Characterisation of buffalo milk, cheese and yoghurt properties
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Foreword

Buffalo are an important domesticated animal globally and produce over 10% of the world’s milk supply. In Australia the industry is a combination of the harvest of feral animals for live export and meat and the husbandry of domesticated animals, for meat and for milk. There is a statutory levy on buffalo slaughtered or exported live that is administered by RIRDC.

This project, supported by buffalo levies, provides some useful information on the properties of Australian buffalo milk and milk products, and compares these properties to bovine milk and bovine products produced under conditions relevant to an Australian manufacturing setting. The data presented may benefit buffalo farmers and manufacturers seeking to better understand and control buffalo milk quality and the properties of milk products, as well as the broader community of dairy researchers.

The research confirmed that Australian buffalo milk has a richer composition than bovine milk, including a higher concentration of fat, protein and total solids. These properties are advantageous for processing, as they lead to a higher yield but they also introduce some challenges in relation to making products such as yoghurt and cheese.

In addition, techniques developed in this study to further characterize buffalo milk, yoghurt or cheese can be used by manufacturers to optimize their processing parameters and to achieve buffalo products with desirable properties.

This report provides valuable information to buffalo producers and to processors of buffalo milk

This report is an addition to RIRDC’s diverse range of over 2000 research publications and it forms part of our Animal Industries RD&E program, which aims to conduct RD&E for new and developing animal industries that contribute to the profitability, sustainability and productivity of regional Australia.

Most of RIRDC’s publications are available for viewing, free downloading or purchasing online at www.rirdc.gov.au. Purchases can also be made by phoning 1300 634 313.

Craig Burns
Managing Director
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About the Authors

Hanh T.H. Nguyen is a PhD student in the Department of Chemical and Biomolecular Engineering, and Bio21 Institute at The University of Melbourne. Her PhD work is supported by funding provided by the University of Melbourne Australian Post Graduate Award, International Postgraduate Research Scholarship, and by the Rural Industries Research and Development Cooperation (RIRDC). She obtained her Master Degree in Food Science from The University of Philippines Los Banos in 2008 before joining Dr Gras’ research group. Her PhD research is on characterization of buffalo milk, yoghurt and cheese properties, and she has published one journal article on this topic. She has actively worked with the buffalo industry and conducted several trials using buffalo milk and ingredients provided by different companies. Recently, she was invited to give an oral presentation on the rheology of buffalo milk products at the Nordic Rheologic Conference in Copenhagen (Denmark).

Lydia Ong is a postdoctoral research fellow in the Department of Chemical and Biomolecular Engineering and Bio21 Institute at The University of Melbourne. Dr Ong’s research interest is in the microstructure of dairy products, including yoghurt and cheese. She received her PhD in 2009 from Victoria University, Australia investigating the ripening and antihypertensive activity of probiotic Cheddar cheese. She is currently working on a project funded by Dairy Innovation Australia Limited on microstructure of curd and Cheddar cheese.

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Sandra E. Kentish is a Professor in the Department of Chemical and Biomolecular Engineering at The University of Melbourne. In October 2012, Professor Kentish was appointed Head of this Department. She is also an invited Professor at the Centre for Water, Earth and the Environment within the Institut National de la Recherche Scientifique (INRS) in Canada. Professor Kentish has broad interests in industrial separations, particularly the use of membrane technology for energy, food and water applications. She has been the Discipline Leader in the CRC for Greenhouse Gas Technologies (CO2CRC) for Membrane Technology since 2003. She has also been a member of the Research Advisory Committee for the National Centre of Excellence in Desalination since 2010.

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Abbreviations

BM: buffalo milk
CM: Bovine milk
CLSM: Confocal laser scanning microscopy
Cryo-SEM: Cryo scanning electron microscopy
MFGM: Milk fat globule membrane
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
CN: Casein
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Executive Summary

What is the report about?

This project provides useful information on the molecular composition, biogenesis, microstructure, physical and chemical properties of Australian buffalo milk and yoghurt. Comparisons were also made with products made from bovine milk produced using conditions standard for Australian manufacturing, that will assist our understanding of buffalo milk products. The insights provided can be used to optimize product quality, minimize defects and increase the consistency of Australian buffalo milk products.

Who is the report targeted at?

This project aims to provide the buffalo industry and producers with greater knowledge of their milk product. The initial target audience is buffalo farmers and buffalo breeders, as well as cheese and yoghurt makers. The information generated may also be of interest to cheese buyers, food processors and consumers of buffalo products. A second target audience is the Australian and international research community.

Where are the relevant industries located in Australia?

The Australian buffalo dairy industry is predominantly located in Queensland (Millaa Millaa and Maleny), Victoria (Yambuk) and in the Northern Territory (Humpty Doo). Key buffalo milk producers and cheese makers from each of these regions were involved in this project. These key producers and other operators in Australia will benefit from this research.

Background

While buffalo milk is widely available globally, little is known about the characteristics of Australian buffalo milk. Only a few studies have characterized the microstructure, physical and chemical properties of buffalo milk products, including yoghurt and soft cheese, and most of these studies have been conducted under conditions not relevant to Australian processing. The research team is experienced in the characterisation of dairy products and study of lactation, and has applied these skills to better understand Australian buffalo milk and milk products.

Aims/objectives

The project aim was to characterise Australian buffalo milk quality and determine characteristics important to cheese and yoghurt making including:

1. Quantitative analysis of major milk components; protein, lipid and carbohydrate
2. Analysis of lipids
3. Analysis of proteins
4. Analysis of fat globule size distribution.

These characteristics were examined across a number of herds, using fresh and frozen milk.

The project examined the arrangement of protein and fat within yoghurt or cheese made from buffalo milk, using a range of advanced microscopy techniques. Following this, the texture of products made from buffalo milk were characterised to better understand how the properties of the raw ingredients
The project also established which genes are expressed in Australian buffalo milk as a function of lactation, by following a single buffalo herd through the periods of colostrum and lactation. The final release of the buffalo genome sequencing initiative, this library will allow the identification of novel buffalo milk genes and compounds associated with milk production traits. In addition, a new class of functional regulatory micro-RNA, only recently identified in the milk of mammals, was investigated in the buffalo. The library may therefore also assist in buffalo breeding and product differentiation.

**Results**

**Key findings**

The composition and properties of Australian buffalo milk are similar to those reported in other international studies of buffalo milk. The milk structure and properties of fat globules, including fat globule size, were also found to be consistent between different regions within Australia. As expected, large differences were found in the composition and physicochemical properties of Australian buffalo milk compared to Australian bovine milk.

**Mammary gene expression and deep sequencing**

Milk cell purification, RNA extractions from skim milk or milk cells and computational analysis of sequencing data were performed in the comparative lactation bioinformatics laboratory at Deakin University. Sequencing of purified RNA was sub-contracted to BGI, a world leader in sequencing service. A deep sequencing strategy was adopted earlier than planned throughout the project, due to difficulties obtaining sufficient RNA yield for the analysis on the more limited bovine platform, and a significant decrease in sequencing cost during the implementation phase of the project. This decision allowed the collection of additional information on buffalo gene sequences and permitted extension of the analysis to characterize the small RNA content of buffalo milk.

**Milk quality**

Yoghurt was produced in the Gras laboratory at the Bio21 Institute at The University of Melbourne. The analysis of yoghurt properties, including the microstructure, rheological and other physical and chemical properties, were carried out using the equipment and facilities available in the Department of Chemical and Biomolecular Engineering and BGI, a world leader in sequencing service. The details of the methods used are provided in the methodology section.

**Methods used**

The project established a gene library of buffalo milk using Deep Sequencing technology, providing a blueprint for the decoding of the bioactive messenger of buffalo milk. In conjunction with the future release of the buffalo genome sequencing initiative, this library will allow the identification of novel genes and compounds associated with the bioactivity of buffalo milk, providing a source of new quality markers and a bioinformatics toolbox for the genetic analysis of buffalo lactation. The data allows comparison of buffalo and bovine gene expression profiles.
The freezing of buffalo milk was found to induce oiling off, to reduce the integrity of the milk fat globule membrane, and to increase the size of some milk fat globules, although no significant effects were observed on physiochemical properties such as the pH, calcium concentration or milk viscosity. The changes observed appear small compared to processes such as homogenisation, although freezing in batches larger than five litres is expected to induce additional damage. The changes observed may not impact on processes such as yoghurt production, but could impact on the production of mature cheeses.

Yoghurt made from Australian buffalo milk is significantly different in physiochemical properties and microstructure compared to yoghurt made from bovine milk. Buffalo yoghurt displayed higher syneresis, was more susceptible to deformation, and was less able to recover the original network structure after deformation. These properties appear to arise from the more porous arrangement of the microstructure within buffalo yoghurt. An adjustment of process parameters during production may be able to optimise some of these properties.

Implications for relevant stakeholders

- The differences observed between Australian buffalo milk and Australian bovine milk composition and properties may be used by farmers and manufacturers to highlight the differences of Australian buffalo milk and milk products
- The similarity between Australian buffalo milk composition or properties and reports in the international literature suggests that the results of some international studies may be directly applied to an Australian context
- The observation that freezing buffalo milk changes selected milk properties suggests that some damage will occur, even if small volumes of milk are frozen rapidly (e.g. 100 ml, 0.54 °C/hour). The effect is smaller than treatments such as homogenisation and may not impact on yoghurt production but could impact on the production of mature cheeses. Buffalo farmers should reduce the volumes of milk frozen in each batch in order to speed the rate of freezing and minimise damage
- The observation that yoghurt made from buffalo milk is more susceptible to deformation than bovine yoghurt, and less able to recover an original structure after deformation, has implications for product transport. The higher syneresis observed for buffalo yoghurt also suggests further optimisation of process parameters could increase product quality
- The application of microscopy techniques, including Cryo Scanning Electron Microscopy and Confocal Laser Scanning Microscopy, has shown how the product microstructure correlates well with the texture and other product properties. These microscopy tools can be used to provide producers with a greater understanding of how the arrangement of protein and fat within buffalo products contributes to product taste and texture.

Recommendations

It is recommended that further research focus on the following questions:

- Could further optimisation of process parameters improve the structure and reduce syneresis in buffalo yoghurt?
- Does damage to the milk fat globule membrane observed in frozen buffalo milk (100 ml or 5L volumes) impact on the properties of targeted model buffalo cheese products?

Are bioactive components present within buffalo milk or colostrum, and can these components be used as health promoting novel ingredients?
Introduction

Buffalo milk is one of the major sources of milk globally, accounting for 13% of total worldwide dairy production in 2009 (International Dairy Federation 2009). This milk is used to produce butter, cream, ghee, condensed milk, cheese and yoghurt. Over 90% of the world buffalo population is found in Asia, mostly in India, China and Pakistan, with medium populations found in Africa and South America, and small populations in Europe and Australia. The Italian buffalo industry is globally important, despite its size, due a history of buffalo dairy production, and research on buffalo genetics and buffalo product quality (International Dairy Federation 2008).

Several international studies have reported the chemical composition of buffalo milk and factors affecting its composition, including lactation stage, season of calving, breed and feeding types (see Chapter 1). A limited number of studies have also examined the effect of processing factors, such as fat and total solids concentrations, on the sensory properties and quality of buffalo yoghurt. There are, however, many other important properties and processing parameters for buffalo milk that have not been investigated. Furthermore, most research has been conducted in countries other than Australia, under conditions not always relevant to Australian manufacturing. This project aimed to address this gap in our understanding, and to assist the Australian buffalo industry.
Objectives

The project aims to characterise Australian buffalo milk quality and determine characteristics important to cheese and yoghurt making including:

1. Quantitative analysis of major milk components; protein, lipid and carbohydrate
2. Analysis of lipids
3. Analysis of proteins
4. Analysis of fat globule size distribution.

These characteristics were examined across a number of herds, using fresh and frozen milk.

The project examined the arrangement of protein and fat within yoghurt or cheese made from buffalo milk using a range of advanced microscopy techniques. Following this, the texture of products made from buffalo milk were characterised to better understand how the properties of the raw ingredients influence the structure and properties of yoghurt or cheese, using techniques established for bovine milk.

The project also established which genes are expressed in Australian buffalo milk as a function of lactation by following a single buffalo herd through the periods of colostrum and lactation time points. In addition, a new class of functional regulatory micro-RNA, only recently identified in the milk of mammals, was investigated in the buffalo. The data allows comparison of buffalo and bovine gene expression profiles.

The project established a gene library of buffalo milk using ‘Deep Sequencing’ technology, providing a blueprint for the decoding of the bioactive messenger of buffalo milk. In conjunction with future release of the buffalo genome sequencing initiative, this library will allow the identification of novel buffalo genes and compounds associated with the bioactivity of buffalo milk, providing source of new quality markers and a bioinformatics toolbox for the genetic analysis of buffalo lactation. The library may therefore also assist in buffalo breeding and product differentiation.
Methodology

Freezing preparation

Fresh raw buffalo milk was obtained from Shaw River (Victoria, Australia). Within six hours of receipt, the milk was frozen using two different volumes to control the freezing rate (Table 3.1). The milk was frozen in a convective air freezer, and the final freezing temperature was -20°C. In the first preparation (treatment A), 100 ml of milk was frozen in a container with dimensions 9.9×6.8×1.5 cm for 20 hours. In the second preparation (treatment B), 5000 mL of milk was frozen in a container with dimensions 35×13×11 cm for 34 hours. The frozen milk was then thawed in a refrigerator at 4°C for approximately 18 hours, before completely thawing in water bath at a temperature of 75-80°C. These thawing conditions were employed to replicate industry practice within Australia. The thawed milk samples were then analysed.

The freezing rate (Fr) was determined using the following equation (Pham 1986):

\[ Fr = \frac{(T_i - T_c)}{10} \]  

where \( Fr \) (°C/s) is the freezing rate, \( T_i \) (°C) is the initial temperature of the milk, \( T_c \) (°C) is the final temperature of the milk and \( t \) is the time taken for the milk to change temperature from \( T_i \) to \( T_c \). In this experiment, \( t \) was determined experimentally and is the time taken for the milk to cool from \( T_i = 0 \) °C to \( T_c = -10 \) °C, the critical temperature range over which ice crystallisation occurs.

It is known that fast freezing rates produce small ice crystals and slower freezing rates produce larger ice crystals. Thus, \( T_i \) and \( T_c \), in equation (1), must be chosen with care to ensure the freezing rate calculated can be related to the ice crystal size. The time taken to pass the freezing stage, where maximal ice crystallisation occurs, is known to have a significant effect on the ice crystal size (Kiani & Sun 2011). According to Li and Sun (2002), maximum ice crystal formation occurs in potatoes and pork, between -1 °C and -8 °C. A similar range of temperature between 0 and -10°C was therefore selected here.

Yoghurt making

Yoghurt was produced from either buffalo milk or bovine milk. Fresh raw buffalo milk was obtained from a dairy farm in Shaw River (VIC, Australia), while pasteurised, homogenised bovine milk was purchased from a local supermarket (Pura Brand, VIC, Australia). Each litre of bovine milk was fortified with 37 g skim milk and 37 g full cream milk powder (Coles, VIC, Australia) to obtain a milk composition (Table 4.1) that enabled the production of yoghurt with a composition similar to a typical of commercial yoghurt (i.e. 3-4% (w/w) fat, 5-6% (w/w) protein 6-8% (w/w) carbohydrate and 150-200 mg of calcium per 100 g of milk). This experimental design, including the choice of non-homogenised and non-fortified buffalo milk, and homogenised and fortified bovine milk, was based on the milk preparations typically used commercially at an industrial scale. Moreover, in a preliminary screening experiment, bovine yoghurt produced from non-homogenised and non-fortified milk produced a soft gel with high syneresis, which offered a poor comparison to buffalo yoghurt and was not representative of a commercial standard.

Four litre samples of buffalo milk or fortified bovine milk were heated at 85°C for 30 minutes in a water bath for pasteurisation, and the milk then cooled to 43°C. Freeze-dried direct vat culture FDV ABT-5 (CHR Hansen, VIC, Australia) containing a mixture of *Streptococcus thermophilus*, *Lactobacillus acidophilus* La-5 and *Bifidobacterium lactis* Bb-12, was then added to the milk. This culture had previously been inoculated in a 100 mL sample of ultra heat treated full cream milk
(Devondale brand, VIC, Australia), and incubated at 43°C for 30 min before addition to the milk for yoghurt production.

The milk was then distributed into plastic containers of two different volumes, 100 mL and 50 mL, for different analyses. Sample containers with a volume of 100 mL were used for chemical, microbiological, textural and rheological analysis. Sample containers with a volume of 50 mL were used for the analysis of syneresis and microstructure to allow centrifugation and easier access to the sample for microscopic analyses. The containers containing the milk samples were transferred and fermented at 43°C in thermostat-controlled water-bath (Qualtex, Watson Victor Ltd., Australia), containing water that had been tempered to 43°C. The fermentation was terminated at a pH of 4.5 and yoghurt samples were immediately stored in a cold room (4°C) for 28 days. During fermentation, the pH, ionic calcium concentration, titratable acidity, lactose concentration, organic acid profile and bacterial growth were investigated (see below). Changes in the rheological properties, gel firmness and organic acid profiles of yoghurt were also evaluated at day 1, day 7 and day 28 of cold storage, while changes in the microstructure and viability of bacteria were assessed at the beginning (day 1) and at the end (day 28) of the storage period.

**Chemical analysis**

**pH measurement**

Changes in sample pH during fermentation were measured using an electrode pH meter (Orion 720A plus, Orion Pacific Pty Ltd., VIC, Australia). Before measurement, calibrations were performed using three standard pH buffer solutions of pH 10.0, 7.0 and 4.0 (Ajax Fine Chem, VIC, Australia).

**Total solids content**

The total solids content of samples was determined according to the standard method of the Association of Analytical Communities (AOAC 2006). Milk samples were dried in a forced convection oven (S.E.M Pty. Ltd., S.A., Australia) at 100 ± 3°C. After drying, the samples were cooled in a desiccator before weighing. This procedure was repeated until the weight of the samples remained unchanged. The total solids content was expressed as a percentage relative to the initial weight of the sample.

**Fat**

The milk fat content of samples was determined using a modified version of the method described by Atwood and Hartmann (1992). Briefly, milk samples were diluted 10 times and warmed to 37°C. A 50 µL aliquot of each sample was mixed with 600 µL ethanol, 100 µL of 2 M hydroxylamine hydrochloride and 100 µL of 3.5 N NaOH and this mixture incubated at room temperature for 30 minutes. After incubation, the mixture was acidified with 120 µL of 3.4 M HCl and this sample added to 100 µL of a ferric chloride-trichloroacetic acid solution, prepared by mixing 10 mL of the solution containing 0.37 M FeCl₃ and 0.1 M HCl with 7.5 g trichloroacetic acid (TCA). The solution was then centrifuged at 1500 g for 5 minutes and the supernatant was dispensed into a 96-well microplate (BD Falcon, N.S.W., Australia). The absorbance of the solution was measured at 540 nm using a spectrophotometer (Fluostar Optima, BMG labtech, Ortenberg, Germany).

**Protein**

The milk protein content of samples was determined using a modified version of the method described by Pesce and Strande (1973). Briefly, a 100 µL sample was mixed with 1 mL of TCA-Ponceau S working reagent, which was prepared from Ponceau - S (Merck Pty Ltd., VIC, Australia)
and TCA (Chem Supply, S.A., Australia), as described by Pesce and Strande (1973). The solution was then centrifuged at 15,800 g for 5 minutes. The supernatant was discarded and the precipitate dissolved in 700 μL of 0.2 N NaOH. Each sample was then transferred to a 96-well microplate (BD Falcon, N.S.W., Australia). The absorbance was measured at 560 nm using the spectrophotometer described above.

**Lactose**

The lactose concentration of samples was determined using high performance liquid chromatography (HPLC) as described previously (Gosling et al. 2009). Briefly, the carbohydrate fraction was collected from the top layer of a mixture of methanol, chloroform and Mili Q water deionized and filtered water, purified to a resistivity of 18.2 mΩ, Millipore, Billerica, MA, U.S.A.). The extracted samples were then separated on an HPLC Shimadzu Prominance system (N.S.W., Australia) equipped with a RID-10A refractive index detector and a 300 x 7.8 mm Rezex RCM-Monosaccharide Ca²⁺ column (Phenomenex, N.S.W., Australia). Milli Q water was used as mobile phase with a flow rate of 0.5 mL.min⁻¹. The column and detector were maintained at 80°C and 40°C respectively. Five standard solutions of lactose were prepared from lactose monohydrate in powder form (Chem Supply, S.A., Australia) using a 0.1 N sodium acetate/lactate buffer (Chem Supply, S.A., Australia).

**Ionic calcium**

The concentration of ionic calcium in samples was determined using an Orion 93 - 20 calcium half-cell electrode in conjunction with an Orion 90 - 02 Ag/AgCl double junction reference electrode (Orion Pacific, VIC, Australia). The electrodes were connected to an Orion 720A+ voltmeter. Samples were placed in a beaker on a magnetic stirrer and the electrodes immersed in the sample for 4 - 5 min to allow stabilisation before measurement. The concentration of ionic calcium was then determined by reference to a calibration curve set up from a series of CaCl₂ standards with concentrations ranging from 0.2 mM to 10 mM. The CaCl₂ standards were prepared from the dihydrate salt (Ajax Fine Chem, VIC, Australia) and potassium chloride (Chem Supply, S.A., Australia) added to achieve constant ionic strength of 0.08 M across the range of standard solutions.

**Total calcium**

The total calcium content in milk samples was determined using inductively coupled plasma optical emission spectrometry (Varian ICP - OES 720, Varian Inc, CA, U.S.A.). Briefly, 10 g of sample was weighed into a porcelain crucible and dried overnight in an oven at 100 ± 3°C. Crucibles containing dry matter were then transferred to a furnace for ashing at 600°C for at least 24 hours. The ash was acidified with 69% (w/w) nitric acid and 32% (w/w) hydrochloric acid and diluted to 10 mL with Milli Q water. Finally, the diluted ash samples were analysed for total calcium by ICP- OES using a wavelength of 373 nm. The concentration of total calcium was determined by reference to a calibration curve of five standard CaCl₂ solutions, prepared from the dihydrate salt (Ajax Fine Chem, VIC, Australia).

**Titratable acidity**

The titratable acidity of yoghurt samples was assessed following the Association of Analytical Communities (AOAC) titration method, using 0.1 N NaOH and phenolphthalein (2% (w/v) in ethanol) as an indicator (AOAC 2006). A 10 g sample of yoghurt was diluted with 10 mL of Milli Q water before titration. Titratable acidity was expressed as a percentage of lactic acid, determined using the following equation:

\[
\% \text{ lactic acid} = (V_{\text{NaOH}} \times 0.09) / m_{\text{sample}}
\]
Where \( V_{NaOH} \) is the volume (mL) of 0.1N NaOH solution required for titration and \( m_{sample} \) is the weight (g) of yoghurt sample.

**Organic acid profile**

The organic acid content of yoghurt samples was determined following the method described by Thi et al. (2012) with some modifications. Briefly, a 4 g sample was mixed with 70 \( \mu \)L of 15.6 M HNO\(_3\) and 4 mL of 0.009 N H\(_2\)SO\(_4\), vortexted for 2 minutes and left to stand for 15 minutes, before the addition of 17 mL of 0.009N H\(_2\)SO\(_4\). After mixing for 15 seconds, the mixture was centrifuged at 3220 g for 30 minutes. The aqueous layer was filtered through a 0.22 \( \mu \)m filter membrane before analysis by HPLC using 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 Pty Ltd, Hercules, CA, U.S.A.). The detector was set to record absorbance at both 220 nm and 290 nm. The mobile phase was 0.009 N H\(_2\)SO\(_4\), delivered at a flow rate of 0.6 mL min\(^{-1}\), and the column maintained at 65°C. Peaks were identified by their retention times and confirmed by spiking with organic acid standards including acetic, citric, formic, hippuric, lactic, propionic, pyruvic, and orotic acids (all supplied by Sigma Aldrich, MO, U.S.A.). The standards were prepared as stock solutions in Milli Q water (except for uric acid and orotic acid which were dissolved in 0.1 N NaOH to ensure solubility). At least four concentrations of each organic acid were used to prepare the calibration curve. The coeluted acids (orotic and citric acid, uric and formic acid) were quantified based on their different absorbance at two different wavelengths (220 nm and 290 nm). Briefly, at 290 nm, the absorbance of citric and formic acid were negligible as compared to the absorbance of uric and orotic acid, which enabled the calculation of concentration of uric and orotic acid at this wavelength. The concentration of citric and formic acid were then obtained by subtraction the total area of absorbance at 220 nm with the area of absorbance of uric and orotic acid.

**Surface potential**

The surface potential was measured following the previously described method (Menard et al. 2010). Milk fat globule surface potential was measured at 25°C, using a Zetasizer Nano Series ZEN 3600 (Malvern Instruments, Worcestershire, UK) with a DTS1060C-clear disposable zeta cell. Milk samples were prepared by suspending 10 \( \mu \)L milk in 20 mL buffer (20 mM imidazole, 50 mM NaCl, 5 mM CaCl\(_2\), pH 7.0). The Smoluchowski approximation, assuming \( f(ka) = 1.50 \), was used. The viscosity of the buffer was 0.886 cp, refractive index was 1.331, and dielectric constant was 78.5. The refractive index of the sample was 1.460 and absorption was 0.001. One minute was allocated for the sample to equilibrate.

**Fat globule size distribution**

The size distribution of the milk fat globule was determined by light scattering (LS) using a Mastersizer 2000 (Malvern Instruments, Malvern, UK) as described by Michalski et al. (2003) and Ong et al. (2010) with some modifications. The refractive indexes of the milk fat and distilled water were set at 1.460 and 1.330, respectively. The milk sample was diluted (1:1) in ethylenediamine tetraacetic acid (EDTA; 50 mM, pH 7) (Merck, Australia) for 2 hours before the analysis, to ensure all casein micelles were dissociated. The mixture was then added gradually into the apparatus circulating cell containing distilled water with 0.05% sodium dodecyl sulphate (SDS; Merck, Australia) to obtain the minimum obscuration of 10%. The volume weighted mean diameter \( D_{[4,3]} \) and the diameter below which 10% (\( D_{v0.1} \)), 50% (\( D_{v0.5} \)), and 90% (\( D_{v0.9} \)) of the volume of particles found were calculated by the Mastersizer 2000 software. Six measurements were carried out for each of the milk preparations.
Syneresis

Syneresis of yoghurt was determined following the method as previously described (Purwandari et al. 2007). Milk fermented in 50 mL centrifuge tubes (BD Falcon, N.S.W., Australia) was centrifuged at 700 g at 8°C for 10 minutes using an Eppendorf centrifuge (Eppendorf 5810R, VIC, Australia). The whey was then gently poured off and weighed. Syneresis was expressed as a weight percentage of the whey separated from the gel over the initial weight of the gel. Three independent samples per trial were used for the determination of syneresis at each time point during storage.

Texture analysis

Texture analysis of yoghurt

The texture of yoghurt was assessed as described by Kumar and Mishra (2003), with some modifications, using a TA.XT-2 texture analyser (Stable Microsystems, Surrey, England) equipped with 2 kg load cell and a 10 mm diameter cylindrical probe. Triplicate yoghurt samples in 120 mL plastic containers (filled with 100 mL of milk and fermented as described in section 2.1) were used to assess yoghurt texture. Samples were kept at 6 - 8°C in an ice box until measurement at room temperature. The contact area was set at 1 mm² and the contact force set at 5 g. The instrument speed was set at 1 mm per second. The compression distance, the distance of penetration from the surface of sample, was set at 20 mm. Data were recorded at a rate of 200 points per second. The firmness of the yoghurt, or gel strength, was determined as the maximum force measured during sample compression.

Rheological properties

Viscosity of milk

Graphs of viscosity as a function of shear rate were obtained with an AR-G2 Controlled Stress Rheometer (TA Instruments Ltd., New Castle, DW, USA) using the parallel plate geometry with 1000 μm fixed gap, and 20 mm radius. The sample tube was inverted a few times before pipetting approximately 1.3 ml on the inset plate. All measurements were conducted at 20° C using a water bath (Julao AWC 100) to maintain the temperature. For all measurements the sample was equilibrated for 2 minutes before data collection began during a shear rate ramp from 10 to 200 s⁻¹ over 10 minutes.

Rheological properties of yoghurt (storage modulus G’ and loss tangent tanδ) during fermentation

The rheological properties of yoghurt samples including the storage modulus (G’) and loss tangent (tan δ) during fermentation were determined using a controlled strain rheometer (Advanced Rheometrics Expansion System, TA Instruments, New Castle, U.S.A.) equipped with a cup 34 mm in diameter and a six-blade vane fixture 32 mm in diameter and 33 mm in height. A 38 mL milk sample was transferred into the cup immediately after inoculation with starter culture. A dynamic time sweep was performed on the samples with an oscillation frequency of 0.1 Hz and a constant strain of 1% at the desired fermentation temperature. The storage modulus (G’) and loss tangent (tanδ) were recorded every 5 minutes until the fermented milk reached a pH of 4.5. The gelation time was defined as the time when the measured modulus exceeded 1 Pa (Lee & Lucey 2004).
Rheological properties of yoghurt (thixotropy, flow behaviour index and consistency index) during storage

The rheological properties of yoghurt during storage were determined using a method described in a previous study (Purwandari & Vasiljevic 2009), with some modifications. The measurements were carried out using the controlled stress rheometer (AR-G2, TA instruments Ltd., New Castle, U.S.A.) fitted with a cone plate (40 mm diameter / 4° angle). All samples were stirred with a spatula 20 times clockwise prior to loading to achieve a homogenous mixture. After the sample was equilibrated to 20°C, the yoghurt was pre-sheared at 500 per second for 60 seconds and then equilibrated for 300 seconds prior to rheological measurements. The flow curves measured the viscosity of the sample as a function of shear rate. The shear rate was increased logarithmically from 0.1 per second to 100 per second in 300 seconds (upward curve) followed by a logarithmic decrease from 100 per second to 0.1 per second in 300 seconds (downward curve). A holding time of 5 seconds at the shear rate of 100 per second was applied to the sample between the increasing and decreasing sweeps. The thixotropy (hysteresis loop area) between the upward and downward flow curves was determined using the Rheology Advantage data analysis software (Version V5.70, TA instruments Ltd., New Castle, U.S.A.). Data obtained from the upward curve were fitted to the modified Ostwald-de Waele model given by the equation:

$$\eta = K. \gamma^{n-1}$$

where \(\eta\) is the apparent viscosity (Pa.s), \(\gamma\) the shear rate (s\(^{-1}\)), \(K\) the consistency coefficient (Pa.s\(^n\)) and \(n\) the flow behaviour index (Purwandari & Vasiljevic 2009).

Microstructural analysis using confocal laser scanning microscopy (CLSM) and cryo scanning electron microscopy (cryo-SEM)

Analysis of buffalo milk using CLSM

Fresh raw milk samples were diluted ten times with phosphate saline buffer (1x PBS buffer). The diluted solution was then mixed with wheat germ agglutinin Alexa Fluor conjugate (WGA488; Invitrogen, Mulgrave, Victoria, Australia) and Fast Green FCF (Sigma-Aldrich, St. Louis, Mo., USA) to achieve a final concentration of 0.01g.L\(^{-1}\) and 0.012g.L\(^{-1}\), respectively. The stained samples were then wrapped in aluminium foil and incubated for at least 2 hours at 4°C. The stained solution (5 µL) was mixed with 20 µL of 0.5% agarose gel solution. Finally, 5 µL of the sample was deposited onto a microscope slide (0.7 mm in depth) (ProSciTech, Thuringowa, Queensland, Australia), covered with a glass coverslip and sealed by nail polish. The samples were then observed in a dark room using an inverted confocal scanning laser microscope (Leica TCS SP2; Leica Microsystems, Heidelberg, Baden-Württemberg, Germany) powered by Ar/Kr and He/Ne lasers with an 100 X oil-immersion objective. The excitation/ emission wavelength of WGA488 and Fast Green FCF were set at 488nm/500-600nm and 633 nm/650-710 nm, respectively. Analysis of the images was then carried out using Imaris image processing software (Bitplane, USA).

Analysis of yoghurt microstructure using CLSM

CLSM was used to assess the microstructure of buffalo yoghurt samples. Milk was fermented in 50 mL centrifuge tubes (BD Falcon, N.S.W, Australia) and then left undisturbed in a cold room at 4°C over the storage period. On the day of analysis, yoghurt was gently taken out of the container by inverting the tube. A thin slice, approximately 2 mm in thickness, was carefully obtained from the middle of the yoghurt sample using a surgical blade. Samples, approximately 3 mm x 3 mm x 2 mm in size, were placed on a microscope slide. Samples were stained with Nile Red and Fast Green FCF (both supplied by Sigma Aldrich, N.S.W., Australia). Stock solutions of Nile Red (1 mg. mL\(^{-1}\)) and

8
Fast Green FCF (1 mg. mL\(^{-1}\)) were diluted 10 times with Milli Q water immediately prior to staining. The diluted staining solutions were added to the samples for 15 minutes at 4°C to allow the stains to penetrate into the sample. Excess stain was removed and sample was covered with a glass coverslip (0.17 mm thick) (ProSciTech, QLD, Australia). Samples were observed using an inverted confocal scanning laser microscope as described above.

**Analysis of yoghurt microstructure using cryo-SEM**

Cryo-SEM was used to assess the microstructure of buffalo yoghurt using a previously described method (Ong et al. 2011). Samples 5 mm x 2 mm x 2 mm in size were mounted on a copper holder attached to a vacuum transfer device (VTD) and then rapidly immersed into a liquid nitrogen slush maintained at -210°C. After freezing, the frozen specimens were immediately transferred into an attached cryo preparation chamber using the VTD. The sample was fractured using a chilled scalpel blade within the chamber, which was maintained at -140°C under a high vacuum conditions (<10\(^{-4}\) Pa). The specimen was then etched to facilitate the removal of ice from the surface of the fractured sample by vacuum sublimation at -95°C for 20 minutes, and coated using a cold magnetron sputter coater with 300 V, 10 mA of sputtered gold/palladium alloy (60/40) for 120 s resulting in a gold layer (~ 6 nm in thickness). Samples were then transferred under vacuum onto a nitrogen gas cooled module, maintained at -140°C and observed using a field emission gun SEM (Quanta, Fei Company, Hillsboro, Oregon, U.S.A.) and a solid state backscattered electron detector (SSD). Two samples were used for cryo-SEM for each trial. Four images were collected for each sample and a typical image presented.

**Microbiological analysis**

The enumeration of bacteria was carried out at the beginning and at the end of yoghurt fermentation when the sample reached a pH of 4.5, and at day 1 and day 28 of the storage, to assess bacterial growth and viability. A 10 g yoghurt sample was diluted in 90 mL of sterile peptone 0.1% (w/w) solution (Oxoid, Basingstoke, U.K.), then serially diluted using 9 mL of sterile peptone solution and added to the media using a pour-plate technique. M17 agar (Oxoid, Basingstoke, U.K.) was used for the enumeration of *Streptococcus thermophilus* (aerobic conditions, 42°C, 48 hours). MRS agar (Oxoid, Basingstoke, U.K.) supplemented with clindamycine (0.1 mg. L\(^{-1}\)) was used for the selective enumeration of *Lactobacillus acidophilus* (anaerobic conditions, 37°C, 72 hours). MRS agar supplemented with cysteine hydrochloride (0.5 g. L\(^{-1}\)), lithium chloride (1 g. L\(^{-1}\)) and dicloxacillin (0.5 mg. L\(^{-1}\)) was used for the enumeration *Bifidobacterium animalis* BB-12. Clindamycine and dicloxacillin were supplied by Sigma Aldrich (N.S.W., Australia) while cysteine hydrochloride and lithium chloride were obtained from Merck (VIC, Australia). The inoculated plates were incubated under anaerobic conditions using an anaerobic gas jar (Oxoid, S.A., Australia) at 37°C for 72 hours. Plates with 25 - 250 colonies were selected for manual counting.

**Mammary gene expression and deep sequencing**

**Milk fractionation**

Buffalo milk and colostrum were collected at the Shaw River Dairy in the state of Victoria, Australia. Approximately 2 to 4 litres of milk or colostrum were collected from different animals at the farm, stored on ice and brought to the lab (3 to 12 hours). The samples were filtered through a large gauge (150 μm) to remove hair and impurities and subjected to centrifugation at 2,000 g for 15 minutes at 4°C to pellet cells and separate fat and skim milk fractions. Cell pellets were washed 2-3 times in PBS before RNA extraction. An estimated 25-40 Million cells were recovered by centrifugation from 2 litres of colostrum or milk (Somatic Cell Count: 0.125-0.2), leading to the final recovery of about 0.5 to 4 ug of total RNA after purification. The RNA profile (Agilent 2100 bioanlyser, RNA 6000
nano-chip) usually presents a large single peak between 1.5 and 4 kilobases. Fractions were stored at -80. A purified exosome fraction was prepared with ExoQuick™ solution (SBI-System Biosciences) following manufacturer instructions.

**RNA preparation and sequencing**

Cellular RNA was prepared with the RNeasy minikit (Qiagen, Sydney, Australia) following manufacturer’s instructions. Total skim milk RNA extractions were performed with the Ambion miRNA Isolation Kit (Life Technologies), according to manufacturer instruction for body fluids. RNA quality and quantity were evaluated on the Agilent Bioanalyser. RNA sequencing (RNA-seq quantification procedure) was contracted from BGI, Shenzen. About 10 to 20 Million reads (50 nucleotide maximum read length), corresponding to over 2 Gbytes of data were obtained for each sample. Poly-A selection or size selection of fragments below 40 nucleotides were applied before mRNA or small RNA sequencing, respectively.

**Bioinformatics analysis**

Because an annotated genome sequence of buffalo is not yet available, read mapping was performed against the closely related bovine genome reference (version UMD3.1 (Zimin et al. 2009)) using Bowtie2 (Langmead & Salzberg 2012) for small RNA and Tophat (Trapnell et al. 2009) for transcriptome data. Cufflink (Trapnell et al. 2010) and SeqMonk software (www.bioinformatics.bbsrc.ac.uk/projects/seqmonk) RPKM quantification pipeline for RNA-seq were used for transcriptome quantification and sequence annotation. Alignment details were also visualized and investigated further in SeqMonk. DSAP (Deep Sequencing Small rna Analysis Pipeline) (Huang et al. 2010) was used for comparative miRNA annotation against the miRBase miRNA reference database (Griffiths-Jones 2006). Functional cluster analysis was done with the online tool DAVID (Huang da et al. 2009a; Huang da et al. 2009b).

**Statistical analysis**

Data were analysed using statistical Minitab software (V16, Minitab Inc., Stage College, PA, U.S.A.). Two way and one way analysis of variance (ANOVA) and Fisher’s paired comparison were used to assess the differences between means, with a significance level of P = 0.05.
1. Literature review

Introduction

Buffaloes belong to the order *Artiodactyla*, suborder *Ruminantia*, family *Bovidae*, tribe *Bovini*. The tribe *Bovini* are then classified into three groups including *Bovini* (cattle), *Bubalina* (Asian buffalo) and *Syncerina* (African buffalo) (Park & Haenlein 2006). The scientific name *Bubalus bubalis* is used to refer to the domesticated buffaloes (Abd El-Salam & El-Shibiny 2011). Buffaloes are considered as multiple purpose animals, as they can be used for draft power, meat and milk. Domesticated buffaloes are further classified into river and swamp buffaloes. River buffaloes are distinguished by their black colour with curved or sickled shaped horns and mostly used for milking. In contrast, swamp buffalo are often used for drafting; they have a grey colour at birth and then turn to a dark grey when fully mature (Park & Haenlein 2006).

Buffalo milk accounts for approximately 13% of total world milk production (International Dairy Federation 2009), and buffaloes are widely distributed, with significant numbers in Southeast Asia, including India and Pakistan. Buffalo milk contains a higher concentration of fat, protein, total solids and minerals than bovine milk, which makes buffalo milk a versatile material for the production of dairy products.

In this literature review, the chemical composition of buffalo milk, factors affecting the composition and preservation methods are discussed. Previous studies on two of the most commonly produced buffalo dairy products: Mozzarella cheese and yoghurt are also reviewed.

Buffalo milk

Chemical composition of buffalo milk

Buffalo milk has a high concentration of fat, protein, lactose and minerals including calcium, magnesium and inorganic phosphate, as well as health promoting conjugated fatty acids (Ahmad et al. 2008a; Bramhapurkar et al. 2007; Braun & Preuss 2008; Menard et al. 2010; Napolano et al. 2007; Patino et al. 2007b). The high concentration of fat, protein and calcium make buffalo milk suitable for the production of several dairy products including cheese, butter, ghee, yoghurt and ice-cream. A comparison of the typical composition reported for buffalo and bovine milk from a number of literature sources is presented in Table 1.1.

Fat

The fat content of buffalo milk varies between 6.4-13.4% (w/w) (Table 1.1). The high fat content contributes to the calorific and nutritive value of the milk, as well as the higher yield typically obtained in butter and ghee production (Bindal & Wadhwa 1993; Menard et al. 2010). Buffalo milk is considered a healthy food, despite the higher fat content because it contains less cholesterol than bovine milk (Mihaiu et al. 2006). Buffalo milk contains a lower concentration of polar lipids (2.6 mg vs. 3.6 mg per gram of total lipid) and the profile of individual phospholipids (expressed as percentage of polar lipids) also differs to bovine milk: buffalo milk has higher percentage of phosphatidyl choline (24.0% (w/w) vs. 21.8% (w/w)), lower percentage of sphingomyelin (24.8% (w/w) vs. 26.9% (w/w)) and similar proportion of phosphatidyl ethanolamine (29.4% (w/w) vs 29.1% (w/w)), phosphatidyl inositol (10.6% (w/w) vs 10.4% (w/w)) and phosphatidyl serine (11.3% (w/w) vs 11.8% (w/w)) (Menard et al. 2010). The fatty acid composition also differs between buffalo and bovine milk (Table 1.2). Palmitic acid (C16:0), oleic acid (C18:1), myristic acid (C14:0) and
stearic acid (C18:0) are the major fatty acids in both milk types. Buffalo milk also contains linoenic acid (C18:3, ω:3), an essential amino acid for human health (Menard et al. 2010).

**Protein**

The concentration of protein in buffalo milk is often higher than in bovine milk; typically 3.8–6.3% w/w for buffalo milk compared to 3.0 – 3.5% w/w for bovine milk (Table 1.1). The protein in buffalo milk is classed into two major groups, known as the casein and whey proteins, with casein comprising more than 80% of total protein (Ariota et al. 2007; Park & Haenlein 2006). Generally, the proteins in buffalo milk show a high homology to those in bovine milk and the exceptions are discussed in the following sections (Abd El-Salam & El-Shibiny 2011; Addeo et al. 1977; D'Ambrosio et al. 2008).
Table 1.1. Chemical composition of buffalo milk (BM) and bovine milk (CM)

<table>
<thead>
<tr>
<th>Type of milk</th>
<th>Country</th>
<th>Breed</th>
<th>Fat (%w/w)</th>
<th>Protein (%w/w)</th>
<th>Lactose (%w/w)</th>
<th>Calcium</th>
<th>Phosphorous</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM</td>
<td>France</td>
<td>Mediterranean breed</td>
<td>7.3</td>
<td>4.6</td>
<td>5.6</td>
<td>-</td>
<td>-</td>
<td>(Menard et al. 2010)</td>
</tr>
<tr>
<td>BM</td>
<td>France</td>
<td>Murrah breed</td>
<td>7.0</td>
<td>4.5</td>
<td>5.2</td>
<td>47.1 mM</td>
<td>27.7 mM</td>
<td>(Ahmad et al. 2008a)</td>
</tr>
<tr>
<td>BM</td>
<td>Germany</td>
<td>Murrah x Mediterranean</td>
<td>7.5-9.6</td>
<td>4.4-5.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Braun &amp; Preuss 2008)</td>
</tr>
<tr>
<td>BM</td>
<td>Italy</td>
<td>Bubalus bubalis</td>
<td>7.4-8.8</td>
<td>4.3-4.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Varricchio et al. 2007)</td>
</tr>
<tr>
<td>BM</td>
<td>Italy</td>
<td>Mediterranean</td>
<td>8.3</td>
<td>4.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Zicarelli 2004)</td>
</tr>
<tr>
<td>BM</td>
<td>Bangladesh</td>
<td>-</td>
<td>6.4</td>
<td>4.5</td>
<td>4.6</td>
<td>-</td>
<td>-</td>
<td>(Faruque &amp; Hossain 2007)</td>
</tr>
<tr>
<td>BM</td>
<td>South African</td>
<td>Syncerus Caffer</td>
<td>13.4</td>
<td>6.3</td>
<td>5.2</td>
<td>-</td>
<td>-</td>
<td>(Osthoff et al. 2009)</td>
</tr>
<tr>
<td>BM</td>
<td>India</td>
<td>Murrah</td>
<td>7.7</td>
<td>3.8</td>
<td>4.8</td>
<td>-</td>
<td>-</td>
<td>(Dubey et al. 1997)</td>
</tr>
<tr>
<td>CM</td>
<td>France</td>
<td>Holstein breed</td>
<td>4.1</td>
<td>3.4</td>
<td>4.8</td>
<td>30.5 mM</td>
<td>19.2 mM</td>
<td>(Ahmad et al. 2008a)</td>
</tr>
<tr>
<td>CM</td>
<td>Brazil</td>
<td>-</td>
<td>3.6</td>
<td>3.3</td>
<td>4.8</td>
<td>0.11-0.13% (w/w)</td>
<td>0.09-0.10% (w/w)</td>
<td>(Santos et al. 2009)</td>
</tr>
<tr>
<td>CM</td>
<td>Australia</td>
<td>Friesian bovine</td>
<td>-</td>
<td>-</td>
<td>4.6-4.8</td>
<td>-</td>
<td>-</td>
<td>(Auldist et al. 2010)</td>
</tr>
<tr>
<td>CM</td>
<td>Australia</td>
<td>-</td>
<td>4.5</td>
<td>3.5</td>
<td>4.6-4.7</td>
<td>0.10 – 0.12 % (w/w)</td>
<td>-</td>
<td>(Ong et al. 2010)</td>
</tr>
<tr>
<td>CM</td>
<td>Australia</td>
<td>Holstein-Friesian</td>
<td>4.2-4.7</td>
<td>3.0-3.5</td>
<td>-</td>
<td>91.0-98.2 mg/100 ml</td>
<td>-</td>
<td>(Solh et al. 2007)</td>
</tr>
</tbody>
</table>
Table 1.2. Fatty acid composition of buffalo milk and bovine milk. Data are presented as the mean ± the standard deviation of the mean (n=3) (Menard et al. 2010).

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Buffalo milk</th>
<th>Bovine milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4:0</td>
<td>2.8 ± 0.5</td>
<td>2.5 ± 0.5</td>
</tr>
<tr>
<td>C6:0</td>
<td>1.9 ± 0.3</td>
<td>2.1 ± 0.4</td>
</tr>
<tr>
<td>C8:0</td>
<td>1.1 ± 0.2</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>C10:0</td>
<td>1.8 ± 0.2</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>C12:0</td>
<td>2.3 ± 0.2</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>C14:0</td>
<td>11.8 ± 0.2</td>
<td>11.1 ± 0.4</td>
</tr>
<tr>
<td>C14:1 c9</td>
<td>0.7 ± 0.0</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>C15:0</td>
<td>1.7 ± 0.1</td>
<td>1.2 ± 0.0</td>
</tr>
<tr>
<td>C15:1 c10</td>
<td>0.4 ± 0.0</td>
<td>0.3 ± 0.0</td>
</tr>
<tr>
<td>C16:0</td>
<td>36.0 ± 1.2</td>
<td>33.8 ± 0.9</td>
</tr>
<tr>
<td>C16:1 c9</td>
<td>1.9 ± 0.1</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>C17:0</td>
<td>0.8 ± 0.0</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>C17:1 c10</td>
<td>0.3 ± 0.0</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>C18:0</td>
<td>9.9 ± 0.2</td>
<td>11.1 ± 0.9</td>
</tr>
<tr>
<td>C18:1 t6,7,8,9</td>
<td>0.4 ± 0.0</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>C18:1 t10</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>C18:1 t11</td>
<td>2.0 ± 0.1</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>C18:1 t12</td>
<td>0.1 ± 0.0</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>C18:1 c9</td>
<td>20.3 ± 0.7</td>
<td>22.1 ± 1.7</td>
</tr>
<tr>
<td>C18:2 c9,12</td>
<td>0.9 ± 0.1</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>C18:3 c9,12, c15 (ω3)</td>
<td>0.7 ± 0.2</td>
<td>0.6 ± 0.0</td>
</tr>
<tr>
<td>C20:0</td>
<td>0.2 ± 0.0</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>C18: 2 c9, t11</td>
<td>0.9 ± 0.1</td>
<td>0.7 ± 0.0</td>
</tr>
</tbody>
</table>

Casein

The casein content in buffalo milk is higher than in bovine milk, 3.86 % - 3.92% w/w versus 2.44 - 3.10% w/w respectively (Ariota et al. 2007; Cecchinato et al. 2012; Litwinczuk et al. 2011). Casein comprises 87%-90% of the total protein in buffalo milk, but only 80%-83% in bovine milk (Bramanti et al. 2003; Jensen 1995). The significance of this difference is that casein proteins are used in cheese making, and are a major determinant of yield.

The caseins in buffalo milk are generally classified into four major types, as occurs for bovine milk: αs1-casein, αs2-casein, β-casein and κ-casein. Some studies also report the presence of γ-casein in buffalo milk, which is formed from the hydrolysis of β-casein by plasmin, an indigenous proteolytic enzyme present in milk (Bramanti et al. 2003; Trieucuot & Addeo 1981).

It is well accepted that the proportions of αs1-casein, αs2-casein, β-casein and κ-casein are different in buffalo milk and bovine milk (Table 1.3); the concentrations of these fractions, however, are not consistent between studies, and likely reflect natural variability between milk sources and the different quantitative techniques used for analysis. The concentration of κ-CN in buffalo and bovine milk is similar, when expressed as a percentage of total casein (Table 1.3) but the total mass of κ-CN is higher per unit volume of buffalo milk. This higher total and κ-casein content is known to increase curd firmness and reduce the amount of enzyme required in processing buffalo cheese (Abd El-Salam & El-Shibiny 2011; Addeo et al. 1977; Cecchinato et al. 2012). It is also reported to create a more branched and interconnected protein network that leads to a firmer curd with more fat and whey solids entrapped during cheese making, resulting in cheese with an improved quality and higher yield (Cecchinato et al. 2012).
The caseins in buffalo show a similar number of amino acids and similar composition of individual amino acids, except for arginine, which was slightly lower in buffalo milk. The rate of hydrolysis by pepsin, trypsin, papain, pancreatin, however, is slower for buffalo casein compared to bovine casein (Ganguli et al. 1964), possibly reflecting differences in sequence or glycosylation.

Table 1.3. Concentration of different caseins in buffalo milk (BM) and bovine milk (CM).

<table>
<thead>
<tr>
<th>Milk type</th>
<th>αs1-casein</th>
<th>αs2-casein</th>
<th>β-casein</th>
<th>γ-casein</th>
<th>κ-casein</th>
<th>Total casein (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM</td>
<td>44c</td>
<td>53</td>
<td>-</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>(Park &amp; Haenlein 2006)</td>
</tr>
<tr>
<td>BM</td>
<td>30</td>
<td>18</td>
<td>34</td>
<td>-</td>
<td>15</td>
<td>-</td>
<td>(Addeo et al. 1977)</td>
</tr>
<tr>
<td>BM</td>
<td>31</td>
<td>13</td>
<td>28</td>
<td>22</td>
<td>7</td>
<td>90</td>
<td>(Bramanti et al. 2003)</td>
</tr>
<tr>
<td>CM</td>
<td>37</td>
<td>8</td>
<td>45</td>
<td>-</td>
<td>10</td>
<td>-</td>
<td>(de Kruif &amp; Huppertz 2012)</td>
</tr>
<tr>
<td>CM</td>
<td>55c</td>
<td>39</td>
<td>-</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>(Park &amp; Haenlein 2006)</td>
</tr>
<tr>
<td>CM</td>
<td>39</td>
<td>8</td>
<td>34</td>
<td>-</td>
<td>15</td>
<td>-</td>
<td>(Addeo et al. 1977)</td>
</tr>
<tr>
<td>CM</td>
<td>37</td>
<td>7</td>
<td>42</td>
<td>6</td>
<td>9</td>
<td>83</td>
<td>(Bramanti et al. 2003)</td>
</tr>
</tbody>
</table>

a. Percentage in weight of total casein.
b. Percentage in weight of total protein.
c. Percentage in weight of total αs1 and αs2-casein.

Whey proteins

The major whey proteins in buffalo milk include: beta-lactoglobulin, alpha-lactalbumin, immunoglobulin and lactoferrin. Alpha-lactalbumin and beta-lactoglobulin comprise a similar proportion of the total protein in buffalo and bovine milk, with 8.4 % (w/w) and 6.4 % (w/w) reported for alpha-lactalbumin and beta-lactoglobulin in buffalo milk and 7.2% (w/w) and 6.7% (w/w) in bovine milk respectively (Park & Haenlein 2006). The concentration of lactoferrin in buffalo milk is significantly higher than in bovine milk (0.32 mg/ml vs. 0.05 mg/ml respectively) (Park & Haenlein 2006), which suggests buffalo milk is a good source for the preparation of this bioactive protein.

Minerals

Buffalo milk possesses a high concentration of calcium and phosphorous (47.1 mM and 27.7 mM, respectively), the two minerals that significantly affect milk coagulation during cheese-making (Ahmad et al. 2008b). A summary of the concentrations of other major minerals is presented in Table 1.4.

Table 1.4. Concentration of major minerals in buffalo milk and bovine milk. Data are presented as mean ± standard deviation of mean (n=2). (Ahmad et al. 2008a)

<table>
<thead>
<tr>
<th>Minerals (mM)</th>
<th>Buffalo milk</th>
<th>Bovine milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>47.1 ± 1.2</td>
<td>30.5 ± 0.8</td>
</tr>
<tr>
<td>Phosphorous</td>
<td>27.7 ± 1.4</td>
<td>19.2 ± 1.0</td>
</tr>
<tr>
<td>Magnesium</td>
<td>7.3 ± 0.2</td>
<td>4.6 ± 0.1</td>
</tr>
<tr>
<td>Sodium</td>
<td>20.3 ± 0.5</td>
<td>17.5 ± 0.4</td>
</tr>
<tr>
<td>Potassium</td>
<td>28.7 ± 0.7</td>
<td>42.0 ± 1.0</td>
</tr>
<tr>
<td>Chloride</td>
<td>16.6 ± 0.8</td>
<td>21.8 ± 1.0</td>
</tr>
<tr>
<td>Citrate</td>
<td>8.3 ± 0.4</td>
<td>8.8 ± 0.4</td>
</tr>
</tbody>
</table>
Free amino acids, vitamins and others

Buffalo milk is also reported to have a higher concentration of free amino acids (5.1 mg/100 mL vs. 4.0 mg/100mL), taurine (5.9 moles/ 100 mL vs. 4.1 moles/100 mL) and vitamin A (340 IU/kg vs. 230 IU/kg) compared with bovine milk (Park & Haenlein 2006).

Factors affecting the composition and quality of buffalo milk

The composition of buffalo milk is influenced by several factors such as the stage of lactation, season of calving, parity, feed type, breed and the health of the individual or herd of buffalos. These factors represent an opportunity that may allow milk quality to be optimised.

Lactation stage

The lactation stage is known to affect the composition of buffalo milk significantly (Braun & Preuss 2008; Dubey et al. 1997; Palmquist et al. 1993; Sekerden et al. 1999). The concentration of fat, protein and casein decreased during the first four months of lactation (reaching 7.1%(w/w), 3.7%(w/w) and 2.9% (w/w), respectively) and then increased gradually throughout the remaining six months of lactation, leading to the highest concentration of fat (8.5% (w/w)) at tenth months of lactation. In contrast, the concentration of lactose increased to 4.9% (w/w) by the fourth month and then decreased gradually during the rest of the lactation to 4.7% (w/w) (Dubey et al. 1997).

The stage of lactation influences the fatty acid profile of buffalo milk. Most fatty acids found during the early stages of lactation were saturated fatty acids, while unsaturated fatty acids, including the health promoting omega-3 and omega-6 fatty acids, were dominant in the later stages of lactation. The late stage of lactation may therefore provide more health benefits to the consumer (Caldeira et al. 2010).

The proportion of individual milk proteins also varied with the stage of lactation (Bonfatti et al. 2012). The concentration of αs1-casein increased during lactation, the concentration of αs2-casein decreased and the concentration of β-CN was maximal between 60 and 160 d of lactation. The concentration of κ-CN was low early in lactation (<60 d) and remained relatively constant in the period of mid and late lactation (Dubey et al. 1997; Patino et al. 2007a; Sekerden et al. 1999).

Calcium and phosphorus concentrations are also dependent on the stage of lactation. The concentration of calcium is highest (1.10 g/kg) early in lactation while the concentration of phosphorous is highest (0.76 g/kg) late in lactation (Patino et al. 2007a).

Season of calving

The season of calving is reported to change the mineral composition of buffaló milk and buffaló calved in the summer produced milk with a higher concentration of calcium and phosphorous (Dubey et al. 1997; Patino et al. 2007a; Sekerden et al. 1999).

Parity

Parity, or the number of times a buffalo has produced a calf, has a profound effect on milk composition, although reports differ (Bonfatti et al. 2012; Dubey et al. 1997). Dubey found the concentration of fat and total solids was highest for the first calf, with no significant difference in the concentration of protein, casein and lactose (Dubey et al. 1997). In contrast, Bonfatti et al. (2012) found the concentration of total casein, whey proteins, αs2-CN, β-CN, and κ-CN decreased while the concentration of glyco-κ-CN increased with subsequent births.
Feed type

Feed type is another important factor that affects buffalo milk composition. Buffalo fed a diet without additional fat, a diet with soybean grain or a diet with soybean oil expressed milk with differing fat content and a different level of saturated and unsaturated fatty acids (Oliveira et al. 2009). The addition of 2% mustard oil to feed also increased the total milk fat conjugated linoleic acid content by 2.85 fold (Kathirvelan & Tyagi 2009). Complete substitution of rice straw with maize silage was also found to increase the concentration of fat and solids non-fat in buffalo milk (Hayashi et al. 2009), illustrating the potential to optimise milk quality by feed type.

Breed

The buffalo breed has been reported to influence milk composition. Misra et al.(2008) investigated the milk composition of four different buffalo breeds including Bhadawari, Mehsana, Murrah and Surti and found Murrah buffalo milk to have the highest protein, casein and fat concentrations, while Mehsana buffalo produced milk with the highest solids non-fat and Bhadawari buffalo the highest total solids content.

Health status and subclinical mastitis

The composition of buffalo milk is affected by the health status of the animal. Buffaloes are generally less susceptible to mastitis compared to bovine animals but are more prone to mastitis during lactation, especially in the first stage of lactation (1-4 months) or the last dry period (10-12 months) (Kavitha et al. 2009). The somatic cell count of the milk is often used as an indicator of mastitis and for quality control, and should not exceed the limit of 400,000 cells/ml for buffalo dairy production (Sharma et al. 2011).

The milk of buffaloes infected with mastitis was found to have a higher pH, electrical conductivity and concentration of sodium, potassium, phosphorous, calcium, magnesium, zinc, iron, total dissolved solids, lactate dehydrogenase, aspartate aminotransferase and alkaline phosphatase, but a decrease in the concentration of fat, protein, lactose and solids non-fat content (Hussain et al. 2012). Studies with bovine and ovine milk have shown that milk with a high somatic cell count used for dairy production can have adverse effects on yoghurt and cheese properties including a decrease in yoghurt gel firmness, increase in syneresis and higher rate of proteolysis and lypolysis in cheese which can lead to an ‘off-flavour’ in the final product (Andreatta et al. 2009; Vivar-Quintana et al. 2006).

The preservation of buffalo milk

Freezing is often used to preserve buffalo milk to assist small scale producers. The effect of freezing on the quality of buffalo milk has not been fully studied, but β-casein fragments are reported to increase due to the hydrolysis of casein in frozen buffalo milk (Luccia et al. 2009). The possible impact of freezing buffalo milk can also be assessed by examining reports of damage for other types of milk.

Adverse effects are reported for frozen ovine milk including fat separation, protein flocculation and the development of an ‘off-flavour’, which reduced shelf-life, yield and the quality of dairy products including cheese or yoghurt (Muir 1984; Needs 1992; Wendorff 2001). Freezing can also disrupt the fat globules in human milk, hydrolyse triglycerides and lead to changes in calcium partitioning as calcium binds to the fat globule membrane (Kent et al. 2009; Wardell et al. 1981).

Yet some studies have found few effects from freezing. Recent studies on frozen ovine milk for example, found no difference in the fatty acid profile, acidity, concentration of peroxide or apparent viscosity of milk and yoghurt products (Katsiari et al. 2002; Zhang et al. 2006).
Several studies have employed fast freezing to reduce the potential damage to milk (Muir 1984; Needs 1992; Wendorff 2001). The use of ultrasound to create small ice crystals during freezing is also reported to lessen adverse effects (Li & Sun 2002).

**Products from buffalo milk**

The composition of buffalo milk is advantageous for the production of yoghurt, cheese, butter, cream and ghee. Yoghurt and cheese are the most common buffalo products in Australia, while the fat rich products such as cream, butter and ghee are more common in India (Bindal & Wadhwa 1993). The literature concerning the production of buffalo yoghurt and Mozzarella is discussed briefly in the next sections.

**Buffalo yoghurt**

Buffalo yoghurt can be produced by the fermentation of lactic acid bacteria, but does not require the addition of skim milk powder or thickener as occurs in the production of bovine yoghurt, due to the high total solids content in buffalo milk (Addeo et al. 2007). In Australia, buffalo yoghurt is normally made from unhomogenized milk, and whey separation is a common defect in the final product. A typical process flow diagram for the production of buffalo or bovine yoghurt following commercial practice is presented in Figure 1.1. Research to date on buffalo yoghurt is limited but several factors are known to affect the quality of buffalo yoghurt including: the fat content, solid non-fat content and storage time (Chawla & Balachandran 1994; Raju & Pal 2009; Yadav et al. 2007).
Figure 1.1. Process Flow Diagram for yoghurt production (Addeo et al. 2007; Tamime & Robinson 2007).

**Buffalo Mozzarella cheese**

Mozzarella cheese is a common buffalo milk product that can be classified based on the milk used for production including: whole buffalo milk Mozzarella, mixed buffalo and bovine milk Mozzarella, and whole bovine milk Mozzarella (Borghese 2005). A further four categories can be applied based on the concentration of fat and moisture: Mozzarella, low moisture Mozzarella, part skim Mozzarella and low moisture part skim Mozzarella (McMahon et al. 1993). Mozzarella cheese must have at least 45% fat in dry weight (FDW) and a moisture content between 52-60% (w/w). Low moisture part skim Mozzarella contains 30-40% FDW and a moisture content of 45-52% (w/w)(McMahon et al. 1993).

Among several Mozzarella cheeses produced from different regions, the “Mozzarella di Bufala Campana” cheese is considered the most famous and has been registered and approved as a Protected Denomination of Origin (or Protected Designation of Origin (POD)) food product since 1996 by the European Union (Brescia et al. 2005). According to European regulation No. 1107/96, the production of the PDO Mozzarella cheese must be produced within three regions of Southern Italy (Campania, Lazio and Apulia), and materials used must be exclusively raw and whole buffalo milk from a Mediterranean breed that has been raised in these areas (Pizzolongo et al. 2007). In addition, the production of DPO Mozzarella must be processed using the traditional method from these regions (Borghese 2005).

In traditional Italian production, raw whole buffalo milk is inoculated with a natural starter culture such as whey from the previous days manufacture. This milk is incubated at 35-37°C and rennet added before the milk coagulates for 40 minutes. The curd is then cut into small cubic pieces of 16 cm³ and left to ripen. This ripening stage takes 4.0-4.5 hours or until the pH reaches 4.9-5.1. After ripening, the whey is drained and curd piled and salted and then stretched in hot water at 85-90°C. The stretched curd is then moulded into small round balls manually. The whey is stored at a temperature of 18-22°C for use the following day (Coppola et al. 1988; Ercolini et al. 2004; Yazici et al. 2010).

Today, buffalo Mozzarella cheese is produced globally using variations on the Italian method. One procedure, suggested by Yazaici and co-authors (2010), is summarized in the process flow diagram in Figure 1.2.

Buffalo Mozzarella is a fresh, soft, white cheese with stringy texture and distinct flavour. It is often sold in small balls approximately 50 – 150 g in weight in a brine solution containing 2-3% NaCl (Borghese 2005) or in cold water.
Buffaloes are a major source of milk globally. Differences in the chemical composition of buffalo milk compared with bovine milk, including the higher concentration of fat, protein, total solids and minerals, make buffalo milk preferable for the production of products such as cheese, yoghurt, butter and ghee. These compositional differences, however, affect both the process for production and the properties of the final dairy product. Consequently, protocols that have been applied for the production of bovine products should be carefully investigated before application to buffalo milk processing. A better understanding of buffalo milk and buffalo milk products is therefore essential.
2. Characterisation of buffalo milk

Introduction

Several international studies have reported that buffalo milk is more nutritious than bovine milk. While the composition of buffalo milk is highly dependent on stage of lactation, breed, geographical location, season and feed type (Kumar & Kansal 2005; Patino et al. 2007a), several general statements can be made. Firstly, the fat content of buffalo milk is typically 6.9-8.7% (w/w), almost twice that of bovine milk (Varricchio et al. 2007). The average protein, lactose and calcium concentration is also typically higher in buffalo milk (4.6% (w/w) vs. 3.35% (w/w), 5.6% (w/w) vs. 4.8% (w/w) and 47.1 mM vs. 30.5 mM respectively (Ahmad et al. 2008b; Braun & Preuss 2008; Upadhyay et al. 2007)). Conversely, the cholesterol concentration is typically lower in buffalo milk (0.65 mg/g vs 3.14 mg/g) (Mihai et al. 2005). These characteristics, specifically the high levels of fat, protein and calcium, make buffalo milk suitable for the production of dairy products including cheese, butter, ghee, yoghurt and ice-cream.

Other differences between buffalo and bovine milk include the size of fat globules; typically 5.0 μm in buffalo milk compared to 3.5 μm in bovine milk (Menard et al. 2010). The lipid composition and fatty acid profiles also differ. Buffalo milk contains a higher percentage of phosphatidyl choline (24.0% (w/w) vs. 21.8% (w/w)) and a significantly higher concentration of linolenic acid (C18:3, ω3) but a lower percentage of sphingomyelin (24.8% (w/w) vs. 26.9% (w/w)) and a similar proportion of phosphatidyl ethanolamine (29.4% (w/w) vs 29.1% (w/w)), phosphatidyl inositol (10.6% (w/w) vs 10.4% (w/w)) and phosphatidyl serine (11.3% (w/w) vs 11.8% (w/w)). Differences in protein composition are also discussed in Chapter 1.

This chapter focuses on the specific physicochemical characteristics of Australian buffalo milk, as previous research is limited. Possible differences between milk samples from different regions of Australia have also not been studied. The research compared the properties of unhomogenized and pasteurized buffalo milk obtained from Victoria, Queensland and the Northern Territory with unhomogenized and pasteurized bovine milk obtained from commercial supplier Parmalat. All milk was stored at 4°C and analysed within two days of receipt. The methods used for analysis are described in the methodology section.

Results and discussion

Chemical composition

Australian buffalo milk pooled from a herd has a significantly higher concentration of protein, fat, total solids and total calcium than bovine milk, as shown in Table 2.1. The pH of buffalo milk was also higher than bovine milk. These results are in agreement with previous studies (Ahmad et al. 2008a; Menard et al. 2010). The concentration of protein, fat and calcium recorded is within the range reported in other international studies of buffalo milk (see Table 1.1 for a comparison), although there are also reports of milk with higher and lower protein or fat content.

The colostrum collected from a single buffalo in the Northern Territory was found to contain less lactose (3.2% (w/v) vs. 5.0% (w/v)) and fat (7.3% (w/v) vs. 7.9% (w/v)) but more protein (8.3% (w/v) vs. 4.1% (w/v)) than the buffalo herd sample (Table 2.1). The fat content reported here for the colostrum is lower than that reported in a previous study, where fat ranged from 11.31-7.51% (w/w) (Coroian et al. 2013). This difference could be due to the stage of lactation, as the fat is reported to gradually decrease from the first day postpartum until day seven. Similarly, the protein content is reported to decrease from ~18% (w/w) in the first four hours postpartum, to around 6.9% (w/w).
within 60 hours (Arain et al. 2008). The concentration of lactose and minerals are consistent with previous reports and increase as lactation proceeds.

Table 2.1. pH and chemical composition buffalo milk obtained from a herd (BM) or individual animal (BM colostrum) and bovine milk (CM). Data are the mean ± the standard deviation of the mean (n = 6) for all analyses.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Buffalo milk (BM)</th>
<th>Bovine milk (CM)</th>
<th>BM colostrum</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>6.73 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.56 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nd</td>
</tr>
<tr>
<td>Protein (% (w/v))</td>
<td>4.1 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.1 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.3 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fat (% (w/v))</td>
<td>7.9 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.6 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.3 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lactose (% (w/v))</td>
<td>5.0 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.7 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.2 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total calcium (mM)</td>
<td>45.8 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.5 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nd</td>
</tr>
<tr>
<td>Total solids (% (w/w))</td>
<td>17.1 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.5 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nd</td>
</tr>
</tbody>
</table>

<sup>abc</sup>Means in the same column with different superscripts are significantly different (P < 0.05) in composition. nd = not determined.<sup>(1)</sup> Buffalo milk was collected from a herd in Victoria, Australia.<sup>(2)</sup> Colostrum was collected from a single buffalo in the Northern Territory (Australia).

**Characterisation of fat globules**

The physicochemical properties of the fat globules found in Australian buffalo milk pooled from herds in Victoria or Queensland, including the charge or surface potential, specific surface area and diameter, are significantly different compared to Australian bovine milk (P < 0.05). The surface potential was more negative for buffalo fat globules and the specific surface area lower, reflecting the larger fat globules in buffalo milk samples. These observations correlate well with the higher fat content determined for buffalo milk samples (7.9% (w/w) vs. 3.6% (w/w) see above).

The results presented here are in agreement with previous study of Menard et al. (2010) who also observed bigger fat globules, a smaller fat globule surface area and a higher absolute surface potential for buffalo milk collected in France compared to bovine milk. The characteristics of the fat globules recorded here is also consistent with those recorded in France including the size (typically 5.0 µm) specific surface area (1.78 m² per gram of fat) and surface potential (-11 mV)(Menard et al., 2012).

No significant differences were observed in the physicochemical properties of buffalo fat globules in herd samples collected from Victoria and Queensland (P > 0.05) (Table 2.2).

Table 2.2. Physiochemical properties of buffalo and bovine milk fat globules. Data are presented as the mean ± SD of the mean (n=6) for all analyses.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Buffalo milk (Victoria)</th>
<th>Buffalo milk (Queensland)</th>
<th>Bovine milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface potential (mV)</td>
<td>-10.25 ± 0.31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nd</td>
<td>-9.2 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Specific surface area (m²/g fat)</td>
<td>1.61 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.62 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.98 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diameter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D[4,3] (µm)</td>
<td>5.5 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.6 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.9 ±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dv0.1 (µm)</td>
<td>2.3 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.2 ± 0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.9 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dv0.5 (µm)</td>
<td>7.7 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.4 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.6 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dv0.9 (µm)</td>
<td>9.2 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.8 ± 0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.3 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

D[4,3] is the volume-weighted mean diameter; Dv0.1, Dv0.5 and Dv0.9 are the diameters below which 10%, 50% and 90% of the volume of particles are found, respectively. <sup>ab</sup>Means in the same column with different superscripts are significantly different (P < 0.05) in composition. nd = not determined.
The size distribution of fat globules in Australian buffalo and bovine milk is shown in Figure 2.1. The size distribution of fat globules in buffalo milk was wider than in bovine milk, ranging from 0.6 µm - 19.9 µm and from 0.6 µm - 11.5 µm respectively. The size distribution observed for Australian buffalo milk was also slightly broader than that observed for French buffalo milk, which had a range of 0.4 µm to 15 µm (Menard et al., 2012), indicating the presence of some larger fat globules in Australian milk.

![Figure 2.1](image)

**Figure 2.1.** Size distribution of fat globules in buffalo milk collected from a herd in Victoria (●), Queensland (■) or bovine milk (○). Data are presented with an (A) linear x-axis or (B) logarithmic x-axis. Data are the average of six replicates (n=6) and error bars are standard deviations of the mean.

**Milk microstructure as observed by confocal laser scanning microscopy (CLSM)**

The structure of Australian buffalo milk at a micron scale, known as the microstructure, was assessed by confocal laser scanning electron microscopy (CLSM). This technique allows individual fat globules within the milk to be visualised after sample staining, as shown in Figure 2.2.
Figure 2.2. CLSM images showing the microstructure of individual fat globules in milk collected from a buffalo herd in Victoria (A), Queensland (B) or bovine milk (C). Milk samples were stained with Nile Red and Fast Green FCF. The Nile Red stained core of the fat globules appears red. The fast green FCF stains milk protein green, although the protein is too dilute to be observed in these images. A 100x objective lens was used to capture the images. The scale bars are 10 µm in length.

The appearance of the fat globules within buffalo milk collected from herds in Victoria and Queensland was similar (Figure 2.2 A and B) and a number of large fat globules were observed in each image. In contrast, the fat globules within bovine milk samples were significantly smaller (Figure 2.2 C), consistent with the measures of fat globule size and size distribution presented above. The microstructure of the fat globules reported in French study of buffalo and bovine milk is also similar to that observed here.

Conclusion

Buffalo milk obtained from herds in different regions of Australia was found to have a similar pH and contained fat globules with similar physicochemical properties including surface potential, specific surface area, size, size distribution and appearance (or microstructure). The colostrum collected from a single buffalo differed in chemical composition to the herd sample reflecting differences that are expected as a function of buffalo lactation.

The properties of Australian buffalo milk are similar to those reported by other international studies. The concentration of protein and fat are within the range typically reported, but higher concentrations are reported elsewhere. The microstructure, average size, specific surface area and surface potential of the fat globules appear similar to international studies, such as those originating from France, although the size distribution of fat globules in Australian milk is slightly broader than in French milk, indicating the presence of some larger fat globules.

Australian buffalo milk was found to differ considerably from Australian bovine milk. Buffalo milk samples collected from a herd had a higher pH and higher concentration of protein, fat, total calcium and total solids. The fat globules in buffalo milk were bigger, the size distribution of fat globules wider, the specific surface area lower and the surface potential more negative. The appearance of buffalo fat globules observed by microscopy was also larger. These differences in chemical composition, physiochemical properties and appearance (or microstructure) suggest that the properties of products made from buffalo milk will be different to those made from bovine milk.
3. The effect of freezing on the quality of buffalo milk

Introduction

The distance between Australian buffalo farms and capital cities is often large and, in some regions, buffalo milk is frozen to preserve milk during transport, and to ensure a continuous supply throughout the year. Freezing has been previously shown to adversely affect the quality of concentrated bovine milk resulting in an ‘oiling-off’ phenomenon, where an oily layer forms on the surface on thawing (Muir 1984). Freezing can also disrupt the milk fat globules membrane that surrounds fat globules, and increase the content of free fatty acids in bovine, ovine and human milk samples (Needs 1992; Wardell et al. 1981; Wendorff 2001). A slow freezing process is thought to cause more damage, due to the formation of larger ice crystals (Muir 1984).

In the early 1900s, several studies examined the effect of freezing milk but there have been few studies since and no specific studies on buffalo milk. Yet freezing may alter the quality of buffalo milk and the texture and quality of buffalo milk products, including yoghurt or Mozzarella cheese. Studies on bovine milk have shown that the effect of frozen storage varies depending on the temperature and length of storage (Wendorff 2001). Good quality yoghurt can be produced from ovine milk frozen and stored at -27°C for up to 12 months, with yoghurt quality comparable to that produced from fresh milk (Wendorff 2001). The yoghurt made from milk stored at -12°C, however, showed visual evidence of protein destabilization and had a lower gel strength. Wendorf et al (2001) also reported that cheeses made from milk that had been frozen had a tendency to develop rancid flavor, but no significant differences in cheese body or texture.

The primary aim of this study was to investigate the effects of freezing on the physicochemical properties of buffalo milk including the pH, concentration of ionic calcium and milk viscosity. Changes to fat globules formed a particular focus and the appearance (or microstructure), size distribution and surface potential of buffalo milk fat globules were determined following freezing.

Fresh raw buffalo milk was obtained from a local farm in Victoria. Within six hours of receipt, the milk was frozen, as described in the methodology section. Briefly, buffalo milk was frozen in either a small volume of 100 mL (treatment A) or a large volume of 5000 mL (treatment B) using a convective air freezer to obtain different freezing rates. The frozen milk was initially thawed at 4°C overnight prior to completely thawing in a water bath at 75-80°C. Fresh buffalo milk was used as the control sample. Physicochemical analyses were carried out as described in the methodology section.

Results and discussion

Freezing rate

The buffalo milk frozen in two containers (100 ml and 5000 ml) froze at different rates, as shown in Table 3.1. The larger volume of milk in treatment B froze at approximately one half the rate observed for the smaller sample in treatment A (0.54 °C/hour c.f. 1.2 °C/hour), resulting in a freezing time that was more than double that recorded for treatment A. The slower freezing rate for the larger sample is expected to result in the formation of larger ice crystals. The formation of a fat layer, known as ‘oiling-off’, was observed in larger samples on thawing,
Table 3.1. Freezing parameters for the two frozen buffalo milk preparations.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Freezing rate (°C/hour)</th>
<th>Freezing time (hours)</th>
<th>Container dimensions (cm)</th>
<th>Container volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment A</td>
<td>1.2</td>
<td>8.3</td>
<td>9.9 × 6.8 × 1.5</td>
<td>100</td>
</tr>
<tr>
<td>Treatment B</td>
<td>0.5</td>
<td>19</td>
<td>35 × 13 × 11</td>
<td>5000</td>
</tr>
</tbody>
</table>

The effect of freezing on the physiochemical properties of buffalo milk

The effect of freezing in treatment A and B on physicochemical properties of buffalo milk including the pH, ionic calcium and milk viscosity was examined.

pH

The pH of buffalo milk was not significantly affected by freezing and a similar pH was observed for milk frozen in treatment A or B and the unfrozen control milk samples, as shown in Figure 3.1.

![Figure 3.1. pH of fresh buffalo milk (control) and milk frozen using treatment A (freezing rate = 1.2°C/hour) or treatment B (freezing rate = 0.54°C/hour). The results are presented as the mean ± standard deviation. Data are obtained from at least two replicates (n≥2).](image)

A previous study of bovine milk found the pH decreased as a result of slow freezing at a rate of 0.1°C/hour (Van Den Berg 1961; Vanden Berg 1961). This was thought to occur due to a precipitation of salts in the supersaturated liquid phase of the frozen milk and the formation of fatty acids from lipolysis that may occur during freezing and thawing (Kent et al. 2009; VanDenberg & Soliman 1969). The impact on milk pH was small, however, if a fast or medium freezing rate of 1°C/hour or 0.25°C/hour was applied (Van Den Berg 1961). These observations are consistent with the data presented here; no significant change in pH was observed in treatment A and B, as the freezing rate was > 0.5°C/hour.
Ionic calcium

The concentration of ionic calcium was not significantly affected by freezing and a similar concentration was observed for milk in treatment A or treatment B and the unfrozen control, as shown in Figure 3.2 (P>0.05). High variability was observed between replicate samples, due to the difficulty of measurements with the calcium electrode.

![Figure 3.2. Ionic calcium concentration of fresh buffalo milk (control) and milk frozen using treatment A (freezing rate = 1.2°C/hour) or treatment B (freezing rate = 0.54°C/hour). The results are presented as the mean ± standard deviation. Data are obtained from at least two replicates (n≥2).](image)

In a previous study on thawed human milk that was partitioned into fat and skim milk fractions, the calcium concentration decreased within the skim milk fractions and increased within the fat layer on freezing (Kent et al. 2009). This increase in calcium concentration within the fat layer is thought to result from the electrostatic binding of calcium to the milk fat globule membrane (Kent et al. 2009). As mentioned previously, a thin layer of fat could be observed in treatment B but this did not appear to significantly correlate with the calcium concentration under the conditions used here.
Viscosity

The viscosity of buffalo milk was not altered by freezing; samples frozen using treatment A or control samples showed shear thinning behaviour, characterised by a decrease in milk viscosity as a function of shear rate and a steady state viscosity at high shear rate, as shown in Figure 3.3.

Figure 3.3. The viscosity of fresh buffalo milk (+) or buffalo milk frozen using treatment A (■). The results are presented as the mean ± standard deviation. Each data point is the average of three replicates (n = 3).

The data presented here is consistent with reports that frozen bovine milk stored at -20 °C for 10 weeks had a similar apparent viscosity to unfrozen control samples (Koschak et al. 1981).

The effect of freezing on the microstructure and properties of buffalo milk fat globules

The effect of freezing on the milk fat globule appearance (or microstructure), size distribution and surface potential following freezing was examined next.

Microstructure

The structure of buffalo milk fat globule membrane at a micron scale, known as the microstructure, was assessed following freezing using confocal laser scanning electron microscopy (CLSM). High resolution images and dyes specific to the outer membrane of the fat globule can be used to visualise the surface and assess potential damage to the fat globule structure as a result of freezing, as shown in Figure 3.4.

A continuous, smooth, undisrupted membrane can be observed on the surface of the raw buffalo milk globules prior to freezing (Figure 3.4A, shown in red), indicating that native glycoproteins are present on the milk fat globule membrane (MFGM) and that this membrane is intact. In samples frozen using treatment A or B (Figure 3.4 B and C), the WGA 488 staining is uneven, resulting in gaps between regions of red stain, suggesting possible damage of the fat globule membrane. Furthermore, where the red WGA 488 stain is absent, point staining of FCF fast green is present suggesting that non-native proteins, such as casein micelles or whey proteins, have absorbed to the damaged area of the fat globule.
Figure 3.4. CLSM images showing the integrity of the fat globule membrane (MFGM) in fresh raw buffalo milk (A), buffalo milk subject to treatment A (B) or buffalo milk subject to treatment B (C). The milk samples were stained with the dyes WGA488 and Fast Green FCF. The WGA488 stained MFGM appears red. The fast green FCF stained milk protein appears green. The images were captured with a 100x objective lens. The scale bars are 5 µm in length. Arrows indicate possible damage to the membrane of the buffalo fat globules.

Several possible mechanisms can lead to fat globule damage during freezing:

- The stress exerted by ice crystals can lead to mechanical damage (Kent et al. 2009)
- Changes in the ionic strength or pH of the unfrozen water fraction in milk may displace the native MFGM proteins from the membrane (Walstra et al. 2006)
- Fat crystallization may lead to deformation and fat crystals can puncture the membrane (Lopez et al. 2002);(Boode & Walstra 1993).

Some or all of these mechanisms may be responsible for the damage observed in this study.

The consequence of fat globule damage may be that globules are less stable, leading to the cream layer observed for some samples, and that they may behave differently during the production of yoghurt or cheese. Previous studies have reported that cheeses made from frozen ovine milk developed a rancid flavor (Wendorff 2001). This may be due to the disruption of the native MFGM, which can lead to lypolysis. Similarly, Cheddar cheese is usually made from unhomogenised bovine milk so as to preserve the native MFGM, which protects the fat from extensive lypolysis during maturation (Ong et al. 2010). It should be noted, however, that the effect of homogenization on the properties of the fat globule and damage to the MFGM is more extensive than that observed in the freezing treatments applied here.

Milk containing damaged fat globules can be effectively used in fresh cheese making and bovine milk with damaged fat globules is typically used in the production of fresh cheese that does not require maturation (Cano-Ruiz & Richter 1997). Frozen Australian buffalo milk could therefore be used for
the production of fresh buffalo mozzarella cheese, although the use of this milk in the production of mature cheeses still requires further investigation.

Size distribution

Freezing increased the size of fat globules measured in buffalo milk samples (P < 0.05) (Table 3.2) but no difference was observed between milk subject to treatment A or treatment B. The average volume weighted mean diameter D[4,3] of fat globules increased from 5.6 μm in raw unfrozen buffalo milk to 6.1 μm - 6.2 μm in frozen samples. Other measures of fat globule diameter, such as Dv[0.5] and Dv[0.9], which indicate the diameters below which 50% or 90% of the volume of particles are found, also increased after freezing. These differences indicate that some fat globules may be coalescing after freezing or that the absorption of protein on the membrane surface has increased the fat globule size.

Table 3.2. The size of fat globules in fresh buffalo milk or milk subject to treatment A (freezing rate = 1.2°C/hour) or treatment B (freezing rate = 0.54°C/hour). Data are presented as the mean ± the standard deviation of the mean, n=6.

<table>
<thead>
<tr>
<th>Diameter (μm)</th>
<th>Control</th>
<th>Treatment A</th>
<th>Treatment B</th>
</tr>
</thead>
<tbody>
<tr>
<td>D[4,3]</td>
<td>5.6 ± 0.1b</td>
<td>6.2 ± 0.2a</td>
<td>6.1 ± 0.2a</td>
</tr>
<tr>
<td>Dv0.1</td>
<td>2.3 ± 0.1b</td>
<td>2.6 ± 0.1a</td>
<td>2.6 ± 0.2a</td>
</tr>
<tr>
<td>Dv0.5</td>
<td>5.2 ± 0.1b</td>
<td>5.6 ± 0.1a</td>
<td>5.5 ± 0.2a</td>
</tr>
<tr>
<td>Dv0.9</td>
<td>9.6 ± 0.1b</td>
<td>11 ± 0.4a</td>
<td>11 ± 0.4a</td>
</tr>
</tbody>
</table>

D[4,3] is the volume-weighted mean diameter; Dv0.1, Dv0.5 and Dv0.9 are the diameters below which 10%, 50% and 90% of the volume of particles are found, respectively. ab Means in the same row with different superscripts are significantly different (P < 0.05).

The size distribution of buffalo milk fat globules was also affected by freezing, as shown in Figure 3.5, consistent with the measurement of average fat globule size above.

![Figure 3.5](image-url)
The increase in fat globule size suggests partial or full coalescence of some buffalo milk fat globules. Three conditions are needed for coalescence to occur (Goff (1997)). These are: partial crystallisation of lipids, contact between fat globules and disruption of the MFGM. In bovine fat globules, lipids crystallise between 40°C to –40°C due to the diversity of triglycerides and fatty acids in bovine milk (Lopez & Ollivon 2009). Buffalo milk fat is expected to crystallise over a similar temperature range.

In this study, the final freezing temperature of -20°C is within the temperature range expected for lipid crystallisation. The fat globules are likely to come into contact during freezing due to the increased concentration of fat in the unfrozen aqueous phase (Saito et al. 1999) and damage to the MFGM was observed (Figure 3.4), suggesting fat coalescence will have occurred. This coalescence may contribute to the ‘oiling–off’ observed in some samples.

**Surface potential**

The surface potential of fat globules in buffalo milk changed from -10.2 ± 0.3 mV in raw buffalo milk to -10.8 ± 0.2 mV in milk subject to treatment A. No significant difference was observed for milk subject to treatment B (-10.3 ± 0.1 mV).

The surface potential has previously been used to assess damage to fat globules in frozen bovine milk (Michalski et al. 2002). A decrease from -13.5 mV to -20 mV was observed when the native MFGM was severely disrupted and the surface potential of absorbed casein micelles contributed to this change. In contrast, a smaller decrease was observed when less damage occurred to the native MFMG resulting in the absorption of fewer casein micelles. The size of the change in surface potential observed here suggests the damage was small, consistent with observations by CLSM and the small changes in fat globule size. The measurement of surface potential appears less sensitive to changes that occurred in treatment B compared to other methods employed above.

The fermentation rate during yoghurt production was also measured following freezing using treatment A and B. This fermentation rate was not found to be affected (data not shown).

**Conclusion**

Two different freezing rates of 1.2°C/hour or 0.54°C/hour (A and B respectively) were found to alter the properties of buffalo milk. While the pH, concentration of ionic calcium and milk viscosity were not altered, changes to the milk fat globules were observed and a fat layer observed on the surface of the milk that received treatment B.

The membrane surrounding the milk fat globule, the MFGM, appeared damaged after either freezing treatment A or B and proteins absorbed to the surface of the fat globule. The size of the fat globules increased and the size distribution was altered following freezing. This was likely due to coalescence between some fat droplets. A change in surface potential was also measured for fat globules subject to treatment A, again indicative of damage to the fat globule and absorption of protein on the fat globule surface.

Few differences were observed between milk samples frozen at 1.2°C/hour or 0.54°C/hour, suggesting that damage can occur even when the volume of milk is small (100 ml) and freezing rate rapid. The extent of damage is expected to be greater if the freezing rate is considerably slower than
recorded for treatment B, which will occur for volumes of buffalo milk frozen in single batches greater than 5L.

The significance of the fat globule damage caused by freezing is not yet clear, and further studies could quantify the extent of ‘oiling-off’ and the fat layer observed on the surface of some samples. The damage caused by freezing using treatment A or B appears minimal compared to other processing techniques such as homogenisation, and the impact will be small if the milk is to be fully or partially homogenised prior to further processing or if the milk is to be used for fresh products such as yoghurt or cheese and a preliminary investigation of fermentation rates suggests that the impact of the damage on processing and product quality without further treatment may be small. Further study is recommended, however, if frozen buffalo milk is to be used to produce matured cheeses or if the volume of milk frozen is to be considerably larger than 5L.
4. Characterisation of buffalo yoghurt - a comparison with bovine yoghurt


Introduction

Yoghurt consumption has increased considerably in the last 35 years, with Australian consumption rising from 1.0 kg to 7.1 kg per capita per year (Australia 2010; Tamime & Robinson 1999). Yoghurt can be produced from a range of milk types, with bovine milk being the most popular raw ingredient and a small volume of buffalo or other milk used for yoghurt production (Tamime & Robinson, 2007). The chemical composition of milk varies considerably between species and this difference impacts on both the processing steps in yoghurt production, as well as the properties of the final product. Buffalo milk, for example, has a higher concentration of fat (8.0% w/w vs. 4.1% w/w), protein (4.6% w/w vs. 3.4% w/w), total solids (17.0% w/w vs. 12.1 % w/w), calcium (47 mM vs. 30.5 mM) and phosphorous (27.7 mM vs. 19.2 mM) (Ahmad et al. 2008a; Braun & Preuss 2008; Menard et al. 2010; Varricchio et al. 2007) than bovine milk.

The set-type buffalo yoghurt found on the Australian market is typically produced with unhomogenised milk. This yoghurt is also made without milk solids fortification or the addition of artificial thickeners, due to the already high solids content of the milk (Addeo et al. 2007). Moreover, buffalo milk powder is not readily available worldwide, which also made the fortification of the buffalo milk more difficult (Tamime and Robinson, 2007). In contrast, bovine milk often requires fortification with skimmed milk powder to increase the solids content and achieve the desired yoghurt firmness and texture (Gun & Isikli 2006; Tamime & Robinson 1999). Furthermore, bovine milk is often homogenised, producing a homogenous product that prevents the formation of a cream layer.

While many studies have examined the structure and properties of bovine yoghurt, few have been conducted on buffalo yoghurt. The effect of fat (Cunha-Neto et al. 2005; Pandya et al. 2004; Raju & Pal 2009), solids content (Chawla & Balachandran 1994; Shiby & Mishra 2008) and storage time on buffalo yoghurt properties has been examined (Yadav et al. 2007) and fortified yoghurt containing fruit pulp, honey and soymilk produced (Ghadge 2008; Kumar & Mishra 2003). These studies have primarily focused on the chemical composition of the final product and have often employed sensory evaluation to evaluate textural properties, which provides only limited insight into the underlying chemical and physical properties of the product.

The viscosity and flow properties of buffalo yoghurt are also not well understood. Many studies have measured the viscosity of buffalo yoghurt at a single shear rate (Raju and Pal, 2009; Cunha-Neto et al., 2005) but as yoghurt is a non-Newtonian fluid, it is expected that the viscosity will be a function of the shear rate applied (Lee and Lucey, 2010). Recently, Bezerra and co-authors (2012) investigated different formulations made from buffalo and goats milk and determined rheological properties including the consistency coefficient K and flow behaviour index n using a range of shear rates from 10 to 1100 s$^{-1}$, however, other important properties such as gelation and thixotropy of buffalo yoghurt have not yet been investigated. Several past studies have also ceased milk fermentation at a pH of 4.8 - 5.0 (Nahar et al., 2007; Yadav et al., 2007; Bezerra et al., 2012), which is significantly higher than the end pH of 4.5 - 4.6 commonly reported in studies for bovine yoghurt (Lee & Lucey 2004;
Robinson & Haddadin 2010; Tamime & Robinson 2007) and the legal requirement for yoghurt production in Australia (a pH of 4.5 or less; Standards 2006).

Comparative analysis of buffalo and bovine yoghurt is likely to be insightful given the differences in bovine and buffalo milk composition and physical properties, yet there have been limited comparative studies. Khanna and Singh (1979) found a higher proteolytic activity, a higher titratable acidity and a better growth of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* when buffalo milk was used in place of bovine milk. Yoghurt made from buffalo milk was also found to be more nutritious than bovine or goat yoghurts due to the higher concentration of protein and fat, although bovine yoghurt was found to have a more acceptable smell and taste (Nahar et al. 2007). Interestingly, buffalo yoghurt contains a higher concentration of acetaldehyde, the major volatile compound in yoghurt, compared to bovine, ewe and goat yoghurts (Erkaya & Sengul 2011), which may be responsible for the distinct aroma. The organic acid profile, which is known to contribute significantly to yoghurt flavour, has also been studied for bovine yoghurt (Adhikari et al. 2002; Donkor et al. 2007; Fernandezgarcia & McGregor 1994; Thi et al. 2012; Tormo & Izco 2004) but not buffalo yoghurt.

While existing studies illustrate some of the possible similarities and differences between bovine and buffalo yoghurt, there has been no comprehensive study on buffalo yoghurt using the range of microscopic techniques now available. This study aimed to characterize the product microstructure and properties of buffalo yoghurt, including the fermentation kinetics, texture, rheological properties, organic acid profile and the growth and viability of probiotic bacteria during fermentation and 28 days of cold storage. A comparison was also made with the properties of fortified bovine yoghurt, in order to provide a better understanding of the nature of buffalo yoghurt and how changes to milk composition alter the properties of dairy products, such as yoghurt. The results of buffalo yoghurt and the comparison with bovine yoghurt obtained in this study will therefore not only be of interest for those researchers working on buffalo products but also offers important insights to the dairy research community.

In this study, the fresh buffalo milk was obtained from a local farm (Victoria, Australia) while the homogenised bovine milk was obtained from a local supermarket (Pura brand, Australia) and fortified with skim milk and full cream milk powder (Coles brand, Victoria, Australia) following normal commercial practice. The yoghurt samples were prepared following the method described in the methodology section. Analyses of physicochemical properties, syneresis, texture and microstructure were also performed as described in the methodology section.

### Results and discussion

Chemical composition of buffalo or fortified bovine milk used for yoghurt production is presented in Table 4.1. After fortification, buffalo and bovine milk are similar in the protein and total solids content and different in the fat, lactose and total calcium concentration.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Lactose (%w/w)</th>
<th>Protein (%w/w)</th>
<th>Fat (%w/w)</th>
<th>Total calcium (mM)</th>
<th>Total solids (%w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffalo milk</td>
<td>5.0 ± 0.2a</td>
<td>4.1 ± 0.4a</td>
<td>7.9 ± 0.3a</td>
<td>45.8 ± 1.0a</td>
<td>17.1 ± 0.4a</td>
</tr>
<tr>
<td>Fortified bovine milk</td>
<td>7.0 ± 0.2a</td>
<td>4.6 ± 0.3a</td>
<td>4.1 ± 0.2b</td>
<td>37.8 ± 1.9b</td>
<td>16.8 ± 0.1b</td>
</tr>
</tbody>
</table>

*abc* Means in the same column with different superscripts are significantly different (P < 0.05) in composition.
The increase of H+ concentration and ionic calcium during fermentation

The concentration of hydrogen ion H+ and ionic calcium increased during the fermentation in both buffalo and fortified bovine yoghurts (Figure 4.1A and B). The increase of H+ concentration was due to the metabolic activities of lactic acid bacteria that produce acid during fermentation. The increase of ionic calcium concentration resulted from the dissociation of calcium from the casein micelles as H+ ion concentration increased.

Figure 4.1A shows that the fermentation time, the time required for the milk to reach a H+ concentration of 3.2 x 10^-5 M (equivalent to a pH of 4.5), was one hour longer for buffalo milk compared to fortified bovine milk (Figure 1A). This longer fermentation time could be due to the lower initial concentration of dissociated H+ in the buffalo milk compared to bovine milk (0.02 x 10^-5 M vs. 0.03 x 10^-5 M, equivalent to pH 6.7 and 6.5, respectively) (P < 0.05) (Figure 4.1A inset). When buffalo milk was pre-acidified to a pH of 6.5, similar to the initial pH of standardised bovine milk, there was no significant difference in fermentation time (data not shown), confirming that difference in the initial milk pH was the major reason for the longer fermentation time for buffalo milk. This result suggests that the standardisation of milk pH could be used to reduce variability in the length of fermentation for buffalo milk products.

![Graphs showing changes in H+ concentration, ionic calcium, lactose concentration, and titratable acidity during fermentation.](image)

**Figure 4.1.** Changes in the concentration of dissociated H+ (A) as a function of fermentation time, and ionic calcium (B), lactose concentration (C) and titratable acidity (D) as a function of H+ ion concentration in buffalo (●) or fortified bovine milk (■). Each data point is the average of six replicates (n=6) and the error bars are the standard deviation of the mean. The inset in (A) shows the first two data points and errors on an enlarged scale.

The concentration of ionic calcium increased during fermentation of both yoghurts (Figure 4.1B), as calcium dissociated from the casein micelles as a result of increased H+ ion concentration. The ionic
calcium concentration recorded at the start of fermentation for both buffalo and fortified bovine milk was similar. However, as fermentation progressed, the level of ionic calcium present in buffalo milk began to exceed that in fortified bovine milk at the same H+ concentration. By the end of the fermentation, the concentration of ionic calcium in buffalo milk was significantly higher (P < 0.05) than that in fortified bovine milk. This difference is possibly due to the higher concentration of total calcium in this milk compared to fortified bovine milk (Table 1). The higher total calcium concentration and similar ionic calcium concentration at the beginning of the fermentation, suggests that buffalo milk contains more colloidal calcium phosphate (CCP), the insoluble form of calcium associated with casein micelles. The equilibrium between CCP and ionic calcium appears different in buffalo milk, particularly at lower pH, where more calcium was dissociated from the casein micelles. This is similar to the results of Ahmad and co-authors (2008), who found more than 82% of total calcium is present as CCP in buffalo milk, while only 72% of total calcium is present as CCP in bovine milk. Similarly, Abd El-Salam (1978) observed a higher concentration of calcium present in the casein micelles of buffalo milk (28.5 – 35.8 g per kg of casein) compared to bovine milk (25.2 – 29.8 g per kg of casein).

**Lactose consumption, acid production and changes in organic acid profiles during fermentation**

During fermentation there was a decrease in lactose concentration and an increase in titratable acidity for both buffalo and bovine milk (Figure 4.1C and D). These changes are due to the metabolic activity of lactic acid bacteria (LAB) in the starter culture, which converts lactose to lactic acid either via the Embden-Meyerhof-Parnas pathway (EMP pathway) or phosphoketolase pathway (PP pathway) in a process of homo or heterofermentation respectively (Kandler 1983). The method of titratable acidity applied here measures all such acidic compounds, with the data presented as lactic acid equivalents.

The amount of lactose consumed by the LAB was not significantly different in buffalo or fortified bovine milk. The titratable acidity of buffalo yoghurt at the end of the fermentation was also similar with 1.08 % and 1.05 % lactic acid equivalents respectively. The titratable acidity of buffalo yoghurt was in the range previously reported, although this range varies significantly from 0.68 % to 1.59 % lactic acid equivalents (Raju & Pal 2009). Differences reported in the literature likely arise from the different methods of yoghurt production (e.g. different end pH or titratable acidity, fermentation time and temperature), as well as differences in the chemical composition of the milk used for yoghurt production. The titratable acidity of buffalo yoghurt and fortified bovine yoghurt were also in agreement with CODEX requirements for yoghurt production (minimum of 0.6 % lactic acid) (Codex standard 243. 2003).

The concentration of organic acids in buffalo and fortified bovine yoghurt during fermentation and storage are presented in Figure 2. Buffalo yoghurt contained a significantly lower concentration of all organic acids, except for pyruvic acid and citric acid. Among the eight organic acids investigated, citric acid and lactic acid were the most abundant acids in both types of yoghurt. The changes in organic acid concentration during fermentation followed similar trends for both buffalo and fortified bovine yoghurt. The concentration of lactic acid, acetic acid and pyruvic acid increased (Figures 4.2C, D and G), while the concentration of other acids remained relatively constant.
Figure 4.2. Changes in the concentration of citric acid (A), orotic acid (B), pyruvic acid (C), lactic acid (D), formic acid (E), uric acid (F), acetic acid (G) and hippuric acid (H) during the fermentation and storage of buffalo (—) or fortified bovine yoghurt (—). Each data point is the average of six replicates (n=6) and the error bars are the standard deviation of the mean. Storage commenced after six hours of fermentation for buffalo yoghurt and five hours for bovine yoghurt.

During the storage period of 28 days at 4°C, the concentration of lactic acid continued to increase for buffalo and fortified bovine yoghurt respectively (Figure 2D). These results are consistent with previous studies, where citric and lactic acids were found to be the dominant acids in bovine yoghurt, with the concentration of lactic acid also increasing on storage (Adhikari et al. 2002; Donkor et al. 2007; Fernandezgarcia & McGregor 1994).

The significant difference in the concentration of some organic acids is likely the consequence of the lower initial concentration of these in buffalo milk (Figure 4.2). The significantly lower concentration of orotic and uric acid in buffalo milk were also reported in previous studies, with values ranging from 1.9 to 2.9 and 0.2 to 0.4 mg/100g for buffalo milk and from 8.3 to 11.3 and 3.5 to 8.7 mg/100g for bovine milk respectively (Fernandezgarcia & McGregor 1994; Larson & Hegarty 1979; Okonkwo & Kinsella 1969; Prakash & Sharma 1986; Sikka et al. 2001; Tormo & Izco 2004; Venkatappaiah & Basu 1952).
The changes in storage modulus $G'$ and loss tangent $\tan \delta$ during fermentation

The changes in the storage modulus $G'$ and loss tangent ($\tan \delta$) measured during yoghurt production for buffalo or fortified bovine milk are presented in Figure 4.3.

![Figure 4.3. Changes in the storage modulus $G'$ (A) and the loss tangent $\tan \delta$ (B) as a function of fermentation time for buffalo (---) or fortified bovine yoghurt (----). Each data point is the average of two fermentations ($n=2$) and the error bars are the standard deviation of the mean. The inset in (A) corresponds to data between 120 - 165 min of fermentation time.]

The point of gelation, defined as the point when the $G'$ reaches 1 Pa, occurred at ~ 150 minutes and ~ 160 minutes for fortified bovine and buffalo milk respectively (Figure 4.3A inset). The slower gelation for buffalo milk is likely the result of the slower acidification observed for non-standardised buffalo milk (Figure 4.1A). Large fluctuations in $\tan \delta$ were observed prior to this point of gelation, indicating instability in the gel. Following gelation, the $G'$ increased and this increase was more rapid for the bovine sample (Figure 4.3A). The $G'$ for the buffalo gel was smaller than for the fortified bovine gel for most time points during fermentation but the $G'$ at the end of fermentation was similar, indicating similar product stiffness. The $\tan \delta$ of the buffalo gel remained almost stable in the range of 0.84 - 1.09, while a significant decrease in $\tan \delta$ was observed between the highest (1.10 ± 0.12) and the lowest value (0.74 ± 0.12) for fortified bovine milk (Figure 4.3B).

The changes in syneresis and gel firmness of buffalo or fortified bovine yoghurt during storage

Significantly higher syneresis was observed for buffalo yoghurt, compared to fortified bovine yoghurt (Figure 4.4A). The syneresis increased as a function of storage time for buffalo yoghurt ($P<0.05$), but syneresis did not increase for the fortified bovine yoghurt.
The large amount of whey separation was also observed by Bezerra and co-authors (2012), with 20.2 mL of whey expelled from 200 g of yoghurt, equivalent to 10.1% (v/w), using the draining method. The higher mass of whey expelled from the buffalo yoghurt here is likely a result of the lower surface area of the fat globules in this preparation compared to the homogenised bovine milk, 1.61 m² per g fat vs. 18.87 m² per g fat, resulting in a less amount of protein absorbed on the surface of fat globules leading to a network with less ability of holding water. This was similar to the findings of previous studies on bovine yoghurt where a decrease of syneresis in yoghurt was observed when the surface area of bovine milk fat globules was increased (Gun & Isikli 2006; Keogh & O’Kennedy 1998) consistent with this hypothesis.

A significant increase of gel firmness (P<0.05) was observed for both buffalo and fortified bovine yoghurt, but no significant difference was found between the two yoghurt types (Figure 4.4B). An increase in yoghurt firmness during storage has also been reported in a previous study of bovine yoghurt (Salvador & Fiszman 2004).

**The changes in rheological properties (thixotropy, flow behaviour index n and consistency coefficient K) of buffalo or fortified bovine yoghurt during storage**

Buffalo yoghurt showed significant differences in the rheological properties including the thixotropy, consistency coefficient K and flow behaviour index n compared with bovine yoghurt (P<0.05) (Figure 4.5).
Buffalo yoghurt was significantly more thixotropic than the yoghurt made from fortified bovine milk throughout the storage period (P < 0.05) (Figure 4.5A). A prolonged storage of 28 days also led to a significant increase in thixotropy for both types of yoghurt (P < 0.05). These results indicate that the structure of buffalo yoghurt is less able to recover from deformation, especially when the yoghurt is stored. A significantly higher consistency K was recorded for buffalo yoghurt (P < 0.05) (Figure 4.5B), indicating buffalo yoghurt is more viscous. The yoghurt viscosity also increased with storage time, as shown by a significant increase in the consistency index K for both buffalo and fortified bovine yoghurt between day 7 and day 28 of storage (P < 0.05). In contrast, the flow behaviour index n, was found to be significantly higher in fortified bovine yoghurt (P < 0.05) (Figure 4.5 C), indicating buffalo yoghurt is more non-Newtonian, more sensitive to shear and more susceptible to a break-down in the network structure.

The differences in rheological properties between buffalo and bovine yoghurt observed in our study explain potential differences in the sensory properties of bovine and buffalo yoghurt. Our data also suggest that more care is required in handling buffalo yoghurt, and that there are potential underlying differences in the network structure of the two yoghurts.

**The changes in microstructure of buffalo or fortified bovine yoghurt during storage**

The microstructure of the bulk network within buffalo and fortified bovine yoghurt was assessed by CLSM for samples stored for 1 and 28 days as shown in Figure 4.6. The structure of the same samples was also observed by cryo-SEM, as shown in Figures 4.7 and 4.8.
Figure 4.6. Microstructure of buffalo (A, B) or fortified bovine yoghurt (C, D) after 1 day of storage (A, C) or 28 days of storage (B, D) as observed by CLSM. Nile Red stained fat appears red and FCF stained protein appears green. The black areas are serum pores. Images were captured using a 63x objective using a 1x digital zoom (upper images) or 5x digital zoom (lower images). The scale bars are 10 μm in length in the upper images and 2 μm in length in the lower images.

The microstructure of both buffalo yoghurt and fortified bovine yoghurt observed by CLSM appears similar (Figure 4.6). Both yoghurts consist of a protein network with integrated fat globules and serum pores (which appear as unstained black regions) (Figure 4.6A1 and C1). The only significant difference observed between the microstructure of the two yoghurts is the appearance of the bigger fat globules within buffalo samples (Figure 4.6A2 and B2), which was expected as the buffalo milk was not homogenized. The microstructure of both yoghurts was affected by storage with larger serum pores appearing after 28 days of storage particularly in the buffalo samples (Figure 4.6B1 vs. A1).

The cryo-SEM images (Figure 4.7A1 - A3) confirm the presence of bigger fat globules within the microstructure of the buffalo yoghurt. The homogenised fat globules in bovine yoghurt are embedded within the protein network (Figure 4.7B1 - B3), whereas the unhomogenised fat globules in buffalo yoghurt appear within the pores of the protein network or are sufficiently large enough to disrupt the network (Figures 4.7A1 - A3). In general, the protein network within buffalo yoghurt appeared more irregular (Figure 4.7A2 and B2) and contained a greater number of serum pores (Figure 4.7A1) consistent with CLSM images (Figure 4.6A1).
Figure 4.7. Microstructure of buffalo (A1 - A3) or fortified bovine yoghurt (B1 - B3) after 1 day of storage as observed by cryo-SEM. Images were captured using a solid state detector at 1000x magnification (left), 4000x magnification (middle) and 16000x magnification (right). The scale bars are 100 µm in length (left), 20 µm in length (middle) or 5 µm in length (right). Arrows indicate the presence of serum pores within the network.

Figure 4.8. Microstructure of buffalo (A1 - A3) or fortified bovine yoghurt (B1 - B3) after 28 days of storage observed by cryo-SEM. Images were captured using a solid state detector at 1000x magnification (left), 4000x magnification (middle) and 16000x magnification (right). The scale bars are 100 µm in length (left), 20 µm in length (middle) or 5 µm in length (right). Arrows indicate the presence of serum pores within the network.

The major change in yoghurt microstructure during storage was the appearance of more serum pores in the network of both buffalo and fortified bovine samples (Figure 4.8A1 and B1). These pores were more numerous in buffalo yoghurt, consistent with observations made by CLSM (Figure 4.6B1 and D1) and may be linked to the increased whey separation (Figure 4.4A) and changes in thixotropy and flow behaviour index observed on storage (Figure 4.5A and C). These images therefore suggest a link between the product microstructure and functional properties.
Bacterial growth and viability during fermentation and storage

Both buffalo and fortified bovine yoghurts were inoculated with the same number of starter culture bacteria. The growth of these three strains during fermentation is presented in Table 4.2, while the viability during storage is summarized in Table 4.3. During fermentation, the number of bacteria increased significantly for each strain in each type of yoghurt (P < 0.05) (Table 2). The most considerable change was found in the population of *Streptococcus thermophilus*, with an increase of ~ 2 log CFU/g (from 7.2 log CFU/g to more than 9.2 log CFU/g) and this strain dominated the microbial population in both yoghurts. *Lactobacillus acidophilus* La-5 and *Bifidobacterium lactis* Bb-12 showed a slower growth, with an increase of less than 1 log CFU/g observed for these populations of bacteria. In general, the bacteria grew better in fortified bovine milk than buffalo milk, except for *Lactobacillus acidophilus* La-5, which showed no significant difference (P > 0.05).

Table 4.2. Bacterial growth during the fermentation of buffalo milk and fortified bovine milk to produce yoghurt. The number of viable bacteria (log CFU/g) were determined by counting colony forming units on poured plates. Data are the mean ± the standard deviation of the mean (n=6). Both yoghurt types were inoculated with the same number of starter bacteria.

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Start of fermentation</th>
<th>End of fermentation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Buffalo milk</td>
</tr>
<tr>
<td>La-5</td>
<td>6.49 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.34 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bb-12</td>
<td>7.19 ± 0.10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.45 ± 0.19&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>St</td>
<td>7.20 ± 0.11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.23 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* La-5=Lactobacillus acidophilus; Bb-12 = Bifidobacterium lactis, St = Streptococcus thermophilus

<sup>abc</sup> Means in the same row with different superscripts represent significant differences between different samples (P < 0.05).

The number of *Lactobacillus acidophilus* La-5 bacteria decreased significantly in both buffalo and fortified bovine yoghurts on storage (P < 0.05) (Table 4.3). In contrast, the number of *Streptococcus thermophilus* appeared constant in both samples and the number of *Bifidobacterium lactis* Bb-12 slightly reduced (P < 0.05) in buffalo yoghurt but unchanged in fortified bovine yoghurt (P > 0.05). The relative numbers of *Lactobacillus acidophilus* La-5 and *Bifidobacterium lactis* Bb-12 also differed between the two yoghurt types, with higher numbers remaining viable in fortified bovine yoghurt, while the number of *Streptococcus thermophilus* was similar.

Previous studies have reported the dominant role of *Streptococcus thermophilus* in yoghurt fermentation, with average viable cell populations ranging from 8 log CFU/g to 9 log CFU/g (Damin et al. 2008; Oliveira et al. 2001; Saccaro et al. 2009). *Streptococcus thermophilus* typically exhibits superior growth and greater viability when mixed with a variety of cultures including *Lb. bulgaricus*, *Lb. acidophilus*, *Lb. casei*, *Lb. rhamnosus* or *Bifidobacterium* spp. (Damin et al. 2008; Dave & Shah 1997; Saccaro et al. 2009). This preferential growth has been observed in a range of milk types including bovine milk, goat milk and soymilk (Bozanic 2002; Bozanic et al. 2011). The better growth and viability of *Streptococcus thermophilus* is likely due to the higher proteolytic activity and lactose utilisation reported for this strain compared to the two other probiotic strains (Marafon et al. 2011; Ozer & Kirmaci 2010; Saccaro et al. 2009).

The significant reduction in viability of *Lactobacillus acidophilus* observed here is consistent with the noted loss of proteolytic activity that is thought to occur for this strain during storage at 4°C (Gilliland & Lara 1988). *Lactobacillus acidophilus* is also sensitive to lactic acid and peroxide produced by other bacterial strains (Dave & Shah 1997; Saccaro et al. 2009). To solve this issue,
several studies have attempted to increase and maintain the number viable of probiotic bacteria in bovine yoghurt by adding more probiotic bacteria at the beginning of the fermentation, encapsulating probiotic bacteria before inoculation, utilising heat-shocked starter cultures or combining *Lactobacillus acidophilus* with the slow acid producing starter culture strains (Bozanic et al. 2011; El-Dieb et al. 2012; Priya et al. 2011).

The higher concentration of milk fat and calcium in buffalo milk are unlikely to affect the viability of *Lactobacillus* and *Bifidobacterium* in the buffalo yoghurt although these components have been reported to stimulate the growth of lactic acid bacteria in previous studies (Tan et al. 2012; Wishon et al. 2010). Differences observed in the microstructure are also unlikely to alter bacterial survival. The viability of the probiotic bacteria could be enhanced by the higher concentration of lactose in bovine yoghurt, as lactose may act as a protective coating. A further factor may be bioactive compounds that may differ in the two milk preparations. The presence of these bioactives and possible mechanism of action is worthy of further study.

### Table 4.3. Changes in the bacterial population within buffalo yoghurt or fortified bovine yoghurt as a function of storage at 4°C. Viable bacteria (log CFU/g) were determined by counting colony forming units on poured plates. Data are the mean ± the standard deviation of the mean (n=6).

<table>
<thead>
<tr>
<th>Yoghurt</th>
<th>Storage time (days)</th>
<th>Bacterial species*</th>
<th>La-5</th>
<th>Bb-12</th>
<th>St</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>7.26 ± 0.12&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.31 ± 0.19&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>9.48 ± 0.14&lt;sup&gt;AA&lt;/sup&gt;</td>
</tr>
<tr>
<td>Buffalo</td>
<td>1</td>
<td></td>
<td>5.17 ± 0.08&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.04 ± 0.17&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>9.30 ± 0.18&lt;sup&gt;AA&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td></td>
<td>7.69 ± 0.09&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>7.90 ± 0.16&lt;sup&gt;TA&lt;/sup&gt;</td>
<td>9.44 ± 0.02&lt;sup&gt;AA&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fortified bovine</td>
<td>1</td>
<td></td>
<td>7.11 ± 0.15&lt;sup&gt;abA&lt;/sup&gt;</td>
<td>7.76 ± 0.29&lt;sup&gt;TaA&lt;/sup&gt;</td>
<td>9.41 ± 0.03&lt;sup&gt;AA&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*La-5 = *Lactobacillus acidophilus*; Bb-12 = *Bifidobacterium lactis*, St = *Streptococcus thermophilus*

<sup>abc</sup> Means in the same column with different superscripts represent significant differences between the same yoghurt type after 28 days of storage (P < 0.05).<sup>ABC</sup> Means in the same column with different superscripts represent significant differences between buffalo yoghurt and bovine yoghurt of the same storage age (P < 0.05).

These results are in agreement with the findings of previous studies on bovine yoghurt, which also reported on the dominant role of *Streptococcus thermophilus*, and the significant decrease in the viability of *Lactobacillus acidophilus* during storage (Damin et al. 2008; Oliveira et al. 2001; Saccaro et al. 2009). The superior growth of *S. thermophilus* is likely due to the higher proteolytic activity and lactose utilisation reported (Marafon et al. 2011; Ozer & Kirmaci 2010; Saccaro et al. 2009) while the significant reduction in viability of *Lactobacillus acidophilus* observed here is consistent with the noted loss of proteolytic activity that is thought to occur for this strain during storage at 4°C (Gilliland & Lara 1988).

The higher viability of the probiotic bacteria observed in fortified bovine yoghurt is likely due to the higher content of lactose in this preparation, which may act as a protective coating. The difference in the presence of some bioactive compounds in the two milk preparations may also contribute to the difference in the survival of the bacteria in the two yoghurt types, which is worthy of further investigation.
Conclusion

Buffalo and bovine yoghurts differ in their microstructure and physicochemical properties, following fermentation and during cold storage. Buffalo yoghurt exhibited a weaker network structure that was more porous, more irregular and more disrupted by large unhomogenised fat globules. This difference in microstructure leads to a significantly higher degree of syneresis, a greater degree of thixotropy, a greater consistency coefficient (K) and a smaller value of flow behaviour index (n). While the gel firmness of both yoghurts was similar, the number of probiotic bacteria was reduced during the cold storage of buffalo yoghurt.

Our results suggest that the total solids content of the milk is not the only factor that may affect the yoghurt quality. Other factors such as the concentration of fat globules, surface area of fat, concentration of lactose, and concentration of calcium can alter the structure and properties of yoghurt. The different microstructure and physiochemical properties observed here for buffalo and bovine yoghurt are also likely responsible for the different sensory properties of these two yoghurts. The different properties observed here suggest a possible route for the production of new dairy products with altered properties.
5. Buffalo milk transcriptomics

Some Preliminary data discussed in this chapter have been published in the proceeding of the World Buffalo Congress, 2013. (Sanjana KURUPPATH, Amit KUMAR, Vengama Naidu MODEPALLI, Ngo Khanh PHUONG, Sally Louis GRAS3, Christophe LEFEVRE. Buffalo milk transcriptomics, 2013).

Introduction

Recent studies have reported transcriptome analysis of mRNA isolated from milk cells or milk fat globules and the presence of small RNA in bovine, human and other milk. In particular, the recent identification of high levels of a large number of micro-RNAs in the milk of eutherian mammals (human, mouse, cow and pig) (Chen et al. 2010; Gu et al. 2012; Hata et al. 2010; Kosaka et al. 2010; Zhou et al. 2012), and the observation that exogenous miRNAs consumed from plant foods may directly influence gene expression in animals (Zhang et al. 2012), raise new questions about the use of miRNAs as biomarkers of lactation status and their putative role in mammary gland physiology, together with their potential effects on growth and development of the young (Kumar et al. 2012). Here, we explore buffalo milk RNA content, showing that RNA isolated from colostrum and milk cells contains high level of milk protein transcripts revealing in part the mammary epithelial cell transcriptome, providing new information on buffalo milk protein genes and their expression during lactation and, that buffalo colostrum and milk contain a large population of miRNA with similarity and differences in composition with the milk of other mammals.

Illumina sequencing technology was used to examine the RNA contents of buffalo milk. All the previous gene expression studies conducted on buffalo have used microarrays. To the best of our knowledge this is the first gene expression study using RNA-seq technology in buffalo. This technology provides a snapshot of all RNA contents in a biological sample at a given time. Unlike microarrays this technology is not dependent on any previous knowledge of genes in the organism and can both precisely quantify the RNA and identify new genes and mRNA transcripts. For this reason and to overcome additional limitations associated with the bovine microarray platform, we decided to take advantage of the decrease of sequencing cost during the establishment of the project and deployed a deep sequencing approach to combine gene characterisation and gene expression analysis in buffalo milk. The composition of colostrum is known to be different from mature milk, in terms of its immunoprotective role in the early days of the newborn (Singh et al. 1993; Uruakpa et al. 2002). Colostrum and mature milk samples were processed to sequence the RNA from somatic milk cells. A global analysis was conducted on the buffalo colostrum and mature milk transcriptomes. At first, highly expressed genes and differentially expressed genes in colostrum and mature milk were identified. Detailed gene ontologies and pathway analysis were conducted using publically available bioinformatics software tools.

Results and discussion

Buffalo colostrum and milk cells transcriptomics

RNA sequencing and bovine genome alignments

In order to test the analysis of gene expression from cells isolated from buffalo milk or colostrum, we chose to perform transcriptome quantification by RNA sequencing. The sequencing of three colostrum and four mature milk RNA sample libraries produced approximately 7 to 11.5 million reads in each sample. The tophat2 program was used to align the reads to the cow UMD 3.1 genome assembly. As shown in table 5.1, a small proportion of reads were filtered out as poor quality
sequences and 45 to 72 percent of individual samples reads aligned to the cow genome, showing that while a buffalo genome sequence reference is not yet available, the bovine genome reference provides a working alternative for the annotation of the RNA sequencing data.

Table 5.1. Summary of sequence reads quality filtering and mapping onto the bovine genome sequence reference UMD 3.1 using Tophat2. CC: colostrum samples, MM: mature milk samples.

<table>
<thead>
<tr>
<th>Milk Samples</th>
<th>CC-1</th>
<th>CC-2</th>
<th>CC-3</th>
<th>MM-1</th>
<th>MM-2</th>
<th>MM-3</th>
<th>MM-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Reads</td>
<td>8794607</td>
<td>11092872</td>
<td>8377306</td>
<td>11027853</td>
<td>10079141</td>
<td>7807251</td>
<td>13200023</td>
</tr>
<tr>
<td>Reads mapped</td>
<td>3990815</td>
<td>8220510</td>
<td>6043630</td>
<td>6733701</td>
<td>5884631</td>
<td>3816871</td>
<td>9075021</td>
</tr>
<tr>
<td>Not mapped</td>
<td>4282493</td>
<td>2823582</td>
<td>2325182</td>
<td>4129425</td>
<td>3423485</td>
<td>3958668</td>
<td>4070467</td>
</tr>
<tr>
<td>Reads filtered out</td>
<td>521299</td>
<td>48780</td>
<td>8494</td>
<td>164727</td>
<td>771025</td>
<td>31712</td>
<td>54535</td>
</tr>
<tr>
<td>% reads mapped</td>
<td>45.4%</td>
<td>74.1%</td>
<td>72.1%</td>
<td>61.1%</td>
<td>58.4%</td>
<td>48.9%</td>
<td>68.8%</td>
</tr>
</tbody>
</table>

Two algorithms were evaluated for the genome mapping of short read sequencing data. Bowtie is basic genome alignment software. Tophat is an extension of Bowtie, designed to improve the resolution with RNA-seq data. It uses genomic annotations to facilitate the detection of exon junctions in order to first align all the RNA-sequence data to mRNAs and then aligns the remaining unmapped sequences to chromosomes. We performed alignments using both tools. Bowtie was much faster than Tophat (version 2), as it aligns RNA sequence data to the chromosomes. In general, we found that Tophat performed better alignments than bowtie, revealing additional details on gene structure. This is particularly relevant when genes encompass short exons such as in the case of casein genes encoding the major milk proteins. As shown in figure 5.1, a comparison of genomic alignments using bowtie and tophat software, tophat found all the annotated exons and suggested exon structure variation in the buffalo compared to the canonical bovine casein-alpha gene. Similar observations have been made possible for a full set of milk protein genes also present in the bovine genome.

Figure 5.1. Comparison of bowtie and tophat alignments on CASA1 gene encoding alpha-casein S1.

In figure 5.1, the top three tracks represent the genomic annotation of casein alpha (CASA1). The top first track represents the region where the gene is found. The second track shows exon regions, which are spliced together to produce the messenger RNA. The third track indicates the regions coding for
the milk protein, excluding non-coding regions of the messenger RNA at both extremities. Below, RNA-seq data alignments for CASA1 are shown for bowtie (upper) and tophat (lower) algorithms. Bowtie missed several short exons, which are apparently correctly reported by Tophat.

**Estimation of gene expression**

The Cufflinks program was used to assemble and calculate gene expression values in each sample based upon the bovine genome annotation. The cow genome assembly encompasses a total of 24,616 annotated genes. After merging these genes with 5,463 newly predicted genes by the cufflinks package based upon the buffalo milk cell sequencing data, a total of 30,079 genes were obtained. As depicted in table 5.2, approximately 2.3 percent of genes were highly expressed incolostrum and mature milk with 695 genes incolostrum and 727 genes in mature milk showing greater than 500 RPKM (Reads Per Kilobase per Million mapped reads) values. Mature milk had a slightly higher number of 7871 genes with medium expression levels (10 – 500 RPKM) compared to 7618 genes incolostrum. As listed in table 5.2,colostrum had the higher number of genes (13949) with low expression less than 10 and greater than 1 RPKM values compared to 12198 genes in mature milk. In total, 27.6 and 28.6 percent of genes showed medium to highly expressed incolostrum and mature milk respectively and approximately 25.9 and 30.8 percent of known bovine orthologs were not detected at all in milk cells. Overall, buffalocolostrum cells had approximately five percent more genes with detectable expression than mature milk, but a greater number of genes with medium to higher expression levels were reported in mature milk, suggesting that the difference in the detection of genes expressed incolostrum and milk cells may only be due to a masking effect of highly expressed milk proteins in milk cells.

<table>
<thead>
<tr>
<th>Gene expression level (RPKM)</th>
<th>Colostrum</th>
<th>Mature milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Highly Expressed (&gt; 500)</td>
<td>695</td>
<td>727</td>
</tr>
<tr>
<td>Medium expressed (10 - 500)</td>
<td>7618</td>
<td>7871</td>
</tr>
<tr>
<td>Low expressed (1 - 10)</td>
<td>13949</td>
<td>12198</td>
</tr>
<tr>
<td>Not expressed (= 0)</td>
<td>7817</td>
<td>9283</td>
</tr>
</tbody>
</table>

For further comparison of gene expression, bovine genome alignments of RNA-seq data were visualized by SeqMonk software. The read count quantitation pipeline was used to quantify the expression of all known bovine genes and datasets were normalized to per million bases units with probe length correction. The data was finally merged intocolostrum and mature milk replicate sets and further normalized by applying the percentile normalization quantitation pipeline. After quality control and meta-analysis of the data, one milk sample (MM-4) was discarded due to poor quality. A box plot of normalized feature probes of the validated sample set is shown in figure 5.2.
Most highly expressed genes in buffalo colostrum and mature milk cells

A list of the most highly expressed genes in colostrum is given in table 5.3 and a similar list for milk cells is shown in table 5.4.

Not so surprisingly, most of the well-known major proteins top the list of most highly expressed genes in colostrum or milk cells. Alpha-S1, alpha-S2, beta and kappa caseins together with beta-lactoglobulin were dominating both transcriptomes. Each represented about 0.7 to 1% of all gene expression in colostrum, and from 0.15 to 0.8% in milk cells.

Other genes highly expressed in colostrum were two genes related to iron metabolism, and two members of the thymosine-beta-4 gene family. FRIH seats on bovine chromosome 29 while FTH1 is on chromosome 15. FRIH encodes ferroxidase, an intracellular iron storage protein in both prokaryotes and eukaryotes (Hentze et al. 1986). FTH1 is annotated as a known processed but non-functional pseudogene in the cow. The status of this gene in the buffalo remains to be confirmed, but high expression in buffalo colostrum cells suggests a possible functional specificity in this organism. Two highly expressed thymosin-beta-4 genes on chromosomes X (TYB4) and 11 (TMSB4X) respectively encode actin sequestering proteins, which plays a role in regulation of actin polymerization and are also involved in cell proliferation, migration, and differentiation (Selmi et al. 2012). These processes are likely to temporarily occur in the mammary gland during the short transition period of colostrum production before the establishment of full lactation after parturition.

Interestingly, in mature milk cells, the whey protein Alpha-lactalbumin was also detected at high level, due to a 7-fold enrichment in milk compared to colostrum. Similarly, the Glycosylation-dependent cell adhesion molecule (GLCM1) gene showed 10-fold higher expression in milk. GLCM1 is a 28 kDa milk glycoprotein also known as lactophorin (Groenen et al. 1995). In addition, acidic ribosomal protein 60S P1 (RLA1) was highly expressed in milk cells. This protein plays an important role during the elongation step of protein synthesis (Rich & Steitz 1987). Finally, two variants of translationally-controlled tumor protein (TCTP) were also present among the ten most highly expressed genes.
expressed genes in both colostrum and milk cells at comparable levels. There are two copies of this gene in the cow genome on chromosomes 25 (2 exons) and 12 (6 exons) coding for same 172 amino acids. This protein has high affinity for calcium, and is preferentially expressed in cells during the early growth phase (Bommer et al. 2002; Bommer & Thiele 2004). It controls microtubules and actin microfilaments and regulates cell shape in normal and cancer cells. It is embryonic lethal when knocked-out in mice, (Bazile et al. 2009). The protein is also known to prevent cell death by binding to calcium, an ion that causes cell death. In lactating mammary glands, where higher cellular activity is required for continued production of milk along with high calcium content of milk, this protein may play an important role in the down-regulation of apoptosis to inhibit program cell death in mammary gland cells (Nagano-Ito & Ichikawa 2012).

**Table 5.3. Genes highly expressed in colostrum cells.**

<table>
<thead>
<tr>
<th>Feature</th>
<th>Description/Ontology terms</th>
<th>Chr.</th>
<th>Colostrum FPKM</th>
<th>Mature Milk FPKM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CASA1</td>
<td>Alpha-S1-casein</td>
<td>6</td>
<td>10512.7</td>
<td>38734.6</td>
</tr>
<tr>
<td>CASB</td>
<td>Beta-casein</td>
<td>6</td>
<td>10424.0</td>
<td>34662.6</td>
</tr>
<tr>
<td>E7E1Q6</td>
<td>beta-lactoglobulin precursor</td>
<td>11</td>
<td>10011.7</td>
<td>80038.9</td>
</tr>
<tr>
<td>CASA2</td>
<td>Alpha-S2-casein</td>
<td>6</td>
<td>7851.0</td>
<td>16234.9</td>
</tr>
<tr>
<td>A3FJ56</td>
<td>kappa-casein precursor</td>
<td>6</td>
<td>7312.0</td>
<td>15002.0</td>
</tr>
<tr>
<td>FRIH</td>
<td>Ferritin heavy chain</td>
<td>29</td>
<td>6266.8</td>
<td>3785.6</td>
</tr>
<tr>
<td>FTH1</td>
<td>Ferritin processed pseudogene</td>
<td>15</td>
<td>5246.2</td>
<td>3145.8</td>
</tr>
<tr>
<td>TYB4</td>
<td>Thymosin beta-4</td>
<td>X</td>
<td>4044.7</td>
<td>1789.9</td>
</tr>
<tr>
<td>TMSB4X</td>
<td>Thymosin beta 4</td>
<td>11</td>
<td>3758.3</td>
<td>1649.3</td>
</tr>
<tr>
<td>TCTP</td>
<td>Translationally-controlled tumor protein</td>
<td>25</td>
<td>3731.1</td>
<td>4262.5</td>
</tr>
</tbody>
</table>
Table 5.4. Genes highly expressed in milk cells.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Description</th>
<th>Chr.</th>
<th>Mature Milk FPKM</th>
<th>Colostrum FPKM</th>
</tr>
</thead>
<tbody>
<tr>
<td>E7E1Q6</td>
<td>beta-lactoglobulin precursor</td>
<td>11</td>
<td>80038.9</td>
<td>10011.7</td>
</tr>
<tr>
<td>CASA1</td>
<td>Alpha-S1-casein</td>
<td>6</td>
<td>38734.6</td>
<td>10512.7</td>
</tr>
<tr>
<td>CASB</td>
<td>Beta-casein</td>
<td>6</td>
<td>34662.6</td>
<td>10424.0</td>
</tr>
<tr>
<td>LALBA</td>
<td>Alpha-lactalbumin</td>
<td>5</td>
<td>19696.5</td>
<td>2968.7</td>
</tr>
<tr>
<td>GLCM1</td>
<td>Glycosylation-dependent cell adhesion molecule 1</td>
<td>5</td>
<td>18059.2</td>
<td>1830.1</td>
</tr>
<tr>
<td>CASA2</td>
<td>Alpha-S2-casein</td>
<td>6</td>
<td>16234.9</td>
<td>7851.0</td>
</tr>
<tr>
<td>A3FJ56</td>
<td>kappa-casein precursor</td>
<td>6</td>
<td>15002.0</td>
<td>7312.0</td>
</tr>
<tr>
<td>RLA1</td>
<td>60S acidic ribosomal protein P1</td>
<td>10</td>
<td>6833.7</td>
<td>3300.1</td>
</tr>
<tr>
<td>TCTP</td>
<td>Translationally-controlled tumor protein</td>
<td>25</td>
<td>4262.5</td>
<td>3731.1</td>
</tr>
<tr>
<td>TCTP</td>
<td>Translationally-controlled tumor protein</td>
<td>12</td>
<td>4163.5</td>
<td>3642.1</td>
</tr>
</tbody>
</table>

Amongst the 150 most highly expressed genes, ribosomal proteins were highly enriched. The ribosome is the main cellular machinery for protein synthesis. Ribosomal proteins accounted for a third of the genes in this group, showing that the milk cell is likely to be very active in protein synthesis. In addition with closer inspection of genomic read mapping, a significant number of novel genes can also be newly identified from the transcriptome data mapped onto the bovine genome, including a large number of strongly expressed novel non-coding RNA and, possibly, new unidentified coding genes. Full characterization of these novel transcripts will need further validation when the genome sequence reference of the buffalo becomes available.

These results show that the characterization and quantification of gene expression is possible with buffalo colostrum or milk as starting materials and confirm that cell transcripts encompass a significant and dominant level of mammary epithelial cell specific transcripts. Thus milk cells transcriptomics provide a useful non-invasive methodology to investigate gene expression during lactation in buffalo or other mammals. This approach reveals the expression of new and known genes contributing to lactation in the buffalo, and allows comparison with data from other sources and other species.
Differential expression analysis of colostrum and mature milk

An intensity difference filter with multiple testing corrections (P-value < 0.05) was used to filter the differentially expressed genes and 156 genes were significantly differentially expressed. From these 77 genes were colostrum enriched and 79 genes were enriched in mature milk. The top 10 colostrum-enriched genes identified are listed in table 5.5, sorted by difference of expression between colostrum and milk. Several of these genes encode peptidase inhibitory functions, including trappin, elafin and plexinlike genes. bTrappin-5 and bTrappin-4 are associated with negative regulation of peptidase activity processes in the extra-cellar region, potentially helping in protecting various proteins secreted in large quantities by the mammary glands. Elafin (A1A4Q6) is a skin derived peptidase inhibitor 3 (Molhuizen et al. 1994) and LOC100295842 is a predicted gene with peptidase inhibitor 3-like potential. The Plexin domain containing protein 1(A6H734) is a disulphide-rich motif. Plexin receptors are involved in the development of neural and epithelial tissues (Basile et al. 2007) and regulate axon guidance, immune function and angiogenesis (Love et al. 2003), thus plexin may be important in the transformation of a non-lactation mammary gland to a lactating mammary gland.

Vascular endothelial growth factor C (Q9XS50) is a gene predicted to play a role in organ morphogenesis and lymph-angiogenesis thorough positive regulation cell division and proliferation (Orpana & Salven 2002). Matrix metalloproteinase-1 (MMP-1) also known as interstitial collagenase and fibroblast collagenase degrades interstitial collagens and is involved in tissue remodeling (Reviglio et al. 2003). Alpha-actinin-2 (F1MRD4) is predicted to play a role in actin crosslink formation and focal adhesion assembly. Thus, genes differentially up-regulated in colostrum predominantly encode secretory proteins present of the extracellular matrix likely to be involved in mammary gland remodeling at the onset of lactation.

The top 10 most enriched genes in mature milk compared to colostrum are listed in table 5.6. The majority of these genes encode extracellular matrix, transport or membrane proteins. The coiled-coil domain containing 108 (CCDC108) is a coiled transmembrane protein. Two proteoglycan genes A4IFV9 and A4IFU0 are major components of the animal extracellular matrix. DnaJ (Hsp40) homolog subfamily C, member 12 (DJC12) is associated with complex assembly, protein folding, and protein export. PEAP is associated with transport, extracellular region and small molecule binding and is predicted to carry a lipocalin-like cytosolic fatty-acid binding protein domain. Beta-crystallin A2 (CRBA2) belong to the crystalline protein family, which are water-soluble structural proteins found in the lens of eye and account for the transparency of structure (Jester 2008). Patatin-like phospholipase domain-containing protein 3 (PNPLA3) is membrane bound and may be involved in the balance of energy usage/storage in adipocytes (Wilson et al. 2006). Energy homeostasis associated gene ENHO is predicted to be involved in the regulation of glucose homestasis and lipid metabolism. Glycosylation-dependent cell adhesion molecule 1 (GLCM1) is an adhesion molecule that accomplishes cell binding by presenting carbohydrate(s) to the lectin domain of L-selectin. GLCM1 has been identified in bovine milk and is believed to contribute to immune protection.
Table 5.5. Genes highly enriched in colostrum cells. (log expression values).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Col.</th>
<th>M.M.</th>
<th>Diff.</th>
</tr>
</thead>
<tbody>
<tr>
<td>bTrappin-5</td>
<td>No description</td>
<td>4.0</td>
<td>-1.6</td>
<td>5.5</td>
</tr>
<tr>
<td>bTrappin-4</td>
<td>No description</td>
<td>5.1</td>
<td>-0.4</td>
<td>5.5</td>
</tr>
<tr>
<td>A1A4Q6</td>
<td>Elafin</td>
<td>3.0</td>
<td>-2.1</td>
<td>5.1</td>
</tr>
<tr>
<td>LOC100295842</td>
<td>No description</td>
<td>3.9</td>
<td>-1.2</td>
<td>5.1</td>
</tr>
<tr>
<td>A6H734</td>
<td>Plexin domain-containing protein 1</td>
<td>-2.1</td>
<td>-6.9</td>
<td>4.9</td>
</tr>
<tr>
<td>Q9XS50</td>
<td>Vascular endothelial growth factor C</td>
<td>-3.5</td>
<td>-8.3</td>
<td>4.8</td>
</tr>
<tr>
<td>LOC100297932</td>
<td>PREDICTED: interstitial collagenase-like</td>
<td>-0.5</td>
<td>-5.0</td>
<td>4.6</td>
</tr>
<tr>
<td>MMP1</td>
<td>Interstitial collagenase precursor</td>
<td>-0.3</td>
<td>-4.6</td>
<td>4.3</td>
</tr>
<tr>
<td>F1MRD4</td>
<td>Alpha-actinin-2</td>
<td>-3.8</td>
<td>-8.0</td>
<td>4.2</td>
</tr>
<tr>
<td>E1BCA0</td>
<td>Uncharacterized protein</td>
<td>-3.4</td>
<td>-7.6</td>
<td>4.2</td>
</tr>
</tbody>
</table>

Table 5.6. Genes highly enriched in milk cells. (log expression values).

<table>
<thead>
<tr>
<th>Feature</th>
<th>Description</th>
<th>milk</th>
<th>Colostrum</th>
<th>Diff.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCDC108</td>
<td>Coiled-coil domain containing 108</td>
<td>1.1</td>
<td>-5.6</td>
<td>6.8</td>
</tr>
<tr>
<td>A4IFV9</td>
<td>Proteoglycan 3</td>
<td>1.5</td>
<td>-5.0</td>
<td>6.5</td>
</tr>
<tr>
<td>E1BLR5</td>
<td>Uncharacterized protein</td>
<td>1.3</td>
<td>-5.1</td>
<td>6.4</td>
</tr>
<tr>
<td>DJC12</td>
<td>DnaJ homolog subfamily C member 12</td>
<td>1.7</td>
<td>-4.3</td>
<td>6.0</td>
</tr>
<tr>
<td>PEAP</td>
<td>lipocalin/cytosolic fatty-acid binding</td>
<td>4.4</td>
<td>-1.1</td>
<td>5.5</td>
</tr>
<tr>
<td>CRBA2</td>
<td>Beta-crystallin A2</td>
<td>1.1</td>
<td>-4.2</td>
<td>5.4</td>
</tr>
<tr>
<td>A4IFU0</td>
<td>Proteoglycan 3</td>
<td>0.5</td>
<td>-4.5</td>
<td>4.9</td>
</tr>
<tr>
<td>PNPLA3</td>
<td>Patatin-like phospholipase domain-containing protein 3</td>
<td>-2.5</td>
<td>-7.4</td>
<td>4.9</td>
</tr>
<tr>
<td>ENHO</td>
<td>Adropin</td>
<td>3.1</td>
<td>-1.7</td>
<td>4.8</td>
</tr>
<tr>
<td>GLCM1</td>
<td>Glycosylation-dependent cell adhesion molecule 1</td>
<td>11.9</td>
<td>7.3</td>
<td>4.6</td>
</tr>
</tbody>
</table>

Gene ontology analysis of buffalo colostrum and milk cell transcriptomes

Functional clustering of the 77 genes identified as colostrum enriched revealed 13 functional clusters with enrichment scores above 1 unit. Functional terms associated with these cluster denoted angiogenesis, blood vessel development and morphogenesis, vasculature and tube development,
extracellular region, signal, secretion, cell junctions, cell-substrate, cell-cell adhesion, followed by plasma membrane, calcium, zinc and metal ion bindings, glycoproteins. Cell migration, mobility and motion ontology terms had were also enriched. All these process mark the changing cellular conditions and processes at the onset of lactation in the mammary gland.

By contrast, 79 milk enriched genes were clustered into 6 David ontology categories. With much higher enrichment scores compared to colostrum (E.S. >4), the most significant cluster contained ontology terms related to milk and mammary gland proteins, casein, extra cellular regions and polymorphism followed by signal, immunoglobins and transmembrane. Other clusters included calcium and iron ion bindings and mitochondrial terms.

Overall, milk showed few clusters with very high enrichment of major milk elements compared to low enrichment scores and wide range of functional enrichments in colostrum enriched genes.

To expend the analysis we harvested larger gene sets for differential functional analysis. When we further relaxed the statistical filter by cancelling the multiple corrections filter, we extracted 2346 genes candidates for differential expression (P-value < 0.05) with 554 genes enriched in mature milk and 1792 colostrum enriched genes. A similar pattern of functional ontology classifications was obtained with the larger set of 1792 colostrum enriched candidates. However the David enrichment scores were generally more significant (E.S. >2.5). Briefly, ontology terms associated with colostrum were: blood vessel development, morphogenesis, angiogenesis and vasculature development - cell motion, migration, mobility and localization - extracellular region - mesenchymal cell differentiation, development and migration - chordate, embryonic and in utero development and tube, blood vessel development and morphogenesis (E.S. 2.65).

With the 552 milk enriched candidate genes the results were similar to the results obtained with the smaller more selective set of 79 genes, with high scores for ontology terms relating to milk, milk proteins, caseins (E.S. 4.82), cofactor, coenzyme binding (3.6), mitochondria related (1.93) and Immunoglobulin like (E.S. 1.62).

**Comparison of buffalo and cow milk transcriptomes**

A transcriptome sequencing study of bovine somatic milk cell was published recently (Wickramasinghe et al. 2012). In this study, milk cell RNA data were obtained at lactation day 15 (early lactation), 90 (mid lactation) and 250 (late lactation). Unfortunately, the RNA-seq data collected was not fully made available and only a short list of highly expressed genes at each stage of lactation was reported. This list was similar to the buffalo milk or colostrum in bovine samples from early and peak lactation, with predominance of the major milk proteins. However genes expression was different during late lactation.

The authors reported 140 genes (gene list not published) in mid-lactation with higher than 500 RPKM values. As described above in buffalo, we found 727 genes with more than 500 RPKM during lactation. A total of 8274 were undetected in the bovine study while we report 9283 genes in this category.

In the absence of original RNA-seq data and a reference of the buffalo genome sequence, it is difficult to perform a global comparison of cow and buffalo milk cell transcriptomes and identify species specific variations, but preliminary comparisons show some overlap between gene expression profiles during lactation.

**Small RNA sequencing of cell-free colostrum and milk**

After cell removal, RNA can also be recovered from the skim milk fractions of Buffalo colostrum and milk. Although the isolation of RNA from the milk fat globule has been reported on bovine and
human milk, we could not recover measurable quantities of RNA from buffalo milk fat. This is likely to be due to rapid degradation during transport from the farm. We estimated typical milk RNA concentration around 200 ng/ml (range 100ng to 1 μg/ml), which is similar to the concentrations reported for other mammals (Maningat et al. 2009), with a slightly higher concentration in colostrum. When the RNA was analysed on the Bioanalyser, the profile showed an abundance of small RNA with a large peak in the micro-RNA region (18-26 nt) representing over 50% of total RNA. To investigate the small RNA content of buffalo milk in more detail, sequencing experiments were conducted.

**Colostrum and milk contain significant amount of small RNA including large amount of miRNA**

Nine small RNA libraries from buffalo milk were sequenced; four milk samples three colostrum samples (CM-1, CM-2 and CM-3), a RNA sample from a purified colostrum exosome fraction (CM-E), and a duplicate sample obtained after size selection between 18 to 80 nucleotides (CM-80) rather than the standard 18 to 40 nucleotide size selection. For each sample approximately 10 to 15 million high quality sequences were obtained. After adaptor removal and various cleaning steps each small RNA library generated approximately 5 to 13 million clean reads, as summarized in table 5.7. Figure 5.3 depicts the length distribution of sequences obtained after trimming. It can be seen that the data consists of a large peak in the region of 19 to 25 base long sequences corresponding to micro-RNA and a second peak for sequences from 30 to 34 bases mainly containing transfer RNA sub-sequences.

**Table 5.7. Summary of small RNA sequencing quality control and filtering**

<table>
<thead>
<tr>
<th></th>
<th>CM-1</th>
<th>CM-2</th>
<th>CM-3</th>
<th>MM-1</th>
<th>MM-2</th>
<th>MM-3</th>
<th>MM-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total reads</td>
<td>11408450</td>
<td>10954359</td>
<td>10901894</td>
<td>10886460</td>
<td>14749581</td>
<td>11442843</td>
<td>11009867</td>
</tr>
<tr>
<td>High quality</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>3'adapter null</td>
<td>0.46%</td>
<td>0.43%</td>
<td>0.48%</td>
<td>0.31%</td>
<td>0.38%</td>
<td>0.54%</td>
<td>0.22%</td>
</tr>
<tr>
<td>Insert null</td>
<td>12.82%</td>
<td>2.29%</td>
<td>10.77%</td>
<td>2.08%</td>
<td>0.74%</td>
<td>24.32%</td>
<td>0.16%</td>
</tr>
<tr>
<td>Clean reads</td>
<td>79.16%</td>
<td>96.74%</td>
<td>52.65%</td>
<td>82.68%</td>
<td>88.86%</td>
<td>43.85%</td>
<td>99.04%</td>
</tr>
</tbody>
</table>
When the data were mapped onto the genome annotation as shown in table 5.8, a majority (50 to 73%) of sequences could indeed be mapped to known micro-RNA genes. In total, 361 and 288 distinct known miRNAs were reported in colostrum and milk respectively with a large majority of 274 miRNAs identified in both samples. In addition, novel miRNAs were predicted by miRanalyzer, a machine-learning algorithm to predict new miRNA from RNA-seq data. This resulted in 428 and 125 novel miRNA candidates newly identified in buffalo colostrum and milk respectively. Most of the colostrum or mature milk specific miRNAs had very low read counts, indicating a large overlap in the miRNA composition of milk and colostrum.

Table 5.9 presents the list of the most highly expressed miRNAs in all samples in decreasing order of expression. There is more variation in individual colostrum samples than mature milk samples, probably denoting the rapidly changes in composition of colostrum likely to have been collected at different time points after parturition. Mir-30b and miR-148a were always highly expressed in all milk samples and accounted for approximately 35% of total miRNA contents. Similarly, the 10 most abundant miRNAs represented approximately 75% of all miRNA reads, showing that a few miRNA species are highly enriched in mammary gland secretions.
Table 5.9. colostrum and milk miRNA ranked by level of secretion in different samples

<table>
<thead>
<tr>
<th>Ranks</th>
<th>CM-1</th>
<th>CM-2</th>
<th>CM-80</th>
<th>MM-1</th>
<th>MM-2</th>
<th>MM-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>miR-30a-5p</td>
<td>miR-191</td>
<td>miR-30a-5p</td>
<td>miR-30a-5p</td>
<td>miR-30a-5p</td>
<td>miR-30a-5p</td>
</tr>
<tr>
<td>2</td>
<td>miR-27b</td>
<td>miR-30a-5p</td>
<td>miR-148a</td>
<td>miR-148a</td>
<td>miR-148a</td>
<td>miR-148a</td>
</tr>
<tr>
<td>3</td>
<td>miR-148a</td>
<td>miR-148a</td>
<td>miR-27b</td>
<td>let-7a-5p</td>
<td>miR-375</td>
<td>miR-375</td>
</tr>
<tr>
<td>4</td>
<td>miR-191</td>
<td>miR-181a</td>
<td>miR-26a</td>
<td>let-7f</td>
<td>let-7f</td>
<td>let-7a-5p</td>
</tr>
<tr>
<td>5</td>
<td>miR-181a</td>
<td>miR-26a</td>
<td>miR-191</td>
<td>miR-375</td>
<td>miR-191</td>
<td>let-7f</td>
</tr>
<tr>
<td>6</td>
<td>miR-26a</td>
<td>miR-92a</td>
<td>miR-181a</td>
<td>let-7a-5p</td>
<td>miR-141</td>
<td>miR-141</td>
</tr>
<tr>
<td>7</td>
<td>miR-141</td>
<td>let-7a-5p</td>
<td>miR-141</td>
<td>miR-191</td>
<td>miR-141</td>
<td>miR-191</td>
</tr>
<tr>
<td>8</td>
<td>miR-186</td>
<td>miR-182</td>
<td>miR-22-3p</td>
<td>miR-181a</td>
<td>miR-21-5p</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>let-7a-5p</td>
<td>miR-27b</td>
<td>let-7a-5p</td>
<td>miR-101</td>
<td>miR-22-3p</td>
<td>miR-181a</td>
</tr>
<tr>
<td>10</td>
<td>let-7f</td>
<td>miR-30d</td>
<td>miR-22-3p</td>
<td>miR-27b</td>
<td>miR-182</td>
<td>miR-143</td>
</tr>
</tbody>
</table>

A list of the most abundant miRNAs identified in colostrum and milk samples is given in the table 5.10.

Table 5.10. the most abundant colostrum and milk miRNA species. Log transformed expression values in all colostrum and mature milk sample or one colostrum exosome enriched sample.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>miR-30a-5p</td>
<td>26.7</td>
<td>miR-30a-5p</td>
<td>35.2</td>
<td>miR-148a</td>
<td>22.5</td>
</tr>
<tr>
<td>2</td>
<td>miR-191</td>
<td>8.9</td>
<td>miR-148a</td>
<td>20.3</td>
<td>miR-30a-5p</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>miR-148a</td>
<td>8.1</td>
<td>miR-375</td>
<td>6</td>
<td>miR-181a</td>
<td>7.9</td>
</tr>
<tr>
<td>4</td>
<td>miR-27b</td>
<td>6.2</td>
<td>let-7f</td>
<td>5</td>
<td>miR-191</td>
<td>5.5</td>
</tr>
<tr>
<td>5</td>
<td>miR-181a</td>
<td>5.3</td>
<td>let-7a-5p</td>
<td>4.7</td>
<td>let-7a-5p</td>
<td>5.4</td>
</tr>
<tr>
<td>6</td>
<td>miR-26a</td>
<td>5.1</td>
<td>miR-141</td>
<td>3.1</td>
<td>miR-27b</td>
<td>4.5</td>
</tr>
<tr>
<td>7</td>
<td>miR-141</td>
<td>3.6</td>
<td>miR-191</td>
<td>2.9</td>
<td>miR-182</td>
<td>3.7</td>
</tr>
<tr>
<td>8</td>
<td>miR-186</td>
<td>3.1</td>
<td>miR-181a</td>
<td>1.9</td>
<td>let-7f</td>
<td>3.5</td>
</tr>
<tr>
<td>9</td>
<td>let-7a-5p</td>
<td>2.9</td>
<td>miR-22-3p</td>
<td>1.8</td>
<td>miR-26a</td>
<td>3.3</td>
</tr>
<tr>
<td>10</td>
<td>miR-182</td>
<td>2.1</td>
<td>miR-21-5p</td>
<td>1.5</td>
<td>miR-141</td>
<td>2.4</td>
</tr>
</tbody>
</table>
**Differential analysis of colostrum and milk miRNA content**

Figure 5.4 shows a scatter-plot of where log normalised expression values in colostrum are plotted against the expression values of identical milk miRNA. A majority of miRNA are found along the diagonal, showing an overall trend indicating comparable miRNA composition of colostrum and milk. However, although most of the high concentration miRNAs are found at comparable levels in colostrum and milk, some miRNAs are apparently enriched in either milk or colostrum. One novel putative miRNA-like sequence was also particularly enriched in milk. When rigorous tests of statistical significance (P < 0.05 with multiple testing correction) were applied to identified miRNA with differential expression in colostrum and milk, mir-375 was the only miRNA significantly enriched in milk and mir-18b was significantly enriched in colostrum. With a less rigorous approach (P<0.05 without correction for multiple testing), the 29 miRNAs listed in table 5.11 were found to be differentially expressed candidates. Among them a cluster of 12 miRNAs on chromosome 21 was found to be milk enriched. By directly comparing probe intensities in the Seqmonk software, 83 and 91 miRNAs were identified with intensity difference over 2-fold in colostrum and mature milk respectively as shown in figure 5.4.

![Figure 5.4. scatter plot of miRNAs quantification in colostrum (horizontal axis) versus milk (vertical axis), showing data points detected as enriched with P-value <0.05 (red) or a selection of colostrum enriched (blue) or milk enriched (green) with over 2-fold differential expression.](image)

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Colostrum</th>
<th>Mature Milk</th>
<th>P-value</th>
<th>Chr</th>
</tr>
</thead>
<tbody>
<tr>
<td>mir-18b</td>
<td>9.5</td>
<td>6.0</td>
<td>0.00</td>
<td>X</td>
</tr>
<tr>
<td>mir-375</td>
<td>16.7</td>
<td>25.6</td>
<td>0.00</td>
<td>2</td>
</tr>
<tr>
<td>mir-411</td>
<td>12.3</td>
<td>20.2</td>
<td>0.00</td>
<td>21</td>
</tr>
<tr>
<td>miRNA</td>
<td>Log expression</td>
<td>Log expression</td>
<td>P-value</td>
<td>Quantity</td>
</tr>
<tr>
<td>---------</td>
<td>----------------</td>
<td>----------------</td>
<td>---------</td>
<td>----------</td>
</tr>
<tr>
<td>mir-122</td>
<td>7.5</td>
<td>17.7</td>
<td>0.00</td>
<td>24</td>
</tr>
<tr>
<td>mir-381</td>
<td>10.6</td>
<td>19.2</td>
<td>0.00</td>
<td>21</td>
</tr>
<tr>
<td>mir-410</td>
<td>10.4</td>
<td>18.0</td>
<td>0.00</td>
<td>21</td>
</tr>
<tr>
<td>mir-409a</td>
<td>9.7</td>
<td>17.4</td>
<td>0.00</td>
<td>21</td>
</tr>
<tr>
<td>mir-24-1</td>
<td>8.8</td>
<td>6.8</td>
<td>0.01</td>
<td>8</td>
</tr>
<tr>
<td>mir-143</td>
<td>18.1</td>
<td>23.2</td>
<td>0.01</td>
<td>7</td>
</tr>
<tr>
<td>mir-3431</td>
<td>10.9</td>
<td>16.8</td>
<td>0.02</td>
<td>X</td>
</tr>
<tr>
<td>mir-486</td>
<td>17.4</td>
<td>21.9</td>
<td>0.02</td>
<td>27</td>
</tr>
<tr>
<td>mir-145</td>
<td>14.1</td>
<td>7.4</td>
<td>0.02</td>
<td>7</td>
</tr>
<tr>
<td>mir-494</td>
<td>7.6</td>
<td>14.2</td>
<td>0.03</td>
<td>21</td>
</tr>
<tr>
<td>mir-376c</td>
<td>7.5</td>
<td>14.0</td>
<td>0.03</td>
<td>21</td>
</tr>
<tr>
<td>mir-487b</td>
<td>7.6</td>
<td>14.2</td>
<td>0.03</td>
<td>21</td>
</tr>
<tr>
<td>mir-369</td>
<td>7.7</td>
<td>14.0</td>
<td>0.03</td>
<td>21</td>
</tr>
<tr>
<td>mir-455</td>
<td>13.7</td>
<td>7.5</td>
<td>0.03</td>
<td>8</td>
</tr>
<tr>
<td>mir-493</td>
<td>9.4</td>
<td>15.0</td>
<td>0.04</td>
<td>21</td>
</tr>
<tr>
<td>mir-34c</td>
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<td>7.5</td>
<td>0.04</td>
<td>15</td>
</tr>
<tr>
<td>mir-299</td>
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<td>0.04</td>
<td>21</td>
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<tr>
<td>mir-340</td>
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<td>19.0</td>
<td>0.04</td>
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<td>0.04</td>
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<tr>
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<td>0.04</td>
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<tr>
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<td>0.04</td>
<td>3</td>
</tr>
<tr>
<td>mir-23b</td>
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<td>0.04</td>
<td>8</td>
</tr>
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<td>mir-374a</td>
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<td>19.3</td>
<td>0.05</td>
<td>X</td>
</tr>
<tr>
<td>mir-101</td>
<td>18.1</td>
<td>21.8</td>
<td>0.05</td>
<td>8</td>
</tr>
<tr>
<td>mir-92</td>
<td>8.9</td>
<td>7.2</td>
<td>0.05</td>
<td>X</td>
</tr>
<tr>
<td>mir-1388</td>
<td>13.9</td>
<td>17.9</td>
<td>0.05</td>
<td>13</td>
</tr>
<tr>
<td>mir-223</td>
<td>9.9</td>
<td>7.3</td>
<td>0.05</td>
<td>X</td>
</tr>
</tbody>
</table>

In general, colostrum enriched miRNAs had log expression values from 7.5 to 15 with few miRNAs above 15, whereas mature milk enriched miRNAs had a larger range of intensity values (log values.
from 10 to 26), and these miRNAs showed greater intensity differences with colostrum. These observations suggest that milk is enriched with some highly expressed miRNAs, including for example mir-122, mir-375 and mir-381. By contrast miRNA enriched in colostrum, such as mir-145, mir-455 and mir-34c, generally represented a lower proportion of the total miRNA population.

**Small RNA profiling of colostrum exosomes**

A number of publications have recently reported that milk miRNA may be packaged inside exosomes, conferring stability and resistance to RNAse activity (Chen et al. 2010; Kosaka et al. 2010). In order to evaluate the contribution of the exosome fraction to colostrum RNA content, we purified exosome from the skim milk using the ExoQuick™ reagent. Starting with 1.5 ml of whey from the same colostrum sample previously used in small RNA sequencing, about 100-300 µl of ExoQuick precipitate was recovered and used for RNA preparation with a yield of 25% of total milk RNA. The small RNA fraction (>40 nt) was subsequently sequenced. From a total of 9,136,568 reads, 8,285,028 (90%) reads could be mapped onto the bovine genome sequence. In general, the small RNA profile of the exosome fraction was very similar to the profile from total colostrum whey. For example, amongst the 235 most highly represented sequences, the majority (133) had quantification within 2-fold change and all contig had differential quantification below 5-fold. Above a threshold of 2-fold, 52 contigs were enriched in the exosome preparations. These represented 20% of total mapped reads in exosome and 8% in whey. A large proportion represented tRNA (10% of total mapped reads, average 3-fold abundance over skim milk) and the set contained 7 miRNAs representing 8% of total mapped reads, including miR-148a which represents alone 6% of mapped reads in the exosome fraction. By contrast, small RNA elevated in the skim milk samples represented only 2% and 5% of all reads in the exosome and whey data respectively. The skim milk profile was poorer in annotated tRNA and contained other known miRNAs where miR-141 was the most abundant. This observation suggests that there is no highly significant difference between small RNA prepared from ExoQuick exosome and skim milk, but points to a slight enrichment (3-fold) of small RNAs, such as tRNA, and selective miRNAs in exosomes. Conversely, a slight (2-3 fold) overall trend in under representation of sequences from miRNA in exosomes could be observed. These results suggest that exosomes may be slightly enriched in small RNA compared to total milk RNA. However the overall difference between exosome and skim milk are small. This may be due to technical variation only or mean either, that most of the colostrum small RNA is present in exosomes or, alternatively, that the ExoQuick preparation does not allow a sufficient purification of exosomes for comparison. This last point is also supported by our results on the yield of RNA in ExoQuick preparations where 25% of the total milk RNA is obtained from 20% of the original volume of milk representing the ExoQuick pellet. However we have also now employed alternative separation methods such as centrifugation and biochemical analysis, which confirmed that a major fraction of small RNA from milk and colostrum, including miRNA, co-purifies in a high molecular weight complex with physicochemical properties compatible with exosomes (data not shown).

**Comparative analysis of milk miRNAs**

In order to identify conserved or specific milk miRNA, a number of small RNA sequencing datasets were retrieved from the publicly available Gene Expression Database (Barrett et al. 2011), including data from previously reported studies on human and pig milk (Gu et al. 2012; Zhou et al. 2012) and processed using the online miRanalyzer miRNA analysis pipeline (Hackenberg et al. 2009) together with data that we generated from buffalo milk. Unfortunately, the sequencing data from a report on bovine milk RNA sequencing (Hata et al. 2010) were not available publicly for direct comparison. Table 5.12 presents the quantification results, expressed in percentage of total miRNA reads mapped, of the top 10 miRNA identified. It can be seen that that miR-148a is consistently present at the highest concentration in the milk of all the species considered, representing from 20% to 50% of all miRNAs sequences. However, while some miRNAs are found at comparable levels in all species (for example miR-30a, mir101, miR-3596d), others are highly enriched in particular species. MiR-486,
miR-185 and miR-103 are enriched in pig, miR-30b in humans or miR-423 in buffalo. It should also be noted that, when multiple data points or duplicate samples are available (pig and human) the abundance of particular miRNA may vary significantly between individual samples from the same species. It remains to be established whether the variation in the estimation represents the genetic variability of milk miRNA between individuals, physiological variation associated with lactation status or, if technical issues such as sequencing depth, RNA quality and annotation methodology are also responsible. However, the results confirm the conserved high abundance of miR-148a in mammalian milk and suggest additional universal milk miRNA markers. They also highlight potential differences between the detailed miRNA content of milk from different species and the requirement for additional experiments to control further for technical, genetic and physiological factors affecting milk miRNA.

**Table 5.12. Comparative abundance of milk miRNA (% of total miRNA content) in buffalo, human and pig milk.**

<table>
<thead>
<tr>
<th>Rank</th>
<th>Buffalo</th>
<th>Human</th>
<th>Pig</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>miR-30a-5p</td>
<td>35.2</td>
<td>24.3 miR-148a-3p</td>
</tr>
<tr>
<td>2</td>
<td>miR-148a</td>
<td>20.3 miR-200a-3p</td>
<td>9.39 miR-30a-5p</td>
</tr>
<tr>
<td>3</td>
<td>miR-375</td>
<td>6 miR-30a-5p</td>
<td>5.71 miR-182</td>
</tr>
<tr>
<td>4</td>
<td>let-7f</td>
<td>5 miR-141-3p</td>
<td>5.29 miR-378</td>
</tr>
<tr>
<td>5</td>
<td>let-7a-5p</td>
<td>4.7 miR-378a-3p</td>
<td>4.38 miR-92a</td>
</tr>
<tr>
<td>6</td>
<td>miR-141</td>
<td>3.1 miR-146b-5p</td>
<td>3.61 miR-30d</td>
</tr>
<tr>
<td>7</td>
<td>miR-191</td>
<td>2.9 miR-101-3p</td>
<td>3.14 miR-30c-5p</td>
</tr>
<tr>
<td>8</td>
<td>miR-181a</td>
<td>1.9 miR-182-5p</td>
<td>2.88 miR-191</td>
</tr>
<tr>
<td>9</td>
<td>miR-22-3p</td>
<td>1.8 miR-375</td>
<td>2.78 let-7c</td>
</tr>
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<td>miR-21-5p</td>
<td>1.5 miR-21-5p</td>
<td>2.60 miR-574</td>
</tr>
</tbody>
</table>

**Discussion**

In this chapter, we have presented preliminary results on the transcriptome analysis of buffalo milk. We have shown that milk cells recovered from fresh milk and colostrum can be analysed by transcriptome sequencing. This provided a large amount of information on buffalo gene sequences and revealed a high level of milk protein gene expression in the harvested cell, showing that a large proportion of milk cells originate from the mammary gland epithelium. Thus, transcriptomics profiling of milk cells provides a non-invasive methodology to investigate the lactation status of buffalo, and characterize the changes in gene expression associated with the transition between colostrum and milk synthesis. We have also shown that colostrum and milk contains a relatively large amount of small RNA, with a large proportion of miRNA. In all, about 300 known and additional novel candidate miRNAs have been characterized in buffalo colostrum and milk. Exosome
purification and sequencing of exosomal RNA supports the notion that, as reported in other species (Hata et al. 2010; Zhou et al. 2012), buffalo colostrum and milk miRNA could be secreted in exosomes. Interestingly, despite high level of milk protein transcripts in the transcriptome of colostrum cells, the expression of miRNA precursors could not be identified in the cell preparation. This suggests that milk miRNA are not synthesised in mammary epithelial cells or that the lifetime of poly-adenylated miRNA precursors is short. A number of highly abundant milk miRNA species are also found at high levels in the milk of other mammals, suggesting that these miRNA sequences could be developed into universal milk miRNA markers. Other milk miRNA may be found at variable levels in the milk of different species and may provide additional specific markers. In addition, variation between the miRNA content of individual animals has been noted within the same species. These observations suggest that milk transcriptomics and miRNA analysis may provide an alternative method to evaluate milk quality and analyse milk origin, as well as lactation physiology. Access to the buffalo genome sequence would also greatly benefit the analysis of sequencing results in the future as the results described here are limited by the use of the bovine genome reference instead.

Finally, the results highlight how further experiments are needed to fully characterise the biogenesis of milk miRNA and identify factors influencing milk miRNA composition and characterize in more details the functional activity of milk miRNA during digestion. Nevertheless, milk transcriptomics, including the integration of cell transcriptome and milk miRNA data within a comparative framework, has the potential to allow the development of a new methodology to analyse lactation in greater detail, while providing new quality biomarkers and novel functional miRNA candidates potentially regulating the development of the young during lactation, or carrying additional health benefits and functionalities associated with the consumption of milk.
Results

The original project objectives have been met. The results obtained in this project include:

1. the characterisation of Australian buffalo milk
2. the characterisation of buffalo milk yoghurt
3. a study of gene expression in buffalo milk.

The details of results against the individual objectives are as follows:

- **Quantitative analysis of major milk components; protein, lipid and carbohydrate and fat globule size distribution across a number of herds using fresh and frozen milk:**
  
  - A quantitative analysis of major components in Australian buffalo milk was performed. The concentration of protein, lipid and carbohydrate (lactose) is provided in Chapter 2. A review of the typical composition reported in the international literature is reported in the literature review in Chapter 1.
  
  - Australian buffalo milk was found to have characteristics largely consistent with the properties reported in other international studies, including the chemical composition and fat globule size, specific surface area and surface potential. Buffalo milk samples collected from different regions of Australia did not show significant differences in the structure of the milk or physical properties of fat globules including fat globule size distribution.

  - Furthermore, the lipid profile including the concentration of cholesterol, polar lipids, profile of individual phospholipids and the fatty acid composition of buffalo milk in comparison with bovine milk is reported in the literature review (Chapter 1). This same chapter discussed the protein profile within buffalo milk including the composition of casein and whey proteins and changes in buffalo milk composition as a function of lactation.

  - The properties of frozen buffalo milk were characterised and compared with the properties raw buffalo milk (Chapter 3). Freezing was found to induce ‘oiling–off’ and the formation of a fat layer on the milk surface for some treatments. Freezing also reduced the integrity of the fat globule membrane and increased the size of some fat globules, although no significant effects were observed on physiochemical properties such as the pH, calcium concentration or milk viscosity.

- **An examination of the arrangement of protein and fat within yoghurt or cheese made from buffalo milk using a range of advanced microscopy techniques and characterisation of the texture of products made from buffalo milk to better understand how the properties of the raw ingredients influence the structure and properties of yoghurt or cheese, using techniques established for bovine milk.**

  - The characterisation of buffalo yoghurt in comparison with bovine yoghurt (Chapter 4) shows significant differences in physiochemical properties and microstructure of the two yoghurt types, due to the differences in their chemical composition.

  - The arrangement of protein and fat within the yoghurt made from buffalo milk was investigated using confocal laser scanning microscopy and cryo-scanning electron microscopy techniques (Chapter 4). Yoghurt produced from buffalo milk shows a
more porous microstructure with a more disrupted and coarser protein network, likely leading to the higher syneresis observed compared to yoghurt produced from homogenised and fortified bovine yoghurt

- Rheological properties, including thixotropy and the flow behaviour index, show that buffalo yoghurt has a poorer texture, which is more sensitive to deformation and less able to recover the original structure after deformation and hence, buffalo yoghurt needs more careful handling compared to bovine yoghurt

- The bigger size of fat globules, and the smaller surface area of fat in buffalo milk, contribute to the high syneresis and poorer texture observed in buffalo yoghurt.

• Gene expression of buffalo milk

- The sequencing of over 100 million short sequences isolated from cells or small RNA secreted in colostrum and milk has uncovered a large amount of new information on genes expressed in somatic milk cells and small RNA secreted in buffalo milk

- Several thousand genes were characterised, and gene expression levels in somatic cells was analysed in detail. The results show a high level of milk protein gene expression in the harvested cells, indicating that a large proportion of milk cells represent exfoliated cells from the mammary gland epithelium and, providing details on buffalo milk protein and other gene sequences and their differential expression in colostrum and milk cells

- The presence in buffalo milk and colostrum of significant amount of miRNA was also confirmed. This new class of secretory RNA was only recently discovered in the milk of other mammals, but has an important functional potential to encode new milk signals, targeting gene expression in the neonate (and possibly the consumer). About 300 known miRNA and several new milk miRNA candidates were discovered in buffalo, filling a gap in the general knowledge of buffalo milk composition. The analysis of miRNA distributions in colostrum and milk samples, together with the comparison with milk from other species reported above, have facilitated the identification of universal, temporal and specific milk miRNA markers. Besides providing new milk and lactation markers, these results will enable the development of new hypothesis about the functional evolution of milk miRNA and the deployment of functional assays to test their bioactivities. The confirmation that buffalo milk miRNA are apparently secreted in exosome-like nano-vesicles should also inspire new studies on the biogenesis and the production of milk miRNA, and further characterisation of the physical, chemical and biological properties of milk exosomes.
Implications

The findings obtained in this study provide buffalo farmers and the buffalo industry with greater information about Australian buffalo milk and buffalo milk products.

The literature review provides a valuable overview of the chemical composition of buffalo milk and factors that affect this composition including the stage of lactation, breed and feed type. This information will be helpful for farmers who wish to optimise and control milk composition by changing the feed type during lactation to achieve a consistent quality of milk with a desired composition.

The chemical analyses, microstructural, textural and rheological techniques used and developed in this study provide further insights into buffalo milk and milk products. These techniques can also be used by producers and manufacturers to further characterise their milk and products or as a quality control tool.

The differences observed between Australian buffalo milk and Australian bovine milk composition and properties may be used by farmers and manufacturers to highlight the differences of Australian buffalo milk and milk products.

The similarity between Australian buffalo milk composition or properties and reports in the international literature suggests that the results of some international studies may be directly applied to an Australian context.

The observation that freezing buffalo milk changes selected milk properties suggests that some damage will occur, even if small volumes of milk are frozen rapidly (e.g. 100 ml, 0.54 °C/hour). The effect is smaller than treatments such as homogenisation, and may not impact on yoghurt production, but could impact on the production of mature cheeses. Buffalo farmers should reduce the volumes of milk frozen in each batch in order to speed the rate of freezing and minimise damage.

The application of microscopy techniques, including Cryo Scanning Electron Microscopy and Confocal Laser Scanning Microscopy, has shown how the product microstructure correlates well with the texture and other product properties. These microscopy tools can be used to provide producers with a greater understanding of how the arrangement of protein and fat within buffalo products contributes to product taste and texture.

The observation that yoghurt made from buffalo milk is more susceptible to deformation than bovine yoghurt, and less able to recover an original structure after deformation, has implications for product transport. The higher syneresis observed for buffalo yoghurt also suggests further optimisation of process parameters could increase product quality.

The discovery of miRNA in buffalo milk opens new opportunities for the development of novel approaches to assay lactation and the quality and functionality of buffalo milk.

Together, these findings could assist in the expansion of production and the market for buffalo products, which will contribute to the development of the Australian buffalo industry.
Recommendations

In this study, it was shown that freezing had a minimal impact on the physicochemical and heological properties of buffalo milk. Further studies are, however, required to investigate the properties of the products produced from frozen milk. Additionally, the freezing experiment was only performed at a volume scale of 5 L. Further studies using a larger volume of buffalo milk e.g. 20 L for freezing may be useful to generate results that are more applicable to the industry practice. Buffalo yoghurt was shown to be more susceptible to deformation and less able to recover the original structure after deformation, especially after long storage. Therefore, buffalo yoghurt should be handled with more care during storage or transportation.

Buffalo yoghurt exhibited a significantly higher syneresis than bovine yoghurt. This defect may affect consumer preference. Therefore, further study to optimize the processing conditions to improve the quality of buffalo yoghurt, especially to reduce the syneresis is recommended.

The viability of probiotic bacteria in buffalo yoghurt was significantly lower than in bovine yoghurt suggesting the potential presence of some bioactive components, including those with antimicrobial properties, in buffalo milk. Further studies in this area and on the application of these health promoting ingredients in food products may benefit buffalo producers and customers.

The authors of this report will also be in contact with key Australian buffalo producers involved in dairy production to discuss project results and possible follow on studies, to meet further industry needs.
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Characterisation of buffalo milk, cheese and yoghurt properties

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