1 **Microstructure and physicochemical properties reveal differences between high**
2 **moisture buffalo and bovine Mozzarella cheeses**

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Abstract

Mozzarella cheese is a classical dairy product but most research to date has focused on low moisture products. In this study, the microstructure and physicochemical properties of both laboratory and commercially produced high moisture buffalo Mozzarella cheeses were investigated and compared to high moisture bovine products. Buffalo and bovine Mozzarella cheeses were found to significantly differ in their microstructure, chemical composition, organic acid and proteolytic profiles but had similar hardness and meltability. The buffalo cheeses exhibited a significantly higher ratio of fat to protein and a microstructure containing larger fat patches and a less dense protein network. Liquid chromatography mass spectrometry detected the presence of only β -casein variant A2 and a single β -lactoglobulin variant in buffalo products compared to the presence of both β -casein variants A1 and A2 and β -lactoglobulin variants A and B in bovine cheese. These differences arise from the different milk composition and processing conditions. The differences in microstructure and physicochemical properties observed here offer a new approach to identify the sources of milk used in commercial cheese products.

1. Introduction

Mozzarella belongs to the *pasta-filata* family, where the cheese is stretched or plasticised in hot water (Jana & Mandal, 2011; Kindstedt, 1993). Traditionally produced in Italy from the milk of water buffalo, this cheese is now manufactured worldwide and can be produced from several sources of milk including bovine, goat or sheep (Kindstedt, Caric, & Milanovic, 2004). The cheese can be classified into two types based on moisture content. These are low moisture Mozzarella cheese (LMMC) with a moisture content between 45-52 % w/w and high moisture Mozzarella cheese (HMMC) with a moisture level > 52 % w/w (Jana & Mandal, 2011; Kindstedt et al., 2004).

The moisture content is a major determinant of the quality and functional properties of Mozzarella cheese (Kindstedt, 1993; McMahon & Oberg, 1998; Rowney, Roupas, Hickey, & Everett, 1999). A high moisture Mozzarella cheese has a soft texture and milky flavour but a poor shreddability. Consequently this cheese is mostly used as a table cheese that is consumed within a few days of production. Low moisture Mozzarella cheese has a firmer body, better shreddability, a longer shelf-life and is normally used as an ingredient for pizza toppings (Kindstedt, 2012). Despite the importance of moisture, most studies have focused on low moisture products made from bovine and buffalo milk (Guinee, Feeney, Auty, & Fox, 2002; Jana & Upadhyay, 1997; Ma, James, Zhang, & Emanuelsson-Patterson, 2013; Rowney et al., 1999; Yazici & Akbulut, 2007) due to the more widespread use of this cheese. A greater understanding of the physicochemical properties of the high moisture Mozzarella cheeses, particularly the differences in these properties arising from different milk sources, is important as this knowledge can be used to assist with quality control and new product development.

The microstructure of a cheese is a key factor in determining the resulting functional properties (Everett & Auty, 2008; Ong, Dagastine, Kentish, & Gras, 2013; Rowney et al., 1999). This structure is known to be affected by processing conditions (Ma, James, Zhang, & Emanuelsson-Patterson, 2011; Ribero, Rubiolo, & Zorrilla, 2009), such as the mechanical and thermal treatments that occur during Mozzarella production that alter the arrangement of fat and protein. Different microscopic techniques have been used to characterise the microstructure of low moisture Mozzarella cheese, including confocal laser scanning microscopy (CLSM), transmission electron microscopy (TEM), scanning electron microscopy (SEM) and cryo-SEM

(Ma et al., 2013; Reid & Yan, 2004; Ribero et al., 2009; Tunick, Van Hekken, Cooke, & Malin, 2002). The microstructure of a high moisture Mozzarella cheese, however, has not been investigated. Furthermore, each of the above techniques has its own advantages and limitations (Ong, Dagastine, Kentish, & Gras, 2011). A combination of multiple microscopy techniques therefore allows a greater understanding of cheese structure.

Studies comparing high moisture buffalo and bovine cheeses are limited. Pagliarini, Monteleone, and Wakeling (1997) have observed that the high moisture Mozzarella cheese made from full fat buffalo milk has significantly different sensorial properties to the equivalent bovine cheese. The buffalo product was identified by its cohesiveness, acid and salty flavour and yoghurt odour, while the bovine cheese was identified by its sweetness together with a milky and creamy flavour and fibrous and elastic texture. Physically, the curds from the two milk types have been found to differ; the curd from buffalo milk exhibited a greater firmness (indicated by a higher storage modulus G') with a higher calcium concentration and a higher yield than the curd produced from bovine milk (Hussain, Bell, & Grandison, 2011; Hussain, Yan, Grandison, & Bell, 2012), whilst the porosity remained similar (Hussain, Grandison, & Bell, 2012). Interestingly, if curd was prepared from ultrafiltered bovine milk that had been standardised to a similar fat and protein concentration as buffalo milk, this product was still less firm than the buffalo equivalent and significantly more porous (Hussain, Bell, & Grandison, 2013a, 2013b), indicating that gross composition is insufficient to explain the differences between these products and highlighting the need to consider differences in individual fat and protein components. The microstructure, texture and other physicochemical properties of the final Mozzarella cheeses made from these two milk types, however, were not further investigated in these studies.

The objective of this study was to characterise the microstructure of high moisture buffalo mozzarella using both CLSM and cryo-SEM techniques and the physicochemical properties of cheese produced both commercially and within a controlled laboratory environment. The study aimed to obtain a better understanding of this cheese and to compare the properties of the high moisture buffalo Mozzarella cheese with bovine Mozzarella cheese to allow a greater insight into the effect of milk type on the quality and functional properties of the cheese. Herein, the term Mozzarella cheese is used to indicate the high moisture variant and wherever different, the details of the cheese are clearly stated.

2. Materials and methods

2.1 Production of buffalo Mozzarella cheese in the laboratory

Buffalo Mozzarella cheese was produced following a previously described method (Fainberg, 2012; Yazici & Akbulut, 2007) with some parameters optimized as a result of laboratory screening experiments. Pasteurised buffalo milk was obtained from a local farm (Shaw River, Yambuk, Australia). The milk was used for cheese making within one day of receipt. Four litres of buffalo milk was warmed to 37 °C before the addition of starter culture TCC-20 (0.072 g.L⁻¹ ~ 0.4 U.L⁻¹, CHR-Hansen, Bayswater, Australia) containing a mixture of *Streptococcus thermophilus* and *Lactobacillus helveticus*. When the milk pH dropped to 6.5, 0.2 ml per L of Chymosin (40 IMCU.L⁻¹, Chymax-plus, CHR-Hansen, Bayswater, Australia) was added and the milk allowed to set for approximately 30 min until an appropriate curd firmness, assessed by a knife test, was obtained. The curd was then cut into small cubes, approximately 2 cm in size and left to heal for 10 min. The curd was gently stirred (~ 30 s) followed by cooking at 42 °C. During cooking, the curd was stirred for 10 min followed by resting for 10 min. This stirring step was repeated until the curd pH reached 5.2, which normally took around 1.5-2 hours. The whey was drained and the curd milled and dry-salted with 2% w/w salt. The curd was then submerged in hot tap water 1:1.5 w:w at 85-90 °C and incubated for 3 min to allow the heat to penetrate into the curd. Half of this water was then decanted and fresh hot water poured onto the curd and left to incubate for another 3 min before stretching. A wooden paddle was used to assist the stretching step in hot water. The cheese curd was moulded into small balls, approximately 80-100 g in size. The cheese balls were finally stored in chilled water in a cold room at 4 °C until further analysis. The cheese production was repeated in three trials on different days and at least two cheese samples were analysed in each trial for each analysis. The shelf life of high moisture traditional buffalo Mozzarella cheese is approximately five to seven days after production (Altieri, Scrocco, Sinigaglia, & Del Nobile, 2005), therefore the laboratory buffalo Mozzarella cheese was characterised on day 1 (BM Lab-D1) and day 7 (BM Lab-D7) of storage.

2.2 Commercial buffalo and bovine Mozzarella cheese collection

The commercial cheeses analysed included two buffalo (BM) and two bovine (CM) cheese products manufactured in Australia, coded as BM-cheese A, B and CM-cheese A and B and one buffalo Protected Designation of Origin cheese produced in Italy (Zanetti Mozzarella di Bufala Campana, purchased in Cora supermarket, Pacé, France). The products manufactured in Australia were used for the characterisation of microstructural and physicochemical properties, while the buffalo cheese purchased in France was only used for the purpose of microstructural comparison. Six cheese samples were analysed for each commercial Mozzarella cheese, except for the moisture and microstructural investigations where three and four samples were used, respectively.

2.3 Chemical compositional analysis

The protein, fat and moisture content of the milk and cheese was determined using the Kjeldahl method (IDF, 2008), the gravimetric method (IDF, 2004a) and the oven drying method (IDF, 2004b) respectively. The minerals, calcium, phosphorous, sodium and ash contents were determined using inductively coupled plasma optical emission spectrometry (Varian ICP - OES 720, Varian Inc, Palo Alto, CA, USA) following an established method (Rice, 2008). The concentration of sugars (lactose, glucose and galactose) was determined by a high performance liquid chromatography (HPLC, Shimadzu Prominence system, Rydalmere, Australia) using a refractive index detector and a 300 x 7.8 mm Rezex RCM-Monosaccharide Ca^{2+} column (Phenomenex, Lane Cove, Australia), as described previously (Gosling et al., 2009). The organic acid profile was determined using an HPLC system equipped with a photo diode array ultra violet detector and a Bio-Rad Aminex HPX 87H cation exchange column connected to a cation H^+ guard column (Bio Rad Laboratories Pty Ltd, Hercules, CA, USA), as previously described (Nguyen, Ong, Lefevre, Kentish, & Gras, 2014). The cheese pH was measured using an electrode pH meter (Orion 720A, Wallsend, Australia).

2.4 Texture analysis

The texture of the Mozzarella cheese was determined following the method described by Zisu and Shah (2005) with some modifications. The measurement was performed using a TA.XT-2 texture analyser (Stable Microsystems, Godalming, England) equipped with a 20 N load cell and a 25.4 mm diameter cylindrical probe. A cylindrical portion was excised from the central part of the cheese ball using a cork borer 20 mm in diameter. A sample 20 mm in height was

then obtained in the middle part of the cylindrical portion. The cheese sample was kept in an enclosed container to prevent dehydration and held at 20°C for at least two hours prior to measurement, in order to allow equilibration to a temperature similar to that of consumption. The contact area and the contact force were set at 1 mm² and 5 g, respectively. The instrument speed was set at 2 mm.s⁻¹. The compression distance, the distance from the surface of sample, was set at 10 mm (50% compression). Data were recorded at a rate of 200 points per second. The cheese hardness was determined as the maximum force measured during sample compression.

2.5 Meltability

Cheese meltability was investigated using a previously described method, with some modifications (Muthukumanrappan, Wang, & Gunasekaran, 1999). Cheese samples (20 mm in diameter and 10 mm in height) were placed in Petri dishes and heated at 130 °C for 10 min. After the melted cheese had cooled to room temperature for 5 min, the minimum and maximum diameters of the spread cheese were measured and the cheese meltability was expressed as the average of the measured maximum and minimum diameters.

2.6 Proteolysis

The proteolysis of the cheese was investigated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The sample preparation and conditions of the gel electrophoresis have previously been described in detail elsewhere (Ong, Henriksson, & Shah, 2006). The electrophoresis system, the sample and running buffers were purchased from Invitrogen (Melbourne, Australia) while the standard molecular weight (Precision Plus Protein-All blue standards), the Coomassie stain and the precast gels were supplied from Biorad (Gladesville, Australia). The protein bands in the gel were visualized using a Fuji Film intelligent Dark Box II with Fuji Film LAS-1000 Lite V1.3 software (Brookvale, Australia).

2.7 Microstructural analysis

The microstructure of the cheese samples was analysed using both confocal laser scanning microscopy (CLSM) and cryo-scanning electron microscopy (cryo-SEM) techniques. For CLSM analysis, samples approximately 3 mm x 3 mm x 2 mm in size were carefully excised from the skin layer (outer surface layer) or from the middle layer (centre) of the cheese balls.

The samples were carefully placed on a flat surface for staining and subjected to CLSM observation as previously described (Ong et al., 2011). Briefly, samples were stained with multiple fluorescent probes including Nile Red (Sigma, MO, USA) for labelling fat and Fast Green FCF (Sigma) for labelling protein. Rh-DOPE (Avanti Polar Lipid, AL, USA) was used for labelling phospholipids within the milk fat globule membrane (MFGM) *in situ* in cheese as previously reported (Lopez, Briard Bion, Beaucher, & Ollivon, 2008). Samples were dual labelled with either Nile Red and Fast Green FCF or Rh-DOPE and Fast Green FCF. Samples were observed using inverted CLSM microscopes (Leica SP2, Leica Microsystems, Heidelberg, Germany) or a Nikon Eclipse-TE2000-C1si (Nikon, Champigny sur Marne, France), with the excitation/ emission wavelengths set at 543 nm/500-600 nm (for Nile Red when using Nikon Eclipse-TE2000-C1si) or 488 nm/500-600 nm (for Nile Red when using Leica SP2), 543 nm/ 565-615 nm (for Rh-DOPE) and 633 nm/ 650-710 nm (for Fast Green FCF). Quantitative image analysis of the microstructure was performed using Imaris image processing software (Bitplane, South Windsor, CT, USA) following a previously described method (Ong, Dagastine, Kentish, & Gras, 2012).

For cryo-SEM analysis, samples approximately 5 mm x 2 mm x 2 mm in size were obtained from the skin and middle of the cheese balls and subjected to a previously described method (Ong et al., 2011). Samples were observed at a spot size of 2 and an acceleration voltage of 10 kV using a field emission scanning electron microscope (Quanta, Fei Company, Hillsboro, OR, USA.).

2.8 Liquid chromatography – mass spectrometry (LC-MS) analysis

A cheese sample was crumbled into small pieces and 0.2 g was added to 1.8 mL of water and 2 mL of working solution 1 containing 0.1 M Bis-Tris Buffer (pH 6.8; Sigma), 6 M guanidine hydrochloride (GndHCl; Sigma), 5.37 mM trisodium citrate (Ajax Finechem, NSW, Australia) and 19 mM DL-Dithiothreitol (Astral Scientific, NSW, Australia). The sample was then incubated at 45°C for 1 hour with shaking for 30 s at 15 min intervals followed by centrifugation at room temperature at 11,000 g for 10 min. The sample was cooled on ice and the fat that formed the top layer removed. An aliquot of 200 µL of sample was mixed with 600 µL of working solution 2 containing 4.5 M GndHCl in a solvent consisting of acetonitrile,

water and trifluoroacetic acid in a ratio of 10:90:1 (v:v:v, pH 2). The sample was then finally filtered through a 0.45 µm membrane before LC-MS analysis.

LC-MS analysis of the cheese extract was performed using an Agilent 1200 series HPLC system (Agilent Technologies, CA, USA) equipped with a photo diode array ultra violet detector (G1315C, Agilent Technologies) coupled to a G6220A Accurate Mass TOF LC/MS (Agilent Technologies). The LC separation was carried out using a Zorbax 300SB-C8 column (4.6 x 150 mm, 3.5 µm; Agilent) where the wavelength for protein detection was set to 214 nm. The elution was performed at 45°C at a flow rate of 0.5 mL/min using a mixture of eluent A (0.1% formic acid in water) and eluent B (0.1% formic acid in 100% acetonitrile). The flow gradient was (i) 0-5 min, 33-35% B, (ii) 5-9 min, 35-37% B, (iii) 9-12 min, 37-38% B, (iv) 12-14 min, 38% B, (v) 14-18 min, 38-39% B, (vi) 18-20 min, 39-40% B, (vii) 20-30 min, 45% B, (viii) 30-31 min, 45-33% B and (ix) 31-40 min, 33% B. To clean the column and minimise the carry over, a blank run followed by a washing step was carried out between samples. Trifluoroethanol was used as the blank with the flow gradient during the first 30 min similar to the above set up for the sample, followed by an increase of solvent B from 45% to 66% at 30-35 min, where the washing step began and lasted for 2 min. The column was then equilibrated for 8 min in 33% B. The sample eluted out of the column in the first 4 minutes was eliminated from the mass spectrometry (MS) analysis to prevent salt flowing to the spectrometer. All mass spectra were acquired in the positive ion mode using a fragmentor voltage of 250 V with the instrument set to scan from m/z 100 – 3200. The Agilent MassHunter Workstation Data Acquisition software was used for equipment control and data acquisition, while the Agilent MassHunter Qualitative Analysis software was used for data processing. Briefly, from the total ion chromatograms (TIC), the whole spectra including all peaks was generated. The spectra at a selected retention time ranges were then deconvoluted based on a charge state deconvolution algorithm with a mass accuracy set at 0.5 Da. The deconvoluted zero-charge spectra show the molecular weight of the protein eluted at a particular time point and the signal abundance of the protein.

2.9 Statistical analysis

Data analysis was performed using Minitab software (V16, Minitab Inc., State College, PA, USA). One way analysis of variance (ANOVA) and Fisher's paired comparison. A significance level of $P = 0.05$ was applied to assess the difference between means.

3. Results and discussion

3.1 Basic chemical composition

Mozzarella cheese made from buffalo milk had significantly higher average fat content (23.2 - 28.7 % w/w) compared to the cheese made from bovine milk (14.9 - 15.2% w/w) for both the laboratory and commercial cheeses used in this study (Table 1). The protein content was similar for all cheeses, resulting in a higher fat: protein ratio for buffalo cheeses. The moisture content varied considerably among products ranging from 53.6% (w/w) to 67.4% (w/w), while the pH was relatively consistent, ranging from 5.3-5.4, with the exception of one sample with a pH of 5.8 (commercial CM-cheese A). The chemical composition in the buffalo cheese produced in the laboratory was within the range observed for commercial samples and no significant difference was observed in the moisture content or pH of the laboratory cheeses during the seven days of storage (Table 1).

The higher fat content of buffalo cheeses observed here is a result of differences in milk composition (Supplementary Figure 1A), as the fat content of buffalo milk is approximately double that in bovine milk (6.7 ± 0.4 % w/v vs. 3.6 ± 0.1 % w/v). Buffalo milk also contains more protein (3.9 ± 0.1 % w/v vs. 3.1 ± 0.2 % w/v); as the magnitude of this difference is smaller this difference did not significantly alter the protein concentration between cheeses. The higher fat: protein ratio observed here for buffalo products is also consistent with the ratios previously reported for buffalo Mozzarella cheeses (1.7-1.8) and bovine cheeses (1.1 -1.2); the moisture range of buffalo and bovine cheese is also consistent with previous reports of 57.8 – 68.7 % (Pagliarini et al., 1997).

3.2 Sugar and organic acid profiles

The concentration of sugars (lactose, glucose and galactose) varied significantly between all products ($P < 0.05$). There was no significant trend in sugar concentration, however, to differentiate buffalo cheeses from bovine cheese products (Figure 1A).

The concentration of acetic, orotic and hippuric acids was lower in buffalo than in the bovine cheeses (Figure 1B) and could be used to differentiate these two products. These differences are likely due to the initial lower concentration of these acids in buffalo milk (Supplementary Figure 1B); they also provide insights into the different flavour profiles as noted for buffalo and bovine Mozzarella cheese products in the previous study (Pagliarini et al., 1997). In contrast, the concentration of major organic acids (citric, formic and lactic acids; Figure 1C) and minor organic acids (pyruvic and uric acids; Figure 1B) did not differ between the products.

The concentrations of sugars and most organic acids of the laboratory cheese were within the range observed in the commercial buffalo cheeses (BM-cheese A and BM-cheese B). The concentrations did not change significantly with storage (laboratory products at day 1 and day 7), except for a slight decrease ($P < 0.05$) in the content of galactose (Figure 1A) and citric acid (Figure 1C), probably due to the metabolic activity of the starter culture that can utilise these substrates.

Interestingly, CM-cheese A showed a significantly higher concentration of lactose (~ 0.9 % w/w) and a negligible concentration of glucose and galactose (< 0.01 % w/w) (Figure 1A). This cheese also exhibited a significantly lower concentration of lactic acid (~ 7 mg/100g) (Figure 1C), which is consistent with the significantly higher pH observed in this product (Table 1). These results suggest that CM-cheese A could have been made via direct acidification or a combination of direct and cultured acidification. Direct acidification involves the addition of one or a mixture of acids, in place of starter cultures (Jana & Mandal, 2011; Joshi, Muthukumarappan, & Dave, 2004). The absence of starter culture activity leads to a lower concentration of metabolic products such as lactic acid. Direct acidification allows the curd to be stretched at $\text{pH} > 5.4$, while the curd is normally left until a pH of ~ 5.2 when using starter cultures (Guinee et al., 2002; Jana & Mandal, 2011; Kindstedt, 1993). This difference is thought to arise from the greater demineralisation that occurs during direct acidification, where calcium is solubilised and transferred from the casein micelles to the whey (Guinee et al., 2002).

3.3 Mineral content

The calcium and phosphorous concentration was similar for all buffalo Mozzarella cheese products, significantly higher ($P < 0.05$) than CM-cheese A and lower than CM-Cheese B (Figure 1D). The calcium and phosphorous content in bovine milk is lower than in buffalo milk (47.1 mM vs. 30.5 mM for calcium and 27.7 mM vs. 19.2 mM for phosphorous) (Ahmad et al., 2008) and likely explains the lower concentrations present in CM-Cheese A. The higher concentration of calcium in CM-cheese B could result from modifications to the cheese production process, such as the addition of calcium or phosphate salt in the brining solution, as has been used in Mozzarella cheese making in previous studies (Jana & Mandal, 2011; Kindstedt, Larose, Gilmore, & Davis, 1996; Luo, Pan, Guo, & Ren, 2013).

The sodium concentration varied significantly across the cheese products (Figure 1D). Such differences can arise from differences in salting conditions, such as the method of salting (dry salting/brine salting), the concentration of the brine solution (in brine salting), the amount of salt added (in dry salting) and the composition of the storage solution.

3.4 Microstructure

The microstructure of cheese samples was investigated within the skin layer, defined as the outermost layer and within the middle central region of the cheese ball, using both confocal laser scanning microscopy (CLSM; Figure 2 and Figure 5) and cryo scanning electron microscopy (cryo-SEM; Figure 3).

The microstructure of the skin and central layers differed. The protein within the skin appeared fibrous, stringy and less dense than in the middle layers in both buffalo and bovine cheeses when examined by both CSLM and cryo-SEM (Figure 2 and Figure 3). The fat in this outer layer appeared as discrete fat globules in small chains or clusters, whereas greater aggregation of fat and partial coalescence of fat droplets was observed within the middle cheese layers. The small fat patches within the skin layer were particularly evident in CSLM images (Figure 2A1-E1) but were also visible by cryo-SEM (Figure 3A1-E1).

The structure of fat differed between buffalo and bovine cheeses, with larger patches of coalesced and aggregated fat appearing in the middle layer of the buffalo cheeses compared to within bovine cheeses (Figure 2A2-C2 c.f. Figure 2D2-E2). This observation was confirmed by quantitative image analysis (Figure 4A-B; $P < 0.05$), which indicated a larger mean volume

for buffalo fat patches, with fewer patches occurring in buffalo than in the bovine cheeses. This difference may arise from a greater susceptibility of the large fat globules in buffalo milk (5.0 vs. 3.5 μm) to rupture during the deformation and shear occurring during the stretching and moulding processes of Mozzarella production (Ménard et al., 2010). The greater concentration of fat in buffalo milk also potentially reduces the proximity of fat globules within the casein network, increasing the propensity for aggregation and coalescence when compared to bovine milk that contains less fat. The sphericity of the fat was not significantly different, however, between the two layers or two types of cheese (Figure 4C).

The milk fat globule membrane (MFGM) was still found to be intact on the surface of several fat globules within the final buffalo cheese product (Figure 5) despite the stretching and processing steps involved in cheese production. The MFGM could be observed in both the skin and middle layers, even in deformed or coalesced fat globules (indicated by the large arrows or large broken arrows respectively). Non-fluorescently labelled lipid domains (indicated by small arrows) were also observed on the surface of some fat globules, *in situ* in cheese, indicating that the heterogeneous distribution of phospholipids previously observed within the native buffalo MFGM (Nguyen et al., 2016; Nguyen et al., 2015) was preserved throughout the cheese making process. This observation is consistent with observations made for Emmental cheese, a lower moisture cheese made from bovine milk (Lopez et al., 2008; Lopez, Camier, & Gassi, 2007).

The MFGM plays an important role in the stability of fat globules and emulsions (Dewettinck et al., 2008) and the buffalo MFGM is rich in proteins reported to be involved in several nutritional and biological processes (Nguyen et al., 2017). The phase separation of polar lipids within the MFGM is also thought to affect the properties of this membrane (Lopez, 2011; Murthy, Guyomarc'H, & Lopez, 2016a, 2016b). The occurrence of the native MFGM with the preserved heterogeneous distribution of the phospholipids within buffalo Mozzarella cheese is likely to impact on the nutritional and functional properties of this product, which warrants further investigation. Further studies could also examine the microstructure of the milk fat globule membrane in bovine Mozzarella cheese, allowing further systematic comparisons and

potentially useful information for the detection of differences between the two Mozzarella cheese types.

3.5 Hardness and meltability

There was no significant difference in the meltability or hardness between buffalo and bovine cheese samples, ($P>0.05$) (Figure 6) despite the differences observed in their chemical composition and microstructure. No changes in these two properties were observed on storage of laboratory cheese products (Figure 6).

The exception was CM-cheese B, which had a higher hardness and lower meltability (Figure 6). This difference may arise from the higher calcium content of this cheese (Figure 1D). The link between calcium content has been well studied for low moisture products (Guinee et al., 2002; Joshi et al., 2004). A systematic decrease in calcium from 0.65% to 0.48%, 0.42% and 0.35% w/w increased cheese meltability by 1.4, 2.1 and 2.6 times, as a result of reduced crosslinks between the casein micelles of the cheese matrix making the cheese softer and easier to melt (Joshi et al., 2004). A similar change in cross-linking most likely explains the differences between CM-cheese B observed here, the first time that this has been reported for the high moisture product. Previous studies have found an increase in Mozzarella cheese hardness and decreased meltability as a result of decreased moisture content (Tunick, 1991).

3.6 Proteolysis pattern

The number and migration of protein bands separated by SDS-PAGE (Figure 7) differed for the bovine (lanes 5-6) and buffalo cheese samples (lanes 1-4). Region A, corresponding to the casein (CN) proteins, contains three bands corresponding to α -CN (likely α_{S1} -CN and α_{S2} -CN) and β -CN for bovine samples but only two bands corresponding to α -CN and β -CN in buffalo samples. The different migration of proteins between the species arises from known differences in primary sequence and phosphorylation of these proteins (Abd El-Salam & El-Shibiny, 2011; D'Ambrosio et al., 2008), which in this case means that α_{S1} and α_{S2} caseins co-migrate for the buffalo cheese.

The low molecular weight proteolytic products present in the cheese also differed between buffalo and bovine products. Region B contained two bands for bovine samples (CM-cheese

A and B) at ~10 kDa and ~ 15 kDa but only one band for buffalo cheese samples (BM-cheese A and B) at ~10 kDa. The additional band in the bovine samples possibly corresponds to a proteolytic product of κ -CN (e.g. para κ -CN) caused by the activity of the residual coagulant or proteinase in the starter culture.

No significant differences in proteolysis were observed in the laboratory buffalo cheese during 7 days of storage (lanes 1 and 2), except for a subtle decrease in the intensity of a faint band corresponding to κ -casein (κ -CN, indicated by the arrow in Figure 7) at ~ 25 kDa, at the end of storage.

3.7 LC-MS analysis

The protein profiles of representative buffalo and bovine cheeses were further characterised using LC-MS, as this method has successfully been used to characterize subtle differences in protein concentration and to detect the adulteration of buffalo dairy products by the addition of other types of milk (Czerwenka, Muller, & Lindner, 2010).

The retention profile and quantity of the proteins present in buffalo and bovine cheese differed significantly (Figure 8). Of particular interest are the proteins β -Lg and β -CN, as these proteins have previously been identified as potential biomarkers to differentiate between buffalo and bovine milk products (Czerwenka et al., 2010; Mishra et al., 2009).

Bovine β -Lg is known to exist as two main variants A and B, while buffalo β -Lg occurs as only one variant, which has similar physico-chemical properties to the bovine β -Lg variant B but differs in its amino acid sequence (Czerwenka et al., 2010; Sen & Sinha, 1961). In the present case, both β -Lg variant A and B were observed in the bovine cheese, while the single buffalo β -Lg was observed in the buffalo cheese (Figure 8A-F).

The β -CN variant A2 appears in the chromatogram for protein extracted from both cheeses (Figure 8A-B). The bovine cheese also contained significant quantities of the β -CN A1 variant, which has a similar mass (Figure 8G-H) despite appearing earlier in the UV chromatographic sequence. This A1 variant was absent from the buffalo cheese. This observation is consistent

with prior studies where β -CN A2 was observed in both buffalo and bovine milk and β -CN A1 also observed in bovine milk (Mishra et al., 2009).

Other proteins were also present in the chromatogram for the buffalo cheese but not the bovine cheese (Figure 8A-B). These proteins may be a useful fingerprint for cheese of this type and are worthy of further study across a broader range of commercial samples.

4. Conclusion

Significant differences were observed in the microstructure and composition of high moisture buffalo and bovine Mozzarella cheese. The fat within buffalo cheese appeared in significantly larger patches within the middle layers of the cheese. The milk fat globule membrane of some fat globules also remained intact with lipid domains still visible within the membrane for both the skin and middle layers of the cheese, potentially impacting on nutritional and functional properties. Buffalo cheese had a higher ratio of fat:protein, a different proteolytic pattern, as well as lower concentrations of acetic, orotic and hippuric acids. Despite these differences, the hardness and meltability of both products was similar. Protein profiles analysed by LC-MS showed that buffalo cheeses contained one peak for β -Lg and a major peak of β -CN variant A2, while bovine cheeses contained two peaks of β -Lg variant A and B and two peaks of β -CN variant A1 and A2. These results can potentially be used to distinguish a buffalo Mozzarella cheese product from a Mozzarella cheese produced from bovine milk or a product made from a mixture of buffalo and bovine milk.

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List of Figures and Legends

Figure 1. Concentration of sugars (A), organic acids (B-C) and minerals (D) in laboratory and commercial buffalo and bovine Mozzarella cheese products. Results are presented as the mean $\pm$ standard deviation of the mean (n=6).

Figure 2. Confocal laser scanning microscopy microstructure of laboratory and commercial buffalo (BM) and bovine (CM) cheese samples showing the cheese skin (A1-E1) and middle layer (A2-E2). (A) Laboratory BM-cheese at day 7, (B) BM-cheese A, (C) BM-cheese B, (D) CM-cheese A, (E) CM-cheese B. Nile Red stained fat appears red, Fast Green FCF stained protein appears green and the white/grey areas are serum pores. Images were captured using a 63x objective at a 2x digital zoom. The scale bars are 5 μ m in length.

Figure 3. Cryo-scanning electron microscopy microstructure of laboratory and commercial buffalo (BM) and bovine (CM) cheese samples showing the skin (A1-E1) and middle layer (A2-E2). (A) Laboratory BM-cheese at day 7, (B) BM-cheese A, (C) BM-cheese B, (D) CM-cheese A, (E) CM-cheese B. Images were captured using a solid state detector at 4000x magnification and the scale bars are 20 μ m in length.

Figure 4. Physical properties of laboratory and commercial buffalo and bovine Mozzarella cheese samples in the skin (■) and middle (■) layers using image analysis of 3D reconstructed CLSM images. (A) mean volume of fat patches, (B) number of fat patches, (C) sphericity of fat patches, (D) volume fraction of protein, (D) volume fraction of fat and (F) porosity. Data are presented as the mean $\pm$ standard deviation of the mean (n=6 for laboratory cheeses and n=4 for commercial cheeses). ^{abc} Means with different superscripts indicate significant difference (P<0.05) between different samples in the middle layer. * indicates significant difference (P<0.05) between the skin and middle layers of the same sample. For the clarity in the Figure, only statistical differences discussed in the manuscript are shown.

Figure 5. Confocal laser scanning microscopy microstructure of the skin (A-B) and middle layer (C-D) of one buffalo Protected Designation of Origin Mozzarella cheese product. RhDOPE stained MFGM appears red and Fast Green FCF stained protein appears green. Images were captured using a 100x objective at a 3x digital zoom (A, C, D) or at a 5x digital

zoom (B). The scale bars are 5 μm (A, C, D) or 3 μm (B) in length. Small arrows indicate the non-fluorescent domains in the MFGM. Large thickened continuous arrows indicate the deformed fat globules and large thickened broken arrows indicate the aggregation of fat globules.

Figure 6. Hardness (A) and meltability (B) of laboratory and commercial buffalo and bovine Mozzarella cheeses. Data are presented as the mean $\pm$ standard deviations (n=6).

Figure 7. Image of SDS-PAGE gel (4% to 12% acrylamide gel) of laboratory and commercial buffalo and bovine Mozzarella cheese samples (STD: standard molecular weight marker; α , β , κ -CN: bovine α , β and κ -caseins respectively; lane 1: BM Lab-D1, lane 2: BM Lab-D7, lane 3: BM-cheese A, lane 4: BM-cheese B, lane 5: CM-cheese A; lane 6: CM-cheese B). Box A indicates the differences in the casein region, where two clear bands are present in buffalo samples and three clear bands in bovine samples. Box B indicates the differences in the proteolysis products where one band is present in buffalo samples and two bands in bovine samples. The arrow indicates the κ -CN band that decreases in intensity during cold storage of the laboratory cheeses due to proteolysis.

Figure 8. LC-MS analytical results obtained for water soluble extracts from bovine and buffalo Mozzarella cheese: total ion chromatograms (A-B), mass spectra and the corresponding deconvoluted mass spectra of β -Lg peaks (C-F) and β -CN peaks (G-J). The insets of F show the mass spectrum of representative interested peak on an enlarged scale.

Figure 1

Figure 2

Figure 3

Figure 4.

Figure 5

A

B

Figure 6

Figure 7

Figure 8

Supplementary Figure 1. Organic acids in buffalo milk (BM, ■) and bovine milk (CM, ■). Data are the average of six replicates (n=6) and error bars are the standard deviation of the mean.

Table 1. Fat, protein, moisture content and pH of laboratory and commercial buffalo (BM) and bovine (CM) Mozzarella cheese products. The data presented are the mean $\pm$ the standard deviation of the mean (n=6 for moisture content and pH; n=3 for fat, protein and the ratio of fat: protein).

Sample	Fat (% w/w)	Protein (% w/w)	Ratio (fat : protein)	Moisture (% w/w)	pH
BM Lab-D1 ¹	28.7 $\pm$ 1.8	16.2 $\pm$ 0.6	1.8 $\pm$ 0.1	55.3 $\pm$ 2.2 ^{cd}	5.3 $\pm$ 0.1 ^b
BM Lab-D7	28.7 $\pm$ 1.8	16.2 $\pm$ 0.6	1.8 $\pm$ 0.1	56.1 $\pm$ 2.1 ^c	5.3 $\pm$ 0.1 ^b
BM-cheese A	23.2 $\pm$ 1.0	13.0 $\pm$ 1.1	1.8 $\pm$ 0.2	58.8 $\pm$ 2.5 ^b	5.3 $\pm$ 0.1 ^b
BM-cheese B	25.9 $\pm$ 1.6	16.2 $\pm$ 1.3	1.6 $\pm$ 0.2	53.6 $\pm$ 2.0 ^d	5.4 $\pm$ 0.1 ^b
CM-cheese A	14.9 $\pm$ 0.6	17.7 $\pm$ 0.9	0.8 $\pm$ 0.1	67.4 $\pm$ 2.2 ^a	5.8 $\pm$ 0.3 ^a
CM-cheese B ²	15.2	17.2	0.9	56.4 $\pm$ 3.9 ^{bc}	5.4 $\pm$ 0.1 ^b

^{abc} Means in the same column with different superscripts are significantly different ($P < 0.05$) in composition. ¹ The fat and protein concentrations of laboratory buffalo Mozzarella cheese were determined only at day 7 but are assumed to be constant throughout the seven days of storage. ² Fat and protein data of this commercial product were obtained from the information stated on the product composition label.