and 5.0% w/v protein were harder than those made from milk with 3.15 % w/v (control) and 4.0% w/v protein. No significant differences were found, however, between the different protein treatments at Week 39, suggesting that any difference in cheese hardness is reduced by this late stage of maturation.

Cohesiveness was significantly affected by the ripening time and **Figure 5b** shows a decrease of with time ($P<0.05$, **Supplementary Table 2**). The cohesiveness appears to be consistent with values found in the literature. (Broome et al. 1998a) noted a cohesiveness of 0.71 and 0.77 (Nsec/Nsec) for 4.0% w/w and 5.0 % w/w cheeses at month 3. However, these authors noted a slight increase in cohesiveness from month 3 to month 6, which are not consistent with our study. Our results are however in agreement with O'Mahony et al. (2005) who noted a decrease in cohesiveness from ~0.56 to ~0.23 (Nsec/Nsec) of control Cheddar cheese ripened from day 1 to day 180. Generally, if the protein network has fewer vertices, then the bonds within the cheese are expected to be weaker. There was a significant correlation between the number of vertices in the protein network and the cohesiveness ($r = 0.70$, $P<0.001$), supporting this finding. In contrast, the impact of the milk protein concentration on cohesiveness was found to be not significant ($P>0.05$, **Supplementary Table 2**).

3.4 Microbiological, sugar and organic acid changes through ripening

The milk protein concentration has no significant effect on the bacterial population (data not shown), consistent with a past study involving the use of ultrafiltered milk to increase milk protein concentration (Acharya and Mistry 2004). Analysis of the bacterial population using a

MALDI biotyper confirmed that the NSLAB microorganisms in this study were predominantly *Lactobacillus paracasei*, with some *Lb. rhamnosus* and *Lb. curvatus* also present, which is in agreement with past studies (Beresford and Williams 2004; Cogan 2002).

The microbial metabolic pathways, available substrates and biochemical environment in the cheese may result in different concentrations of organic acids and sugars during ripening. However, no effects were observed due to differences in milk protein concentration except for small changes in the rate of accumulation of citric acid (Supplementary Table 3).

Lactose was not detected in any of the cheese samples at any time points in this study. This is consistent with the high number of lactic acid bacteria detected initially in the cheese (**data not shown**), which may have consumed any remaining lactose at the early stages of ripening. The lack of lactose in any of the cheese samples may also explain the pH results. Generally, the pH of the cheese was found to increase slightly with time. It was significantly affected ($P<0.05$, **Supplementary Table 2**) by the ripening time, with the pH being significantly higher at Week 39 than at other time points ($P<0.05$, **Supplementary Figure 3**). These results are in agreement with those obtained by Shakeel Ur et al. (2004) for cheese with low residual lactose.

The glucose level was significantly affected by time ($P<0.05$**)**, while the galactose level was not significantly affected by either time or protein treatment ($P>0.05$) (**Supplementary Figure 4, Supplementary Table 2**). The level of variability of the data, however, prevents any firm conclusions concerning the sugar concentration. The galactose concentration in the cheese (2 to 8 mg/g cheese) is consistent with past studies (St Gelais 1991; Upreti et al. 2006).

Most of the organic acids monitored during ripening were found to be only significantly affected by the ripening time ($P<0.05$, **Figure 6**, **Supplementary Table 3**, **Supplementary Figures 5-6**). Of particularly interest is citric acid. As the ripening period increased, the amount of citric acid generally increased (**Figure 6b**). The initial amount of acid was similar to that noted in a study by Bouzas et al. (1991) who reported a concentration of 2.2 mg/g (0.22 % w/w) citric acid for a 60-day old commercial Cheddar cheese. The increase observed here is consistent with past studies on full fat Cheddar cheese (McGregor and White 1990), although decreases have also been observed in the literature (Bouzas et al. 1993; Lues and

Botha 1998). Lues and Botha (1998) noted a decrease from ~0.91 mg/g in 6-10 week old Cheddar to ~0.89 mg/g in 8-10 months old Cheddar (0.091 to 0.089 % w/w). Of interest in the present case is that the citric acid in the cheese with the highest protein concentration increased more slowly than for the other cheeses (**Figure 6b** and **Supplementary Table 3**, M*T, $P<0.05$)

Acetic acid was also of particular interest to this study. Generally, the acetic acid concentration increased with time and the acid content was significantly higher at Weeks 26 and 39 when compared to earlier time-points ($P<0.05$, **Figure 6c**). This trend is in agreement with studies who noted a rise in acetic acid in Cheddar cheese throughout ripening (Bouzas et al. 1993; Marsili 1985). Bouzas et al (1993) suggested acetic acid may be produced from the metabolism of citrate, lactose and amino acids and suggested that changes in acetic acid concentration could be used as an indicator of the degree of activity by heterofermentative organisms. Ong and Shah (2008) also observed that the acetic acid content of a control Cheddar cheese ripened at 8°C was ~ 0.09% at week 24, which is consistent with the acid concentration of 0.1 % w/w observed in this study at week 26 for the 4% w/w milk protein cheese. Overall the level of acetic acid is quite low and is unlikely to impact the flavour characteristics of the cheese.

4. CONCLUSION

Quantitative analysis of the CLSM images shows a decrease in the number of vertices in the protein network, as well as a slight increase in the porosity of the cheese with time. These results indicate proteolysis increases with time and that textural properties such as cohesiveness could be expected to decrease with time. As expected, significant correlations were obtained between the cohesiveness and the number of vertices ($r = 0.70$, $P<0.001$), as well as between the concentration of TCA-SN to TN and number of vertices ($r = -0.66$, $P<0.001$).

The hardness was different for cheeses made with different milk protein concentrations during ripening, consistent with cryo-SEM microscopy images, which show thicker protein network for the higher milk protein treatments and confocal image analysis, which showed slightly lower porosity. Overall, however, the effect of time during the ripening process had a greater effect than the milk protein concentration. By Week 39, there were no quantitative differences in hardness. These findings suggest that the effect of the protein concentration on the cheese texture becomes minimal after 39 weeks, when an adequate ripening period is provided. The greater firmness observed at Week 26, for cheese made from 4.8% w/w or 5.8% w/w milk protein, might also prove beneficial for particular end uses or selected consumers.

5. ACKNOWLEDGEMENTS

The authors would acknowledge the Australian Government for providing the Australian Postgraduate Award (APA) scholarship and Dairy Innovation Australia (DIAL) for financial support. We also thank Murray Goulburn for the supply of cheese-milk and for providing access to the equipment and plant for the cheese-making process in Cobram (Victoria, Australia). The staff at Murray Goulburn are acknowledged for their help during the trials in Cobram and we thank Warrnambool Cheese and Butter and Lion for their involvement in the project. We also thank the Particulate Fluids Processing Centre (PFPC) and the Bio21 Institute for access to equipment and Mr Roger Curtain for his help in operating the scanning electron microscope in cryo mode. Ms Rachel Knight of Bioscreen (Australia) provided access to and advice regarding the operation of the MALDI biotyper. The authors would also thank the Department of Mathematics and Statistics (University of Melbourne, Australia) and Ms Rachel Sore for statistical advice .

REFERENCES

Acharya MR & Mistry VV (2004) Comparison of effect of vacuum-condensed and ultrafiltered milk on Cheddar cheese. Journal of Dairy Science, 87(12), 4004-4012.

AOAC (1990). *Official methods of analysis (15th ed.)*. Arlington, VA, USA. **Vol. 2**.

Australian Standard (1989). *Methods of chemical and physical testing in the dairy industry – determination of pH.* Australian Standard 2300.1.6.

Auty MAE, Twomey M, Guinee TP & Mulvihill DM (2001) Development and application of confocal scanning laser microscopy methods for studying the distribution of fat and protein in selected dairy products. Journal of Dairy Research, 68(03), 417-427.

Beresford T & Williams A (2004) The microbiology of cheese ripening. In: Fox PF, McSweeney PLH, Cogan TM and Guinee TP (Eds.), Cheese: Chemistry, physics and microbiology, pp 287-317. Elsevier Academic Press, London, UK.

Blonk JCG & van Aalst H (1993) Confocal scanning light microscopy in food research. Food Research International, 26(4), 297-311.

Bouzas J, Kantt CA, Bodyfelt F & Torres JA (1991) Simultaneous determination of sugars and organic acids in Cheddar cheese by high-performance liquid chromatography. Journal of Food Science, 56(1), 276-278.

Bouzas J, Kantt CA, Bodyfelt FW & Torres JA (1993) Time and temperature influence on chemical aging indicators for a commercial Cheddar cheese. Journal of Food Science, 58(6), 1307-1313.

Brickley CA, Auty MAE, Piraino P & McSweeney PLH (2007) The effect of natural Cheddar cheese ripening on the functional and textural properties of the processed cheese manufactured therefrom. Journal of Food Science, 72(9), C483-C490.

Broome M, Tan S, Alexander M & Manser B (1998a) Low-concentration-ratio ultrafiltration for Cheddar cheese manufacture: 2. Effect on maturation. Australian Journal of Dairy Technology, 53(1), 11-16.

Broome M, Tan S, Alexander M & Manser B (1998b) Low-concentration-ratio ultrafiltration for Cheddar cheese manufacture: Part 1: Effect on seasonal cheese composition. Australian Journal of Dairy Technology, 53(1), 5.

Buffa MnN, Trujillo AJ, Pavia M & Guamis B (2001) Changes in textural, microstructural, and colour characteristics during ripening of cheeses made from raw, pasteurized or high-pressure-treated goats’ milk. International Dairy Journal, 11(11–12), 927-934.

Chevanan N & Muthukumarappan K (2007) Effect of calcium and phosphorus, residual lactose, and salt-to-moisture ratio on the melting characteristics and hardness of Cheddar cheese during ripening. Journal of Food Science, 72(4), E168-E176.

Chevanan N, Muthukumarappan K, Upreti P & Metzger LE (2006) Effect of calcium and phosphorus, residual lactose and salt-to-moisture ratio on textural properties of Cheddar cheese during ripening. Journal of Texture Studies, 37(6), 711-730.

Cogan TM (2002) Cheese | microbiology of cheese. In: Hubert R (Eds.), Encyclopedia of dairy sciences, pp 306-314. Elsevier, Oxford.

Everett DW & Auty MAE (2008) Cheese structure and current methods of analysis. International Dairy Journal, 18(7), 759-773.

Famelart MH, Le Graet Y, Michel F, Richoux R & Riaublanc A (2002) Evaluation of the methods of measurement for functional properties of Emmental cheeses from the West of France. Évaluation des méthodes d'appréciation des propriétés fonctionnelles des fromages d'Emmental de l'Ouest de la France, 82(2), 225-245.

Fox PF, Guinee TO, Cogan TM & McSweeney PLH (2000) Overview of cheese manufacture. (Eds.), Fundamentals of cheese science, pp 10-18. Aspen Publication, Gaithersburg MA, USA.

Gosling A, Alftren J, Stevens GW, Barber AR, Kentish SE & Gras SL (2009) Facile pretreatment of bacilius circulans beta-galactosidase increases the yield of galactosyl oligosaccharides in milk and lactose reaction systems. Journal of Agricultural and Food Chemistry, 57(24), 11570-11574.

Govindasamy-Lucey S, Jaeggi JJ, Bostley AL, Johnson ME & Lucey JA (2004) Standardization of milk using cold ultrafiltration retentates for the manufacture of parmesan cheese. Journal of Dairy Science, 87(9), 2789-2799.

Green ML (1985) Effect of milk pretreatment and making conditions on the properties of Cheddar cheese from milk concentrated by ultrafiltration. Journal of Dairy Research, 52(04), 555-564.

Green ML, Glover FA, Scurlock EMW, Marshall RJ & Hatfield DS (1981a) Effect of use of milk concentrated by ultrafiltration on the manufacture and ripening of Cheddar cheese. Journal of Dairy Research, 48(02), 333-341.

Green ML, Turvey A & Hobbs DG (1981b) Development of structure and texture in Cheddar cheese. Journal of Dairy Research, 48(02), 343-355.

Guinee TP, Auty MAE & Fenelon MA (2000) The effect of fat content on the rheology, microstructure and heat-induced functional characteristics of Cheddar cheese. International Dairy Journal, 10(4), 277-288.

Guinee TP, O'Callaghan DJ, Mulholland EO & Harrington D (1996) Milk protein standardization by ultrafiltration for Cheddar cheese manufacture. Journal of Dairy Research, 63(02), 281-293.

Guinee TP, O'Kennedy BT & Kelly PM (2006) Effect of milk protein standardization using different methods on the composition and yields of Cheddar cheese. Journal of Dairy Science, 89(2), 468-482.

Guinee TP, Pudja PD & Mulholland EO (1994) Effect of milk protein standardization, by ultrafiltration, on the manufacture, composition and maturation of Cheddar cheese. Journal of Dairy Research, 61(01), 117-131.

Hakon W (1935) Volume, shape, and roundness of quartz particles. The Journal of Geology, 43(3), 250-280.

Halmos AL, Pollard A, Sherkat F & Seuret MG (2003) Natural Cheddar cheese texture variation as a result of milk seasonality. Journal of Texture Studies, 34(1), 21-40.

Hassan A, Johnson ME & Lucey JA (2004) Changes in the proportions of soluble and insoluble calcium during the ripening of Cheddar cheese. Journal of Dairy Science, 87(4), 854-862.

Hassan AN, Frank JF & Elsoda M (2003) Observation of bacterial exopolysaccharide in dairy products using cryo-scanning electron microscopy. International Dairy Journal, 13(9), 755-762.

Kefford B, Christian MP, Sutherland BJ, Mayes JJ & Grainger C (1995) Seasonal influences on Cheddar cheese manufacture: Influence of diet quality and stage of lactation. Journal of Dairy Research, 62(03), 529-537.

Lawrence RC, Creamer LK & Gilles J (1987) Texture development during cheese ripening. Journal of Dairy Science, 70(8), 1748-1760.

Lopez C, Camier B & Gassi J-Y (2007) Development of the milk fat microstructure during the manufacture and ripening of Emmental cheese observed by confocal laser scanning microscopy. International Dairy Journal, 17(3), 235-247.

Lues JFR & Botha WC (1998) Relationships amongst South African processed, young and matured Cheddar cheese pertaining to organic acid content and non-starter population. Food Research International, 31(6–7), 449-457.

Marsili R (1985) Monitoring chemical changes in Cheddar cheese during aging by high performance liquid chromatography and gas chromatography techniques. Journal of Dairy Science, 68(12), 3155-3161.

McGregor JU & White CH (1990) Effect of enzyme treatment and ultrafiltration on the quality of lowfat Cheddar cheese. Journal of Dairy Science, 73(3), 571-578.

McSweeney PLH (2004) Biochemistry of cheese ripening: Introduction and overview. In: Patrick F. Fox PLHMTMC and Timothy PG (Eds.), Cheese: Chemistry, physics and microbiology, pp 347-360. Academic Press.

O'Keeffe AM (1984) Seasonal and lactational influences on moisture content of Cheddar cheese. Irish Journal of Food Science and Technology, 8(1), 27-37.

O'Mahony JA, Lucey JA & McSweeney PLH (2005) Chymosin-mediated proteolysis, calcium solubilization, and texture development during the ripening of Cheddar cheese. Journal of Dairy Science, 88(9), 3101-3114.

O'Mahony JA, McSweeney PLH & Lucey JA (2006) A model system for studying the effects of colloidal calcium phosphate concentration on the rheological properties of Cheddar cheese. Journal of Dairy Science, 89(3), 892-904.

Ong L, Dagastine R, Kentish S & Gras S (2013) Microstructure and composition of full fat Cheddar cheese made with ultrafiltered milk retentate. Foods, 2(3), 310-331.

Ong L, Dagastine RR, Kentish SE & Gras SL (2011) Microstructure of milk gel and cheese curd observed using cryo scanning electron microscopy and confocal microscopy. LWT - Food Science and Technology, 44(5), 1291-1302.

Ong L, Dagastine RR, Kentish SE & Gras SL (2012) The effect of pH at renneting on the microstructure, composition and texture of Cheddar cheese. Food Research International, 48(1), 119-130.

Ong L, Henriksson A & Shah NP (2006) Development of probiotic Cheddar cheese containing *Lactobacillus acidophilu*s, *Lb. Casei, Lb. Paracasei* and *bifidobacterium* spp. And the influence of these bacteria on proteolytic patterns and production of organic acid. International Dairy Journal, 16(5), 446-456.

Ong L & Shah NP (2008) Influence of probiotic *Lactobacillus acidophilus* and *L. Helveticus* on proteolysis, organic acid profiles, and ace-inhibitory activity of Cheddar cheeses ripened at 4, 8, and 12 °C. Journal of Food Science, 73(3), M111-M120.

Pommert JF, Klaebe A, Perie J, Lebugle A & Puech J (1988) Observation and analysis of crystalline phases in processed cheese. Journal of Food Science, 53(5), 1367-1369.

Rogers NR, McMahon DJ, Daubert CR, Berry TK & Foegeding EA (2010) Rheological properties and microstructure of Cheddar cheese made with different fat contents. Journal of Dairy Science, 93(10), 4565-4576.

Rousseau M & Le Gallo C (1990) Scanning electron microscopic study of the structure of Emmental cheese during manufacture. Lait, 70(1), 55-66.

Shakeel Ur R, Waldron D & Fox PF (2004) Effect of modifying lactose concentration in cheese curd on proteolysis and in quality of Cheddar cheese. International Dairy Journal, 14(7), 591-597.

St Gelais D (1991) Sugar and organic-acid concentrations during ripening of Cheddar cheese-like products. Milchwissenschaft, 46(5), 288.

Upreti P, McKay LL & Metzger LE (2006) Influence of calcium and phosphorus, lactose, and salt-to-moisture ratio on Cheddar cheese quality: Changes in residual sugars and water-soluble organic acids during ripening. Journal of Dairy Science, 89(2), 429-443.

Zeppa G, Conterno L & Gerbi V (2001) Determination of organic acids, sugars, diacetyl, and acetoin in cheese by high-performance liquid chromatography. Journal Of Agricultural And Food Chemistry, 49(6), 2722-2726.

List of Figures and Table legends

Figure 1: Cryo-SEM microstructure of cheese made with a milk protein concentration of 3.7% w/w (A, E, I, M), 4% w/w (B, F, J, N), 4.8% w/w (C, G , K, O) or 5.8% w/w (D, H, L, P) after ripening at 8^{o}C at Week 1 (A to D), Week 13 (E to H), Week 26 (I to L) or Week 52 (M to P). Images were captured using a solid state detector at a magnification of 8000 x and scale bars of 10 µm or 20 µm were used. Protein network is indicated by the white arrows. Selected fat globules are labelled FG. The black regions are discussed in the text.

Figure 2: Confocal microscopy images of the microstructure of cheese made with a milk protein concentration of 3.7% w/w (A, E, I), 4% w/w (B, F, J), 4.8% w/w (C, G , K) or 5.8% w/w (D, H, L) after ripening at 8^{o}C at Week 1 (A to D), Week 26 (E to H) or Week 39 (I to L). Fat is stained red (Nile Red) and protein green (FCF). Regions coloured black represent pores which correspond to aqueous phase or air pockets. Images were captured using 63x objective lens using a 2x digital zoom and the scale bars are 10 µm in length. A junction between fused curd fragments is shown in D.

Figure 3: Changes in the a) percentage volume of protein b) number of vertices in the protein network c) porosity in cheese samples and d) equivalent diameter of fat globules (µm), made using milk with a nominal protein concentration of 3.7% w/w (◆), 4% w/w (■), 4.8% w/w (▲) or 5.8% w/w (☒) after ripening at 8^{o}C at Weeks 1, 4, 13, 26 and 39. The results are expressed as the mean ± the standard deviation of the mean (n=3). The image analysis of the cheeses was performed over a volume fraction of 140000 µm^{3}.

Figure 4: Changes in the ratio of a) water soluble to total nitrogen (WSN/TN) and b) Tricholaroacetic acid soluble to total nitrogen (TCA-SN/TN) of cheese samples made using

milk with a nominal protein concentration of 3.7% w/w (◆), 4% w/w (■), 4.8% w/w (▲) or 5.8% w/w (☒) after ripening at 8^{o}C at Weeks 1, 4, 13, 26 and 39. The results are expressed as the mean ± the standard deviation of the mean (n=3).

Figure 5: Changes in the a) hardness and b) cohesiveness of cheese samples made using milk with a nominal protein concentration of 3.7% w/w (◆), 4% w/w (■), 4.8% w/w (▲) or 5.8% w/w (☒) after ripening at 8^{o}C at Weeks 1, 4, 13, 26 and 39. The results are expressed as the mean ± the standard deviation of the mean (n=3).

Figure 6: Changes in the concentration of a) lactic acid, b) citric acid and c) acetic acid in cheese samples made using milk with a nominal protein concentration of 3.7% w/w (◆), 4% w/w (■), 4.8% w/w (▲) or 5.8% w/w (☒) after ripening at 8^{o}C at Weeks 1, 4, 13, 26 and 39. The results are expressed as the mean ± the standard deviation of the mean (n=3).

Supplementary Figure 1: Changes in the a) sphericity and b) fat count in cheese samples made using milk with a nominal protein concentration of 3.7% w/w (◆), 4% w/w (■), 4.8% w/w (▲) or 5.8% w/w (☒) after ripening at 8^{o}C at Weeks 1, 4, 13, 26 and 39. The results are expressed as the mean ± the standard deviation of the mean (n=3). The image analysis of the cheeses was performed over a volume fraction of 140000 µm^{3}.

Supplementary Figure 2: Cryo-SEM microstructure of cheese made with a milk protein concentration of a) 3.7% or b) 4.8% w/w after ripening at 8^{o}C at Week 13. Images were captured using a solid state detector at a magnification of 8000 x and a scale bar of 20 µm was used. Mineral deposits are circled in white.

Supplementary Figure 3: Change in the pH of cheese samples made using milk with a nominal protein concentration of 3.7% w/w (◆), 4% w/w (■), 4.8% w/w (▲) or 5.8% w/w (⊠) after ripening at 8^{o}C at Weeks 4, 13, 26 and 39. Data presented is the mean ± the standard deviation of the mean (n=3).

Supplementary Figure 4: Change in a) glucose concentration (mg/g cheese) and b) galactose concentration (mg/g cheese) of cheese samples made using milk with a nominal protein concentration of 3.7% w/w (◆), 4% w/w (■), 4.8% w/w (▲) or 5.8% w/w (⊠) after ripening at 8^{o}C at Weeks 1, 4, 13, 26 and 39. The results are expressed as the mean ± the standard deviation of the mean (n=3).

Supplementary Figure 5: Changes in the concentration of a) formic acid, b) propionic acid, c) butyric acid and d) pyruvic acid in cheese samples made using milk with a nominal protein concentration of 3.7% w/w (◆), 4% w/w (■), 4.8% w/w (▲) or 5.8% w/w (⊠) after ripening at 8^{o}C at Weeks 1, 4, 13, 26 and 39. The results are expressed as the mean ± the standard deviation of the mean (n=3).

Supplementary Figure 6: Changes in the concentration of a) orotic acid and b) uric acid in cheese samples made using milk with a nominal protein concentration of 3.7% w/w (◆), 4% w/w (■), 4.8% w/w (▲) or 5.8% w/w (⊠) after ripening at 8^{o}C at Weeks 1, 4, 13, 26 and 39. The results are expressed as the mean ± the standard deviation of the mean (n=3).

Table 1. Effect of milk protein concentration, ripening time and their interaction on the microstructure of the cheese during ripening.

Supplementary Table 1: Composition and cheese yield for Cheddar cheese made with different milk protein concentrations. These data have previously been reported by Ong et al. (2013) who described the manufacture and microstructure of the pressed Cheddar cheese, prior to the commencement of this study. The composition and yield data for the Cheddar cheese were analysed using one way analysis of variance and Tukey's paired comparison with a significance level of 0.05 in Minitab (Minitab Inc, Pennsylvania, USA).

Supplementary Table 2: Effect of milk protein concentration, ripening time and their interaction on the pH, microbial population, proteolysis, sugar content and texture of the cheese

Supplementary Table 3: Effect of milk protein concentration, ripening time and their interaction on the organic acid content of the cheese

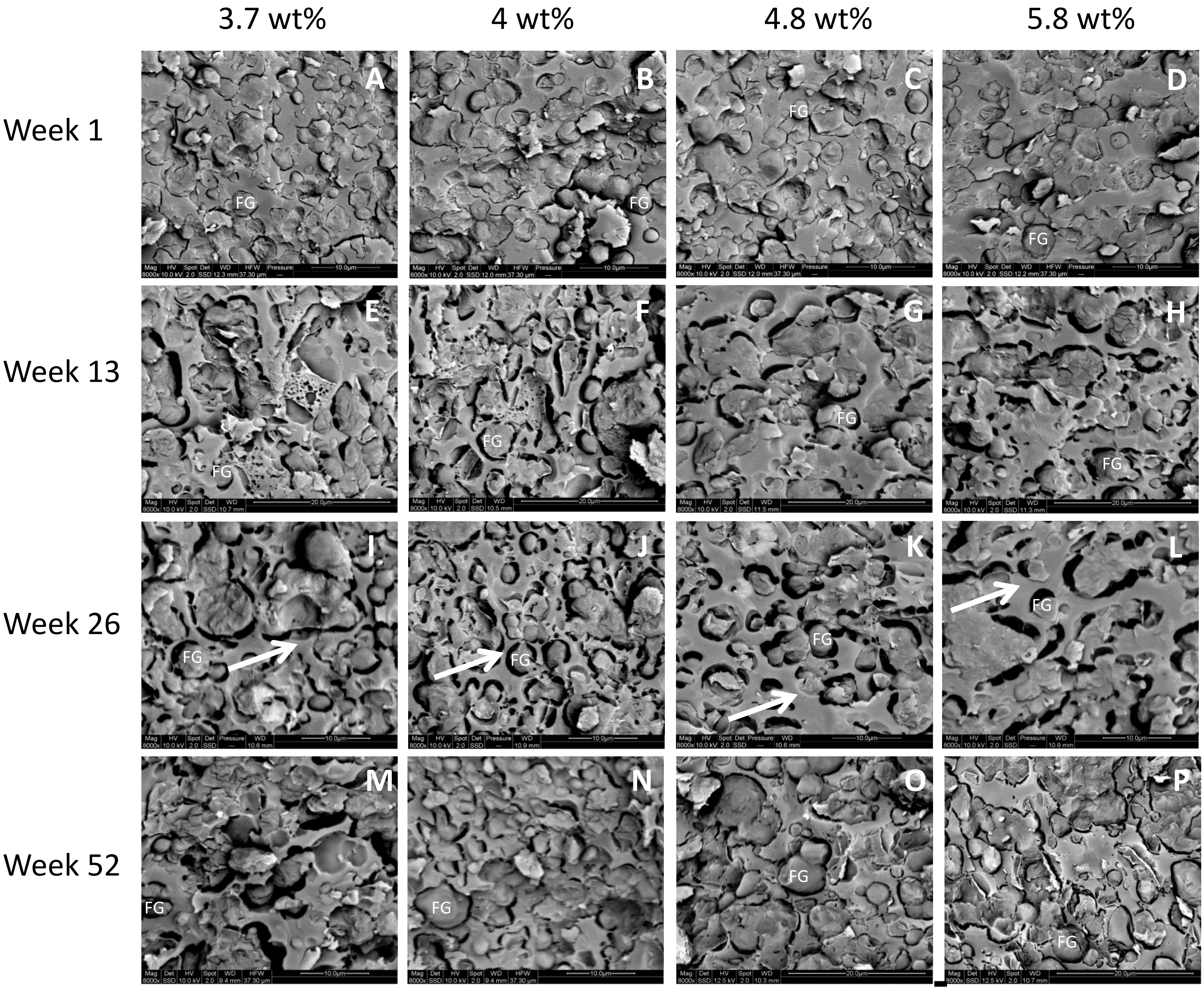

3.7 wt%
4 wt%
4.8 wt%
5.8 wt%
Week 1
Week 13
Week 26
Week 52
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
FG
10.0µm
20.0µm

Fig. 1

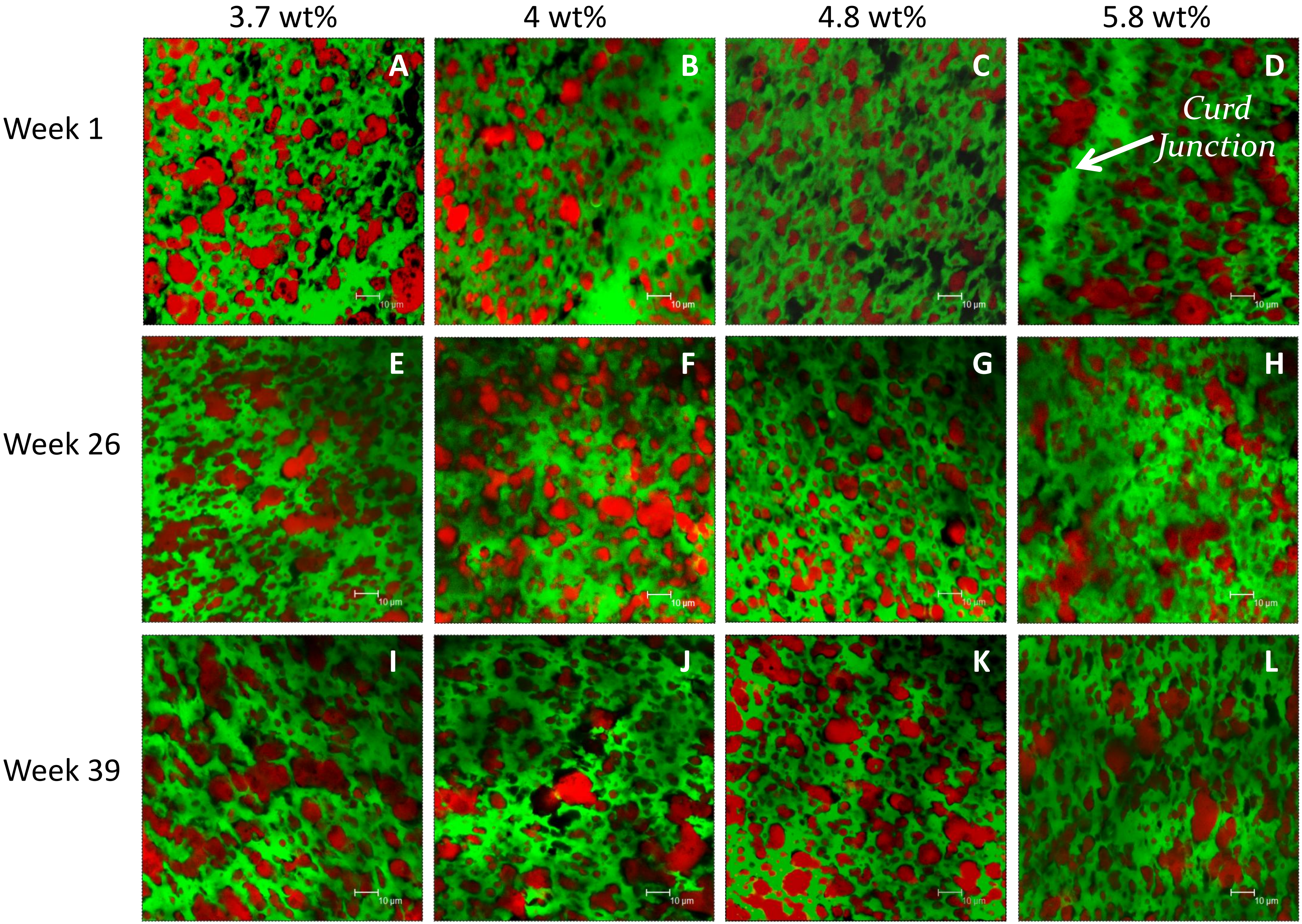

3.7 wt%
4 wt%
4.8 wt%
5.8 wt%
Week 1
Week 26
Week 39
A
B
C
D
Curd Junction
E
F
G
H
I
J
K
L
10 µm

Fig. 2

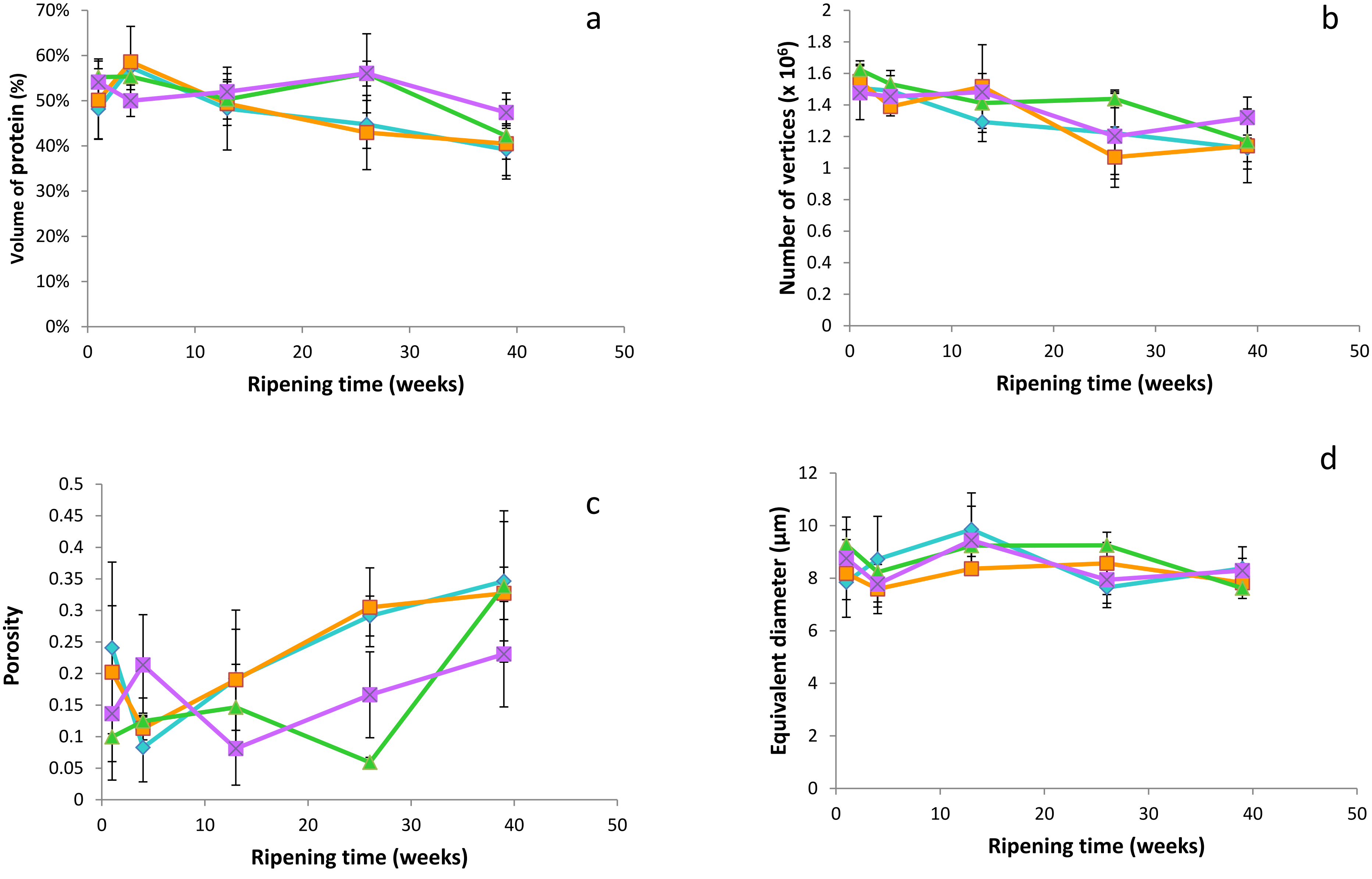

a
Volume of protein (%)
70%
60%
50%
40%
30%
20%
10%
0%
0
10
20
30
40
50
Ripening time (weeks)
b
Number of vertices (x 10^6)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
Ripening time (weeks)
c
Porosity
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
10
20
30
40
50
Ripening time (weeks)
d
Equivalent diameter (µm)
12
10
8
6
4
2
0
0
10
20
30
40
50
Ripening time (weeks)

Fig. 3

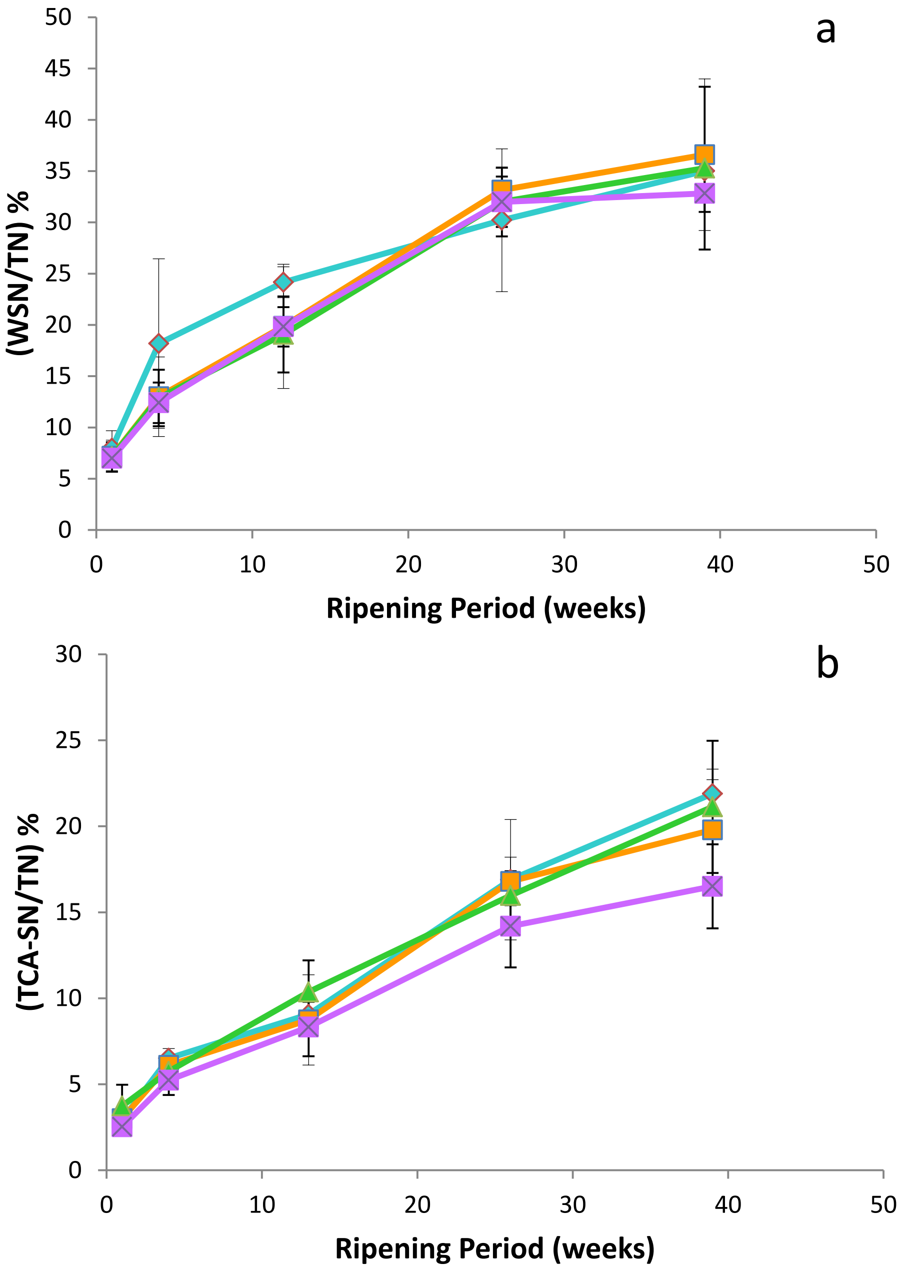

a
50
45
40
35
30
25
20
15
10
5
0
0
10
20
30
40
50
(WSN/TN) %
Ripening Period (weeks)
b
30
25
20
15
10
5
0
0
10
20
30
40
50
(TCA-SN/TN) %
Ripening Period (weeks)

Fig. 4

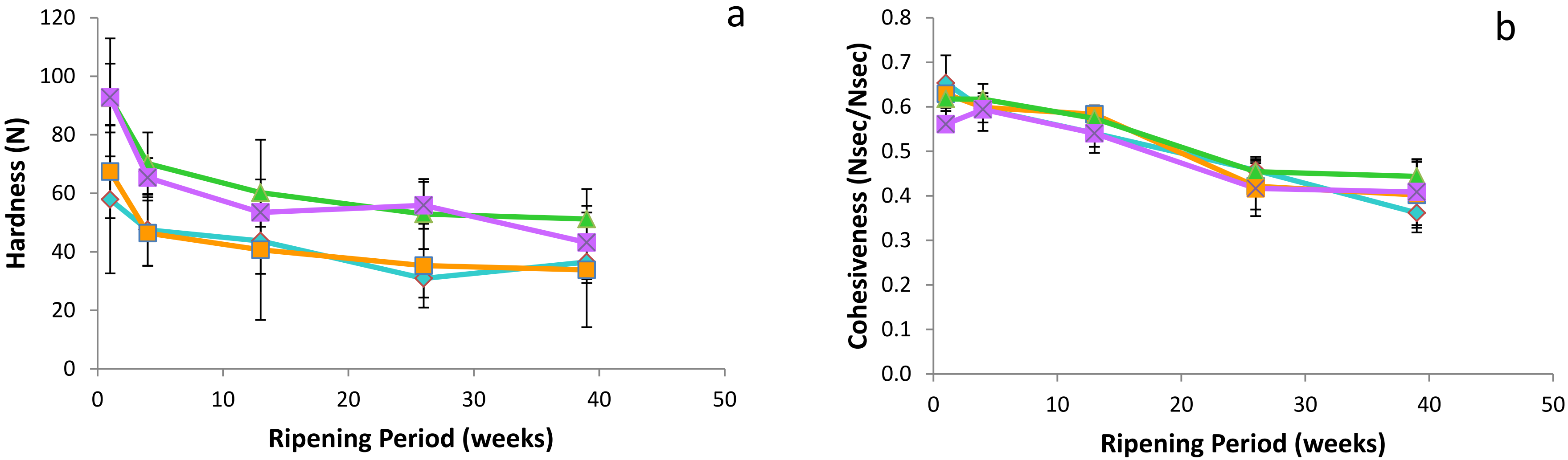

a
Hardness (N)
120
100
80
60
40
20
0
0
10
20
30
40
50
Ripening Period (weeks)
b
Cohesiveness (Nsec/Nsec)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
Ripening Period (weeks)

Fig. 5

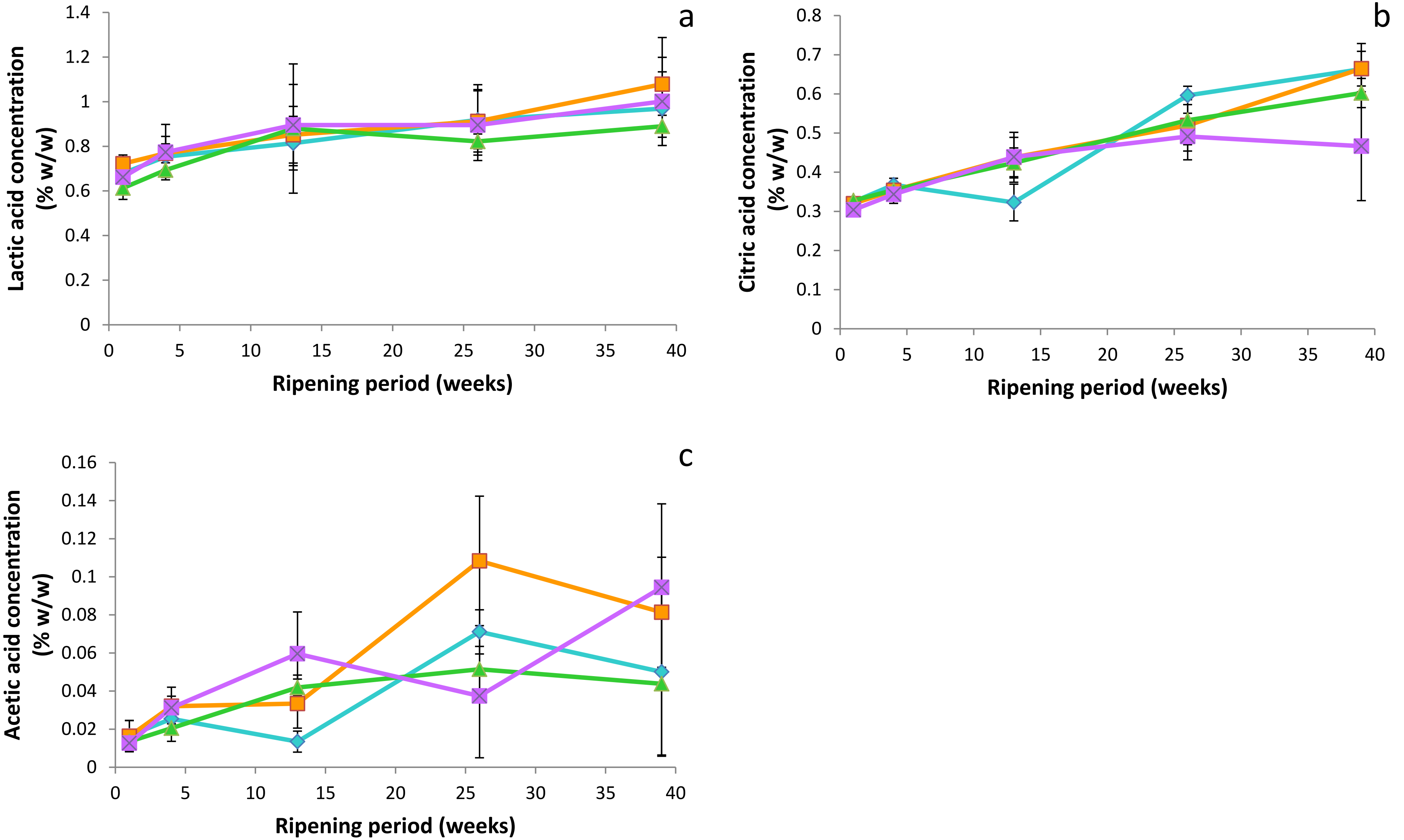
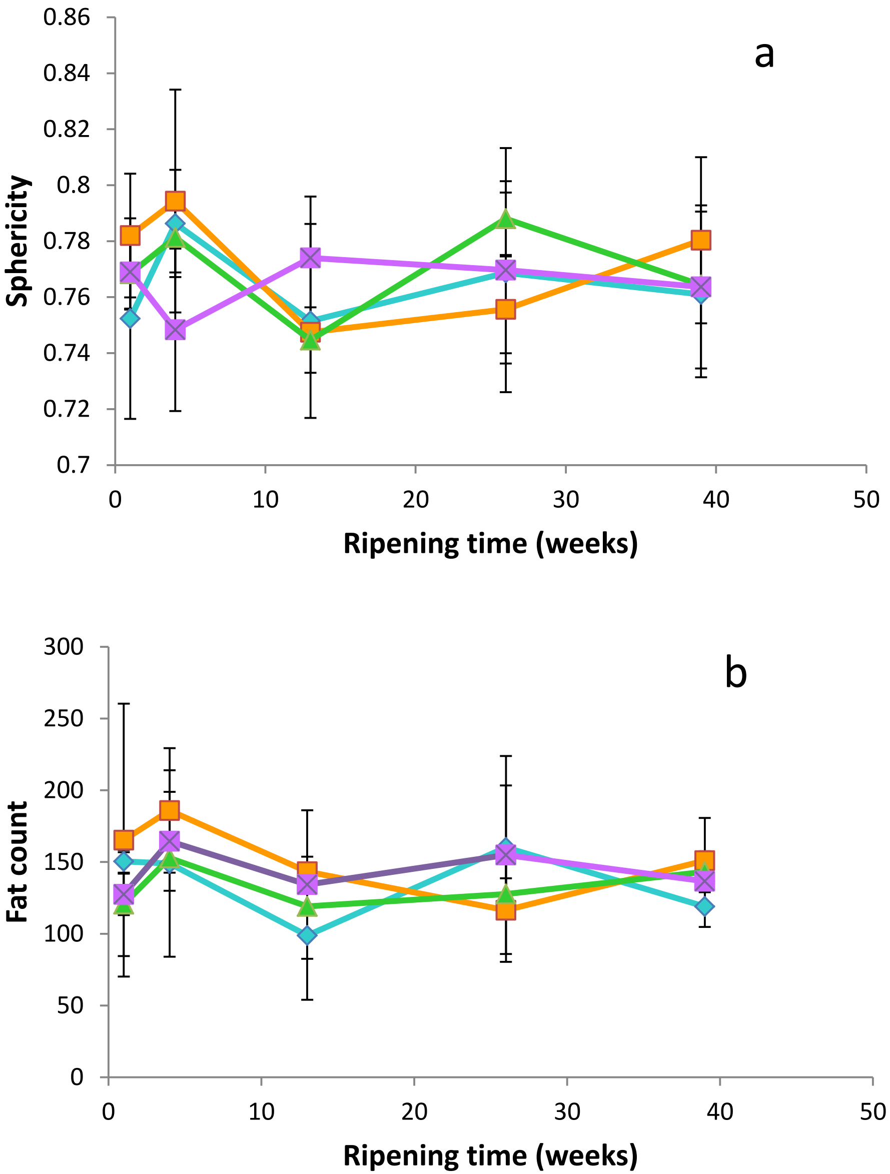
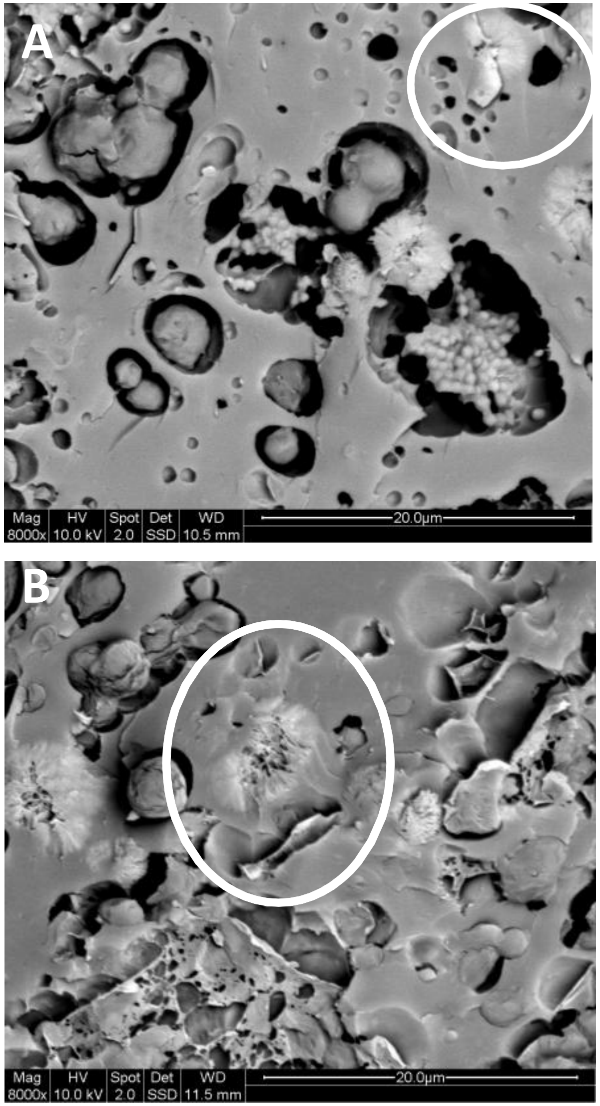

a
Lactic acid concentration (% w/w)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
Ripening period (weeks)
0
5
10
15
20
25
30
35
40
b
Citric acid concentration (% w/w)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Ripening period (weeks)
0
5
10
15
20
25
30
35
40
c
Acetic acid concentration (% w/w)
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
0.16
Ripening period (weeks)
0
5
10
15
20
25
30
35
40

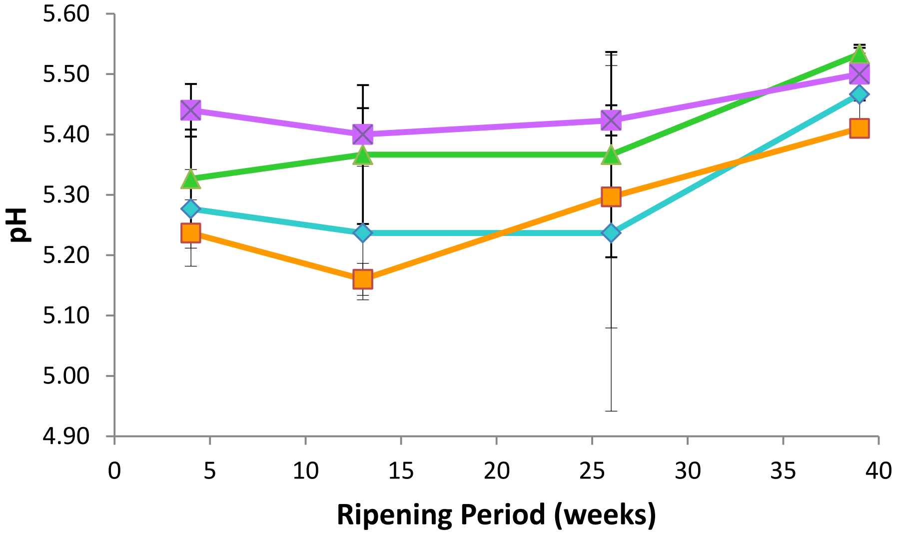
Fig. 6



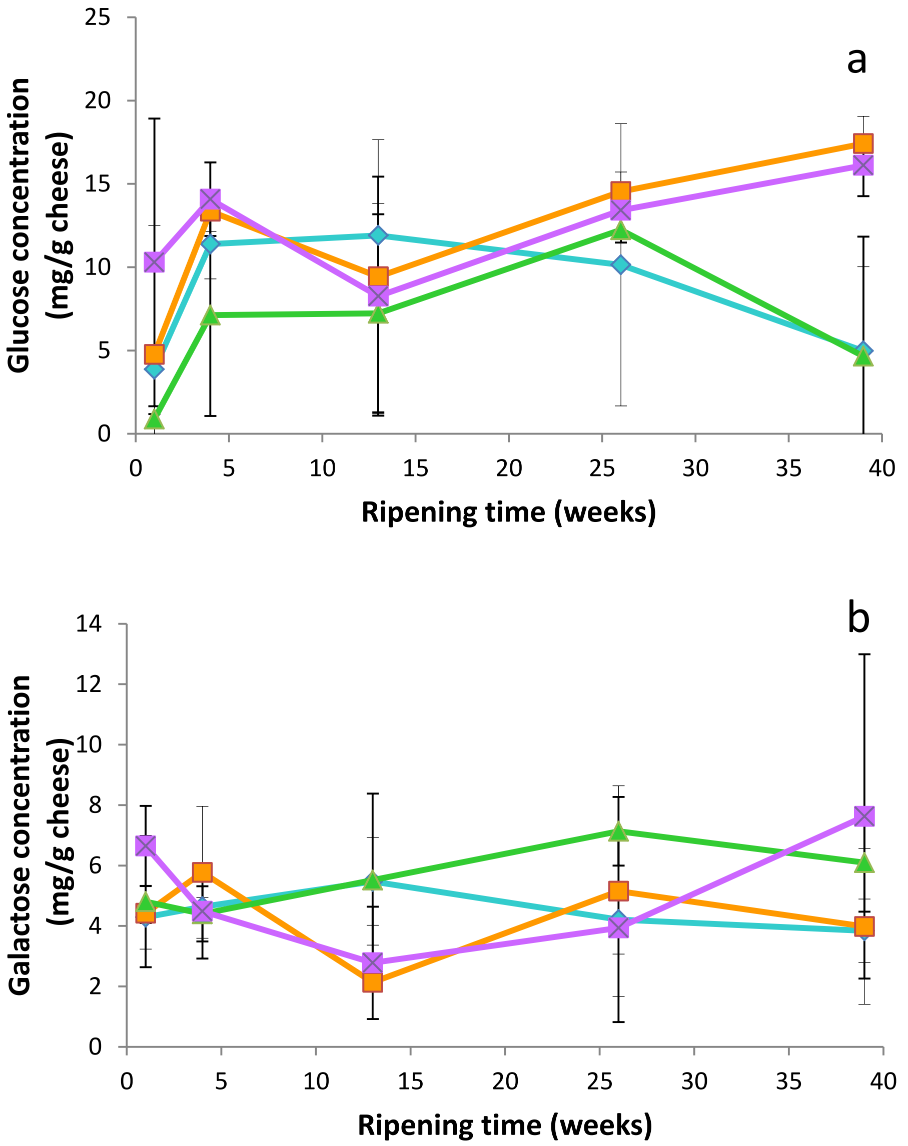
Supplementary Fig. 1



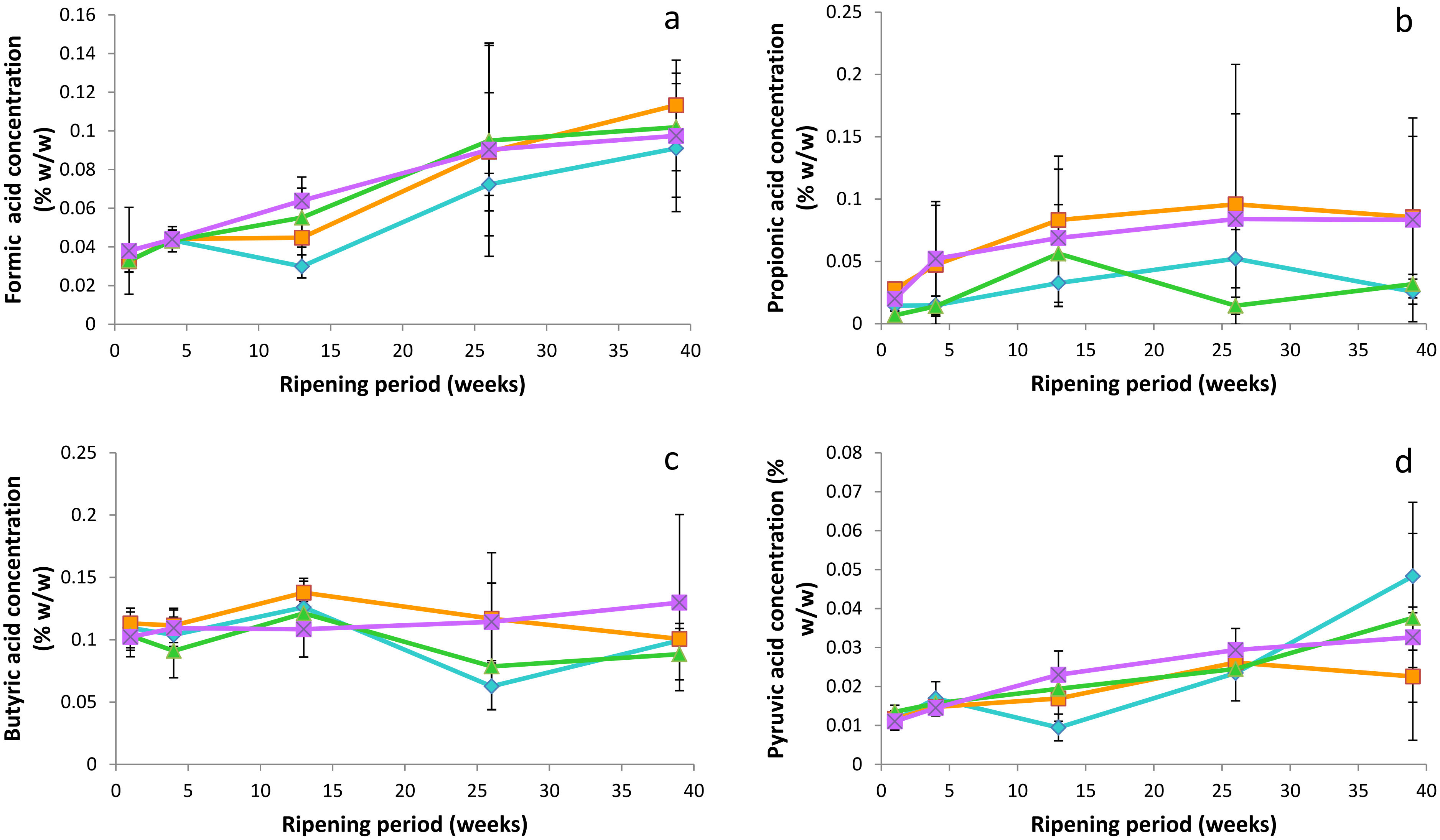
Supplementary Fig. 2



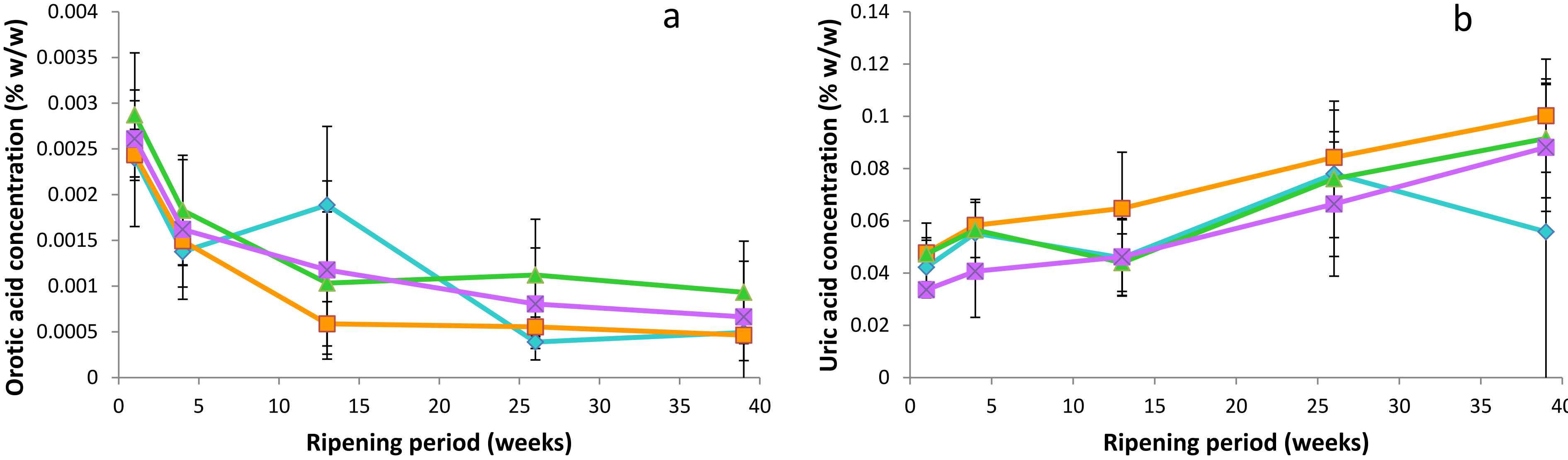
Supplementary Fig. 3



Supplementary Fig. 4



Supplementary Fig. 5



Supplementary Fig. 6

Table 1. Effect of milk protein concentration, ripening time and their interaction on the microstructure of the cheese during ripening.

Variables and Interactions	Probability, P [a]					
	% Volume of protein in cheese section	**Number of vertices in protein section[b]**	**Porosity**	**Number of fat globules[b]**	**Equivalent diameter of fat globules**	**Sphericity of fat globules**
Milk Protein Concentration (M)	NS	NS	NS	NS	NS	NS
Ripening Time (T)	**<0.001**	**<0.001**	**<0.001**	NS	**0.026**	NS
M*T	NS	NS	**0.008**	NS	NS	NS

[a] *The statistics are significant when $P<0.05$*

[b] *The image analysis of the cheeses was performed over a volume fraction of 140000 μm^3.*

NS The statistics are not significant ($P>0.05$)

Supplementary Table 1: Composition and cheese yield for Cheddar cheese made with different milk protein concentrations. These data have previously been reported by Ong et al. (2013) who described the manufacture and microstructure of the pressed Cheddar cheese, prior to the commencement of this study. The composition and yield data for the Cheddar cheese were analysed using one way analysis of variance and Tukey's paired comparison with a significance level of 0.05 in Minitab (Minitab Inc, Pennsylvania, USA).

Nominal milk protein concentration (% w/w)	**3.7**	**4**	**4.8**	**5.8**
Protein (% w/w)	24.5 ± 0.5^{a}	24.2 ± 0.2^{a}	25.0 ± 0.4^{a}	25.5 ± 0.4^{a}
Fat (%w/w)	33.5 ± 0.7^{b}	34.7 ± 0.5^{ab}	35.8 ± 0.9^{a}	36.2 ± 0.4^{a}
Moisture (% w/w)	37.0 ± 1.0^{a}	36.5 ± 0.3^{a}	34.5 ± 0.5^{b}	34.1 ± 0.5^{b}
Salt (% w/w)	1.47 ± 0.06^{a}	1.43 ± 0.15^{a}	1.55 ± 0.18^{a}	1.52 ± 0.08^{a}
Yield (total cheese per kg of cheese-milk, % w/w)	10.7 ± 0.3^{d}	12.0 ± 0.3^{c}	14.0 ± 0.8^{b}	17.0 ± 0.3^{a}

Results are expressed as mean ± standard deviation of mean (n = 3). ^{ab}; means across a single row with different superscripts are significantly different (P < 0.05).

Supplementary Table 2: Effect of milk protein concentration, ripening time and their interaction on the pH, proteolysis, sugar content and texture of the cheese

Variables and Interactions	Probability, P[a]						
	pH	**WSN/TN**	**TCA-SN/TN**	**Glucose**	**Galactose**	**Hardness**	**Cohesiveness**
Milk Protein Concentration (M)	NS	NS	**0.049**	NS	NS	**0.030**	NS
Ripening Time (T)	**<0.001**	**<0.001**	**<0.001**	**0.002**	NS	**<0.001**	**<0.001**
M*T	NS	NS	NS	NS	NS	NS	NS

[a] *The statistics are significant when P<0.05*

NS The statistics are not significant (P>0.05)

Supplementary Table 3: Effect of milk protein concentration, ripening time and their interaction on the organic acid content of the cheese

Variables and Interactions	Probability, P [a]								
	Citric	**Lactic**	**Formic**	**Acetic**	**Propionic**	**Butyric**	**Pyruvic**	**Orotic**	**Uric**
Milk Protein Concentration (M)	**0.011**	NS	NS	NS	NS	NS	NS	NS	NS
Ripening Time (T)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.003**	**0.017**	**<0.001**	**<0.001**	**<0.001**
M*T	**0.008**	NS	NS	**0.038**	NS	NS	NS	NS	NS

[a] *The statistics are significant when P<0.05*

NS The statistics are not significant (P>0.05)